Gain of Function $Na_V1.7$ Mutations in Idiopathic Small Fiber Neuropathy

Catharina G. Faber, MD, PhD,[1] Janneke G. J. Hoeijmakers, MD,[1] Hye-Sook Ahn, PhD,[2,3] Xiaoyang Cheng, PhD,[2,3] Chongyang Han, PhD,[2,3] Jin-Sung Choi, PhD,[2,3]* Mark Estacion, PhD,[2,3] Giuseppe Lauria, MD, PhD,[4] Els K. Vanhoutte, MD,[1] Monique M. Gerrits, PhD,[5] Sulayman Dib-Hajj, PhD,[2,3] Joost P. H. Drenth, MD, PhD,[6] Stephen G. Waxman, MD, PhD,[2,3] and Ingemar S. J. Merkies, MD, PhD[1,7]

Objective: Small nerve fiber neuropathy (SFN) often occurs without apparent cause, but no systematic genetic studies have been performed in patients with idiopathic SFN (I-SFN). We sought to identify a genetic basis for I-SFN by screening patients with biopsy-confirmed idiopathic SFN for mutations in the *SCN9A* gene, encoding voltage-gated sodium channel $Na_V1.7$, which is preferentially expressed in small diameter peripheral axons.
Methods: Patients referred with possible I-SFN, who met the criteria of $\geq$2 SFN-related symptoms, normal strength, tendon reflexes, vibration sense, and nerve conduction studies, and reduced intraepidermal nerve fiber density (IENFD) plus abnormal quantitative sensory testing (QST) and no underlying etiology for SFN, were assessed clinically and by screening of *SCN9A* for mutations and functional analyses.
Results: Twenty-eight patients who met stringent criteria for I-SFN including abnormal IENFD and QST underwent *SCN9A* gene analyses. Of these 28 patients with biopsy-confirmed I-SFN, 8 were found to carry novel mutations in *SCN9A*. Functional analysis revealed multiple gain of function changes in the mutant channels; each of the mutations rendered dorsal root ganglion neurons hyperexcitable.
Interpretation: We show for the first time that gain of function mutations in sodium channel $Na_V1.7$, which render dorsal root ganglion neurons hyperexcitable, are present in a substantial proportion (28.6%; 8 of 28) of patients meeting strict criteria for I-SFN. These results point to a broader role of $Na_V1.7$ mutations in neurological disease than previously considered from studies on rare genetic syndromes, and suggest an etiological basis for I-SFN, whereby expression of gain of function mutant sodium channels in small diameter peripheral axons may cause these fibers to degenerate.
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Small nerve fiber neuropathy (SFN) is a relatively common disorder of thinly myelinated and unmyelinated nerve fibers recently recognized as a distinct clinical syndrome.[1] The clinical picture is typically dominated by onset in adulthood of neuropathic pain, often with a burning quality, and autonomic symptoms.[2–6] The diagnosis of pure SFN, in which small diameter nerve fibers are affected but large diameter fibers are spared, is usually made on the basis of the clinical picture, preservation of large fiber functions (normal strength, tendon reflexes, and vibration sense), and normal nerve conduction studies (NCS), and is confirmed by demonstration of reduced intraepidermal nerve fiber density (IENFD) or abnormal quantitative sensory testing (QST).[7] Despite intensive search for underlying causes such as diabetes mellitus, impaired glucose tolerance, Fabry disease, celiac disease, sarcoidosis, human immunodeficiency virus (HIV), and other systemic illnesses that may be

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Address correspondence to Dr Faber, Department of Neurology/School of Mental Health and Neuroscience, Maastricht University Medical Center, Maastricht, PO-Box 5800, 6202 AZ, Maastricht, the Netherlands. E-mail: c.faber@mumc.nl

Current address for Dr Choi: College of Pharmacy, Catholic University of Korea, Bucheon, South Korea.

From the [1]Department of Neurology, University Medical Center Maastricht, Maastricht, the Netherlands; [2]Department of Neurology, Yale University School of Medicine, New Haven, CT; [3]Center for Neuroscience and Regeneration Research, Veterans Affairs Medical Center, West Haven, CT; [4]Neuromuscular Diseases Unit, IRCCS Foundation, Carlo Besta, Milan, Italy; [5]Department of Clinical Genomics, University Medical Center Maastricht, Maastricht, the Netherlands; [6]Department of Gastroenterology and Hepatology, Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands; and [7]Department of Neurology, Spaarne Hospital, Hoofddorp, the Netherlands.

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treatable,[5,8] the proportion of patients with idiopathic SFN (I-SFN), in which no cause can be identified, remains substantial, ranging in different series from 24% to 93%.[5,6,9] Observations of autosomal dominant inheritance suggest a genetic origin for the small fiber involvement that is seen in burning feet syndrome.[10] However, no specific gene has been linked to, or mutations identified in, patients with adult onset I-SFN.

Voltage-gated sodium channel $Na_V1.7$ is preferentially expressed in dorsal root ganglion (DRG) and sympathetic ganglion neurons[11,12] and their axons,[13] and opens in response to small depolarizations close to resting potential.[14] Gain of function mutations in the *SCN9A* gene encoding $Na_V1.7$ have been found to cause the painful disorders inherited erythromelalgia (IEM)[15,16] and paroxysmal extreme pain disorder (PEPD),[17] which are characterized by increased excitability of DRG neurons, and loss of function mutations of $Na_V1.7$ have been linked to channelopathy-associated insensitivity to pain.[18] Sodium channel mutations have not, however, been linked to axonal degeneration. Reasoning that $Na_V1.7$ is present in small diameter peripheral axons,[13] in this study we asked whether mutations in the *SCN9A* gene could be found in a clinically well-defined cohort of patients with biopsy-confirmed I-SFN.

Our results demonstrate, for the first time, the presence of sodium channel mutations in a substantial proportion of patients with I-SFN, show that these missense mutations occur in an ion channel that is preferentially expressed in peripheral axons and share the common feature of rendering DRG neurons hyperexcitable, and point to a broader role of $Na_V1.7$ mutations in neurological diseases than previously considered from studies on rare genetic hyperexcitability syndromes.

Patients and Methods

Patients

INCLUSION/EXCLUSION CRITERIA: PRESELECTION. To accrue a cohort of patients with I-SFN, we initially assessed all patients aged $\geq$18 years seen at Maastricht University Medical Center neurological clinic with a clinical diagnosis of SFN between 2006 and 2009 and excluded those in whom, after full workup, a cause for SFN was identified. All patients with a clinical diagnosis of I-SFN were asked to participate in this study. Eligibility criteria were normal strength, tendon reflexes, and vibration sense; normal NCS; and presence of at least 2 of the following symptoms: burning feet, allodynia, diminished pain and/or temperature sensation, dry eyes or mouth, orthostatic dizziness, bowel disturbances (constipation/diarrhea/gastroparesis), urinary disturbances, sweat changes (hyper-/hypohidrosis), accommodation problems and/or blurred vision, impotence, diminished ejaculation or lubrication, hot flashes, and palpitations. Exclusion criteria were symptoms or signs of large nerve fiber involvement (muscle weakness, loss of vibration sense, hypo-/areflexia), abnormal NCS, and history or detection after screening of illnesses known to cause SFN, including diabetes mellitus, impaired glucose tolerance, hyperlipidemia, liver, kidney, or thyroid dysfunction, monoclonal gammopathy, connective tissue disorders, sarcoidosis, Sjogren syndrome, amyloidosis, Fabry disease (alpha-galactosidase, in females combined with GLA gene sequencing), celiac disease, HIV, alcohol abuse, hemochromatosis, antiphospholipid syndrome, B6 intoxication, and neurotoxic drugs (eg, chemotherapy). Patients were not screened for mutations associated with hereditary sensory and autonomic neuropathy, which usually has an early onset and clinical characteristics[19] that are different from those seen in our cohort of patients with I-SFN, or for antibodies to peripherin, which have recently been associated with small fiber neuropathy.[20]

FINAL PATIENT SELECTION, BIOPSY, AND QST CONFIRMATION OF SFN. Of 248 patients initially screened following referral with a suspected clinical diagnosis of SFN, 44 patients met inclusion/exclusion criteria and underwent skin biopsy and QST. From this group, 28 met strict criteria for I-SFN (ie, reduced IENFD and abnormal QST compared to normative values). *SCN9A* gene analysis was carried out in all 28 patients with biopsy-confirmed I-SFN. The current study describes 8 patients, from this group of 28 patients with I-SFN, who were found to carry a mutation in the *SCN9A* gene (Fig 1).

Clinical Characterization

SKIN BIOPSY. Punch biopsy (10cm above lateral malleolus) specimens were fixed (2% paraformaldehyde-lysine-sodium periodate at 4°C), cryoprotected, and stored at −80°C in cryoprotective solution (20% glycerol) before sectioning (50μm).[21] The numbers of individual nerve fibers crossing the dermal–epidermal junctions were analyzed in each of 3 sections, immunostained with polyclonal rabbit antiprotein gene product-9.5 antibody (Ultraclone; Wellow, Isle-of-Wight, UK), by bright field microscopy using a stereology workstation (Olympus [Tokyo, Japan] BX50, PlanApo oil-objective $\times$40/numerical aperture (NA) = 1.0). Linear quantification of intraepidermal nerve fiber density was compared with available age- and gender-adjusted normative values.[22]

QST. QST, performed in accordance with previous guidelines,[23] using a TSA-2001 (Medoc, Ramat-Yishai, Israel) instrument, assessed thresholds at the dorsum of both feet and thenar eminences, using ascending/descending (warm/cool) thermal ramp stimuli delivered through a thermode.[24] Heat pain modality was also examined. Results were compared with reported normative values.[25] Measurements were considered abnormal when Z values exceeded 2.5. A sensory modality was classified as abnormal if results of both method of limits and method of levels were abnormal.[26]

SFN SYMPTOM INVENTORY QUESTIONNAIRE. The validated SFN Symptom Inventory Questionnaire (SIQ) includes 13 questions (sweating abnormalities, sudden diarrhea, constipation, urination problems (eg, incontinence), dry eyes and/or mouth, orthostatic dizziness, palpitations, hot flashes,

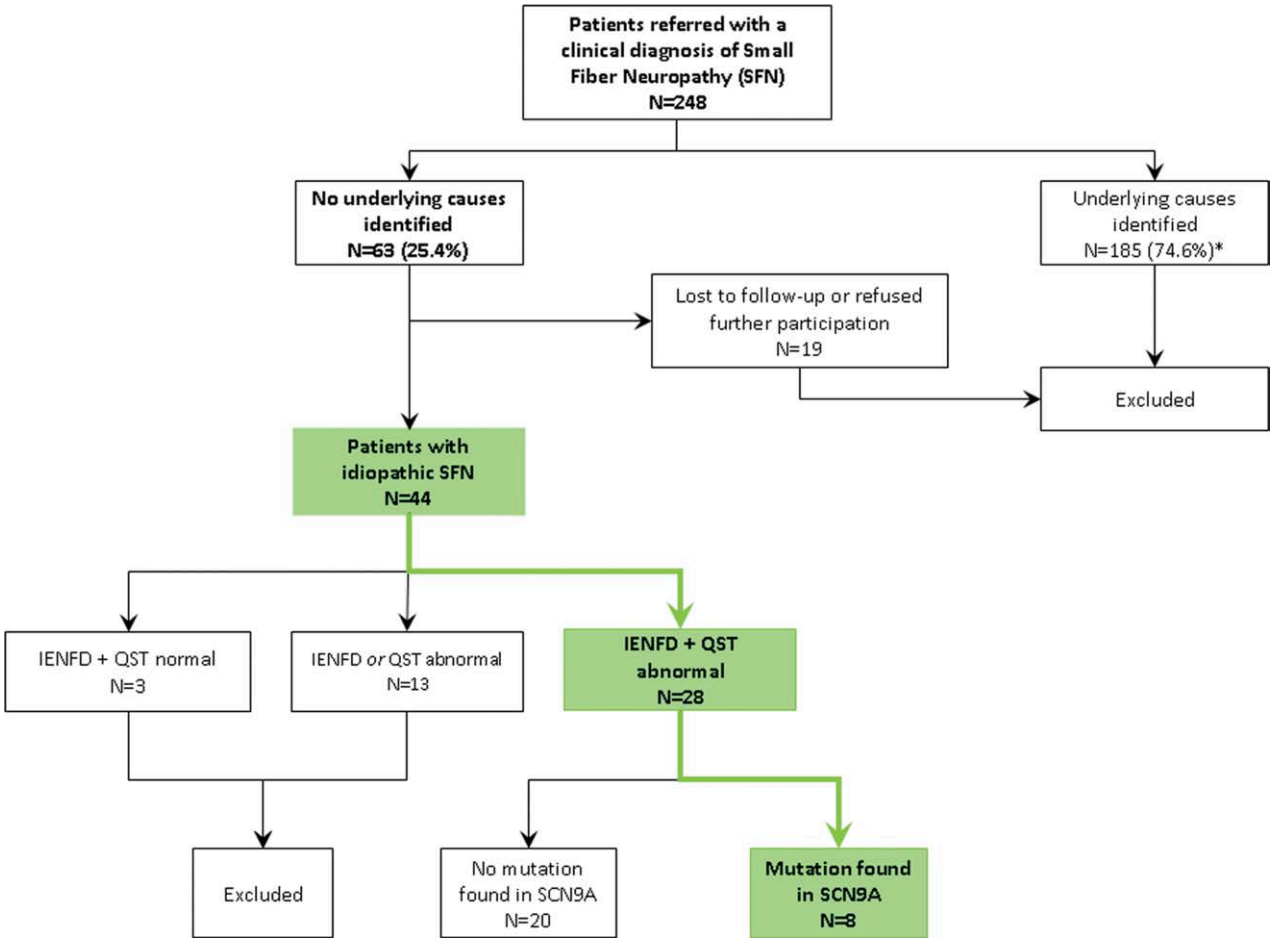

FIGURE 1: *Causes identified for SFN: sarcoidosis, n = 150; medication, n = 9; hemochromatosis, n = 5; diabetes mellitus, n = 4; thyroid dysfunction, n = 4; alcohol abuse, n = 4; gammopathy related, n = 3; hypercholesterolemia, n = 2; vitamin B6 intoxication, n = 1; Lyme disease, n = 1, Wegener granulomatosis, n = 1; antiphospholipid syndrome, n = 1. The Maastricht University Medical Hospital is a referral center for sarcoidosis in the Netherlands. IENFD = intraepidermal nerve fiber density; QST = quantitative sensory testing.

skin sensitivity of legs, burning feet, sheet intolerance, and restless legs; each having 4 response options: 0 = never, 1 = sometimes, 2 = often, 3 = always) derived from the SIQ[22] and a composite autonomic symptoms scale.[27]

NEUROPATHIC PAIN SCALE/VISUAL ANALOGUE PAIN SCALE. The Neuropathic Pain Scale (NPS) was used to assess neuropathic pain, each of 10 qualities being scored from 0 (no pain) to 10 (most intense pain imaginable).[28] The Visual Analogue Pain Scale (VAS) ranges from 0 (no pain) to 100 (most severe pain).[29]

SCN9A Mutation Analysis

EXON SCREENING. Genomic DNA was extracted from 300μL whole blood using the Puregene genomic DNA isolation kit (Gentra-Systems, Minneapolis, MN). 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.[30] Genomic sequences were compared with reference $Na_V1.7$ cDNA (NM_002977.3) to identify sequence variations,[31] using Alamut Mutation-Interpretation Software (Interactive-Biosoftware, Rouen, France). A control panel of DNA from 100 healthy Dutch (Caucasian) individuals (200 chromosomes) was screened for all new mutations.

Functional Analysis

Previous studies have demonstrated the importance of profiling the effects of $Na_V1.7$ mutations on channel function (voltage clamp), and on DRG neuron firing properties (current clamp).[15,32] This multimodal analysis of 7 mutations was carried out by 5 electrophysiologists using previously published voltage clamp and current clamp methods in HEK293 cells[16] and DRG neurons,[33,34] transfected with $Na_V1.7$ wild-type (WT) or mutant channels as described previously.[34] To minimize inherent culture to culture variation and to overcome the possibility of error introduced by pooling the results of experiments on DRG neurons harvested from multiple animals and cultured over many months, each mutant was compared with contemporaneous controls (WT $Na_V1.7$ expressed in cultures of cells prepared, transfected, and recorded under identical

TABLE 1: Clinical Description of Patients with Small Nerve Fiber Neuropathy and *SCN9A* Mutations

Patient	Age at Referral, yr/Gender	Age at Onset Symptoms, yr	Initial Symptom(s) + Location	Later Symptoms	Aggravated by Warmth/ Relieved by Cold	Family History	Medication	IENFD (corresponding normative value)	QST Impaired Modality		*SCN9A* Mutation
									Thenar	Foot	
1	54/M	24	Pain and paresthesias, feet and hand	52 years: burning feet, "electrical current" in soles, and redness feet; ↑ with exercise and interfered with walking	No/no	Brother* similar complaints; grandfather (deceased) painless burns and difficulty walking	No effect, pregabalin and amytriptyline	1.0/mm (≥3.2/mm)	Warmth-R, cold-R, warmth-L, cold-L	Warmth-R, cold-R, heat pain-R, warmth-L, cold-L, heat pain-L	c.554G>A (R185H)
2	24/F	23	Tingling feet, lower legs, and hands	2 months later: continuous severe pain in feet; occasionally: dry mouth and orthostatic dizziness	No/no	Father similar complaints	No relief from acetaminophen, anticonvulsants, antidepressants, mexiletine, opioids	4.9/mm (≥6.7/mm)	Warmth-L, cold-L	Warmth-R, cold-R, warmth-L, cold-L	c.554G>A (R185H)
3	63/F	22	Painful muscles persisting to present	58 years y.o.: severe burning pain, initially soles, later feet/hands; 61 y.o.: patchy skin redness, dry eyes, dry mouth, orthostatic dizziness; 62 y.o.: tenderness and burning of scalp, burning pain of lips, mouth, and trunk	Yes/no	Sister (78 y.o.) similar complaints	Some relief from pregabalin and duloxetine	2.8/mm (≥3.3/mm)	—	Cold-R, cold-L	c.1867G>A (D623N)
4	51/F	14	Burning pain, hot flashes, and itching of face, lower legs, and feet	Complaints ↑ with exercise, ↓ by cooling; dry mouth, dry eyes, blurred vision, orthostatic dizziness, alternating constipation/diarrhea, hyperhidrosis, palpitations, episodic swallowing difficulties; redness of hands; 49 y.o.: joint and muscle pain	Yes/yes	Father (deceased), sister* and 2 sons, similar complaints	Slight relief from amytriptyline	3.4/mm (≥4.1/mm)	Warmth-L	Warmth-L, cold-L	c.2215A>G (I739V)
5	39/M	37	Stabbing pain in the whole body	2 months later: burning pain in feet/lower legs followed by lower arms; numbness in feet bilaterally; hyperhidrosis in feet,	No/no	Unremarkable	No effect from pregabalin	4.5/mm (≥4.7/mm)	Warmth-L	Warmth-R, warmth-L	c.2 159T>A (I720K)

TABLE 1 (Continued)

Patient	Age at Referral, yr/Gender	Age at Onset Symptoms, yr	Initial Symptom(s) + Location	Later Symptoms	Aggravated by Warmth/ Relieved by Cold	Family History	Medication	IENFD (corresponding normative value)	QST Impaired Modality		*SCN9A* Mutation
									Thenar	Foot	
				dry mouth, episodic diarrhea, blurred vision							
6	70/F	68	Stabbing pain and redness in the feet, slowly extending to lower legs, hands, and lower arms	Symptoms restrict daily activities; dry eyes and orthostatic dizziness	No/no	Unremarkable	No effect from pregabalin	2.3/mm (≥2.7/mm)	Warmth-R, warmth-L	Cold-R, cold-L	c.4596G>A (M1532I)
7	22/M	16	Burning pain of feet and lower legs	Complaints ↑ with exercise and interfered with standing; orthostatic dizziness, dry mouth, dry eyes, constipation; sought psychiatric treatment for these symptoms	Yes/no	Unremarkable	No relief with gabapentin	4.0/mm (≥5.4/mm)	—	Warmth-L	c.2794A>C M932L and c.2971G>T (V991L)
8	51/M	32	Excruciating pain in teeth and jaw triggered by cold + heat, sometimes radiating to temporomandibular joint; also, pain behind the eyes	Multiple tooth extractions did not provide pain relief; myalgia triggered by exercise, persisting 5–6 days; pain ↑ by cold and ↓ by warmth (better in summer); occasional swollen feet; for 35 years: stomach cramps and diarrhea; for several years: dry mouth, dry eyes, reduced urinary sensation, and intermittent hesitation	No/no	Sister with rheumatoid arthritis had burning hands	Pain bearable with acetaminophen; no relief antidepressants, NSAIDs	1.6/mm (≥3.2/mm)	—	Warmth-R, cold-R	c.684C>G (I228M)

The obtained QST scores were compared with the reported normative values by Yarnitsky and Sprecher.[25] A sensory modality was classified as abnormal if the results of both method of limits and method of levels were abnormal. See also Supplementary Table S-1 for corresponding findings in relation to normative data.
↓ = reduced; ↑ = increase; * = refused participation; F = female; IENFD = intraepidermal nerve fiber density; L = left; M = male; NSAID = nonsteroidal anti-inflammatory drug; QST = quantitative sensory testing; R = right; y.o. = years old.

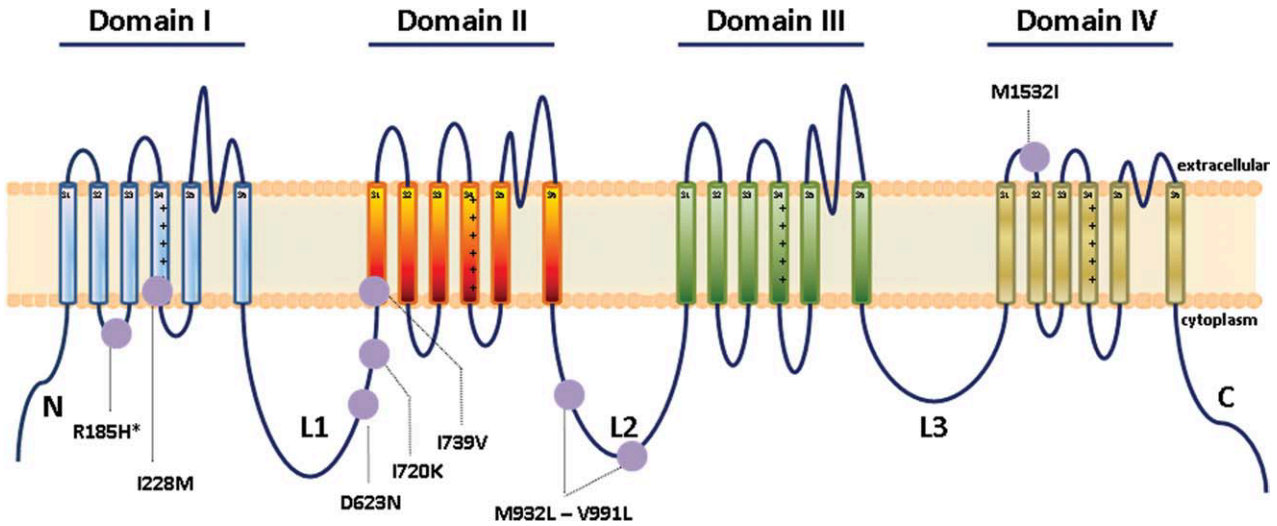

FIGURE 2: Schematic sodium channel showing the locations of the $Na_V1.7$ mutations found in patients with idiopathic small nerve fiber neuropathy. Mutation R185H was found in 2 patients.

conditions by the same electrophysiologist).[15] Previous studies have demonstrated that some $Na_V1.7$ mutations do not produce biophysical changes after expression within heterologous systems such as HEK293 cells, but do produce functional changes after expression in DRG neurons, where voltage clamp is more difficult to achieve due to neurite outgrowth, but the channels are expressed in a native cell background.[33,34] Voltage clamp analysis was therefore carried out after transfection into HEK293 cells together with β-1 and β-2 subunits[16] or, if biophysical changes were not detected in this cell background, within adult small ($<30\mu$m diameter) DRG neurons.[33,34] If changes were not found in activation, fast inactivation, slow inactivation, or ramp current, the proportion of cells producing resurgent current, which has been found to be enhanced by some $Na_V1.7$ mutations[35] and the amplitude of resurgent current, was assessed as previously described.[35] Current clamp analysis was carried out after transfection into DRG neurons.[33,34]

Study Design

The study was approved by medical ethical committees at Yale University and Maastricht University Medical Center. All aspects of the study were explained and written informed consent obtained prior to study. After examination, patients completed the SFN-SIQ, NPS, and VAS in random order. The study was performed between December 2008 and March 2011. Normative values of IENFD were obtained in an earlier study.[22]

Data Analysis

Clinical characteristics are descriptively presented. Electrophysiological data were analyzed using PulseFit 8.74 (HEKA Electronics, Lambrecht, Germany) or ClampFit (Molecular Devices, Sunnyvale, CA) and Origin 8.1 (Microcal, Northampton, MA), and presented as means ± standard error. Statistical significance was determined by unpaired Student *t* tests (voltage clamp except resurgent currents; current clamp except firing frequency and spontaneous activity), Mann-Whitney test (firing frequency), or 2-proportion *z* test (comparison of proportion of cells producing resurgent currents or spontaneous activity).

Results

Patient Selection and SCN9A Analysis

Of 248 Dutch patients referred with a suspected clinical diagnosis of SFN and screened, underlying causes were identified in 185 patients. Nineteen patients were lost to follow-up or refused participation. Forty-four patients met inclusion/exclusion criteria and underwent skin biopsy and QST. From this group, 28 Dutch Caucasian patients met strict criteria for I-SFN (ie, reduced IENFD compared with age- and gender-adjusted normative values,[22] plus abnormal QST and no apparent cause) and underwent *SCN9A* gene analysis (see Fig 1 and Supplementary Fig).

Eight (28.6%) of these 28 patients with biopsy-confirmed I-SFN had mutations in *SCN9A* (Table 1, Supplementary Table S-1, and Fig 2). In each case, the mutation was missense (c.554G>A, p.R185H in 2 unrelated patients; c.1867G>A, p.D623N; c.2215A>G, p.I739V; c.2159 T>A, p.I720K; c.4596G>A, p.M1532I; c.2794A>C, p.M932L + c.2971G>T, p.V991L; c.684C>G, p.I228M), and the patient was heterozygous for the mutation. None of these mutations was found in a control panel (DNA from 100 healthy Caucasian Dutch individuals; 200 chromosomes).

Clinical characteristics of the 8 patients with *SCN9A* mutations (Table 2) were similar to those of the 20 patients without *SCN9A* mutations (Supplementary Table S-2). Here, we describe these 8 patients with I-SFN and *SCN9*A mutations.

General Characteristics

Mean age of these 8 patients with *SCN9A* mutations was 32.4 (standard deviation [SD], 20.7; median, 23.5;

TABLE 2: SFN Symptoms Inventory Questionnaire Findings in Patients with *SCN9A* Novel Mutations

Patient	Mutation	Sweating	Diarrhea	Constipation	Micturation Problems	Dry Eyes	Dry Mouth	Orthostatic Dizziness	Palpitations	Hot Flashes	Skin Hyperesthesia	Burning Feet	Sheet Intolerance	Restless Legs
1	R185H	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	1[b]	2[b]	3[b]	3[b]
2	R185H	0[a]	0[a]	0[a]	0[a]	0[a]	1[b]	1[b]	0[a]	0[a]	3[b]	3[b]	2[b]	2[b]
3	D623N	0[a]	1[b]	1[b]	0[a]	1[b]	2[b]	2[b]	2[b]	0[a]	2[b]	2[b]	2[b]	2[b]
4	I739V	3[b]	2[b]	1[b]	2[b]	2[b]	3[b]	1[b]	1[b]	3[b]	2[b]	2[b]	2[b]	2[b]
5	I720K	3[b]	1[b]	0[a]	1[b]	1[b]	2[b]	0[a]	0[a]	1[b]	2[b]	1[b]	1[b]	1[b]
6	M1532I	0[a]	0[a]	0[a]	0[a]	1[b]	0[a]	1[b]	1[b]	0[a]	3[b]	3[b]	1[b]	3[b]
7	M932L + 1[b] V991L	0[a]	2[b]	1[b]	1[b]	1[b]	1[b]	0[a]	1[b]	1[b]	1[b]	2[b]	0[a]	0[a]
8	I228M	1[b]	3[b]	1[b]	2[b]	2[b]	3[b]	1[b]	1[b]	2[b]	2[b]	2[b]	1[b]	1[b]

[a]Absence (score 0) of corresponding SFN-related symptom.
[b]Presence of SFN-related symptom, with variable intensity (score 1 = sometimes present; score 2 = often; score 3 = always present).SFN = small nerve fiber neuropathy.

range, 14–68 years; 4 females/4 males). Mean duration of symptoms was 14.5 (SD, 16; range, 1–37) years. Three patients reported similar complaints in family members, but detailed information was not available; family history was unremarkable in 5 patients (see Table 1). Mean age of the 20 patients without *SCN9A* mutations was 42.7 (SD, 15.4; median, 44; range, 7–67 years; 11 females/9 males).

Clinical Features

PAIN. All 8 patients complained of pain. Six (patients 1–5, 8) had VAS scores >50, and 5 (patients 2–6) had scores of >5 on at least 7 of the 10 NPS questions, indicating severe pain. Intensity and quality of pain tended to vary from patient to patient. Pain intensity and quality for the 2 patients (patients 1, 2) carrying the c.554G>A, p.R185H mutation were different from each other. Patient 1 reported less pain compared to patient 2.

Pain began in the distal extremities (feet > hands) in most patients. However, patients 3 (c.1867G>A, p.D623N) and 5 (c.2159T>A, p.I720K) initially experienced pain throughout the body with muscle ache, before developing distal pain. Pain was aggravated by warmth in 3 of the 8 patients, but not in the other 5. Cooling relieved pain in 1 patient, but not in the other 7. Patient 8 (c.684C>G, p.I228M) initially experienced excruciating pain in the teeth/jaw triggered by cold and heat, and pain behind both eyes, not relieved by multiple tooth extractions. He subsequently developed myalgia, aggravated by cold and relieved by warmth, which could persist for 5 to 6 days after light physical activity, and intermittent foot swelling, and was unable to work.

AUTONOMIC DYSFUNCTION. Seven of the 8 patients reported autonomic complaints. In 5 patients, 6 or more of the 9 SFN-SIQ autonomic complaints were present. Orthostatic dizziness, palpitations, dry eyes, and dry mouth were more common (see Table 2). Autonomic complaints were most prominent in patients 4 (c.2215A>G, p.I739V) and 8 (c.684C>G, p.I228M) (see Table 1). Patient 4 experienced dry mouth/eyes, blurred vision, orthostatic dizziness, alternating constipation/diarrhea, hyperhidrosis, palpitations, hot flashes, and swallowing difficulties, followed by widespread joint/muscle pain. Patient 8 had a 35-year history of stomach cramps/diarrhea, and dry mouth/eyes and reduced urinary sensation/hesitation for several years.

Autonomic symptoms were absent in 1, and much less prominent in the second, of the 2 patients with R185H mutation (see Table 2).

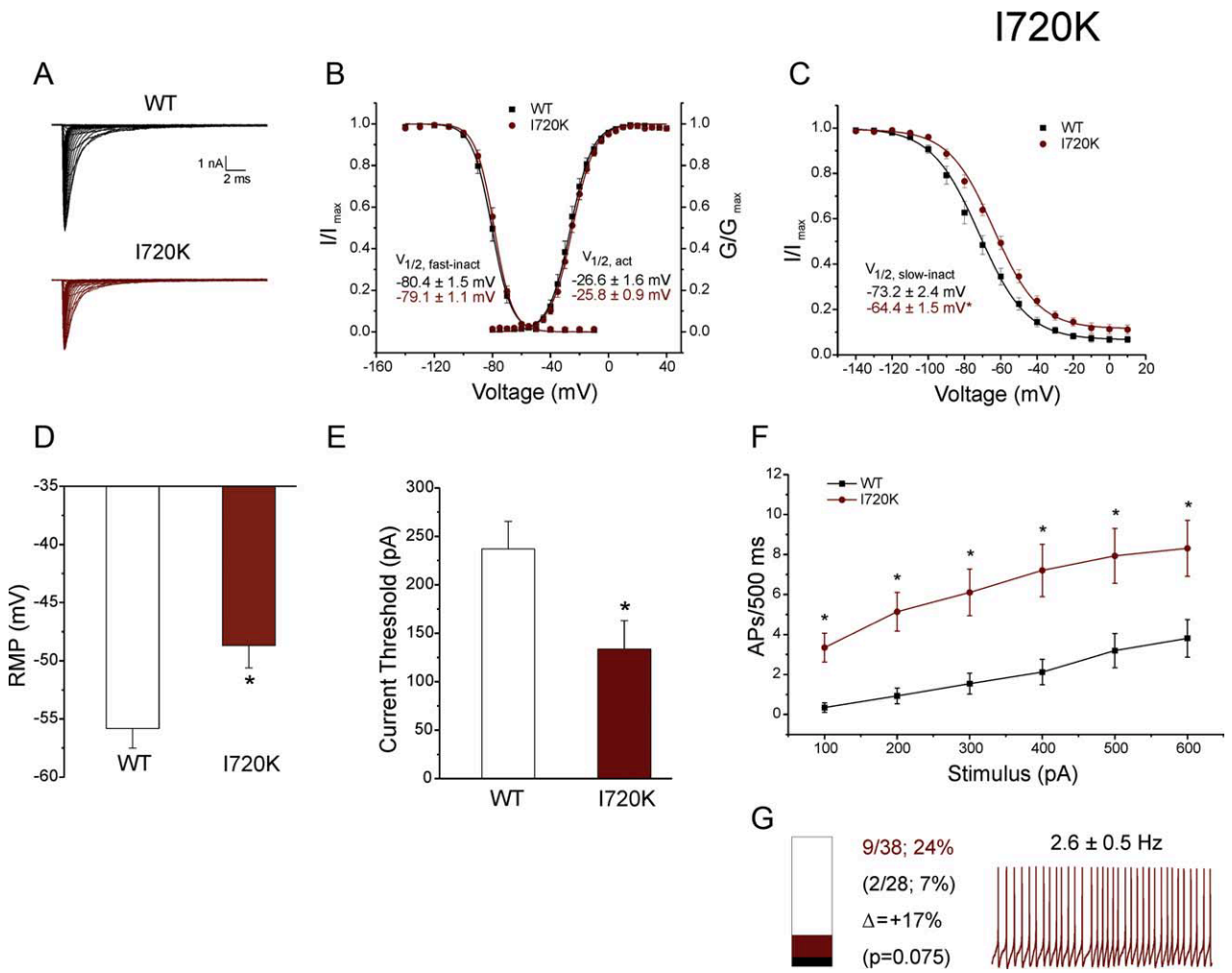

FIGURE 3: Electrophysiological analysis of I720K mutation. (A) Representative current traces recorded from HEK 293 cells expressing wild type (WT) (top) or I720K (bottom), evoked by voltage steps (100 milliseconds) from −80 to 40mV in 5mV increments, from a holding potential of −120mV. (B) Activation and steady state fast inactivation for WT *(black squares)* and I720K *(red circles)*. Fast inactivation was examined using a series of 500-millisecond prepulses from −140 to −10mV followed by test pulses to −10mV. Left inset: midpoint values for fast inactivation ($V_{1/2,\ fast\text{-}inact}$) of WT (black) and I720K (red). Right inset: midpoint values for activation ($V_{1/2,\ act}$) of WT (black) and I720K (red). (C) Steady state slow inactivation of WT *(black squares)* and I720K *(red circles)*. Slow inactivation was assessed using a 20-millisecond pulse to −10mV after a 30-second prepulse to potentials from −140 to 10mV followed by a 100-millisecond pulse to −120mV to remove fast inactivation. Inset: midpoint values of slow inactivation ($V_{1/2,\ slow\text{-}inact}$) (WT: black; I720K: red); *$p < 0.05$. (D) Resting membrane potential (RMP) of dorsal root ganglion (DRG) neurons expressing WT (−55.8 ± 1.7, n = 26) or I720K (−48.7 ± 1.9, n = 29); *$p < 0.05$. (E) Current threshold of DRG neurons expressing WT (237 ± 28, n = 26) or I720K (134 ± 30, n = 29) to 500-millisecond stimuli; $p < 0.05$. (F) Comparison of mean firing frequency in DRG neurons expressing WT and I720K across a range of current injections from 100 to 600pA; *$p < 0.05$. (G) Bar graph showing the proportion of spontaneous firing cells for DRG neurons expressing I720K (red) and WT channels (black); numbers to the right of the bar graph show mean values for WT (lower value in parentheses) and I720K (upper value). The recording on the right shows spontaneous firing (10 seconds) of representative DRG neuron expressing I720K; the numbers above the trace show average ± standard deviation frequency of spontaneous action potentials. $V_{1/2}$ represents voltage midpoint, I/I represents normalized current, and G/G represents normalized conductance for fast-activation, slow-inactivation, and activation. APs = action potentials.

IENFD and QST Findings

There was a decrease in IENFD below the 5th percentile for age- and sex-matched controls[22] in all 8 patients with *SCN9A* mutations (see Table 1). Supplementary Figure 1 shows the IENFD findings in patient 8 juxtaposed to an age- and gender-matched control subject.

On QST, 5 patients displayed abnormal warm and cold sensation, 1 patient displayed abnormal warm sensation, and 2 displayed abnormal cold sensation. One patient displayed reduced heat pain (see Table 1; Supplementary Table S-1). More abnormalities were seen in the foot (21 of 48 sensory qualities tested, 43.8%) compared with the hand (10 of 48, 20.8%).

Functional Characterization of $Na_V1.7$ Mutations

Voltage clamp analysis of the mutant channels from patients with I-SFN showed that they were all gain of

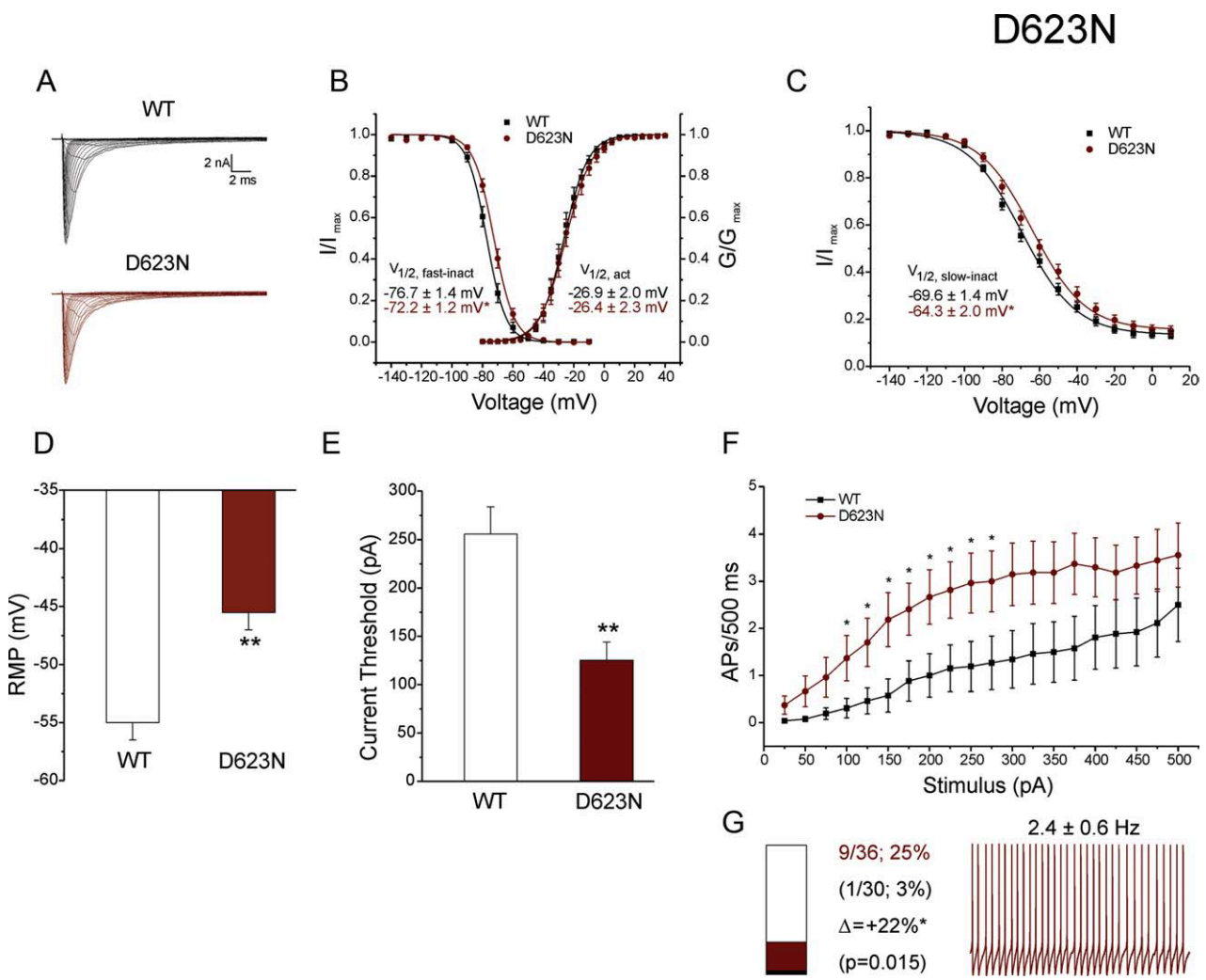

FIGURE 4: Electrophysiological analysis of D623N mutation. (A) Representative current traces recorded from dorsal root ganglion (DRG) neurons expressing wild type (WT) (top) or D623N (bottom), evoked by voltage steps (100 milliseconds) from −80 to 40mV in 5mV increments, from a holding potential of −100mV. (B) Activation and steady state fast inactivation for WT *(black squares)* and D623N *(red circles)*. Fast inactivation was examined using a series of 500-millisecond prepulses from −140 to −10mV followed by test pulses to −10mV. Left inset: midpoint values for fast inactivation ($V_{1/2,\ fast\text{-}inact}$) of WT (black) and D623N (red). Right inset: midpoint values for activation ($V_{1/2,\ act}$) of WT (black) and D623N (red). (C) Steady state slow inactivation of WT *(black squares)* and D623N *(red circles)*. Slow inactivation was assessed using a 20-millisecond pulse to −10mV after a 30-second prepulse to potentials from −140 to 10mV followed by a 100-millisecond pulse to −120mV to remove fast inactivation. Inset: midpoint values of slow inactivation ($V_{1/2,\ slow\text{-}inact}$) (WT: black; D623N: red); *$p < 0.05$. (D) Resting membrane potential (RMP) of DRG neurons expressing WT (−55.0 ± 1.5, n = 29) or D623N (−45.5 ± 1.5, n = 27), **$p < 0.01$. (E) Current threshold of DRG neurons expressing WT (256 ± 28, n = 29) or D623N (125 ± 19, n = 27) to 200-millisecond stimuli; **$p < 0.01$. (F) Comparison of mean firing frequency in DRG neurons expressing WT and D623N across a range of current injections from 25 to 500pA; *$p < 0.05$. (G) Bar graph showing the proportion of spontaneous firing cells for DRG neurons expressing D623N (red) and WT channels (black); numbers to the right of the bar graph show mean values for WT (lower value in parentheses) and D623N (upper value); *$p < 0.05$. The recording on the right shows spontaneous firing (10 seconds) of representative DRG neuron expressing D623N; the numbers above the trace show the average ± standard deviation frequency of spontaneous action potentials. $V_{1/2}$ represents voltage midpoint, I/I represents normalized current, and G/G represents normalized conductance for fast-activation, slow-inactivation, and activation. APs = action potentials.

function, and that they impaired slow inactivation (p.I720K, p.M1532I, p.I228M, p.I739V), depolarized slow and fast inactivation (p.D623N), or enhanced resurgent currents (p.M932L/V991L, p.R185H). None of these mutations exhibited the hyperpolarized activation or enhanced ramp currents characteristic of IEM[15] or the incomplete fast inactivation characteristic of PEPD[17] mutations of $Na_V1.7$. Current clamp analysis demonstrated that all 7 mutations rendered DRG neurons hyperexcitable. Here we present the functional profiling of 3 representative mutant channels from patients with I-SFN. Functional profiling of the other 4 mutations yielded similar results (unpublished results).

I720K: Impaired Slow Inactivation and DRG Neuron Hyperexcitability

Voltage clamp analysis of I720K mutant channels following expression in HEK293 cells demonstrated impaired slow inactivation (Fig 3C). Current densities (WT: 375

± 68pA/pF, n = 18; I720K: 228 ± 35pA/pF, n = 22), activation $V_{1/2}$ ($V_{1/2}$ represents voltage midpoint) (WT: −26.6 ± 1.6mV, n = 12; I720K: −25.8 ± 0.9mV, n = 13), fast inactivation $V_{1/2}$ (WT: −80.4 ± 1.5mV, n = 13; I720K: −79.1 ± 1.1mV, n = 13), and ramp currents (WT: 0.8 ± 0.2%, n = 9; I720K: 0.7 ± 0.1%, n = 10) for HEK293 cells transfected with WT or I720K were not significantly different. Slow inactivation was impaired for I720K mutant channels, with a depolarized $V_{1/2}$ (WT: −73.2 ± 2.4mV, n = 6; I720K: −64.4 ± 1.5mV, n = 7; $p < 0.05$; see Fig 3C). Impaired slow inactivation increases the number of channels available for activation at potentials positive to −100 mV, including potentials close to resting potential of DRG neurons.

The I720K mutation had clear functional effects on DRG neurons, which were rendered hyperexcitable by the mutant channels (see Fig 3). I720K produced a depolarizing shift in resting membrane potential (WT: −55.8 ± 1.7mV, n = 26; I720K: −48.7 ± 1.9mV, n = 29; $p < 0.05$). I720K increased excitability of DRG neurons, with a 43% reduction in current threshold to 500-millisecond stimuli (WT: 237 ± 28pA, n = 26; I720K: 134 ± 30pA, n = 29; $p < 0.05$). I720K significantly increased the number of action potentials evoked by 500-millisecond depolarizing stimuli at all intensities tested, from 100 to 600pA. I720K produced a trend toward an increase in the proportion of spontaneously firing cells (9 of 38 [24%] vs 2 of 28 [7%] for cells transfected with WT channels) that did not reach statistical significance ($p = 0.075$); mean frequency of spontaneous activity in cells transfected with I720K was 2.6 ± 0.5Hz (n = 9), with 4 of 9 spontaneously firing cells showing continuous firing at a frequency of >1Hz throughout the 30-second recording period.

D623N: Impaired Fast and Slow Inactivation and DRG Neuron Hyperexcitability

D623N mutant channels did not display gating abnormalities following expression in HEK293 cells but, when assessed by voltage clamp after expression in DRG neurons, demonstrated impaired fast inactivation and slow inactivation (Fig 4A–C). Current densities (WT: 407 ± 90pA, n = 12; D623N: 474 ± 121pA, n = 10), activation $V_{1/2}$ (WT: −26.9 ± 2.0mV, n = 12; D623N: −26.4 ± 2.3mV, n = 10), and ramp currents (WT: 2.6 ± 0.4%, n = 15; D623N: 2.1 ± 0.3%, n = 17) were not significantly different. The $V_{1/2}$ of fast inactivation (WT: −76.7 ± 1.4mV, n = 13; D623N: −72.2 ± 1.2mV, n = 13; $p < 0.05$) and slow inactivation (WT: −69.6 ± 1.4mV, n = 12; D623N: −64.3 ± 2.0mV, n = 11; $p < 0.05$) were depolarized for D623N mutant channels (see Fig 4B, C). Impaired fast inactivation and slow inactivation increase the number of channels available for activation.

Current clamp recording showed that D623N mutant channels rendered DRG neurons hyperexcitable and produced aberrant spontaneous firing in 25% of neurons (see Fig 4). D623N produced a depolarizing shift in resting membrane potential (WT: −55.0 ± 1.5mV, n = 29; D623N: −45.5 ± 1.5mV, n = 27; $p < 0.01$) and a 51% reduction in current threshold to 200-millisecond stimuli (WT: 256 ± 28pA, n = 29; D623N: 125 ± 19pA, n = 27; $p < 0.01$). D623N significantly increased the number of action potentials evoked by 500-millisecond depolarizing stimuli ranging from 100 to 275pA. D623N produced an increase in the proportion of spontaneously firing cells (9 of 36 [25%] for DRG neurons transfected with this mutant channel; 1 of 30 [3%] for cells transfected with WT channels, $p < 0.05$). Mean frequency of spontaneous activity in cells transfected with D623N was 2.4 ± 0.6Hz (n = 9); 4 of 9 spontaneously firing cells showed continuous firing at a frequency of >1Hz throughout the 30-second recording period.

M932L/V991L: Increased Resurgent Currents and DRG Neuron Hyperexcitability

M932L/V991L mutant channels, assessed by voltage clamp after expression in DRG neurons (Fig 5A–D), enhanced the generation of resurgent currents. Voltage clamp analysis of M932L/V991L mutant channels, both in HEK293 cells and DRG neurons, did not reveal a significant effect of the mutation on activation, fast inactivation, slow inactivation, ramp currents, or deactivation. Current densities (WT: 440 ± 49pA/pF, n = 32; M932L/V991L: 541 ± 86pA/pF, n = 23), activation $V_{1/2}$ (WT: −20.2 ± 0.6mV, n = 16; M932L/V991L: −20.4 ± 1.2mV, n = 11), fast inactivation $V_{1/2}$ (WT: −68.5 ± 0.6mV, n = 26; M932L/V991L: −68.5 ± 0.6mV, n = 17), slow inactivation $V_{1/2}$ (WT: −66.1 ± 1.0mV, n = 24; M932L/V991L: −63.8 ± 1.7mV, n = 16), ramp currents (WT: 1.61 ± 0.16%, n = 19; M932L/V991L: 1.85 ± 0.27%, n = 14), and deactivation (no significant differences in deactivation measured between −100 and −50mV at 5mV intervals) for DRG neurons transfected with WT and M932L/V991L were not significantly different. However, a higher percentage of DRG neurons expressing M932L/V991L (5 of 10 cells, 50%; $p < 0.05$) compared to cells expressing WT channels (1 of 11 cells, 9%; see Fig 5D) produced resurgent currents, a change that would be expected to produce repetitive firing.

Current clamp recording showed that M932L/V991L mutant channels made DRG neurons hyperexcitable. Mean resting potential was significantly depolarized

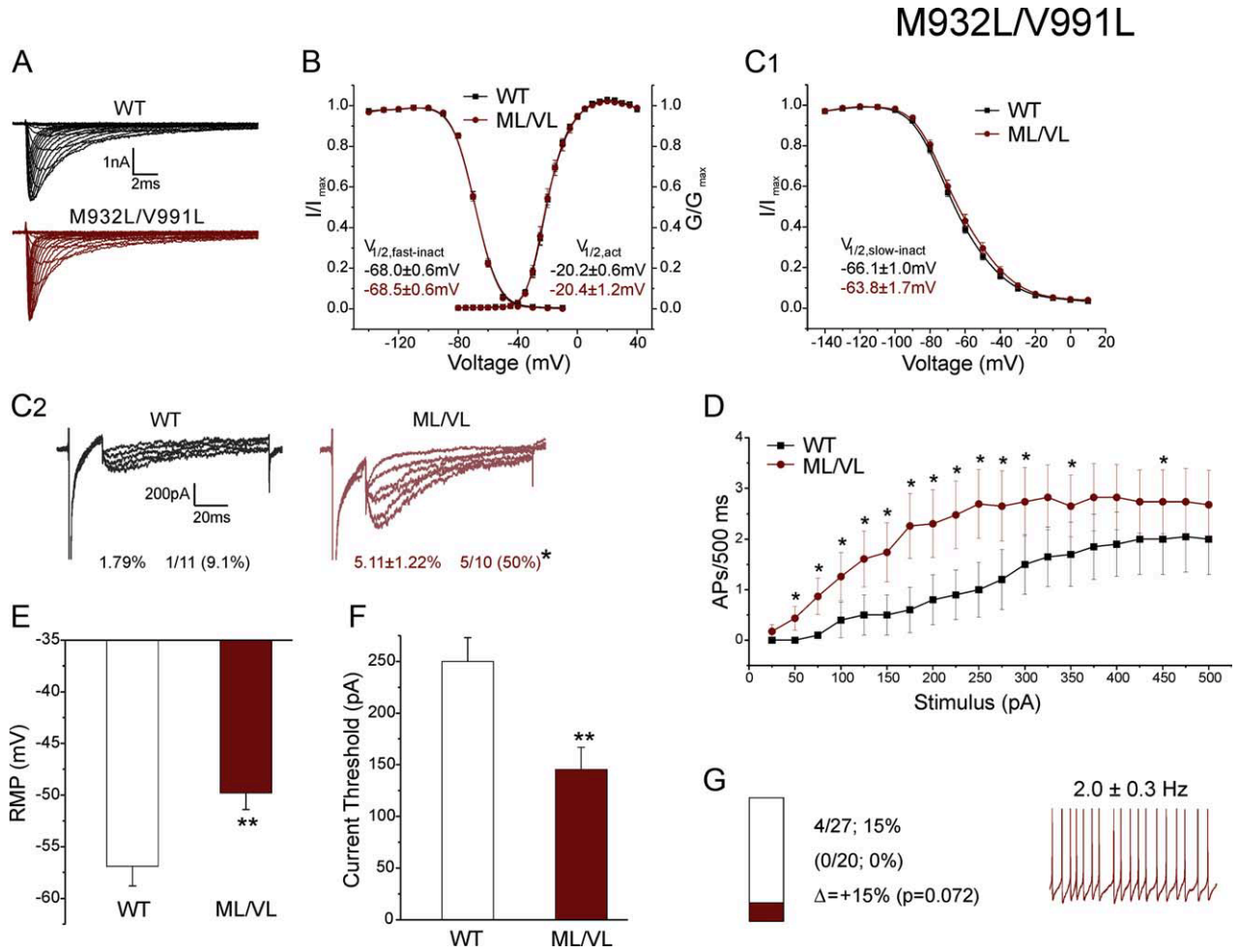

FIGURE 5: Electrophysiological analysis of M932L/V991L mutation. (A) Representative current traces recorded from dorsal root ganglion (DRG) neurons expressing wild type (WT) (top) or M932L/V991L (bottom) (unless otherwise noted, protocols are the same as in Figs 3 and 4). (B) Activation and steady state fast inactivation for WT *(black squares)* and M932L/V991L (ML/VL; *red circles*). Inset shows midpoint values for fast inactivation ($V_{1/2,\ fast\text{-}inact}$) and activation ($V_{1/2,\ act}$) of WT (black) and M932L/V991L (red), respectively. (C) Steady state slow inactivation of WT *(black squares)* and M932L/V991L *(red circles)*. Inset: midpoint values of slow-inactivation ($V_{1/2,\ slow\text{-}inact}$) (WT: black; M932L/V991L: red). (D) Resurgent currents recorded from DRG neurons expressing WT (left) or M932L/V991L (right). Resurgent currents were assessed with a 2-step protocol that initially depolarized the membrane to +30mV for 20 milliseconds before testing for resurgent sodium currents by hyperpolarizing the membrane potential in −5mV increments from 0 to −80mV for 100 milliseconds, then returning to the holding potential of −100mV. Current amplitude (normalized to peak current evoked by a +30mV depolarization) (left) and proportion of cells producing resurgent current (right) are shown below traces; *$p < 0.05$. (E) Resting membrane potential (RMP) of DRG neurons expressing WT (−56.9 ± 1.9mV, n = 20) or M932L/V991L (−49.8 ± 1.6mV, n = 23); **$p < 0.01$. (F) Current threshold of DRG neurons expressing WT (250 ± 23pA, n = 20) or M932L/V991L (145 ± 22pA, n = 23) to 200-millisecond stimuli; **$p < 0.01$. (G) Comparison of mean firing frequencies of DRG neurons expressing WT and M932L/V991L across the range of current injections from 25 to 500pA; *$p < 0.05$. (H) Bar graph showing the proportion of spontaneous firing cells for DRG neurons expressing M932L/V991L (red); numbers to the right of the bar graph show mean values for WT (lower value in parentheses) and M932L/V991L (upper value); $p = 0.072$. The recording on the right shows spontaneous firing (10 seconds) of representative DRG neuron expressing M932L/V991L; the numbers above the trace show average ± standard deviation frequency of spontaneous action potentials. $V_{1/2}$ represents voltage midpoint, I/I represents normalized current, and G/G represents normalized conductance for fast-activation, slow-inactivation, and activation. APs = action potentials.

(WT: −56.9 ± 1.9mV, n = 20; M932L/V991L: −49.8 ± 1.6mV, n = 23, $p < 0.01$, and threshold was significantly decreased (WT: 250 ± 23pA, n = 20; M932L/V991L: 145 ± 22pA, n = 23, $p < 0.01$ in DRG neurons expressing M932L/V991L (see Fig 5). The number of action potentials evoked by 500-millisecond depolarizing stimuli was increased at all stimulus strengths between 50 and 300pA for cells expressing M932L/V991L channels, compared to cells expressing WT. M932L/V991L produced a trend toward an increase in the proportion of spontaneously firing cells (4 of 27 [15%] vs 0 of 20 [0%] for cells transfected with WT channels) that did not reach statistical significance ($p = 0.072$); mean frequency of spontaneous activity in cells

transfected with M932L/V991L was 2.0 ± 0.3Hz, with all 4 spontaneously firing cells showing continuous firing at a mean frequency of >1Hz throughout the 30-second recording period.

Discussion

Despite careful clinical assessment, an underlying cause cannot be found in a substantial number (24 to >90% in different series) of patients with SFN.[5,6,9] In this study we show, in 8 of 28 (28.6%) patients with skin biopsy- and QST-confirmed I-SFN, missense mutations in the *SCN9A* gene, which encodes a voltage-gated sodium channel, $Na_V1.7$, that is present within small peripheral nerve fibers. Electrophysiological analysis demonstrated gain of function changes in the mutant channels and showed that the mutations share the common feature of rendering DRG neurons hyperexcitable.

Our findings of gain of function mutations of Nav1.7 in 28.6% of patients with I-SFN are based on an analysis of 28 Dutch Caucasian patients who met criteria that included no history or detection on screening of disorders known to cause SFN, and confirmation of the diagnosis of SFN by abnormal QST, and by reduced IENFD on skin biopsy. These patients were derived from a larger group of 248 patients referred with a clinical diagnosis of SFN for evaluation at an academic medical center. Aside from any selection bias inherent in referral to an academic medical center, and from any bias introduced by our inclusion/exclusion criteria, which yielded a study cohort of 28 patients meeting stringent criteria for I-SFN, we believe that our sample may be representative of the general Dutch Caucasian population of patients with SFN. Although there were no other distinguishing clinical characteristics, age of onset of symptoms was younger (although not statistically significant) for patients with *SCN9A* mutations than for patients without *SCN9A* mutations.

Mutations in the *SCN9A* gene have been previously linked to IEM, a rare inherited disorder characterized by distal burning pain,[15] and PEPD, characterized by perineal, periocular, and perimandibular pain.[17] Some of our patients with SFN also reported burning feet and hands, or pain around the eyes and jaw. However, despite this apparent similarity, our patients exhibited clinical characteristics typical for small fiber neuropathy[1–6] and differed from patients with prototypical EIM and PEPD in multiple ways: (1) Autonomic dysfunction is common in SFN, and severe autonomic symptoms were seen in almost all of our patients. Except for skin reddening, autonomic symptoms are not prominent in IEM.[15,36] (2) Location and onset of pain and related complaints were distributed throughout the body in our patients with I-SFN, whereas in IEM pain is mainly located in the distal extremities. In patient 3 (D623N), painful muscles from early childhood preceded distal complaints, whereas patient 5 (I1720K) experienced initial pain throughout the entire body, and patient 8 (I228M) initially experienced severe jaw pain. (3) Whereas IEM is characterized by erythema of the involved areas,[15] half of our patients did not display this sign. (4) Our patients did not display the aggravation of symptoms by warmth and relief by cold that are characteristic of IEM.[15,37] Five of our 8 patients denied aggravation by warmth, and 7 had no relief by cold. Patient 8 reported that cold increased symptoms and warmth relieved them. (5) The $Na_V1.7$ mutations that we profiled did not display the hyperpolarized activation and enhanced ramp responses characteristic of IEM mutations[15] or the incomplete fast inactivation[17] characteristic of PEPD mutations. The present results demonstrate that $Na_V1.7$ mutations, distinct from those that have been associated with IEM[15] and PEPD,[17] occur in a substantial proportion of patients with I-SFN.

$Na_V1.7$ is preferentially expressed within DRG and sympathetic ganglion neurons[11,12] and their axons, including small diameter ($<0.5\mu$m) intracutaneous axon terminals, where it is coexpressed with other sodium channel subtypes ($Na_V1.6$/$Na_V1.8$/$Na_V1.9$) and the sodium–calcium exchanger NCX.[13] $Na_V1.7$ channels modulate the excitability of these neurons by opening and producing a Na^+ current in response to small depolarizations close to resting potential, thus bringing the neuron closer to the activation potential of other sodium channel isoforms.[14] The $Na_V1.7$ mutations that we found in patients with SFN impaired slow inactivation, depolarized fast and slow inactivation, or enhanced resurgent currents. Each of the mutations rendered DRG neurons hyperexcitable.

Sodium channel activity has been shown to trigger axonal degeneration via calcium-importing reverse sodium–calcium exchange in axons under conditions where the ability to extrude sodium is exceeded.[38,39] Degeneration of nonmyelinated axons has been described in hypoxic neuropathy,[40] and the distal pains reported by some of our patients are similar to the acral paresthesias that have been linked to low Na/K adenosine triphosphatase levels in peripheral nerves in chronic mountain sickness.[41] Although there is no reason to believe that the patients we have described suffered from systemic hypoxia, Na^+ influx is known to impose an energetic load on neurons and neuronal processes,[42] and increased activity of mutant $Na_V1.7$ channels would be expected to have an especially large effect on small diameter intracutaneous axons, where NCX is present,[13] due to their

high surface to volume ratio and input resistance, low capacitance per unit length, and shorter wavelength.[43,44] Consistent with a role of sodium channels in I-SFN, action potential activity at physiological frequencies can sensitize axons to otherwise reversible metabolic insults, and can produce degeneration of these axons[45] that is attenuated by sodium channel blockers.[46]

In conclusion, we demonstrate the occurrence of missense mutations in the *SCN9A* gene encoding the $Na_V1.7$ sodium channel, in a substantial proportion (28.6%) of patients with biopsy- and QST-confirmed I-SFN, and show that these mutations render DRG neurons that give rise to small axons hyperexcitable. Expression of $Na_V1.7$ and NCX in small diameter axons may cause these fibers to degenerate in response to gain of function changes produced by $Na_V1.7$ mutations such as those described in this paper. Our results suggest that these mutations may predispose to the development of channelopathy-associated SFN. *SCN9A* gene analysis might be considered for patients with SFN in whom other causes are excluded, particularly patients with younger ages of onset. In terms of treatment, existing nonspecific sodium channel blockers, $Na_V1.7$-selective blockers when available, and inhibitors of NCX2 merit study as therapeutic approaches that might slow or halt axonal degeneration in I-SFN.

Authorship

C.G.F. and J.G.J.H. are first authors. S.G.W. and I.S.J.M are senior authors.

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Potential Conflicts of Interest

C.G.F.: grants/grants pending, Prinses Beatrix Fonds. E.K.V.: grant, Baxter fellowship. S.D.-H.: consultancy, Regeneron Pharmaceuticals, Vertex Pharmaceuticals, Guidepoint Global; patent, sodium channel NaV1.9 (Yale holds); stock/stock options, Trans Molecular, Pfizer. S.G.W.: consultancy, Bristol Myers Squibb, Vertex Pharmaceuticals, ChromoCell, DaiNippon Sumitomo Pharm, Cardiome Pharm; grants/grants pending, Pfizer Research, Trans Molecular; patents, NaV1.9 sodium channel (Yale owns); stock/stock options, Trans Molecular. I.S.J.M.: travel support, Peripheral Nerve Society; board membership, steering committee member ICE trial that was published in 2008 plus steering committee member for CSL Behring CIDP study; grants/grants pending, GBS/CIDP International foundation grant for the PeriNomS study, Talents program grant for the PeriNomS study, Peripheral nerve society grant for the PeriNomS study.

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