Themed Section: Recent Advances in Targeting Ion Channels to Treat Chronic Pain

RESEARCH PAPER

Reverse pharmacogenomics: carbamazepine normalizes activation and attenuates thermal hyperexcitability of sensory neurons due to Na$_v$1.7 mutation I234T

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BACKGROUND AND PURPOSE
Pharmacotherapy for pain currently involves trial and error. A previous study on inherited erythromelalgia (a genetic model of neuropathic pain due to mutations in the sodium channel, Na$_v$1.7) used genomics, structural modelling and biophysical and pharmacological analyses to guide pharmacotherapy and showed that carbamazepine normalizes voltage dependence of activation of the Na$_v$1.7-S241T mutant channel, reducing pain in patients carrying this mutation. However, whether this approach is applicable to other Na$_v$ channel mutants is still unknown.

EXPERIMENTAL APPROACH
We used structural modelling, patch clamp and multi-electrode array (MEA) recording to assess the effects of carbamazepine on Na$_v$1.7-I234T mutant channels and on the firing of dorsal root ganglion (DRG) sensory neurons expressing these mutant channels.

KEY RESULTS
In a reverse engineering approach, structural modelling showed that the I234T mutation is located in atomic proximity to the carbamazepine-responsive S241T mutation and that activation of Na$_v$1.7-I234T mutant channels, from patients who are known to respond to carbamazepine, is partly normalized with a clinically relevant concentration (30 μM) of carbamazepine. There was significantly higher firing in intact sensory neurons expressing Na$_v$1.7-I234T channels, compared with neurons expressing the normal channels (Na$_v$1.7-WT). Pre-incubation with 30 μM carbamazepine also significantly reduced the firing of intact DRG sensory neurons expressing Na$_v$1.7-I234T channels. Although the expected use-dependent inhibition of Na$_v$1.7-WT channels by carbamazepine was confirmed, carbamazepine did not enhance use-dependent inhibition of Na$_v$1.7-I234T mutant channels.

CONCLUSION AND IMPLICATIONS
These results support the utility of a pharmacogenomic approach to treatment of pain in patients carrying sodium channel variants.

LINKED ARTICLES
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Abbreviations
DRG, dorsal root ganglion; IEM, inherited erythromelalgia; MEA, multi-electrode array

Introduction

Pharmacogenomics has, as a major goal, the matching of specific medications with particular patients on the basis of their genetic background. The voltage gated **sodium channel Na$_v$1.7** sets the gain on dorsal root ganglion (DRG) sensory neurons (Waxman, 2006) and plays a major role in the pathophysiology of pain in humans (Dib-Hajj *et al.*, 2013). Over the past decade, it has become clear that point mutations that hyperpolarize the voltage dependence of activation of Na$_v$1.7 channels cause hereditary pain disorders characterized by severe distal limb pain (Cummins *et al.*, 2004; Dib-Hajj *et al.*, 2005; Faber *et al.*, 2012; Hoeijmakers *et al.*, 2012; Huang *et al.*, 2014). Pain in most of inherited erythromelalgia (IEM) patients is not relieved by pharmacotherapy. However, an IEM family carrying the Na$_v$1.7-V400M mutation was found to be responsive to **carbamazepine** which normalized activation of the mutant channel, shifting its activation in a depolarizing direction towards wild-type (WT) values (Fischer *et al.*, 2009). Yang *et al.* capitalized on this finding, using the V400M mutation as a seed to predict, by structural modelling, that another Na$_v$1.7 mutation, S241T, should also be responsive to treatment with carbamazepine, confirming responsiveness of DRG sensory neurons carrying S241T mutant channel *in vitro* (Yang *et al.*, 2012). In a double-blind, placebo-controlled follow-up clinical study, we showed that treatment of patients carrying the S241T mutation with carbamazepine produced substantial pain relief (Geha *et al.*, 2016). However, whether this pharmacogenomic approach is applicable to other Na$_v$1.7 channel mutations has not been established.

Here, we followed up a report of pain relief by carbamazepine in a patient with severe pain due to the Na$_v$1.7 mutation I234T (Meijer *et al.*, 2014). This patient reported warmth-induced pain attacks, similar to the vast majority of IEM patients carrying Na$_v$1.7 mutations. We have used a reverse engineering approach to show that the Na$_v$1.7-I234T mutation is located in proximity to the carbamazepine-responsive V400M and S241T mutations within the folded channel protein, and revealed that carbamazepine partly normalized hyperpolarized activation of the mutant channel and attenuated physiologically relevant thermally induced hyperexcitability of DRG sensory neurons expressing Na$_v$1.7-I234T channels. Our demonstration of carbamazepine-induced restoration of channel activation and neuronal excitability towards WT values supports the notion that a pharmacogenomic approach to pain treatment is an achievable objective.

Methods

Structural modelling

Structural modelling was carried out as described in our previous reports (Yang *et al.*, 2012) (Yang *et al.*, 2013). Sequence alignment was performed using Clustal Omega and refined manually (Yarov-Yarovoy *et al.*, 2012). Individual models for the four transmembrane domains of human hNa$_v$1.7 channel were generated with GPCR-ITASSER (Roy *et al.*, 2011) (Zhang *et al.*, 2015) by using templates including bacterial sodium channels (Protein Data Bank ID code 3RVY) (Payandeh *et al.*, 2011). Four individual domains were assembled in a clockwise order viewed from the extracellular side (Dudley *et al.*, 2000) (Li *et al.*, 2001). The four domain complex structural model was refined by fragment-guided molecular dynamics simulation (Zhang *et al.*, 2011). PyMol (Schrödinger, New York, NY, USA) was used to generate figures from the models.

Plasmid preparation and HEK293 cell transfection

A channel construct encoding eGFP with a 'StopGo' 2A linker (Atkins *et al.*, 2007) in-frame with the N-terminus of human Na$_v$1.7-WT channel that has been rendered resistant to tetrodotoxin (TTX-R) by the Tyr362Ser substitution has been previously described (Huang *et al.*, 2014) (Yang *et al.*, 2016). The I234T mutation was created using a site-directed mutagenesis kit (Agilent). These TTX-R derivatives of hNa$_v$1.7 were used to establish HEK293 cell lines stably expressing Na$_v$1.7-I234T or Na$_v$1.7-WT. HEK293 cells were maintained in DMEM/F-12 supplemented with 10% FBS (HyClone) in an incubator with 5% CO$_2$ at 37°C. HEK293 cells were seeded on glass coverslips coated with poly-L-lysine (BD Biosciences, San Jose, CA, USA) in a 24-well plate.

Voltage clamp recording of HEK293 cells

Whole-cell voltage clamp recordings were obtained from HEK293 cells expressing WT or mutant Na$_v$1.7 channels using an EPC-10 amplifier and the PatchMaster program (v 53; HEKA Elektronik, Holliston, MA, USA) at room temperature. The extracellular solution contained the following (in mM): 140 NaCl, 3 KCl, 1 MgCl$_2$, 1 CaCl$_2$, 20 dextrose and 10 HEPES, pH = 7.3 with NaOH (320 mOsm adjusted with dextrose). The pipette solution contained the following (in mM): 140 Cs-fluoride, 10 NaCl, 1 EGTA, 10 HEPES, 20 dextrose, pH = 7.3 with CsOH (310 mOsm adjusted with dextrose). Patch pipettes had a resistance of 1–2 MΩ when filled with pipette solution. Series resistance and prediction compensation (80–90%) was applied to reduce voltage errors. The recorded currents were digitized at a rate of 50 kHz after passing through a low-pass Bessel filter setting of 10 kHz. After achieving whole-cell configuration, a 5 min equilibration period was applied before starting the recording.

Voltage clamp recording data were analysed using Fitmaster (HEKA Elektronik) and OriginPro (Microcal Software, Northampton, MA, USA). For activation curves to be generated, cells were held at −120 mV and stepped to potentials of −80 to 40 mV in 5 mV increments for 100 ms. Peak inward currents were extracted by Origin automatically and fitted with a Boltzmann function (BoltzIV) to determine the voltage at half-activation (V$_{1/2}$), activation curve slope at half-activation (Z) and reversal potential (E$_{Na}$) for each successful recording. Conductance was calculated as $G = I/(Vm − E_{Na})$, normalized by the maximum conductance value and fitted with Boltzmann equation (Cummins *et al.*, 2004; Dib-Hajj *et al.*, 2005). To examine the use-dependent effect of carbamazepine on the WT and I234T channels, we depolarized HEK293 cells expressing either Na$_v$1.7-WT or Na$_v$1.7-I234T mutant channels to −10 mV from a −120 mV holding potential at a frequency of 5, 10 and 20 Hz, with pre-incubation with DMSO or 30 μM carbamazepine (Choi *et al.*, 2009; Estacion *et al.*, 2010).

Isolation and transfection of primary DRG sensory neurons

All animal care and experimental studies were approved by the Veterans Administration Connecticut Healthcare System Institutional Animal Care and Use Committee. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). Dorsal root ganglia (DRGs) were harvested from 4- to 6-week-old female and male Sprague Dawley rats and dissociated as described previously (Dib-Hajj *et al.*, 2009b) (Huang *et al.*, 2013). Briefly, DRGs were dissociated with a 20 min incubation in 1.5 mg·mL^{-1} collagenase A (Roche, Indianapolis, IN, USA) and 0.6 mM EDTA, followed by an 18 min incubation in 1.5 mg·mL^{-1} collagenase D (Roche), 0.6 mM EDTA and 30 U·mL^{-1} papain (Worthington Biochemical, Lakewood, NJ, USA). DRGs were then centrifuged and triturated in 0.5 mL of DRG media containing 1.5 mg·mL^{-1} BSA (low endotoxin) and 1.5 mg·mL^{-1} trypsin inhibitor (Sigma, St. Louis, MO, USA). After trituration, 2 mL of DRG media was added to the cell suspension, which was filtered with a 70 μm nylon mesh cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA). The mesh was washed twice with 2 mL of DRG media. Neurons were then transfected with Na$_v$1.7-I234T or Na$_v$1.7-WT using a Nucleofector IIS (Lonza, Basel, Switzerland) and Amaxa Basic Neuron SCN Nucleofector Kit (VSPI-1003).

Multi-electrode array (MEA) recording

MEA experiments were carried out according to our recently developed protocol (Yang *et al.*, 2016). Briefly, dissociated neurons were maintained at 37°C in a 5% CO$_2$ incubator for 3 days before MEA recordings. Action potential activity in these neurons was assessed using a multi-well MEA system (Maestro, Axion Biosystems, Atlanta, GA, USA). A 12-well recording plate was used, embedded with a total of 768 electrodes. For each experiment, three wells (with ~192 available electrodes for recording) were used to assess neurons expressing Na$_v$1.7-WT or Na$_v$1.7-I234T mutant channels. For potential variations to be minimized during the recording, neurons expressing WT or I234T constructs, derived from DRG sensory neurons pooled from two rats on the same day by the same investigator, were plated in the same 12-well MEA recording plate. Additionally, we always analyse sister cultures prepared in parallel by the same technician, in a head-to-head comparison. For experiments with carbamazepine, neurons were pretreated with either carbamazepine (30 μM) or DMSO for more than 30 min in its normal culture medium as described previously (Yang *et al.*, 2012). Measurement of the mean firing frequency and the number of active electrodes with DMSO or carbamazepine treatment was made from the same culture, providing an assessment on the efficacy of carbamazepine on the same neurons.

The investigator was blinded to the identity of the constructs expressed in the DRG sensory neurons during the recording. A spike detection criterion of >6 standard deviations above background signals was used to separate action potential spikes from noise. We defined active electrodes as registering >1 recorded spike over a 200 s period (Yang *et al.*, 2016). MEA data were analysed using Axion Integrated Studio AxIS2.1 (Axion Biosystems) and NeuroExplorer (Nex Technologies, Madison, AL, USA).

For assessment of the effects of carbamazepine on DRG sensory neurons expressing either Na$_v$1.7-WT or Na$_v$1.7-I234T mutant channels under different temperatures, the precise temperature control of the MEA system was utilized, which enables continuous monitoring of neuronal firing during temperature ramps. DRG sensory neurons expressing either Na$_v$1.7-WT or Na$_v$1.7-I234T channels were plated on the same MEA plate for temperature ramp study and assessed by an investigator blinded to the identity of the constructs. Three different temperatures (33, 37 and 40°C) were used for the study, and each temperature was maintained for 7–10 min to allow analysis of steady-state neuronal firing at each temperature.

Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Results are presented as mean ± SEM. Unless otherwise noted, statistical significance was determined using Student's *t*-test. Use-dependent inhibition analysis was done using two-factor ANOVA with replication and Bonferroni *post hoc* test. We accepted statistical significance when $P < 0.05$.

Materials

All chemicals used in this study, including carbamazepine, were supplied by Sigma-Aldrich.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b).

Results

The I234T mutation is located in atomic proximity to the carbamazepine-responsive S241T mutation within the folded sodium channel structure

Using the carbamazepine-responsive V400M Na$_v$1.7 mutation as a seed, we previously observed that the S241T mutation, located in proximity to V400M in the channel folded structure, responds to carbamazepine (Yang *et al.*, 2012). We hypothesized that this atomic proximity of mutations within the channel 3D structure might be an indicator of their responsiveness to carbamazepine, which was confirmed at the channel level (by voltage clamp) and at the cell level (by current-clamp) *in vitro* (Yang *et al.*, 2012), and in patients with IEM carrying this mutation (Geha *et al.*, 2016). This finding encouraged us to hypothesize that structural modelling might identify additional candidate mutations of Na$_v$1.7 channels for further biophysical and pharmacological analysis. Among existing disease-causing IEM mutations of Na$_v$1.7 channels, we identified the I234T mutation as a primary candidate using this approach (Figure 1A). As shown in the structural model of the Na$_v$1.7 channel, I234 is located in the same
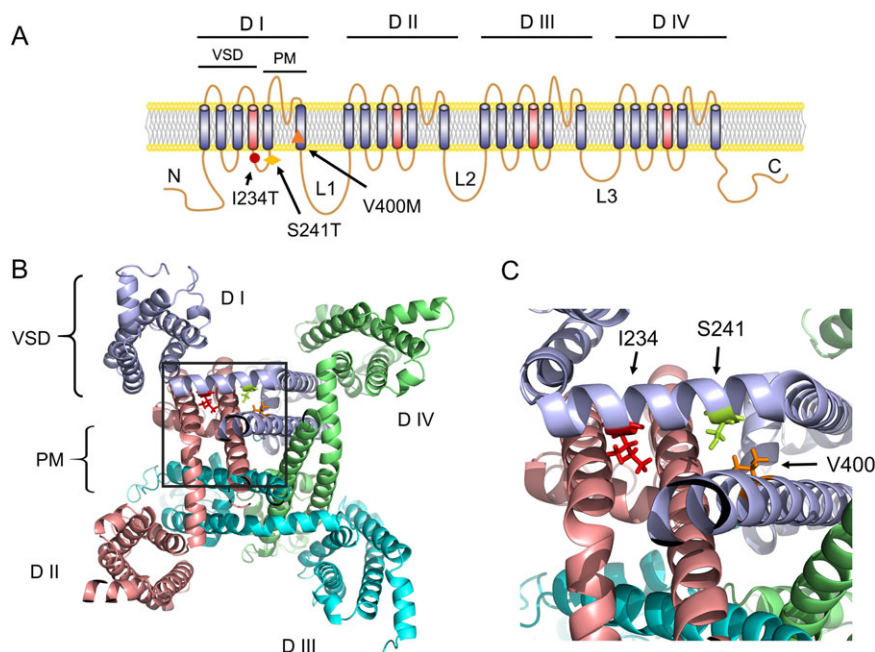
Figure 1

Structural modelling of the I234T of human Na$_v$1.7 channels. (A) Diagram of the human Na$_v$1.7 channel topology showing the location of I234T, S241T and V400M, all carbamazepine-responsive mutations, in the domain I (D I). (B) Cytosolic view of the 3D structural model of human Na$_v$1.7 transmembrane domains. (C) Close-up cytosolic view of the boxed area of panel (B). I234, S241 and V400 are shown as stick model. VSD, voltage-sensing domain; PM, pore module. L1/2/3, intracellular loop 1/2/3.

S4–S5 linker as S241 (Figure 1B). Upon closer examination, we found that the side chain of I234 was located within ~6.5 Å of S241, pointing towards the S6 helix with the same orientation of S241 (Figure 1C). Extrapolating from our previous demonstration that the proximity to V400M, a carbamazepine-responsive mutation, identified S241T as a potential carbamazepine-responsive mutation, here we hypothesized that the proximity of I234 to S241 and the orientation of their side chains also serves as a potential indicator of responsiveness to carbamazepine.

Carbamazepine partly corrects hyperpolarized activation of Na$_v$1.7-I234T mutant channel

To determine whether carbamazepine could also normalize the biophysical properties of the Na$_v$1.7-I234T mutant channel as it did for Na$_v$1.7-S241T channels, we studied the voltage dependence of activation of Na$_v$1.7-I234T channels using voltage clamp recording. Compared with Na$_v$1.7-WT, the Na$_v$1.7-I234T channel displays a remarkable ~18 mV hyperpolarizing shift in the V$_{1/2}$ of activation (Ahn *et al.*, 2010), making the mutant channel easier to open in response to small depolarizing stimuli. To determine whether carbamazepine corrected this hyperpolarizing shift of channel activation, we pre-incubated the Na$_v$1.7-I234T cells with either DMSO or a clinically relevant concentration (30 μM) of carbamazepine for at least 30 min or longer before recording (Fischer *et al.*, 2009; Yang *et al.*, 2012). Carbamazepine or DMSO was maintained in the external bath solution during the recording. Representative current traces from neurons

expressing Na$_v$1.7-WT or Na$_v$1.7-I234T channels are shown in Figure 2A, B. Notably, pre-incubation with 30 μM carbamazepine produced a significant depolarizing shift (~6 mV) in the V$_{1/2}$ of activation compared with that with DMSO (I234T with DMSO: -41.48 ± 0.7 mV, $n = 11$; I234T with carbamazepine: -35.69 ± 1.29 mV, $n = 12$; $P < 0.05$, Student's t-test; Figure 2C). This type of partial correction of V$_{1/2}$ of activation was seen in Na$_v$1.7-S241T and Na$_v$1.7-V400M channels pretreated with carbamazepine (Fischer *et al.*, 2009; Yang *et al.*, 2012). I234T channels respond to carbamazepine in a similar manner to S241T channels, suggesting that a partial correction of activation V$_{1/2}$ may confer carbamazepine responsiveness to DRG sensory neurons expressing this mutant channel.

To determine whether carbamazepine might have a similar effect on the Na$_v$1.7-WT channel, we studied the activation of WT Na$_v$1.7 channels with or without carbamazepine pretreatment. Interestingly, we found that carbamazepine does not shift the activation of WT Na$_v$1.7 (Na$_v$1.7-WT DMSO: -25.2 ± 1.3 mV, $n = 8$; Na$_v$1.7-WT carbamazepine: -23.0 ± 1.2 mV, $n = 9$; $P > 0.05$) (Figure 2D), suggesting that the carbamazepine effect on channel activation is mutant-specific.

We also assessed whether carbamazepine affects fast inactivation of the Na$_v$1.7-I234T mutant channel. As found previously for the V400M and S241T mutant channels, carbamazepine did not change the fast inactivation of I234T mutant channels (I234T DMSO: -75.4 ± 1.2 mV; I234T carbamazepine: -74.5 ± 1.3 mV, $P > 0.05$).
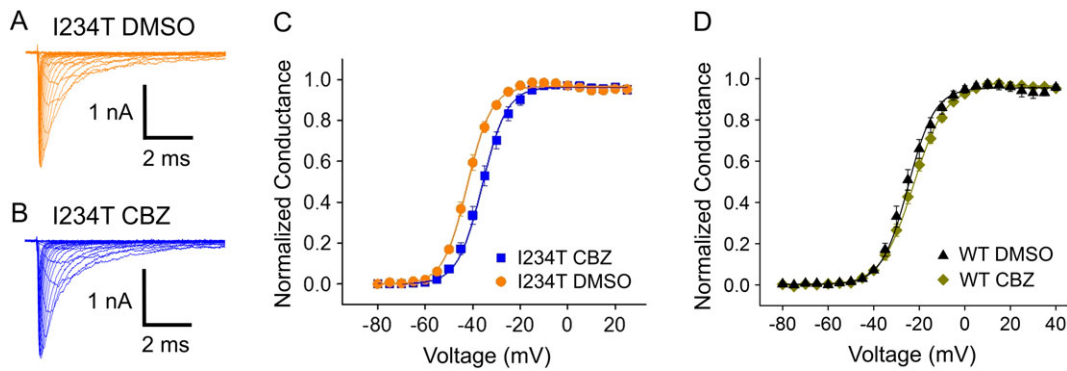
Figure 2

Carbamazepine depolarizes the voltage dependence of activation of the Na$_v$1.7-I234T mutant channel. (A, B) Representative current traces recorded from HEK293 cells expressing Na$_v$1.7-I234T mutant channels with either (A) DMSO or (B) carbamazepine (CBZ) pre-incubation. (C) The voltage dependence of activation curves of Na$_v$1.7-I234T mutant channels treated with DMSO or carbamazepine were plotted and fitted with Boltzman equation. A depolarizing shift of activation of ~6 mV was observed when Na$_v$1.7-I234T mutant channels was pre-incubated with carbamazepine. (D) The voltage dependence of activation curves of Na$_v$1.7-WT channels treated with DMSO or carbamazepine were plotted and fitted with Boltzman equation. No significant difference was found between the curves from Na$_v$1.7-WT channels incubated with DMSO and those incubated with carbamazepine.

Na$_v$1.7-I234T mutation increases the firing of intact DRG sensory neurons across a physiological temperature range

The hyperpolarizing shift of activation of Na$_v$1.7-I234T channels suggests that the expression of this mutation may cause hyperexcitability of DRG sensory neurons. To assess the firing properties of DRG sensory neurons expressing Na$_v$1.7-I234T mutant channels, we used MEA recording, which provides a non-invasive, high-throughput, extracellular

recording approach that can monitor the firing of intact neurons, at three physiological temperatures: skin temperature (33°C), core body temperature (37°C), and non-noxious warmth (40°C) (Yang *et al.*, 2016).

We observed a marked difference in response to increasing temperature between DRG sensory neurons expressing Na$_v$1.7-WT channels and Na$_v$1.7-I234T mutant channels. Firing frequencies, which are represented by colours as a heatmap (Figure 3A–F), were clearly different between DRG
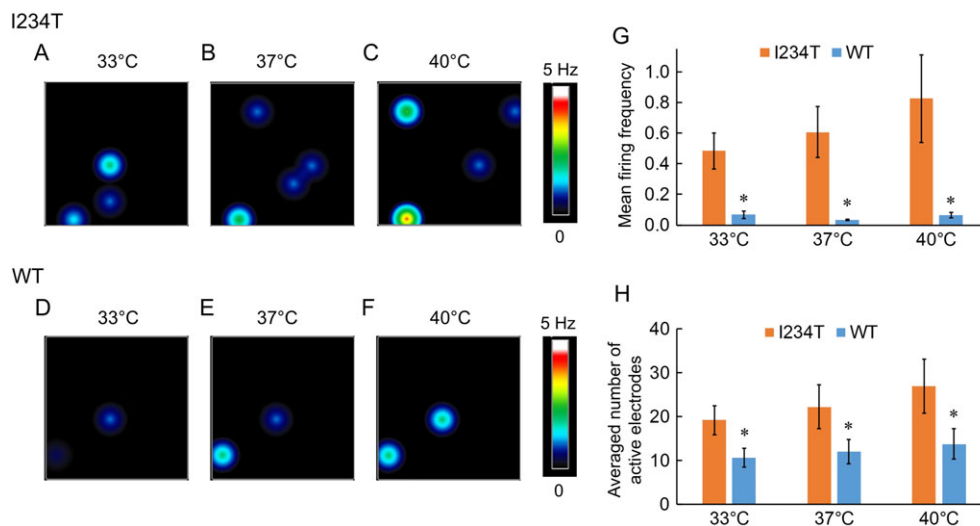

Figure 3

DRG sensory neurons expressing Na$_v$1.7-I234T mutant channels display increased firing. (A–F) Heatmap of representative MEA recordings from DRG sensory neurons expressing Na$_v$1.7-I234T (A–C) or WT channels (D–F). The firing frequency of each active electrode is colour-coded: white/ red represents high firing frequency; blue/black represents low firing frequency. Each circle represents an active electrode within an 8 × 8 electrode array. (G) Mean firing frequency of neurons expressing Na$_v$1.7-WT channels and Na$_v$1.7-I234T mutant channels at 33, 37 and 40°C. *$P < 0.05$, significantly different from WT; I234T, $n = 4$ experiments with eight rats; WT, $n = 5$ experiments with 10 rats. (H) Averaged number of active electrodes from neurons expressing Na$_v$1.7-WT or Na$_v$1.7-I234T channels at these three temperatures. *$P < 0.05$, significantly different from WT.

neurons expressing $Na_v1.7$-WT and $Na_v1.7$-I234T channels. The firing level of DRG sensory neurons expressing $Na_v1.7$-WT channels was fairly low across all three temperatures. In contrast, the expression of I234T mutant channels significantly increased the firing of DRG sensory neurons at all three temperatures (Figure 3G).

We found that the number of active electrodes for DRG sensory neurons expressing $Na_v1.7$-WT channels was also lower than for those expressing $Na_v1.7$ I234T mutant channels at each experimental temperature (Figure 3H). Although neurons expressing I234T mutant channel exhibited a trend for increased firing frequency and number of active electrodes with increased temperature, this difference did not reach statistical significance. Taken together, our data indicate that expression of $Na_v1.7$-I234T mutant channels causes hyperexcitability of DRG sensory neurons by increasing the firing frequency and the number of active neurons.

Carbamazepine reduces the firing of intact DRG sensory neurons expressing $Na_v1.7$-I234T mutant channels across a physiological temperature range

To determine whether carbamazepine has an inhibitory effect on the firing of DRG sensory neurons expressing $Na_v1.7$-I234T channels, we assayed the firing of DRG sensory neurons with or without pre-incubation with a clinically relevant concentration of carbamazepine (30 μM) using MEAs at 33, 37 and 40°C. We found that carbamazepine markedly attenuated the firing of DRG sensory neurons expressing I234T mutant channels (Figure 4A–F) in terms of both mean firing frequency and average number of active electrodes across all three temperatures (Figure 4G,H).

These results indicate that a clinically relevant concentration of carbamazepine indeed inhibited firing of DRG sensory

neurons expressing $Na_v1.7$-I234T channels, by reducing both the number of active neurons and the firing frequency of neurons across a physiological temperature range.

Carbamazepine did not significantly reduce the firing of intact DRG sensory neurons expressing $Na_v1.7$-WT channels

To assess whether the inhibitory effect of carbamazepine on the firing of DRG sensory neurons expressing Na1.7-I234T mutant channels is specific, we performed similar MEA experiments on DRG sensory neurons expressing $Na_v1.7$-WT channels. We found that carbamazepine had no significant effect on the firing of DRG sensory neurons expressing $Na_v1.7$-WT channels, compared with DMSO (Figure 5A–G). Additionally, the number of active electrodes remained relatively unchanged with DMSO or carbamazepine treatment, for all conditions (Figure 5H). These data suggest that the main effect of carbamazepine on DRG sensory neurons expressing $Na_v1.7$-I234T mutant channels was mutant-specific.

The effect of carbamazepine is not mediated via use-dependent inhibition of the channel peak current

Local anaesthetics, anticonvulsants (e.g. carbamazepine) and tricyclic antidepressants exhibit use-dependent inhibition against voltage-gated sodium channels (Tanelian and Brose, 1991; Kuo et al., 1997; Kuo, 1998; Yang et al., 2010). In the case of V400M, S241T and I234T channels, we found that carbamazepine acts via a novel mechanism by depolarizing activation of mutant channels (Fischer et al., 2009; Yang et al., 2012). Here, we wanted to further determine whether carbamazepine might have a classical use-dependent inhibitory effect on the $Na_v1.7$-I234T mutant channel. To examine the use-dependent effect of carbamazepine on the WT and
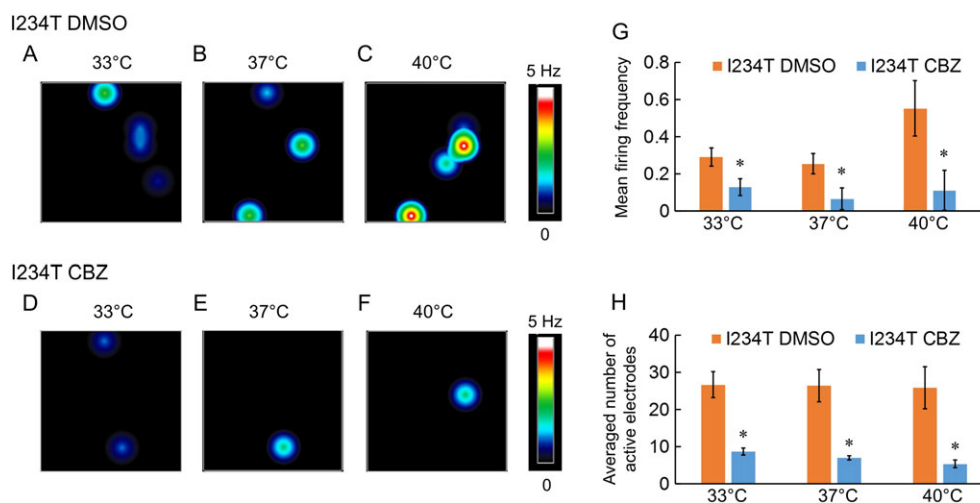

Figure 4

Carbamazepine reduces the firing of DRG sensory neurons expressing $Na_v1.7$-I234T mutant channels. (A–F) Heatmap of a representative MEA recording of DRG sensory neurons expressing $Na_v1.7$-I234T with DMSO or carbamazepine (CBZ) pre-incubation. Carbamazepine produces a pronounced reduction in the number of active electrodes and mean firing frequency. (G) mean firing frequency of neurons expressing $Na_v1.7$-I234T channels with DMSO or carbamazepine pre-incubation at all three temperatures (33, 37 and 40°C). *$P < 0.05$, significantly different from DMSO; $n = 6$ experiments with 12 rats. (H) Averaged number of active electrodes from neurons expressing $Na_v1.7$-I234T channels with DMSO or carbamazepine pre-incubation at all three temperatures (33, 37 and 40°C). *$P < 0.05$, significantly different from DMSO.
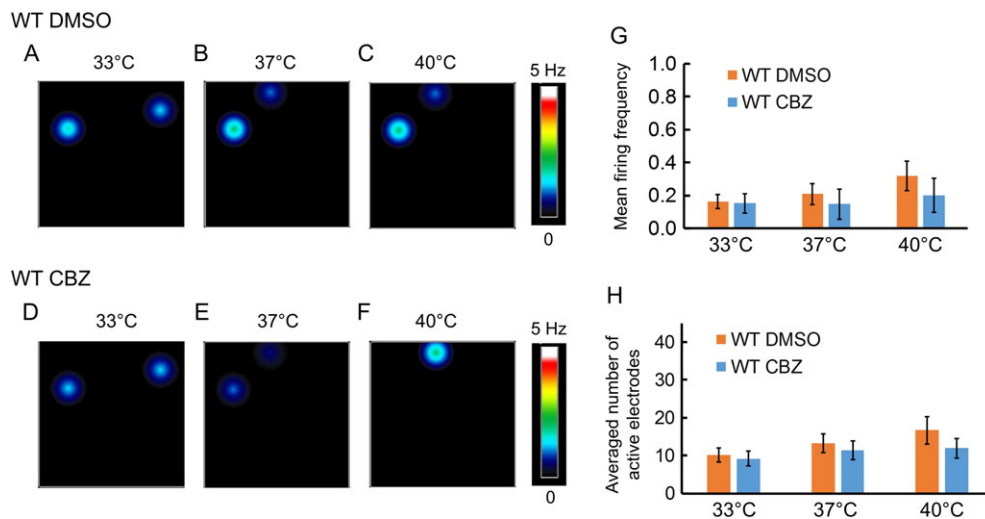
Figure 5

Carbamazepine does not reduce the firing of DRG sensory neurons expressing Na$_v$1.7-WT channels significantly. (A–F) Heatmap of a representative MEA recording of DRG sensory neurons expressing Na$_v$1.7-WT channels with DMSO or carbamazepine (CBZ) pre-incubation. Carbamazepine did not significantly affect the firing frequency of these neurons. (G) Mean firing frequency of neurons expressing Na$_v$1.7-WT channels with pre-incubation with DMSO or carbamazepine, at all three temperatures (33, 37 and 40°C). $n = 5$ experiments with 10 rats. (H) Averaged number of active electrodes from neurons expressing Na$_v$1.7-WT channels with DMSO or carbamazepine pre-incubation at all three temperatures (33, 37 and 40°C).

I234T channels, we depolarized HEK293 cells expressing either Na$_v$1.7-WT or Na$_v$1.7-I234T mutant channels to −10 mV from a −120 mV holding potential at a frequency of 20 Hz with the pre-incubation of DMSO or 30 μM carbamazepine.

We found that 30 μM carbamazepine indeed produced a notable use-dependent inhibition of Na$_v$1.7-WT channels, compared with DMSO control at 20 Hz frequency (Figure 6A, B). For Na$_v$1.7-WT channels, use-dependent current amplitude at the 30th pulse was reduced to a significantly greater extent after carbamazepine than after DMSO (Figure 6E). On the other hand, Na$_v$1.7-I234T channels displayed a stronger use-dependent fall-off with DMSO pre-incubation, similar to that with 30 μM carbamazepine pre-incubation (Figure 6C, D, F). Additionally, we studied use-dependent inhibition of Na$_v$1.7-I234T channels at other frequencies (5 and 10 Hz) and found that carbamazepine did not affect use-dependent inhibition at these frequencies either (at 5 Hz, I234T DMSO: 91.2 ± 1.3%; I234T carbamazepine: 91.1 ± 1.1%; $n = 9$, $P > 0.05$. At 10 Hz, I234T DMSO: 84.3 ± 1.9%; I234T carbamazepine: 84.7 ± 2.0%; $n = 8$, $P > 0.05$). Taken together, our data demonstrate that a clinically relevant concentration of carbamazepine produces a use-dependent inhibition of the Na$_v$1.7-WT channel but not of the Na$_v$1.7-I234T mutant channel, suggesting that use-dependent current reduction is not likely to be a contributing factor for carbamazepine-mediated effects on the firing of DRG sensory neurons expressing Na$_v$1.7-I234T mutant channels.

Discussion

IEM, a painful disorder caused by gain-of-function mutations in Na$_v$1.7 channels, provides a genetic model of neuropathic pain (Drenth and Waxman, 2007; Dib-Hajj *et al.*, 2009a; Dib-Hajj *et al.*, 2013). Pharmacotherapy of IEM remains generally ineffective, and the mainstay of treatment at this time is identification and avoidance of triggers of pain episodes, combined with cooling of the distal extremities when episodes occur. However, sodium channel blockers have been used with some success in certain patients. **Lidocaine** and **mexiletine** have been reported to be efficacious in some patients, although their effects are transient (Harty *et al.*, 2006). In a child with another Na$_v$1.7 mutation, treatment with mexiletine was effective for 1 month before termination due to concerns about side effects (Choi *et al.*, 2009).

The selection of a pharmacological therapy for most of these IEM patients has been thus far based on trial and error. Building upon the observation that carbamazepine normalizes activation in the V400M IEM mutant Na$_v$1.7 channel and that carbamazepine monotherapy produces pain relief in patients carrying this mutation (Fischer *et al.*, 2009), a pharmacogenomic approach aided by structural modelling and *in vitro* functional assays (Yang *et al.*, 2012) predicted the efficacy of treatment of patients carrying the S241T mutation of Na$_v$1.7 channels (Geha *et al.*, 2016), suggesting that pharmacogenomically guided therapy for pain might be applied more broadly.

Initially identified in a 5-year-old British girl with episodes of severe intermittent pain, the I234T mutation substitutes a highly conserved isoleucine 234 by threonine at the N-terminus of the linker between S4 and S5 in domain I (D1/S4–S5) of the Na$_v$1.7 channel. When studied by voltage clamp, this mutation hyperpolarizes activation by ~18 mV (Ahn *et al.*, 2010). A trial of carbamazepine in this patient was terminated because of side effects (Ahn *et al.*, 2010). A second patient of French-Canadian ancestry carrying the same mutation, also with a history of severe intermittent pain, was subsequently reported (Meijer *et al.*, 2014) (Kim
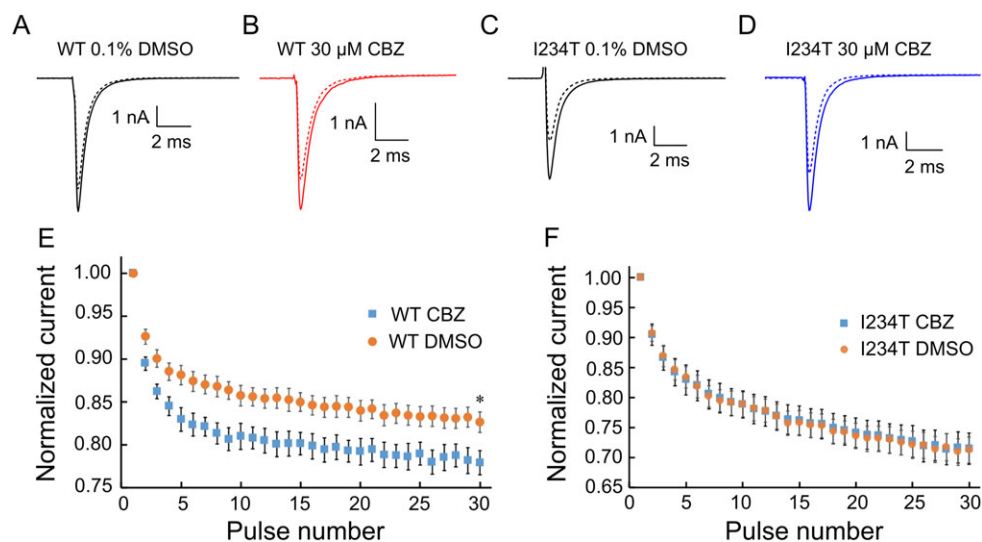
Figure 6

The effect of carbamazepine is not mediated via a use-dependent inhibition of $Na_v1.7$-I234T mutant channels. The use-dependent block, defined as the ratio of the peak from the 30th pulse normalized to the peak of the first pulse, at a frequency of 20 Hz recording, is determined for each cell. (A, B) Representative first and last (30th) current traces recorded from HEK293 cells expressing $Na_v1.7$-WT channels with either (A) DMSO or (B) carbamazepine (CBZ) pre-incubation. (C, D) Representative first and last (30th) current traces recorded from HEK293 cells expressing $Na_v1.7$-I234T mutant channels with either DMSO (C) or carbamazepine (D) pre-incubation. (E, F) Use-dependent block curves of $Na_v1.7$-WT and $Na_v1.7$-I234T mutant channels at a frequency of 20 Hz recording were plotted. (E) Use-dependent fall-off of peak $Na_v1.7$-WT current with the pre-incubation of DMSO or 30 μM carbamazepine.*$P < 0.05$, significantly different from DMSO; $n=9$. (F) Use-dependent fall-off of peak $Na_v1.7$-I234T current with the pre-incubation of DMSO or 30 μM carbamazepine.

et al., 2015). Episodes of severe pain, associated with sweating and erythema of the lower limbs and hands, lasting 30–60 s, recurred multiple times per hour. In response to treatment with carbamazepine, at a therapeutic dose (30 mg·kg^{-1}) with adequate blood levels, the episodes became less frequent and less intense (Meijer *et al.*, 2014). This case represents the third mutation of IEM for which patients respond to treatment with carbamazepine and has provided the opportunity to test our approach using structural modelling and *in vitro* pharmacological analysis.

As a comparator, we have studied the $Na_v1.7$-F1449V mutation from a family that has been anecdotally reported to be non-responsive to carbamazepine. We reported that carbamazepine does not shift the activation of $Na_v1.7$-F1449V channels, nor does carbamazepine reduce the firing of DRG sensory neurons expressing these channels (Yang *et al.*, 2012). We would note, however, that anecdotal reporting of lack of clinical efficacy should be interpreted with caution. Ethical considerations preclude a formal clinical study on patients to test a drug that has been predicted not to be efficacious based on *in vitro* pharmacology.

A dose–response correlation for carbamazepine was obtained in our previous study on the V400M $Na_v1.7$ mutation (Fischer *et al.*, 2009), and 30 μM was chosen as a clinically relevant concentration to use in *in vitro* studies on the V400M (Fischer *et al.*, 2009), S241T (Yang *et al.*, 2012) and I234T channels (this current study). This concentration is consistent with therapeutic ranges of carbamazepine suggested in previous reports (Tomson *et al.*, 1980; O'Dougherty *et al.*, 1987; Breton *et al.*, 2005). More importantly, we recently studied the effect of carbamazepine in patients with IEM

and observed a clinical response (reduction in pain) with serum concentrations of carbamazepine of ~3.6–6.0 mg·L^{-1}, corresponding to 15.2–25.4 μM (Geha *et al.*, 2016).

In our previous study, we used mutant cycle analysis to study the coupling between S241T and V400M because these two residues are located 159 amino acids away from each other, in the S4–5 linker and S6 transmembrane regions of domain I of the channel. The conventional view, which the S4–5 linker may exert an effect on the S6 transmembrane segment (Payandeh *et al.*, 2011), prompted an empirical assessment of energetic coupling. By contrast, I234 and S241 are located within the same helix, so that mutant cycle analysis is not needed.

In the current study, we found that DRG sensory neurons expressing $Na_v1.7$-WT channels do not display a notable temperature-related response, when studied via MEA recording. This phenomenon has been observed in our previous studies (Yang *et al.*, 2016). We suggest that other sensory neurons in the culture that do not normally respond to temperature may mask the effect of temperature-sensitive neurons (Yang *et al.*, 2016).

A characteristic action of carbamazepine on mutant $Na_v1.7$ channels, identified from carbamazepine-responsive individuals with IEM, is that it shifts the voltage dependence of activation in the direction of WT channels. We found that in all three mutants (V400M, S241T and I234T), incubation with carbamazepine for at least 30 min is required, to affect the channel activation. We hypothesize that the I234T, S241T or V400M mutations may have an unusual allosteric effect on the channel and that carbamazepine may act on these mutant channels via an action as a chaperone that

'corrects' the folding of the mutant channels in a similar manner to the rescue of folding defective proteins by binding to their inhibitors, which may require additional time (Yang *et al.*, 2012).

In the present study, voltage clamp experiments showed that pre-incubation of cells expressing the Na$_v$1.7-I234T mutant channel with clinically relevant concentrations of carbamazepine (30 μM) shifted the $V_{1/2}$ of activation of the Na$_v$1.7-I234T mutant channel ~6 mV in a depolarizing direction, thereby partly returning activation to its normal voltage-dependence. The magnitude of this shift is comparable with that observed when similar treatment was applied to cells expressing the V400M (Fischer *et al.*, 2009) and S241T (Yang *et al.*, 2012) IEM mutations. Carbamazepine treatment of DRG sensory neurons expressing these mutant channels reduced hyperexcitability (Yang *et al.*, 2012; Geha *et al.*, 2016) (and this study). Importantly, the magnitude of carbamazepine-mediated reduced excitability of DRG sensory neurons expressing Na$_v$1.7-I234T channels was substantially greater than the effect of carbamazepine on DRG sensory neurons expressing Na$_v$1.7-WT channels. These findings argue that pain relief in these patients is due, at least in part, to an effect of carbamazepine on the responsive mutant Na$_v$1.7 channels that reduces peripheral barrage rather than a non-specific effect on sodium channels.

The mode of action of clinically relevant concentrations of carbamazepine, which induced a depolarizing shift of activation, returning its voltage dependence towards that of WT channels (Fischer *et al.*, 2009; Yang *et al.*, 2012), is distinct from its widely accepted activity as a use-dependent inhibitor of sodium channels (Tanelian and Brose, 1991; Kuo *et al.*, 1997; Kuo, 1998; Yang *et al.*, 2010). Indeed, we confirmed here the use-dependent blockade of WT Na$_v$1.7 channels by carbamazepine. By contrast, I234T mutant channels displayed a use-dependent fall-off in the current which was not enhanced by treatment with carbamazepine. These data suggest that the classical blockade mechanism by carbamazepine (use-dependent inhibition of hyperactive channels) does not play a detectable role in reducing the firing of DRG sensory neurons expressing Na$_v$1.7-I234T mutant channels.

One possible explanation for the effect of carbamazepine on specific Na$_v$1.7 mutations is that it acts as a chemical chaperone, in a manner analogous to the effect of carbamazepine, **phenytoin** and mexiletine on folding-defective **Na$_v$1.1** and **Na$_v$1.5** sodium channels (Valdivia *et al.*, 2004; Tan *et al.*, 2006; Rusconi *et al.*, 2007; Rusconi *et al.*, 2009) which rescue channel activity. However, unlike these previous studies, carbamazepine has an effect after about 30 min on cells expressing the Na$_v$1.7 mutant channel, rather than the ~24 h incubation that was used in the Na$_v$1.1 and Na$_v$1.5 mutant channels. The novel mode of action of carbamazepine on I234T, S241T and V400M channels, normalizing voltage dependence of activation, is also distinct from its effect in rescuing current density of folding-defective channels. For these reasons, we suggest that the effect of carbamazepine on the Na$_v$1.7 mutant channels involves a mechanism, other than those involved with folding-defective Na$_v$1.1 and Na$_v$1.5 channels or reflects a channel-specific response to treatment.

Examination of carbamazepine classic binding using the **Na$_v$1.2** channel model suggests that carbamazepine interacts with three key residues of the Na$_v$1.2 channel (Leu^{1465} of DIII-S6, Phe^{1764} of DIV-S6 and Tyr^{1771} of DIV-S6) (Lipkind and Fozzard, 2010). The corresponding residues in the Na$_v$1.7 channel are Leu^{1438} of DIII-S6, Phe^{1737} of DIV-S6 and Tyr^{1744} of DIV-S6 respectively. In the structural model of Na$_v$1.7 channels, these residues face the ion-conducting pathway, just as in the Nav1.2 channel model. Given the orientations and locations of these residues, no obvious interactions between these residues and the V400M, S241T or I234T mutants could be found. We hypothesize that the classic interaction of carbamazepine with the three residues in the ion-conducting pathway reflects its traditional mechanism of action via high affinity binding to the inactivated state of sodium channels, while the I234T mutation, like V400M and S241T, may allosterically affect the binding of carbamazepine to the channel.

In conclusion, we show here that structural modelling and *in vitro* functional assays demonstrate that the voltage dependence of Na$_v$1.7-I234T channels, from patients with IEM who responded to treatment with carbamazepine, is shifted to more depolarized potentials, closer to the value of WT channels, by clinically relevant concentrations of this agent. Carbamazepine treatment of DRG sensory neurons expressing the Na$_v$1.7-I234T mutant channel led to significant reduction in mutant-induced hyperexcitability in response to a physiologically relevant thermal stimulus, in agreement with amelioration of pain in patients carrying the I234T mutation. Our data also support the conclusion that the efficacy of carbamazepine in patients with the V400M, S241T and I234T mutations is due to a direct action on the mutant Na$_v$1.7 channels in primary afferents, rather than a pan-sodium channel effect of this drug. These data more generally support a pharmacogenomic approach, guided by structural modelling and functional assessment *in vitro*, to treatment of neuropathic pain, and suggest that the carbamazepine-induced depolarizing shift of voltage dependence activation might provide a predictor of efficacy in patients carrying Na$_v$1.7 channel variants.

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Author contributions

Y.Y., S.D.D.-H. and S.G.W. designed research; Y.Y., T.A. and L.C. performed research; Y.Y., T.A., L.C., P.E., S.D.D.-H. and S.G.W. analysed data; Y.Y., P.E., S.D.D.-H. and S.G.W. wrote the paper with inputs from T.A. and L.C.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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