Alternative splicing may contribute to time-dependent manifestation of inherited erythromelalgia

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The Na\textsubscript{v}1.7 sodium channel is preferentially expressed in nocioceptive dorsal root ganglion and sympathetic ganglion neurons. Gain-of-function mutations in Na\textsubscript{v}1.7 produce the nocioceptor hyperexcitability underlying inherited erythromelalgia, characterized in most kindreds by early-age onset of severe pain. Here we describe a mutation (Na\textsubscript{v}1.7-G616R) in a pedigree with adult-onset of pain in some family members. The mutation shifts the voltage-dependence of channel fast-inactivation in a depolarizing direction in the adult-long, but not in the neonatal-short splicing isoform of Na\textsubscript{v}1.7 in dorsal root ganglion neurons. Altered inactivation does not depend on the age of the dorsal root ganglion neurons in which the mutant is expressed. Expression of the mutant adult-long, but not the mutant neonatal-short, isoform of Na\textsubscript{v}1.7 renders dorsal root ganglion neurons hyperexcitable, reducing the current threshold for generation of action potentials, increasing spontaneous activity and increasing the frequency of firing in response to graded suprathreshold stimuli. This study shows that a change in relative expression of splice isoforms can contribute to time-dependent manifestation of the functional phenotype of a sodium channelopathy.

Keywords: pain; SCN9A; sodium channel; channelopathy; splice variants

Abbreviations: cDNA = complementary DNA; CSS = complete saline solution; DRG = dorsal root ganglion; GFP = green fluorescent protein; HEPES = N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; PKA = protein kinase A; SCN9A = gene encoding voltage-gated sodium channel Na\textsubscript{v}1.7; TTX = tetrodotoxin

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Introduction

Twelve gain-of-function mutations of voltage-gated sodium channel Na\textsubscript{v}1.7 (Dib-Hajj \textit{et al.}, 2007; Cheng \textit{et al.}, 2008; Choi \textit{et al.}, 2009; Fischer \textit{et al.}, 2009; Han \textit{et al.}, 2009) have been shown to induce hyperexcitability of dorsal root ganglion (DRG) neurons in inherited erythromelalgia, an autosomal dominant disorder characterized by episodic severe burning pain in the extremities, triggered by warmth (Dib-Hajj \textit{et al.}, 2007; Drenth and Waxman, 2007). Most patients with inherited erythromelalgia manifest symptoms beginning in early childhood (prior to 5–6 years of age); occasional families, however, begin to show symptoms at older ages (Burns \textit{et al.}, 2005; Dib-Hajj \textit{et al.}, 2007; Drenth and Waxman, 2007). Why a gain-of-function channelopathy should manifest variable age-onset is not been clear.

Sodium channel Na\textsubscript{v}1.7 is preferentially expressed in DRG and sympathetic ganglion neurons (Sangameswaran \textit{et al.}, 1997; Toledo-Aral \textit{et al.}, 1997; Djouhri \textit{et al.}, 2003). Slow closed-state inactivation permits Na\textsubscript{v}1.7 to respond to slow, subtle depolarizations (including ramp stimuli) similar to generator potentials in DRG neurons (Dib-Hajj \textit{et al.}, 2005; Harty \textit{et al.}, 2006). The nomenclature 5N and 5A was based on studies of brain channels (Chatelier \textit{et al.}, 2008). Mutations from patients with early-onset inherited erythromelalgia, experimentally expressed within the neonatal-short Na\textsubscript{v}1.7 (Na\textsubscript{v}1.7\textsubscript{NS}) isoform, have been shown to hyperpolarize the voltage-dependence of activation, slow deactivation and increase the channel’s response to ramp stimuli (Dib-Hajj \textit{et al.}, 2007; Cheng \textit{et al.}, 2008; Choi \textit{et al.}, 2009; Fischer \textit{et al.}, 2009; Han \textit{et al.}, 2009). At the cellular level, these mutations in the Na\textsubscript{v}1.7\textsubscript{NS} variant lower the threshold for generation of action potentials and increase the frequency of firing in response to graded suprathreshold stimulation in DRG neurons (Dib-Hajj \textit{et al.}, 2005; Harty \textit{et al.}, 2006; Rush \textit{et al.}, 2006).

Here we report a new mutation in Na\textsubscript{v}1.7, present in all affected patients, from a family with inherited erythromelalgia in which some patients first manifested disease symptoms in adulthood. We hypothesized that the late onset of symptoms in some patients is due to a preferential functional effect of the mutation on an adult isoform of the channel. Our voltage-clamp studies show that the mutation alters gating properties of Na\textsubscript{v}1.7\textsubscript{AL} but not Na\textsubscript{v}1.7\textsubscript{NS}, when expressed in DRG neurons. Using current-clamp, we show that expression of the mutant Na\textsubscript{v}1.7\textsubscript{AL} but not Na\textsubscript{v}1.7\textsubscript{NS} channels, renders DRG neurons hyperexcitable. Our results suggest that a change in relative expression of Na\textsubscript{v}1.7 splice isoforms provides a basis for the adult age-onset of pain in some members of this kindred.

Materials and methods

Patients

The proband is a Caucasian Dutch male with severe burning pain in his feet, hands and sometimes face, with onset at the age of 24 years. Informed consent was obtained according to an approved Institutional Review Board and venous blood was collected from the patient and family members for genomic screening.

Exon screening

Genomic DNA was purified from venous blood. For the gene encoding voltage-gated sodium channel Na\textsubscript{v}1.7 (SCN9A), genomic DNA from 100 Caucasian individuals in The Netherlands and 50 Caucasian individuals from the USA (Coriell Institute, Camden, NJ) was used as a normal population control. Coding exons and flanking intronic sequences, as well as exons encoding 5’ and 3’ untranslated sequences within the complementary DNA (cDNA) sequence were amplified and sequenced as described previously (Drenth \textit{et al.}, 2005). Genomic sequences were compared to the reference Na\textsubscript{v}1.7 cDNA (Klugbauer \textit{et al.}, 1995) to identify sequence variation. A SCN10A mutation that causes R1268Q substitution in sodium channel Na\textsubscript{v}1.8, found in the proband, was not found in other affected family members. Nevertheless, this Na\textsubscript{v}1.8 mutation was studied by patch-clamp recordings.

Plasmids

The human Na\textsubscript{v}1.7/NS insert (carrying the neonatal exon 5, E5N, and short loop 1) was cloned into a mammalian expression vector (Klugbauer \textit{et al.}, 1995) and converted to tetrodotoxin-resistance (TTX-R) (hNa\textsubscript{v}1.7\textsubscript{NS}; designated WT\textsubscript{NS} hereinafter) by Y362S substitution (Herzog \textit{et al.}, 2003). The two amino-acid substitutions in adult exon 5 (E5A), L201V and N206D (Raymond \textit{et al.}, 2004) were introduced using QuickChange XL II site-directed mutagenesis (Stratagene, La Jolla, CA); the longer L1 was subcloned into the resulting plasmid to generate Na\textsubscript{v}1.7\textsubscript{ALS}/AL (designated WT\textsubscript{AL} hereinafter). The nomenclature 5N and 5A was based on studies of brain channels Na\textsubscript{v}1.2, Na\textsubscript{v}1.3 and Na\textsubscript{v}1.6, where the abundance of templates carrying the alternate exons was determined experimentally (Sarao \textit{et al.}, 1991; Gustafson \textit{et al.}, 1993; Plummer \textit{et al.}, 1998). While it has been reported that all four alternative Na\textsubscript{v}1.7 transcripts are co-expressed in an adult DRG sample (Raymond \textit{et al.}, 2004), validation of this finding in a larger sample and assessment of abundance of the Na\textsubscript{v}1.7\textsubscript{AL} splice isoform in embryonic and neonatal DRG neurons have not yet been reported. Therefore, we use the term E5A conservatively and in keeping with the conventions in sodium channel research. The G616R mutation was introduced into WT\textsubscript{NS} and WT\textsubscript{AL} using QuickChange XL II site-directed mutagenesis (Stratagene).

Full-length inserts of the different clones were sequenced at the Howard Hughes Medical Institute/Keck Biotechnology Centre at Yale University. Sequence analysis using BLAST (National Library of Medicine) and Lasergene (DNAStar, Madison, WI) confirmed the inserts to be devoid of un-intended mutations.

Primary sensory neuron isolation and transfection for voltage clamp

DRG neurons were cultured from mice and transfected by biolistics as described previously (Dib-Hajj \textit{et al.}, 2009). Animal studies followed a...
therefore, these persistent TTX-resistant currents are not significant previously showed that Na v1.8-null DRG neurons do not express fast-conducted 24–36 h after transfection, providing ample time for successful biolistic transfection. Electrophysiological studies were conducted from GFP-labelled cells. Within 24 h the cells usually showed expression of GFP, indicating successful biolistic transfection. Electrophysiological studies were conducted 24–36 h after transfection, providing ample time for robust channel expression; therefore, most of the cells that expressed GFP also expressed fast-inactivating TTX-resistant sodium currents. We previously showed that Na1.8-null DRG neurons do not express fast or slow-inactivating TTX-resistant sodium currents (Cummins et al., 1999, 2001). Some Na1,8-null DRG neurons express persistent TTX-resistant sodium currents (Cummins et al., 1999), but these typically display amplitudes <500 pA after several days in culture and run down quickly in whole-cell configuration (Cummins et al., 2001); therefore, these persistent TTX-resistant currents are not significant under the culture and recording conditions used in this study.

**Voltage-clamp electrophysiological recordings**

Whole-cell patch-clamp recordings were conducted from GFP-labelled DRG neurons of ≤25 μm diameter at room temperature (~21 °C) using Axopatch 200B amplifiers (Molecular Devices, Sunnyvale, CA). Micropipettes (0.6–0.9 MΩ) were pulled from capillary glass (PG10165–4, WPI, Sarasota, FL) with a Flaming-Brown P80 micropipette puller (Sutter Instruments, Novato, CA), polished on a microforge. To optimize space clamp, only isolated cells with soma diameter of ≤25 μm were recorded. Capacity transients were cancelled and series resistance compensated (>90%) in all experiments. Average series resistance was 1.7 ± 0.1 MΩ (n = 66) and estimated maximum voltage-error after series resistance compensation was 2.0 ± 0.2 mV. The pipette solution contained (in mM): 140 CsF, 1 EGTA, 10 NaCl and 10 HEPES, pH 7.3 (adjusted to 310 mOsm/l with sucrose). The bath solution contained (in mM): 140 NaCl, 3 KCl, 1 MgCl₂, 1 CaCl₂, 20 TEA-Cl, 10 glucose and 10 HEPES, pH 7.3 with NaOH (adjusted to 320 mOsm/l with sucrose). An amount of 0.1 mM CdCl₂ was added to block endogenous Ca²⁺ currents. TTX at 300 nM was included in the bath solution to isolate TTX resistant currents from endogenous tetrodotoxin-sensitive Na⁺ currents, which are completely blocked by this TTX concentration (Cummins et al., 2001; Herzog et al., 2003). For protein kinase A (PKA) phosphorylation studies, cells were held in the bath solution containing either vehicle (distilled water) or 1 mM 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP, Calbiochem, San Diego, CA) for 15 min prior to the experiments.

Whole-cell patch-clamp recordings followed standard protocols (Cummins et al., 2009). The pipette potential was zeroed before seal formation and voltages were not corrected for liquid junction potential. Leakage current was digitally subtracted online using hyperpolarizing control pulses, applied after the test pulse, of one-Nth test pulse amplitude (P/N subtraction). Whole-cell currents were filtered at 5 kHz and acquired at 50–100 kHz using Clampex 8.2 software (Molecular Devices). Recordings were started at least 4 min after establishing whole-cell configuration to allow currents to stabilize.

Cells were held at ~100 mV, a voltage at which most of the persistent current is inactivated to minimize possible contamination by Na,1,9 currents (Cummins et al., 1999). Additionally, transfected DRG neurons in culture can produce extensive neurites, which may pose problems of space-clamp during whole-cell patch-clamp recording. We usually did not see those with recombinant channels, but to minimize the space clamp problems we determined current density by measuring peak current amplitude from 0 to 2 ms after initiating the depolarization pulse, and excluded from further analysis cells with peak current (<2 nA) or when the secondary peak amplitude was ≥5%
of primary peak. Activation curves were constructed with membrane potential held at −100 mV and a series of 100 ms test pulses from −80 to +40 mV in increments of 5 mV.

Voltage-dependent activation curves were fitted using the Boltzmann distribution equation:

$$G = \frac{G_{\text{max}}}{1 + \exp\left(\frac{V_{1/2} - V_m}{k}\right)}$$

where $G$ is voltage-dependent sodium conductance, $G_{\text{max}}$ is maximum conductance, $V_{1/2}$ is membrane potential at half-maximal conductance and $k$ is the slope factor. Reversal potential for each current was estimated by extrapolating the linear ascending segment of the I–V curve to the voltage axis.

The voltage-dependent steady-state inactivation relationship was investigated with a standard two-pulse protocol. For maximum recovery from slow inactivation, interpulse time between test potentials was 10 s; the test pulses were preceded by a 500 ms preconditioning pulse ranging from −140 to 0 mV. Normalized curves ($I/I_0$) were fitted using the Boltzmann equation:

$$\frac{I}{I_0} = \frac{1}{1 + \exp\left(\frac{V_{1/2} - V_m}{k}\right)}$$

where $I_0$ is peak Na⁺ current at tested pulse measured from the most negative preconditioning pulse potential, $V_m$ is the preconditioning pulse potential, $V_{1/2}$ is membrane potential at half-maximal $I$ and $k$ is the slope factor.

**Current-clamp recordings**

Small (≤25 μm diameter) GFP-labelled DRG neurons were used for current clamp recording 36–48 h after transfection, using Multiclamp 700B amplifiers (Molecular Devices). Electodes had a resistance of 1.2–1.6 MΩ when filled with pipette solution containing (in mM): 140 KCl, 0.5 EGTA, 5 HEPES and 2 Mg-ATP, pH 7.3 with KOH (adjusted to 315 mOsm/l with sucrose). Pipette tips were wrapped with Parafilm to reduce pipette capacitance, minimizing the errors of filtering the membrane potential signal. Extracellular solution contained (in mM): 140 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 10 HEPES, pH 7.3 with NaOH (adjusted to 320 mOsm/l with sucrose). Recordings were made within 20 min after establishing whole-cell configuration to minimize possible time-dependent channel rundown in the absence of GTP in the pipette.

Whole-cell configuration was obtained in current-clamp mode after compensation of electrode capacitance and series-resistance using computer-controlled compensation circuits. Except for neurons producing spontaneous action potentials at resting cells, cells with stable resting potentials more negative than −35 mV were used for data collection. All experiments were conducted at resting potential. Input resistance was determined by the slope of a line fit to hyperpolarizing responses to current steps of 5–40 pA. Current-threshold was determined using a series of brief (2 ms) current injections that increased in 10 pA increments. Action potentials were filtered at 10 kHz and acquired at 100 kHz, using Clampex 10.2. To determine voltage threshold, action potential amplitude and time-to-action potential peak ($t_{\text{peak}}$), short current injections (2 ms) were used so that the action potential was uncontaminated by injected current. Long current injections (500 ms) were applied to examine repetitive firing. It is known that TTX-sensitive sodium currents participate in action potential initiation in DRG neurons (Blair and Bean, 2002) and slow closed-state inactivation of Nav1.7 make the channels available in response to slow deep depolarizations (Cummins et al., 1998). To mimic slow depolarizing inputs, ramp current injection (0–0.5 nA in 500 ms) was also applied to study time-to-first action potential.

**Experimental protocols and data analysis**

Data were analysed using Clampfit 10.2 software and OriginPro 7.5 (Microcal, Northampton, MA). Shapiro–Wilk normality test and Student’s unpaired t-test in OriginPro 7.5 software was used for statistical analysis of the data. For comparison of spontaneous action potentials, we used two-proportion z-test. Data are presented as mean ± SEM.

**RNA isolation**

Adult (4–6 weeks) and neonatal (P2–P4) Sprague–Dawley rats were rendered unconscious using CO₂ asphyxiation and decapitated. L4–L6 lumbar DRG were quickly removed and placed in tubes on dry ice. Total RNA was immediately isolated from the ganglia using the RNeasy Fibrous Tissue Kit (Qiagen, Valencia, CA) and RNA was eluted in 50 μl of RNase-free water and the concentration determined using UV spectroscopy; the A$_{260}$/A$_{280}$ ratio for each RNA sample was >1.95 indicating high purity. Prior to carrying out reverse transcriptase or real-time polymerase chain reaction (PCR) the RNA samples were also treated with two units of RNase-free DNase I for 30 min at 37°C and re-purified to remove any potential genomic DNA contamination.

**Quantitative real-time polymerase chain reaction**

Real-time PCR experiments were calibrated using standard curves for each of the four Na$_{\text{v}}$1.7 splice variants. Standard curves were constructed using 10-fold serial dilutions of a 10 ng splice variant-specific plasmid DNA. Splice-variant clones were generated by amplifying a 1.6 kb segment spanning exon 4 to exon 12 of the rat Na$_{\text{v}}$1.7 transcript from a DRG cDNA template of neonatal or adult rat, and ligating the resulting amplicon into a PCR cloning vector, pGEM-T Easy (Promega, Madison, WI). First, a 1 μg sample of adult rat DRG RNA was reverse transcribed into cDNA using 200 units of Superscript III reverse transcriptase (Invitrogen), 250 ng of RETROscript random decamers (Ambion, Austin, TX) and 1 μl 25 mM deoxynucleotide triphosphates (Roche). The fragment spanning exons 4–12 was amplified in a standard single-tube reverse transcriptase (RT)-PCR reaction consisting of 100 ng of cDNA, 2.5 μl 10× PCR Buffer 3 (Roche), 0.5 μl 25 mM deoxynucleotide triphosphates, 1 μl forward primer (SCN9A4F 5′ CGCTGGGAGAATTCCACCTTCCTCGGT 3′), 1 μl reverse primer (SCN9A12R 5′ CAGTAAGCTGATCAAGGAGTCTGTCTTC 3′) and two units DNA polymerase. Before continuing, a sample of the PCR reaction was analysed on a 1% agarose gel in order to determine whether the amplicon was the correct size. The PCR product was ligated into the pGEM-T Easy PCR cloning vector using a 3:1 insert:vector ratio. JM109 High Efficiency Competent Cells (Promega) were transformed using 2 μl of the ligation reaction and isolates with recombinant plasmid were identified using blue/white screening on Luria Bertani-ampicillin selection plates. Identity of clones with the different splice isoforms was confirmed by restriction enzyme analysis and sequencing of the insert.

Standard curves were generated for each splice variant using a 10-fold serial dilution of 10 ng of plasmid DNA. TaqMan® primer-probe assays (Supplementary Table 1), designed to recognize the unique splice junctions on each transcript (Raymond et al., 2004),
were used to detect each splice variant. Primer/probe sets were obtained from Applied Biosystems. PCR reactions were carried out in triplicate using the TaqMan® RNA-to-Ct™ 1-Step Kit (Applied Biosystems). A master mixture was prepared and aliquoted so that each 10 μl reaction contained 5 μl of 2× TaqMan® reverse transcriptase-PCR mix, 0.5 μl of 20× custom primer-probe assay and 0.25 μl of reverse transcriptase enzyme mix. An amount of 4.25 μl of template RNA was then added to each well. The cycling protocol on Eppendorf Mastercycler® ep replex PCR cyclers in twincet semi-skirted white real-time PCR 96 well-plates consisted of 15 min at 48°C for reverse transcription, 10 min at 95°C to activate the DNA polymerase and 40 cycles of 15 s at 95°C and 60 s at 60°C. For reactions using plasmid DNA the 15 min 48°C reverse transcription step was omitted. The standard curves were linear across 10 orders of magnitude and the efficiency for each target was 0.98. A standard curve for the 18S ribosomal RNA (rRNA) housekeeping gene was also prepared using 100 ng of adult rat total RNA and the TaqMan® 18S rRNA endogenous control primer-probe assay (Applied Biosystems). The standard curve was linear across eight orders of magnitude and the efficiency was 1.04. PCR reactions were carried out in triplicate using the TaqMan® RNA-to-Ct™ 1-Step Kit (Applied Biosystems).

The abundance of alternatively spliced transcripts was determined using 50 ng of template RNA from either neonatal (P2–P4) or adult (4–6 weeks) rat DRG. Initial levels of individual splice isoform transcript levels were determined using 50 ng of template RNA from either neonatal (P2–P4) or adult (4–6 weeks) rat DRG. Initial levels of individual splice isoform transcripts were determined using 50 ng of template RNA from three different animals for each developmental stage. CT values for the target gene and the housekeeping gene were comparable between individual experiments. PCR reactions in the absence of reverse transcriptase did not produce a detectable fluorescent signal before 35 amplification cycles. In addition, specificity of the primer-probe assays and size of the resulting amplicons were confirmed by analysing reaction products on a 2% agarose gel. Values for the abundance of each alternative transcript were calculated by assuming that the sum of each splicing event at a given site is equal to 1.0 (e.g. exon 5N + exon 5A = 1.0) (Raymond et al., 2004). The F statistic associated with one-way ANOVA was used to test the null hypothesis of no differences between conditions, and a post hoc Tukey’s test was conducted to examine significance between the groups at P level of 0.05. Descriptive data are presented as mean±SEM. The ANOVA and post hoc analyses were conducted using Origin 8.0 (Microcal).

Results

Clinical data

The proband (Fig. 1A, II1) is a Caucasian Dutch 51-year-old male with severe burning pain in his feet, hands and sometimes face, with onset of symptoms in the feet at the age of 24 years. At the age of 44 years, pain severity intensified and the symptoms extended to hands, arms and sometimes face and ears, with the patient grading the severity of his symptoms as 8 on a scale of 10 (1 = no pain; 10 = very intense pain). Cooling of extremities ameliorates pain. Based on the clinical data and exclusion of other causes, the patient was diagnosed with adult-onset erythromelalgia. At the age of 48, the pain became unbearable and quality of life deteriorated with the patient finding it difficult to stand, walk or sleep. Initially, the patient responded favourably to lidocaine infusions, but this treatment subsequently lost efficacy. Mexiletine and fentanyl matrix and lidocaine cream (25 mg/g) on his hands provide partial relief. Elevation of the feet also provides partial relief, but burning pain has progressively increased despite all medical and rehabilitative measures.

The patient (II1) has a family history of similar symptoms. He has four children, two of whom (Fig. 1A, III3, III4) show pain symptoms, while the oldest male child (III1, 18-years-old) and the female child (III2, 17-years-old) do not report pain symptoms. The affected children began showing pain symptoms at the ages of 6 years (III4, now 14-years-old) and 8 years (III3, now 10-years-old). Both affected children report increasing pain in the feet that has begun to limit their mobility. The patient’s father was reported to have a similar disorder but is deceased; information about the age of onset is not available. The patient’s mother (I1, 82-years-old) has reported by guest on April 29, 2016 http://brain.oxfordjournals.org/ Downloaded from

![Figure 1](http://brain.oxfordjournals.org/) Inheritance pattern of the G616R mutation in loop 1 of Na1.7. (A) Inheritance of erythromelalgia in three generations of a Dutch family. Circles and squares denote females and males, respectively. The proband is indicated by an arrow. Blackened symbols indicate subjects showing pain symptoms. A plus symbol denotes subjects heterozygous for the 1846G>A mutation in exon 11, which leads to G616R substitution in the channel polypeptide, and a minus symbol denotes subjects without the mutation. Numbers after plus signs indicate age of onset of pain, where known. The ‘+’ next to the plus symbol for the female in generation III indicates that, at age 17, pain has not appeared. (B) Sequence alignment of L1 loop from orthologues of Na1.7 from different mammalian species: Hs = Homo sapiens, accession number Q15858; Mam = Macaca mulatta, accession number XP_001101662, predicted sequence; Cf = Canis familiaris, accession number XP_859026, predicted sequence; Oc = Oryctolagus cuniculus, accession number Q28644; Bt = Bos taurus, accession number XP_001252616, predicted sequence; Ec = Equus caballus, accession number XP_001496473, predicted sequence; Md = Monodelphis domestica, accession number XP_001367438, predicted sequence; Rn = Rattus norvegicus, accession number NP_579823; Mm = Mus musculus, accession number Q62205.
Identification of a mutation in Na\textsubscript{v}1.7

All known exons of SCN9A were amplified from genomic DNA from the proband and his family (four children), and their sequences were compared to Na\textsubscript{v}1.7 cDNA (Klugbauer et al., 1995). Proband and control templates produced amplicons of the same size which were purified and sequenced. Sequence analysis identified c.1846G>A mutation in exon 11 (E11) which substitutes glycine (G) with arginine (R) at codon 616 (G616R) of the polypeptide, located in loop 1 (L1) joining domains I and II. G616R substitution is present in all individuals with pain symptoms (II1, II3, and III4) of this family (Fig. 1A), but is not present in the unaffected mother (II2) or the unaffected brother (III1). The proband’s daughter (III2) carries the G616R mutation but shows no pain symptoms at age 17, suggesting a possible delay in the appearance of symptoms similar to her father.

While certain regions of L1 show significant amino-acid sequence conservation (mostly the membrane-proximal regions) the sequence surrounding G616 is not highly conserved among sodium channels. However, G616 is conserved in all orthologues of Na\textsubscript{v}1.7 from the different mammalian species reported to date (Fig. 1B).

The c.1846G>A mutation destroys the restriction site for BstI (CCNNNNN/NNGG) in the E11 amplicon (495 bp), which facilitated screening of control samples. The amplicon from the asymptomatic parent and sibling produced the expected wild-type pattern, while the proband showed an additional band consistent with the loss of the BstI site (data not shown). Restriction analysis of the amplicons from 150 Caucasian control samples (300 chromosomes) showed only the expected wild-type pattern. The c.1846G>A mutation segregated with inherited erythromelalgia symptoms except for the proband’s daughter (III2) who carries the mutation but, at age 17, has not experienced pain symptoms of inherited erythromelalgia to date.

Activation and steady-state inactivation for WT\textsubscript{NS} and G616R\textsubscript{NS} in neonatal DRG neurons do not differ

To study the mutation in the neonatal-short (NS) splice variant of Na\textsubscript{v}1.7, the TTX-resistant versions of the hNa\textsubscript{v}1.7\textsubscript{R}, neonatal short splice variants of wild-type hNa\textsubscript{v}1.7\textsubscript{R} and its mutant G616R (termed WT\textsubscript{NS} and G616R\textsubscript{NS} in this paper, respectively) were transiently expressed in neonatal DRG neurons, a mammalian neuronal cell background, from P1 to P5 Na\textsubscript{v}1.8-null mice. These neurons, which lack functional, slow TTX-resistant Na\textsubscript{v}1.8 channels (Akopian et al., 1999), and express very low levels of persistent TTX-resistant sodium current, provide an expression system in which sodium channels of interest, such as WT\textsubscript{NS} and G616R\textsubscript{NS}, can be studied in isolation after other TTX-sensitive sodium channels are blocked with 300 nM TTX (Cummins et al., 1999).

Figure 2 shows families of currents from DRG neurons transfected with WT\textsubscript{NS} and G616R\textsubscript{NS} elicited using a series of depolarizations from a holding potential of −100 mV. This protocol produced robust fast-activating and fast-inactivating sodium currents, with an average peak current density of 642 ± 179 pA/pF (n = 11) for neurons expressing WT\textsubscript{NS} and 513 ± 202 pA/pF (n = 13) for neurons expressing G616R\textsubscript{NS}. Figure 2C shows the voltage-dependent activation and steady-state inactivation curves for those recombinant channels. To minimize the contamination of the apparent small persistent TTX-resistant current, cells displaying <2 nA of peak current were excluded from the analysis. The voltage-dependent activation and inactivation (estimated by fitting the data with a Boltzmann function) in DRG neurons expressing G616R\textsubscript{NS} (V\textsubscript{1/2} = −27.1 ± 1.7 mV for activation; V\textsubscript{1/2} = −74.8 ± 2.9 mV for inactivation) were not significantly different from those in neurons expressing WT\textsubscript{NS} (V\textsubscript{1/2} = −28.1 ± 2.0 mV for activation; V\textsubscript{1/2} = −74.1 ± 2.4 mV for inactivation). Time-to-peak (a measure of activation kinetics) and inactivation kinetics were not different for WT\textsubscript{NS} and G616R\textsubscript{NS} (data not shown).

Voltage dependence of steady-state inactivation is depolarized for G616R\textsubscript{AL} in adult DRG neurons

To study the effects of the mutation in adults, adult-long (AL) splicing variants of wild-type hNa\textsubscript{v}1.7\textsubscript{R} and its mutant G616R
density was 804 ± 177 pA/pF (n = 21) for neurons transfected with WT AL and 1124 ± 191 pA/pF (n = 12) for neurons transfected with G616RAL. Time to peak and inactivation kinetics were not different for WT AL versus G616RAL. The midpoints of activation curves in neurons expressing G616RAL (−29.1 ± 2.0 mV) were similar to those in neurons expressing WT AL (−29.0 ± 1.7 mV). However, the steady-state inactivation curves in neurons expressing G616RAL were significantly different from those in neurons expressing WT AL. The midpoint of steady-state inactivation for WT AL (V1/2 = −75.0 ± 2.0 mV, n = 9) was ~6 mV more positive and the slope (k = 6.1 ± 0.4 mV) was shallower than for WT AL currents (V1/2 = −80.7 ± 1.3 mV, P = 0.023; k = 5.1 ± 0.2 mV, P = 0.025, n = 11). The availability for G616RAL was larger than for WT AL at a range of potentials close to resting potential (Fig. 3C, inset) and thus the predicted window currents in a range surrounding resting membrane potentials are larger for G616RAL than for WT AL.

### Alteration of inactivation by the G616R mutation is splice variant-dependent, not cell background-dependent

The experiments described above did not determine whether the shift of steady-state inactivation curve results from the change of DRG neuron background (neonatal versus adult) or the effect of splice variant itself (NS versus AL). To address this question, we asked whether G616RAL would alter voltage-dependence, compared to WT AL, when expressed in neonatal DRG neurons. Figure 3D shows the voltage-dependent activation and steady-state inactivation curves for WT AL and G616RAL expressed within neonatal DRG neurons. The midpoints of activation were −32.1 ± 1.4 mV for WT AL and −31.2 ± 2.3 mV for G616RAL. However, similar to the results when WT AL and G616RAL were expressed in adult DRG neurons, the midpoint of steady-state inactivation was shifted 5 mV more positive for the mutant channel (WT AL: −78.3 ± 1.2 mV, n = 11; G616RAL: −73.4 ± 1.5 mV, n = 13; P = 0.020), and the slope was shallower for G616RAL (k = 6.0 ± 0.3 mV) than for WT AL (k = 4.9 ± 0.2 mV, P = 0.006), similar to our results when these two constructs were expressed in adult DRG neurons. These results show that alteration of inactivation by the G616R mutation is splice variant dependent, not cell background dependent.

### Protein kinase A does not alter activation or steady-state inactivation of wild-type or G616RAL channels

Previously, PKA activation was reported to regulate the short versus long L1 splice isomers of Na1.7 differentially by shifting in a hyperpolarizing direction the voltage-dependence of activation of Na1.7 channels carrying the short but not the long loop 1 (Chatelier et al., 2008). Since G616R manifested an effect on steady-state channel inactivation in the long splice isoform but not the short isoform, we investigated the hypothesis that the G616RAL mutant channels may be modulated by PKA activation. PKA was activated by treatment with 1 mM 8-Br-cAMP for 15 min (term WT AL and G616RAL, respectively) were examined after expression in adult DRG neurons from 4- to 6-week-old Na1.8-null mice. Figure 3 shows families of currents from DRG neurons transfected with WT AL and G616RAL. The average peak current

![Figure 3](http://brain.oxfordjournals.org/)

**Figure 3** Comparison of voltage-dependent activation and steady-state inactivation for WT AL and G616RAL expressed in DRG neurons. Representative current traces recorded from adult DRG neurons transfected with WT AL and G616RAL.
in bath solution prior to starting patch-clamp recordings, conditions which have been shown to activate PKA and modulate voltage-gated sodium currents in DRG neurons (Chatelier et al., 2008). Our data (Supplementary Fig. 1) show that neither activation nor steady-state inactivation of WT_AL or G616RAL were changed by PKA activation under the conditions used here. Our results from expression of WT_AL in DRG neurons are in agreement with the previous report (Chatelier et al., 2008) of a lack of an effect of PKA activation on these Na_v1.7 splice-isoforms in human embryonic kidney-293 cells, and show that the G616R mutation does not change this property.

Mutant adult-long isoform, but not mutant neonatal-short isoform, renders DRG neurons hyperexcitable

We used current clamp recording to test the hypothesis that expression of G616RAL, but not of G616RNS, would make DRG neurons hyperexcitable. For expression of the WT_NS splice variant and its G616RNS mutant, we used DRG from postnatal 1- to 5-day-old rats as a neonatal neuronal background for functional analysis. For expression of the WT_AL splice variant and its G616RAL mutant, we used DRG from 4- to 6-week-old rats as an adult neuronal background for functional analysis. Mean input resistance and average resting membrane potentials of DRG neurons expressing WT_NS and G616RNS were not significantly different (Table 1). Mean input resistance of neurons expressing WT_AL and G616RAL were also not significantly different. However, the resting membrane potentials of neurons expressing G616RAL were significantly depolarized (~5 mV) compared to those of neurons expressing WT_AL (Table 1 and Fig. 4A). Moreover, >30% of DRG neurons expressing G616RAL produced spontaneous action potentials (Table 1 and Fig. 4B), while <5% neurons expressing WTNS, G616RNS and WT_AL generated spontaneous action potentials. The proportion of spontaneous spiking neurons for G616RAL was significantly larger than that for WT_AL (P = 0.013). In this experiment, we did not inject a bias current to achieve a constant holding membrane potential or to stabilize the membrane potential for comparison of data across cells, because the G616RAL mutation changed availability of the sodium channels, which is highly voltage-dependent, near the resting membrane potential. Since the resting membrane potential determines the availability of Na_v1.7, a change of resting membrane potential induced by a bias current would distort the effect of mutation. Because it is difficult to measure the response to injected current in neurons producing spontaneous action potentials, we also excluded these neurons from our analysis of injected current threshold and action potential firing in response to injected current.

Current threshold, i.e. the injected current required to generate the all-or-none action potential, was significantly lower for DRG neurons expressing G616RAL (783 ± 78 pA) than for neurons expressing WT_NS and G616RNS (Table 1 and Fig. 4B). Moreover, 43% of DRG neurons expressing G616RAL produced spontaneous action potentials with a spectrum of frequencies from low (left) to high (right) at the resting membrane potential.

Table 1 Resting membrane potential and action potential characterization for DRG neurons expressing WT_NS, G616RNS, WT_AL and G616RAL hNa_v1.7 channels

<table>
<thead>
<tr>
<th></th>
<th>WT_NS (n=25)</th>
<th>G616RNS (n=26)</th>
<th>WT_AL (n=23)</th>
<th>G616RAL (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells with spontaneous firing</td>
<td>0 (0%)</td>
<td>1 (3.8%)</td>
<td>1 (4.3%)</td>
<td>7 (33.3%)*</td>
</tr>
<tr>
<td>R_in (GΩ)</td>
<td>1.46 ± 0.19</td>
<td>1.40 ± 0.15</td>
<td>0.40 ± 0.06</td>
<td>0.45 ± 0.07</td>
</tr>
<tr>
<td>Resting membrane potential (mV)</td>
<td>−53.5 ± 1.3</td>
<td>−52.8 ± 1.1</td>
<td>−51.6 ± 1.7</td>
<td>−46.2 ± 1.1*</td>
</tr>
<tr>
<td>Action potential amplitude (mV)</td>
<td>100.3 ± 3.2</td>
<td>100.7 ± 2.7</td>
<td>97.9 ± 4.2</td>
<td>94.4 ± 3.6</td>
</tr>
<tr>
<td>t_peak (ms)</td>
<td>6.8 ± 0.7</td>
<td>6.0 ± 0.8</td>
<td>8.3 ± 1.9</td>
<td>13.3 ± 4.3</td>
</tr>
<tr>
<td>Voltage threshold (mV)</td>
<td>−23.7 ± 1.3</td>
<td>−21.4 ± 1.4</td>
<td>−21.5 ± 1.4</td>
<td>−24.5 ± 1.6</td>
</tr>
<tr>
<td>Current threshold (pA)</td>
<td>594 ± 55</td>
<td>548 ± 35</td>
<td>1243 ± 141</td>
<td>783 ± 78*</td>
</tr>
</tbody>
</table>

*P = 0.013, 0.025 and 0.018 for cells with spontaneous firing and resting membrane potential, and current threshold, respectively, compared with WT_AL.

Figure 4 Resting membrane potential and spontaneous action potentials. (A) The representative resting membrane potentials were measured for 10 s without any stimulation immediately after establishing whole-cell configuration. (B) Seven out of twenty-one neurons expressing G616RAL produced a spontaneous action potentials a spectrum of frequencies from low (left) to high (right) at the resting membrane potential.
expressing WTAL (1243 ± 141 pA; P = 0.018), while current thresholds for neurons expressing WTNS (1240 ± 140 pA) and G616RNS (1240 ± 139 pA) were similar. The mean action potential peak, time-to-peak (tpeak) and mean voltage threshold (i.e. voltage for take-off of an all-or-none action potential) for the neurons expressing wild-type were not significantly different from those for neurons expressing its mutants (Table 1 and Fig. 5). Because neurons that generated spontaneous action potentials were excluded from this analysis, our measurement of the change of current threshold in neurons expressing G616RAL is, if anything, an underestimate.

To assess repetitive action potential firing, 500 ms current stimuli (100–400 pA in 100 pA increments) were injected (Fig. 6). Neurons expressing WTNS and G616RNS produced similar numbers of action potentials in response to all tested stimuli (Fig. 6A). In contrast, neurons expressing G616RAL tended to generate a larger number of action potentials than neurons expressing WTAL (Fig. 6B). The number of action potentials produced by cells expressing G616RAL (4.6 ± 1.1) was significantly larger than neurons expressing WTAL (1.6 ± 0.7, P = 0.034) at 300 pA current injection. Since neurons producing spontaneous action potentials were excluded from this analysis, if anything it underestimates the increase in firing rate produced by the adult isoform mutant channel.

We also studied the effects of slow ramp current injections from 0 to 0.5 nA over 500 ms on firing (Fig. 7), to mimic a slow depolarization. The number of action potentials evoked by the 0.5 nA ramp was greater for G616RAL (2.5 ± 0.8) than for WTAL (1.1 ± 0.5), G616RNS (0.6 ± 0.3) and WTNS (1.9 ± 0.7). Time to first action potential (tpeak) for neurons expressing G616RAL (235.6 ± 49.8 ms) was significantly shorter than for neurons expressing WTAL (406.1 ± 33.8 ms; P = 0.006), whereas the time to peak for neurons expressing WTNS (379.5 ± 39.7 ms) and G616RNS (432.6 ± 30.1 ms) were not significantly different.

Taken together, our current clamp experiments indicate that the G616R mutation in the adult splicing variant of hNa1.7, but not in the neonatal splicing variant, depolarizes resting membrane potential and lowers current threshold, increases the firing rate in response to injected current and increases the proportion of DRG neurons displaying spontaneous action potential firing at resting potential, i.e. produces DRG neuron hyperexcitability.

**Na1.8-R1268Q mutation in the proband but not in other affected family members**

A second mutation, R1268Q, was also found in the Na1.8 channel in the proband. This mutation was not present in his two...
affected children (III3 and III4), or his G616R-carrier asymptomatic daughter III2. Ninety-eight ethically matched control alleles were also screened but only wild-type DNA sequence was found. While this mutation did not segregate with disease phenotype, we nevertheless profiled it functionally. Our voltage-clamp analysis revealed an 11 mV depolarizing shift in the voltage-dependence of activation, and no change in steady-state inactivation (Supplementary Fig. 2). The depolarizing shift in activation, if anything, might be expected to decrease nociceceptor excitability. Our current-clamp analysis, however, did not reveal a significant difference in threshold or firing frequency of DRG neurons transfected with wild-type Na\textsubscript{v}1.8 versus R1268Q mutant Na\textsubscript{v}1.8 channels (Supplementary Figs 3 and 4, Tables 2 and 3).

Differential expression of \textit{Na\textsubscript{v}1.7\textsubscript{A}} versus \textit{Na\textsubscript{v}1.7\textsubscript{N}}, and \textit{Na\textsubscript{v}1.7\textsubscript{S}} versus \textit{Na\textsubscript{v}1.7\textsubscript{L}}

Since our electrophysiological results showed a functional effect of the G616R mutation in the \textit{Na\textsubscript{v}1.7\textsubscript{AL}} but not in the \textit{Na\textsubscript{v}1.7\textsubscript{NS}} splice isoforms, we compared the levels of transcripts carrying E5N versus E5A and E11S versus E11L within neonatal (P2–P4) and adult (4- to 6-week-old) rat DRGs, as previously described (Raymond \textit{et al.}, 2004). The data in Fig. 8 show the abundance of each splice variant as a percentage of the sum of splicing events at that site. In neonatal rat DRG neurons the expression level of \textit{Na\textsubscript{v}1.7} transcripts with the neonatal form of exon 5 (E5N: 62.2 ± 5.8%) is significantly higher than the level of \textit{Na\textsubscript{v}1.7} transcripts with the adult form of exon 5 (E5A: 37.8 ± 5.8%; P < 0.05). Our results show that, by adulthood, the expression of splice variants carrying E5A increases to 49.6 ± 7.2% (the level of E5N decreases to 50.4 ± 7.2%) although this level is not significantly different from that in the neonatal tissue (E5A: 37.8 ± 5.8%; P > 0.05). Our data also show a significant increase in the expression of the splice variant carrying E11L in adult versus neonatal rat DRG neurons (neonates: 43.9 ± 0.8%; adult: 69.4 ± 0.2%; P < 0.05). There is also a corresponding reduction in the expression of the splice variant carrying E11S between neonatal and adult rat DRG neurons (neonate: 56.1 ± 0.8%; adult: 30.6 ± 0.2%; P < 0.05). Thus, the E5A/E5N ratio changes from 0.63 in neonatal rat DRG to 1.0 in adult rat DRG, and the
E11L/E11S ratio changes from 0.72 in neonatal rat DRG to 2.2 in adult rat DRG.

Discussion

Inherited erythromelalgia, an autosomal dominant disorder characterized by severe burning pain triggered by warmth, is produced by gain-of-function mutations in sodium channel Na\textsubscript{v}1.7 (Dib-Hajj \textit{et al.}, 2007; Cheng \textit{et al.}, 2008; Han \textit{et al.}, 2009), which is preferentially expressed in DRG neurons including nociceptors (Sangameswaran \textit{et al.}, 1997; Toledo-Aral \textit{et al.}, 1997; Djouhri \textit{et al.}, 2003). Inherited erythromelalgia mutations that have been studied by current-clamp render DRG neurons hyperexcitable (Dib-Hajj \textit{et al.}, 2005; Harty \textit{et al.}, 2006; Rush \textit{et al.}, 2009). In most families with inherited erythromelalgia, patients begin to manifest pain in early childhood. Here we describe a novel Na\textsubscript{v}1.7 mutation, G616R, in a family with inherited erythromelalgia in which some family members did not develop pain until adulthood, and present evidence that indicates that switching of expression of Na\textsubscript{v}1.7 splice isoforms can contribute to time-dependent disease expression.

As a result of alternative splicing of exons 5 and 11, four splice isoforms of Na\textsubscript{v}1.7 (Na\textsubscript{v}1.7\textsubscript{NS}, Na\textsubscript{v}1.7\textsubscript{NL}, Na\textsubscript{v}1.7\textsubscript{AS} and Na\textsubscript{v}1.7\textsubscript{AL}) carrying either neonatal (E5N) or adult (E5A) variants of exon 5, and short (E11S) or long (E11L) variants of exon 11 that encodes part of loop 1, with an 11 amino-acid extension in the long form, are expressed within adult DRG neurons (Raymond \textit{et al.}, 2004). E5 splicing, which produces E5N or E5A variants, has been described for other sodium channels including Na\textsubscript{v}1.2, Na\textsubscript{v}1.3 and Na\textsubscript{v}1.6 with, in general, a transition from predominant expression of a neonatal form in the foetal/neonatal nervous system, to predominant expression of the adult form in adulthood (Sarao \textit{et al.}, 1991; Gustafson \textit{et al.}, 1993; Plummer \textit{et al.}, 1998). We have observed an increase in relative expression of the Na\textsubscript{v}1.7\textsubscript{NL} splice isoform, and an apparent increase in relative expression of Na\textsubscript{v}1.7\textsubscript{AS} within mature rat DRG neurons, but whether this type of transition occurs for Na\textsubscript{v}1.7 in humans is not yet known.

We report a family with adult-onset inherited erythromelalgia in some family members, in which all affected members harbour the G616R Na\textsubscript{v}1.7 mutation, and demonstrate a preferential effect of this mutation on the adult Na\textsubscript{v}1.7 splice variant (G616R\textsubscript{AL}) that may contribute to delayed onset of clinical disease. We show, using voltage-clamp experiments, that the G616R mutation affects channel gating properties within the adult splice variant, but not the neonatal splice variant. We demonstrate, using current-clamp, that the G616R\textsubscript{AL} mutant channel, but not G616R\textsubscript{NS}, makes DRG neurons hyperexcitable. Our results also show that expression of G616R\textsubscript{AL}, but not the developmental stage of the host cell, provides a basis for DRG neuron hyperexcitability.

The inherited erythromelalgia mutations studied to date hyperpolarize activation (Dib-Hajj \textit{et al.}, 2007; Cheng \textit{et al.}, 2008; Choi \textit{et al.}, 2009; Fischer \textit{et al.}, 2009; Han \textit{et al.}, 2009). In contrast, the G616R\textsubscript{AL} mutant channel depolarizes steady-state inactivation, similar to Na\textsubscript{v}1.7 mutations that cause paroxysmal extreme pain disorder, which is characterized by perirectal, ocular and mandibular pain (Fertleman \textit{et al.}, 2006; Dib-Hajj \textit{et al.}, 2008; Jarecki \textit{et al.}, 2008). Depolarized steady-state inactivation, without a change of activation, enhances channel availability and increases window current rather than lowering activation threshold, as shown for previous inherited erythromelalgia mutations (Harty \textit{et al.}, 2006). A larger window current would be expected to depolarize resting potential, consistent with our current-clamp data. When studied by current-clamp, DRG neurons expressing both inherited erythromelalgia (Dib-Hajj \textit{et al.}, 2005; Harty \textit{et al.}, 2006; Rush \textit{et al.}, 2009) and paroxysmal extreme pain disorder mutations (Dib-Hajj \textit{et al.}, 2008; Estacion \textit{et al.}, 2008) display increased firing frequency in response to graded suprathreshold stimulation. Why one group of mutations causes limb pain and another group of mutations causes perirectal, peri orbital and mandibular pain is not known, although increased DRG neuron excitability appears to be linked to pain in both groups of patients. Notably, the effects of G616R on the channel (depolarization of inactivation) are different from those associated with many of the mutations linked to inherited erythromelalgia (hyperpolarization of activation). This raises the possibility that the age of onset of pain may be more variable because the mutation has a different effect on the channel.

Despite the presence of conserved motifs in L1, the sequence of L1 is divergent among sodium channels. This diversity has been linked to isoform-specific behaviour, e.g. modulation of current density in response to PKA/protein kinase C (Fitzgerald \textit{et al.}, 1999; Scheuer and Catterall, 2006) or p38 microtubule-associated protein kinase activation (Wittmack \textit{et al.}, 2005; Hudmon \textit{et al.}, 2008). The alternative splice isoforms of Na\textsubscript{v}1.7 (Na\textsubscript{v}1.7\textsubscript{NS}, Na\textsubscript{v}1.7\textsubscript{NL}, Na\textsubscript{v}1.7\textsubscript{AS} and Na\textsubscript{v}1.7\textsubscript{AL}) display small differences in gating properties, without significant difference in voltage-dependence of steady-state inactivation. The longer L1 has been reported to attenuate PKA-mediated modulation of channel activation (Chatelier \textit{et al.}, 2008). The G616R substitution occurs 31 amino acids N-terminal to the L1 extension residues, at a site that is highly conserved in mammalian Na\textsubscript{v}1.7 orthologues (Fig. 1), suggesting conserved function(s). However, our data show that the G616R mutation does not change the effect of PKA on activation or steady-state inactivation.

Our results indicate that the G616R mutation depolarizes steady-state inactivation within the adult, but not the neonatal isoform, of Na\textsubscript{v}1.7. Cell background can also contribute to the physiological profile of a given sodium channel. For example, functional properties of Na\textsubscript{v}1.3 (Cummins \textit{et al.}, 2001), Na\textsubscript{v}1.6 (Raman and Bean, 1997; Cummins \textit{et al.}, 2005) and Na\textsubscript{v}1.8 (Blair and Bean, 2003; Choi \textit{et al.}, 2007) differ, depending on the cell-type where the channel is expressed. However, we found that the G616R mutation alters Na\textsubscript{v}1.7 gating properties irrespective of whether it was expressed within adult or neonatal DRG neurons. Our results show that the gating shift is dependent on the splice variant in which the G616R mutation is expressed, but not on the host neuron's developmental age.

The proband under study first manifested pain at age 24 years and his daughter who is heterozygous for the G616R mutation has not manifested pain, even at age 17 years. Most families with inherited erythromelalgia are characterized by onset of pain in the first decade, usually prior to age 6 years, and in these families, the mutation expressed in the Na\textsubscript{v}1.7 neonatal splice variant...
produces gain-of-function changes, including a hyperpolarizing shift in activation, and DRG neuron hyperexcitability (Dib-Hajj et al., 2007; Cheng et al., 2008; Choi et al., 2009; Fischer et al., 2009; Han et al., 2009), which were not seen when the G616R mutation was expressed in the Na\textsubscript{v}1.7 neonatal splice variant. We recently characterized a Na\textsubscript{v}1.7 mutation (Q10R) in a family with delayed-onset (second decade) inherited erythromelalgia, which shifts Na\textsubscript{v}1.7 activation, but to a smaller degree, and makes DRG neurons hyperexcitable, but also to a smaller degree compared to mutations associated with early-onset inherited erythromelalgia (Han et al., 2009). The effect of the Q10R mutation was comparable within Na\textsubscript{v}1.7\textsubscript{NS} and Na\textsubscript{v}1.7\textsubscript{AL}, suggesting that other factors, e.g. differences in expression of modifier genes, or in central gating mechanisms or central sensitization, may contribute to delayed-onset (Han et al., 2009). In contrast, for the G616R mutation described here, our results may provide a different explanation for clinical onset in adulthood in some family members, related to changes in expression of Na\textsubscript{v}1.7 splice variants as age increases.

The proband in this family harbours a Na\textsubscript{v}1.8 mutation (R1268Q) in addition to the G616R Na\textsubscript{v}1.7 mutation. This Na\textsubscript{v}1.8 mutation is not present in two affected sons or the asymptomatic G616R-carrier daughter, demonstrating that it is not a prerequisite for development of early-onset inherited erythromelalgia or delayed onset of the symptoms to the second decade; both of the affected sons harbour only the G616R Na\textsubscript{v}1.7 mutation. Nonetheless, because onset of pain occurred late (age 24 years) in the father, we considered the possibility that the R1268Q Na\textsubscript{v}1.8 mutation might have had a protective effect. We consider this possibility to be unlikely for several reasons: (i) the father is heterozygous with respect to Na\textsubscript{v}1.8, carrying one wild-type allele and one R1268Q mutant allele which increases the channel activation threshold, and it is known (Akopian et al., 1999) that Na\textsubscript{v}1.8 does not manifest haploinsufficiency and that loss of one Na\textsubscript{v}1.8 allele does not affect DRG neuron excitability; and (ii) the proband’s daughter remains asymptomatic at age 17 years while carrying the G616R Na\textsubscript{v}1.7 mutation without the R1268Q Na\textsubscript{v}1.8 mutation. Consistent with a small contribution, if any, of the R1268Q Na\textsubscript{v}1.8 mutation to the proband’s clinical picture, we modelled his heterozygous Na\textsubscript{v}1.8 status by transfecting DRG neurons with wild-type or R1268Q mutant Na\textsubscript{v}1.8, and detected no significant difference in threshold or firing frequency. Co-expression of G616R\textsubscript{AL} and Na\textsubscript{v}1.8-R1268Q did not alter the excitability of DRG neurons, compared to neurons transfected with G616R\textsubscript{AL} and Na\textsubscript{v}1.8-WT (Supplementary Fig. 4). Thus, it appears that the Na\textsubscript{v}1.7 G616R substitution is the pathogenic sodium channel mutation in this kindred.

The late onset of symptoms in members of this family who carry the G616R mutation, with one member first manifesting disease at age 24 years, another remaining asymptomatic at age 17 and two children exhibiting symptoms in the second half of the first decade, contrasts with earlier onset among families carrying other inherited erythromelalgia mutations, e.g. onset in the largest multigeneration family with inherited erythromelalgia at 1–6 years (mean 3 years) (Dib-Hajj et al., 2005). A wide range of age of onset of symptoms (13–27 years) has been reported in families with hypokalaemic periodic paralysis caused by mutations in SCN4A (Miller et al., 2004), and in a calcium channelopathy with a mutation in CACNA1S (5–27 years) (Houinato et al., 2007). In the present family with inherited erythromelalgia, it is not known whether differences in the developmental pattern of splice-variant expression in different family members, or other factors such as modifier genes, contribute to different ages of onset of pain.

In summary, our results indicate that the physiological effects of a sodium channel mutation can depend on alternative splicing of the mutated channel, and suggest that developmental switching of splice isoforms contributes to delayed onset of disease with symptoms appearing only after expression of a sufficient proportion of channels in the permissive splice variant.

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Supplementary material

Supplementary material is available at Brain online.

References


