clear cells of patients with AD,[19,20] implicate the inhibition of brain IL-1 as a novel mechanism of action for the beneficial effect of AChE inhibitors in AD.

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Effects of $Ca_v3.2$ Channel Mutations Linked to Idiopathic Generalized Epilepsy

Houman Khosravani, MSc,[1] Christopher Bladen, BSc,[1,2] David B. Parker, PhD,[2] Terrance P. Snutch, PhD,[3] John E. McRory, PhD,[1] and Gerald W. Zamponi, PhD[1]

Heron and colleagues (Ann Neurol 2004;55:595–596) identified three missense mutations in the Cav3.2 T-type calcium channel gene (CACNA1H) in patients with idiopathic generalized epilepsy. None of the variants were associated with a specific epilepsy phenotype and were not found in patients with juvenile absence epilepsy or childhood absence epilepsy. Here, we introduced and functionally characterized these three mutations using transiently expressed human Cav3.2 channels. Two of the mutations exhibited functional changes that are consistent with increased channel function. Taken together, these findings along with previous reports, strongly implicate CACNA1H as a susceptibility gene in complex idiopathic generalized epilepsy.

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A hallmark of generalized epileptic disorders is the generation of synchronous spike-wave discharges (SWDs) recorded bilaterally over both brain hemispheres at seizure onset. Idiopathic generalized epilepsy (IGE) refers to a spectrum of generalized epilepsies such as childhood absence (CAE), juvenile absence (JAE), and juvenile myoclonic epilepsies (JME).[1] Upon seizure onset,

From the [1]Cellular and Molecular Neurobiology Research Group, Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta; [2]NeuroMed Technologies, Inc.; and [3]Michael Smith Laboratories, University of British Columbia, Vancouver, British Columbia, Canada.

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Address correspondence to Dr Zamponi, Department of Physiology and Biophysics, University of Calgary, 3330 Hospital Drive NW, Calgary, T2N 4N1, Canada. E-mail. zamponi@ucalgary.ca

electroencephalogram recordings show bilateral SWDs in the range of 3 to 6Hz. There usually are no radiological findings and patients are otherwise neurologically normal. A strong genetic component is known to be involved in IGEs, and a family history of epilepsy can be as high as 40% in cases with JME.[2] Almost all of the idiopathic epilepsies with a known molecular basis are channelopathies involving either voltage-gated (eg, potassium, sodium, and calcium) or ligand-gated ($GABA_A$) ion channels.[3]

Generalized seizures and SWD generation are believed to involve interactions between thalamic and cortical structures. However, recent evidence suggests that the neocortex is the first structure involved at seizure onset with rapid recruitment of thalamic structures.[4,5] Upon seizure initiation, the cortex and thalamus engage in a complex interplay that underlies SWDs,[4] which are mechanistically known to involve the actions of T-type calcium channels[6,7] in the form of low-threshold calcium spikes. There are three known genes (subtypes) encoding for different T-type channels ($Ca_v3.1$–$Ca_v3.3$) which exhibit distinct biophysical characteristics.[8] The thalamus expresses predominantly the $Ca_v3.1$ T-type channel isoform, whereas the neocortex appears to express multiple T-type channel subtypes, including $Ca_v3.2$.

The role of T-type channels in SWD epilepsies is highlighted by the anticonvulsive effects of ethosuximide, an inhibitor of T-type Ca^{2+} currents, in the treatment of absence seizures.[9] Furthermore, thalamic neurons from transgenic mice lacking a subtype of T-type channels fail to fire in burst mode, and these mice are resistant to pharmacologically induced absence seizures.[10] In a related study, mice lacking P/Q-type Ca^{2+} channels also experience absence seizures with 3 to 5Hz SWDs, which can be abolished with an additional knockout of the $Ca_v3.1$ T-type channel.[11] Increased T-type channel expression also has been observed in a rat strain (GEARS) that exhibits spontaneous absence seizures.[12]

A recent study by Heron and colleagues[13] has identified three missense mutations and a single nonsense mutation in the *CACNA1H* ($Ca_v3.2$) T-type calcium channel gene in 9 of 192 patients with IGE that were not found in control subjects. Furthermore, in a previous study by Chen and colleagues,[14] 12 different missense mutations were found in the *CACNA1H* gene in 14 of 118 children with CAE, and the functional effects on ion channel gating properties were demonstrated by our group.[15] In this study, we have generated each of the three missense mutations reported by Heron and colleagues[13] and have characterized their functional consequences using exogenous expression of the wild-type and mutated human $Ca_v3.2$ T-type channels in HEK293 (tsA-201) cells. We find that the mutations result in small, but statistically significant, changes in the activation and inactivation rates of the channel. Given that T-type calcium channels are expressed both in the thalamus and neocortex, these functional effects may account for altered seizure thresholds in patients with IGE.

Materials and Methods

Experimental protocols have been described previously in detail.[15] Site-directed mutagenesis of $Ca_v3.2$ in pcDNA-3 was conducted via QuickChange mutagenesis (Stratagene, La Jolla, CA), using the entire $Ca_v3.2$-pcDNA-3 as the template plasmid followed by sequencing of the entire coding sequence.

Tissue culture and transfection of tsA-201 cells was described by us previously in detail.[15,16] $Ca_v3.2$ channel subunits (8μg) and eGFP marker (1μg) cDNA were transfected into cells via the calcium phosphate method. For recording, cells were bathed in a solution containing 5mM barium.[15] Microelectrodes were pulled (2–4MΩ) and filled with a 108mM $CsCH_3SO_4$ internal solution.[15] Data were acquired at room temperature using an Axopatch 200B amplifier and pClamp 9.0. Series resistance was compensated to 80%. Data are plotted as mean ± standard error of the mean, and statistical analyses were conducted using analysis of variance where p value less than 0.05 was considered as significant.

Results and Discussion

We introduced the three recently identified missense mutations (A480T, P618L, G755D, all of which are located in the I-II linker region of the channel) into human $Ca_v3.2$ cDNA. Each of the mutant channels expressed well in HEK tsA-201 cells, exhibited current densities similar to those obtained for the wild-type channel, and produced current waveforms typically observed with expressed T-type channels (Fig 1A). None of the mutations mediated significant differences in the shape or position of the current-voltage (I-V) relation (see Fig 1B, D) or changes in voltage-dependence of inactivation (see Fig 1C, D). In neurons, we would expect the variant channels to activate and inactivate at similar membrane potentials as compared with the wild-type $Ca_v3.2$ channel.

The time course of activation was significantly accelerated in the P618L mutant at potentials more positive than −30mV (Fig 2A), suggesting that this variant might conduct greater inward current during brief membrane depolarizations. Both P618L and G755D exhibited significantly altered rates of inactivation. For depolarizations to modest membrane potentials (ie, −20mV), the time course of inactivation was significantly accelerated (see Fig 2B), whereas that of the A480T mutant was not. This suggests that the P618L mutant is able to both activate and inactivate faster in response to changes in membrane potential, thereby allowing for greater channel availability in response to varying depolarizations above −20mV when compared with the wild type. Recovery from inactivation was not

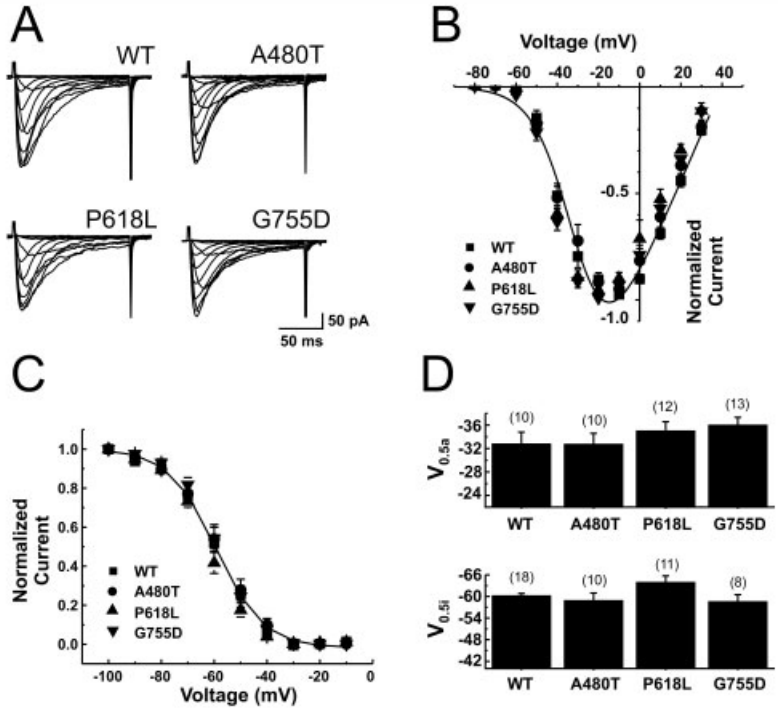

Fig 1. (A) Families of raw current traces obtained with wild-type (WT) and mutant (A480T, P618L, and G755D) $Ca_v3.2$ channels. The currents were elicited by stepping from a holding potential of −110mV to a set of test potentials. (B) Ensemble of whole-cell current-voltage (I-V) relations obtained with wild-type and mutant $Ca_v3.2$ channels. Each individual current voltage relation was normalized to a peak value of one, and the data points reflect means of the normalized amplitudes. The solid line is a fit using the Boltzmann equation to the wild-type data. (C) Ensemble steady state inactivation curves obtained with wild-type and mutant $Ca_v3.2$ channels. The currents were elicited by inactivating the channel population (−10mV for 1.5 seconds) followed by a step to the peak activation current. The line is a fit with the Boltzmann relation to the wild-type data. Mean half inactivation potentials are obtained from fits to individual state inactivation curves. (D) Mean half-activation ($V_{0.5a}$) and half-inactivation ($V_{0.5i}$) potentials obtained with wild-type and mutant channels. The half-activation potentials were determined via Boltzmann fits to individual whole-cell current voltage relations. Numbers of cells recorded are denoted in parentheses.

significantly affected by any of the mutations (see Fig 2C). Taken together, two of the three mutants investigated exhibited relatively small, albeit statistically significant altered channel kinetics, which in a native neuronal environment likely would contribute to altered firing behavior.

Although we did not investigate the result of the nonsense mutation reported by Heron and colleagues,[13] expression of this mutant channel results in a premature termination within the I-II linker, resulting in the translation of only $Ca_v3.2$ domain I, and thus likely a nonfunctional channel. We note that transfection of domain I of $Ca_v3.2$ into NG108-15 cells results in an approximately 50% reduction in native T-type channel amplitude.[17] This suggests the possibility that a similar dominant negative effect might occur in thalamic or cortical neurons.

Results in this study showing relatively minor biophysical effects of channel mutations associated with human disease are not without precedent. For example, several mutations in the $Ca_v2.1$ P/Q-type calcium channel found in patients with familial hemiplegic migraine do not appear to obviously alter channel function.[18] A recent study on the same channel has identified a novel mutation (E147K) associated with absence epilepsy that segregates in an autosomal dominant fashion in successive generations; yet, no changes in gating were observed as result of this mutation. However, coexpression of the mutant with the native channel resulted in slightly decreased peak current amplitudes.[19] Finally, in our previous study, examining mutations in the $Ca_v.3.2$ T-type channel associated with CAE,[15] two of the five mutations investigated resulted in no detectable gating effects.

Genetic association studies have improved greatly in identifying genes and mutations therein involved in disease processes. In the context of idiopathic epilepsies, most of the ion channel defects that have been identified typically account for a small fraction of families with sporadic cases presenting with the specific syndrome in question.[3] This suggests that ion channel variants with large biophysical effects may account for a subset of individuals with the polygenic substrate associated with the idiopathic epilepsies. The reported

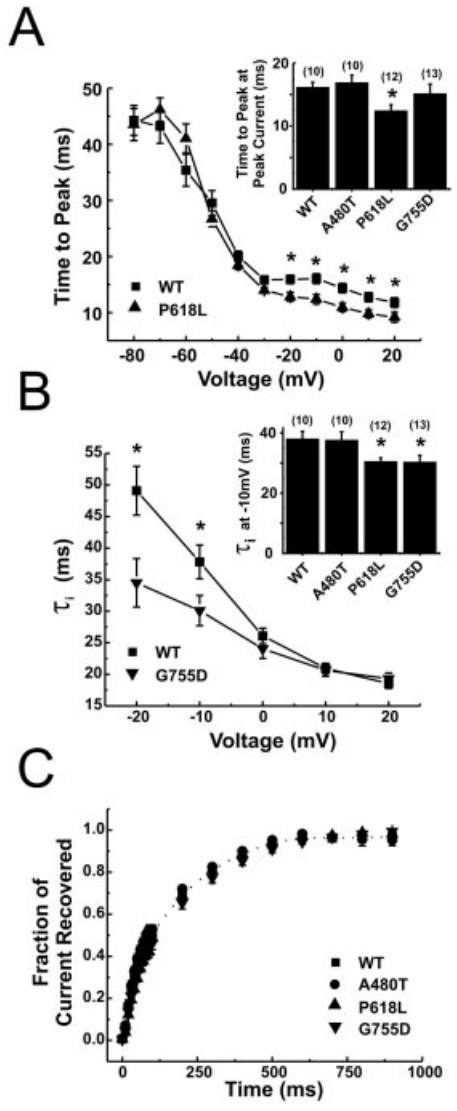

Fig 2. (A) Time to peak for wild-type (WT) and P618L mutant $Ca_v3.2$ channels at various test potentials; asterisks *denote statistically significant deviations (*$p < 0.05$, t *test).* (inset) *Mean time to peak values obtained for wild-type and mutant $Ca_v3.2$ channels at the peak voltage of the I-V relation. P618L shows a statistically significant decrease in mean time to peak (*$p < 0.05$, *analysis of variance). (B) Time constants of inactivation for wild-type and G755D mutant $Ca_v3.2$ channels, obtained from mono-exponential fits to raw current traces at various test potentials.* (inset) *Mean time constant of inactivation obtained at a test potential of −10mV (peak current) for wild-type and mutant $Ca_v3.2$ channels. P618L and G755D show a statistically significant decrease in inactivation time constants. (C) Recovery from inactivation for wild-type and mutant $Ca_v3.2$ channels, normalized to 1 (full current recovered). No statistically significant differences were observed in mean time constants for recovery. Asterisks denote statistical significance relative to the wild type (*$p < 0.05$, *analysis of variance). Numbers of cells recorded are denoted in parentheses.*

mutations in $Ca_v3.2$ are an example of this, whereas in the study by Heron and colleagues[13] none of the CAE patients (34 of 192) exhibited any of the mutations previously reported by Chen and colleagues.[14] Nevertheless, this suggests that the *CACNA1H* gene is an active locus involved in the IGEs. It is also important to consider the possibility of additive effects of genetic variation, perhaps within the same ion channel family and subtype, as likely to underlie the common forms of IGE. Alternatively, an explanation for the presence of a seizure disorder in patients may be caused by changes in brain development as a result of the mutations and not directly causal to detectable biophysical changes.

In summary, our data constitute only the second report on functional changes attributable to naturally occurring mutations in T-type calcium channels. The discovery of these mutations in a subset of affected individuals, but not in a larger number of control subjects, suggests that their presence is functionally significant in relation to the diseased state. These mutations and their functional changes may act synergistically with other factors such as other ion channels and intracellular modulators, all of which are capable of a spectrum of activity modes in the epileptic brain. Thus, it remains to be determined as to whether the physiological effects of $Ca_v3.2$ T-type calcium channel mutations are linked directly to SWD generation in structures such as the neocortex and thalamus that are known to be involved in the idiopathic epilepsies.

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Severe Neuropathy with Leaky Connexin32 Hemichannels

Grace S. Lin Liang, MD,[1] Marta de Miguel, MB,[2] Juan M. Gómez-Hernández, PhD,[2] Jonathan D. Glass, MD,[3] Steven S. Scherer, MD, PhD,[4] Mark Mintz, MD,[5] Luis C. Barrio, MD, PhD,[2] and Kenneth H. Fischbeck, MD[6]

X-linked Charcot-Marie-Tooth disease is one of a set of diseases caused by mutations in gap junction proteins called connexins. We identified a connexin32 missense mutation (F235C) in a girl with unusually severe neuropathy. The localization and trafficking of the mutant protein in cell culture was normal, but electrophysiological studies showed that the mutation caused abnormal hemichannel opening, with excessive permeability of the plasma membrane and decreased cell survival. Abnormal leakiness of connexin hemichannels is likely a mechanism of cellular toxicity in this and perhaps other diseases caused by connexin mutations.

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At least 10 different human genetic diseases are caused by mutations in connexin genes, which encode the protein subunits of gap junction channels. Many of these diseases are caused by a loss of connexin function. Others are dominantly inherited, consistent with a toxic effect of the mutations. X-linked Charcot-Marie-Tooth disease (CMTX), a hereditary neuropathy characterized by progressive muscle weakness and atrophy, sensory loss, and reduced nerve conduction velocities,[1] is caused by mutations in connexin32 (Cx32).[2,3] Male subjects usually develop symptoms during adoles-

From the [1]Department of Neurology, Parkinson's Disease and Movement Disorders Center, Department of Neurology, Penn Neurological Institute, University of Pennsylvania Medical Center, Philadelphia, PA; [2]Unit of Experimental Neurology, Department of Research, Hospital, Madrid, Spain; [3]Department of Neurology, Emory University School of Medicine, Whitehead Biomedical Research Building, Atlanta, GA; [4]Department of Neurology, University of Pennsylvania Medical Center, Philadelphia, PA; [5]Bancroft Neurosciences Institute, Cherry Hill, NJ; and [6]Neurogenetics Branch, National Institute of Neurological Disease and Stroke, National Institutes of Health, Bethesda, MD.

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Address correspondence to Dr Liang, Parkinson's Disease and Movement Disorders Center, Department of Neurology, Penn Neurological Institute, 330 South Ninth Street, Philadelphia, PA 19107. E-mail: liangg@pahosp.com

