Inherited Pain

**SODIUM CHANNEL NAV1.7 A1632T MUTATION CAUSES ERYTHROMELALGIA DUE TO A SHIFT OF FAST INACTIVATION**

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Mirjam Eberhardt‡§, Julika Nakajima†, Alexandra B. Klinger‡, Cristian Neacsu‡, Kathrin Hühne‡, Andrias O. O’Reilly‡, Andreas M. Kist‡, Anne K. Lampa‡, Kerstin Fischer**§, Jane Gibson‡§, Carla Nau**§‡, Andreas Winterpacht‡, and Angelika Lampert†‡§

From the †Institute of Physiology and Pathophysiology, Friedrich-Alexander-Universität Erlangen-Nürnberg, Universitätsstrasse 17, 91054 Erlangen, Germany, the ‡Department of Anesthesiology and Intensive Care Medicine, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, the §Department of Human Genetics Friedrich-Alexander Universität Erlangen-Nürnberg, Schwabachanlage 10, 91054 Erlangen, Germany, the ¶South East of Scotland Clinical Genetic Service, Western General Hospital, Edinburgh EH4 2XU, United Kingdom, the **Department of Anesthesiology Friedrich-Alexander Universität Erlangen-Nürnberg, Krankenhausstrasse 12, 91054 Erlangen, Germany, the §§Fife Rheumatic Diseases Unit, Whyteman’s Brae Hospital, Kirkcaldy, KY1 2ND, United Kingdom, the ‡‡Department of Anesthesiology and Intensive Care, University Medical Center Schleswig-Holstein, Campus Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany, and the ‡§Institute of Physiology, Rheinisch-Westfälische Technische Hochschule Aachen University, Paulwelsstrasse 30, 52074 Aachen, Germany

**Background:** Mutations in the sodium channel Nav1.7 cause the inherited pain syndromes IEM and PEPD.

**Results:** The new IEM mutation A1632T impairs channel inactivation, whereas an IEM/PEPD crossover mutation (A1632E) at the same position additionally increases resurgent sodium currents.

**Conclusion:** Reduced inactivation without increased resurgent currents induces symptoms of IEM.

**Significance:** Resurgent currents are likely to determine whether a mutation leads to IEM or PEPD.

Inherited erythromelalgia (IEM) causes debilitating episodic neuropathic pain characterized by burning in the extremities. Inherited “paroxysmal extreme pain disorder” (PEPD) differs in its clinical picture and affects proximal body areas like the rectal, ocular, or jaw regions. Both pain syndromes have been linked to mutations in the voltage-gated sodium channel Nav1.7. Electrophysiological characterization shows that IEM-causing mutations generally enhance activation, whereas mutations leading to PEPD alter fast inactivation. Previously, an A1632T mutation of a patient with overlapping symptoms of IEM and PEPD was reported (Estacion, M., Dib-Hajj, S. D., Benke, P. J., Te Morsche, R. H., Eastman, E. M., Macala, L. J., Drenth, J. P., and Waxman, S. G. (2008) NaV1.7 Gain-of-function mutations as a continuum. A1632T displays physiological changes associated with erythromelalgia and paroxysmal extreme pain disorder mutations and produces symptoms of both disorders. J. Neurosci. 28, 11079–11088), displaying a shift of both activation and fast inactivation. Here, we characterize a new mutation of Nav1.7, A1632T, found in a patient suffering from IEM. Although transfection of A1632T in sensory neurons resulted in hyperexcitability and spontaneous firing of dorsal root ganglia (DRG) neurons, whole-cell patch clamp of transfected HEK cells revealed that Nav1.7 activation was unaltered by the A1632T mutation but that steady-state fast inactivation was shifted to more depolarized potentials. This is a characteristic normally attributed to PEPD-causing mutations. In contrast to the IEM/PEPD crossover mutation A1632E, A1632T failed to slow current decay (i.e. open-state inactivation) and did not increase resurgent currents, which have been suggested to contribute to high-frequency firing in physiological and pathological conditions. Reduced fast inactivation without increased resurgent currents induces symptoms of IEM, not PEPD, in the new Nav1.7 mutation, A1632T. Therefore, persistent and resurgent currents are likely to determine whether a mutation in Nav1.7 leads to IEM or PEPD.

Sodium channels are important for action potential initiation and propagation in sensory neurons (1). Gain-of-function mutations in the gene SCN9A encoding for the voltage-gated sodium channel subtype Nav1.7 lead to distinct pain syndromes, which present as severe, debilitating neuropathy. Inherited (or primary) erythromelalgia (IEM; 2 MIM 133020) is characterized by episodic reddening of and burning pain in the hands and feet (2). Its onset is during adolescence, and patients often resort to submerging the limbs in ice-cold water for relief, which can lead to further complications like hypothermia, tissue damage, and wound infections (3, 4).

Paroxysmal extreme pain disorder (PEPD, MIM 167400) begins in early infancy with attacks of excruciating pain in proximal body areas like the rectal, ocular, or jaw regions provoked

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† To whom correspondence should be addressed: Institute of Physiology, RWTH Aachen University, Paulwelsstr. 30, 52074 Aachen, Germany, Tel.: 49-9131-85-22-888 or 49-241-80-88810; E-mail: alampert@ukaachen.de.

2 The abbreviations used are: IEM, inherited erythromelalgia; PEPD, paroxysmal extreme pain disorder; DRG, dorsal root ganglion/ganglia; ANOVA, analysis of variance; pA, picoampere; pF, picofarad.
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by light touch, bowel movements, startling, or eating. Patients with PEPD can also suffer from autonomic dysfunction leading to poor feeding and reflux, vomiting, tonic attacks, breath holding spells, and bradycardia that sometimes requires insertion of a pacemaker (5, 6).

Over 20 different IEM mutations have been discovered in Nav1.7, and almost all mutations investigated so far result in a hyperpolarizing shift of activation, allowing Nav1.7 to open at lower potentials compared with the wild type. This left shift of activation enhances excitability, intuitively explaining the pain phenotype.

On the other hand, all of the 10 known PEPD mutations in Nav1.7 induce a depolarizing shift of steady-state fast inactivation, hampering channel closure during an action potential. This prolonged sodium current may lower the activation threshold for the following action potential, and, indeed, both right shift of activation and left shift of inactivation have been shown to induce hyperexcitability (7). One reported IEM mutation shifts steady-state fast inactivation to more depolarized potentials in the adult long splice variant (8).

Sodium channels may undergo an open channel block at depolarized potentials by an endogenous blocking particle, most likely the C terminus of the β4 subunit. Repolarization may cause the release of this block, allowing resurgent currents to flow (9). These depolarizing currents arise in the decaying phase of an action potential and have been suggested to contribute to high-frequency firing in physiological and pathological conditions (10, 11). PEPD mutations have been shown to enhance resurgent currents in Nav1.7, most likely because of the slowing of fast inactivation kinetics, providing more time for the blocking particle to interact with the open channel (11–13) and, thereby, priming the channel for resurgent currents (11).

Here we characterize a new mutation of Nav1.7, A1632T, found in a patient presenting with IEM symptoms, using whole-cell voltage-clamp recordings of transfected HEK cells. Surprisingly, the voltage dependence of channel activation was unaltered by this mutation, but steady-state fast inactivation was shifted to more depolarized potentials, a characteristic normally attributed to PEPD. An A1632E mutation of a patient with overlapping symptoms of IEM and PEPD has been reported previously (5) that displays both a shift of activation and fast inactivation. We introduced Thr/Glu/Asp/Val substitutions of the Ala-1632 residue, and our results suggest that there is a differential impact on persistent and resurgent currents by the introduction of either a negative charge or hydroxyl moiety.

EXPERIMENTAL PROCEDURES

Patient—Detailed histories and clinical examinations of the patient and her mother were obtained, and blood samples and buccal swabs were obtained with their written consent.

SCN9A Sequence Analysis—DNA was extracted from blood lymphocytes and oral mucosa epithelial cells. All coding exons, including exon-intron boundaries of the SCN9A gene, were amplified by PCR using gene-specific intronic primers (primer sequences are available on request). Direct cycle sequencing was carried out using Big Dye Terminator v3.1 sequencing on an ABI3730 automatic sequencer (Applied Biosystems). The resulting sequences were compared with the NCBI reference sequences NM_002977 and NT_005403 (http://www.ncbi.nlm.nih.gov).

Computer Modeling—A homology model of hNav1.7 was generated on the basis of the crystal structure of NavAb (14) (PDB code 3RVY). Sequences were aligned using ClustalW (15), 50 models were produced using MODELLER, and the best model according to the internal scoring function of MODELLER was validated following visual inspection and assessment of stereochemistry using the web server for quantitative evaluation of protein structure (volume, area, dihedral angle reporter: VADAR) web server (17).

HEK Cell Culture and Transfection—The A1632D, A1632E, A1632K, A1632T, and A1632V mutations of hNav1.7 were generated using the QuickChange XL site-directed mutagenesis kit (Stratagene). All mutations were introduced into the neonatal short splice variant of human Nav1.7 in a modified pcDNA3 vector. HEK293 cells were maintained in DMEM (Invitrogen) including 10% FBS, 1.0 g/liter glucose, and 1% penicillin/streptomycin (PAA Laboratories GmbH). HEK293 cells were plated on 3.5-cm dishes (Falcon Corning, IBD) and transiently transfected with Nanofectin (PAA Laboratories GmbH) on the following day according to the protocol of the manufacturer using 1 μg of either WT or mutant (A1632E, A1632T, A1632D, A1632K, and A1632V) hNav1.7 DNA and 0.5 μg of EGFP-C1 (Clontech Laboratories, Inc.). Cells were recorded 1–2 days after transfection.

Electrophysiology: Whole-cell Voltage Clamp—Whole-cell voltage clamp recordings of transfected HEK cells were performed using glass electrodes with tip resistances of 1.5–2.0 MΩ, manufactured with a Zeitz DMZ-puller (Zeitz Instruments GmbH, Martinsried, Germany), filled with an internal solution comprising 140 mM CsF, 10 mM NaCl, 10 mM HEPES, 1 mM EGTA, and 15 mM glucose (adjusted to pH 7.4 with CsOH). The external bathing solution comprised 140 mM NaCl, 3 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 10 mM HEPES, and 20 mM glucose (pH 7.4) adjusted with NaOH. Membrane currents were acquired at room temperature 21 ± 2°C using a HEKA EPC-10USB amplifier (HEKA Electronics, Lambrecht, Germany), low pass-filtered at 10 kHz, and digitized at 100 kHz. The pipette potential was zeroed prior to seal formation, and capacitive transients were compensated using C-fast for pipette capacity correction and, subsequently, C-slow for cell-capacity compensation (PatchMaster, HEKA Electronics). The series resistance was compensated by 50–70%, and leak pulses were applied following the test pulse to subtract the mean leak current digitally online corresponding to the P/4 test pulse procedure. PatchMaster/FitMaster software (HEKA Elektronics) was used for acquisition and off-line analysis.

Voltage protocols were carried out after current stabilization, and equilibration was established. Standard current-voltage (I-V) curves were recorded using 100-ms pulses from a holding potential of −120 mV to a range of potentials (−100 to +80 mV) in 10-mV steps, with 5 s at −120 mV between pulses. Conductance-voltage curves were obtained by calculating the conductance (G) at each voltage (V) using the equation G = I/V − Vrev, with Vrev being the reversal potential, determined for each cell individually. Conductance-voltage curves were fit-
ted with a Boltzmann equation: $G_{Na} = G_{Na,max}/(1 + \exp [(V_m - V_{1/2})/k])$, where $G_{Na}$ is the voltage-dependent sodium conductance, $G_{Na,max}$ is the maximal sodium conductance, $V_{1/2}$ is the potential at which activation is half-maximal, $V_m$ is the membrane potential, and $k$ is the slope factor.

Time to peak was measured from pulse onset to maximal current at potentials between $-50$ and $+50$ mV. To calculate fast inactivation time constants, the current decay of traces obtained with the activation protocol was fitted with a single exponential function. Persistent current was determined as the mean current between 40 and 50 ms after pulse onset in the activation protocol.

Voltage dependence of steady-state fast inactivation was measured using a series of prepulses ($-150$ to $-30$ mV) lasting 500 ms, followed by a 40-ms depolarization to $-20$ mV to assess the available non-inactivated channels. Peak inward currents at the test pulse were normalized to the maximal inward current, the available non-inactivated channels. Peak inward currents at 500 ms, followed by a 40-ms depolarization to $20$ mV for 40 ms, at which available channels were assessed.

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The rate of deactivation was measured using a short (0.4-ms) depolarization pulse to $20$ mV, followed by a 100-ms repolarizing pulse to potentials ranging from $-120$ to $-30$ mV. Decaying currents were then fitted with a single exponential function using FitMaster software (HEKA Electronics). To evoke ramp currents, slow depolarization pulses were applied from a holding potential of $-140$ to $+70$ mV at a rate of 0.2 mV/ms.

To measure resurgent currents in HEK293 cells, 100 $\mu M$ B4 peptide (KKLITIFILKKTREK, PSL GmbH, Heidelberg, Germany (18)) was added to the internal solution immediately before starting the measurements. The voltage protocol consisted of a 20-ms depolarization voltage step from $-120$ to $+30$ mV, followed by 10-mV steps of 500-ms duration from $-80$ to $+20$ mV. The resurgent current quantity was measured by detecting the peak inward current following the prepulse.

To assess steady-state slow inactivation, a series of 60-s prepulses ranging from $-130$ to $+10$ mV was followed by a 100-ms step to $-120$ mV to remove fast inactivation and a test pulse to $20$ mV for 40 ms, at which available channels were assessed.

**DRG Culture, Transfection, and Current Clamp Recordings**—Dorsal root ganglia (DRG) of 4- to 8-day-old rat pups were excised and transferred into DMEM solution containing 50 $\mu g/ml$ gentamicin (Sigma Aldrich, Germany). As described previously (19), ganglia were treated with 1 mg/ml collagenase and 0.1 mg/ml protease for 30 min (both from Sigma Aldrich, Germany) and subsequently dissociated using a fire-polished, silicone-coated Pasteur pipette. Neurons were immediately transfected with the hNav1.7 WT or A1632T mutation and EGFP by electroporation with a Nucleofector II (Lonza, Cologne, Germany), plated on poly-D-lysine-coated (200 $\mu g/ml$, Sigma Aldrich) coverslips and cultured in TNB100 cell culture medium supplemented with TNB100 lipid-protein complex and 100 $\mu g/ml$ streptomycin, penicillin (all from Biochrom, Berlin, Germany). Following 24 h of culture, cells were measured in current clamp mode using an Axioptach 200A amplifier (Molecular Devices).

**Statistics**—Statistical data analysis was generally done by ANOVA following Fisher’s least significant difference (LSD) or Tukey-HSD post hoc tests as noted using Statistica 7 software (StatSoft, Tulsa, OK). Unpaired Student’s $t$ test was used to compare current clamp data measured from A1632T and wild type-transfected DRG neurons. All data are presented as mean $\pm$ S.E. * and # indicate $p < 0.05$.

**RESULTS**

**Clinical Picture**—The 22-year-old patient first presented at the age of 17 with recurrent episodes of burning pain, redness, warmth, and swelling in her feet. Her hands are similarly but less often affected, and she has no symptoms in the face, arms, or ears. The symptoms have been present since early childhood and are triggered by warmth, exercise, tight shoes, wearing socks, and alcohol but not spicy foods. She also complains of triphasic color changes of the skin in the winter, affecting the fingers and toes. She does not report allodynia and hyperalgesia and has no episodes of rectal, ocular, or submandibular pain. Her condition interferes with walking, sports, and sleep, but she still manages to attend work. She tried amitryptiline, nifedipine, fexofenadine, and iloprost without success but gets some, albeit incomplete, relief from Carbamazepine (CBZ). Her mother also describes recurrent episodes of burning pain, redness, warmth, and swelling in her feet, with her hands similarly but less frequently affected. She remembers the age of onset as around the age of 3 or 4 but describes that her symptoms now, at the age of 50, are improved markedly. Triggers are similar to those in her daughter, and, again, she does not complain about symptoms of allodynia and hyperalgesia or episodes of rectal, ocular, or submandibular pain. Her condition does interfere with walking and sports and, occasionally, with sleep, but she manages to attend work, and she does not use medication.

**Molecular Genetic Analysis Identified the p.A1632T Mutation in Nav1.7**—We sequenced the complete coding region of the SCN9A gene in DNA extracted from peripheral blood of the patient and her mother. In both samples, we detected a heterozygous guanosine to adenosine transition in exon 26 (c.4894G$\rightarrow$A), resulting in the substitution of alanine to threonine at position 1632 (p.A1632T) of the corresponding protein sequence (NM_002977) (Fig. 1A). Because the adenosine peak of the mutant (c.4894G$\rightarrow$A) was significantly lower in the sequence of the mother compared with the daughter, we suspected a mosaic status for this mutation. Therefore, we extracted DNA from the oral mucosa of the mother and sequenced the exon of interest (exon 26). Again, the mutant adenosine peak was reduced significantly compared with the sequence of the daughter, but it was clearly present on both strands, confirming a mosaic status of this mutation in the mother (Fig. 1A). The A1632T mutation substitutes a residue of the domain IV S4-S5 linker, which is situated in the transmembrane region of Nav1.7 (Fig. 1B).

**IEM Mutation A1632T Shifts Steady-state Fast Inactivation to More Depolarized Potentials**—WT Nav1.7 and its IEM mutation, A1632T, expressed well in HEK293T cells and whole-cell voltage clamp recordings, revealed fast-gating sodium currents...
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Because all IEM mutations described up to now almost exclusively shift activation to more hyperpolarized potentials, we assessed the voltage dependence of activation using the protocol shown in Fig. 2A, top panel. The results obtained from A1632T-mutated channels did not show any difference to the WT. The midpoint of half-maximal activation was not shifted by this IEM mutation (Fig. 2, B and D; WT, $-27.09 \pm 1.11$ mV, $n = 18$; A1632T, $-27.18 \pm 1.14$ mV, $n = 25$). As a measure for the speed of channel opening, time to peak current from pulse onset was determined, and no change was detected for the IEM mutation A1632T (data not shown).

Deactivation refers to the closing of the activation gate of the channel. To measure time constants of deactivation, channels were activated by a brief depolarization and repolarized before a significant amount of fast inactivation could occur (also see “Experimental Procedures”). As published previously (5), and in line with all PEPD mutations measured so far, single exponential fits of the decaying currents revealed a significantly slower time constant for A1632E compared with WT channels at $+100$ mV and over a voltage range from $+80$ to $-30$ mV (Fig. 2D). The A1632T mutant channels, however, closed faster than WT channels at $-60$ and $-30$ mV ($p < 0.04$, ANOVA following HSD post hoc test, $n = 18–27$ each, Fig. 2C).

Following activation, Nav1.7 inactivates within milliseconds, facilitating membrane repolarization. To assess the voltage dependence of steady-state fast inactivation, 500-ms prepulses ranging from potentials of $+150$ to $-10$ mV were applied, and available channels were assessed (see “Experimental Procedures”). Remarkably, steady-state fast inactivation was significantly shifted to more depolarized potentials for A1632T ($-70.77 \pm 1.26$ mV; Fig. 2, D and E, red square) compared with the Nav1.7 wild type ($-78.1 \pm 1.1$ mV, red square, $p < 0.001$, $n = 13–24$ each). This is likely to produce an enhancement of excitability but is normally expected to be linked with PEPD and not IEM.

FIGURE 1. The erythromelalgia patient and her mother carry the A1632T mutation. A, sequencing results from blood samples (b) and oral mucosa (om) of a healthy control person (c), the patient (p), and the mother of the patient (m). The patient is heterozygous for the c.4894G->A mutation (gray frame), whereas the mother is mosaic for this base change (m/b, m/om). Results of forward (fw) and reverse (rev) primers are shown and compared with the NCBI reference sequence NT_005403 (upper line). B, top panel, transmembrane topology map of Nav1.7. The 1632 position is labeled with a star. IFM = Ile-Phe-Met, inactivation gate. Lower panel, three-dimensional homology model of Nav1.7 with S4-S5, S5, P-loop, and S6 helices of the pore module shown as a ribbon. Ala-1632 is presented in black space-fill and labeled with an arrow.
Steady-state slow inactivation, on the other hand, was not altered (Fig. 3A).

In line with results published previously, the A1632E mutation gave rise to ramp currents upon slow depolarization (0.2 mV/s) that were larger than those of WT channels (13.67 ± 1.73% of peak inward current for A1632T compared with 4.86 ± 0.96% for the WT, Fig. 3B). Cells expressing A1632T channels displayed ramp currents of about the same size as the WT (5.51 ± 0.9% of peak inward current, respectively; p < 0.001; ANOVA following HSD post hoc test; n ≥ 17).
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A1632T Enhances Excitability in Small Sensory Neurons—To assess the impact of the IEM-linked A1632T mutation on neuronal excitability, rat DRG neurons were transfected with the Nav1.7 WT or A1632T and investigated by current clamp. The resting membrane potential was similar for small neurons transfected with the Nav1.7 WT or A1632T (−41.03 ± 1.87 mV and −41.8 ± 2.54 mV, respectively; \( p = 0.81 \); mean cell capacitance of the WT, 24.54 ± 1.39 pF and of A1632T, 23.45 ± 1.68 pF; \( p = 0.62 \)). 62.5% of A1632T-transfected neurons presented with spontaneous action potential discharge, which is 42.5% more compared with the Nav1.7 WT (Fig. 4, A and B). The firing threshold was reduced for A1632T compared with the Nav1.7 WT, as assessed by the amount of current injected to elicit a single action potential (Fig. 4, A and B; A1632T, 105.38 ± 15.59 pA; WT, 170.63 ± 16.34 pA; \( p = 0.008; n = 12 \) each; all Student’s t tests). Analysis of all recorded neurons shows that A1632T-expressing neurons fire more action potentials during 1 s for almost each amount of current injected in 5-pA increments between 5 and 95 pA (Fig. 4C, \( p = 0.045, n = 12 \) each).

**A Polar or Negatively Charged Amino Acid at Position 1632 Supports a Rightward Shift of Steady-state Fast Inactivation**—A mutation at position Ala-1632 that substitutes negatively charged glutamate (Glu) is linked to a clinical picture that is composed of both PEPD and IEM symptoms (5). We confirmed a prominent shift in both voltage dependence of activation and steady-state fast inactivation for this mutation (Fig. 2D; A1632E \( V_{1/2} \) of activation, −32.12 ± 1.11 mV; \( p = 0.042; V_{1/2} \) of fast inactivation, −65.12 ± 1.09 mV, blue □, \( p < 0.001 \) compared with the WT).

To assess the biophysical influence of residue size, polarity, and charge at position 1632, we substituted the small and nonpolar alanine with the slightly larger, nonpolar valine (Val), the larger and negatively charged aspartate (Asp), or the positively charged lysine (Lys, Fig. 2A, insets). All three constructs produced sodium currents in HEK cells (Fig. 2A; current densities: A1632V, 139.9 ± 15.7 pA/pF; A1632D, 70.0 ± 8.2 pA/pF; A1632K, 177.1 ± 21.9 pA/pF). The negatively charged A1632D substitution shifted voltage dependence of activation to more negative potentials, thereby mimicking the effects of the IEM- and PEPD-linked A1632E mutation (Fig. 2B, and C, deactivation of A1635D, −32.17 ± 0.91 mV; \( p = 0.039; \) green ○). Similarly, steady-state fast inactivation was significantly shifted to more depolarized potentials for A1632D (Fig. 2, D and E; \( V_{1/2} \) of steady-state fast inactivation for A1632D, −63.67 ± 1.62 mV; A1632K, −78.57 ± 0.98 mV; A1632V, −75.61 ± 0.98 mV). Interestingly, the A1632K mutation, which introduces a positive charge and the longest side chain compared with the other mutations, did not affect activation or fast inactivation at all.

**A1632E (Linked to IEM and PEPD), but Not A1632T (Linked to IEM Only), Increases Persistent and Resurgent Currents**—Because the shift of steady-state fast inactivation of the IEM- and PEPD-linked A1632E mutation was also observed in the new...
IEM-only A1632T mutation, the question arises as to which gating changes are responsible for the typical PEPD symptoms. Impaired fast inactivation is often accompanied by slower kinetics of current decay. Therefore, we fitted the decaying phase of evoked inward currents with a single exponential function and determined the decay time constants. As shown in Fig. 5A, introduction of a negative charge at position Ala-1632 (Glu or Asp) led to a slower current decay over voltages ranging from −100 mV to +10 mV (p < 0.02 each, ANOVA and LSD post hoc test), whereas the decay time constants of A1362T were not different from the WT (p > 0.17, Fig. 5A).

Hampered channel inactivation could lead to persistent sodium currents, which were determined as the mean current during a period of 10 ms, starting 40 ms after pulse onset and normalized to the peak inward current of each cell (Fig. 5B, top panel). Although the A1632T mutation did not influence persistent currents, these currents were larger for A1632D (green ●) and A1632E (blue □) compared with WT Nav1.7 (■) over a voltage range of between −50 and +20 mV (Fig. 5B, bottom panel; p < 0.048, respectively; n ≥ 12 each, ANOVA following LSD post hoc test).

FIGURE 4. Sensory neurons transfected with the IEM mutation A1632T display enhanced excitability. A, stimulation protocol and representative current clamp recordings from DRG neurons expressing Nav1.7 WT (black lines, left) or the A1632T mutation (red lines, right) when current was injected at the action potential threshold (top panel) or two times threshold (bottom panel); B, the injected current to elicit an action potential is reduced (n ≥ 13 each; p < 0.008) and spontaneous firing is increased in A1632T-transfected (AT) neurons, whereas the resting membrane potential remains unchanged (n ≥ 15 each; p = 0.81). C, A1632T-transfected neurons fire more action potentials during 1 s for almost each amount of injected current tested (n ≥ 12 each; *, p < 0.05; all Student’s t test for independent samples).

Slowed transition from the open to the inactivated state, as observed for A1632E, promotes the occurrence of resurgent currents (13). To measure this current in a heterologous expression system, a peptide with the sequence of the C-terminal section was added to the pipette solution (13). In contrast to A1632T (Fig. 5C, top panel, red) and WT Nav1.7 (black), A1632E (blue) exhibited clearly detectable resurgent currents when 100 μM β4 peptide was present in the internal recording solution. Mean peak resurgent currents within the first 20 ms after repolarization were larger in cells expressing A1632E (Fig. 5C, blue □) compared with those expressing A1632T (red □) or Nav1.7 WT (■) over a voltage range of between −60 and −10 mV (p < 0.032 each; n ≥ 8; ANOVA following LSD post hoc test). Thus, the electrophysiological difference between the IEM-linked A1632T and the IEM- and PEPD-linked A1632E IEM Mutation A1632T Shifts Inactivation

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mutation is not the shift of steady-state fast inactivation (because both display this change) but an enhancement of persistent and resurgent current.

**DISCUSSION**

In this study, we identified and characterized the Nav1.7 A1632T mutation in a patient with a typical clinical picture of IEM whose mother, who is more mildly affected, is mosaic for the same mutation. Despite its mainly IEM-like clinical phenotype, the A1632T mutation shifts the voltage dependence of steady-state fast inactivation to more depolarized potentials, which has hitherto been considered the defining characteristic of PEPD mutations. This modified fast inactivation may produce two effects that enhance excitability. More channels may be available at resting membrane potential, and an action potential may be prolonged because of reduced sodium channel inactivation. DRG neurons transfected with the A1632T mutation become hyperexcitable and show spontaneous activity and display a lowered current threshold and increased firing rates upon current injection. Remarkably, the A1632E mutation in the same position produces an overlapping clinical phenotype of IEM and PEPD (5) and shifts both activation and steady-state fast inactivation. We were able to mimic these results by introducing an alternative negative charge, A1632D, suggesting that the insertion of the negative charge in this position is affecting activation and destabilizes the fast inactivation of the channel. In contrast to A1632T, A1632E slows channel deactivation and current decay, induces ramp and persistent currents, and also gives rise to resurgent currents. Because these are the only differences between the electrophysiological phenotype of A1632T (IEM-linked) and A1632E (IEM- and PEPD-linked), we suggest that it is these current characteristics and not solely a shift of steady-state fast inactivation that evokes the PEPD phenotype.

The mother of the patient, who is a mosaic for the A1632T mutation, is also affected by IEM, but her clinical symptoms are milder in comparison to her daughter. To our knowledge, this is the first description of a mosaic for a heterozygous SCN9A missense mutation that causes a clinical phenotype. A single case of mosaicism (heterozygous p.L858F) in SCN9A linked to IEM has been described, but the individual was clinically asymptomatic (20). It may be possible that differences in the degree or distri...
bution of the mosaicism may play a role. However, the electrophysiological phenotype of Nav mutations also depends on the cellular expression background, and some effects may be hidden in transfected HEK cells or DRG neurons (21).

A negative shift of the activation of the Nav1.7 channel is currently accepted to cause the clinical picture of IEM (22). Nevertheless, the age of disease onset as a measure for clinical severity correlates best to the sum of the shift of activation and to that of steady-state fast inactivation (23). Surprisingly A1632T does not shift activation, but steady-state fast inactivation, to more positive potentials. In addition to our data, there are several reports in the literature suggesting a reconsideration of the tight link between a shift of Nav1.7 activation and the clinical picture of IEM. Choi et al. (8) reported that the IEM mutation G616R of Nav1.7, when introduced into the adult long splice variant, showed normal activation properties but, like the A1632T mutation reported here, a depolarized shift of steady-state fast inactivation. The well-characterized IEM-mutation I848T (24) was recently re-examined at 35 °C (4) because elevated ambient temperature is known to induce symptoms in IEM patients. The reported hyperpolarizing shift of activation (measured at room temperature) was lost in I848T-mutated channels compared with wild-type Nav1.7, whereas a depolarizing shift of inactivation remained at 35 °C. This suggests that the IEM symptoms at increased temperature of patients carrying the I848T mutation are due to a shift of steady-state fast inactivation, not activation, supporting our findings.

The aforementioned IEM mutations that shift fast inactivation properties demonstrate that impairment of steady-state fast inactivation is probably not the exclusive cause of PEPD. Instead, as suggested earlier (13), the slowing of current decay, i.e. slower open-state inactivation, appears to be a major feature of PEPD mutations. Persistent and resurgent currents may appear as a consequence of the slower binding of the inactivation particle in these mutations, thus allowing more time for the open channel blocker to dock to the pore (Fig. 5C).

Jareki et al. (11) and Theile et al. (13) showed that resurgent currents may be recorded from PEPD-mutated Nav1.7, but not WT or IEM-mutated Nav1.7, when expressed in DRG neurons or HEK cells. Although channel decay was unaltered in the IEM mutants, there was a strong correlation of slowed decay time constants and resurgent current amplitude in the PEPD mutants. The IEM-linked mutation A1632T investigated here, as well as the IEM- and PEPD-linked A1632E mutation, shift steady-state fast inactivation to more depolarized potentials, but only the latter slowed current decay kinetics and increased persistent and resurgent currents (Fig. 5). This adds more evidence to the hypothesis that slowed fast inactivation kinetics and increased persistent and resurgent currents are electrophysiological characteristics of PEPD.

Few IEM-linked mutations induce slowed current decay kinetics. Only one of them (del955 (25)) also produces persistent currents, but it was not tested for resurgent currents. The Nav1.7 G856D mutation displays persistent and resurgent currents but is linked to a syndrome of pain, dysautonomia, and acromesomelia, leaving its pathophysiological impact to be determined (26). The I234T mutation, which slows slight current decay, was classified as an IEM mutation, although the clinical picture shows some features of PEPD (mechanical stimulation of the sacrum and being seated causes attacks of pain in the child) (27).

Ramp currents are evoked by slow depolarizations mimicking weak natural stimuli and are likely to support hyperexcitability. Although ramp currents are described to be enhanced in all PEPD mutants investigated to date, some IEM mutations do not have ramp currents (23). The A1632E mutation, in contrast to the A1632T mutation, slows current decay (Fig. 5A), which indicates impaired open-state inactivation. This may not only favor binding of the open channel blocker but also the generation of ramp currents. Nevertheless, some IEM mutations speed up current decay and have ramp currents. Therefore, the link between these two types of currents needs further investigation. The A1632T mutation, on the other hand, displays reduced steady-state fast inactivation and no alterations of open-state inactivation (Figs. 2E and 5A). At potentials more negative than the opening threshold of the channel, steady-state fast inactivation measures closed-state inactivation and would not support the binding of the open channel blocker, in line with our results (Fig. 5C).

Interestingly, the A1632T mutation deactivates faster than WT, which may be due to its more depolarized fast inactivation. The A1632E mutation, on the other hand, along with most IEM mutations, deactivates more slowly and may thus contribute to its IEM phenotype.

Three-dimensional modeling studies locate A1632 at the turn between the S4-S5 linker and the S5 helix of domain IV (Fig. 1B). Structural comparison of bacterial voltage-gated channels crystallized in different conformational states indicates that this region, termed the “S5 gating hinge,” is flexible (14, 28, 29). Also, the structure of NavCt (30), which was resolved in a closed and putatively inactivated state, suggests that this region plays an intricate role in pore gating. A mutation in position Ala-1632 close to this gating hinge may thus interfere with the S5 gating hinge and modify inactivation, especially because domain IV contains binding determinants for the inactivation particle (31).

The Thr/Asp/Glu mutations of Ala-1632 produced a significant shift of fast inactivation, whereas substitution with hydrophobic valine or the large, positively charged lysine did not modify fast inactivation. A common property of threonine, aspartate, and glutamate is the ability of the side chain hydroxyl or carboxylate group to accept a hydrogen bond. It is possible that the formation of a novel hydrogen bond in the domain IV S5 gating hinge region of Thr/Asp/Glu mutant channels interferes with the normal gating-related conformational changes. Consequently, the rate at which the receptor site for the inactivation particle forms may be slowed, or, alternatively, this receptor may be allosterically perturbed. We speculate that either or both of these processes could produce the observed depolarizing shift of fast inactivation in the Thr/Asp/Glu mutants.

Characterization of the new IEM-linked A1632T mutation revealed a shift of steady-state fast inactivation but no change of activation properties. Comparison with the mutation A1632E, which is linked to IEM and PEPD, suggests that we need to reconsider our current understanding of the electrophysiologi-
cal basis of these two neuropathic pain syndromes. Our data suggest that it is the size of the resurgent and persistent sodium currents that determines whether gain-of-function mutations of Nav1.7 induce PEPD or IEM.

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