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# A new $Na_v1.7$ mutation in an erythromelalgia patient ☆

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ABSTRACT

Gain-of-function missense mutations of *SCN9A* gene, which encodes voltage-gated sodium channel $Na_v1.7$, alter channel's biophysical properties causing painful disorders which are refractory to pharmacotherapy in the vast majority of patients. Here we report a novel *SCN9A* mutation (ca.T3947C) in exon 20 in a 9 year old patient, not present in 200 ethnically-matched control alleles; the mutation substitutes the invariant valine 1316 residue within DIII/S5 by alanine (V1316A). Voltage-clamp studies show that $Na_v1.7$ V1316A mutation hyperpolarizes activation (−9 mV), and enhances response to ramp stimuli (3-fold), changes that are predicted to cause hyperexcitability of DRG neurons. V1316A also hyperpolarizes steady-state slow-inactivation (−9.9 mV), which is predicted to attenuate the effect of this mutation on DRG neuron firing. These changes are consistent with previously characterized Erytheromelalgia associated mutations of $Na_v1.7$.



## 1. Introduction

Genetic and functional studies have shown that sodium channel $Na_v1.7$ is a major contributor to pain [1–3]. Autosomal dominant gain-of-function mutations in $Na_v1.7$ that shift activation in a hyperpolarizing direction produce inherited erythromelalgia (IEM) [4–20], while those that impair fast-inactivation cause paroxysmal extreme pain disorder (PEPD) [14,21–23]. Interestingly, one subject with an overlap of IEM and PEPD symptoms was found to harbor a $Na_v1.7$ mutation which hyperpolarized activation and depolarized inactivation [24]. Current-clamp studies have shown that these gain-of-function mutations cause hyperexcitability of DRG neurons [5,8,10,14,17,22,25]. In contrast, autosomal recessive loss-of-function mutations of $Na_v1.7$ lead to congenital indifference to pain but without detectable cognitive, motor, cardiac or sympathetic deficits [26–31]. Thus $Na_v1.7$ has become an attractive target for the development of new and more effective therapeutics to treat pain without major undesirable side effects.

IEM is generally manifested as episodes of burning pain together with erythema and mild swelling in the hands and feet [1,2]. Pharmacological therapies (including treatment with sodium channel blockers) are ineffective in most IEM cases, and cooling of affected extremities is commonly used to obtain temporary relief of the symptoms. However, pharmacological treatment in a few cases have been partially effective [8,32] or produce long-term efficacy [16,19]. While the lack of pharmacological efficacy in one case [11] could be attributed to lower affinity of binding of local anesthetics to the mutant $Na_v1.7$ channel compared to wild type channels, the mechanism underlying the favorable response in the other cases is not well understood.

We report here the identification of a new mutation in a sporadic case of IEM. The biophysical properties of the $hNa_v1.7$-V1316A mutant show gain-of-function changes consistent with an IEM diagnosis and probably contribute to the pathophysiology of her symptoms.

## 2. Methods

### 2.1. Patient

The patient was a 9-year-old female at the time of diagnosis. Family consent was obtained according to the institutional review

☆ This article quantifies the biophysical parameters of a new Nav1.7 mutation found in a patient exhibiting symptoms of erythromelalgia.

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board protocol and blood samples were then obtained and analyzed for mutations in *SCN9A*.

### 2.2. Exon screening

Genomic DNA was extracted from blood samples of the patient. *SCN9A* exons and immediately-flanking intron ends from the proband were amplified and sequenced as previously described [33]. Genomic sequences were compared to reference $Na_v1.7$ cDNA [34] to identify sequence variation.

### 2.3. Plasmids and transfection

The plasmid carrying the wild-type human $Na_v1.7_R$ insert and methods to introduce the mutation have been described previously [4,5]. V1316A mutant channels were transfected into human embryonic kidney (HEK293) cells using Lipofectamine 2000, and stable cell lines were generated by continuous exposure to the selectable marker G418 as described previously [7].

### 2.4. Whole-cell patch-clamp recordings

Whole-cell patch-clamp recordings were performed at room temperature with Axopatch 200B amplifiers (Molecular Devices, Foster City, CA) using the following solutions: internal (mM), 140 CsF, 1 EGTA, 10 NaCl, and 10 HEPES, pH 7.3, adjusted to 310 mOsmol/l with sucrose and CsOH; external (mM), 140 NaCl, 3 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 20 HEPES, pH 7.3, adjusted to 320 mOsmol/l with sucrose and NaOH. Recordings were started 5 min after establishing whole-cell configuration to allow currents to stabilize. Currents were elicited from a holding potential of −120 mV, filtered at 5 kHz, and acquired at 50 kHz using pClamp 10.2 (Axon Instruments). Voltage errors were minimized using 90% series resistance compensation in all recordings. The liquid junction potential was not corrected.

Student's unpaired *t* tests were used with the criterion for statistical significance set at 0.05. Descriptive data are presented as the mean ± SEM. Data were analyzed using Clampfit 10.2 software and Origin 8.5.

## 3. Results

### 3.1. Clinical description

The proband is a female who was 9 years old at the time of first examination, with a three year history of paroxysmal erythema and a tolerable feeling of burning in her feet. Pain episodes occurred 4–5 times per day which could be relieved by cooling. Symptoms were triggered by exercise or exposure to warmth. Her hands were not affected. The symptoms were aggravated in summer and relieved in winter, and remained unchanged with age. She has no sibling and there is no family history of similar symptoms.

Treatment with oral mexiletine 25 mg bid (about 3 mg/kg per day) showed little effect, but after taking CBZ 100 mg bid (about 10 mg/kg per day) for 2–3 days, the burning sensation and skin redness totally vanished. However, the patient became drowsy which led her to sleep for more than 10 h per day. So after maintaining this regimen for 20 days, the dose of CBZ was reduced to 50 mg tid (150 mg/day). In the following 15 days, the burning sensation never occurred and the mild redness of feet seldom appeared, with the drowsiness markedly improved. Inexplicably, CBZ lost effectiveness in relieving symptoms after an eight month treatment course.

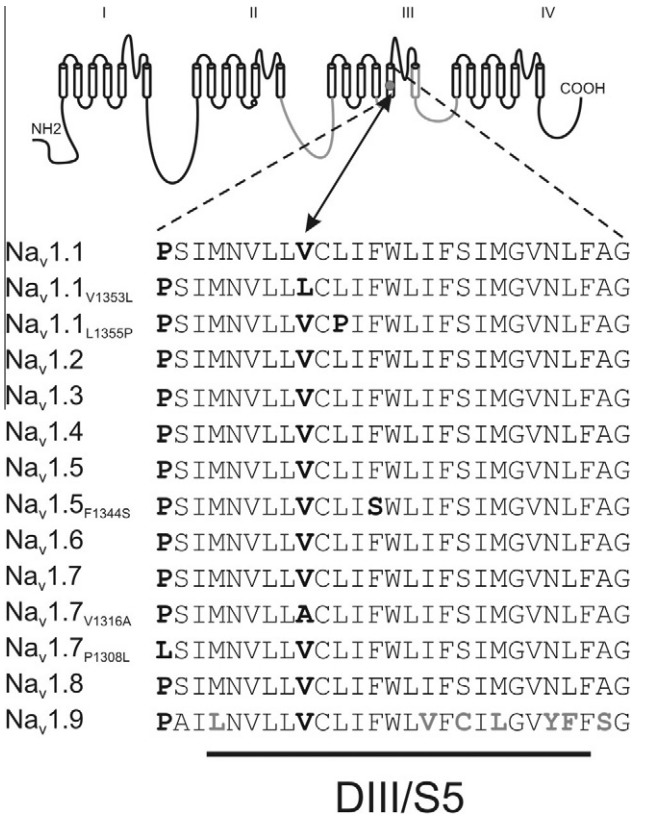


**Fig. 1.** Schematic of V1316A location. V1316 is conserved in the nine members of the $Na_v1$ family of voltage-gated sodium channels. This region of the Nav1.7 channel is the site for other pain-associated mutations. The location of P1308L, another IEM mutation at the hinge of DIII/S4-5 linker and DIII/S5, is shown for comparison. Also mutations in DIII/S5 of $Na_v1.1$ and $Na_v1.5$ are shown for comaprison.

Sequence analysis of *SCN9A* demonstrated a T to G substitution (c. 3947T>G) in exon 20 in the proband. This single nucleotide mutation causes the substitution of valine 1316 with alanine (V1316A) in transmembrane segment 5 in domain III (Fig. 1). This substitution was not found in 200 alleles from normal ethnically-matched controls, or in the 1000 genome project (http://browser.1000genomes.org/Homo_sapiens/Gene/Variation_Gene/Table?db=core;g=ENSG00000169432;r=2:167051695-167232503#NON_SYNONYMOUS_CODING), indicating that this mutation is not a polymorphism.

### 3.2. V1316A mutation lowers voltage threshold for activation

Stable HEK293 cell lines expressing wild-type (WT) $hNa_v1.7_R$ or the mutant channel $hNa_v1.7_R$/V1316A (V1316A) were established and used in voltage-clamp recordings. Fig. 2A and B shows representative sodium currents recorded from cells expressing WT and V1316A channels, respectively. Peak current densities (Fig. 2C) of WT (266 ± 37 pA/pF; $n = 15$) and V1316A (123 ± 12 pA/pF; $n = 21$, $p < 0.005$) indicate that the V1316A cell line expressed current at a significantly lower level. The reduced current density of the mutant channel may be related to site of plasmid integration within the genome of HEK293 cells or to a mutation-induced effect on channel trafficking or stability. The voltage-dependence of activation was examined using a series of depolarizing test pulses from a holding potential of −120 mV. As seen in the mean normalized current–voltage relationships (Fig. 2D), WT channels activate at potentials positive to −40 mV and peak near −5 mV, while V1316A channels activate at potentials positive to −50 mV and

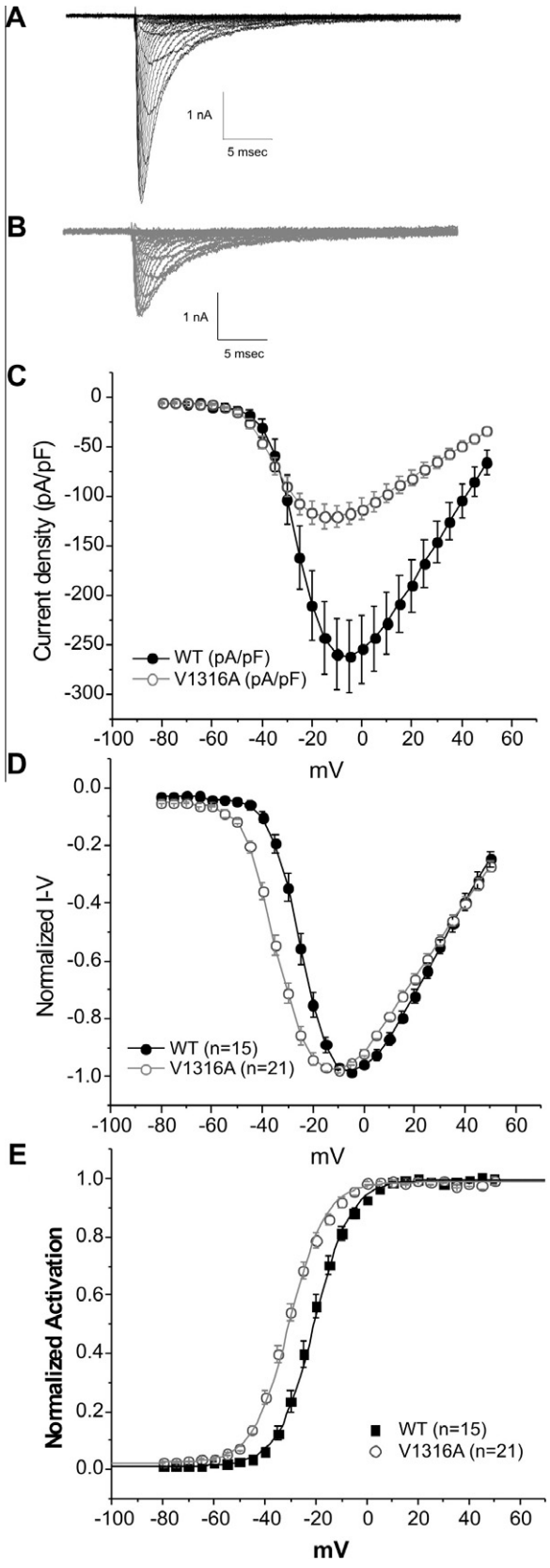


**Fig. 2.** Activation properties of V1316A in HEK293 cells. Electrophysiological analysis of V1316A mutation: representative current traces recorded from HEK293 cells expressing wild type WT (panel A) or V1316A (panel B), evoked by voltage steps (100 ms) from −80 to +40 mV in 5 mV increments, from a holding potential of -120 mV. (C) Average current density I–V for V1316A (open circles) and WT (filled circles). (D) Normalized I–V for V1316A (open circles) and WT (filled circles). (E) Normalized G–V for V1316A (open circles) and WT (filled circles).

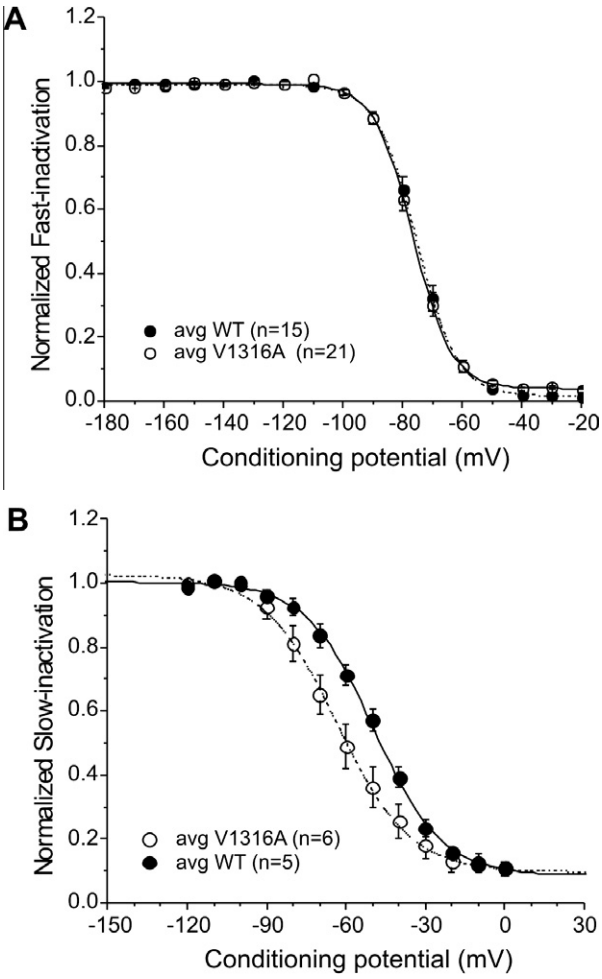


**Fig. 3.** Inactivation properties of V1316A in HEK cells. Electrophysiological analysis of V1316A mutation: (A) fast inactivation of V1316A (open circles) and WT (filled circles) was examined using a series of 500 ms conditioning pulses from −180 to −20 mV followed by test pulses to −10 mV. (B) Steady state slow inactivation of V1316A (open circles) and WT (filled circles). Slow inactivation was assessed using a 30 s conditioning pulse to potentials from −120 to 0 mV followed by a 100 ms pulse to −120 mV to remove fast-inactivation and then the peak inward current during a 20 ms test pulse to −10 mV was measured.

peak near −15 mV. When the voltage-dependent activation curves were fitted with Boltzmann function (Fig. 2E), the midpoint of activation of V1316A (−30.8 ± 1.1 mV; $n = 21$) was found to be significantly more hyperpolarized than in WT (−21.5 ± 1.7 mV; $n = 15$, $p < 0.001$) with a shallower slope (V1316A, 7.2 ± 0.2 mV; WT, 6.6 ± 0.2 mV, $p = 0.02$).

### *3.3. V1316A mutation hyperpolarizes voltage-dependence of slow inactivation but not fast-inactivation*

In contrast to the differences in the voltage-dependence of activation, the voltage-dependence of fast-inactivation was similar for WT and V1316A channels (Fig. 3A). The midpoint of steady-state fast-inactivation (measured with 500 ms prepulses) was not significantly different for WT (−75.4 ± 1.3 mV; $n = 15$) and V1316A (−76.4 ± 1.1 mV; $n = 21$) channels. The slope of the steady-state inactivation curve for V1316A (6.3 ± 0.3 mV) was not significantly different from the slope for WT channel (5.8 ± 0.2 mV) as well.

Slow-inactivation (30-s conditioning pulses) was enhanced for V1316A compared to WT channels (Fig. 3B). Boltzman fits revealed a 9.9 mV hyperpolarizing shift of $V_{1/2}$ of slow-inactivation for

V1316A channels (−59.8 ± 1.6 mV, $n = 5$) compared to WT channels (−49.9 ± 1.5 mV, $n = 6$, $p < 0.005$).

### 3.4. V1316A mutation causes an increase in the ramp current

We examined the currents elicited in WT and V1316A channels by slow ramp depolarizations (0.2 mV/ms from −100 to 20 mV over 600 ms). The peak inward ramp current for V1316A channels was significantly larger than for WT channels (Fig. 4). Expressed as a percentage of peak current, the ramp currents were 2.73 ± 0.57% ($n = 9$) for V1316A channels and 1.07 ± 0.13% ($n = 8$, $p < 0.05$) for WT channels.

## 4. Discussion

Gain-of-function mutations of the peripheral sodium channel $Na_v1.7$ in individuals with IEM cause neuronal proexcitatory changes which underlie pain symptoms, with most patients not responsive to treatment including the use of non-selective sodium channel blockers [1,2]. We report in this study a novel mutation in $Na_v1.7$, V1316A, in a patient with early-onset IEM, which is absent in 200 ethnically-matched control alleles and in the 1000 genome project database. We demonstrate that V1316A mutation produces proexcitatory gain-of-function effects, hyperpolarizes activation and enhances response to ramp stimuli. We also show that V1316A hyperpolarizes steady-state slow-inactivation, which is predicted to ameliorate the mutant-induced DRG neuronal hyperexcitability. While the patient responded favorably to treatment with carbamazepine for eight months, this monotherapy was discontinued when unexpectedly efficacy was lost.

The S5/S6 transmembrane segments of the four domains of sodium channels form the inner lining of the channel's pore [35,36], thus mutations of these segments are likely to produce channelopathies. Indeed, IEM mutations in $Na_v1.7$ DII/S5 (A863P and V872G) [8,16], and the IEM mutation P1308L at the hinge of the DIII/S4-5 linker and S5 in $Na_v1.7$ [14] have previously been shown to induce a hyperpolarizing shift of activation. We have shown here that V1316A in $Na_v1.7$ DIII/S5 causes a significant hyperpolarizing shift in activation and increase in ramp current, both proexcitatory changes that are common to all previously reported IEM mutations [1]. Computer simulations have shown that hyperpolarized activation is sufficient to induce neuronal hyperexcitability [11], which has been validated experimentally because expression of IEM mutant $Na_v1.7$ causes increased DRG neuronal excitability in all tested mutations thus far [1]. Mutant $Na_v1.7$-induced DRG neuron hyperexcitability is consistent with the exaggerated pain phenotype in IEM patients.

The 26 residues of DIII/S5 transmembrane segment are identical in sodium channels $Na_v1.1$-$Na_v1.8$ (Fig. 1), supporting a strict structure–function relationship of this segment; $Na_v1.9$ is the only exception with 7 substitutions (Fig. 1), which may contribute to the distinct hyperpolarized activation of this channel [37]. Thus it is not surprising that mutations in DIII/S5 of $Na_v1.1$ (V1353L [38] and L1355P [39]), $Na_v1.5$/F1344S [40], and $Na_v1.7$/V1316A (this study) are linked to neuronal and cardiac channelopathies. Surprisingly the seemingly conservative substitution V1353L residue in $Na_v1.1$ from a patient with severe myoclonic epilepsy in infancy (SMEI) [38], which is located at the corresponding position of V1316 in $Na_v1.7$ (Fig. 1), rendered $Na_v1.1$ channels nonfunctional [41]. A second mutation, L1355P, was found in another SMEI patient [39], but was not characterized functionally. However, L1355P is expected to be a loss-of-function mutation consistent with the $Na_v1.1$-mediated SMEI phenotype [42]. The mutation F1344S in DIII/S5 of $Na_v1.5$ was found in a patient with Brugada syndrome and functional expression demonstrated a depolarized shift in channel activation [40], consistent with the clinical phenotype. The V1316A substitution, unlike the $Na_v1.1$/V1353L, did not render the channel non-functional, suggesting that DIII/S5 is intolerant of a bulkier side chain at this position. Alternatively, the effects of mutations in DIII/S5 on channel function are isoform-dependent. Future experiments are needed to distinguish between these hypotheses.

Small molecule sodium channel use-dependent blockers [43] are among first line treatments for neuropathic pain [44], but pharmacotherapy for IEM patients is generally ineffective [2], and most patient resort to environmental measures to cool their extremities in order to ameliorate their pain. It is possible that lack of clinical efficacy of the non-selective sodium channel blockers is related to

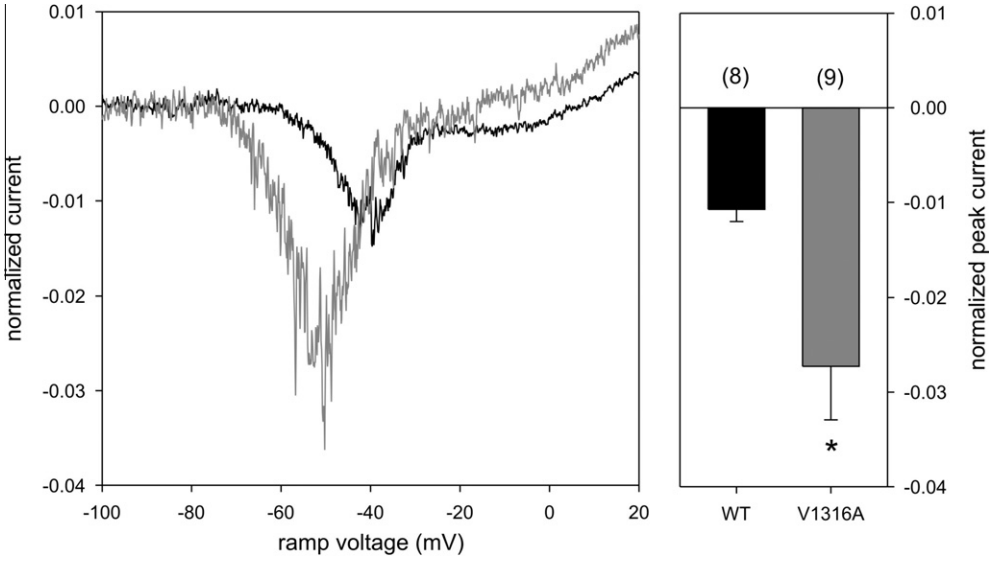


**Fig. 4.** Slow ramp responses of V1316A are enhanced. Slow ramps measure the current elicited to a smoothly increasing ramp of voltage over the range of −100 to 20 mV at a rate of 0.2 mV/ms (600 ms duration). The peak inward current recorded during the ramp is normalized to the peak inward current recorded during the activation protocol for each cell. The slow ramp response (left panel) of a selected V1316A cell (grey line) illustrates the enhanced slow ramp response compared to a selected WT cell (black line). For display purposes, the data were additionally filtered during analysis from 5 kHz to 500 Hz. The average slow ramp peak current (right panel) is enhanced nearly 3-fold for V1316A channels (grey bar, $n = 9$) compared to WT channels (black bar, $n = 8$).

the significant cognitive, motor and cardiac side effects of these drugs at doses needed to alleviate pain in patients with erythromelalgia. However, a few subjects have reported pain relief upon treatment with sodium channel blockers. Lidocaine and mexiletine have produced temporary relief in one subject [8,32], and long-term relief in another [16]. CBZ monotherapy has been reported to be effective in long-term pain relief in several members of a family with the IEM mutation V400M [19], and in combination with gabapentin in another subject carrying the I848T mutation [45]. A systematic drug efficacy assessment of patients with IEM is lacking, because the small number of affected individuals and their widespread geographic distribution makes controlled drug therapy trials logistically challenging and resource-intensive.

This patient harboring the V1316A mutation in her $Na_v1.7$ channel reported temporary relief upon CBZ treatment. This relief lasted for eight months after which CBZ treatment became ineffective. The mechanisms for the initial, favorable response to CBZ and the subsequent loss of efficacy in this patient are unknown and require further study. The CBZ $IC_{50}$ of block of V1316A channels was comparable to that of WT channels, and pre-incubation with CBZ did not correct the voltage-dependence of activation as was previously shown for the V400M [19] and S241T [46] Nav1.7 mutant channels (Data not shown). Shifts of the voltage-dependence of slow-inactivation have been previously observed in other IEM-associated mutations of Nav1.7 [4,15,18]. The approximately 10 mV leftward shift observed here for V1316A would predict a reduced availability of the mutant channels compared to wild-type channels. This anti-excitatory change in gating properties may modulate the extent of DRG neuron excitability which we have correlated with less severe symptoms or later onset of pain symptoms [1]. We have quantitatively demonstrated this in a recent study of an IEM-associated mutation of Nav1.7 (delta955) [15] which showed a nearly −40 mV shift of slow-inactivation yet still exhibited a pain phenotype.

In summary, the shifts of biophysical properties induced by the V1316A mutation are consistent with other inherited Erythermelalgia mutations of Nav1.7. The left-shifted activation voltage-dependence and the enhanced slow ramp response are common to all IEM mutations characterized to date. These shifts make these mutant channels even more sensitive to subthreshold membrane depolarizations and contribute to DRG hyperexcitability.

## 5. Disclosure

The authors report no conflicts of interest. Research Funding from the Medical Research Service and Rehabilitation Research Service, Dept. of Veterans Affairs and by grants from the National Multiple Sclerosis Society and the erythromelalgia Association (SDH and SGW); National Program for New Century Excellent Talents in University (NCET-06-0015) and National Natural Science Foundation (Grant 30400168) (YY). The Center for Neuroscience and Regeneration Research is a Collaboration of the Paralyzed Veterans of America, and the United Spinal Association with Yale University.

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