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A novel de novo mutation of *SCN8A* ($Na_v1.6$) with enhanced channel activation in a child with epileptic encephalopathy

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ABSTRACT

Rare de novo mutations of sodium channels are thought to be an important cause of sporadic epilepsy. The well established role of de novo mutations of sodium channel *SCN1A* in Dravet Syndrome supports this view, but the etiology of many cases of epileptic encephalopathy remains unknown. We sought to identify the genetic cause in a patient with early onset epileptic encephalopathy by whole exome sequencing of genomic DNA. The heterozygous mutation c. 2003C>T in *SCN8A*, the gene encoding sodium channel $Na_v1.6$, was detected in the patient but was not present in either parent. The resulting missense substitution, p.Thr767Ile, alters an evolutionarily conserved residue in the first transmembrane segment of channel domain II. The electrophysiological effects of this mutation were assessed in neuronal cells transfected with mutant or wildtype cDNA. The mutation causes enhanced channel activation, with a 10 mV depolarizing shift in voltage dependence of activation as well as increased ramp current. In addition, pyramidal hippocampal neurons expressing the mutant channel exhibit increased spontaneous firing with PDS-like complexes as well as increased frequency of evoked action potentials. The identification of this new gain-of-function mutation of $Na_v1.6$ supports the inclusion of *SCN8A* as a causative gene in infantile epilepsy, demonstrates a novel mechanism for hyperactivity of $Na_v1.6$, and further expands the role of de novo mutations in severe epilepsy.

Introduction

Early-onset epileptic encephalopathy is a heterogenous disorder characterized by seizures, developmental delay and progressive loss of neurological function. Since most patients lack a family history, linkage studies have not been feasible. Whole genome and exome sequencing has provided important tools for identifying the genetic basis of this devastating disorder. We identified and functionally validated a gain-of-function mutation in *SCN8A* in a patient with epileptic encephalopathy and SUDEP (Veeramah et al., 2012). This de novo mutation, p.Asn1768Asp in sodium channel $Na_v1.6$, results in impaired channel inactivation and neuronal hyperactivity. We now describe the distinct functional consequence of a second de novo mutation of *SCN8A* identified in a patient with a more severe epileptic encephalopathy. Since our initial report, large-scale exome sequencing projects have identified eleven de novo mutations of *SCN8A* in singleton patients with epilepsy and/or intellectual disability (Carvill et al., 2013; Epi4K Consortium et al., 2013; Rauch et al., 2012; reviewed in O'Brien and Meisler, 2013). Another de novo mutation of *SCN8A* was recently identified in a child with epileptic encephalopathy and congenital anomalies (Vaher et al., 2013). Since genetic evidence of linkage is not possible for de novo mutations, functional studies and analysis of evolutionary conservation and population frequency can contribute to assessing the pathogenicity of these mutations.

In addition to *SCN8A*, mutations associated with epilepsy have been identified in *SCN1A*, *SCN2A*, and *SCN3A*, the other pore-forming α-subunits of voltage-gated sodium channels that are expressed at high levels in the CNS (Helbig and Lowenstein, 2013; Kullmann, 2010; Lerche et al., 2013; Meisler et al., 2010). De novo mutations of *SCN1A* account for more than 80% of Dravet Syndrome, and inherited mutations contribute to generalized epilepsy with febrile seizures plus (Hirose et al., 2013; Meisler et al., 2010). Inherited and de novo mutations of *SCN2A* have been linked to benign familial neonatal-infantile seizures

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and other epileptic disorders (Shi et al., 2012). Gain-of-function mutations in *SCN3A* have been associated with cryptogenic partial epilepsy (Estacion et al., 2010; Holland et al., 2008; Vanoye et al., 2013).

$Na_v1.6$ is widely expressed in CNS and PNS neurons (Caldwell et al., 2000). Within neurons, $Na_v1.6$ is concentrated at the axon initial segment in both excitatory (Liu et al., 2013; Ogiwara et al., 2013; Oliva et al., 2012) and inhibitory neurons (Lorincz and Nusser, 2008), where it plays a critical role in regulating neuronal excitability (Hu et al., 2009; Lorincz and Nusser, 2008; Vacher et al., 2008). Consistent with this role in neuronal excitability, many types of neurons from *Scn8a* null mice lacking $Na_v1.6$ exhibit reduced excitability, including retinal ganglion cells, hippocampal CA1 cells, and cerebellar Purkinje cells (Raman et al., 1997; Royeck et al., 2008; Van Wart and Matthews, 2006; reviewed in O'Brien and Meisler, 2013). $Na_v1.6$. is also the major sodium channel at mature nodes of Ranvier (Boiko et al., 2001; Caldwell et al., 2000; Kaplan et al., 2001; Lorincz and Nusser, 2010), and reduced $Na_v1.6$ results in slowed nerve conduction velocity (Kearney et al., 2002). $Na_v1.6$ produces a tetrodotoxin-sensitive (TTX-S) fast-inactivating current (Sangameswaran et al., 1997; Smith et al., 1998), a persistent current (Rush et al., 2005; Smith et al., 1998), and a resurgent current (Cummins et al., 2005; Raman et al., 1997) and has been associated with rapid burst firing in cerebellar Purkinje neurons (Khaliq et al., 2003; Raman et al., 1997). *Scn8a* null mice exhibit juvenile lethality, demonstrating the essential role of $Na_v1.6$ in vivo (Burgess et al., 1995; Meisler et al., 2004; Sharkey et al., 2009). We report here the identification and functional validation of a de novo $Na_v1.6$ mutation in a patient with epileptic encephalopathy. Our data support the inclusion of *SCN8A* as a causative gene in infantile epilepsy.

Methods

Exome sequencing

Exome sequencing was carried out by the Medical Genetics Laboratories/Whole Genome Laboratory at Baylor College of Medicine (www.bcm.edu/geneticlabs). Variants were analyzed using in-house developed software. A relevant variant of unknown significance (VUS) in the *SCN8A* gene was reported based on previously published reports implicating this gene in epileptic encephalopathy (Veeramah et al., 2012) and intellectual disability (Trudeau et al., 2006).

Generation and expression of the mutant cDNA

The de novo *SCN8A* mutation c. 2300C>T (p.Thr767Ile) was introduced into the tetrodotoxin (TTX)-resistant derivative of the $Na_v1.6$ cDNA clone $Na_v1.6_R$ (Herzog et al., 2003). In this cDNA, the amino acid substitution Y371S confers TTX-resistance and makes it possible to specifically study $Na_v1.6$ in a biologically appropriate neuronal cell background (Dib-Hajj et al., 2009). Comparisons between TTX-resistant wildtype and mutant cDNAs have facilitated the delineation of biophysical changes produced by human mutations in $Na_v1.3$, $Na_v1.6$ and $Na_v1.7$ (Dib-Hajj et al., 2013; Estacion et al., 2010; Veeramah et al., 2012).

After site-directed mutagenesis to introduce the p.Thr767Ile mutation, the 6-kb open reading frame of the cDNA was completely sequenced to confirm the absence of additional mutations. Expression of the mutant protein was confirmed by transfection into HEK293 cells and analysis by Western blot with rabbit polyclonal anti-$Na_v1.6$ (Alomone ASC-009, 1:100). To evaluate the functional consequences of the p.Thr767Ile (T767I) mutation, the wildtype and mutant cDNAs were tested in the neuronal cell line ND7/23 and in transfected hippocampal pyramidal neurons, as previously described (Sharkey et al., 2009; Veeramah et al., 2012).

Voltage-clamp recording

Forty-eight hours after transfection, ND7/23 cells with robust green fluorescence were selected for recording. In the presence of 300 nM TTX, endogenous sodium currents are blocked (Sharkey et al., 2009; Wittmack et al., 2004) and currents derived from $Na_v1.6_R$ or T767I channels can be studied in isolation. Whole-cell voltage-clamp recordings were performed at room temperature (20–22 °C) using an HEKA EPC800 amplifier (HEKA Electronik). Fire-polished electrodes (0.6–1.3 MΩ) were fabricated from borosilicate glass micropipettes (World Precision Instruments). The pipette potential was adjusted to zero before seal formation, and liquid junction potential was not corrected. Capacity transients were canceled and voltage errors were minimized with 60–90% series resistance compensation. Currents were acquired with Clampex 10.2 at 5 min after establishing whole-cell configuration, sampled at a rate of 50 kHz, and filtered at 10 kHz. Activation and steady-state fast-inactivation of channels were obtained as previously described (Sharkey et al., 2009).

Current-clamp recording in transfected hippocampal neurons

Animal protocols complied with NIH guidelines, and were approved by the VA-Connecticut Healthcare System Animal Use Committee. Sprague–Dawley rat pups of either sex between postnatal days 10 (P10) and 14 (P14) were deeply anesthetized by ketamine/xylazine (100/10 mg/kg, i.p.) and killed by decapitation. Hippocampal neurons were isolated, transfected and recorded as previously described (Estacion et al., 2010; Veeramah et al., 2012). Recordings were performed on transfected hippocampal pyramidal neurons based on the morphology of small diameter (10–15 μm) triangular cell bodies that also exhibited green fluorescence. All recordings were performed between 40 h and 50 h post-transfection. Whole-cell current-clamp recordings were performed using the HEKA EPC800 amplifier, digitized using the Digidata 1440A interface and controlled using pCLAMP software, as described previously (Estacion et al., 2010). HEKA EPC-800 patch-clamp amplifier provides a low-frequency voltage-clamp (LFVC) circuit. LFVC is a mode in which a target resting membrane potential (−80 mV in this study) is specified; the clamp injects a continually updated holding current during a current-clamp recording to maintain the neuron resting potential near the target value.

Data analysis

Data were analyzed using Clampfit 10.2 (Molecular Devices) and Origin 8.5 Pro (Microcal Software), and presented as means ± SE. The Mann–Whitney nonparametric test was used to analyze current density data. Student's t test, except where ANOVA is specifically noted, was used to assess the statistical significance in characteristics of WT $Na_v1.6_R$ and the p.Thr767Ile mutant channels.

Results

Clinical description

The proband exhibits profound developmental delay, intellectual disability and intractable epilepsy. Myoclonic jerks and stiffness were observed in the neonatal period after an uncomplicated full term birth. Hyperekplexia was confirmed by EEG and clonazepam treatment was initiated. Seizures began at two weeks of age, when an EEG showed multifocal epileptiform activity with a normal background and the MRI was normal. An extensive genetic and metabolic workup was negative. Daily tonic and myoclonic seizures continue, in addition to rare tonic clonic seizures. At two years of age, an EEG revealed multifocal epileptiform activity with background slowing, and an MRI detected delayed myelination. The longest seizure-free period has been 2 weeks. The patient was first treated with levetiracetam, with subsequent addition

of phenobarbital. Addition of lacosamide resulted in some improvement. After a recent hospital admission for increased seizures, oxcarbazepine was added.

On physical examination at three years of age, the patient is minimally responsive with no obvious dysmorphic features. There is profound central hypotonia with some spasticity in the extremities. Reflexes are 3+ with ankle clonus and the patient can lift extremities against gravity spontaneously. The patient does not roll over, has complete head lag, and does not fixate or track. Feeding is by gastric tube. There have been multiple hospital admissions for pneumonia.

Mutation identification

There was no family history of seizures or developmental delay and there are two healthy older siblings. Genetic testing for mutations in the glycine receptor subunits GLRA1 and GLRB and the Rett Syndrome gene *MECP2* were negative. Whole exome sequencing was carried out in the Medical Genetics Laboratories at the Baylor College of Medicine and revealed a heterozygous c.2300C>T variant in the *SCN8A* gene resulting in the novel protein variant p.Thr767Ile in $Na_v1.6$. The mutation was confirmed by Sanger Sequencing. Examination of maternal and paternal DNA by Sanger Sequencing did not detect the variant, indicating that it results from a de novo mutation, although the possibility of mosaicism in either parent cannot be eliminated. The mutated threonine residue is located within the first transmembrane segment of domain II of the channel (DII/S1; Fig. 1A). This residue is evolutionarily conserved in vertebrate orthologs and invertebrate homologs of *SCN8A* (Fig. 1B). Threonine 767 is invariant in all of the human sodium channels, which diverged from a common ancestor more than 400 million years ago (Zakon et al., 2011) (Fig. 1C). The extensive evolutionary conservation, the location of the mutation in a transmembrane segment, and the replacement of the polar threonine residue with a bulkier hydrophobic side chain, predict a deleterious effect of this mutation on channel activity. To test this prediction, the mutation was introduced into the $Na_v1.6$ cDNA for functional characterization.

Voltage-clamp recordings of wildtype $Na_v1.6$ and T767I currents

Whole-cell voltage-clamp recordings were performed on ND7/23 cells expressing either wild-type $mNa_v1.6_R$ (WT) or $mNa_v1.6_R$-T767I (T767I). The activation properties of the currents expressed by these cells were tested by holding the cells at −120 mV and then applying 100 msec test pulses to potentials between −80 and +50 mV in 5 mV increments. Representative inward currents recorded by this protocol are shown for WT and T767I channels (Fig. 2A). The current–voltage plots derived from peak inward responses from individual cells were averaged together and shown in Fig. 2B. Cells with a peak amplitude >500 pA were included in measuring current density. The peak current density for T767I channels (−64 ± 10 pA/pF, n = 14) was reduced approximately 2.5-fold compared to WT channels (−167 ± 67 pA/pF, n = 11).

Voltage-dependence of activation and fast-inactivation

The voltage-dependence of activation and fast-inactivation was examined by transforming the peak current versus voltage (I–V) curves into conductance versus voltage (G–V) and fit to a Boltzmann function which directly gives the voltage midpoint ($V_{1/2}$) as well as the slope factor (k) of the voltage-dependent response. The Boltzmann fits for both activation and fast-inactivation were derived for each cell individually and then averaged. The averages of the normalized G–V curves for activation are significantly different with the $V_{1/2Act}$ of T767I shifted by 10.8 mV in a hyperpolarized direction (WT, n = 11: $V_{1/2}$ = −17.9 ± 1.3 mV, k = 7.3 ± 0.2; T767I, n = 14: $V_{1/2}$ = −28.7 ± 1.2 mV, k = 6.8 ± 0.6, $p < 0.001$) as shown in Fig. 2C. The average of the fits for fast-inactivation revealed similar properties for WT and T767I channels (WT, n = 11: $V_{1/2}$ = −65.9 ± 1.4 mV, k = 6.0 ± 0.4; T767I, n = 14: $V_{1/2}$ = −64.8 ± 1.6 mV, k = 7.0 ± 0.6) (Fig. 2D). The 10 mV shift in voltage dependence of activation is sufficiently large to predict significantly increased excitability of neurons expressing the mutant channel in vivo.

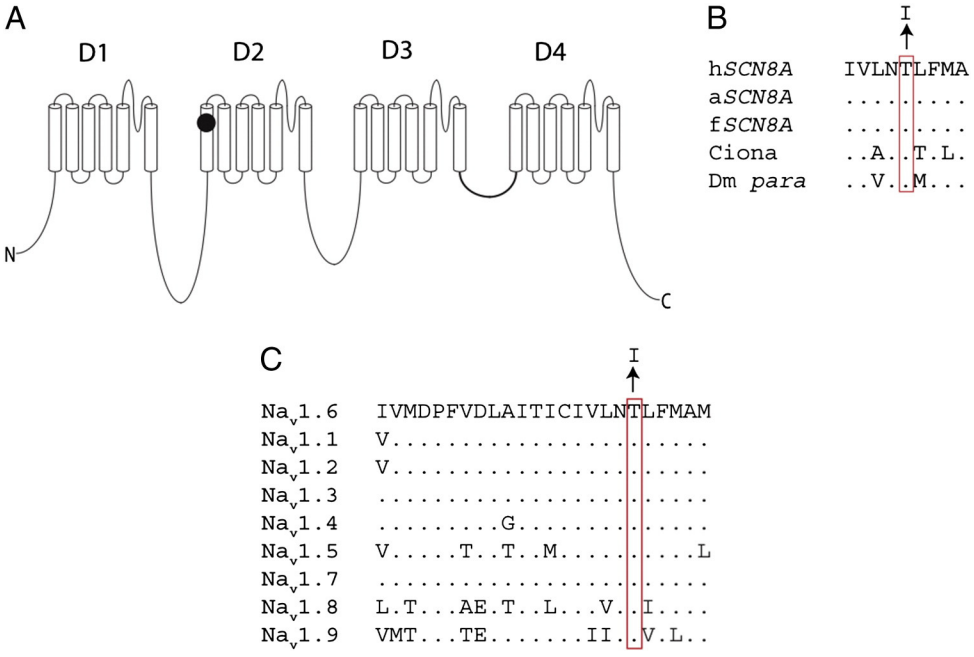

Fig. 1. Location of the p.Thr767Ile mutation in $Na_v1.6$ and evolutionary conservation of the mutated residue. (A) Threonine 767 is located in transmembrane segment 1 of domain II of the voltage-gated sodium channel alpha subunit. (B) This threonine residue is conserved in the vertebrate *SCN8A* channel from reptile (a, anotole) and fish (f, Fugu) and in the homologous invertebrate channel from *Drosophila* and *Ciona*. (C) Threonine (T) at position 767 is conserved in the members of the human voltage-gated sodium channel gene family, including muscle and cardiac channels *SCN4A* and *SCN5A*, respectively.

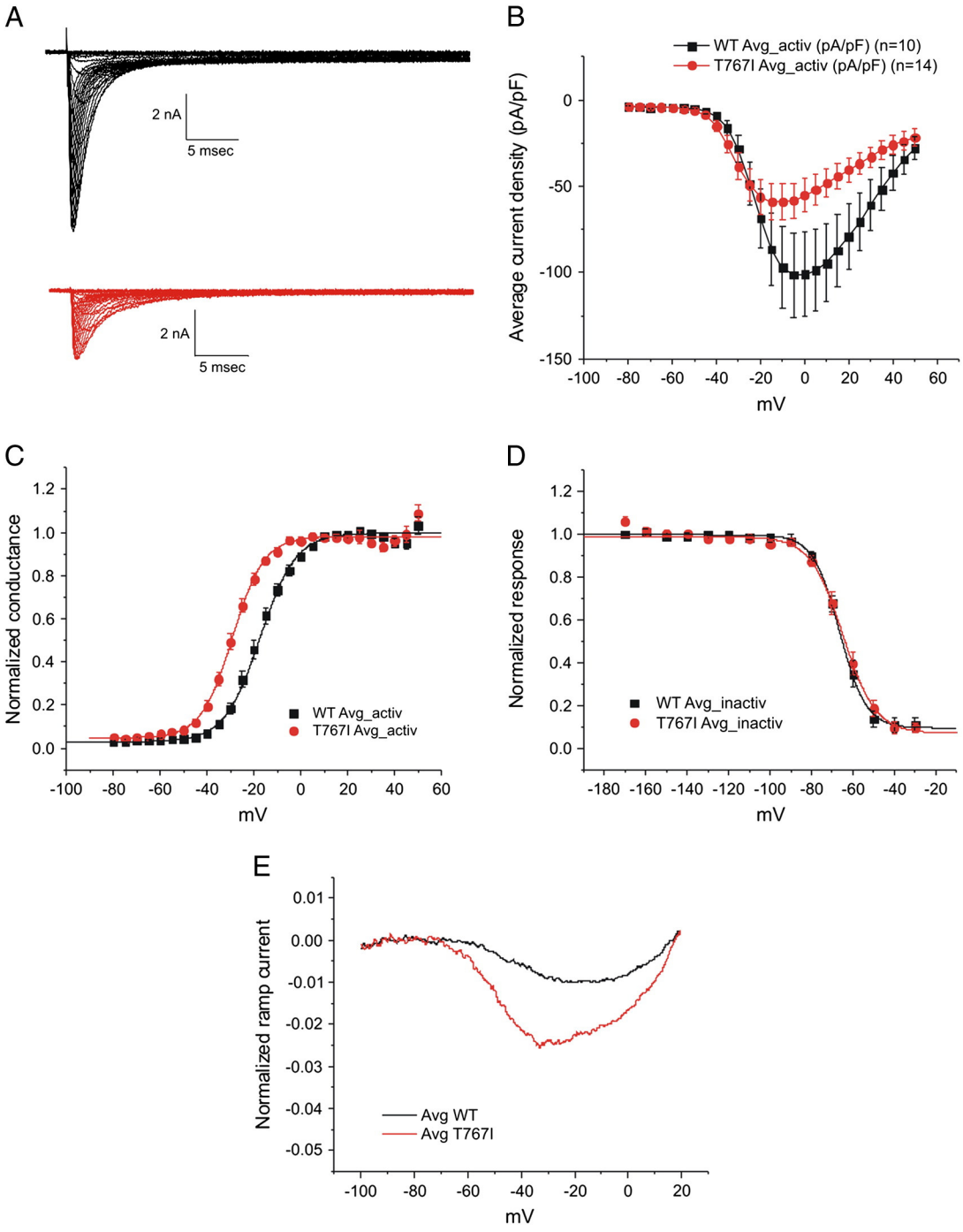

Fig. 2. Voltage-clamp analysis of $Na_v1.6$ p.Thr767Ile mutant channels. (A) Representative traces recorded from ND7/23 cells expressing $mNa_v1.6_R$ (WT, black lines) and T767I (red lines) in response to the activation stimulation protocol (100 msec duration pulses). (B) The peak currents are normalized for cell capacitance and then are averaged together to obtain the activation current–voltage (I–V) relation for cells expressing WT (black symbols) or T767I (red symbols) as described in the Methods section. (C) The G–V curves for both WT (black symbols) and T767I (red symbols) cell are normalized and then averaged to obtain the Boltzmann fit for activation data, which shows hyperpolarizing shift of activation of T767I channels compared to WT channels. (D) The responses to the fast-inactivation protocol are analyzed to obtain the voltage-dependence of fast-inactivation as described in the Methods section. The normalized and averaged and the Boltzmann's fit are shown for both WT (black symbols) and T767I (red symbols). The voltage-dependence of fast-inactivation of T767I channels is not different from that of WT channels. Error bars are standard error of the mean (SEM). (E) The averaged currents evoked during a slow ramp stimulus beginning at −100 mV and ending at 20 mV over a duration of 600 ms are shown from WT (black line, n = 6) and T767I (red line, n = 10) expressing cells. The traces have been normalized to the maximal peak current recorded from that cell. T767I channels produce a larger normalized ramp current which peaks at a more hyperpolarized voltage compared to that of WT channels.

Responses to a ramp stimulus

We evaluated the response of WT and T767I channels to slow depolarizations using a slow ramp protocol from a holding potential of −100 mV to +20 mV over 600 ms for a ramp rate of 0.2 mV/ms. To compare the response in different cells, the response was normalized to the peak inward current recorded during the activation I–V protocol. The averaged response of WT and T767I channels is shown in Fig. 2E. The peak ramp current of T767I averaged 3.1 ± 0.4% of the peak current (n = 10), which is a 3-fold increase over the average ramp response of

WT channels (1.1 ± 0.2%, n = 6; p = 0.003). This change is predicted to increase channel activity in the range of subthreshold depolarizations and enhance the probability of action potential firing.

Current-clamp recording in pyramidal hippocampal neurons

To assess the effect of the p.Thr767ILe mutation on excitability, hippocampal neurons from wildtype mice (P10–P14) were transfected with either the WT or T767I construct and current-clamp recordings were used to assess the pattern of firing, including action potential threshold and evoked firing frequency in pyramidal neurons. Input resistance of neurons transfected with wildtype channels (1.06 ± 0.53 GΩ) was not significantly different from that of neurons expressing the T767I mutant channels (1.25 ± 0.14 GΩ; p > 0.05) (Table 1). Since the input resistance of the transfected hippocampal pyramidal cells is high and in the same range as seal resistance, it is not informative to measure actual resting membrane potential in the current-clamp mode because of potential errors that could be as high as 50% of the recorded value. Instead, we used on-line LFVC (see Methods). LFVC holds the membrane potential of the transfected neurons near a target potential of −80 mV so that differences in firing activity are unlikely to be due to differences of resting membrane potential, and can rather be attributed to the altered properties of the mutant channels.

A notable difference between neurons transfected with T767I compared to neurons transfected with WT channels was an increase in the number of neurons that exhibited spontaneous firing. T767I transfected neurons showed spontaneous firing of action potentials in 6 of 15 cells while only 1 of 17 WT transfected neurons fired spontaneously. The majority of the T767I transfected neurons with spontaneous activity also showed fluctuations of resting membrane potential that elicited bursts of action potentials (Fig. 3, panel A1). Paroxysmal depolarizing shift-like complexes (PDS; Fig. 3 panel A2) were present in 4 of 6 spontaneously active neurons expressing the T767I mutation. In addition to spontaneous firing, the evoked firing of transfected pyramidal neurons was examined. The threshold for the first action potential for cells expressing T767I mutant channels was 25 ± 6 pA (n = 13) which is less than half of the threshold in cells expressing WT channels (64 ± 13 pA; n = 8) (p < 0.05) (Figs. 4A, B). Basic properties of the action potentials elicited at threshold were not different for cells expressing either $Na_v1.6$-WT or T767I (Table 1). The average number of spikes elicited in response to graded 1 second step current injections was significantly increased (ANOVA, p < 0.05) in hippocampal neurons expressing the T767I compared to WT channels (Fig. 4C). All of the observed changes are predicted to increase the activity of neurons expressing the mutant channel.

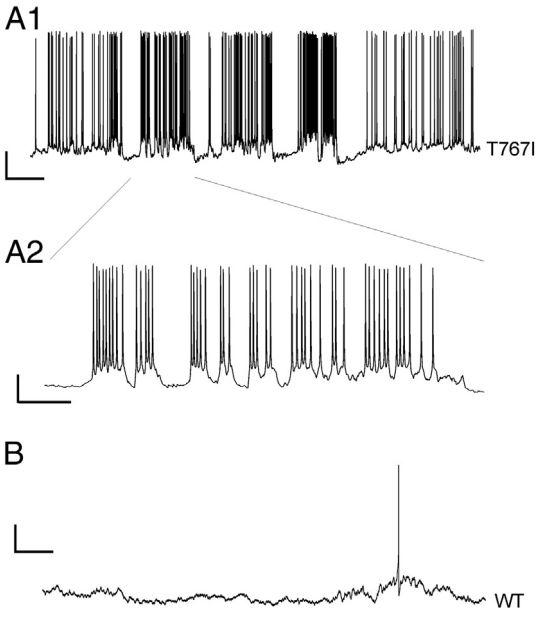

Fig. 3. Neurons expressing $Na_v1.6$-T767I exhibit an increase in spontaneous activity. Continuous segments of current-clamp recording from hippocampal pyramidal neurons illustrate patterns of spontaneous action potential firing seen in a subset of cells expressing T767I channels. (A1) Trace from a representative spontaneously active cell. Scale bar indicates 20 mV vertical, 5 s horizontal. (A2) A small region of the trace was expanded to illustrate firing behavior riding on top of transient depolarizations of the membrane potential, paroxysmal depolarizing shift-like complexes, characteristic of epileptic discharges. Scale bar indicates 20 mV vertical, 1 s horizontal. (B) Trace from a hippocampal pyramidal cell expressing $Na_v1.6$-WT with the same scaling as panel A1 for comparison. Scale bar indicates 20 mV vertical, 5 s horizontal.

Discussion

The severity of epileptic encephalopathy in the patient and the absence of a family history of neurological disorders suggested that the underlying cause might involve a dominant de novo mutation. Whole exome sequencing of the proband identified the de novo variant p.Thr767Ile in $Na_v1.6$. Functional testing demonstrated that this is a gain-of-function mutation, with a significant shift in the voltage dependence of channel activation in a hyperpolarizing direction. In addition, the ramp current is increased three-fold compared to that of the WT channels. These properties are predicted to increase excitability of neurons expressing the mutant channel, and this was confirmed using current-clamp recordings in pyramidal hippocampal neurons. Our data provide compelling evidence that p.Thr767Ile causes hyperexcitability of excitatory neurons resulting in the severe epileptic encephalopathy in this patient.

The evolutionary conservation of the threonine 767 residue in DII/S1 and the invariance of this residue in the nine human paralogs of the voltage-gated sodium channel gene suggest an important contribution to channel function. The replacement of the polar threonine residue by the branched hydrophobic isoleucine residue in a transmembrane domain also predicts a functional effect on channel properties. Indeed, voltage-clamp recording demonstrates that the half-activation voltage of the mutant channel is shifted by 10.8 mV in a hyperpolarizing direction, indicating that the channel will activate in response to weaker stimuli compared to WT channels. Additionally, the 3-fold increase and hyperpolarizing shift of the voltage of the peak ramp current of the mutant channel indicates that it can boost depolarization by weaker stimuli compared to WT channels, thus increasing the probability that neurons reach the threshold for the all-or-none action potential. These proexcitatory changes in the gating properties of the p.Thr767Ile mutant channel may be somewhat attenuated by the reduction in the current density of mutant channels. The dominance of the proexcitatory changes, however, is manifested at the neuronal level by the significant increase in the fraction of hippocampal neurons expressing p.Thr767Ile channels that display spontaneous firing (40%), compared to those expressing WT channels (6%), and by the lower threshold for evoked single action potential and increased number of spikes in response to a graded stimulus in neurons expressing the mutant channel. Thus neurons that express the mutant p.Thr767Ile channels in addition to their endogenous channels are more excitable than those expressing wildtype channels, consistent with an epileptic phenotype. Regardless of the precise ratio of mutant to wildtype channels in a heterozygous neuron, the premature opening of mutant channels at hyperpolarized potential is predicted to result in neuronal hyperexcitability.

Table 1
Properties of action potentials recorded from pyramidal neurons expressing either WT $Na_v1.6$ or T767I mutant channels.

Action potential	Threshold	Amplitude	Slope	Half-width
WT	64 ± 13 pA	82 ± 5 mV	18 ± 4 mV/ms	4.8 ± 0.7 ms
T767I	25 ± 6 pA*	86 ± 5 mV	33 ± 12 mV/ms	5.0 ± 0.5 ms

* p < 0.05 (ANOVA).

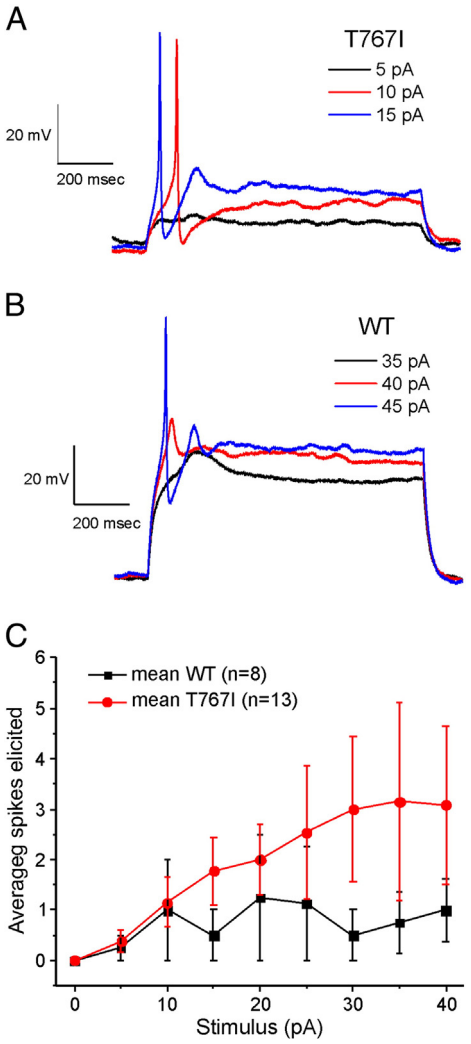

Fig. 4. Neurons expressing $Na_v1.6$-T767I are hyperexcitable compared to those expressing the WT channel. Selected traces illustrating action potentials elicited near threshold stimulus current injections for $Na_v1.6$-T767I expressing (A) and $Na_v1.6$-WT expressing (B) pyramidal hippocampal neurons. (C) The average number of spikes elicited to one second long current injections of 0 pA to 40 pA in 5 pA increments from hippocampal pyramidal neurons expressing WT (black symbols, N = 8) or T767I (red symbols, N = 13) channels. The increase of the response for T767I expressing neurons was significant by ANOVA ($p < 0.05$). Data presented as mean ± SEM.

Our data support the contribution of gain-of-function mutations of $Na_v1.6$ that increase excitatory pyramidal neuron excitability. A role for $Na_v1.6$ in excitatory neurons and susceptibility to epileptic seizures is consistent with recent studies of the RNA binding protein CELF4, that is expressed primarily in excitatory neurons of the cerebral cortex and hippocampus of the mouse. The knockout of *Celf4* is proepileptic, and the complex seizure disorder in the *Celf* mutant mice has been linked to increased sodium currents and increased concentration of $Na_v1.6$ at the axon initial segment of excitatory neurons (Sun et al., 2012). Similarly, in the period following electrical induction of status epilepticus in the mouse, there is an increase in the abundance of $Na_v1.6$ at the axon initial segment (Hargus et al., 2013). Heterozygous *Scn8a* null mice exhibit spike-wave discharges and absence seizures (Papale et al., 2009), suggesting that loss-of-function mutations may also cause some forms of epilepsy, possibly due to a differential impact on inhibitory neurons where $Na_v1.6$ is also expressed (Lorincz and Nusser, 2008). However, reduced abundance of $Na_v1.6$ in heterozygous null mice is also protective against kainate-induced epilepsy and reduces seizures in compound heterozygotes carrying an *SCNA1* Dravet mutation (Blumenfeld et al., 2009; Martin et al., 2007; Papale et al., 2009). The pro-seizure effects of increased $Na_v1.6$ activity and the protective effect of haploinsufficiency both support the pathogenicity of gain-of-function mutations of human $Na_v1.6$ that increase channel activity in excitatory pyramidal neurons.

The clinical phenotype of the patient described here is more severe than in the previous case of de novo *SCN8A* mutation (Veeramah et al., 2012), with seizure onset at 2 weeks compared with six months, impaired development rather than developmental delay and regression, and a different seizure profile. Both of the $Na_v1.6$ mutations result in gain-of-function attributes which make them pathogenic in the heterozygous state. Pyramidal hippocampal neurons expressing either of these mutant channels are hyperexcitable compared to neurons expressing wildtype channels. Nonetheless the changes in channel properties are distinct at the biophysical level, with impaired channel inactivation and increased persistent current in the previous case and enhanced activation in the present case. Both of the *SCN8A* mutations also result in increased ramp current, which has also been reported for epileptogenic mutations of *SCN3A* (Vanoye et al., 2013). The data indicate that alterations in multiple parameters can lead to neuronal hyperexcitability in epileptic encephalopathy, and demonstrate the importance of functional evaluation of multiple mutations for understanding genotype/phenotype relationships. Comparison of neuronal firing patterns in mouse models of the two gain-of-function mutations could provide insight into the cellular origin of seizures and the more severe phenotypic effect of the p.Thr767Ile mutation.

Although candidate gene sequencing has become a clinical diagnostic tool for patients with epilepsy, the majority of sporadic cases remain of unknown etiology. The advent of next generation sequencing methods has accelerated the unbiased approach to discovery of new targets in epilepsy research. Since our report of the first de novo gain-of-function mutation in *SCN8A* (Veeramah et al., 2012), an additional eleven de novo variants have been observed in patients with epileptic encephalopathy or intellectual disability; these include R1617Q in domain III/S4 (Rauch et al., 2012), R662C in L1, L1331V in DIII/S5 and R1872Q in the C-terminus (Carvill et al., 2013). In silico analysis predicts that several of these new mutations may be pathogenic (Carvill et al., 2013; Rauch et al., 2012). The combination of genetic and functional data provided here for the T767I mutation provides compelling evidence that it is a second pathogenic mutation in $Na_v1.6$ in a patient with epileptic encephalopathy. Functional analysis of additional mutations will provide a broader understanding of the relationship between molecular lesions and clinical course. The rapid increase in identification of *SCN8A* mutations suggests that it will prove to be an important causative gene in infantile epilepsy.

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