A novel gain-of-function Na\textsubscript{v}1.7 mutation in a carbamazepine-responsive patient with adult-onset painful peripheral neuropathy

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Abstract
Voltage-gated sodium channel Na\textsubscript{v}1.7 is a threshold channel in peripheral dorsal root ganglion (DRG), trigeminal ganglion, and sympathetic ganglion neurons. Gain-of-function mutations in Na\textsubscript{v}1.7 have been shown to increase excitability in DRG neurons and have been linked to rare Mendelian and more common pain disorders. Discovery of Na\textsubscript{v}1.7 variants in patients with pain disorders may expand the spectrum of painful peripheral neuropathies associated with a well-defined molecular target, thereby providing a basis for more targeted approaches for treatment. We screened the genome of a patient with adult-onset painful peripheral neuropathy characterized by severe burning pain and report here the new Na\textsubscript{v}1.7-V810M variant. Voltage-clamp recordings were used to assess the effects of the mutation on biophysical properties of Na\textsubscript{v}1.7 and the response of the mutant channel to treatment with carbamazepine (CBZ), and multi-electrode array (MEA) recordings were used to assess the effects of the mutation on the excitability of neonatal rat pup DRG neurons. The V810M variant increases current density, shifts activation in a hyperpolarizing direction, and slows kinetics of deactivation, all gain-of-function attributes. We also show that DRG neurons that express the V810M variant become hyperexcitable. The patient responded to treatment with CBZ. Although CBZ did not depolarize activation of the mutant channel, it enhanced use-dependent inhibition. Our results demonstrate the presence of a novel gain-of-function variant of Na\textsubscript{v}1.7 in a patient with adult-onset painful peripheral neuropathy and the responsiveness of that patient to treatment with CBZ, which is likely due to the classical mechanism of use-dependent inhibition.

Keywords
Neuropathic pain, sodium channel, Nav1.7, mutation, DRG neuron, carbamazepine

Introduction
Chronic pain is a frequent and major unmet global challenge.\textsuperscript{1,2} Current treatments for chronic pain include sodium channel blockers, but these are often not very effective and are accompanied by side effects that limit their use even when relief is initially observed.\textsuperscript{3} Although general guidelines have been developed, treatment remains largely dependent on a trial-and-error strategy for individual patients. A more complete understanding of underlying mechanisms for pain might lead to more informed choice of therapy.

Voltage-gated sodium channel Na\textsubscript{v}1.7 has been established as a critically important “pain channel” in several conditions.
human pain disorders. Na\(_{\text{a},1.7}\) is preferentially expressed in peripheral somatic and visceral sensory neurons within dorsal root ganglia (DRG) and in sympathetic ganglion neurons\(^4\) and is characterized by a slow rate of closed-state inactivation, enabling channels to activate in response to small, slow depolarizations, thereby amplifying small stimuli.\(^9\) Thus, Na\(_{\text{a},1.7}\) has been considered to act as a threshold channel which can set the gain of nociceptors.\(^7\) Dynamic clamp studies\(^8\) and pharmacological block using Na\(_{\text{a},1.7}\)-selective blockers\(^9\) have provided strong evidence supporting the role of Na\(_{\text{a},1.7}\) in regulating both threshold for action potentials in rodent and human DRG sensory neurons and neurotransmitter release from peripheral and central terminals of mouse primary afferents.\(^9,10\) Knockout of Na\(_{\text{a},1.7}\) increases pain threshold in animal models,\(^10\)–\(^13\) further supporting the contribution of this channel to pain.

Genetic studies in humans have provided compelling evidence for the role of Na\(_{\text{a},1.7}\) in rare Mendelian pain disorders and in more common painful peripheral neuropathy. Familial and sporadic dominant gain-of-function mutations in Na\(_{\text{a},1.7}\) have been identified in patients with the painful disorders inherited erythromelalgia (IEM) and paroxysmal extreme pain disorder (PEPD).\(^4,5\) Studies of more common painful peripheral neuropathy have identified gain-of-function variants in patients with idiopathic small fiber neuropathy (SFN)\(^14\) and in patients with painful diabetic neuropathy.\(^15\) Patients with IEM or Na\(_{\text{a},1.7}\)-related painful peripheral neuropathy experience attacks of excruciating pain, usually most intense in the distal extremities (feet and hands), which in IEM are exacerbated by mild warmth or exercise and relieved by cooling. Most patients with IEM are resistant to pharmacotherapy and do not report relief with any available agents, while patients with PEPD generally respond to treatment with carbamazepine (CBZ).\(^16\) Although Na\(_{\text{a},1.7}\)-selective blockers have started to be tested in clinical studies,\(^17\) they have not yet reached clinical use. However, pharmacogenomic studies have revealed a novel mode of action of CBZ which depolarizes activation of several mutant Na\(_{\text{a},1.7}\) channels, and pain in patients with these specific Na\(_{\text{a},1.7}\) mutations is relieved by CBZ\(^18,19\) suggesting that a personalized medicine approach might become available in the foreseeable future.

These studies suggest that identification of Na\(_{\text{a},1.7}\) mutations in patients with painful peripheral neuropathy will not only lead to better understanding of pathophysiological mechanisms underlying pain in these patients but may also inform decisions about treatment. We report here the identification of a novel rare variant of Na\(_{\text{a},1.7}\) in a patient with adult-onset pain symptoms and functional assessment of the effect of the mutation on the channel properties and on the excitability of DRG neurons that express this mutant channel. We also assessed the sensitivity of the channel to a clinically achievable concentration of CBZ in order to explore mechanistic basis for the responsiveness of the patient to CBZ.

**Materials and methods**

### Patient enrollment and genomic analysis

The patient first presented to neurology clinic at the age of 53 with a chief complaint of episodic painful dysesthesias. The study participant signed informing consent and underwent clinical assessment which consisted of clinical history, neurological examination, imaging of the entire neuraxis, laboratory tests of blood and cerebrospinal fluid (CSF), and a complete serum paraneoplastic antibody panel (Mayo, Mayo Clinic Laboratories, Rochester, MN, USA). Cardiology evaluation revealed supraventricular tachycardia and atrial fibrillation, and an ablation procedure was performed. Treatment with flecainide caused a marked worsening of dysesthesias and was terminated, and eventually a pacemaker was placed.

Whole exome sequencing was performed at Baylor Miraca Genetics Laboratories (Houston, TX), and the variants that were detected in SCN5A and SCN9A were confirmed by Sanger sequencing. Rare variants that were present at <1% allele frequency in the Exome Variant Server (exome sequencing project [ESP], http://evs.gs.washington.edu/EVS), Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org), and 1000 Genomes (http://phase3browser.1000genomes.org) were selected for further analysis.

#### Plasmids and transfection of HEK293 cells

The human adult-long splice Na\(_{\text{a},1.7}\) isoform complementary DNA (cDNA) has been previously described.\(^14\) Briefly, the cDNA was cloned into a mammalian expression vector and converted to a tetrodotoxin-resistant phenotype by Y362S substitution (hNa\(_{\text{a},1.7}\)R/AL, hereafter referred to as WT). The hNa\(_{\text{a},1.7}\)R/AL-V810M missense mutation (hereafter referred to as V810M) was introduced using QuickChange XL site-directed mutagenesis (Stratagene, San Diego, CA). Human embryonic kidney 293 (HEK293) cells seeded onto 12 mm poly-d-lysine/laminin coated glass coverslips (Corning, Corning, Inc., Corning, NY, USA) were cotransfected with either WT or V810M plasmids (0.8 µg/well) and human β1 and β2 subunits (0.2 µg/well each) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). HEK293 cells were maintained under standard culture conditions (37°C with 5% CO\(_2\)) in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium.
supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (hereafter referred to as DRG medium).

**Voltage-clamp electrophysiology**

Whole-cell voltage-clamp recordings were obtained at room temperature 24 h after transfection using an EPC-9 amplifier and PatchMaster software (HEKA Elektronik, Holliston, MA, USA). We alternated recordings from cells expressing either WT or V810M channels on the same day. Patch electrodes were pulled from fire-polished borosilicate glass capillaries (1.65/1.1 mm OD/ID; World Precision Instruments, Inc., Sarasota, FL, USA) using a P-97 puller (Sutter Instrument Company, Novato, CA, USA) and had a resistance of 0.8–1.3 MΩ when filled with intracellular solution, which contained (in mM): 140 CSF, 10 NaCl, 1.1 ethylene glycol tetraacetic acid, 10 hydroxyethyl piperazineethanesulfonic acid (HEPES) (pH 7.3 with CsOH, adjusted to 310 mOsm with dextrose). Extracellular solution contained (in mM): 140 NaCl, 3 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES (pH 7.3 with NaOH, adjusted to 320 mOsm with dextrose). Pipette potentials were adjusted to zero prior to gigaseal formation, and no adjustments were made for liquid junction potential. Holding potential was set to –120 mV. Voltage errors were minimized using 80–90% series resistance compensation, and only cells with a voltage error <3 mV after compensation were included for analysis. Leak currents were subtracted using the P/6 method, except during use-dependence protocols.

Recordings began following a 5-min equilibration period after establishing whole-cell configuration. To measure activation, cells were stepped from the holding potential of –120 mV to potentials ranging from –10 mV increasing in 10 mV increments followed by a 100 ms hyperpolarization to –120 mV. Cells were then depolarized to –10 mV for 20 ms. Peak inward currents obtained from steady-state slow inactivation protocols were normalized to the maximum current amplitude and fit to the Boltzmann equation,  

\[ I/I_{\text{max}} = 1/(1 + \exp[(V_m - V_{1/2})/k]) \]

where \( V_{1/2} \) is the midpoint of slow inactivation, and \( k \) is the slope factor. Protocols to determine steady-state slow inactivation involved 30-s prepulses ranging from –130 mV to +30 mV increasing in 10 mV increments followed by a 100 ms hyperpolarization to –120 mV. Cell currents were then depolarized to –10 mV for 20 ms. Peak inward currents obtained from steady-state slow inactivation protocols were normalized to the maximum current amplitude and fit to the Boltzmann equation,  

\[ I/I_{\text{max}} = 1/(1 + \exp[(V_m - V_{1/2})/k]) \]

where \( V_{1/2} \) is the midpoint of slow inactivation, and \( k \) is the slope factor. To measure use-dependence, a train of thirty 20-ms pulses to –10 mV were conducted at a frequency of 20 Hz. Peak inward currents obtained from use-dependence protocols were normalized to the maximum current amplitude.

**Isolation and transfection of primary DRG neurons**

All animal care and experimental studies followed protocols approved by the Veterans Administration Connecticut Healthcare System Institutional Animal Care and Use Committee. Dorsal root ganglion (DRG) neurons from male and female Sprague-Dawley neonatal rat pups (P0-P5) were harvested and dissociated as previously described. In brief, DRG neurons were dissociated with a 20-min incubation in 1.5 mg/mL collagenase A (Roche Diagnostics, Indianapolis, IN, USA) and 0.6 mM ethylenediaminetetraacetic acid (EDTA), followed by another 20-min incubation in 1.5 mg/mL collagenase D (Roche Diagnostics, Indianapolis, IN, USA), 0.6 mM EDTA, and 30 U/mL papain (Warthington Biochemical Corp., Lakewood, NJ, USA). Dissociated cells were then centrifuged and triturated in 0.5 mL DRG medium containing 1.5 mg/mL bovine serum albumin (low endotoxin) and 1.5 mg/mL trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Finally, DRG neurons were transfected with either WT or V810M plasmids (2.5 μg)
using a Nucleofector IIS (Lonza, Basel, Switzerland) and Amaxa Basic Neuron SCN Nucleofector Kit (VSP1-
1003). Transfected neurons were given 5 min at 37°C to 
recover in Ca2+-free DMEM before being seeded onto 
12 well multi-electrode array (MEA) plates (Axion 
Biosystems, Atlanta, GA) coated with poly-d-lysine (50 
µg/mL)/laminin (10 µg/mL). DRG medium (1.5 mL per 
well) supplemented with 50 ng/mL each of mouse nerve 
growth factor (Alomone Labs, Jerusalem, Israel) and glial 
cell line-derived neurotrophic factor (PeproTech, Rocky 
Hill, NJ, USA) was added to cells.

**MEA recording**

MEA recordings were obtained as previously 
described.21,22 Briefly, dissociated and transfected 
DRG neurons were maintained in standard culture condi-
tions (37°C with 5% CO2) for at least three days prior 
to recording. Spontaneous firing activity of these neu-
rons was assessed using a multi-well MEA system 
(Maestro, Axion Biosystems). A 12-well recording 
plate was used, with each well containing 64 low-noise 
individual embedded microelectrodes with integrated 
ground electrodes, forming an 8 × 8 recording grid of 
electrodes across a 2 × 2-mm area. Each electrode had 
a diameter of 30 µm with 200 µm center-to-center spac-
ing between individual electrodes. For each experiment, 
three wells each (~192 available electrodes) of either WT 
or V810M were prepared and seeded. To minimize var-
ation, DRG tissue from two different animals were 
pooled, prepared, and transfected with WT and 
V810M plasmids in parallel by the same investigator in 
each experiment. The investigator performing the MEA 
recordings was blinded to the identity of the channel 
expressed in each well until after recordings were com-
pleted. Three independent experiments were conducted. 
To provide environmental control during recordings, an 
ECmini unit was used to maintain CO2 concentra-
tions around MEA cultures with a low flow of premixed gas 
(5% CO2, 20% O2, balance nitrogen), and temperature 
was maintained at a physiological level of 37°C. A spike 
detection criterion of >6 standard deviations above 
background signal was used to distinguish action poten-
tials from noise. Active electrodes were defined as regis-
tering >1 recorded spike over a period of 200 s.

**Drug studies**

CBZ (Sigma-Aldrich) was dissolved in dimethyl sulfox-
ide (DMSO) to make a 30-mM (1000×) stock solution. 
Working solutions were mixed fresh daily. Transfected 
HEK293 cells were preincubated for 30 min in standard 
culture conditions (37°C with 5% CO2) with either 
30 µM CBZ or 0.1% DMSO vehicle in serum-free 
DMEM/F12 medium (Thermo Fisher, Waltham, MA) as previously described.18,22 CBZ or DMSO concen-
tration was maintained in extracellular solution 
during recordings.

**Data analysis and statistics**

Voltage-clamp data were analyzed using FitMaster 
(HEKA Elektronik), Origin 2017 (OriginLab 
Corporation, Northampton, MA, USA), and 
GraphPad Prism 7.01 (GraphPad Software, La Jolla, 
CA, USA) software. MEA data were analyzed using 
Axion Integrated Studio AxIS2.1 (Axion Biosystems), 
NeuroExplorer (Nex Technologies, Colorado Springs, 
CO, USA), and Origin 2017 (OriginLab Corporation, 
Northampton, MA, USA). Unless otherwise noted, sta-
tistical significance was assessed using two-sample t test. 
Statistical significance for kinetics of deactivation and 
use-dependence data were evaluated using two-factor 
analysis of variance with repeated measures and 
Bonferroni post hoc test. Descriptive data are expressed 
as mean ± standard error. Data were considered signifi-
cant at p < 0.05.

**Results**

**Clinical phenotype and molecular genetics**

The patient presented to neurology clinic at the age of 53 
with a chief complaint of episodic painful dysesthesias. 
He had a history of itching paresthesias in the soles and 
heels beginning in his early 40s. At around age 48, with-
out clear provocation, he developed severe pain down 
the left leg, cramping feeling in the left hand, and numb-
ness/paresthesias in the left face. Magnetic resonance 
imaging of the head and neck were normal. Around 
the same time, he developed episodes of supraventricular 
tachycardia and atrial fibrillation and underwent an 
ablation procedure.

At the age of 53, the neurological symptoms wors-
ened. The patient developed cramps in the hands and 
forearms and very painful burning in the feet, which 
then ascended to the knees and became more diffuse. 
The sensory disturbances affected all four limbs as well 
as the back, head, and face. The chest and abdomen 
were relatively unaffected, although he reported episodes 
of anal pain. Strength, vision, cognition, and bulbar 
function were normal. Imaging of the entire neuraxis 
and CSF examination were normal.

Brief trials of gabapentin, duloxetine, pregabalin, 
nortriptyline, and narcotics and a gluten-free diet were 
tried without benefit. A short empiric trial of oral corti-
costeroids seemed to improve his symptoms, and over 
the course of a year, symptoms improved markedly but 
did not completely resolve. Eventually, he was able to 
stop all symptomatic medication and returned to a high
level of function. At the age of 57, symptoms returned without clear inciting event. He experienced burning pain and paresthesia as well as chest pains and labile blood pressure. Supraventricular tachycardia and atrial fibrillation were treated with a second ablation procedure. Treatment with flecainide caused worsening of dysesthesias. Ultimately, he had pacemaker placed.

Diffuse neuropathic pain has continued in a relapsing/remitting pattern; pain attacks lasting for weeks are in some cases provoked by cold or vibratory stimulation, with improvement on warm days. Common symptoms during attacks are burning and stinging sensations in the face, buttocks, legs, and fingers. Low-dose amitriptyline and nortriptyline provided some benefit for nighttime symptoms. Mexiletine was not tolerated. At the age of 59, he started treatment with CBZ. The maximum tolerated dose of 300 mg per day significantly reduced frequency and severity of pain attacks.

Whole exome sequencing of the patient’s DNA identified variants in **SCN9A**, the gene which encodes sodium channel Nav1.7, and in **SCN5A**, the gene which encodes Na\(_{\text{v}}\)1.5. The patient showed heterozygous mutations in Na\(_{\text{v}}\)1.7 (c. 2428G\(\rightarrow\)A) and Na\(_{\text{v}}\)1.5 (c. 1844G\(\rightarrow\)A). These variants were confirmed using Sanger targeted sequencing of exons of both genes. The Na\(_{\text{v}}\)1.7 (c. 2428G\(\rightarrow\)A) variant substitutes valine (V) by methionine (M) at position 810 (p. V810M). The variant allele is very rare (0.008%, ESP; 0.03%, ExAC; 0.02%, 1000 Genomes) and was classified in these databases as of unknown significance. Although V810, which is located at the C-terminal end of transmembrane segment 3 in domain II (DII/S3) in Na\(_{\text{v}}\)1.7, is shared with Na\(_{\text{v}}\)1.1 and Na\(_{\text{v}}\)1.4, the equivalent residue is methionine in Na\(_{\text{v}}\)1.2, Na\(_{\text{v}}\)1.3, and Na\(_{\text{v}}\)1.6; leucine in Na\(_{\text{v}}\)1.8; and alanine in Na\(_{\text{v}}\)1.9. However, V810 is highly conserved in orthologues of Na\(_{\text{v}}\)1.7 in mammalian species in which this channel has been identified (Figure 1). The conservation of a valine residue at this position in Na\(_{\text{v}}\)1.7 in different mammalian species suggests that its substitution may cause functional changes to the channel.

**Voltage-clamp recordings of WT and V810M currents**

To determine whether the V810M mutation alters voltage-dependent properties of Na\(_{\text{v}}\)1.7, HEK293 cells were transiently cotransfected with either WT or V810M channels and human \(\beta\)1 and \(\beta\)2 subunits, and voltage-clamp recordings performed 24 h after transfection. Representative whole-cell currents from WT and V810M mutant channels are shown in Figure 2(a). The average peak inward current density was significantly increased for V810M by 1.5-fold as compared to WT channels (\(p = 0.03\); Table 1, Figure 2(b)).

Normalized voltage-dependence of activation curves are shown in Figure 2(c). Voltage midpoints (\(V_{1/2}\)) of activation were calculated from Boltzmann fits of normalized conductance (Figure 2(d)) and show that the V810M mutation hyperpolarizes activation by 3 mV (\(p = 0.04\); Table 1). The slope factor of voltage-dependent activation, which quantifies the steepness of the Boltzmann fits of normalized conductance, was unaffected by the V810M mutation (Table 1). There was no significant difference in reversal potentials between V810M and WT channels (WT: 71.2 \(\pm\) 2.4 mV, \(n = 18\); V810M: 68.2 \(\pm\) 1.5 mV, \(n = 16\)). We assessed kinetics of deactivation, which represents the transition of the channel from the open state to the closed state and found that V810M currents showed significantly slowed deactivation kinetics relative to WT channels over a range of potentials from –65 to –40 mV (\(p = 0.0005\); Bonferroni comparisons: –65 mV, \(p = 0.02\); –60 mV, \(p = 0.004\); –55 mV, \(p < 0.0001\); –50 mV, \(p < 0.0001\); –45 mV, \(p < 0.0001\); –40 mV, \(p < 0.0001\); Table 1, Figure 2(e)). The increase in peak current density, a 3-mV hyperpolarized shift in activation, and slowed deactivation kinetics of V810M all represent gain-of-function changes of the mutant channel.

V810M did not alter voltage-dependence of steady-state fast inactivation (Table 1, Figure 3(a)) or steady-state slow
Figure 2. V810M increases peak current, hyperpolarizes voltage-dependent activation, and delays deactivation of Na\textsubscript{1.7}.
(a) Representative whole-cell current traces from HEK293 cells transiently transfected with WT (black) or V810M (red) channels. Cells were held at –120 mV and stepped to potentials ranging from –80 to +40 mV in 5 mV increments for 100 ms with 5 s between pulses. (b) Current density for WT (black squares, \(n = 18\)) and V810M (red circles, \(n = 16\)) channels showing a significant increase in peak current density for V810M. Current density was calculated by normalizing peak currents to capacitance. (c) Normalized peak current-voltage relationship for activation of WT (black squares, \(n = 18\)) and V810M (red circles, \(n = 16\)) channels showing a significant hyperpolarized shift in activation of V810M. (d) Voltage-dependent activation of WT (black squares, \(n = 18\)) and V810M (red circles, \(n = 16\)) channels showing a small but significant hyperpolarized shift in activation of V810M. Conductance curves were normalized to the maximum conductance value and fit to a Boltzmann equation. (e) Deactivation time constants for WT (black squares, \(n = 17\)) and V810M (red circles, \(n = 11\)) channels showing significantly slowed kinetics of deactivation for V810M over a voltage range of –65 to –40 mV. Data are expressed as means ± SEM.

Table 1. Biophysical properties of WT and V810M channels.

<table>
<thead>
<tr>
<th>Na\textsubscript{1.7}</th>
<th>Current density</th>
<th>Activation (mV)</th>
<th>Steady-state fast inactivation (mV)</th>
<th>Steady-state slow inactivation (mV)</th>
<th>Deactivation (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pA/pF</td>
<td>(V_{1/2}) k n</td>
<td>(V_{1/2}) k n</td>
<td>(V_{1/2}) k n</td>
<td>–40 mV n</td>
</tr>
<tr>
<td>WT</td>
<td>380.1 ± 42.2</td>
<td>–18.5 ± 0.7 7.7 ± 0.1 18</td>
<td>–83.7 ± 1.4 6.7 ± 0.2 18</td>
<td>–72.2 ± 1.7 14.0 ± 0.6 8</td>
<td>0.26 ± 0.01 17</td>
</tr>
<tr>
<td>V810M</td>
<td>577.6 ± 80.3*</td>
<td>–21.5 ± 1.2 7.4 ± 0.3 16</td>
<td>–85.5 ± 1.2 6.7 ± 0.2 17</td>
<td>–69.8 ± 1.7 14.5 ± 0.6 7</td>
<td>0.32 ± 0.01** 11</td>
</tr>
</tbody>
</table>

*\(p < 0.05\), **\(p < 0.0001\) significantly different from WT; \(n\) represents the number of cells from which recordings were acquired.
inactivation (Table 1, Figure 3(b)). Furthermore, the mutation had no effect on use-dependent inhibition at 20 Hz (ratio of peak current of the 30th pulse normalized to peak current of the first pulse: WT: 76.5 ± 1.6%, n = 16; V810M: 73.1 ± 2.1%, n = 16).

**MEA recordings of WT and V810M spontaneous firing**

The increase in current density and the gain-of-function changes to voltage-dependent properties of V810M suggest that this mutation may alter excitability in DRG neurons. To assess the effects of V810M on excitability of DRG neurons, we used MEA recording, which allows for a high-throughput, noninvasive approach for recording from intact neurons. Recordings were performed at the physiological core body temperature of 37°C and revealed significant differences between DRG neurons expressing WT and V810M across multiple metrics of excitability, including total number of spikes per well (WT: 256.9 ± 72.9, n = 8 wells from 3 experiments, 6 rats; V810M: 1924.9 ± 590.8, n = 9 wells from 3 experiments, 6 rats; p = 0.02); mean firing frequency (WT: 0.013 ± 0.004 Hz, n = 8 wells from 3 experiments, 6 rats) and V810M (red fill, n = 9 wells from 3 experiments, 6 rats) channels, showing a significant increase in mean firing frequency by V810M-expressing neurons. (d) Average number of active electrodes per well (WT: 4.3 ± 1.0, n = 8 wells from 3 experiments, 6 rats; V810M: 10.2 ± 2.0, n = 9 wells from 3 experiments, 6 rats; p = 0.02; Figure 4).

**Effects of CBZ on voltage-dependent properties of V810M**

In an attempt to explain the mechanistic basis for CBZ-responsiveness of the patient, we performed voltage-clamp experiments in the presence of either a clinically achievable concentration of 30 μM CBZ or 0.1% DMSO vehicle control. Voltage-clamp studies revealed that
orthologues of Na\textsubscript{v}1.7 in various mammalian species. V810M allele is rare, and V810 is highly conserved in variant of unknown significance. However, the Na\textsubscript{v}1.7-channel and it was previously classified in genomic databases as a

gested that the V810M mutation might be pathogenic, prediction among other Na\textsubscript{v} channel isoforms would have sug-

ceeded that the V810M substitution near the C-terminus of the DII/S3 nor the lack of conserva-

tion of the entire neuraxis was normal, as was CSF

lung and responding to treatment with CBZ. Carriers of Na\textsubscript{v}1.5 mutations have not reported

somatic pain symptoms; therefore, the G615E mutation was not considered as a contributor to the pain phenotype in this patient.

The sensory disturbances in this patient affected all limbs as well as the back, head, and face, although the chest and abdomen were relatively unaffected. Imaging of the entire neuraxis was normal, as was CSF

examination, which is consistent with a functional and not necessarily structural pathophysiological mechanism. Interestingly, pain attacks could be provoked by cold or vibratory stimulation, and symptoms improved with warmth. Although the prototypical cases of burning pain in individuals carrying mutations in Na\textsubscript{v}1.7 report pain attacks triggered by warmth and relieved by cooling, a natural history study of 13 individuals with IEM carrying well-characterized mutations in Na\textsubscript{v}1.7 identified an individual who reported cold triggers of pain, even when other members of their family who carry the same mutation reported cold-induced relief. It is likely that additional genetic and epigenetic factors contribute to this individual-to-individual variability in the response to environmental triggers among patients carrying mutations in Na\textsubscript{v}1.7, but these factors are not well understood at this time.

An additional cardiac comorbidity is present in this patient. He carried a rare variant in Na\textsubscript{v}1.5 (0.02%, ExAC database), which substitutes glycine (G) by glutamic acid (E) at position 615 (G615E) in the Na\textsubscript{v}1.5 channel. This variant has been previously reported in patients with irritable bowel syndrome (IBS), both with or without cardiac symptoms, and functional testing showed that it confers loss-of-function attributes on the channel (reduced peak current amplitude and depolarizing shift of activation). Although the patient in our study reported cardiac deficits that required placement of a pacemaker, he did not report symptoms of IBS. Carriers of Na\textsubscript{v}1.5 mutations have not reported somatic pain symptoms; therefore, the G615E mutation was not considered as a contributor to the pain phenotype in this patient.

The V810M allele is rare and is represented in different databases at <0.02%. V810 is located at the C-terminal end of transmembrane segment 3 in domain II (DII/S3) in Na\textsubscript{v}1.7 but is not highly conserved among other members of the sodium channel family. A valine at the corresponding position is conserved only in Na\textsubscript{v}1.1 and Na\textsubscript{v}1.4, and the equivalent residue is methionine in Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, and Na\textsubscript{v}1.6. However, a valine residue is

CBZ had no detectable effect on activation of either WT or V810M channels (Table 2, Figure 5(a)). Furthermore, steady-state fast inactivation of neither WT nor V810M was affected by CBZ (Table 2). However, both WT and V810M showed a significant increase in use-dependent inhibition in the presence of CBZ (WT: \( p = 0.0001 \); V810M: \( p = 0.002 \); Table 2, Figure 5(e)). Taken together, these data suggest that CBZ acts in this patient with the V810M mutation via the classical mechanism of use-dependent inhibition and not by activation modulation as previously described for other gain-of-function Na\textsubscript{v}1.7 mutations.\textsuperscript{18,22,23}

### Discussion

We describe here a patient with painful peripheral neuropathy who carries the Na\textsubscript{v}1.7-V810M variant. This patient presented with clinical symptoms of severe distal pain and responded to treatment with CBZ. Neither the position of the V810M substitution near the C-terminus of the DII/S3 nor the lack of conservation among other Na\textsubscript{v} channel isoforms would have suggested that the V810M mutation might be pathogenic, and it was previously classified in genomic databases as a variant of unknown significance. However, the Na\textsubscript{v}1.7-V810M allele is rare, and V810 is highly conserved in orthologues of Na\textsubscript{v}1.7 in various mammalian species (Figure 1), which suggested a possible conserved function. Functional testing revealed gain-of-function attributes that the V810M substitution conferred on the channel, and expression of V810M mutant channels in DRG neurons rendered them hyperexcitable. Taken together, these genetic, clinical, and functional assessments are consistent with the designation of this variant as “pathogenic” or “probably pathogenic” based on classification criteria developed for sequence variants in voltage-gated ion channels.\textsuperscript{24}

The sensory disturbances in this patient affected all limbs as well as the back, head, and face, although the chest and abdomen were relatively unaffected. Imaging of the entire neuraxis was normal, as was CSF examination, which is consistent with a functional and not necessarily structural pathophysiological mechanism. Interestingly, pain attacks could be provoked by cold or vibratory stimulation, and symptoms improved with warmth. Although the prototypical cases of burning pain in individuals carrying mutations in Na\textsubscript{v}1.7 report pain attacks triggered by warmth and relieved by cooling, a natural history study of 13 individuals with IEM carrying well-characterized mutations in Na\textsubscript{v}1.7 identified an individual who reported cold triggers of pain, even when other members of their family who carry the same mutation reported cold-induced relief.\textsuperscript{25} It is likely that additional genetic and epigenetic factors contribute to this individual-to-individual variability in the response to environmental triggers among patients carrying mutations in Na\textsubscript{v}1.7, but these factors are not well understood at this time.

An additional cardiac comorbidity is present in this patient. He carried a rare variant in Na\textsubscript{v}1.5 (0.02%, ExAC database), which substitutes glycine (G) by glutamic acid (E) at position 615 (G615E) in the Na\textsubscript{v}1.5 channel. This variant has been previously reported in patients with irritable bowel syndrome (IBS), both with or without cardiac symptoms, and functional testing showed that it confers loss-of-function attributes on the channel (reduced peak current amplitude and depolarizing shift of activation).\textsuperscript{26} Although the patient in our study reported cardiac deficits that required placement of a pacemaker, he did not report symptoms of IBS. Carriers of Na\textsubscript{v}1.5 mutations have not reported somatic pain symptoms; therefore, the G615E mutation was not considered as a contributor to the pain phenotype in this patient.

The V810M allele is rare and is represented in different databases at <0.02%. V810 is located at the C-terminal end of transmembrane segment 3 in domain II (DII/S3) in Na\textsubscript{v}1.7 but is not highly conserved among other members of the sodium channel family. A valine at the corresponding position is conserved only in Na\textsubscript{v}1.1 and Na\textsubscript{v}1.4, and the equivalent residue is methionine in Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, and Na\textsubscript{v}1.6. However, a valine residue is

<table>
<thead>
<tr>
<th>Na\textsubscript{v}1.7</th>
<th>Drug</th>
<th>Activation (mV)</th>
<th>Steady-state fast inactivation (mV)</th>
<th>Use dependence (20 Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V\textsubscript{1/2}</td>
<td>k</td>
<td>n</td>
<td>V\textsubscript{1/2}</td>
</tr>
<tr>
<td>WT</td>
<td>DMSO</td>
<td>–18.6 ± 0.8</td>
<td>8.2 ± 0.3</td>
<td>15</td>
</tr>
<tr>
<td>CBZ</td>
<td>–20.2 ± 1.0</td>
<td>7.8 ± 0.2</td>
<td>12</td>
<td>–99.9 ± 1.5</td>
</tr>
<tr>
<td>V810M</td>
<td>DMSO</td>
<td>–20.9 ± 0.5</td>
<td>7.8 ± 0.2</td>
<td>15</td>
</tr>
<tr>
<td>CBZ</td>
<td>–21.5 ± 0.8</td>
<td>7.5 ± 0.3</td>
<td>13</td>
<td>–88.1 ± 1.9</td>
</tr>
</tbody>
</table>

Note: CBZ: carbamazepine; DMSO: dimethyl sulfoxide.

*\( p < 0.01 \) significantly different from DMSO; \( n \) represents the number of cells from which recordings were acquired.
conserved at this position in orthologues of Na\textsubscript{v}1.7 in mammalian species, except for the rat where it is replaced by isoleucine, which has a branched aliphatic side chain similar to valine (Figure 1). Importantly, functional assessment has shown that V810M substitution confers gain-of-function attributes on the channel. The gain-of-function attributes of the V810M substitution in Na\textsubscript{v}1.7 are consistent with the view that this residue contributes to the gating of Na\textsubscript{v}1.7. The presence of methionine at the corresponding position in three voltage-gated sodium channels suggests that the V810M substitution in Na\textsubscript{v}1.7 may have uncovered an isoform-dependent effect for this residue.

Pain has been linked to hyperexcitability of DRG neurons.\textsuperscript{27–30} We assessed the effect of the expression of V810M mutant channels on the excitability of the DRG neurons. MEA recordings have the benefit of assessing the excitability of intact neurons and in this case demonstrated enhanced excitability of DRG neurons expressing the V810M mutant channels. The gain-of-function attributes at the neuronal level included total number of spikes per well, mean firing frequency, and number of active electrodes (Figure 4). Our MEA data supports the pathogenicity of the V810M mutation and provide further evidence for a mechanistic basis for pain in this individual.

Although the patient has carried the mutation from birth, he began to manifest symptoms in his early 40s, and the symptoms increased in severity by the age of 53, with no clear precipitating events. Gain-of-function mutations of Na\textsubscript{v}1.7 have been identified in patients with adult-onset SFN\textsuperscript{14} and in patients with type 2 diabetic neuropathy and pain.\textsuperscript{15} Gain-of-function mutations in Na\textsubscript{v}1.8\textsuperscript{31–33} and Na\textsubscript{v}1.9\textsuperscript{34,35} have also been found in patients with adult-onset SFN. In a family with the gain-of-function Na\textsubscript{v}1.7-G616R mutation, pain developed in the proband in his mid-20s, while his children developed pain before the age of 10.\textsuperscript{36} The nature of the compensatory factors that prevent pain prior to this age of onset in our patient, as in these other cases, are unknown, but the phenomenon is well-established.

Based on the patient’s successful response to CBZ manifested as reduced frequency and severity of pain attacks, we assessed the mechanism of action of this drug on V810M. CBZ is the first-line treatment for trigeminal neuralgia,\textsuperscript{37} and subjects with Na\textsubscript{v}1.7-related PE PD are generally responsive to treatment with CBZ.\textsuperscript{38,39} The responsiveness of these patients to CBZ may be due to the classical effect of this drug as a potent state- and use-dependent blocker of neuronal Na\textsubscript{v} channels.\textsuperscript{40} Recently, a new mode of action of CBZ as an activation modulator in three IEM mutations in Na\textsubscript{v}1.7 has been described,\textsuperscript{19,22,23} where CBZ acts by depolarizing activation. The observed increase in use-
dependent inhibition by CBZ coupled with a lack of an effect on activation of V810M channels suggest that CBZ produces pain relief in this patient via its classical use-dependent mechanism and not via its novel mode of action as an activation modulator. The favorable response of the patient to treatment with CBZ is also suggestive that the V810M mutation did not alter the high-affinity local anesthetic binding site where CBZ binds. It is important to note that CBZ is known to act on other Na\textsubscript{v} channels, and whether its efficacy in relieving pain in this patient is due to Na\textsubscript{v}1.7 modulation, effects on other Na\textsubscript{v} channel isoforms, or a combination of the two, cannot be definitively determined.

In summary, we report here the identification of a novel rare variant of Na\textsubscript{v}1.7 in a patient with adult-onset pain symptoms. Functional assessment of mutant channels demonstrated that the V810M mutation conferred gain-of-function attributes on the channel and rendered DRG neurons that express the mutant channels hyperexcitable. We also assessed the sensitivity of the channel to a clinically achievable concentration of CBZ. Voltage-clamp recordings showed that CBZ did not depolarize activation of the mutant channel but produced use-dependent inhibition which may have contributed to the pain relief reported by the patient.

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Author Contributions
TA, ME, SDD-H, and SGW designed the research; TA, ME, BRS, and SV performed the research; TA, ME, SDD-H, and SGW analyzed the data; TA, SV, SDD-H, and SGW wrote the paper.

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