Small nerve fibres, small hands and small feet: a new syndrome of pain, dysautonomia and acromesomelia in a kindred with a novel NaV1.7 mutation

Janneke G. J. Hoeijmakers, 1,* Chongyang Han, 2,3,* Ingemar S. J. Merkies, 1,4 Lawrence J. Macala, 2,3 Giuseppe Lauria, 5 Monique M. Gerrits, 6 Sulayman D. Dib-Hajj, 2,3 Catharina G. Faber, 1,† and Stephen G. Waxman 2,3,†

1 Department of Neurology, University Medical Centre Maastricht, 6202 AZ Maastricht, The Netherlands
2 Department of Neurology, Yale University School of Medicine, New Haven, CT 06510, USA
3 Centre for Neuroscience and Regeneration Research, Veterans Affairs Medical Centre, West Haven, CT 06516, USA
4 Department of Neurology, Spaarne Hospital, 2130 AT Hoofddorp, The Netherlands
5 Neuromuscular Diseases Unit, IRCCS Foundation, Carlo Besta, 20133 Milan, Italy
6 Department of Clinical Genetics, University Medical Centre Maastricht, 6202 AZ Maastricht, The Netherlands

*These authors contributed equally to this work.
†These authors are co-senior authors.

Correspondence to: Stephen G. Waxman, M.D., Ph.D., Neuroscience and Regeneration Research Centre, VA Connecticut Healthcare System, 950 Campbell Avenue, Building 34, West Haven, CT 06516, USA
E-mail: stephen.waxman@yale.edu

The NaV1.7 sodium channel is preferentially expressed within dorsal root ganglion and sympathetic ganglion neurons and their small-diameter peripheral axons. Gain-of-function variants of NaV1.7 have recently been described in patients with painful small fibre neuropathy and no other apparent cause. Here, we describe a novel syndrome of pain, dysautonomia, small hands and small feet in a kindred carrying a novel NaV1.7 mutation. A 35-year-old male presented with erythema and burning pain in the hands since early childhood, later disseminating to the feet, cheeks and ears. He also experienced progressive muscle cramps, profound sweating, bowel disturbances (diarrhoea or constipation), episodic dry eyes and mouth, hot flashes, and erectile dysfunction. Neurological examination was normal. Physical examination was remarkable in revealing small hands and feet (acromesomelia). Blood examination and nerve conduction studies were unremarkable. Intra-epidermal nerve fibre density was significantly reduced compared to age- and sex-matched normative values. The patient’s brother and father reported similar complaints including distal extremity redness and pain, and demonstrated comparable distal limb under-development. Quantitative sensory testing revealed impaired warmth sensation in the proband, father and brother. Genetic analysis revealed a novel missense mutation in the SCN9A gene encoding sodium channel NaV1.7 (G856D; c.2567G > A) in all three affected subjects, but not in unaffected family members. Functional analysis demonstrated that the mutation hyperpolarizes (−9.3 mV) channel activation, depolarizes (+6.2 mV) steady-state fast-inactivation, slows deactivation and enhances persistent current and the response to slow ramp stimuli by 10- to 11-fold compared with wild-type NaV1.7 channels. Current-clamp analysis of dorsal root ganglion neurons transfected with G856D mutant channels demonstrated depolarized resting potential, reduced current threshold, increased repetitive firing in response to suprathreshold stimulation and increased spontaneous firing. Our results
demonstrate that the G856D mutation produces DRG neuron hyperexcitability which underlies pain in this kindred, and suggest that small peripheral nerve fibre dysfunction due to this mutation may have contributed to distal limb under-development in this novel syndrome.

Keywords: small nerve fibre neuropathy; small fibre neuropathy; SCN9A mutation; acromesomelia; neuropathic pain; sodium channel

Abbreviations: DRG = dorsal root ganglion

Introduction

Small fibre neuropathy is a disorder of thinly myelinated and unmyelinated nerve fibres and is clinically characterized by burning pain and autonomic complaints (Gorson and Ropper, 1995; Holland et al., 1998; Lacomis, 2002; Devigili et al., 2008). The diagnosis of pure small fibre neuropathy, in which small-diameter nerve fibres are affected but large-diameter fibres are spared, is usually made on the basis of the clinical picture, together with preservation of large fibre functions (normal strength, tendon reflexes and vibration sense) and normal nerve conduction studies, and is confirmed by demonstration of reduced intra-epidermal nerve fibre density or abnormal quantitative sensory testing (Tesfaye et al., 2010). We have recently described the presence of novel functional variants in SCN9A, the gene encoding for the NaV1.7 sodium channel, in ~30% of patients with biopsy-confirmed idiopathic small fibre neuropathy (Faber et al., 2011). NaV1.7 is preferentially and abundantly expressed within dorsal root ganglion (DRG) and sympathetic ganglion neurons (Toledo-Aral et al., 1997; Rush et al., 2006) and their small-diameter peripheral axons (Persson et al., 2010) where it amplifies small depolarizations and modulates excitability (Herzog et al., 2003). The biophysical changes in NaV1.7 channels produced by SCN9A-related small fibre neuropathy variants published to date include impaired slow-inactivation, impaired slow- and fast-inactivation, or enhanced resurgent currents (Faber et al., 2011).

In this report, we describe a kindred with clinical features of small fibre neuropathy that produced distal extremity pain, together with a remarkable phenotype of small forearms, hands, lower legs and feet (acromesomelia). Each of the three affected subjects harbouring a novel mutation in SCN9A (c.2567 G > A; G856D) produces an ensemble of functional changes including enhanced activation, ramp current and persistent current. The mutant channels increase DRG neuron excitability. Our results demonstrate that the G856D mutation produces DRG neuron hyperexcitability that underlies pain in this kindred, and suggest the hypothesis that this NaV1.7 mutation produces peripheral nerve dysfunction that may contribute to limb under-development.

Materials and methods

Anthropometrics

Twelve anthropometric body dimensions were measured in the index patient and family members (father, mother and two brothers) and compared with reported age- and gender-matched Dutch health controls (see Supplementary Fig. 1 for definitions of the measurements) (Steenbekkers and van Beijsterveldt, 1998; Molenbroek, 2004). All measurements were performed twice to assure accuracy. Measurements were completed by one observer (J.G.J.H.). Stature was measured using a wall-attached ruler (cm). The subject was requested to stand straight-up against the wall, feet together and barefooted. A tape measure (cm) was used for all other variables. Shoulder height sitting, elbow height sitting, elbow-grip length, buttock-popliteal distance, buttock–knee distance and popliteal height were measured with the subject sitting straight-up, with the posterior parts of the buttocks against the back of a chair, the popliteal fossa against the front of the chair, knees 90° flexed and the feet resting on the floor (patient sitting in a vertical plane adjustable chair). The shoulders were relaxed with the upper arms hanging alongside the body, and forearms in a horizontal plane (Supplementary Fig. 1). Hand length and hand width (with and without thumb) were measured while the hand was extended by the subject and resting on an examination couch. Foot length and foot width were measured with the subject standing straight-up, the feet slightly apart and weight evenly distributed. All findings were compared with published normative values (Steenbekkers and van Beijsterveldt, 1998; Molenbroek, 2004).

Quantitative sensory testing

Quantitative sensory testing was performed as previously described (Hoitsma et al., 2003; Reulen et al., 2003; Shy et al., 2003; Lauria et al., 2010b; Faber et al., 2011). In brief, quantitative sensory testing thresholds for warm, cool and heat pain modalities were assessed using a TSH-2001 device (Medoc) at the dorsum of both feet and thenar eminences, and a modality was classified as abnormal if results of both method-of-limits and method-of-levels were abnormal (Hoitsma et al., 2003; Reulen et al., 2003).

Intra-epidermal nerve fibre density

For assessment of intra-epidermal nerve fibre density, a punch biopsy was taken 10 cm above the lateral malleolus. The number of individual nerve fibres crossing the dermal-epidermal junction was counted in three randomly taken sections (50 μm) after fixing the biopsy by subjecting it to cryoprotective solution and immunostaining with polyclonal rabbit anti-protein-gene-product-9.5 antibody (PGP9.5; Ultraclone), using bright-field microscopy with a stereology workstation (Olympus BX50, PlanApo oil-objective × 40/NA = 1.0) (Lauria et al., 2010a). Intra-epidermal nerve fibre density findings were compared with published normative data (Lauria et al., 2010a).

SCN9A mutation analysis

Genomic DNA was extracted from blood using the Puregene® genomic DNA isolation kit (Gentra-Systems). All coding exons and
flanking intronic sequences, and exons encoding 5′- and 3′-untranslated sequences within the complementary DNA, were amplified and sequenced as described previously (Drenth et al., 2005). Genomic sequences were compared with reference Na\textsubscript{\textit{v}}1.7\textsubscript{K} complementary DNA (NM_002977.3; Klugbauer et al., 1999) to identify sequence variations, using Alamut Mutation-Interpretation Software (Interactive-Biosoftware). A control panel of DNA from 100 healthy Dutch (Caucasian) individuals (200 chromosomes) was screened for all new mutations. The NCBI SNP database, the Human Gene Mutation Database (HGMDD\textsuperscript{5}) and the 1000 genomes project (www.1000genomes.org/data) were also screened.

**Functional analysis**

**Voltage-clamp analysis**

The plasmid carrying the tetrodotoxin-resistant version of human Na\textsubscript{\textit{v}}1.7\textsubscript{K} was described previously (Herzog et al., 2003). The G856D mutation was introduced into hNa\textsubscript{\textit{v}}1.7\textsubscript{K} using QuickChange XL site-directed mutagenesis (Stratagene). Wild-type or G856D mutant hNa\textsubscript{\textit{v}}1.7\textsubscript{K} channels were co-transfected with the human β1 and β2 subunits into HEK293 cells, grown under standard culture conditions (5% CO\textsubscript{2}, 37°C) in Dulbecco’s Modified Eagle’s Medium supplemented with 10% foetal bovine serum, by Optifect\textsuperscript{16} transfection reagent (Invitrogen).

Whole-cell patch-clamp recordings were obtained at room temperature (~21°C), 24 h after transfection using an EPC-9 amplifier and Pulse 8.5 (HEKA) with 0.8–1.5 MΩ electrodes. Voltage errors were minimized using 80–90% series resistance compensation and linear leak subtraction; capacitance artefact was cancelled using computer-controlled circuitry. Recordings were started 5 min after establishing whole-cell configuration. The pipette solution contained: 140 mM CsF, 1 mM EGTA, 10 mM NaCl and 10 mM HEPES, pH 7.3 with CsOH (adjusted to 315 mOsm with dextrose). The bath solution was 140 mM NaCl, 3 mM KCl, 1 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2} and 10 mM HEPES, pH 7.3 with NaOH (adjusted to 320 mOsm with dextrose).

**Transfection of dorsal root ganglion neurons and current-clamp recordings**

The protocol for care and sacrifice of rats used in the study was approved by the Veterans Administration Connecticut Healthcare system IACUC. DRG from 4- to 8-week-old Sprague-Dawley rats were harvested and dissociated as previously described (Choi et al., 2010). Sodium channel and Green Fluorescent Protein (GFP) constructs (channel:GFP ratio of 5:1) were electroporated into DRG neurons using Rat Neuron Nucleofector Solution (Amaxa) with wild-type Na\textsubscript{\textit{v}}1.7\textsubscript{K} and G856D mutant derivative as described previously (Dib-Hajj et al., 2005). Transfected DRG neurons were incubated at 37°C in Ca\textsuperscript{2+}-free culture medium (Dulbecco’s Modified Eagle’s Medium) for 24 h after transfection using an EPC-9 amplifier and Pulse 8.5 (HEKA) with 0.8–1.5 MΩ electrodes. Voltage errors were minimized using 80–90% series resistance compensation and linear leak subtraction; capacitance artefact was cancelled using computer-controlled circuitry. Recordings were started 5 min after establishing whole-cell configuration. The pipette solution contained: 140 mM CsF, 1 mM EGTA, 10 mM NaCl and 10 mM HEPES, pH 7.3 with CsOH (adjusted to 315 mOsm with dextrose). The bath solution was 140 mM NaCl, 3 mM KCl, 1 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2} and 10 mM HEPES, pH 7.3 with NaOH (adjusted to 320 mOsm with dextrose).

**Results**

**Clinical description**

A 35-year-old male presented with episodic cramps, erythema and burning pain. At the age of 10 years, he experienced episodes of redness of the skin in both hands that lasted for hours. A few years later, he developed burning pain in his hands. These symptoms were triggered by warmth, exercise or taking a warm bath, and were relieved by cold water. In adulthood, pain episodes were also triggered by alcohol. Initially, the episodes occurred in the evening. However, there was a gradual increase in frequency and duration of pain episodes, which could occur at any time during the day. The redness of the skin and the pain also expanded to the feet, cheeks and ears. The intensity and hotness (‘burning’) of pain were scored 8 and 9, respectively, on a 10-point scale (Galer and Jensen, 1997). The patient also experienced episodic spontaneous sweating, bowel disturbances (diarrhoea or constipation), episodic dry eyes and mouth, hot flashes, and erectile dysfunction. The patient did not use any drugs that are known to cause neuropathy. Treatment with aspirin, a beta-blocker and pregabalin was ineffective.

On physical examination, no signs of large nerve fibre involvement were found. As shown in Fig. 1A, the patient had profound redness of the distal forearms and hands. Remarkably, his hands, lower legs and feet were small when compared to age- and gender-matched normative values (Figs 1B and 2; Table 1; see Supplementary Fig. 1 for explanation of anthropometric measurements) (Steenbekkers and van Beijsterveldt, 1998; Molenbroek, 2004). Nerve conduction studies were normal. The patient was given a clinical diagnosis of small fibre neuropathy and was subsequently subjected to additional investigations. Tables 2 and 3
Quantitative sensory testing and intra-epidermal nerve fibre density measurements in the index patient

Quantitative sensory testing showed impaired warmth sensation at the thenar eminence bilaterally (Table 4). Intra-epidermal nerve fibre density at the lateral malleolus was reduced (intra-epidermal nerve fibre density: 5.0/mm) compared to age- and gender-matched normative values (median 10.3/mm, 5th percentile \(\geq 5.2/mm\) (Lauria et al., 2010a), confirming the diagnosis of small fibre neuropathy.

DNA analysis in the index patient

Sequence analysis (Faber et al., 2011) showed a novel mutation (c.2567G \(\rightarrow\) A; G856D) in the SCN9A gene. This mutation substitutes a negatively charged aspartic acid for a highly conserved non-polar glycine within the S4–S5 linker of domain II of the channel (Fig. 4B). The mutation was not found in a control panel of DNA from 100 healthy Dutch controls (Caucasian individuals (200 chromosomes), and has not been reported in the NCBI SNP database, the HGMD database, or in the 1000 genomes project.

Findings in proband’s family members

The pedigree of this family with G856D mutation is presented in Fig. 4A. Tables 2–4 summarize the findings in the father and Brother A of the index patient, who displayed symptoms that were less severe than in the proband (Tables 2 and 3). Quantitative sensory testing was abnormal in both family members (warmth sensation was impaired at the thenar eminence for both; in addition, the father demonstrated impaired cold sensation at the thenar and foot) (Table 4). The intra-epidermal nerve fibre density on the other hand was normal. Remarkably, anthropometric data in both the father and brother demonstrated findings quite similar to those in the index patient, with acral and distal limb under-development (Table 1). SCN9A analysis in these two family members with small fibre neuropathy demonstrated the same mutation (c.2567G \(\rightarrow\) A; G856D) as in the index patient (Fig. 4). The unaffected mother and Brother B, without small fibre neuropathy complaints and normal posture, did not harbour the mutation.

Functional analyses

Functional characterization of Na\(_{\alpha}\)1.7 mutations

Effects of the mutation on channel properties were assessed by whole-cell voltage clamp after transient transfection of HEK293 cells with wild-type hNa\(_{\alpha}\)1.7\(_{\text{R}}\) or the mutant channel G856D together with hß1 and hß2 subunits. Figure 5A(1) shows representative Na\(_{\alpha}\)1.7 sodium currents recorded from cells expressing wild-type channels, and Fig. 5A(2) from cells expressing G856D mutant channels. Current density of G856D mutant channel (118 ± 13 pA/pF; \(n=28\)) was significantly smaller (\(P<0.001\)) than that of wild-type (504 ± 61 pA/pF; \(n=25\)).

The voltage-dependence of channel activation was examined using depolarizing test pulses to +60 mV from a holding potential of –120 mV. The threshold for activation was 10–15 mV more negative for G856D mutant channels than for wild-type channels.
### Table 1  
**Body proportions: findings of index patient and family members with G856D SCN9A mutation (Molenbroek, 2004)**

| Anthropometric measurements (see Supplementary Fig. 1 for explanation) | Measure | Mean (mm) (31–60 years) | 5th percentile cut-off (31–60 years) | Index patient (mm)/% value | Proband’s brother (mm)/% value | Mean (mm) (> 60 years) | 5th percentile cut-off (> 60 years) | Proband’s father (mm)/% value |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Stature | 1770 | 1645 | 1665/8.1 | 1670/45.3 | 1729 | 1619 | 1610/3.9 |
| 2 | Shoulder height sitting | 612 | 561 | 580/15.1 | 550/2.2 | 596 | 545 | 510/0.3 |
| 3 | Elbow height sitting | 253 | 207 | 235/26.5 | 180/0.5 | 237 | 189 | 170/1 |
| 4 | Elbow-grip length | 352 | 318 | 340/28.5 | 340/28.5 | 347 | 317 | 330/17.1 |
| 5 | Hand length | 194 | 178 | 160/0.1 | 175/2.8 | 191 | 175 | 180/13.8 |
| 6 | Hand width (with thumb) | 119 | 111 | 90/0.1 | 90/0.1 | 111 | 103 | 110/42 |
| 7 | Hand width (without thumb) | 91 | 84 | 70/0.1 | 70/0.1 | 90 | 82 | 80/2.2 |
| 8 | Buttock-popliteal distance | 503 | 459 | 450/2.4 | 500/4.4 | 499 | 458 | 470/12.5 |
| 9 | Buttock-knee distance | 631 | 579 | 510/0.1 | 590/10.4 | 625 | 579 | 570/2.4 |
| 10 | Popliteal height | 481 | 429 | 370/0.1 | 430/5.4 | 470 | 427 | 440/11.7 |
| 11 | Foot length | 266 | 241 | 180/0.1 | 210/0.1 | 267 | 246 | 240/1.8 |
| 12 | Foot width | 101 | 91 | 70/0.1 | 80/0.1 | 100 | 90 | 90/4.8 |

The numbers in bold are <5th percentile cut-off normative values (Molenbroek, 2004).

### Table 2  
**Clinical description of index patient and family members with G856D SCN9A mutation**

<table>
<thead>
<tr>
<th>Age at referral/ gender</th>
<th>Age at onset symptoms</th>
<th>Initial symptom(s) + location</th>
<th>Later symptoms</th>
<th>Precipitating factors/relieving factors</th>
<th>Medication</th>
<th>Intra-epidermal nerve fibre density + (corresponding normative value)</th>
<th>Quantitative sensory testing impaired modality</th>
<th>SCN9A mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Index patient</strong></td>
<td>35/male</td>
<td>10 years</td>
<td>Redness hands and wrists, burning pain</td>
<td>Beginning at age ~25 years, redness skin expanded to the feet, cheeks and ear region and became painful</td>
<td>↑ with warmth, exercise, or drinking alcohol. ↓ by cooling.</td>
<td>No effect Aspirin, beta-blocker and Pregabalin</td>
<td>5.0/mm (≥ 5.2/mm)</td>
<td>Warth-L</td>
</tr>
<tr>
<td><strong>Brother A</strong></td>
<td>32/male</td>
<td>Childhood</td>
<td>Redness hands and feet</td>
<td>Recent onset moderate burning pain</td>
<td>↑ rising temperatures</td>
<td>–</td>
<td>11.1/mm (≥ 5.2/mm)</td>
<td>Warth-L</td>
</tr>
<tr>
<td><strong>Father</strong></td>
<td>68/male</td>
<td>Childhood until age 20 years</td>
<td>Redness hands and feet</td>
<td>Redness hands and feet</td>
<td>–</td>
<td>–</td>
<td>3.7/mm (≥ 2.8/mm)</td>
<td>Warth-L Cold-L</td>
</tr>
</tbody>
</table>

Quantitative sensory testing scores were compared with normative values reported by Yarnitsky and Sprecher (1994). A sensory modality was classified as abnormal if the results of both method-of-limits and method-of-levels were abnormal. Note: the mother and a second brother (indexed as Brother B) did not have complaints, had no physical abnormalities (normal anthropometrics) and did not harbour the G856D mutation.

L = left; R = right; ↑ = increase; ↓ = reduced.
Fig. 5B). The midpoint of activation for G856D (estimated by fitting with a Boltzmann function) was significantly shifted by $-9.3 \pm 0.6$ mV ($n = 27$) compared with wild-type channels ($-22.7 \pm 0.8$ mV, $n = 23$; $P < 0.001$). We measured the kinetics of deactivation, which reflect the transition from the open to the closed state, using single exponential fits to estimate the time of current decay at potentials from $-120$ to $-40$ mV after briefly activating the channels at $-20$ mV for 0.5 ms. As shown in Fig. 5C, the rates of current decay of G856D mutant channels were significantly slower than those of wild-type channels across all deactivation potentials tested.

Steady-state fast-inactivation was measured using a series of 500-ms prepulses from $-150$ to $0$ mV followed by 40-ms test pulses at $-10$ mV. The midpoint of fast inactivation for G856D mutant channels ($-72.8 \pm 0.8$ mV, $n = 27$) was shifted 6.2 mV in a depolarizing direction compared to that of wild-type channels ($-79.0 \pm 1.0$ mV, $n = 22$; $P < 0.001$) (Fig. 5D). The combination of the depolarizing shift in fast-inactivation and the hyperpolarizing shift in activation results in increased overlap between activation and fast-inactivation, which predicts a large window current.

We measured slow-inactivation with 30-s prepulses, followed by 100-ms pulses to $-120$ mV to allow recovery from fast-inactivation, and then a 20-ms test pulse to $0$ mV to determine the fraction of available channels. The steady-state slow-inactivation curve of G856D mutant channels was shifted in a hyperpolarizing direction (Fig. 5E). The midpoint was $-62.9 \pm 1.3$ mV for wild-type ($n = 13$) and $-78.0 \pm 1.1$ mV for G856D mutant channels ($n = 16$; $P < 0.001$).

Table 3: Small nerve fibre neuropathy symptoms inventory questionnaire findings

<table>
<thead>
<tr>
<th>sweating</th>
<th>diarrhoea</th>
<th>constipation</th>
<th>micturition problems</th>
<th>dry eyes</th>
<th>dry mouth</th>
<th>orthostatic dizziness</th>
<th>palpitations</th>
<th>hot flashes</th>
<th>skin hyperaesthesia</th>
<th>burning feet</th>
<th>sheet intolerance</th>
<th>restless legs</th>
</tr>
</thead>
<tbody>
<tr>
<td>index patient</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>brother A</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>father</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Green indicates absence (score 0) of corresponding small fibre neuropathy-related complaint; a red box indicates the presence of small fibre neuropathy-related symptom, with variable intensity (score 1: sometimes present; score 2: often and a score 3: always present). The proband’s father (aged 68 years) experienced redness of the feet and hands until his early twenties.
Table 4  Quantitative sensory testing, findings and corresponding normative data

<table>
<thead>
<tr>
<th></th>
<th>Theminoright</th>
<th>Theminoleft</th>
<th>Dorsumfootright</th>
<th>Dorsumfootleft</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Warmthsensation</td>
<td>Cold sensation</td>
<td>Heatpain</td>
<td>Warmthsensation</td>
</tr>
<tr>
<td>Index patient</td>
<td>37.2</td>
<td>37.7</td>
<td>20.0</td>
<td>29.7</td>
</tr>
<tr>
<td>Normal values</td>
<td>&lt;33.2</td>
<td>&lt;35.3</td>
<td>&gt;30.1</td>
<td>&gt;28.3</td>
</tr>
<tr>
<td>Brother A</td>
<td>32.8</td>
<td>37.8</td>
<td>30.5</td>
<td>29.3</td>
</tr>
<tr>
<td>Normal values</td>
<td>&lt;33.2</td>
<td>&lt;35.3</td>
<td>&gt;30.1</td>
<td>&gt;28.3</td>
</tr>
<tr>
<td>Father</td>
<td>37.0</td>
<td>37.6</td>
<td>28.0</td>
<td>28.9</td>
</tr>
<tr>
<td>Normal values</td>
<td>&lt;33.4</td>
<td>&lt;35.8</td>
<td>&gt;30.1</td>
<td>&gt;27.5</td>
</tr>
</tbody>
</table>

Thresholds were assessed at the dorsum of the foot and thenar eminence bilaterally, using ascending (warm) and descending (cool) thermal energy ramp stimuli delivered through a thermode. Heat pain modality was also examined. Obtained values were compared with reported normative values (Yarnitsky and Sprecher, 1994). A measurement was considered abnormal (bold) when its Z-value exceeded 2.5. A sensory modality was classified as abnormal if the results of both method-of-limits and method-of-levels were abnormal (red). MLE = method-of-levels; MLI = method-of-limits.

Figure 4  (A) Pedigree showing index patient (arrow) and family members. Family members that underwent SCN9A analysis are depicted in bold. Filled symbols represent affected family members (index patient, father and Brother A) with small fibre neuropathy complaints and novel SCN9A mutation G856D. Open symbols represent the mother and Brother B having no small fibre neuropathy complaints, normal anthropometrics, and no SCN9A mutation (square = male; circle = female; + = G856D mutation present; − = G856D mutation absent). (B) The G856D mutation substitutes an amino acid at a site that is conserved in all mammalian sodium channels (human sodium channels shown).
Figure 5 Voltage-clamp analysis of NaV1.7/G856D mutant channels. (A) Representative current traces recorded from HEK293 cells expressing wild-type (WT) (A1) or G856D (A2) channels, evoked by voltage steps (100 ms) from −80 to 60 mV in 5 mV increments, from a holding potential of −120 mV. (B) Normalized peak current–voltage relationship curves for wild-type (black squares, n = 23) and G856D (red circles, n = 27) channels. G856D shifts threshold for channel activation by 10–15 mV in a hyperpolarizing direction. (C) Time constants for tail current deactivation. Cells were held at −120 mV and tail currents were generated by a brief 0.5-ms depolarization to −20 mV followed by a series of repolarizations ranging from −120 to −40 mV, *P < 0.05. G856D channels (red circles, n = 19) deactivate significantly slower than wild-type channels (black squares, n = 13). (D) Steady-state fast-inactivation for wild-type (black squares, n = 22) and G856D (red circles, n = 27) channels, examined using a series of 500-ms prepulses from −150 to 0 mV followed by test pulses to −10 mV. G856D mutation shifts V1/2 for fast-inactivation by 6.2 mV in a depolarized direction. The voltage-dependence of activation was obtained by converting current to conductance, and fitting Boltzmann functions to data shown in B. G856D mutation shifts V1/2 of activation by −9.3 mV. (E) Steady-state slow-inactivation curves for wild-type (black squares, n = 13) and G856D (red circles, n = 16), assessed using a 20-ms pulse to −10 mV after a 30-s prepulse to potentials ranging from −130 to 10 mV followed by a 100-ms pulse to −120 mV to remove fast-inactivation. G856D mutation enhances steady-state slow-inactivation with the V1/2 shifting by −15.1 mV. (F) Time constants for development of closed-state inactivation, measured by holding at −120 mV, prepulsing membrane potential to the inactivation potential for increasing durations, then stepping to −10 mV to determine the fraction of current inactivated during the prepulse. Data were fit with single exponential. G856D mutation (red circles) slows the development of closed-state inactivation significantly compared with wild-type (black squares) from −80 to −60 mV. (G) Representative ramp currents, elicited with 600-ms ramp depolarization from −120 to 0 mV for wild-type (black) and G856D mutant (red). G856D mutation increases ramp current amplitude and shifts the voltage at which the peak of ramp current occurs. (H) The traces elicited by pulses to −20 mV (the same two cells as in A), and the currents were normalized to peak inward currents. G856D-expressing (red line) cell produces larger persistent current than wild-type-expressing (black line) cell. Inset: The end of the 100-ms trace and vertical axis were expanded to show the difference in persistent current between wild-type and G856D mutant channels at the end of 100-ms recording. (I) Comparison of persistent currents between cells expressing wild-type channels (n = 11) and cells expressing G856D mutant channels (n = 9) for activation depolarization step pulses from −80 to 40 mV.
Recovery from fast-inactivation (repriming) was assessed using pairs of pulses with an incrementally increasing recovery time at six different recovery potentials (−120, −110, −100, −90, −80 and −70 mV). G856D mutation did not change the repriming kinetics at any of these six potentials (data not shown). The development of closed-state inactivation was slower for G856D mutant channels, and the time constants were significantly longer than those of wild-type channels at −80, −70 and −60 mV (Fig. 5F).

Ramp currents, reflecting the channel’s response to the small, slow depolarizations, were evaluated by a slow ramp protocol with a depolarization from −120 to 0 mV over 600 ms. The recorded current was expressed as a percentage of the peak inward current obtained during the activation protocol. For cells expressing wild-type channels, the amplitude of ramp current was 0.73 ± 0.1% (n = 10). In contrast, for cells expressing G856D mutant channels, the ramp current amplitude was 8.42 ± 0.49% (n = 19; P < 0.001), an 11-fold increase compared to wild-type. Compared with wild-type channels (−39.6 ± 1.3 mV, n = 10), the peak ramp current of G856D mutant channels occurred at more negative potentials (−50.6 ± 1.1 mV, n = 19; P < 0.001) (Fig. 5G), a similar shift as was observed for step depolarization.

We assessed the effect of the mutation on persistent currents, i.e. currents that are not inactivated at the end of 100-ms depolarization pulses, expressed as a percentage of peak current amplitude elicited by step depolarizations. As shown in Fig. 5H (traces elicited by pulses to −20 mV, from the same cells as shown in Fig. 5A(1) and (2)), mutant channels produced markedly larger persistent currents than wild-type channels. The average persistent current amplitude of G856D at −20 mV (normalized to peak of transient current) was 3.51 ± 0.91% (n = 9), >10 times larger than that of wild-type channels (0.32 ± 0.11%, n = 11; P < 0.01) (Fig. 5I). Notably, cells expressing G856D mutant channels produced enhanced persistent currents [1.21 ± 0.25% (n = 9), compared with 0.13 ± 0.06% (n = 11) for wild-type; P < 0.01] even around −55 mV, close to the resting membrane potential of DRG neurons (Fig. 5I). These changes would be expected to increase the excitability of neurons expressing mutant G856D channels.

To assess the effect of the G856D mutation on DRG neuron excitability, we expressed wild-type and G856D mutant channels in small DRG neurons (22–28 μm diameter) and performed current-clamp recordings. Input resistance was similar between DRG neurons expressing wild-type channels (1164 ± 82 MΩ, n = 32) and DRG neurons expressing G856D mutant channels (1246 ± 129 MΩ, n = 21; P > 0.05). However, resting membrane potential of DRG neurons, which expressed G856D mutant channels (−49.7 ± 1.1 mV, n = 21) was significantly depolarized by 6.8 mV compared with DRG neurons expressing wild-type channels (−56.5 ± 0.1 mV, n = 32; P < 0.001).

Current threshold, the injection stimulus required to produce a single all-or-none action potential, was reduced in DRG neurons expressing G856D. Figure 6A shows traces from a representative DRG neuron expressing wild-type channels. In response to ≤220 pA sub-threshold current injections, the neuron only generated small, graded membrane potential depolarization. The first all-or-none action potential required a stimulus of 225 pA (current threshold for this neuron). Figure 6B shows recordings from a representative DRG neuron, which expressed G856D mutant channels. For this neuron, the current injection required to produce the first all-or-none action was 100 pA. Figure 6C presents a comparison of current threshold between these two groups of neurons. Expression of G856D (109 ± 16 pA, n = 21) reduced current threshold significantly compared with wild-type NaV1.7 (222 ± 19 pA, n = 32; P < 0.001). There were no significant differences of either voltage threshold (the voltage at which action potential take-off occurs) (wild-type: −21.2 ± 1.2 mV, n = 32; G856D: −23.9 ± 1.3 mV, n = 21; P > 0.05) or action potential amplitude (wild-type: 107.1 ± 2.3 mV, n = 32; G856D: 104.4 ± 2.0 mV, n = 21; P > 0.05) between these two groups of DRG neurons.

To evaluate repetitive action potential firing, we injected DRG neurons with a series of 500-ms current stimuli from 25 to 500 pA in 25 pA increments. DRG neurons expressing G856D mutant channels fired more action potentials compared with neurons expressing wild-type channels at all stimulus levels. Figure 6D–I show the responses of two representative DRG neurons that expressed wild-type and G856D mutant channels, respectively, to 500-ms current steps at 1 x, 2 x and 3 x current thresholds for the respective neuron. DRG neurons expressing wild-type channels generated a single spike in response to current injections at both 1- and 2-fold current threshold, and generated two spikes in response to stimuli at 3-fold current threshold. In contrast, neurons expressing G856D mutant channels tended to fire with multiple action potentials in response to stimuli at 2- and 3-fold threshold. Figure 6J compares the responses of DRG neurons expressing G856D mutant channels and wild-type NaV1.7 channels over a range of graded suprathreshold stimulation levels.

We also found that G856D mutation produced an increase in the proportion of spontaneously firing DRG neurons. Figure 6K shows an example of sustained spontaneous firing from a representative DRG neuron expressing G856D mutant channels. Figure 6L displays 2 s of spontaneous firing from this neuron on an expanded time-base. As the bar graph in Fig. 6L shows, only 3% (1/33) of DRG neurons expressing wild-type channels displayed spontaneous firing. In contrast, 36% of DRG neurons expressing G856D displayed spontaneous firing (12/33 cells, 36.4%), significantly more than for wild-types (P < 0.05, z-test).

Taken together, the current-clamp results show that the G856D mutation depolarizes resting membrane potential of DRG neurons, increases excitability of these cells as evidenced by reduced current threshold, increases firing rates in response to suprathreshold stimulation and increases the proportion of cells that fire spontaneously in the absence of stimulation.

**Discussion**

The NaV1.7 sodium channel is abundantly expressed within DRG and sympathetic ganglion neurons (Toledo-Aral et al., 1997; Rush et al., 2006) and in their small-diameter axons within peripheral nerves (Persson et al., 2010). Single amino acid substitutions of NaV1.7 that produce gain-of-function have been associated with
Figure 6  Current-clamp analysis of effects of G856D mutant channels on DRG neuron excitability. (A and B) Representative action potential (AP) traces recorded from DRG neuron expressing wild-type (WT) (A) or G856D mutant (B) channels. Action potentials were elicited by 200-ms step depolarizing current injections from resting membrane potential. (C) Comparison of current threshold for DRG neurons expressing wild-type and G856D mutant channels. Expression of G856D channels reduces current threshold significantly. Current threshold: wild-type: 222 ± 19 pA, n = 32; G856D: 109 ± 16 pA, n = 21. *** P < 0.001. (D–F) Responses of a representative DRG neuron expressing wild-type channels to 500-ms depolarization current steps that are 1 × (D), 2 × (E) and 3 × (F) the current threshold of this neuron. (G–I) Responses of a representative DRG neuron expressing G856D mutant channels to 500-ms depolarization current steps that are 1 × (G), 2 × (H) and 3 × (I) the current threshold of this neuron. (J) Comparison of responses (number of impulses evoked by a 500-ms stimulus) in DRG neurons expressing wild-type (black square, n = 32) and G856D channels (red circle, n = 21) across a range of step current injections from 25–500 pA, *P < 0.05. (K) Representative recording showing spontaneous firing of DRG neuron expressing G856D mutant channels. Trace was recorded for 30 s without any current injection. (L) Top: Bar graph showing the proportion of spontaneously firing DRG neurons expressing G856D (red) and wild-type channels (black); numbers to the right of the bar graph show values for wild-type (black font) and G856D (orange font); ×12 indicates 12-fold increase; *P < 0.05. Bottom: 2 s of spontaneous firing for the same neuron as in (K), shown on an expanded time-base.
three classes of disorders: (i) \(Na_v1.7\) mutations that enhance activation have been shown to produce inherited erythromelalgia (Cummins et al., 2004; Dib-Hajj et al., 2005, 2010; Han et al., 2006; Choi et al., 2010); (ii) mutations of \(Na_v1.7\) that impair fast-inactivation produce paroxysmal extreme pain disorder (Fertleman et al., 2006); and (iii) single amino acid substitutions of \(Na_v1.7\) that impair slow-inactivation, impair fast- and slow-inactivation, or enhance resurgent currents have been found in \(\sim 30\%\) of patients with idiopathic small fibre neuropathy (Faber et al., 2011).

In the present article, we describe a novel syndrome of painful small fibre neuropathy, small hands and small feet, in a family housing a \(Na_v1.7\) mutation that enhances activation, impairs fast-inactivation and markedly enhances persistent current and the response to slow ramp stimuli. The patient described here complained of burning pain and redness of the hands and feet, triggered by warmth and relieved by cold, similar to inherited erythromelalgia (Dib-Hajj et al., 2010) and to one of the previously described patients with idiopathic small fibre neuropathy associated with a \(Na_v1.7\) variant (Faber et al., 2011). However, unlike most patients with inherited erythromelalgia (Drenth et al., 2005), the present patient also displayed profound autonomic dysfunction, a common clinical feature of small fibre neuropathy (Gorson and Ropper, 1995; Holland et al., 1998; Lacomis, 2002; Devigili et al., 2008). Demonstration of decreased intra-epidermal nerve fibre density on skin biopsy and abnormal quantitative sensory testing confirmed the diagnosis of small fibre neuropathy in the index patient, who also displayed under-development of the hands and feet. Intra-epidermal nerve fibre density was normal in the proband’s father and brother; however, they also displayed distal extremity pain and redness, met diagnostic criteria for small fibre neuropathy on quantitative sensory testing in all three affected patients in the context of abnormal function of small-diameter axons innervating our observations of small nerve fibre loss in the index patient, which also displayed under-development of the hands and feet. Intra-epidermal nerve fibre density was normal in the proband’s father and brother; however, they also displayed distal extremity pain and redness, met diagnostic criteria for small fibre neuropathy on quantitative sensory testing in all three affected patients in the context of abnormal function of small-diameter axons innervating

The patients described in this article are notable in displaying acromesomelia, or under-development of distal hands and feet (Langer and Garrett, 1980). Limb morphogenesis is a complex process involving multiple signalling cascades that have been partially explicated at the molecular and genetic levels. Several gene mutations have been associated with syndromal and non-syndromal limb defects (Gurrieri et al., 2002; Barham and Clarke, 2008; Lyons and Ezaki, 2009; Decker et al., 2011). The role of sodium channels in limb development is not understood, and no relationship between mutations in sodium channels and small limbs has previously been described.

\(Na_v1.7\) is co-expressed with \(Na_v1.6, Na_v1.8\) and \(Na_v1.9\) and the sodium–calcium exchanger NCX2 in small-diameter DRG neurons and their distal terminals (Persson et al., 2010), where a high surface-to-volume ratio and high input impedance are expected to enhance the effect of sodium channel activity (Waxman et al., 1989; Donnelly, 2008). In non-myelinated axons, the energetic cost of action potential conduction per impulse per unit length is \(\sim 10\)-fold higher than in larger myelinated axons (Wang et al., 2008). Sodium channel blockers have been shown to have a protective effect, limiting the degree of axonal degeneration, in multiple axon injury models (Stys et al., 1992a; Kapoor et al., 2003; Lo et al., 2003; Bechtold et al., 2004, 2005). It remains to be determined whether the hyperexcitability produced in DRG neurons by \(G856D\) poises small axons to degenerate, or whether the robust persistent current of \(G856D\) mutant channels can trigger reverse sodium–calcium exchange, which injures small-diameter axons, as has been demonstrated in CNS white matter (Stys et al., 1992b, 1993).

Anthropometric assessments have not been carried out in individuals harbouring \(Na_v1.7\) mutations that substitute other amino acids within the domain II S4–S5 linker (Cummins et al., 2004; Han et al., 2006; Estacion et al., 2008), close to \(G856\), or more generally in patients harbouring \(Na_v1.7\) variants (Dib-Hajj and Waxman, 2010; Faber et al., 2011). While we cannot rule out the possibility that acromesomelia in the three affected individuals is the result of a second mutation or chance association, the segregation of the \(G856D\) mutation with acromesomelia, and the similar topographic pattern of distal limb pain and redness and distal limb under-development in this kindred suggest that the \(G856D\) mutation may possibly contribute to acromesomelia. Although \(Na_v1.7\) has not been reported in bone or cartilage cells at any stage of development, \(Na_v1.2\) sodium channels are expressed in osteoblasts during embryogenesis (Black et al., 1995), and we cannot exclude an effect of the \(G856D\) mutation on limb development due to transient expression of \(Na_v1.7\) within non-neuronal cells at some stage of ontogenesis. Alternatively, our observations of small nerve fibre loss in the index patient, and of abnormal function of small-diameter axons innervating the limb as evidenced by the clinical picture and abnormal quantitative sensory testing in all three affected patients in the context of a sodium channel mutation that markedly enhances persistent current and DRG neuron excitability, suggest the possibility that dysfunction or injury of peripheral nerve fibres may have interfered with normal limb development.
Impaired limb growth has been reported following denervation (brachial plexus neurectomy) in newborn rabbits (Alharby, 2010). Reddening of the skin in the present patient might be interpreted as suggesting altered vasomotor function, possibly due to an effect of the mutation on sympathetic ganglion neurons that express NaV1.7 (Rush et al., 2006). Innervation of bone vasculature (Hurrell, 1937; Sherman, 1963; Bjurholm et al., 1988; Hukkanen et al., 1993) raises the possibility that altered vascular perfusion may have affected limb morphogenesis during development. Bones are innervated by small myelinated and unmyelinated sensory and sympathetic nerves (Hurrell, 1937; Sherman, 1963; Bjurholm et al., 1988; Hill and Elde, 1991; Mach et al., 2002; Jimenez-Andrade et al., 2010; Castaneda-Corral et al., 2011), some of which contain peptides such as calcitonin gene-related peptide (Jimenez-Andrade et al., 2010; Castaneda-Corral et al., 2011). Calcitonin gene-related peptide has been implicated as a modulator of the metabolism of bone cells, since it regulates osteoclast and osteoblast function, thus modulating bone formation and resorption (Hohmann et al., 1986; Bjurholm et al., 1988; Hill and Elde, 1991; Hukkanen et al., 1993; Mach et al., 2002; Jimenez-Andrade et al., 2010; Castaneda-Corral et al., 2011). In addition, nerve fibres are frequently located in the epiphyseal region, a location that has been interpreted as suggesting that they may participate in the regulation of the epiphyseal growth plate (Bjurholm et al., 1988; Hill and Elde, 1991).

Several disorders are known to cause both neuropathy and limb defects in the embryo. An example is the teratogenic effect of thalidomide, a sedative and hypnotic drug formerly used as an anti-emetic in pregnancy, which has been linked to severe limb deformities in children, especially bone deformities such as phocomelia (Vargesson, 2009). Several models have been postulated to explain the limb teratogenesis produced by thalidomide. One model suggests a direct effect on sensory nerves or neural crest, which may produce an embryonic peripheral neuropathy resulting in impaired trophic function of the nerves with consequent under-development of the limbs (McCredie and McBride, 1973; McCredie and McLeod, 1974; McCredie et al., 1984). Another example is the teratogenicity of maternal varicella-zoster infection, which may lead to a peripheral neuropathy that is associated with hypoplastic limbs (Savage et al., 1973). A similar mechanism has been suggested in congenital malformations in infants of diabetic mothers (Rajbhandari et al., 2002; Botek et al., 2010). Taken together with these earlier studies, the coexistence of small fibre neuropathy and acromesomelia in this kindred with a gain-of-function NaV1.7 mutation leads us to speculate that dysfunction of small nerve fibres innervating the limbs may have adversely affected limb morphogenesis at some stage during development.

In conclusion, we describe a novel syndrome of distal pain, dysautonomia, small hands and small feet in a kindred with a novel missense SCN9A mutation that causes multiple gain-of-function changes in NaV1.7, including markedly enhanced persistent current. Future studies will be needed to test the hypothesis that peripheral nerve dysfunction due to this mutation can contribute to impaired limb development.

Acknowledgements

We thank E. Lapré for constructing Supplementary Fig. 1. We thank Els K. Vanhoutte, Lynda Tyrrell, Peng Zhao and Palak Shah for dedicated assistance.

Funding

‘Profilieringsfonds’ University Hospital Maastricht (partial) and Rehabilitation Research Service and Medical Research Service, Department of Veterans Affairs. The Centre for Neuroscience and Regeneration Research is a collaboration of the Paralyzed Veterans of America with Yale University.

Supplementary material

Supplementary material is available at Brain online.

References

Small fibre neuropathy and acromesomelia with Na⁺,1.7 mutation

Brain 2012; 135: 345–358


