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De Novo KCNB1 Mutations in Epileptic Encephalopathy

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Abstract

Background—Numerous studies have demonstrated increased load of *de novo* copy number variants (CNVs) or single nucleotide variants (SNVs) in individuals with neurodevelopmental disorders, including epileptic encephalopathies, intellectual disability and autism.

Methods—We searched for *de novo* mutations in a family quartet with a sporadic case of epileptic encephalopathy with no known etiology to determine the underlying cause using high

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Declaration of Interests:

Ali Torkamani declares he is a cofounder and stock holder of Cypher Genomics, Inc.

Author Contributions:

Experiments were conceived by A.T., C.S.B., J.A.K. and A.L.G. Patient phenotyping and review performed by R.L.B., J.R.F., J.C., S.G. and S.N. Sequencing performed by G.O. Sequence data analysis and statistical interpretation performed by A.T. and J.A.K. Functional evaluation of mutations performed and analyzed by K.B., B.S.J., C.G.V., A.L.G., and J.A.K. Manuscript was written by A.T., A.L.G and J.A.K., and reviewed by all authors.

coverage whole exome sequencing (WES) and lower coverage whole genome sequencing (WGS). Mutations in additional patients were identified by WES. The effect of mutations on protein function was assessed in a heterologous expression system.

Results—We identified a *de novo* missense mutation in *KCNB1* that encodes the K_v2.1 voltage-gated potassium channel. Functional studies demonstrated a deleterious effect of the mutation on K_v2.1 function leading to a loss of ion selectivity and gain of a depolarizing inward cation conductance. Subsequently, we identified two additional patients with epileptic encephalopathy and *de novo* *KCNB1* missense mutations that cause a similar pattern of K_v2.1 dysfunction.

Interpretation—Our genetic and functional evidence demonstrate that *KCNB1* mutation can result in early onset epileptic encephalopathy. This expands the locus heterogeneity associated with epileptic encephalopathies and suggests that clinical WES may be useful for diagnosis of epileptic encephalopathies of unknown etiology.

INTRODUCTION

Epileptic encephalopathies are a heterogeneous group of severe childhood-onset epilepsies characterized by refractory seizures, neurodevelopmental impairment, and poor prognosis.¹ The developmental trajectory is normal prior to seizure onset, after which cognitive and motor delays become apparent. Ongoing epileptiform activity adversely affects development and contributes to functional decline. Therefore, early diagnosis and intervention may improve long term outcomes.^{2,3,4}

Recently, there has been significant progress in identifying genes responsible for epileptic encephalopathies and *de novo* mutations have been reported in approximately a dozen genes, including *SCN1A*, *SCN2A*, *SCN8A*, *KCNQ2*, *HCN1*, *GABRA1*, *GABRB3*, *STXBP1*, *CDKL5*, *CHD2*, *SYNGAP1*, and *ALG13*.⁵⁻⁹ The majority of mutations reported are in genes encoding voltage-gated ion channels, neurotransmitter receptors and synaptic proteins. There is significant phenotype heterogeneity, with mutations in the same gene resulting in different clinical presentations, as well as locus heterogeneity, with mutations in different genes resulting in the same syndrome. Further, epileptic encephalopathy genes have substantial overlap with genes responsible for other neurodevelopmental disorders, including autism and intellectual disability.¹⁰⁻¹²

Due to the considerable phenotype and locus heterogeneity, it is difficult to predict appropriate candidate genes for testing in a particular patient. Therefore hypothesis-free approaches such as whole exome sequencing (WES) or whole genome sequencing (WGS) may be useful for uncovering causative variations in epileptic encephalopathies of unknown etiology. Thus, we aimed to identify the underlying genetic cause of epileptic encephalopathy in the proband by WES and WGS of a family quartet.

METHODS

Study Subjects

Study participants included ID9, parents ID9F and ID9M, and an unaffected sister ID9S. Adults provided written informed consent, with additional assent by ID9 and ID9S, under a

protocol approved by the Scripps institutional review board. Consent for release of medical information for individual 2 was obtained from the parents. Clinical details are described below and summarized in Table 4.

Individual ID9 is a 9-year-old female with epileptic encephalopathy, hypotonia, developmental delays, cognitive impairment, and intermittent agitation. Pre- and perinatal histories were unremarkable, although hypotonia and excessive somnolence were noted early. Motor milestones were delayed, with sitting at 9 months, walking at 20 months, and persistent clumsiness. Language acquisition was delayed with regression at age 18 months. Motor, language and behavior have fluctuated but overall there has been forward developmental progress. Onset of generalized tonic-clonic seizures (GTCS) was at 4.75 years of age, although behavioral manifestations likely representing other seizure types were present earlier. Multiple seizure semiologies were reported including rare GTCS, head drops, and more common facial twitching with drooling, eye fluttering, gagging, vomiting and stiffening. Rare GTCS are controlled with levetiracetam and clonazepam, but other seizure types have been poorly controlled with multiple therapies that were ineffective or limited by side effects (Table 4). In addition, the patient experiences sumatriptan-responsive migrainous episodes consisting of headache, abdominal discomfort, photophobia and lethargy.

Brain MRI showed subtle asymmetric volume loss in left hippocampus. Recent seven day video-EEG monitoring revealed mild diffuse slowing and abundant bi-hemispheric multifocal epileptiform discharges more prominent in right temporal and midparietal regions. Electroclinical seizures began in the left hemisphere, with two in centro-parietal areas and one without clear localization. Magnetoencephalography showed frequent epileptiform spikes during sleep (648 spikes observed over 50 minutes), with frequent trains of spikes. Source modeling showed epileptiform spike activity arising from bilateral posterior perisylvian regions. The first cluster of spikes (~55%) originated from the anterior-inferior aspect of the left parietal lobe, extending to the left superior temporal gyrus. The second cluster of spikes (~45%) originated from the right temporal-parietal junction. No propagations were observed.

Muscle biopsy showed type 1 fiber predominance with mild generalized hypertrophy. There was slight elevation of plasma guanidinoacetate 2.5 (0.3-2.1 μ M) but not in the range typically associated with disease. There was mild elevation of cerebrospinal fluid (CSF) pyruvate with normal lactate [2.06 (0.5-2.2 mM)/147 (0-75 μ M)]. CSF 5-methyltetrahydrofolate was mildly reduced 36 (40-128 nM). Folinic acid therapy has had unclear impact. Extensive additional work-up was normal and included: karyotype, fragile X and Angelman syndrome testing, CGH Oligo-SNP Array, mitochondrial DNA Southern blot and mitochondrial DNA sequencing, plasma acylcarnitine, CPK, uric acid, biotinidase, ammonia, Vitamin B12, folate, homocysteine, folate receptor antibodies, lymphocyte pyruvate dehydrogenase activity, urine organic acids, urine creatine and guanidinoacetate, CSF glucose, protein, amino acids, neurotransmitter metabolite levels and pterins, muscle carnitine, CoQ10, and electron transport chain complex analysis.

Individual 2 presented as a 2-yr-10-month male with poor seizure control, developmental delay, absent speech, stereotyped handwringing movements, and progressive in-turning of feet requiring orthotic support. Prenatal and perinatal histories were normal. Development plateaued at 6 months and seizures began at 8 months of age, with hypsarrhythmic EEG for which he was treated with ACTH. Although seizures were resistant to conventional treatment (Table 4), a gluten/casein/sugar/starch-free diet begun at 2.5 years of age resulted in seizure reduction to 3/day despite marked spike activity demonstrable on EEG. At 4 years of age, seizures worsened and L-carnitine was added with subsequent amelioration. At 5 years of age, EEG was persistently abnormal with epileptiform discharges of multifocal origin including bursts of diffuse polyspikes, diffuse polyspike-wave, right temporal spike and wave, left occipital spikes and diffuse polyspike bursts lasting up to 4 minutes. Brain MRI studies at 9 months did not show structural, neuronal migration, or white matter abnormalities. He began walking at 2.5 years, and is presently interactive socially, though nonverbal. Extensive additional work-up was normal (Table 4). Genetic testing for mutations associated with *SCN1A*, *MECP2*, *CDKL5*, *FOXG1*, *ARX*, Fragile X, Pitt-Hopkins and Angelman syndromes were negative. Tests for plasma and cerebrospinal fluid (CSF) amino acid concentrations, urine organic acid levels, CSF neurotransmitter levels, lysosomal enzymes, very long chain fatty acids (VLCFA), neuronal ceroid lipofuscinosis (NCL) 1 & 2, urine sulfocysteine, congenital disorders of glycosylation (CDG), and oligo array were all normal. Clinical WES was performed by GeneDx and reported variants were confirmed by Sanger sequencing.

Individual 3 (Coriell ND27062) was ascertained by the Epilepsy Phenome/Genome Project¹³ as part of a patient cohort with infantile spasms and/or Lennox-Gastaut syndrome.⁵ Seizure onset occurred during the first year of life and seizure types include tonic-clonic, atypical absence, atonic, infantile spasms and focal dyscognitive (Table 4). EEG findings were unspecified, while imaging studies were reported as normal.

Whole Exome, Whole Genome Sequencing, Variant Calling, and Filtration

Genomic DNA was extracted from blood using the QiaAmp system (Qiagen, Valencia, CA). Enriched exome libraries were prepared using the SureSelect XT enrichment system (Agilent, Santa Clara, CA). WES was performed on an Illumina HiSeq2500 instrument with indexed, 100-bp, paired-end sequencing. Reads were mapped to the hg19 reference genome using BWA¹⁴, variant calling and quality filtration was performed using GATK best practices variant quality score recalibration.¹⁵⁻¹⁷ Mean coverage of 97 to 124-fold was achieved for each subject with 94-95% of the target exome covered by >10 reads (Table 1). Libraries for low-pass WGS were prepared using the NEBNext DNA Library Prep System (New England Biolabs, Ipswich, MA). WGS was performed on an Illumina HiSeq2500 with 100-bp indexed, paired-end sequencing. Mean coverage of 4 to 7-fold was achieved for each subject with ~64.3% of the genome covered by >5 reads (Table 1). Copy number variants (CNVs) were identified by CNVNator.¹⁸

Variant annotation was performed using the Scripps Genome Annotation and Distributed Variant Interpretation Server (SG-ADVISER) (<http://genomics.scripps.edu/ADVISER/>) as previously described.¹⁹ A series of filters were applied to derive a set of candidate disease-

causing variants (Table 2): (1) population-based filtration removed variants present at >1% allele frequency in the HapMap²⁰ or 1,000 Genomes²¹, NHLBI Exome Sequencing Project (<http://evs.gs.washington.edu/EVS/>), or Scripps Welllderly populations (individuals over the age of 80 with no common chronic conditions sequenced on the Complete Genomics platform); (2) annotation-based filtration removed variants in segmental duplication regions that are prone to produce false positive variant calls due to mapping errors²²; (3) functional impact-based filtration retained only variants that are non-synonymous, frameshift, nonsense, or affect canonical splice-site donor/acceptor sites, and (4) inheritance-based filters removed variants not present in the trio in a manner consistent with affection status. Following filtering, retained variants were confirmed by Sanger sequencing.

Locus Specific Mutation Rate Estimate

The *KCNB1* locus specific mutation rate was determined as described.²³ Human and chimpanzee alignments of the protein coding portion of exons and intronic essential splice sites were considered. The *KCNB1* mutation rate per site is 2.71×10^{-3} differences per bp of aligned sequence. Assuming a divergence time of 12 million years between chimpanzee and human and a 25 year average generation time, the *KCNB1* locus specific mutation rate per site per generation is 5.65×10^{-9} . The probability of observing *de novo* mutation events was estimated using the Poisson distribution:

$$P(X; \mu) = \left[(e^{-\mu}) (\mu^X) \right] / X!$$

where X is the number of *de novo* events observed and μ is the average number of *de novo* events based on the locus specific mutation rate.

Plasmids and Cell Transfection

Mutations were introduced into full-length human K_V2.1 cDNA engineered in plasmid pIRES2-Ds-Red-MST²⁴ by QuikChange mutagenesis (Agilent). Wildtype human K_V2.1 cDNA was subcloned into the pIRES2-smGFP expression vector. Expression of wildtype and mutant K_V2.1 in CHO-K1 cells was achieved by transient transfection using FUGENE-6 (Roche) and 0.5 µg of total cDNA (1:1 mass ratio). Expression of wildtype alone was achieved by transfection with pIRES2-smGFP-WT-K_V2.1 plus empty pIRES2-DsRed-MST, while expression of mutant alone was performed with pIRES2-DsRed-MST-mutant-K_V2.1 and empty pIRES2-smGFP. Co-expression of mutant and wildtype was achieved by co-transfection with pIRES2-smGFP-WT-K_V2.1 and pIRES2-DsRed-MST-mutant-K_V2.1 or pIRES2-dsRed-MST-WT-K_V2.1. Following transfection cells were incubated for 48 hours before use in experiments.

Cell Surface Biotinylation

Proteins on the surface of CHO-K1 cells transfected with wildtype and/or mutant K_V2.1 were labeled with cell membrane-impermeable Sulfo-NHS-Biotin (Thermo Scientific). Following quenching with 100 mM glycine, cells were lysed and centrifuged. Supernatant was collected and an aliquot was retained as the total protein fraction. Biotinylated surface proteins (100 µg per sample) were recovered from the remaining supernatant by incubation

with streptavidin-agarose beads (Thermo Scientific) and eluted in Laemmli sample buffer. Total (1 μ g per lane) and surface fractions were analyzed by Western blotting using mouse anti-Kv2.1 (1:500; NeuroMab, clone K89/34), mouse anti-transferrin receptor (1:500; Invitrogen, #13-6800), rabbit anti-calnexin (H70)(1:250; Santa Cruz, sc-11397) primary antibodies, and peroxidase-conjugated mouse anti-rabbit IgG (1:100,000 Jackson ImmunoResearch) and goat anti-mouse IgG (1:50,000, Jackson ImmunoResearch) secondary antibodies. Blots were probed for each protein in succession, stripping in between with Restore Western Blot Stripping Buffer (Pierce). Western blot analysis was performed in triplicate on samples from three independent transfections. The order of anti-Kv2.1 and anti-transferrin receptor antibodies was alternated, with transferrin receptor probed first in two of three experiments. Selectivity of biotin labeling for cell surface was confirmed by probing with calnexin following detection of Kv2.1 and transferrin receptor. Calnexin signal was consistently present in total protein lanes and absent in surface fraction lanes. Densitometry was performed using NIH ImageJ software. To control for protein loading, Kv2.1 bands were normalized to the corresponding transferrin receptor band. For each genotype, the normalized values were then expressed as a ratio of surface:total expression. Normalized total, surface and surface:total ratios were compared between genotypes using one-way ANOVA.

Electrophysiology

Whole cell patch-clamp recordings were performed as previously described²⁴, except that recording solutions were altered to achieve appropriate voltage-control. The external solution contained (in mM): 132 XCl [where X is Na⁺ except when molar substitution for K⁺, Rb⁺ or N-methyl-D-glucamine (NMDG⁺) is indicated in the figure], 4.8 KCl, 1.2 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES, pH 7.4. The internal solution contained (in mM): 20 K-aspartate, 90 NMDG-Cl, 1 MgCl₂, 1 CaCl₂, 11 EGTA, 10 HEPES, and 5 K₂ATP, pH 7.3. When cells expressing mutant channels alone or co-expressed with wildtype Kv2.1 (Kv2.1-WT) were held at -80 mV, they exhibited large currents that prevented adequate voltage control. Therefore, a holding potential of -30 mV was used for experiments. Whole cell currents were measured from -80 to +60 mV (in 10 mV, 500 msec long steps) from a holding potential of -30 mV followed by a 500 msec step to 0 mV (tail currents). Voltage-dependence of activation was evaluated from tail currents measured 10 msec after stepping to 0 mV from -40mV to +30mV and fit to the Boltzmann equation. Kinetic analysis of activation rate was performed by exponential fit of the first 50 msec of current induced after a voltage step from the holding potential.

For cation selectivity experiments, recording solutions were altered as follows: sucrose dilution was performed by adding 300 mM sucrose solution 10:1 (v/v) to the external solution described above. For determining permeability ratios, the internal solution was modified to contain (in mM): 110 K-aspartate, 1 MgCl₂, 1 CaCl₂, 11 EGTA, 10 HEPES, and 5 K₂ATP, pH 7.3 and equimolar replacement of extracellular sodium with the monovalent cations K⁺, Rb⁺ and NMDG⁺. The permeability ratio (P_X/P_{Na}) was calculated from measured reversal potentials (E_{rev}) according to the following equation²⁵:

$$E_{rev} = (RT/F) \ln \left(P_x [X^+]_o / PK^+ [Na^+]_i \right)$$

where R is the gas constant, T is absolute temperature, F is Faraday's constant, X^+ is the monovalent cation in the extracellular solution, and P_X is permeability of the X^+ cation.

Data for each experimental condition were collected from 3 transient transfections, and analyzed and plotted using Clampfit (Molecular Devices) and Graphpad Prism5 (Graphpad software). Currents were normalized for membrane capacitance and shown as mean $\pm$ SEM, and number of cells used for each experimental condition is listed in Table 5. Statistical significance was determined using unpaired Student's *t* test (Graphpad). *P* values are provided in the figures or figure legends.

RESULTS

We employed WES (~100X coverage) and WGS (~5X coverage) of the proband (ID9), unaffected father (ID9F), unaffected mother (ID9M), and unaffected sister (ID9S) to identify the molecular cause of an epileptic encephalopathy. Filtering of WES variants was done under the assumption that disease in ID9 was the result of a heterozygous *de novo* mutation, but we also considered simple and compound recessive models. Variants discovered by WES were processed through a series of population-, variant annotation-, functional-impact-, and inheritance-based filters, to identify a set of candidate disease-causing variants (Table 2). Sequence coverage and detailed variant data are presented in Table 1 and Supplemental Table S1. CNVs were also interrogated by WGS, however, no CNVs consistent with disease segregation were identified. This process identified *de novo* missense variants in 2 candidate genes, *KCNB1* and *MLST8* (Table 3). Under other genetic models, we identified a homozygous missense variant in *HRNBP3* and compound heterozygous variants in *NLRP1* and *BAHCCI* (Table 3). Of all the identified variants, the *KCNB1* variant was deemed the most likely candidate based on the *de novo* inheritance pattern, the function of *KCNB1* and its relationship to other epilepsy genes, and the predicted deleterious consequence on protein function by multiple algorithms (Table 3). *KCNB1* encodes the alpha subunit of the Kv2.1 voltage-gated potassium channel, a delayed rectifier potassium channel that is an important regulator of neuronal excitability. The S347R variant is located in the pore domain that is necessary for ion selectivity and gating (Fig. 1).

We identified two additional unrelated patients with epileptic encephalopathy and *de novo* missense variants in *KCNB1* discovered by WES. Individual 2 presented with a sporadic epileptic encephalopathy of unknown cause (Table 4). After a series of negative genetic and metabolic tests, he was referred for clinical WES. From that analysis it was determined that he had a single *de novo* missense variant in *KCNB1*. The variant G379R, located in the selectivity filter of Kv2.1, was predicted to be deleterious by functional impact algorithms (Fig. 1; Table 4). Additional inherited variants included a heterozygous splice site mutation in the *NPC2* gene (IVS4+1 G>A) inherited from his unaffected father and a variant of unknown significance in *GRIN2A* (A1276G) inherited from his unaffected mother. Neither of these transmitted variants was thought to be causative for the principal phenotypes of

individual 2, although they may contribute by modifying overall expression of the clinical phenotype. Inheritance of NPC type 2 is generally recessive, and the clinical phenotype of individual 2 was not consistent with NPC type 2. The *GRIN2A* A1276G variant is a known SNV that was inherited from the unaffected mother and exists in the general population (0.1% MAF in European Americans). A1276G is a conservative substitution in an alternatively spliced portion of the *GRIN2A* gene at a position that does not show a high degree of evolutionary conservation and was predicted to be benign by multiple functional impact algorithms (Provean: neutral (−0.78); SIFT: tolerated (0.284; Polyphen2: benign (0.376)).

Individual 3 was recently reported as part of an epileptic encephalopathy exome sequencing study by the Epi4K consortium.⁵ She presented with early-onset epileptic encephalopathy and cerebral palsy (Table 4). A *de novo* missense variant in *KCNB1* was reported for individual 3, with no additional *de novo* variants reported.⁵ The variant T374I is located in the pore domain of K_v2.1 and was predicted to be deleterious by functional impact algorithms (Fig. 1; Table 4).

Given the locus-specific mutation rate of *KCNB1* (5.65×10^{-9} mutation rate/base/generation), the probability of identifying three independent mutations is low ($P < 1.1 \times 10^{-13}$), providing statistical evidence that these variants may be pathogenic. The altered residues show a very high degree of evolutionary conservation (Fig. 1a), with T374 and G379 being invariant through the ancestral KcsA bacterial potassium channel. Further, all three *KCNB1* variants are located in the functionally important pore domain of the K_v2.1 channel protein. Serine 347 is located in the pre-pore transmembrane segment and threonine 374 is located in the pore helix. Glycine 379 is part of the critical “GYG” motif that defines the potassium selectivity filter (Fig. 1a-b).

Effects of the *KCNB1* variants on K_v2.1 channel function were evaluated following transient expression in CHO-K1 cells. Expression of each mutant in CHO-K1 cells resulted in total and cell surface expression similar to the wildtype channel, with no significant genotype-dependent differences in total ($F_{3,8}=1.767$, $p=0.213$), surface ($F_{3,8}=0.017$, $p=0.997$) and surface:total ($F_{3,8}=0.266$, $p=0.848$) expression of K_v2.1. This indicates that the mutations do not interfere with protein expression or trafficking to the cell surface (Fig. 1c). Expression of K_v2.1-WT resulted in large voltage-dependent potassium currents with characteristic outward rectification and late inactivation (Fig. 2b-c). In contrast, expression of each of the three mutants yielded small currents with linear current-voltage relationships (Fig. 2b-c). These aberrant currents were blocked by gadolinium (Gd³⁺), strongly suggesting that the currents are pore-mediated (Fig. 3). Based upon the external and internal K⁺ concentrations used in these experiments, the theoretical reversal potential (E_{rev}) for K⁺-selective currents is −47 mV. Expression of the mutant channels produced currents with depolarized E_{rev} (S347R: -23.2 ± 4.8 mV; G379R -14.0 ± 4.5 mV; T374I -16.5 ± 5.5 mV) indicating that the mutations affect ion selectivity. To test ion selectivity, the external solution was diluted 1:10 with 300 mM sucrose. Under these conditions, a depolarizing shift in E_{rev} would indicate anion selectivity, while a hyperpolarizing shift would indicate cation selectivity. Dilution of the extracellular solution produced a hyperpolarizing shift in E_{rev} confirming the current conducted was cation-selective (Fig. 4). Changes in cation selectivity

were determined by measuring changes in E_{rev} following molar replacement of extracellular sodium with monovalent cations. All three mutants exhibited loss of K^+ selectivity, with K^+/Na^+ permeability ratios of 0.9 (Fig. 4) compared to the reported 14:1 ratio for Kv2.1-WT.²⁶

To investigate the effects of the mutant channels in a heterozygous background, we co-expressed each mutant with Kv2.1-WT channel and compared to the wildtype channel expressed alone. Co-expression of Kv2.1-WT with T374I, S347R, or G379R resulted in reduced current measured at test potentials ranging from 0 to +60 mV (Fig. 2c), depolarizing shifts in the voltage-dependence of steady-state activation (Fig. 2d; Table 5; Fig. 5), and greater time constants of activation (τ) measured from +30 to +60 mV test potentials (Fig. 2e; Fig. 5). The observed changes in kinetic parameters suggest that the mutant and wildtype subunits can form heterotetrameric channels.

DISCUSSION

Co-occurrence of *de novo* variants in *KCNB1* in three independent patients with overlapping clinical phenotypes that include epileptic encephalopathy with associated cognitive and motor dysfunction provides strong genetic evidence that the *KCNB1* variants are likely pathogenic. Further evidence for a pathogenic effect of the *KCNB1* mutations comes from functional studies of mutant Kv2.1 channels. All three mutations, located within the pore domain of Kv2.1, resulted in channels with similar dysfunctional features.

Previous studies demonstrated that mutations in the pore region can result in altered ion selectivity.²⁷⁻³⁰ Consistent with this, each Kv2.1 mutant exhibited voltage-independent, nonselective cation currents. When co-expressed with wildtype channels, all Kv2.1 mutants induced depolarizing shifts in the voltage-dependence of activation and reduced current density at more depolarized voltages. Further, co-expression of the Kv2.1 mutants with wildtype channels resulted in inward currents in the voltage range where Kv2.1 channels are normally closed, as evidenced by large inward currents observed when using a holding potential of -80 mV. These gain-of-function and dominant-negative functional defects are predicted to result in depolarized resting membrane potential and impaired membrane repolarization, with increased cellular excitability as a net consequence.

Kv2.1 is the main contributor to delayed rectifier potassium current in pyramidal neurons of the hippocampus and cortex.³¹⁻³⁵ Delayed rectifier potassium current is critical for membrane repolarization under conditions of repetitive stimulation and acts to dampen high frequency firing. Reduction of delayed rectifier potassium current by *Kcnb1* deletion in mice results in reduced thresholds to induced seizures, but not spontaneous seizures.³⁶ This suggests that loss of Kv2.1 function predisposes neuronal networks to hyperactivity, resulting in a modest increase in seizure risk. In contrast, our results demonstrate that gain-of-function and dominant-negative effects result in epileptic encephalopathy. A similar phenomenon is observed with *KCNQ2* wherein heterozygous loss-of-function mutations result in benign familial neonatal seizures, while mutations with dominant-negative effects result in epileptic encephalopathy.³⁷ This suggests that variable functional defects resulting

from different mutations in the same gene contribute to the pleiotropic effects observed for genes associated with neurodevelopmental disorders.

In summary, our genetic and functional evidence identify mutation of *KCNB1* as a cause of epileptic encephalopathy. This expands the considerable locus heterogeneity associated with epileptic encephalopathies⁵⁻⁶, suggesting that clinical exome sequencing may be useful for molecular diagnosis. Rapid genetic diagnosis is beneficial for appropriate disease management and may improve long-term outcomes in epileptic encephalopathies.²⁻⁴

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

K_V2.1 mutations identified in three individuals with epileptic encephalopathy. (a)

Evolutionary conservation of K_V2.1. Multiple alignment of K_V2.1 species orthologs (Clustal

Omega³⁸). Mutated amino acids are shaded and functional sub-domains of the pore region

are indicated. (b) Location of mutations mapped onto the crystal structure of a K_V2.1/K_V1.2 chimera (PDB 29R9).³⁹ The left panel shows a channel tetramer from the extracellular side.

S347 (green) lies at the interface between the voltage sensor (blue) and pore (white)

domains. T374 (teal) lies adjacent to the selectivity filter, while G379 (red) lies in the

selectivity filter. (c) Mutant K_V2.1 proteins are expressed and trafficked to the cell surface.

Cell surface expression was measured using cell surface biotinylation of CHO-K1 cells

transfected with wildtype or mutant K_V2.1. Total and surface fractions of K_V2.1 were

detected with anti-K_V2.1 antibody. Endogenous transferrin receptor levels were measured as

a loading control. The blot was probed first with anti-transferrin receptor, stripped, and then

re-probed with anti-K_V2.1.

Figure 2.

Functional consequence of Kv2.1 mutations. (a) CHO-K1 cells were held at -30mV and whole cell currents were recorded from -80 to +60 mV in 10 mV steps for 500 msec followed by a 500ms step to 0mV to record tail currents. (b) Average whole-cell current traces recorded from non-transfected CHO-K1 cells and CHO-K1 cells transiently expressing wildtype or mutant (G379R, S347R, T374I) Kv2.1 channels, or co-expressing wildtype plus mutant channels. (c) Current density-voltage relationships measured from CHO-K1 cells expressing mutant, wildtype, or co-expressing wildtype and mutant Kv2.1

channels. Currents were normalized to cell capacitance (pF). **(d)** Voltage dependence of steady-state activation. Tail currents were normalized to peak amplitude and fit with Boltzmann function. Biophysical parameters of voltage-dependence are detailed in Table 5. **(e)** The time constant of activation was determined from exponential fit of individual current traces.

a

Figure 3.

GdCl₃ block of mutant Kv2.1 channels. (a) Representative traces of control and Gd³⁺ block at +60 mV of wildtype ($86 \pm 2.1\%$, $n=5$) or mutant channels (S347R ($94 \pm 3.7\%$, $n=3$), G379R ($79 \pm 4.2\%$, $n=4$), T374I ($94 \pm 2.5\%$, $n=3$)).

Figure 4.

Ion selectivity of mutant $K_v2.1$ channels. **(a)** Reversal potentials were determined by linear fit to $y=mx+b$ in control bath and after bath was diluted 10-fold in 300mM sucrose solution. **(b)** Change in reversal potential after equimolar substitution of extracellular monovalent Na^+ .

Figure 5.

Expanded view of whole-cell current traces for evaluation of activation kinetics of wildtype K_V2.1 channel alone or co-expressed with mutant channels. Expanded view of the first 50 msec of whole-cell currents following voltage change from -80mV to $+60\text{mV}$ and normalized to peak current recorded from CHO-K1 cells transiently expressing K_V2.1-WT (a) or co-expressing wildtype and mutant K_V2.1 channels ((b) G379R, (c) S347R, (d) T374I).

Table 1

Whole exome and whole genome sequencing coverage

Sample	Whole Exome Sequencing		Whole Genome Sequencing	
	Coverage (Mean)	% Target Exome with at least 10 Reads	Coverage (Mean)	% Genome Redundancy of 5 Reads
ID9 – Proband	109	94.5%	4.1	37.9%
ID9F – Father	109	94.6%	6.0	66.7%
ID9M – Mother	124	94.9%	6.9	75.2%
ID9M – Sister	97	94.2%	7.2	77.4%

Table 2

WES Variant Filtration for individual ID9

Variant Type	# in Family
Total Variants	101,797
Rare Variants (<1% allele frequency)	18,475
Not in Segmental Duplication	13,463
Nonsynonymous, Truncating, or Splice Site	1,053
<i>De Novo</i> Inheritance	2
Homozygous or Compound Heterozygous Inheritance	3

Table 3

Inheritance of validated candidate variants in individual ID9

Gene	ID1 Status	Amino Acid Change	WES Genotype				Predicted Consequence		
			ID9	ID9F	ID9M	ID9S	Provean	SIFT	Polyphen 2
<i>KCNBI</i>	<i>de-novo</i>	S347R	01	00	00	00	deleterious (-4.996)	damaging (0.003)	probably damaging (0.995)
<i>MLST8</i>	<i>de-novo</i>	Q302R	01	00	00	00	neutral (-2.12)	tolerated (0.203)	benign (0.114)
<i>HRNBP3</i>	homozygous	Y5H	11	01	01	00	deleterious (-3.54)	damaging (0.000)	benign (0.446)
<i>NLRP1</i>	compound heterozygous	V939M	01	00	01	01	neutral (-2.08)	damaging (0.006)	probably damaging (0.982)
		R834C	10	01	00	00	neutral (-0.46)	tolerated (0.216)	benign (0.000)
<i>BAHCCI</i>	compound heterozygous	A941T	01	00	01	01	neutral (-1.38)	tolerated (0.215)	benign (0.009)
		E1990K	10	01	00	00	neutral (-0.91)	tolerated (0.168)	possibly damaging (0.900)

Genotypes: 0 - reference allele, 1 - alternate allele

Table 4
Clinical features in three individuals with epileptic encephalopathy and KCNB1 missense mutations.

Individual	Current Age (sex)	Age of onset	Seizure types	Seizure control with anti-epileptic therapies? (treatments)	Developmental Delay	Brain MRI	EEG	Other	Family history of epilepsy	Presumed causal variant	Amino Acid Change	Predicted Consequence Proven (score) SIFT (score) Polyphen2 (score)
ID9	9 yo (female)	4 yrs	Tonic-clonic; Tonic; Atonic; Focal; Focal with secondary generalization	No (Topiramate, clobazam, levetiracetam, Depakote, carbamazepine, trileptal, lacosamide, clonazepam, oxcarbazepine)	Yes (motor and language)	Subtle volume loss in left hippocampus	Mild diffuse slowing and abundant bi-hemispheric multifocal epileptiform discharges	Hypotonia; Strabismus; Migraine;	No	Chr20: 47991056 G/T	S347R	Deleterious (-4.996) Damaging (0.003) Probably Damaging (0.995)
2	7 yo (male)	8 months	Tonic-clonic; Atonic; Focal; Infantile spasms	No (ACTH, Topiramate, Depakote, pyridoxine; ketogenic diet)	Yes (motor and language)	normal	Hypsarhythmia; diffuse polyspikes, diffuse polyspike-wave, right temporal spike and wave, left occipital spikes and series of bursts of diffuse polyspikes	Hypotonia; Strabismus; Tremulousness; Non-verbal; Stereotyped handwringing movements; In-turning of feet	No	Chr20: 47990962 C/T	G379R	Deleterious (-7.926) Damaging (0.000) Probably Damaging (1.000)
3 (Coriell ND27062)	5 yo (female)	0 years	Tonic-clonic; Atonic; Focal dyscognitive Atypical absence; Infantile spasms;	unknown	Yes (unspecified)	normal	unspecified	Cerebral palsy	Yes (Absence epilepsy in great uncle)	Chr20: 47990976 G/A	T374I	Deleterious (-5.945) Damaging (0.000) Probably Damaging (0.999)

Table 5

Voltage-dependence of activation for wildtype and mutant Kv2.1 channels.

	Mean $V_{1/2} \pm \text{S.E.M.}$	Slope factor $\pm \text{S.E.M.}$	n
Kv2.1-WT	-4.1 ± 1.5	4.4 ± 0.4	12
Kv2.1-WT + G379R	$1.3 \pm 2.0^*$	5.2 ± 0.6	8
Kv2.1-WT + S347R	$2.8 \pm 1.6^*$	5.9 ± 0.5	8
Kv2.1-WT + T374I	$1.9 \pm 1.5^*$	5.7 ± 0.2	7

*
 $P < 0.05$