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FULL-LENGTH ORIGINAL RESEARCH

Functional variants in *HCN4* and *CACNA1H* may contribute to genetic generalized epilepsy

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Summary

Objective: Genetic generalized epilepsy (GGE) encompasses seizure disorders characterized by spike-and-wave discharges (SWD) originating within thalamo-cortical circuits. Hyperpolarization-activated (HCN) and T-type Ca^{2+} channels are key modulators of rhythmic activity in these brain regions. Here, we screened *HCN4* and *CACNA1H* genes for potentially contributory variants and provide their functional analysis.

Methods: Targeted gene sequencing was performed in 20 unrelated familial cases with different subtypes of GGE, and the results confirmed in 230 ethnically matching controls. Selected variants in *CACNA1H* and *HCN4* were functionally assessed in tsA201 cells and *Xenopus laevis* oocytes, respectively.

Results: We discovered a novel *CACNA1H* (p.G1158S) variant in two affected members of a single family. One of them also carried an *HCN4* (p.P1117L) variant inherited from the unaffected mother. In a separate family, an *HCN4* variant (p.E153G) was identified in one of several affected members. Voltage-clamp analysis of *CACNA1H* (p.G1158S) revealed a small but significant gain-of-function, including increased current density and a depolarizing shift of steady-state inactivation. *HCN4* p.P1117L and p.G153E both caused a hyperpolarizing shift in activation and reduced current amplitudes, resulting in a loss-of-function.

Significance: Our results are consistent with a model suggesting cumulative contributions of subtle functional variations in ion channels to seizure susceptibility and GGE.

KEY WORDS: *HCN4*, T-type Ca^{2+} channels, Thalamo-cortical circuits, Generalized epilepsy.

Epilepsy is a prevalent neurological disorder with a lifetime incidence of up to 3%.[1] The most common inherited form of epilepsy is genetic generalized epilepsy (GGE), which encompasses four major subtypes: childhood and juvenile absence epilepsy (CAE/JAE), juvenile myoclonic epilepsy (JME), and epilepsy with generalized tonic-clonic

Key Points

- *HCN4* and *CACNA1H* are highly expressed in the thalamo-cortical loop involved in generation of generalized seizures
- Both genes were sequenced in a cohort of patients with generalized epilepsy
- Functional analysis of one *CACNA1H* revealed gain-of-function, and two *HCN4* variants showed an overall loss-of-function
- These subtle but distinctive functional changes may contribute to seizure susceptibility in the affected individuals

seizures on awakening (EGTCA).[2] Some genetic models suggest GGE is a polygenic disorder where a number of genetic variations with small-to-moderate effects, which alone are insufficient to cause epilepsy, are required to precipitate seizures.[3] GGE mutations have been described in several, mainly ion channels–encoding, genes.[4–8] Additionally, structural genetic variations, common single nucleotide polymorphisms (SNPs), and ultra-rare variants have also been associated with GGE.[9–15]

GGE is characterized by spike-and-wave discharges (SWDs) on electroencephalogram (EEG). SWDs are thought to originate from within the thalamo-cortical loop that is composed of neurons in three brain regions: the thalamic reticular nucleus, the thalamic relay nuclei, and the cortex.[16] Pacemaker ion channels, such as T-type Ca^{2+} and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, are critical modulators of oscillatory behavior in this network,[17,18] which makes them good candidates for genetic studies in GGE. Three distinct T-type Ca^{2+} channel isoforms are encoded by *CACNA1G* ($Ca_V3.1$), *CACNA1H* ($Ca_V3.2$), and *CACNA1I* ($Ca_V3.3$). Previous studies have identified variants in *CACNA1H*, particularly in CAE but also in other GGE seizure types, including JAE and JME.[19–22] Variants of *CACNA1G* have also been implicated in GGE,[23] but so far no association with *CACNA1I* has been reported.[24] More recent large-scale sequencing studies do not support a major role of these channels in genetic architecture of common epilepsies.[9,10,12,15,25]

Four HCN channel subtypes, HCN1–HCN4 are encoded by *HCN1*, *HCN2*, *HCN3,* and *HCN4*, respectively. Several epilepsy-associated variants in *HCN* genes have been recently described. Di Francesco et al.[26] reported a homozygous *HCN2* mutation p.E515K in a patient with sporadic GGE, and a deletion (719–721ΔP) and p.S126L mutation in *HCN2* have been associated with febrile seizure syndromes.[27,28] Nava and colleagues detected several de novo *HCN1* mutations in patients with early-onset epileptic encephalopathy.[29]

In this study, we embarked on the genetic analysis of the GGE-associated *CACNA1H* gene as well as the *HCN4* gene, which are among the channels expressed at high levels in the thalamus.[18,30] The sequencing was done in 20 independent patients with GGE core phenotypes, including patients from the $GEFS^+$ (genetic epilepsy with febrile seizures) spectrum.[31] We also provide functional analysis of the detected variants to assess their potential impact on the biophysical properties of affected channels and understand how they may contribute to seizure genesis in these GGE families.

Materials and Methods

Clinical and genetic analysis

Twenty patients with core GGE phenotypes, including some with febrile seizures, were recruited. All patients and relatives or their legal representatives gave written informed consent to participate in this study. Ethical approval was obtained from the responsible local authorities. The clinical information for the two families in which the mutations have been detected is presented in the Appendix S1.

Genomic DNA was extracted from peripheral blood leukocytes using a salting-out method. Polymerase chain reaction (PCR) was performed with 50-ng genomic DNA, 10 pmol of each primer, 200 μM dNTP, 50 mM Tris-HCl, 15 mM ammoniumsulfate, 2.5 mM $MgCl_2$, 5% dimethyl sulfoxide (DMSO), 0.75 U AccuTaq Polymerase (Sigma-Aldrich) in a total volume of 25 μl. Primers were designed to amplify the entire coding region and adjacent intron sequences of the candidate genes. PCR was performed in an MJ Research thermocycler with the following conditions: 35 cycles of denaturation at 95°C for 30 s, annealing temperatures ranging from 62°C to 90°C for 30 s, and extension at 68°C degree for 90 s. The amplicons were purified and subsequently sequenced.

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For all missense variants, we assessed genotype and allele frequencies in a total of 230 ethnically matched controls using specific restriction digestion assays. All available members of the families were genotyped for the cosegregation analysis.

Functional analysis

Full-length cDNA encoding the HCN4 channel (NM_005477) was derived from a human cDNA library (Life Technologies) and subcloned into the pGEMHE vector for *in vitro* cRNA transcription. Cloning of the human Cav3.2 has been previously described.[32] Site-directed mutagenesis in HCN4 and Cav3.2 was performed using overlap PCR strategy or Quick Change kit (Stratagene). *In vitro* synthesis of *HCN4* wild type (WT) and mutant cRNAs was performed using the mMessage mMachine T7 transcription kit (Ambion).

Patch clamp recordings

For transient expression of Cav3.2, we used tsA201 cells grown in DMEM/F-12 (Life Technologies) supplemented with 10% fetal bovine serum. Cells were transfected with either WT or mutant channel using the calcium phosphate method. Cotransfection with CD8 marker plasmid was used to control for transfection efficiency and selection of cells for electrophysiological analysis.

Whole cell patch-clamp recordings were performed at room temperature using Axopatch 200 A amplifier. Data were sampled at 10 kHz and filtered at 3–5 kHz. Whole cell Ca^{2+} currents were recorded in extracellular solution containing (in mM): 10 $CaCl_2$, 10 HEPES, 6 CsCl, 140 TEA-Cl; pH was adjusted to 7.4 with CsOH. The intracellular solution contained (in mM): 1 $MgCl_2$, 10 HEPES, 10 EGTA, 135 CsCl, with pH adjusted to 7.4 using CsOH. Patch pipettes were pulled from borosilicate glass (DMZ-Universal Puller) and had a resistance of 2–5 MΩ when filled with intracellular solution. Series resistance was compensated to 65–80%, resulting in maximal residual voltage error below 5 mV during measurements.

HCN4 two-electrode voltage clamp recordings

Oocytes from *Xenopus laevis* were prepared as previously described.[28] Briefly, 50 nl of cRNA-encoding HCN4 subunit (12.5 ng/μl; concentration confirmed spectrophotometrically and by gel analysis) was injected into stage 5/6 oocytes using the Roboocyte (Multi Channel Systems) and incubated for 2 days at 15°C prior to experimentation. Oocytes were perfused with a bath solution containing (in mM): 96 KCl, 2 NaCl, 2 $MgCl_2$, and 10 HEPES (pH 7.5 using KOH). For voltage clamp recordings, oocytes were impaled with two glass electrodes containing 1.5 M potassium acetate (I) and 0.5 M KCl (V) and clamped at a holding potential of −30 mV. All experiments were performed at room temperature.

Data analysis

Detailed electrophysiological protocols and data analysis are presented in the Appendix S2. Data are presented as mean ± SEM. Statistical differences were obtained using unpaired t test with post hoc test for multiple comparisons or one-way ANOVA (Prism 6, GraphPad Prism Software, La Jolla, CA, U.S.A.).

Results

New variants detected in GGE

A systematic search for variants in *CACNA1H* and *HCN4* genes was performed in a sample of 20 unrelated patients with GGE. Seizure subtypes represented among the patients included CAE, JAE, JME, and epilepsy with generalized tonic-clonic seizures (EGTCS). Detected nonsynonymous missense variations are presented in Table 1. Out of the 13 variants detected in *CACNA1H*, one novel variant, p.G1158S, appeared only in two GGE patients of Family 1 (Figs 1A, S1A) and not in 230 controls. This variant was also found in another sample of 80 GGE cases, in a patient with EGTCS phenotype and is present at low frequency (1/13,148 alleles) in the ExAC database.[33] Furthermore, of the five newly detected *HCN4* gene variants in our sample, two were not detected in the 230 tested controls, but one (p.P1117L) appeared in ExAC in 63/19,948 alleles (Table 1). The p.P1117L variant was detected in one of the two affected members of Family 1 carrying the *CACNA1H* p.G1158S variant and was inherited from the unaffected mother (Figs 1A, S1A). The second variant, p.E153G, was identified in only one of several affected members of Family 2 (Figs 1B, S1B). Alignments of sequences among different species revealed high amino acid conservation at positions 1158 and 153 within the Cav3.2 and HCN4 protein, respectively (Fig. 2A). Predicted localization of these variants within the affected channel proteins is shown in Fig. 2B.

In Family 1, the *CACNA1H* p.G1158S variant is inherited by both affected children from their father, who experienced syncope of unknown etiology. The child carrying only the *CACNA1H* mutation had generalized tonic-clonic seizures on awakening (GTCA) and absence seizures from 12 years of age. His sister, carrying both the *CACNA1H* and the *HCN4* variant, experienced GTCA starting from 26 years of age. The clinical phenotype in Family 2 included febrile, generalized tonic-clonic, and absence seizures, representative of $GEFS^+$. The *HCN4* mutation carrier had only one seizure at the age of 17. There were no cardiac pathologies or arrhythmias reported for the patient with syncope or any of the carriers of *HCN4* variants.

***CACNA1H* variant causes a gain-of-function**

Whole cell patch-clamp recordings in tsA201 cells revealed that, compared to the WT $Ca_V3.2$, the p.G1158S channels showed a significant increase in the current density

Table 1. Overview of *CACNA1H* and *HCN4* gene variants detected in this study, with highlighted analyzed variants[a]

Nr.	Exon	Nucleotide substitution	Amino acid substitution	Patients[b] wt:mut/wt:mut	Controls[b] wt:mut/wt:mut	ExAC[33]
CACNA1H						
1	4	c.450G>T	p.E150D	97:3:0	99:1:0	No
2	7	c.937A>G	p.M313V[48]	70:28:2	61:30:9	14726/90236 homozyg 1106
3	9	c.1919C>T	p.P640L[21]	45:49:6	47:36:17	10391/23896 homozyg 2026
4	9	c.1991T>C	p.V664A[21]	50:40:10	49:44:7	1322/5608 homozyg 149
5	10	c.2362C>T	p.R788C[21]	85:15:0	83:12:5	9548/107658 homozyg 525
6	**17**	**c.3472G>A**	**p.G1158S**	**98:2:0**	**230:0:0**	**1/13148 0 homozyg**
7	33	c.5612G>A	p.R1871Q[37]	78:21:1	88:11:1	2525/25780 107 homozyg
8	34	c.5897C>T	p.A1966V[37]	94:6:0	96:4:0	380/17716 13 homozyg
9	34	c.5921A>G	p.E1974G[37]	96:4:0	97:3:0	744/18188 27 homozyg
10	34	c.6013C>T	p.R2005C[21]	76:23:1	86:13:1	1358/14960 74 homozyg
11	35	c.6179G>A	p.R2060H[21]	77:21:2	73:25:2	9751/56274 718 homozyg
12	35	c.6230G>A	p.R2077H[21]	25:51:24	17:46:37	63097/95200 20660 homozyg
13	35	c.6322G>A	p.A2108T	98:2:0	99:1:0	141/96230 0 homozyg
HCN4						
1	1	c.107G>A	p.G36E	17:3:0	88:12:0	848/13832 28 homozyg
2	**1**	**c.458A>G**	**p.E153G**	**19:1:0**	**250:0:0**	**No**
3	8	c.2648C>G	p.P883R	19:1:0	95:5:0	892/101748 9 homozyg
4	8	c.3337A>G	p.M1113V	19:1:0	97:3:0	467/18868 2 homozyg
5	**8**	**c.3350C>T**	**p.P1117L**	**19:1:0**	**250:0:0**	**63/19948 0 homozyg**

[a]Analyzed variants are highlighted in bold type.
[b]Ratio of individuals carrying both WT, WT and mutant, and both mutant alleles.

at more depolarized potentials (Figs. 3A,B). Whereas no significant changes were found in the voltage dependence of activation, the steady-state inactivation curve of the mutant was shifted by +5 mV to more depolarized potentials in the presence of Ca^{2+} as charge carrier (Figs. 3A–C). There were no significant differences in the recovery from inactivation (Fig. 3D), or activation, inactivation, and deactivation kinetics between mutant and WT channels (Table S1). Thus, the p.G1158S mutation causes a small but significant gain-of-function.

HCN4 variants result in a loss-of-function

Robust currents could be recorded in *X. laevis* oocytes expressing WT channels and the p.E153G and p.P1117L epilepsy variants (Fig. 4A). Current amplitudes were not significantly different between the variants and the WT channel (Fig. 4B). However, conductance-voltage relationship revealed a left shift in activation for both the p.E153G and p.P117L relative to WT, consistent with a loss-of-function (Fig. 4C). Boltzmann functions fit to the data points confirm this, revealing a significant shift in $V_{1/2}$ for both GGE variants (Fig. 4C inset). Wild type and the p.E153G variant had a similar slope (WT = 7.3 ± 0.2, n = 21 vs. p.E153G = 7.5 ± 0.2, n = 19; p = 0.56), but the slope of the p.P1117L variant curve was significantly different from that of the WT (WT = 7.3 ± 0.2, n = 21 vs. p.P1117L = 8.1 ± 0.2, n = 30, p = 0.02). Time to half maximal activation of the two epilepsy variants was not different from the WT across a range of voltages (Fig. S2).

Discussion

We report here three functionally relevant novel variants in *CACNA1H* and *HCN4* that were found in four individuals with GGE, one of them carrying one variant in both genes. As expected from previous studies in mouse and

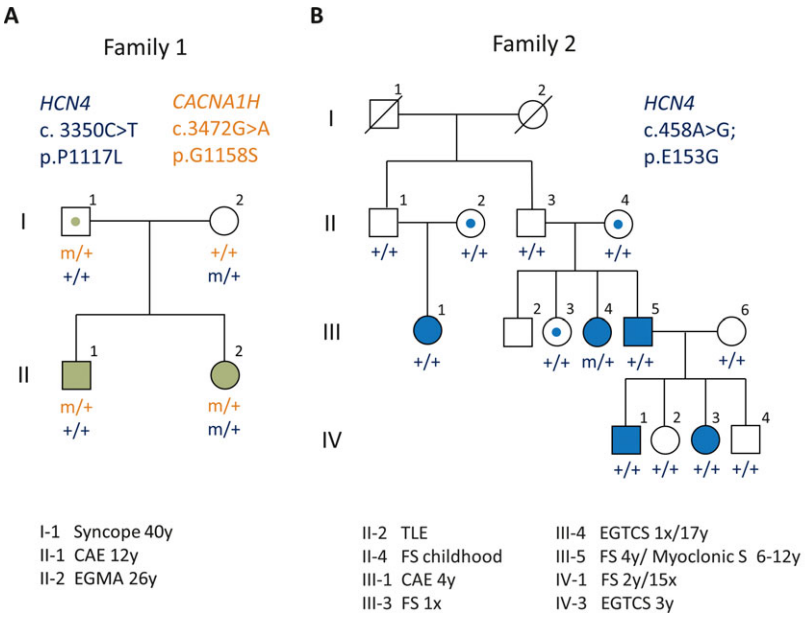

Figure 1.
Pedigrees of the two GGE families. (**A**) Pedigree of Family 1 showing a complete cosegregation of the *CACNA1H* variant (orange) with the GGE phenotype and the inheritance pattern of the *HCN4* variant (blue). (**B**) Pedigree of Family 2 in which the second *HCN4* mutation p.E153G was identified in a single patient; EGMA, epilepsy with grand mal seizures on awakening; TLE, temporal lobe epilepsy; FS, febrile seizures; CAE, childhood absence epilepsy; EGTCS, epilepsy with generalized tonic-clonic seizures.
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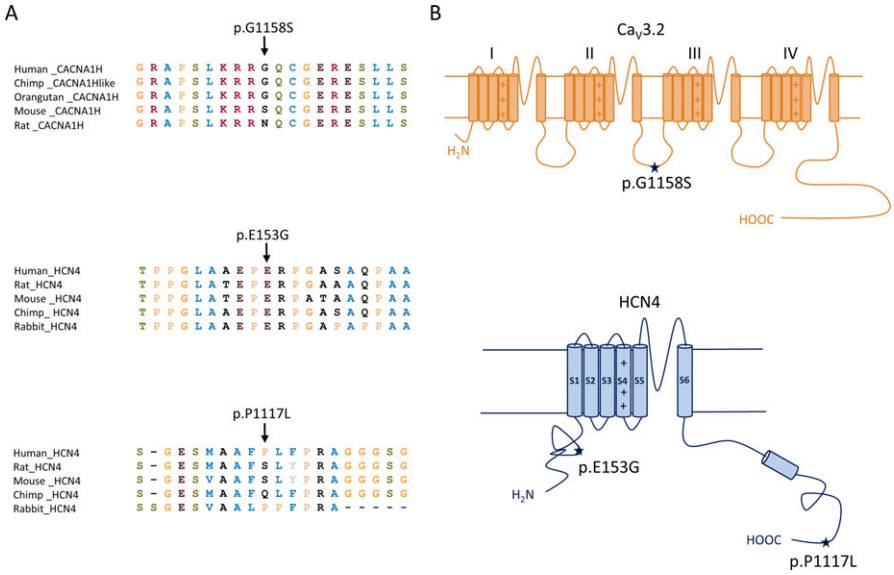

Figure 2.
Conservation and localization of affected amino acids. (**A**) Alignment of protein sequences showing the conservation of affected amino acids in Cav3.2 (encoded by *CACNA1H*) and HCN4 proteins among different species. (**B**) Predicted localization of detected variants within the Cav3.2 and HCN4 channel proteins.
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humans[18,30] and discussed further below, the *CACNA1H* variant caused a gain-of-function, whereas both *HCN4* variants caused a loss-of-function. The effects of variants were small, and they were found in only a single affected individual (*HCN4* variants) or in a small family with only one affected sib-pair (*CACNA1H* variant). Our results are consistent with the hypothesis of a polygenic disease model in which multiple variants that cause small-to-moderate

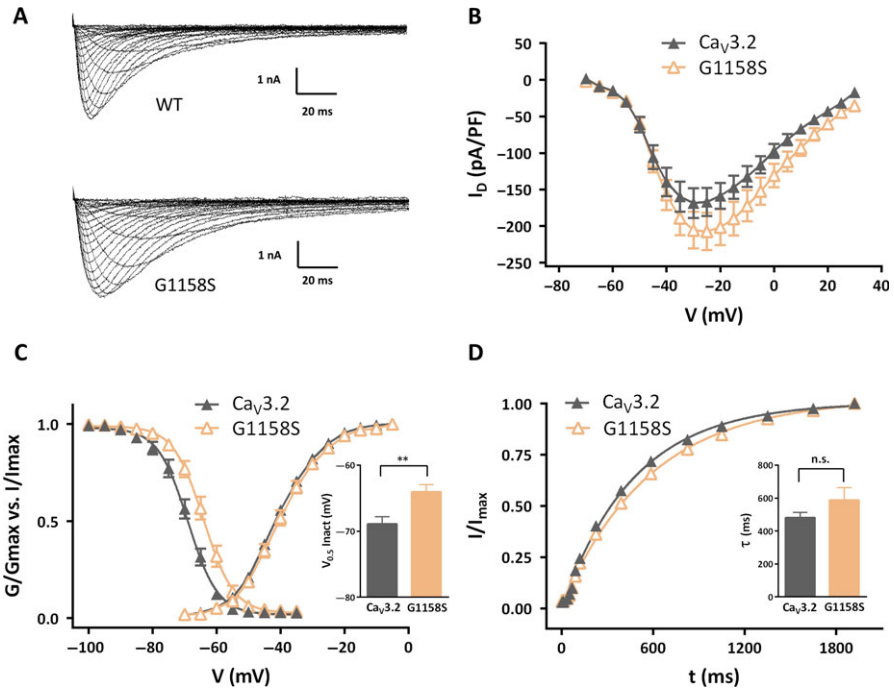

Figure 3.

Functional analysis of the *CACNA1H* variant in tsA201 cells. (**A**) Whole cell currents of WT Cav3.2 and G1158S channels elicited by depolarizing the membrane between −70 mV and 70 mV in 5-mV steps from a holding potential of −90 mV. (**B**) Current density for the Cav3.2 WT and G1158S mutant channels at different potentials. (**C**) Steady-state activation and inactivation for Cav3.2 WT and G1158S mutant channels obtained by standard protocols using a holding potential of −90 mV. Recordings were performed with Ca^{2+} as the charge carrier. Parameters for activation were as follows: WT – $V_{0.5}$ = −41.4 ± 0.8 mV, k = −6.7 ± 0.3 mV (n = 13); G1158S – $V_{0.5}$ = −40.8 ± 0.8 mV, k −1.3 ± 0.3 (n = 10). For the inactivation (inset), parameters were: WT – $V_{0.5}$ = −69.2 ± 1.1 mV, k = 4.3 ± 0.2 mV (n = 11); G1158S – $V_{0.5}$ = −64.1 ± 1.1 mV, k = −4.4 ± 0.1 (n = 7), **p < 0.01, unpaired t test. (**D**) Recovery from inactivation for Cav3.2 WT and G1158S mutant channels. Curves represent monoexponential fits to the averaged data (n = 4–12). Inset: mean time constant for recovery from inactivation for Cav3.2 WT and G1158S mutant, obtained from monoexponential fits to the normalized current recovery curve (n = 4–12, n.s. = not significant).

effects on protein function cumulatively contribute to epileptogenesis rather than any particular variant being disease causing in isolation. We, therefore, suggest that both *CACNA1H* and *HCN4* may act as susceptibility genes in GGE, in terms of additive contributions of subtle functional variations to overall seizure susceptibility.

The genetic architecture of GGE is yet to be fully explained. Although microdeletions present the most common genetic alteration predisposing to GGE,[10,13] large-scale genetic efforts are only beginning to better resolve the contribution of common and rare variants to GGE.[9,10,12,15] We acknowledge that small families tested here are insufficient to provide statistically valid genetic evidence and that the variants may have been discovered by chance alone. Once fully validated statistical models of GGE are developed, the implications of our results will become clearer.

Initial reports have described numerous *CACNA1H* variants associated with GGE,[19–22,34] particularly CAE. CAE is also a feature of the family harboring the *CACNA1H* p.G1158S mutation. However, recent large-scale studies of common and rare variants in GGE have not revealed a major impact of *CACNA1H* variants,[9,12,15] and *de novo* mutations in this gene have been linked to early-onset hypertension and primary aldosteronism.[35,36] This has prompted the reassessment of the role of *CACNA1H* in epilepsy. In this regard, our retrospective analysis of 19 previously identified *CACNA1H* variants found in patients and families presenting with different GGE forms[37] revealed the presence of all but two variants in the ExAC database. The allele frequency varied between 10^{-5}% and 10%, with the majority of variants (14/19) having frequency of <1%. No distinctive correlation between allele frequency and reported functional effects could be established.

The functional impact of variants in *CACNA1H* associated with GGE is variable, although a net gain-of-function, as found here, is generally considered to be the basis of increased excitability as observed in primary neuronal cultures recombinantly expressing a *CACNA1H* epilepsy-associated variant.[38] This is consistent with pharmacological and physiological data implicating T-type Ca^{2+} channels in GGE: (1) the first-line anti-absence drug ethosuximide

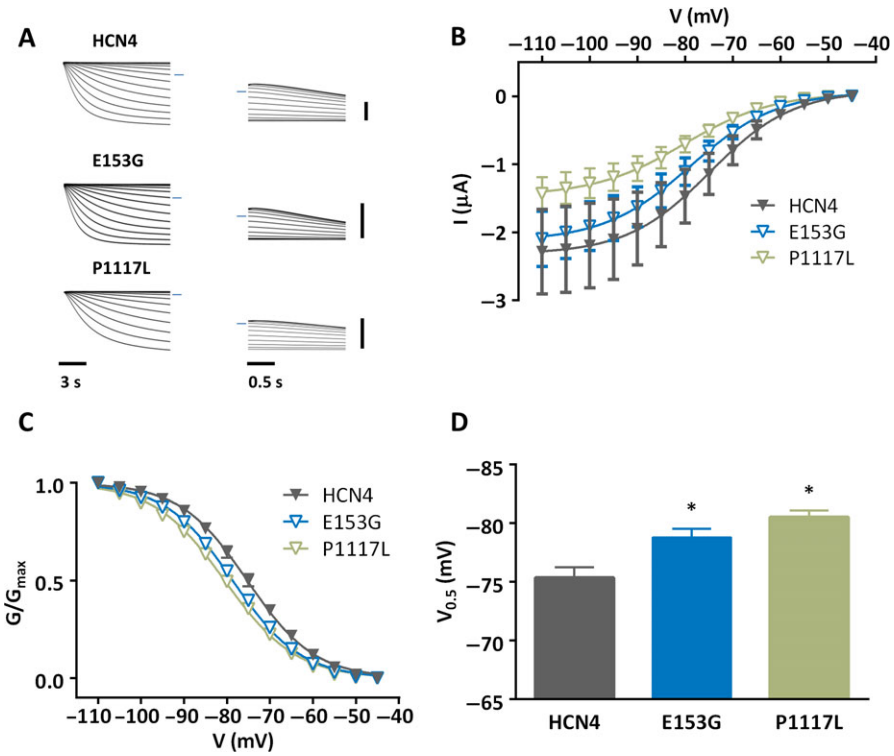

Figure 4.

Electrophysiological characterization of *HCN4* epilepsy variants. (**A**) Steady-state (left) and tail (right) currents from oocytes expressing HCN4 WT and E153G and P1117L epilepsy variants. Vertical scale bar represents 1 μA. (**B**) Average tail current–voltage (I–V) relationship of WT (n = 21), E153G (n = 19), and P1117L (n = 30) channels. (**C**) Activation curve constructed from average normalized tail currents and fit with the Boltzmann equation for HCN4 WT and E153G and P1117L variants. (**D**) Bar graph of the average half-activation voltage ($V_{0.5}$) for each situation.*$p < 0.05$, one-way ANOVA.

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blocks T-type Ca^{2+} channels, suggesting that increased channel function leads to hyperexcitability;[39] and (2) physiological experiments in rat models of GGE report increased T-type Ca^{2+} currents in reticular thalamic neurons.[40,41] Our results are therefore consistent with the idea that gain-of-function *CACNA1H* variants may contribute to neuronal hyperexcitability in GGE. Taken together, these data corroborate that *CACNA1H* is not a gene of major effect in GGE, but its variants might act as susceptibility factors contributing to epilepsy phenotypes.[37]

Several studies have implicated HCN channels in epilepsy,[26–29] but only limited evidence links HCN4 channels to increased neuronal network excitability. A seizure-associated up-regulation of HCN4 expression occurs in the rat pilocarpine model of temporal lobe epilepsy.[42] Furthermore, Paz and colleagues have shown in a cortical stroke model that thalamo-cortical neurons switch from a predominant HCN2 to a predominant HCN4 channel expression.[43] Whether these changes in HCN4 expression are part of the pathogenic mechanism is not clear. Here we describe two *HCN4* variants that cause a hyperpolarizing shift in the voltage dependence of activation resulting in reduced function within a physiologically relevant voltage range. Investigations in the *HCN2* knockout mouse, which displays SWDs, reveal that thalamo-cortical neurons are more hyperpolarized because of a reduction in Ih.[44] The more-hyperpolarized resting membrane potential increases the availability of T-type Ca^{2+} channels and subsequent burst firing, a hallmark of SWDs.[45] Future experiments are required to determine whether HCN4 channels can contribute to excitability in a similar manner.

Unraveling the genetic architecture of GGE is possibly complicated by its polygenic nature. Computational studies have suggested that multiple small changes in the biophysical properties of ion channels that alone cannot alter network excitability can in concert cause aberrant network hyperexcitability.[46] We assume that all variants studied here are contributing factors to GGE in individual patients and that even in larger families each affected member may present with a different combination of genetic alterations, which in sum could cause the disease.

Genetic alterations in *HCN4* have been linked to cardiac disease. In all reported cases, genetic variants associated with dysrhythmia cause a loss-of-function.[47] Our data demonstrate for both *HCN4* variants a subtle hyperpolarizing shift in activation, resulting in loss-of-function, but

cardiac disease was not observed in either family. The subtle effect of mutations on HCN4 function may not be sufficient to cause dysrhythmia in these families. In addition, the fact that epilepsy was not reported in patients with cardiac dysrhythmias[47] suggests that *HCN4* variant alone is unlikely to cause epilepsy, but rather presents a modifier.

In conclusion, we identified and characterized variants in *CACNA1H* and *HCN4* as putative susceptibility factors in GGE. Alterations in these two genes may work in concert to increase neuronal network excitability in thalamo-cortical networks and contribute to seizure susceptibility in the affected individuals.

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Disclosure

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Clinical picture.

Appendix S2. Supplementary Methods.

Table S1. Time constants of activation, inactivation, deactivation, and recovery from inactivation at indicated membrane potentials.

Figure S1. Chromatograms presenting the Sanger sequencing results for the affected genes in Family 1 (A) and Family 2 (B).

Figure S2. Time to half maximal activation for WT and the two *HCN4* epilepsy variant channels across a range of voltages.