Extended Spectrum of Idiopathic Generalized Epilepsies Associated with *CACNA1H* Functional Variants

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Objective: The relationship between genetic variation in the T-type calcium channel gene *CACNA1H* and childhood absence epilepsy is well established. The purpose of this study was to investigate the range of epilepsy syndromes for which *CACNA1H* variants may contribute to the genetic susceptibility architecture and determine the electrophysiological effects of these variants in relation to proposed mechanisms underlying seizures.

Methods: Exons 3 to 35 of *CACNA1H* were screened for variants in 240 epilepsy patients (167 unrelated) and 95 control subjects by single-stranded conformation analysis followed by direct sequencing. Cascade testing of families was done by sequencing or single-stranded conformation analysis. Selected variants were introduced into the CACNA1H protein by site-directed mutagenesis. Constructs were transiently transfected into human embryo kidney cells, and electrophysiological data were acquired.

Results: More than 100 variants were detected, including 19 novel variants leading to amino acid changes in subjects with phenotypes including childhood absence, juvenile absence, juvenile myoclonic and myoclonic astatic epilepsies, as well as febrile seizures and temporal lobe epilepsy. Electrophysiological analysis of 11 variants showed that 9 altered channel properties, generally in ways that would be predicted to increase calcium current.

Interpretation: Variants in *CACNA1H* that alter channel properties are present in patients with various generalized epilepsy syndromes. We propose that these variants contribute to an individual's susceptibility to epilepsy but are not sufficient to cause epilepsy on their own. The genetic architecture is dominated by rare functional variants; therefore, *CACNA1H* would not be easily identified as a susceptibility gene by a genome-wide case–control study seeking a statistical association.

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The idiopathic epilepsies are thought to have a largely genetic basis, and there has been considerable success in identifying causative genes in families with autosomal dominant inheritance of epilepsy.[1] However, these families account for only a small fraction of individuals with epilepsy. The majority of cases are either sporadic or occur within families with recurrence risks far below that expected for dominant Mendelian traits, suggesting that there is a polygenic, complex, or multifactorial basis to the disease. In the rare large epilepsy families with autosomal dominant inheritance, there is variability in phenotypic expression and incomplete penetrance, again suggesting that additional factors to the dominant gene of large effect play a role in the phenotype of affected family members. We postulate a large but unknown number of epilepsy susceptibility genes within the human gene pool. The combination of a sufficient number of variants in these genes, together with environmental factors, will result in multifactorial epilepsy or epilepsy with complex genetics.[2,3]

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Identification of the genes involved in epilepsy with complex genetics has proved difficult, with only four strongly suspected so far from functional characterization. The GABA receptor subunit gene *GABRD* has been shown to contain variants likely to increase seizure susceptibility.[4] A polymorphism in the promoter of the GABA receptor subunit gene *GABRB3* is associated with childhood absence epilepsy (CAE) in some cohorts and has been shown to affect transcription efficiency.[5,6] A truncation mutation of the potassium channel subunit gene *KCND2* has been identified in a patient with temporal lobe epilepsy (TLE), as well as some unaffected family members, suggesting that it is a probable susceptibility allele rather than a dominant mutation of large effect.[7] Variants in the T-type calcium channel gene *CACNA1H* have been associated with CAE in Chinese patients,[8,9] and these variants alter channel function in ways consistent with epileptogenesis.[10–12] Two *CACNA1H* variants in patients with febrile seizures (FS) and myoclonic-astatic epilepsy (MAE) also alter channel function,[13,14] suggesting that variation in this gene plays a more general role in epilepsy susceptibility.

We now report a broader screen for *CACNA1H* variants conducted to determine the range of epilepsy syndromes with complex genetics in which genetic variation in *CACNA1H* could play a role in susceptibility. Electrophysiological studies of 11 of the variants identified showed that 9 exhibit biophysical alterations that may contribute to epilepsy susceptibility.

Subjects and Methods

Patients

One hundred and ninety-two patients were screened for exons 3 to 8 and 12 to 35 of *CACNA1H*. Exons 9 to 11 had been screened previously for these patients.[13] A further 48 patients were screened for exons 3 to 35 of *CACNA1H*. Two-hundred and forty patients (167 unrelated) were screened in total. Their clinical phenotypes included 37 CAE, 14 juvenile absence epilepsy (JAE), 31 juvenile myoclonic epilepsy, 5 juvenile myoclonic epilepsy evolved from CAE, 20 other idiopathic generalized epilepsies (IGEs), 55 MAE, 17 (FS), 28 generalized epilepsy with febrile seizures plus (GEFS+), 5 other generalized epilepsies, 11 TLE, 3 other focal epilepsies, 10 miscellaneous epilepsy patients, and 4 unaffected family members of epilepsy patients. Relatives of patients with amino acid changes were tested for the relevant variant(s). Patients were predominantly white. The study was approved by the Human Ethics Committee of Austin Health. Control subjects (n = 95) were anonymous blood donors.

Molecular Genetic Analysis

Single-stranded conformation analysis (SSCA) screening was conducted using the GelScan 2000 (Corbett Research, Sydney, Australia). Gels contained 4% (35:1) polyacrylamide, 2% (wt/vol) glycerol, and 0.6xTBE. Samples showing bandshifts were sequenced using BigDye 3.1 (ABI; Applied Biosystems, Foster City, CA). (Details of primers and reaction conditions used for polymerase chain reaction [PCR] are available on request from the authors.) Although single-stranded conformation analysis will not detect 100% of variants, we identified sufficient novel variants in patients with a range of epilepsy phenotypes for a detailed electrophysiological analysis of potential susceptibility alleles of *CACNA1H*.

Assay for R2005C Variant

A BstUI site (CGCG) was introduced into the R allele by PCR using the primers 5′-GTCCTGTGCCTCCC-TCCAGATCCCA-3′ and 5 ′-AGCAGCCGGCTGAGACT-GGGGGCGC-3′. PCRs contained 0.25U Taq DNA polymerase (Invitrogen, La Jolla, CA), 200μM of each deoxyribonucleotide triphosphate (dNTP), 1.5mM $MgCl_2$, 15ng/μl of each primer and 90ng template DNA in a 10μl reaction volume. PCRs were cycled through 11 cycles of 94°C for 15 seconds, 60°C for 15 seconds, and 72°C for 15 seconds, followed by 26 cycles of 94°C for 15 seconds, 55°C for 15 seconds, and 72°C for 15 seconds. After cycling, 2 units BstUI (New England Biolabs, Beverly, MA) diluted in 10μl 2x digestion buffer was added to each PCR. The digests were incubated at 60°C for 2 hours. Fragments were analyzed by electrophoresis on 3% agarose gels. Incomplete digestion of the 117bp PCR product indicated that the C allele was present.

Site-Directed Mutagenesis

Site-directed mutagenesis of the human $Ca_v3.2$ calcium channel α1 (accession NM_001005407) subunit in pcDNA(Zeo)3.1 was performed using the Quick change mutagenesis kit (Stratagene, La Jolla, CA). Mutagenesis was performed, for 11 missense changes, using 2 different strategies that utilized different templates for a subset of the variants. For four variants (A876T, A1059S, E1170K, and Q1264H), a 903bp fragment (flanked by *Bsr*GI and *Age*I restriction sites) from the $Ca_v3.2$ coding sequence was amplified using PCR and inserted into the pGEM-T-easy vector (Promega, Madison, WI) for subsequent mutagenesis. For each variant, the entire insert was sequenced to confirm the presence of the mutation and to check for any PCR-introduced errors. The insert was then reintroduced into the wild-type $Ca_v3.2$ complementary DNA, and both the insert and flanking regions were resequenced to ensure proper ligation. For the remaining seven variants (R788C, G983S, T1606M, A1705T, T1733A, R1892H, and R2005C), the full-length $Ca_v3.2$ complementary DNA was used as a mutagenesis template, and the entire coding region was sequenced after mutagenesis to ensure the presence of the mutation and the lack of mutagenesis errors.

Cell Culture and Transient Transfection

Tissue culture and transfection of tsA-201 cells was described by us previously in detail.[14] In brief, human embryo kidney cells were grown to 85% confluence at 37°C (5% CO_2) in Dulbecco's modified Eagle's medium (+10% fetal bovine serum, 200U/ml penicillin, and 0.2mg/ml streptomycin; Invitrogen). Cells were dissociated with trypsin (0.25%)-EDTA and plated on glass coverslips. Mutant or wild-type $Ca_v3.2$

channel α1 subunits (8μg) and CD8 marker (1μg) DNA were transfected into cells by the calcium phosphate method. Cells were transferred to 30°C 24 hours after transfection, and recordings were conducted a day later.

Electrophysiology and Data Analysis

Before recordings, cells were transferred into an external bath solution of 2mM calcium (in mM): 128 CsCl, 2 $CaCl_2$, 1.5 $MgCl_2$, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 25 D-glucose; pH was adjusted to 7.4 with CsOH. Borosilicate glass pipettes were pulled and polished to 2.5 to 4MΩ resistance and filled with internal solution (in mM): 135 CsCl, 10 EGTA, 2 $CaCl_2$, 10 HEPES, and 1 $MgCl_2$; pH was adjusted to 7.4 with CsOH. Data were acquired at room temperature using either an Axopatch 200B or MultiClamp 700B amplifier and pClamp 9.2 software (Molecular Devices, Palo Alto, CA), low-pass filtered at 1kHz, and digitized at 10kHz. Series resistance was compensated to 80%. Data analysis and off-line leak subtraction were performed in Clampfit 9.2 (Molecular Devices), and all curves were fit using Origin analysis software (OriginLab) and in-house software developed in Matlab (Mathworks, Sherborn, MA). Current-voltage (IV) plots were fitted using the Boltzmann equation, $I = (V-E_{rev})*G* (1/(1+\exp(-(V-V_{0.5a})/S))$, where E_{rev} is the reversal potential, G is the maximum slope conductance, $V_{0.5a}$ is the half-maximal activation potential, and S is the slope factor. Individual inactivation curves were fitted with the Boltzmann equation, $I_n = X+(1-X)/(1+\exp(-z*(V_{0.5i}-V)/25.6$, where I_n is the normalized fraction of available current, X is the noninactivating fraction of current, z is the slope factor, and $V_{0.5i}$ is the half-inactivating potential. Time constants for activation and inactivation were obtained from monoexponential fits to the raw current data. Time constants for recovery from inactivation, τ_r, were obtained by monoexponential fits to the time course of recovery from inactivation. These data were obtained by applying an inactivating conditioning pulse followed by a variable recovery period preceding the test pulse. Time-to-peak current was calculated from the current onset time (posttransient) to the peak current. All averaged data are plotted as mean ± standard error of the mean, and numbers in parentheses reflect numbers of cells tested. Statistical analysis was performed using one-way analysis of variance (unless otherwise stated), where $p < 0.05$ was considered as significant.

Results

More than 100 sequence variants were identified in our patients and control subjects for the portion of the gene screened. These included 50 variants in the introns and 3′ untranslated region; 30 synonymous coding variants; and 32 variants causing amino acid changes. Nineteen of the amino acid changes have not been previously described. All amino acid changes identified are described in Supplementary Table 1, and the novel variants are shown in Figure 1.

Site-directed mutagenesis was used to introduce each of 11 amino acid changes into the human $Ca_v3.2$ calcium channel complementary DNA sequence. Ten of the novel variants and the previously known polymor-

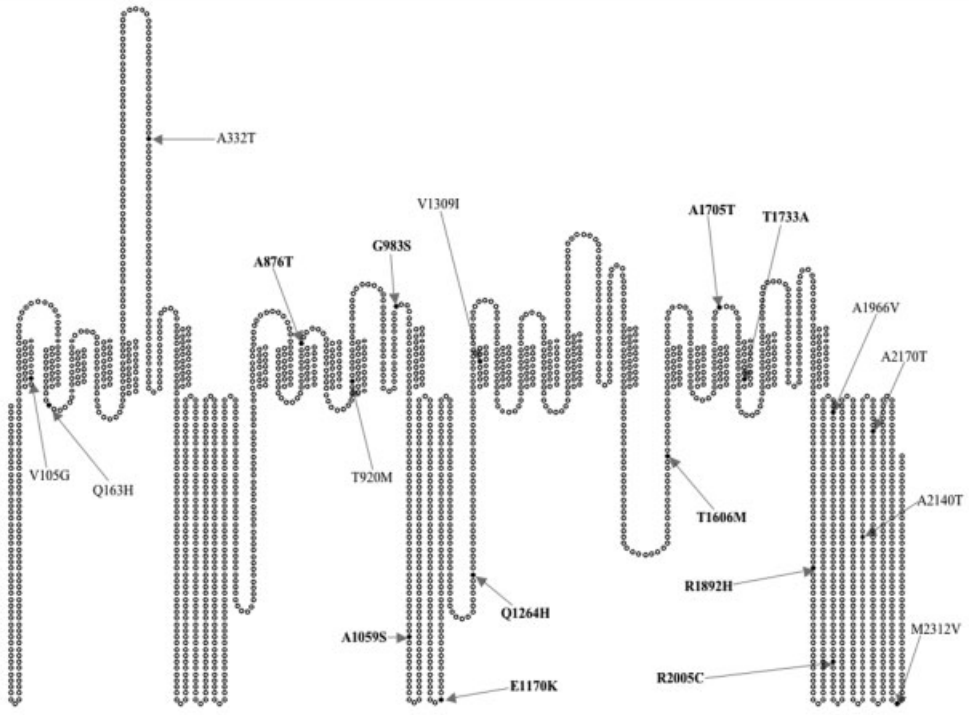

Fig 1. Structure of the CACNA1H protein showing the locations of the novel variants identified in epilepsy patients. The variants labeled in bold were analyzed by electrophysiology.

phism p.R788C were analyzed. The novel variants selected for analysis fulfilled at least one of the following criteria: alteration of a conserved amino acid; partial segregation with affection status in a family; absence in control subjects or location in a region of the protein of known functional significance. (These variants are described in the Table. For the 10 novel variants, the conservation of the altered amino acids in T-type calcium channel sequences is shown in Supplementary Figure 2.). The mutants were transiently expressed in tsA-201 cells and characterized via whole-cell patch clamp. Changes in channel function were observed for 9 of the 11 variants tested (Fig 2). For every channel variant, the voltage dependences, rates of activation and inactivation, and the time constant for recovery from inactivation were determined and compared with those of wild-type channels. None of the mutants showed changes in the time course of inactivation, but several displayed alteration in the other gating characteristics. Figure 2 highlights only properties in which statistically significant changes were observed for a given mutant.

Variants Resulting in Altered Channel Function

The variant p.A876T is seen in one family with FS, CAE, symptomatic generalized epilepsy, and TLE, in which it segregates with affection status (Fig 3, Family C), and not in control subjects. This variant alters a conserved amino acid in transmembrane domain II and affects two biophysical parameters of channel activity. First, there is a depolarizing shift in the half-inactivation potential (ie, the membrane potential at which half of the channels can be activated by a membrane depolarization) of the channel (see Fig 2C). This results in an increase in the fraction of channels that are available for opening, thus causing a gain of function. Second, the time course of recovery from channel inactivation (see Fig 2E) is faster, hence the channels are less likely to accumulate in a nonconducting state during successive membrane depolarizations, which in this case would be consistent with increased channel function.

The variant p.G983S was observed in a single patient with MAE. This change cosegregates with the polymorphism p.R2005C, which is described later. Both variants were inherited from the patient's father, who himself was unaffected but who had a family history of epilepsy (see Supplementary Fig 1, Family J). The p.G983S change alters a highly conserved amino acid in the pore loop of transmembrane domain II (see Supplementary Fig 2) and would be expected to significantly alter channel properties. Electrophysiological studies showed that this variant slows the recovery from inactivation (see Fig 2E) and shifts the half-inactivation potential toward more negative voltages (see Fig 2C). These effects are opposite to those seen with p.A876T and are therefore consistent with a loss of channel function.

The variant p.A1059T was seen in three families (see Fig 3, Family B; see Supplementary Fig 1, Families E and M) and one control subject. Affected individuals have a range of generalized epilepsies, and one has TLE after FS. Susceptibility variants are not unexpected in control subjects in whom there are insufficient num-

Table. **Details of Novel Variants Analyzed by Electrophysiology**

Amino Acid Change	Associated Phenotypes	Conserved Amino Acid?	Segregation with Affection Status?	Present in Control Subjects?	Domain
p.A876T	CAE, FS, TLE, SGE	Yes	Yes	No	DII-S3
p.G983S	MAE	Yes	No	No	DII P-loop
p.A1059S	CAE, JME, MAE, IGE, FS, TLE	Yes	Partial	Yes	DII-DIII loop
p.E1170K	MAE, FS	Yes	Partial	No	DII-DIII loop
p.Q1264H	CAE, MAE, FS+	No	No	No	DII-DIII loop
p.T1606M	FS, IGE, CAE, JAE	Yes	Partial	No	DIII-DIV loop
p.A1705T	MAE, FS+	Yes	Partial	Yes	DIV
p.T1733A	CAE, FS	Yes	No	No	DIV-S4
p.R1892H	CAE, JAE, photosensitivity	No	Yes	No	C terminus
p.R2005C		Yes	No	Yes	C terminus

CAE = childhood absence epilepsy; FS = febrile seizures; TLE = temporal lobe epilepsy; MAE = myoclonic-astatic epilepsy; JME = juvenile myoclonic epilepsy; IGE = idiopathic generalized epilepsy; JAE = juvenile absence epilepsy; SGE = symptomatic generalized epilepsy.

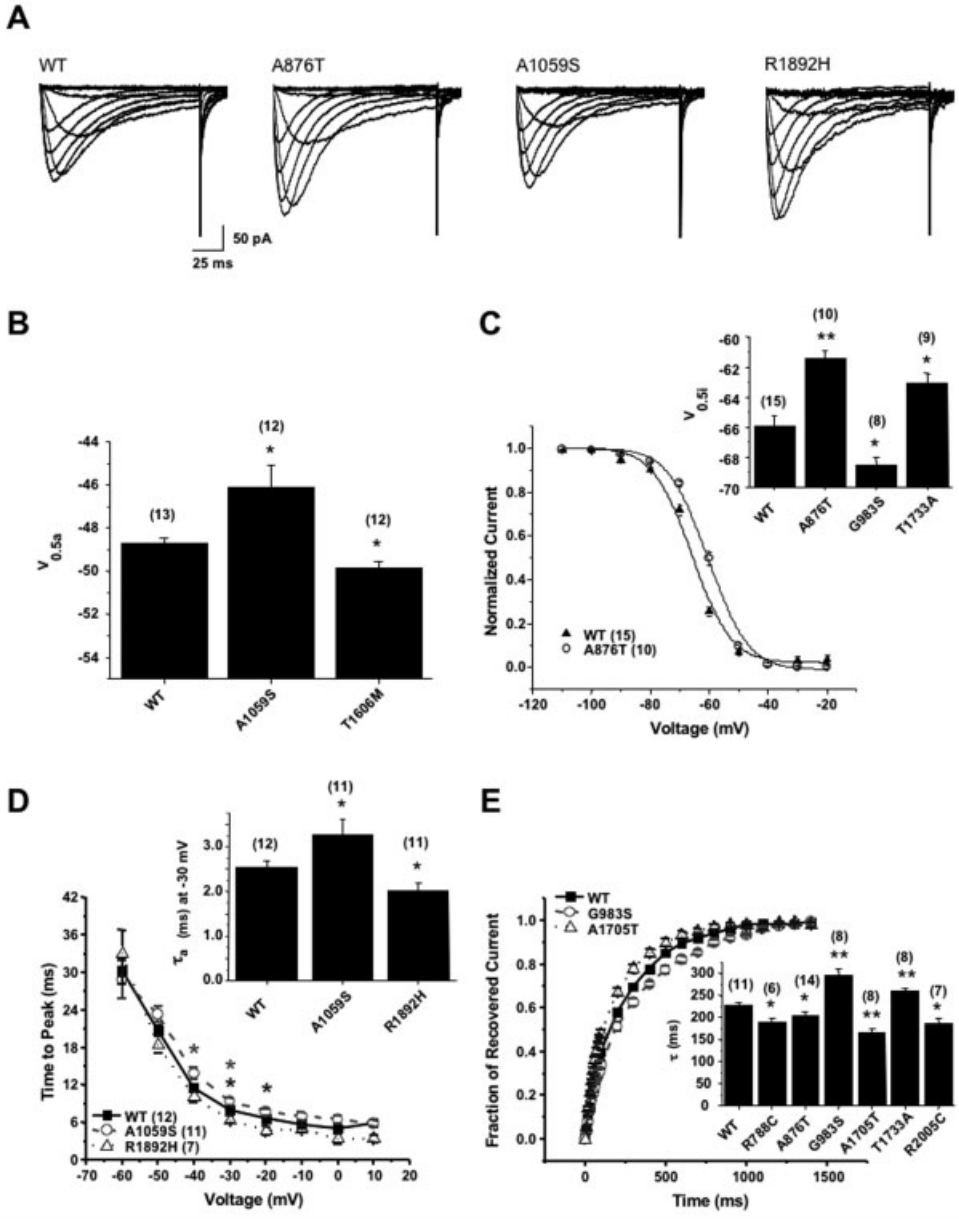

Fig 2. (A) Families of whole-cell currents obtained with wild-type channels and 3 of the 11 mutants characterized in this study. The currents were elicited by step depolarizations from a holding potential of −120mV to various test potentials. Note that all channels display the typical current waveforms of T-type calcium channels. (B) Half-activation potentials observed with wild-type $Ca_v3.2$ and two $Ca_v3.2$ variants with significant alterations in this parameter. Data were obtained from fits to current voltage relations. (C) Steady-state inactivation curves obtained with wild-type $Ca_v3.2$ and the A876T mutant. Data were fitted with the Boltzmann equation (see Methods). (inset) Comparison of the half-inactivation potentials observed with wild-type channels and those mutants that showed a significant change in this parameter. (D) Comparison of the voltage dependences of the time to peak for the wild-type channel and the two mutants that showed a significant change in this parameter. (inset) Comparison of the time constants for activation of these channel variants at a test potential of −30mV. (E) Time course of recovery from inactivation for the wild-type channel and two selected mutants with altered recovery time constants. (inset) Comparison of the time constants for recovery from inactivation for wild-type channels and those mutants that showed a significant change in this parameter. In all panels, error bars denote standard errors, numbers in parentheses indicate numbers of experiments, and single *and* double asterisks *denote statistical significance at the 0.05 and 0.01 levels, respectively.*

bers of susceptibility alleles for the individual to cross the seizure threshold and therefore have epilepsy. It alters an amino acid in the intracellular loop between transmembrane domains II and III and slightly, but significantly raises the threshold of channel activation as evident from a small depolarizing shift in the half-

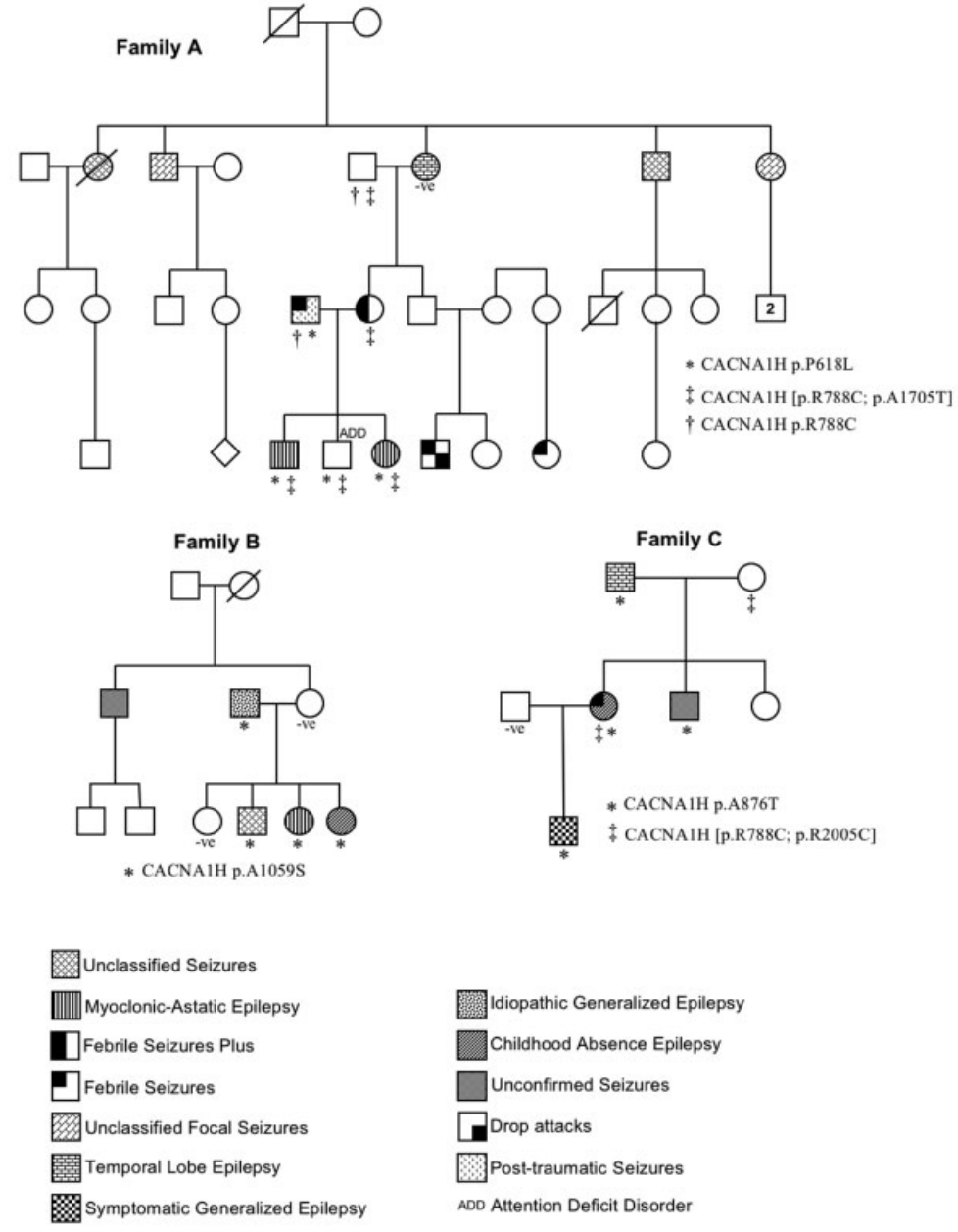

Fig 3. Pedigrees of three selected families with variants in CACNA1H. *These pedigrees demonstrate the range of phenotypes that may be associated with a single variant and the range of segregation patterns that are seen for the variants. Two of these families have multiple variants. It is possible that the combination of variants is contributing to the more severe phenotypes seen in some individuals.*

activation potential (ie, the voltage at which half of the channels are activated; see Fig 2B). Moreover, the channel was slower to activate (see Fig 2D), altogether indicating a slight reduction in channel function.

The variant p.T1606M was observed in two epilepsy families with FS, CAE, IGE, JAE, and unclassified epilepsy, and was not present in control subjects. In both families, the variant is transmitted from an unaffected parent to the affected children (see Supplementary Fig 1, Families G and K). This variant alters an amino acid in the intracellular loop between transmembrane domains III and IV. This variant causes a small, hyperpolarizing shift in the half-activation potential (see Fig 2B) and would be expected to cause a small gain of function, and thus increase in calcium current.

The variant p.A1705T was seen in two epilepsy families with MAE and GEFS+ (see Fig 3, Family A; see Supplementary Fig 1, Family F) and one sporadic MAE patient, and was absent in control subjects. In both families, the change cosegregates with the polymorphism p.R788C. The sporadic patient also carries both changes, but DNA was not available from any relatives

to allow analysis of the cosegregation of the two changes. When tested alone, the p.A1705T variant decreased the time of recovery from inactivation (consistent with a small gain of function), but did not alter any of the other parameters measured (see Fig 2E). Although this alteration of kinetics is small, it is possible that the difference would have been larger if the change had been tested with the p.R788C variant in the same construct. One of the families, Family A, has two rare variants in *CACNA1H,* p.A1705T and p.P618L, as well as the p.R788C polymorphism (see Fig 3). Each of the rare variants comes from a marry-in to the family, and patients who have both rare variants perhaps not coincidentally have a more severe phenotype than their parents who each carry one rare variant. However, their unaffected sibling also has both variants. This family would be expected to have other epilepsy susceptibility alleles, as well as the *CACNA1H* variants, because both of these come from marry-ins and there are affected family members who do not have either variant.

The variant p.T1733A was seen in a single epilepsy family with CAE (see Supplementary Fig 1, Family L) and did not segregate with epilepsy in the two affected siblings in this family. This variant alters an amino acid in the S4 segment of transmembrane domain IV, which forms part of the voltage sensor. It caused a depolarizing shift in half-inactivation potential (see Fig 2C), yet a slowing of recovery from inactivation (see Fig 2E), thus mediating opposing effects on channel function.

The variant p.R1892H was seen in a single family with CAE and JAE (see Supplementary Fig 1, Family D) and was not present in control subjects. The change was transmitted from a mother with photosensitivity to her four affected children and was absent in the fifth unaffected child. This variant was slightly faster to activate in response to membrane depolarization (see Fig 2D), and thus was consistent with a gain of function.

The variant p.R2005C is a polymorphism that was observed in 17.4% of patients and 14.6% of control subjects (C allele frequencies 8.7 and 7.3%, respectively). Although the frequency of this polymorphism is increased in the patient population, this increase is not statistically significant ($\chi^2 = 0.36$; $p > 0.5$). The change alters an amino acid that is conserved in CACNA1H and CACNA1G sequences (see Supplementary Fig 2), suggesting that it is important for channel function. Functional analysis showed that the p.R2005C variant decreases the time-constant of recovery from inactivation (see Fig 2E), leading to a small gain of function.

The known polymorphism p.R788C was seen in both patients and control subjects. The frequency of the C allele was 11.5% in patients and 9.8% in control subjects ($\chi^2 = 0.33$; $p > 0.5$). Although the frequency of this polymorphism was not significantly increased in the patient population, the change decreases the time-constant of recovery from inactivation (see Fig 2E), leading to a small gain of function.

Other Variants

Two of the variants tested were not found to alter those channel properties examined. Both of these variants are in the intracellular loop between transmembrane domains II and III. The variant p.E1170K, which alters a highly conserved amino acid (see Supplementary Fig 2), is seen in a single family with GEFS+ (see Supplementary Fig 1, Family H) and not in control subjects. The variant p.Q1264H was seen in monozygotic twins with CAE and their unaffected mother (see Supplementary Fig 1, Family I), in one MAE patient, and in one FS+ patient, but was not present in control subjects.

Discussion

The T-type calcium channels are involved in the thalamocortical network,[15] and variants in the gene coding for one of these channels, *CACNA1H,* have been related to CAE and rare cases with IGE.[16] We have screened 240 individuals from 167 IGE and GEFS+ families for variants in the *CACNA1H* gene and have identified 19 novel variants causing amino acid changes. Functional analysis was done for 10 of these as well as one previously known polymorphism. Nine of the variants altered channel kinetics consistent with epileptogenesis. These variants were observed in patients with a range of epilepsy syndromes including classic IGEs (CAE, JAE, juvenile myoclonic epilepsy, IGE with generalized tonic-clonic seizures), GEFS+ (MAE, FS), and TLE. Genes common to both classic IGEs and GEFS+ have previously been shown including GABA receptor subunit genes *(GABRG2, GABRD),*[1,4] and mutations in the sodium channel subunit gene *SCN1B* are associated with both GEFS+ and TLE.[1]

All of the variants were also observed in unaffected individuals. In some families, the variant segregated with epilepsy, whereas in others it did not. This variation in segregation patterns is consistent with susceptibility alleles and departs from the pattern of vertical inheritance seen in monogenic autosomal dominant epilepsies. These results suggest that variants in *CACNA1H* underlie a range of generalized epilepsy phenotypes with complex genetics in addition to CAE, but alone do not cause epilepsy. Other factors such as the involvement of other genes or environmental factors may allow a variant to manifest itself in one individual but not in another, even though both subjects may have the same T-type calcium channel variant.

Most of the variants we have identified are predicted to cause a gain of function; that is, they cause an increase in calcium current. A direct consequence of increased T-type channel activity may result in increased spiking behavior in neurons that exhibit this rebound

bursting, thereby contributing to the generation of epileptiform discharges. This effect can occur in both excitatory and inhibitory neuronal elements. In excitatory neurons, increased burst-mode activity directly affects network behavior, such as during spike-wave discharges. In the case of inhibitory interneurons, increased T-type channel–mediated bursting can act to synchronize the activity of excitatory neurons, thereby recruiting them into epileptiform activity. Furthermore, in early development, GABAergic synaptic transmission is excitatory; hence, increased bursting activity in interneurons may contribute to epileptogenesis.[17] Two variants (p.G983S and p.A1059S) exhibited a loss of channel function. One variant (p.T1733A) displayed changes in gating behavior that are less clear. The variants that have been previously reported in CAE patients are predicted to cause a gain of function, leading to increased calcium influx and hyperexcitability.[10–12] Although the occurrence of loss of function variants of *CACNA1H* in epilepsy appears to be paradoxical, these variants may contribute to seizures by altering the timing of neuronal firing patterns. Alteration in T-type currents resulting in a loss of function can also manifest its effects by altering the balance between excitatory and inhibitory neuronal elements. It is also important to note that the phenotypic expression of a particular variant may depend on its interaction with other ionic currents such as those mediated by calcium-activated potassium channels. These effects are of particular relevance in the case of *CACNA1H* variants, which are seen only in epilepsy with polygenic inheritance.

Of the variants examined here, six are localized to intracellular regions of the channels such as the II-III and III-IV loops, as well as the C terminus. The II-III linker region has been implicated as a hotspot of second messenger regulation by calmodulin kinases and G-protein $\beta\gamma$ subunits,[18,19] raising the possibility that variants in this region may alter channel regulation by intracellular signaling molecules such as kinases, G proteins, and phosphatases. Such a mechanism might account for the phenotype observed with two of the II-III linker variants that were biophysically silent in our experiments. Moreover, changes in intracellular linkers may affect the association of the channel with other cellular proteins that are involved in maintaining channel function, and their targeting to, and retention in, the plasma membrane. Indeed, effects on channel targeting have previously been described for other CAE variants found in the I-II linker region of the human $Ca_v3.2$ calcium channel,[20] and it will be of interest to determine whether any of the variants examined here mediate similar effects. The notion that the variants produced relatively mild biophysical changes, and in some cases did not affect channel gating, fits with such putative mechanisms. In addition, it is important to consider that the variants may affect posttranslational modifications of the channel protein or alter the extent of transcript splicing as has been reported recently for $Ca_v3.2$ mutations linked to CAE.[21]

We have extended the range of phenotypes associated with functionally relevant *CACNA1H* variants to include a wide range of generalized epilepsies and rarely TLE. CAE is part of this spectrum, and the variants previously reported in CAE were proposed as epilepsy susceptibility alleles.[10–12] This is also the first report of *CACNA1H* variants in white CAE patients. Chioza and colleagues' study[22] of 220 European CAE patients did not identify any variants in *CACNA1H.* However, that study looked specifically for 29 of the variants that Chen and coworkers[8] identified in the Han Chinese population. Nineteen of the amino acid changes identified here have not been reported previously, which reinforces the extent of genetic variation present at this locus. Only about one quarter of the variants we identified were also observed in the Han Chinese population.[8,9] This suggests that the majority of the variants in this gene are present at low frequencies and are population specific, with only relatively common polymorphisms being seen in multiple populations.

Variants in *CACNA1H* with measurable functional effects have been reported in patients with autism spectrum disorders.[23] This suggests that epilepsy and autism spectrum disorders may have susceptibility alleles in common. Mutations of the voltage-gated sodium channel genes *SCN1A* and *SCN2A* have been reported both in patients with epilepsy[24–28] and autism.[29] One-third of patients with autism also have epilepsy,[23] strongly suggesting that there are common mechanisms based on shared genetic determinants underlying the two disorders. Some of our epilepsy patients with *CACNA1H* variants also have other neurological disorders such as intellectual disability, learning difficulties, and attention deficit disorder. It is possible that the variants are contributing to all of these features. However, this is difficult to prove given that these disorders are relatively common and considerable variation in *CACNA1H* is to be expected in the normal population.

Variants in *CACNA1H* contribute to epilepsy in a manner consistent with the polygenic heterogeneity model.[1–3] In this model, there are an unknown number of rare susceptibility alleles within the human gene pool, and the combination of a sufficient number of these within the genome of an individual will result in the manifestation of disease. Occasional more frequent polymorphisms may also be involved, such as the variants p.R788C and p.R2005C in *CACNA1H,*[11] the p.R220H allele of *GABRD,*[4] and a promoter polymorphism in *GABRB3.*[5] However, the effect of these variants in individuals is likely to be small. This model would explain why many association studies for epilepsy, and brain disorders in general, have been unsuccessful: Any variant with an effect will be too rare within each epilepsy syn-

drome to give a positive result in an association study without extremely large sample sizes.[30]

Here we have provided functional evidence that variants in *CACNA1H* are also associated with CAE in the white population and have extended the phenotypes associated with this gene to include other epilepsies with complex genetics. All epilepsy-related variants so far described in *CACNA1H* appear to be susceptibility alleles, which contribute to disease but alone are not sufficient to cause it. Hence, *CACNA1H* variants do not segregate with epilepsy in large families. Further characterization of the role of *CACNA1H* in idiopathic epilepsy can be achieved by the identification of further variants in patients with a range of epilepsy phenotypes and correlating that with functional properties of the protein. Intuitively, the population genetics of any epilepsy gene should encompass the extreme possibilities of pathogenic mutations of large effect and functionally neutral polymorphisms, with the complete spectrum between the two. Cis and trans interactions between variants may lead to an additional layer of complexity.

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