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De Novo Mutations in *SLC1A2* and *CACNA1A* Are Important Causes of Epileptic Encephalopathies

Epi4K Consortium*

Epileptic encephalopathies (EEs) are the most clinically important group of severe early-onset epilepsies. Next-generation sequencing has highlighted the crucial contribution of de novo mutations to the genetic architecture of EEs as well as to their underlying genetic heterogeneity. Our previous whole-exome sequencing study of 264 parent-child trios revealed more than 290 candidate genes in which only a single individual had a de novo variant. We sought to identify additional pathogenic variants in a subset ($n = 27$) of these genes via targeted sequencing in an unsolved cohort of 531 individuals with a diverse range of EEs. We report 17 individuals with pathogenic variants in seven of the 27 genes, defining a genetic etiology in 3.2% of this unsolved cohort. Our results provide definitive evidence that de novo mutations in *SLC1A2* and *CACNA1A* cause specific EEs and expand the compendium of clinically relevant genotypes for *GABRB3*. We also identified EEs caused by genetic variants in *ALG13*, *DNM1*, and *GNAO1* and report a mutation in *IQSEC2*. Notably, recurrent mutations accounted for 7/17 of the pathogenic variants identified. As a result of high-depth coverage, parental mosaicism was identified in two out of 14 cases tested with mutant allelic fractions of 5%–6% in the unaffected parents, carrying significant reproductive counseling implications. These results confirm that dysregulation in diverse cellular neuronal pathways causes EEs, and they will inform the diagnosis and management of individuals with these devastating disorders.

Introduction

Epileptic encephalopathies (EEs) frequently begin in infancy or childhood and often carry a poor prognosis. They are typically characterized by multiple seizure types and abundant epileptiform activity and are associated with developmental delay or regression. Genetic studies in recent years have established that de novo mutations are responsible for many EE phenotypes. These studies have also highlighted the marked genetic heterogeneity of the EEs, emphasizing the value of sequencing large cohorts to identify multiple individuals with mutations in the same gene to establish a specific gene's role in disease causation. Identification of multiple individuals harboring mutations in a given gene allows us to define the phenotypic spectrum of that disease and enables recognition of co-morbidities, prognosis, and therapeutic response.

Our initial whole-exome sequencing (WES) study of 264 parent-child trios focused on the specific EEs of infantile spasms (IS) and Lennox-Gastaut syndrome and revealed 329 de novo variants in 305 genes.¹ Although 19 individuals had de novo mutations in previously reported epilepsy-associated genes, we also identified a statistically significant enrichment of mutations in *GABRB3* (MIM: 137192) and *ALG13* (MIM: 300776), establishing their role in the etiology of EEs.¹ Similarly, *DNM1* (MIM: 602377) was confirmed as an EE-associated gene in our subsequent study.² For more than 290 genes, a de novo variant was identified in only a single individual, and there was insufficient evidence to establish association with disease causation. Identifying a significant enrichment of cases with de novo variants in an individual gene is

required for that gene's role in EEs to be established; ideally, such identification would be combined with functional validation to support pathogenicity.

In this study, we employ massively parallel targeted sequencing of 27 candidate and recently described EE-associated genes in a large cohort of 531 individuals with a range of EEs. We hypothesized that if the genes in which a de novo variant in the original study was identified were causative, then we might identify additional de novo variants in a large cohort of individuals with EE. The identification of an enrichment of cases with the same genetic etiology would confirm the role of a specific gene in causing EE. We have identified individuals with a de novo mutation in seven of the 27 genes; several mutations were recurrent. In particular, we define an entity of *SLC1A2* encephalopathy and highlight its importance as a glutamatergic gene associated with EE. We also emphasize the emerging importance and diagnostic value of *CACNA1A* (MIM: 601011) and *GABRB3* as recurring genes associated with EEs.

Methods

Gene Selection for Targeted Sequencing

From the original Epi4K cohort,¹ we selected ~10% of the 290 genes in which at least a single de novo variant had been identified, and we used these genes for targeted sequencing. Selection was based on expert panel review and the following criteria: a gene in which more than one proband in the original study had a de novo variant (*ALG13*, *GABRB3*, *DNM1*, *HDAC4*); a gene recently described as associated with EE (*GNAO1*);³ gene or a closely related gene family member in which mutations cause epilepsy or related disease (*CACNA1A*, *GABRB1*, *GRIN1*,

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Table 1. Epilepsy Syndromes in Cohort of Individuals with Unsolved EEs

Diagnosis	Number
Epileptic encephalopathy unclassified	205
Infantile spasms	90
Epilepsy with myoclonic-atonic seizures	80
Lennox-Gastaut syndrome	41
Early-onset epileptic encephalopathy (seizure onset \leq 3 months)	33
Epilepsy aphasia syndrome	20
Dravet syndrome (<i>SCN1A</i> negative)	15
Febrile infection-related epilepsy syndrome	14
Epileptic encephalopathy with continuous diffuse spike waves during slow-wave sleep	13
Epilepsy of infancy with migrating focal seizures	8
Atypical benign partial epilepsy of childhood	7
Early myoclonic encephalopathy	4
Early infantile epileptic encephalopathy (Ohtahara syndrome)	1
Total individuals	531

MTOR, *PIK3AP1*, *SLC1A2*, *IQSEC2*, *SLC35A2*); a gene predicted to have protein-protein interaction with a known epilepsy-associated gene (*HNRNPH1*, *NEDD4L*); genomic location is in an epilepsy-related CNV (*YWHA*G); or intolerance to functional variation on the basis of a residual variation intolerance score (RVIS)⁴ < 25% and biological plausibility based on predicted protein function (*DNAJC6*, *EMILIN3*, *TRIM32*, *RALGAPB*, *VSP37A*, *EPHB1*, *NFASC*, *PLXNA1*, *SMURF1*, *THOC2*, *TRIM32*, and *ZFH3*).

Cohort

The cohort comprised 531 individuals with EEs of unknown cause. Most of these individuals had been tested for mutations of known and novel candidate genes as well as pathogenic copy-number variants,^{5–8} so the cohort represented a highly selected group of individuals without mutations in known epilepsy genes. Detailed phenotypic characterization was performed with the help of a validated seizure questionnaire,⁹ together with review of medical records and EEG, imaging, and investigation results. Each individual underwent phenotypic analysis and was classified into a specific epilepsy syndrome when possible.¹⁰

All individuals or, in the case of minors or those with intellectual disability, their parents or legal guardians gave consent for participation in the study. The study was approved by the Human Research Ethics Committee of Austin Health, Melbourne, Australia and by the Institutional Review Board at the University of Washington.

Design of the Molecular Inversion Probe and Library Preparation

Molecular inversion probes (MIPs) were designed as previously described⁵ to capture the coding exons plus a minimum of five base pairs of flanking sequence of intronic/exonic boundaries in each of the 27 genes. Hybridization and amplification were performed as previously described.^{5,6,8}

Variant Calling

We sequenced libraries on an Illumina HiSeq according to the manufacturer's instructions to generate 100 base pair paired-end reads. Raw reads were mapped to the human genome (GRCh37/hg19) with the Burrows-Wheeler Aligner (BWA v0.7.8), and variant calling was performed with the Genome Analysis Toolkit (GATK v3.1.1). Duplicate reads were marked with the Picard toolkit (v.1.113). SeattleSeq (v137) was used for annotating variants. Only variants that were predicted to affect protein sequence (missense, nonsense, frameshift, or splice variants) were assayed further. CADD, PolyPhen, and GERP scores were evaluated, but no variants were excluded on the basis of in silico predictions. Either a second independent MIP capture or Sanger sequencing was used for validating and testing whether a variant was de novo or inherited. A read-depth of 50 \times was required for accurate variant calling.

Criteria for Pathogenicity

Variants in recently identified EE-associated genes were considered pathogenic if they arose de novo (or from a somatic mosaic parent) and had never been observed in the publically available control datasets (ExAC v0.3, ESP6500, 1000 Genomes). For variants in genes that have not previously been associated with EE, we assessed the number of de novo changes observed versus the number predicted to determine whether it reached statistical significance on a per-gene basis.¹¹

In cases where the inheritance was not known because parental DNA was unavailable for an established EE-associated gene, the significance of a variant was based either on whether it was a recurrent mutation or on convergent information provided by the in silico tools CADD, PolyPhen-2, and GERP. The in silico tools had to be in agreement (CADD > 25, PolyPhen-2 > 0.9, and GERP > 5) in order for a variant to be classified as "likely pathogenic." We took a more conservative approach to novel variants with limited inheritance information for genes not yet established as causative and classified these as variants of unknown significance regardless of in silico predictions. We used microsatellite markers to test the relatedness within families in which the proband had a "de novo" mutation and to rule out, when possible, sample duplication in the case of recurrent mutations ($n = 5/7$ recurrent mutations tested). For one inherited GABRB3 variant (RefSeq accession number NM_000814.4: c.470C>T [p.Thr157Met]), review of the pedigree showed that the variant segregated in a family with genetic epilepsy and febrile seizures plus (GEFS+).

Statistical Analysis

Mutation enrichment was assayed in the EE-associated genes *SLC1A2* and *CACNA1A* with denovolyzer.¹¹ In brief, using weighted per-gene mutation rates, we calculated the number of expected de novo mutations in our cohort of 531 individuals.¹¹ We used denovolyzer to calculate the probability of observing more de novo missense mutations than would be expected by chance and corrected for the 27 genes we examined.

Results

We sequenced 27 candidate or newly described EE-associated genes in a cohort of 531 individuals with unsolved EE representing a wide range of epilepsy syndromes (Table 1). On average, 88% of the target (27 genes) was

Table 2. Pathogenic and Likely Pathogenic Variants in EE-Associated Genes Identified in This Study

Gene	Proband (Sex)	Inheritance	Inferred Effect	GRCh37/hg19 Genomic Coordinate	cDNA Change	Protein Change	CADD	Diagnosis
SLC1A2	EG1291 (F)	de novo	pathogenic ^a	chr11:g.35336636	NM_004171.3: c.244G>C	p.Gly82Arg	26.0	EOEE
	T23159 (F)	de novo	pathogenic	chr11:g.35336626	NM_004171.3: c.254T>C	p.Leu85Pro	22.3	EME
GABRB3	EG0254 (M)	de novo	pathogenic ^a	chr15:g.26866564	NM_000814.4: c.358G>A	p.Asp120Asn	36.0	MAE
	EG0258 (M)	segregates with GEFS+ family	pathogenic	chr15:g.26828553	NM_000814.4: c.470C>T	p.Thr157Met	24.4	DS-like
	T22598 (F)	de novo	pathogenic ^b	chr15:g.26825603	NM_000814.4: c.545A>T	p.Tyr182Phe	32.0	EE
	T25111 (F)	de novo	pathogenic	chr15:g.26812818	NM_000814.4: c.745C>A	p.Gln249Lys	29.7	EE
	EG1542 (M)	de novo	pathogenic	chr15:g.26812796	NM_000814.4: c.767T>A	p.Leu256Gln	17.6	EOEE
	T23950 (F)	unknown	likely pathogenic	chr15:g.26806281	NM_000814.4: c.878T>A	p.Leu293His	25.1	EOEE
T25708 (M)	de novo	pathogenic	chr15:g.26806246	NM_000814.4: c.913G>A	p.Ala305Thr	35.0	LGS	
CACNA1A	EG1371 (M)	de novo	pathogenic	chr19:g.13566019	NM_023035.2: c.301G>C	p.Glu101Gln	19.1	EIMFS
	T21924 (F)	unknown, father unavailable	VOUS ^c	chr19:g.13476262	NM_023035.2: c.653C>T	p.Ser218Leu	16.6	EOEE
	T23039 (F)	de novo	pathogenic ^a	chr19:g.13414398	NM_023035.2: c.2137G>A	p.Ala713Thr	22.1	EOEE
	T24139 (M)	mosaic mother	pathogenic ^a	chr19:g.13414398	NM_023035.2: c.2137G>A	p.Ala713Thr	22.1	EOEE
	EG1519 (F)	de novo	pathogenic	chr19:g.13368235	NM_023035.2: c.4531G>T	p. Ala1511Ser	20.2	EOEE
DNM1	T24107 (F)	mosaic parent	pathogenic ^a	chr9:g.130982480	NM_004408.2: c.709C>T	p.Arg237Trp	19.9	EE
GNAO1	T25023 (M)	de novo	pathogenic ^a	chr16:g.56385408	NM_020988.2: c.836T>A	p.Ile279Asn	28.6	EOEE
IQSEC2	T17563 (F)	de novo	pathogenic	chrX:g.53279555	NM_001111125.1: c.2203C>T	p.Gln735*	39.0	SGE
ALG13	T22647 (F)	unknown, father unavailable	pathogenic ^a	chrX:g.110928268	NM_001099922.2: c.320A>G	p.Asn107Ser	21.0	EOEE

Abbreviations are as follows: DS, Dravet syndrome; EE, epileptic encephalopathy; EIEE, early infantile epileptic encephalopathy; EIMFS, epilepsy of infancy with migrating focal seizures; EME, early myoclonic encephalopathy; EOEE, early-onset epileptic encephalopathy; GEFS+, Generalized epilepsy with febrile seizures plus; ID, intellectual disability; IS, infantile spasms; LGS, Lennox-Gastaut syndrome; MAE, epilepsy with myoclonic-atic seizures; SGE, symptomatic generalized epilepsy; VOUS, variant of unknown significance.

^aRecurrent mutation that has previously been reported as causing EE.

^bPreviously reported.¹²

^cClinical Variant (rs121908225) associated with Familial hemiplegic migraine type 1 with progressive cerebellar ataxia.

covered at 50× or higher; there was a per-gene range of 69.65% (*GRIN1*) to 99.04% (*GABRB1*) (Figure S1).

Pathogenic or likely pathogenic variants were discovered in seven of the 27 genes (*ALG13*, *CACNA1A*, *DNM1*, *GABRB3*, *GNAO1*, *IQSEC2*, and *SLC1A2*), accounting for 17/531 cases, or 3.2% of the entire cohort (Table 2). Mutations in *GABRB3* (n = 7) and *CACNA1A* (n = 4) accounted for the largest proportion of individuals. Recurrent mutations were identified in six genes (*ALG13*, *CACNA1A*, *DNM1*, *GABRB3*, *GNAO1*, and *SLC1A2*). An additional six variants of uncertain significance (VOUS) were detected (Table S1).

We identified two individuals with de novo mutations in *SLC1A2* (MIM: 600300), establishing this as an EE-associated gene (p = 0.0052). Previously, only a single individual with IS and a de novo missense mutation had been reported.¹ *SLC1A2* encodes Excitatory Amino Acid Trans-

porter 2 (EAAT2), which is one of the major glutamate transporters expressed in astroglia and is responsible for clearing glutamate from the extracellular space at the synapse.¹³ One individual from our study has the same missense mutation (NM_004171.3; c.244G>C [p.Gly82Arg]) as the original IS individual, and the other person harbors a novel c.254T>C mutation (p.Leu85Pro; NM_004171.3) (Figure S2). Microsatellite testing confirmed that the two people with the p.Gly82Arg variant were different individuals. These mutations cluster in the first extracellular domain of this transmembrane protein.

Our individuals with *SLC1A2* mutations presented with an extremely severe phenotype, characterized by seizure onset in the first week of life and profound developmental impairment, without detectable regression. The original IS individual had seizure onset in the neonatal period (Table 3). All 3 individuals had multiple seizure types

Table 3. Clinical Features of Individuals with SLC1A2 Mutations

Identifier	Age, Gender	Epilepsy Syndrome	Development prior to Seizure Onset	Age at Seizure Onset	Seizure Type at Onset	Development after Seizure Onset	Other Seizure Types	Age of Seizure Offset	EEG	Neuroimaging	Other Features	Medications ^a
Individuals Identified in This Cohort												
T23159	17 yr, F	EME	never normal	2 days	myoclonic (virtually continuous multifocal twitching, particularly in sleep)	profound ID	tonic (generalized and focal); myoclonic SE; possible NCSE	ongoing	multifocal; slow background	3 months—normal; 1 yr—almost complete absence of myelination of cerebrum but normal myelination of cerebellum and brain stem, thin CC, T2 hyperintensity in lentiform nuclei 3 yr—cerebral atrophy, complete absence of myelination of subcortical and periventricular white matter, normal myelination of cerebellum, brain stem, thalami and basal ganglia, thin CC, thinning of cortical gray matter, no cerebellar atrophy; 13 yr—extreme supratentorial atrophy involving cortex, deep gray matter and white matter; sparing of posterior fossa; brainstem and cerebellum are of normal size and signal intensity	Severe asymmetric quadriparesis; gastrostomy; bilateral nephrocalcinosis; amenorrhoea; delayed dentition; frequent bradycardia and hypothermia exacerbated by sleep and anesthesia; progressive microcephaly with bitemporal narrowing; severe growth failure (15 kg at 16 yr); hirsutism; severe kyphoscoliosis; joint contractures with tapering fingers	<u>CBZ</u> , <u>LTG</u> , <u>LCM</u> , pyridoxine, <u>CZP</u> , <u>AZD</u> , <u>VPA</u> , <u>PB</u> , <u>PHT</u> , prednisolone, <u>VGB</u> , <u>KD</u> , <u>TPM</u>
EG1291	6 yr, F	EOEE	never normal	5 days	epileptic spasms	profound ID	myoclonic; myoclonic SE; tonic; tonic-clonic; focal	ongoing	multifocal (no hypsarrhythmia)	6 months—delayed myelination; 3 yr—progress in myelination, frontal atrophy, thin CC	mild-moderate generalized hypotonia; father had two seizures as an adult, no further seizures while on LTG	<u>LEV</u> , <u>VPA</u> , pyridoxine, <u>VGB</u> , prednisolone, <u>ZNS</u> , <u>KD</u>
Individuals Identified in Epi4K Consortium et al., (2013)¹												
n	8 yr, F	IS	unknown	1 month	focal tonic; myoclonic	some regression; severe ID		unknown	hypsarrhythmia; disorganized background; bifrontal central frequent discharges	normal	blindness; bilateral hamstring contractures; torticollis; extensor plantar response on L and flexor on R; head lag; axial hypotonia; mild appendicular hypertonia	<u>VGB</u> , <u>OCBZ</u> , <u>TPM</u> , <u>PB</u> , <u>LEV</u>

Abbreviations are as follows: AZD, acetazolamide; CBZ, carbamazepine; CC, corpus callosum; CLB, clobazam; CLZ, clorazepate; CZP, clonazepam; EE, epileptic encephalopathy; EME, early myoclonic encephalopathy; EOEE, early onset epileptic encephalopathy; F, female; ID, intellectual disability; IS, infantile spasms; KD, ketogenic diet; L, left; LCM, lacosamide; LEV, levetiracetam; LTG, lamotrigine; NCSE, non-convulsive status epilepticus; NG, nasogastric; OCBZ, oxcarbazepine; PB, phenobarbitone; PHT, phenytoin; R, right; SE, status epilepticus; TPM, topiramate; VGB, vigabatrin; VPA, valproate; and ZNS, zonisamide.

^aMedications that were current at the last follow-up are underlined.

with prominent myoclonic and tonic seizures as well as spasms. One (T23159) had profound growth failure with microcephaly (at age 17 years: weight 15 kg, height and head circumference 49 cm), which was not observed in the remaining two cases.

We identified seven individuals with mutations in *GABRB3*, a gene previously reported to have de novo mutations in four individuals with Lennox-Gastaut syndrome (LGS) or infantile spasms (IS).¹ The seven newly identified individuals presented with somewhat heterogeneous phenotypes of LGS (1), epilepsy with myoclonic-atonic seizures (1), and unclassified epileptic encephalopathy (4). The seventh case had features reminiscent of Dravet syndrome but could be distinguished by the absence of typical Dravet features, including generalized spike wave activity and hemi-clonic and focal seizures, although these features are not universal in Dravet syndrome (Table 4). For this case, the variant segregated in a family with genetic epilepsy and febrile seizures plus (GEFS+). Ten of the eleven individuals who carried a *GABRB3* de novo missense mutation had seizure onset by 1 year of age; three of these had had normal development up until this point, six had had slow development, and details were unknown for one individual. The eleventh child had developmental delay and experienced seizure onset at 12 years (Table 4). Five had severe to profound intellectual disability, three had mild to moderate disability, and the degree of cognitive impairment was unclear in the remaining three. Predominant seizure types were myoclonic, tonic, absence, and generalized tonic-clonic seizures. One individual had epileptic spasms.

Five of the eleven pathogenic *GABRB3* variants clustered in the transmembrane domains at evolutionarily conserved sites (average GERP 5.54 ± 0.4, nucleotide resolution). Six pathogenic variants, including the recurrent mutation, are dispersed throughout the N-terminal extracellular domain at well-conserved residues (average GERP 5.38 ± 0.4, nucleotide resolution). One variant in *GABRB3* was classified as “likely pathogenic” because inheritance information was limited, but the amino acid change from a polar to a basic residue in the transmembrane domain (Figure S3) and a CADD score > 25 and PolyPhen-2 of 0.992 (probably damaging) predicted a “likely damaging” effect on protein function.

Pathogenic mutations in *CACNA1A* were identified in four probands and one affected sibling. Deep sequencing of the parents with two affected children revealed that the unaffected mother was a mosaic carrier with a 6.3% mutant allelic fraction in lymphocyte-derived DNA. Two probands and the affected sibling have a recurrent mutation (RefSeq NM_023035.2; c.2137G>A [p.Ala713Thr]) first identified in our original study.¹ This number of de novo missense mutations in our cohort represents a significant enrichment ($p = 8.0 \times 10^{-5}$), providing clear evidence that de novo mutations in *CACNA1A* cause epileptic encephalopathy.

Of the six infants with *CACNA1A* mutations, five, including the sibling pair, began having seizures on the

first day of life; the siblings additionally made in utero seizure-like movements described as “butterfly wings rapidly flapping.” Seizures were recognized by 2 hr of age in three of these five infants, and the remaining individual had seizure onset at 4 weeks of age (Table 5). Three individuals, including the sibling pair, initially had myoclonic seizures, and three presented with convulsive seizures with tonic or tonic-clonic features. Multiple seizure types developed in each case and included focal, tonic, and tonic-clonic seizures. Status epilepticus occurred in all, including at seizure onset in one case. Reflex myoclonic seizures triggered by touch and noise were observed in the siblings, as well as in our previously described individual with LGS.¹ EEG studies showed multifocal epileptiform activity in three individuals, generalized spike wave and polyspike wave in two individuals, and multifocal slowing in one. Development was delayed in all individuals; five had severe intellectual disability (ID), and one moderate ID. Cerebral palsy with variable features was observed in all (Table 5). Ophthalmic findings included strabismus (1) and nystagmus (2). Motor features included ataxia (3), tremor (2), and athetosis (1).

We also identified three affected individuals with a recurrent mutation in one of three genes: *DNM1*, *GNAO1*, or *ALG13* (Table S2). The proband with the *DNM1* mutation (NM_004408.2; c.709C>T [p.Arg237Trp]), which appeared to be a de novo mutation, had a similarly affected sibling who carried the same mutation. Deep sequencing of the unaffected parents confirmed somatic mosaicism in the father and revealed a 5.5% mutant allelic fraction in blood-derived DNA. The siblings had onset of seizures at 1 month and 4 months of age and displayed multiple seizure types, severe to profound developmental delay, and prominent movement-disorder features.

A recurrent mutation in *GNAO1* (NM_020988.2; c.836T>A [p.Ile279Asn]) was identified in a 2-year-old boy with early-onset epileptic encephalopathy. All 11 previously reported individuals with mutations in *GNAO1* have been female despite the fact that *GNAO1* is located on chromosome 16. In the boy described here, seizures might have begun in utero; unusual movements were noted from 32 weeks' gestation. Apnoeic episodes began at 26 min of age and averaged 20 episodes per day. At 9 days, focal clonic seizures of the face were noted. Unusual jerky movements, involving the eyes as well, began at 4 months and were followed by extensor spasms. Tonic seizures developed at 16 months. Development was never normal, and regression subsequently occurred in association with spasms and hypsarrhythmia, evolving to profound developmental impairment.

We also report on a female with a recurrent mutation (NM_001099922.2; c.320A>G [p.Asx107Ser]) in *ALG13*, which is located on the X chromosome. The *ALG13* p.Asx107Ser variant had previously arisen de novo in seven other reported females; the father of the female we report on here was not available.^{1,14–16} Microsatellite testing confirmed that the female we report on here was not related

Table 4. Clinical Features of Individuals with *GABRB3* Mutations

Identifier	Age, Gender	Epilepsy Syndrome	Development prior to Seizure Onset	Age at Seizure Onset	Seizure Type at Onset	Development after Seizure Onset
Individuals Identified in This Cohort						
T25111	19 yr, F	EE	delayed at 6 months	12 yr	tonic-clonic	severe ID
T22598	SUDEP 2 yr 11 month, F	EE	delayed	6 months	facial grimace and horizontal head shaking with retained awareness	delayed
T25708	14 yr, M	LGS	never normal; feeding via NG tube from 5 months	5 months	altered facial expression, choking, stiffening of arms, eye rolling	severe ID
T23950	7 yr, M	EOEE	never normal	1 month	myoclonic	profound ID
EG0258	9 yr, M	Dravet syndrome-like	normal	8 months	febrile; tonic-clonic	mild ID; delayed at 2.5 yr
EG1542	3 yr, M	EOEE	unknown	1 day	tonic-clonic	delayed at 3 months; severe ID
EG0254	12 yr, M	MAE	normal	1 yr	febrile; tonic-clonic	mild ID; delayed at 2.5 yr
Individuals Identified in Epi4K Consortium et al. (2013)¹						
dr	13 yr, F	IS	normal	5 months	IS	mild delay
gs	18 yr, F	LGS	delayed	10 months	focal impaired awareness	severe ID
jw	20 yr, M	LGS	mildly delayed	10 months	IS	mild ID
jr	11 yr, M	LGS	delayed	10 months	IS (some letters say FS; shortly after birth mother noticed jerking movements)	moderate ID

ACTH, adrenocorticotropic hormone; ADHD, attention deficit hyperactivity disorder; AZD, acetazolamide; CBZ, carbamazepine; CC, corpus callosum; CLB, clonazepam; CLZ, clonazepam; C-PAP, continuous positive airway pressure; CSF, cerebral spinal fluid; CT, computed tomography; CSE, convulsive status epilepticus; CZP, clonazepam; d, days; DZP, diazepam; EE, epileptic encephalopathy; EIEE, early infantile epileptic encephalopathy; EIMFS, epilepsy of infancy with migrating focal seizures; EME, early myoclonic encephalopathy; EOEE, early onset epileptic encephalopathy; ETX, ethosuxamide; F, female; FBM, felbamate; FIAS, focal impaired awareness seizure; FS, febrile seizures; GBP, gabapentin; GORD, gastro-esophageal reflux disease; GPFA, generalized paroxysmal fast activity; GSW, generalized spike wave; ID, intellectual disability; IS, infantile spasms; KD, ketogenic diet; kg, kilograms; L, left; LCM, lacosamide; LEV, levetiracetam; LTG, lamotrigine; LGS, Lennox-Gastaut Syndrome; M, male; MAD, modified Atkins diet; MDZ, midazolam; NCSE, non-convulsive status epilepticus; NG, nasogastric; NICU, neonatal intensive care unit; NZP, nitrazepam; OCBZ, oxcarbazepine; PB, phenobarbitone; PFA, paroxysmal fast activity; PHT, phenytoin; PRM, primidone; PSW, polyspike wave; PEG, percutaneous endoscopic gastrostomy; R, right; RFM, rufinamide; sec, seconds; SE, status epilepticus; SGE, symptomatic generalized epilepsy; SPECT, single-photon emission computed tomography; STP, stiripentol; SSW, slow spike wave; SUDEP, sudden unexpected death in epilepsy; TPM, topiramate; VGB, vigabatrin; VNS, vagus nerve stimulation; VPA, valproate; ZNS, zonisamide.

^aMedications that were current at the last follow-up are underlined.

to the two individuals we reported previously.¹ Our subject showed minimal responsiveness prior to onset of tonic seizures at 1–2 months, and spasms developed at 4 months. She had profound impairment, hypotonia, and choreoathetoid movements. Seizures were refractory; vigabatrin increased spasms and exacerbated her movement disorder. There are limited data on other individuals with *ALG13* mutations, but common features include infantile spasms

(7/7), severe to profound intellectual disability (6/7), visual impairment (2/7), and hypotonia (2/7).

Finally, we identified a 42-year-old woman with a de novo nonsense mutation in *IQSEC2* (NM_001111125.1; c.2203C>T [p.Gln735*]), a gene associated with X-linked intellectual disability.¹⁷ She had preceding mild developmental delay when she presented at 5 years with nonconvulsive status epilepticus with absence seizures,

Other Seizure Types	Seizure Offset	EEG
absence; atonic; tonic-clonic; CSE; focal impaired awareness evolving to bilateral convulsion; tonic	ongoing	generalized; atypical fast background; right and bisynchronous epileptiform activity; PFA; multifocal
tonic-clonic; focal with retained awareness; focal SE; atonic; atypical absence; tonic	ongoing until death	multifocal; slow background
tonic-clonic; atypical absence; tonic; myoclonic; episodes of kicking or thrashing of limbs and body; bouts of laughter lasting <3 min	ongoing	almost continuous GSW; slow background; multifocal; SSW; GPFA
tonic; focal motor; clonic	unknown (lost contact)	multifocal sharp waves
tonic-clonic; absence; myoclonic; atonic	ongoing	frontal, temporal, and central sharp waves; frontal and temporal R sharp waves
spasms; focal motor; tonic	ongoing	hypsarrhythmia; polymorphous delta intermixed with polyspikes; diffuse background slowing
myoclonic-atonic; atonic; myoclonic; absence	1.5 yr	2.5–3 Hz GSW; diffuse slowing
myoclonic; focal impaired awareness	unknown	hypsarrhythmia evolved from multifocal discharges; background slowing
tonic-clonic; CSE	unknown	generalized slowing; GSW; bilateral synchronous L > R sharp waves or spikes; L temporal or generalized onset to seizures; L temporal paroxysmal features; bilateral independent hemispheric seizures; bilateral independent sharp waves or spike in temporal and occipital regions
tonic-clonic; atypical absence; myoclonic; atonic	unknown	2 Hz sharp-spike wave; bilateral occipital slow wave; asymmetric photic driving; poorly organized background; multifocal
FS; tonic-clonic; tonic; atypical absence; myoclonic; atonic	unknown	generalized 2 Hz discharges; irregular spikes with myoclonic jerks and accounting for 40%–50% of sleep recording; slow background

(Table continued on next page)

tonic-clonic seizures, and drop attacks, associated with regression. She later developed myoclonic seizures.

Discussion

Our study of 531 individuals with unsolved EEs highlights the value of large-scale studies to secure the role of novel

disease-associated genes, given the marked genetic heterogeneity underlying the EEs.¹⁸ We performed targeted sequencing of 27 genes drawn from more than 290 genes identified as having a de novo variant in a cohort of 264 individuals with two specific EEs: infantile spasms and Lennox-Gastaut syndrome. We identified 17 (3.2%) individuals with a causative mutation. By finding additional individuals with de novo, and sometimes recurrent,

Table 4. Continued

Neuroimaging	Other Features	Medications^a
normal	pain insensitivity; transient balance issues; ataxia; dyspraxia; behavioral issues; aggression; autism; gum hypertrophy (PHT use)	<u>LTG</u> , <u>VPA</u> , <u>ZNS</u> , <u>PHT</u> , CLB, CZP, CBZ, LCM, LEV, TPM
incomplete myelination; plagiocephaly	autistic features	PB, CBZ, MAD
8 months—mild prominence of ventricles and subarachnoid spaces; 2 yr—atrophy and hypomyelination of central white matter and ventricles; 6 yr—delayed but progressing myelination, empty sella with flattened pituitary gland; 11 yr— normal	PEG feeding; microcephaly; scoliosis; cerebral palsy; visual and hearing impairment; sleep disturbance	<u>LTG</u> , <u>LEV</u> , <u>VPA</u> , PB
cortical atrophy; thin CC; increased T2 in globus pallidus	congenital unilateral ptosis; constipation; hypotonia; drooling; dysmorphic features	VGB, PB, TPM
normal	severe ADHD; fever sensitive seizures	LTG, LEV, STP
hypomyelination	diffuse hypotonia; dyskinesia	PB, VGB, hydrocortisone, VPA, LEV, CLB, KD
normal	stereotyped behavior; learning disability	VPA, LTG, CLB, ETX, TPM (seizure free on LEV + VPA + TPM)
normal	recurrent infections; epiphora	TPM, ACTH
normal	hypertonia in legs, especially ankles	PB, CBZ, VPA, PHT, CZP, TPM, FBM
normal	ADHD; mood lability; impulsivity; distractibility; hyperactive behavior; acting out; aggression; psychosis; drooling; clumsiness; mild dysmorphism	VPA, TPM, LTG, CBZ, LEV, FBM, VNS
small acute infarct in splenium of CC	sleeping difficulties; ADHD; impulsive behavior; echopraxia; distractibility; hypotonia but normal strength; dyspraxia; dysarthria; dyspraxic fine and gross motor skills	Prednisolone, ZNS, LTG, AZD, CZP, LEV, VPA

mutations, we strengthen the case for pathogenicity of that gene. Our results provide evidence for *SLC1A2* mutations as a cause of EE, and we refine the phenotypes of EEs associated with mutations in *GABRB3* (7/531, 1.3%) and *CACNA1A* (4/531, 0.8%), which accounted for the largest proportion of individuals with mutations identified in our cohort. As larger cohorts of individuals with specific genetic EEs are assembled, the efficacy (or exacerbation) of

specific anti-epileptic therapies can be established. Moreover, understanding the functional consequences of specific mutations will pave the way to precision medicine.

We report conclusive evidence that mutations in *SLC1A2* cause an EE; the phenotype is extremely severe and involves seizure onset in the first week of life; multiple seizure types, including tonic and myoclonic seizures; and profound impairment. *SLC1A2* encodes one of the major

Table 5. Clinical Features of Individuals with CACNATA Mutations

Identifier	Age, Gender	Epilepsy Syndrome	Development prior to Seizure Onset	Age at Seizure Onset	Seizure Type at Onset	Development after Seizure Onset	Other Seizure Types
Individuals identified in this cohort							
T24139 (brother of T24629)	5 yr, M	EOEE; initially diagnosed with hyperekplexia	C-PAP ventilation for 4 hr after birth	at birth (abnormal movements in utero)	jittery movements at birth; myoclonic seizures in NICU	regression at 2 yr; severe ID	tonic-clonic; focal tonic; focal impaired awareness; CSE; ongoing myoclonic seizures (spontaneous and reflex to noise and touch)
T24629 (sister of T24139)	15 yr, F	EOEE, initially diagnosed with hyperekplexia	delayed	5 hr (abnormal movements in utero)	focal myoclonic	severe ID	tonic-clonic; focal; CSE; tonic
T23039	7 yr, F	EOEE	Normal until 4mth	1–2 hr	tonic-clonic	severe ID	myoclonic; focal CSE; focal impaired awareness
EG1371	4 yr, M	EIMFS	delayed	4 weeks	tonic with focal features	severe ID	asymmetric tonic; focal motor; focal NCSE
EG1519	12 yr, F	EOEE	delayed	1 day	CSE	moderate ID	2 additional CSE at 14 months and 24 months without intervening seizures Tonic with R hemifacial clonic and L eye deviation
Individuals Identified in Epi4K Consortium et al. (2013)¹							
EPGP011141	19 yr, F	EOEE → LGS	unremarkable	1 hr	myoclonic	severe ID; regression	tachypnoeic episode; tonic-clonic; focal impaired awareness; CSE; drop attacks; myoclonic (reflex to loud noise); tonic

ACTH, adrenocorticotropic hormone; ADHD, attention deficit hyperactivity disorder; AZD, acetazolamide; CBZ, carbamazepine; CC, corpus callosum; CLB, clonazepam; CLZ, clonazepam; C-PAP, continuous positive airway pressure; CSF, cerebral spinal fluid; CT, computed tomography; CSE, convulsive status epilepticus; CZP, clonazepam; d, days; DZP, diazepam; EE, epileptic encephalopathy; EIEE, early infantile epileptic encephalopathy; EIMFS, epilepsy of infancy with migrating focal seizures; EME, early myoclonic encephalopathy; EOEE, early onset epileptic encephalopathy; ETX, ethosuxamide; F, female; FBM, felbamate; FIAS, focal impaired awareness seizure; FS, febrile seizures; GBP, gabapentin; GORD, gastro-esophageal reflux disease; GPFA, generalized paroxysmal fast activity; GSW, generalized spike wave; ID, intellectual disability; IS, infantile spasms; KD, ketogenic diet; kg, kilograms; L, left; LCM, lacosamide; LEV, levetiracetam; LTG, lamotrigine; LGS, Lennox-Gastaut syndrome; M, male; MAD, modified Atkins diet; MDZ, midazolam; mth, months; NCSE, non-convulsive status epilepticus; NG, nasogastric; NICU, neonatal intensive care unit; NZP, nitrazepam; OCBZ, oxcarbazepine; PB, phenobarbitone; PFA, paroxysmal fast activity; PHT, phenytoin; PRM, primidone; PSW, polyspike wave; PEG, percutaneous endoscopic gastrostomy; R, right; RFM, rufinamide; SE, status epilepticus; SGE, symptomatic generalized epilepsy; SPECT, single-photon emission computed tomography; STP, stiripentol; SSW, slow spike wave; TPM, topiramate; VGB, vigabatrin; VNS, vagus nerve stimulation; VPA, valproate; and ZNS, zonisamide.

^aMedications that were current at the last follow-up are underlined.

(Table continued on next page)

glutamate transporters, EAAT2. Mutations in *Slc1a2* in mice lead to impaired glutamate uptake, and the resulting excess glutamate leads to subsequent excitotoxicity.¹⁹ Null mice exhibit lethal spontaneous epileptic seizures, and very few animals survive beyond 13 weeks.¹⁹ Determining whether our mutations cause haploinsufficiency will require functional studies; if they do, recently identified translational enhancers of EAAT2 might offer a novel therapeutic approach.^{20,21}

Our studies have now identified a total of 11 individuals with *GABRB3* mutations over a wide phenotypic spectrum, accounting for more than 1% of unsolved EEs. Seizure onset, including onset of myoclonic, tonic, absence, and general-

ized tonic-clonic seizures, began in the first year of life in 10/11 cases; spasms were rare. Early development could be normal or delayed; intellectual outcome varied from severe to mild. Recent case reports include a boy with an EOEE attributed to an in-frame insertion in *GABRB3* and another boy with a de novo p.Thr287Ile substitution.^{22,23}

CACNA1A encodes the alpha1 subunit of the Ca_v2.1 P/Q-type calcium channel and was first reported as a gene involved in episodic ataxia type 2 (OMIM: 18500), spinocerebellar ataxia 6 (OMIM: 18036), and familial hemiplegic migraine type 1 (OMIM: 145100). *CACNA1A* is among the 2% most intolerant genes in the human genome.⁴ Although deletions that include *CACNA1A* and

Table 5. Continued

Age at Seizure Offset	EEG	Neuroimaging	Other Features	Medications ^a
Individuals identified in this cohort				
ongoing	multifocal; diffuse slowing; R tempoparietal spikes	normal	hypertonia; hyperreflexia; transient ventricular tachycardia; hyperkeratosis of feet; gum hypertrophy (PHT use); 2 small café au lait lesions	<u>LTG</u> , <u>TPM</u> , <u>LEV</u> , <u>PB</u> , <u>MDZ</u> , <u>CLZ</u> , <u>CZP</u> , <u>PHT</u> , lignocaine, pyridoxine, propranolol, pyridoxal-phosphate
ongoing	multifocal; beta activity; R > L sharp and slow waves	2 days—suggestive of hypoxic damage in periventricular area; 4 yr—normal	hypertonia; hyperreflexia; tremor; nystagmus; adducted thumbs; gum hypertrophy (PHT use); increasing number and size of café au lait lesions over time; athetoid movements at 12 yr; head-hitting mannerism	<u>LTG</u> , <u>VPA</u> , <u>TPM</u> , <u>LEV</u> , <u>PB</u> , <u>CBZ</u> , <u>CZP</u> , <u>MDZ</u> , <u>PHT</u> , <u>DZP</u>
ongoing	GSW; PSW; mild slowing bi-mid central frontal area	4 days—normal; 18 months—bimesial temporal lobe increased T2 signal	hyperreflexia; ataxia; very active	<u>VPA</u> , <u>AZD</u> , <u>CZP</u> , <u>PB</u> , <u>CLB</u> , <u>TPM</u> , <u>LTG</u> , <u>LEV</u> , <u>ETX</u>
ongoing	migration of rhythmic left predominant 2–4 Hz bi-occipital activity to right; 5–6 Hz theta activity; L > R posterior quadrant slowing	2 months—normal	alternating convergent strabismus; cerebral palsy	<u>LEV</u> , <u>STP</u> , <u>CBZ</u> , <u>VPA</u> , <u>TPM</u> , <u>RFM</u> , <u>VGB</u> , <u>ZNS</u>
ongoing	bi-frontal, L fronto-central, and temporal slowing	normal	mild diffuse hypotonia; ataxic gait; intention tremor	<u>TPM</u> , <u>LTG</u> , <u>CLB</u> , <u>VPA</u> , <u>LEV</u> , <u>KD</u>
Individuals Identified in Epi4K Consortium et al. (2013)¹				
ongoing	GSW; PSW; generalized slowing	CT newborn period: normal; 5 weeks: normal; SPECT 7 yr—areas of mildly decreased radiopharmaceutical uptake in L frontal, parietal, and temporal lobes	bilateral alternating esotropia; unsustained nystagmus on lateral gaze bilaterally; ataxic gait; autism; generalized hypotonia; 15 yr—contractures	<u>VPA</u> , <u>VGB</u> , <u>CLB</u> , <u>FBM</u> , <u>CBZ</u> , <u>GBP</u> , <u>KD</u> , <u>LTG</u> , <u>AZD</u> , <u>TPM</u> , <u>VNS</u> , <u>DZP</u> , <u>PHT</u> , <u>PB</u> , <u>ZNS</u> , levocarnitine

a single truncating mutation have been associated with EE,^{24–26} we show that de novo missense mutations can also cause a severe EE in six individuals. Seizures began on the first day of life in 5/6 individuals and typically included focal, tonic, and tonic-clonic seizures. Myoclonic seizures, sometimes with a prominent reflex component, were observed. Severe ID was usual, and a range of motor features were observed. All individuals had missense mutations that clustered in the transmembrane domains of this multi-pass transmembrane protein (Figure S4).

Seven of our 17 (41%) mutations were recurrent, in that they had been reported in at least one affected individual. The *DNM1* p.Arg237Trp variant arises in the context of a CpG dinucleotide, a known mutation hotspot.²⁷ The recurrence of the *SLCIA2* p.Gly82Arg variant might be accounted

for by a homopolymer stretch of guanines. Sequence context does not appear to explain the recurrence of the other mutations. The most notable example of recurrence is the p.Asn107Ser variant in *ALG13*, which has now been reported in seven affected females. Although loss of *ALG13* function causes glycosylation defects in boys, two females had normal glycosylation levels according to biochemical testing,^{14,15} suggesting an alternative mechanism.

In two out of 14 families in which we identified a mutation and were able to test both parents, we found the apparently de novo mutation in a similarly affected sibling. Deep sequencing in both sets of parents confirmed low-level somatic mosaicism of around 6% in one parent. This finding mirrors a recent Dravet syndrome study in which, in ~10% of cases, one parent of a child with an

apparently de novo *SCN1A* mutation had low, but detectable, levels of mosaicism.²⁸ This has critical implications for calculations of recurrence risk, for reproductive choices, and for prenatal counseling.

There are several possible limitations to our study. First, from the phenotypic viewpoint, the discovery cohort included individuals with IS or LGS, whereas the EEs included in this cohort were much more diverse (Table 1). Of the 531 individuals screened, 90 had IS and 41 had LGS, meaning that >75% of the cohort had other EEs. If mutations in some of these genes specifically cause IS or LGS, our cohort might be too small to allow detection of additional mutations. However, because the majority of mutation-carrying individuals in our cohort did not have IS or LGS, our findings highlight the importance of screening broader populations of individuals with EEs to define the phenotypic spectrum of new genetic diseases.

From a molecular standpoint, we acknowledge that mutations might remain undetected as a result of suboptimal coverage of a given gene or in a given individual. Some genomic regions are less amenable to sequencing with the molecular inversion probe technology.²⁹ Moreover, our conservative requirement of 50× coverage for variant calling could result in reduced sensitivity. Finally, it is important to note that not all 329 de novo variants identified in our original WES study are pathogenic. The reason for the lack of mutations in some candidate genes in our panel could be that these genes are not relevant to EE pathogenesis, or perhaps they explain a far smaller fraction of the EE population. Sequencing additional individuals and combining large datasets will facilitate the identification of additional EE-associated genes among the candidate genes from the original study.

In summary, we identified mutations in 17/531 probands. Pathogenic variants were identified in 7 of the 27 genes selected for targeted sequencing. There are six additional variants of unknown significance (Table S1), but determining whether these variants are pathogenic will require further genetic and functional studies. *GABRB3* and *CACNA1A* explained the largest proportion of cases in this study and provided a molecular cause for 1.3% (7/531) and 0.8% (4/531) of individuals, respectively; we also confirm *SLC1A2* as an EE-associated gene. Importantly, our study highlights the role of recurrent mutations, the relevance of parental mosaic transmission, and the genetic and phenotypic heterogeneity of the EEs.

Supplemental Data

A list of affiliations for Epi4K Consortium members, four figures, and two tables are available in the Supplemental Data at <http://dx.doi.org/10.1016/j.ajhg.2016.06.003>.

Consortia

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Web Resources

Burrows-Wheeler Aligner, <http://bio-bwa.sourceforge.net/>
DenovolyzeR, <http://jamesware.github.io/denovolyzeR/>
Exome Aggregate Consortium (ExAC) Browser, <http://exac.broadinstitute.org/>
GATK, <https://www.broadinstitute.org/gatk/>
OMIM, <http://www.omim.org>
Picard, <http://broadinstitute.github.io/picard>
SeattleSeq Annotation, <http://snp.gs.washington.edu/SeattleSeqAnnotation137/>

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