

TRPV4 mutations and cytotoxic hypercalcemia in axonal Charcot-Marie-Tooth neuropathies



C.J. Klein, MD
Y. Shi, MD, PhD*
F. Fecto, MD*
M. Donaghy, MB, PhD
G. Nicholson, MD
M.E. McEntagart, MD
A.H. Crosby, MD
Y. Wu, PhD
H. Lou, MD
K.M. McEvoy, MD, PhD
T. Siddique, MD
H.-X. Deng, MD, PhD
P.J. Dyck, MD

Address correspondence and reprint requests to Dr. Christopher J. Klein, Department of Neurology and Division of Peripheral Nerve Diseases, Mayo Clinic Foundation, Rochester, MN 55905
klein.christopher@mayo.edu
or Dr. Han-Xiang Deng, Davee Department of Neurology and Clinical Neurosciences, Northwestern University Feinberg School of Medicine, Chicago, IL 60611
h-deng@northwestern.edu

Editorial, page 856

Supplemental data at
www.neurology.org

ABSTRACT

Objective: To improve understanding of TRPV4-associated axonal Charcot-Marie-Tooth (CMT) neuropathy phenotypes and their debated pathologic mechanism.

Methods: A total of 17 CMT2C phenotypic families with vocal cord and diaphragmatic involvement and 36 clinically undifferentiated CMT2 subjects underwent sequencing analysis of the coding region of *TRPV4*. Functional studies of mutant proteins were performed using transiently transfected cells for TRPV4 subcellular localization, basal and stimulated Ca²⁺ channel analysis, and cell viability assay with or without channel blockade.

Results: Two *TRPV4* mutations R232C and R316H from 17 CMT2C families were identified in the ankyrin repeat domains. The R316H is a novel de novo mutation found in a patient with CMT2C phenotype. The family with R232C mutation had individuals with and without vocal cord and diaphragm involvement. Both mutant TRPV4 proteins had normal subcellular localization in HEK293 and HeLa cells. Cells transfected with R232C and R316H displayed increased intracellular Ca²⁺ levels and reversible cell death by the TRPV channel antagonist, ruthenium red.

Conclusion: TRPV4 ankyrin domain alterations including a novel de novo mutation cause axonal CMT2. Individuals with the same mutation may have nondistinct CMT2 or have phenotypic CMT2C with vocal cord paresis. Reversible hypercalcemic gain-of-function of mutant TRPV4 instead of loss-of-function appears to be pathologically important. The reversibility of cell death by channel blockade provides an attractive area of investigation in consideration of treatable axonal degeneration. *Neurology*® 2011;76:887-894

GLOSSARY

CMT = Charcot-Marie-Tooth; **dSMA** = distal spinal muscular atrophy; **HMSN2C** = hereditary motor and sensory neuropathy 2C; **PI** = propidium iodide; **SPSMA** = scapulo-peroneal spinal muscular atrophy; **TRPV4** = transient receptor potential vanilloid 4.

Hereditary motor and sensory neuropathy 2C (HMSN2C) or Charcot-Marie-Tooth type 2C (CMT2C) is a dominantly inherited axonal neuropathy with diaphragmatic and vocal cord paresis.¹ The occurrence of voice and diaphragmatic involvements provided for subclassification beyond the broad classification of HMSN2 (CMT2). Our initial chromosomal localization to 12q24.1-q24.3² and confirmatory localization³ further validated its differentiation from other CMT subtypes. This localization also led us to speculate that CMT2C was allelic to scapulo-peroneal spinal muscular atrophy (SPSMA)⁴ and possibly other disorders which colocalized to this same chromosomal region including congenital distal spinal muscular atrophy (dSMA).² In SPSMA, scapular wasting was clinically distin-

e-Pub ahead of print on February 2, 2011, at www.neurology.org.

*These authors contributed equally to this work.

From the Department of Neurology and Division of Peripheral Nerve Diseases (C.J.K., K.M.M., P.J.D.) and Department of Laboratory Medicine and Pathology (Y.W.), Mayo Clinic Foundation, Rochester, MN; Davee Department of Neurology and Clinical Neurosciences (Y.S., F.F., H.L., T.S., H.-X.D.), Northwestern University Feinberg School of Medicine, Chicago, IL; Department of Clinical Neurology (M.D.), John Radcliffe Hospital, Oxford, England; Molecular Medicine Laboratory & ANZAC Research Institute (G.N.), Concord Hospital, Australia; and Department of Medical Genetics (M.E.M., A.H.C.), St George's University of London, London, England.

Study funding: Supported by the NIH (K08 NS065007 to C.J.K., NS050641, and T32 AG20506), the Les Turner ALS Foundation, Vena E. Schaff ALS Research Fund, Harold Post Research Professorship, Herbert and Florence C. Wenske Foundation, the David C. Asselin MD Memorial Fund, Help America Foundation, and Les Turner ALS Foundation/Herbert C. Wenske Foundation Professorship (T.S.). H.L. is supported by the Chinese Medical Association (08020530131).

Disclosure: Author disclosures are provided at the end of the article.

guishable from CMT2C but both had laryngeal palsy. In both disorders, childhood presentations had more severe disease with respiratory failure. Sensory nerve involvements were most apparent in CMT2C while only infrequent vibratory loss was observed in SPSMA. We recently identified 2 mutations in transient receptor potential vanilloid 4 (TRPV4) as causative in the originally described CMT2C (R269H) and SPSMA (R316C) kindreds.⁵ At the same time, 2 other TRPV4 mutations (R315W and R269C) were described by different groups.^{6,7} The R315W mutation was found in one family with diverse phenotypes, i.e., CMT2C, SPSMA, and dSMA. Collectively these mutations have also been found in other unrelated kindreds along with 2 additional mutations (R232C, V620I).⁸

TRPV4 is a transmembrane Ca²⁺-permeable, nonselective cation channel and responsive to mechanical force, osmotic concentration, and increased temperature.⁹ The potential for calcium neurotoxicity is emphasized by earlier reviews in neurodegeneration¹⁰ with suggested roles in AD,¹¹ motor neuron disease,¹² and PTEN-induced putative kinase (PINK1)-associated PD.¹³ Additionally, L-type calcium channel blockers have recently been shown as neuroprotective in development of idiopathic PD.¹⁴

Earlier functional analysis of TRPV4 has yielded conflicting results.⁵⁻⁷ Two studies^{5,6} showed mutant proteins have normal subcellular localization with neurotoxic gain of function from increased Ca²⁺ influx, whereas another study⁷ suggested TRPV4 mutants lost membrane localization and had decreased stimulus-dependent channel activity utilizing HeLa cells.⁷ Cell-line-specific differences may explain divergent results as we utilized HEK293 cells. All the studied mutations occurred in ankyrin repeat domains, raising the question of whether mutant proteins could lose the binding ability to PIP2, a cell membrane phospholipid, thus leading to the altered stimulus-dependent channel activity.¹⁵ Complex pathogenesis is further highlighted by the bone disorders associated with *TRPV4* mutations including brachyolmia,¹⁶ spondylometaphyseal dysplasia Kozlowski type metatropic

dysplasia,¹⁷ and now spondylo-epiphyseal dysplasia.¹⁸ The variable phenotypes such as short trunk, scoliosis, and small hands occur in these disorders but detailed neuromuscular testing has not been reported. Toxic gain of function of TRPV4 was suggested to be responsible for their pathogenesis, but a large number of mutations discovered in a wide spectrum of phenotypes including SPSMA, CMT2C, distal SMA, and HMN with vocal cord paralysis and clinically undifferentiated HMSN2 suggests that additional phenotype modifier or more complex pathogenic mechanism exists. Some individuals with CMT2C and SPSMA had bony abnormalities beyond what would be expected with a primary neurogenic process, possibly linking these primary bone disorders with neuronal degenerative pathogenesis.^{5,6}

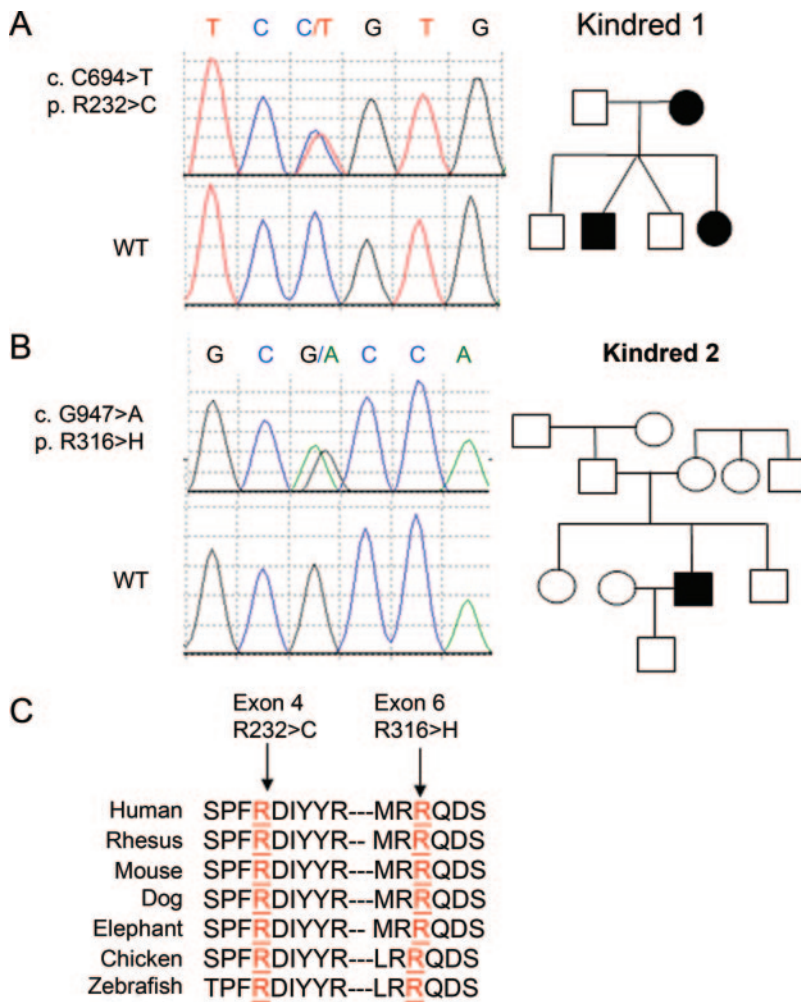
We report findings from exon sequencing of *TRPV4* in 17 families with CMT2C phenotypes and 36 nondistinct CMT2 individuals without proximal vocal cord or diaphragm involvement. Protein localization experiments and functional analysis were performed using both HEK293 and HeLa cells to determine whether discovered mutants have loss or gain of TRPV4 function.

METHODS Patients. We identified 17 families with CMT2C phenotypes and 36 cases of nondistinct CMT2 individuals and families where genetic cause was unknown. The families were not known to be related. All persons had clinical examinations with sensory testing and nerve conduction studies verifying the axonal nature of their motor and sensory involvements with exclusion of acquired etiologies. Among patients with CMT2C, a shortness of breath, a breathy voice, or asthma-like wheeze on exertion was typical. Phrenic nerve conduction studies, diaphragm needle EMG, pulmonary function tests, direct visualization of vocal cords, and speech pathology were employed in ascertainment. Family history or clinical involvements were confirmed by direct examination in kindred evaluation.

CMT2C kindred 1 (R232C) with varied vocal cord involvement. A 43-year-old man and his family, reported previously,¹⁹ presented with 4 years of diminished manual dexterity, difficulty walking, and a 2-year history of dysphonia. He had distal amyotrophy, pes cavus foot deformity, left lateral rectus palsy, and left vocal cord paralysis by visualization. Generalized areflexia and loss of sensory vibration sense were detected in the legs of all 3 affected. His mother and sister were asymptomatic, but distal muscle weakness was elicited and vocal cord and diaphragm involvement was absent. All 3 affected persons showed evidence of an axonal polyneuropathy with varied extent of sensory involvement on electrophysiology testing and no bony dysmorphic features (figure 1A).

CMT2C kindred 2 (R316H). A 30-year-old man presented with shortness of breath which limits him in mild exercise in-

Figure 1 Mutations of TRPV4 in Charcot-Marie-Tooth (CMT) 2C and CMT2 pedigrees



Wild-type (WT) sequences are shown in the lower panels of each case. All examined and affected carried the corresponding mutation not found in unaffected persons and our 800 controls. (A) Heterozygous mutation c.C694>T, resulting in amino acid change p.R232>C, was identified in exon 4 of the TRPV4 gene. The affected male had vocal cord involvement which was not found in his mother or sister. (B) Heterozygous mutation c.G947>A, resulting in amino acid change p.R316>H, was identified in exon 6 of the TRPV4 gene in this CMT2C de novo patient; the mutation was not found in his genetically confirmed parents or other family members. (C) Evolutionary conservation of amino acids in the mutated regions of TRPV4 among different species is shown. 232R and 316R are labeled in red and the surrounding TRPV4 amino acids sequences of human (*H sapiens*) and its orthologues in rhesus (*Macaca mulatta*), mouse (*M musculus*), dog (*Canis lupus familiaris*), elephant (*Elephantidae*), chicken (*Gallus gallus*), and zebrafish (*Danio rerio*) are aligned. The mutated amino acids are indicated by arrows on the top.

cluding walking. There was also a weakness of voice that precluded yelling dating back to his childhood. He suffered chronically with fatigue in long speeches with reduced volume. There was distal foot and hand atrophy. Sensory symptoms of paresthesias, dysesthesias, or sensory loss were denied; sensory loss was documented in distal extremities to light touch, vibration, temperature, and pain. He had moderate ankle dorsiflexion weakness and could not walk on his heels. Mild abductor weakness of the intrinsic hand muscles accompanied distal areflexia and proximal hyporeflexia. Quantitative sensory testing (heat, pain, cooling, and vibration) showed that large (256-Hz vibration) and small fiber sensory loss occurred in the feet and hands.

He had a hoarseness of voice, and on direct visualization of vocal cords had complete paresis of the left vocal cord with diminished abduction on the right. Pulmonary function testing showed reduced functional vital capacity (FVC 55% of predicted) and forced expiratory volume (FEV1 52% of predicted) consistent with neuromuscular failure.

The electrophysiological features were of a chronic motor predominant axonal denervating process with chronic reinnervation. The motor nerve conductions were preserved with peroneal compound motor action potential amplitude being 4.8 mV with conduction velocity of 49 m/s. Ulnar motor amplitude was 8.1 mV with conduction velocity of 67 m/s. The sural sensory amplitude was 7 μ V with conduction velocity of 47 m/s. The radial and median sensory amplitudes were reduced at 15 and 8 μ V with normal distal latencies. The phrenic responses were reduced in amplitude, 0.3 (left) and 0.8 mV (right), with associated prolonged latencies 9.0 and 9.3 msec. Blink responses R1, R2 ipsilateral to supraorbital trigeminal stimulation were normal. On needle examination motor units were long in duration with reduced recruitment more prominent in distal compared to proximal musculature, but also with diaphragm and paraspinal muscles involved.

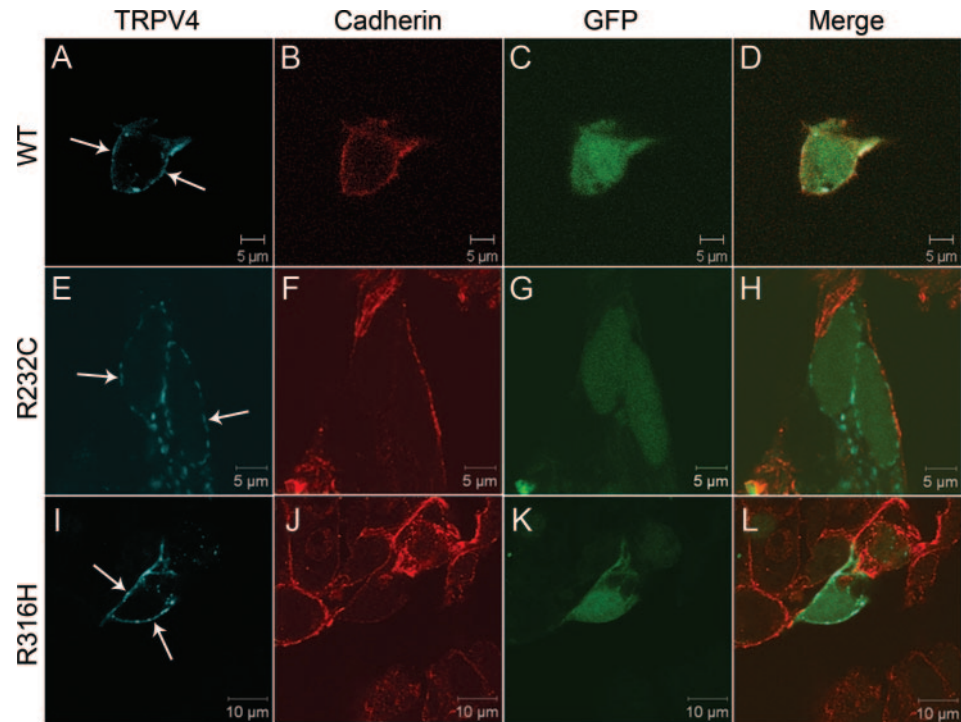
His genetically confirmed parents were unaffected by neuropathy as determined by normal clinical examinations. His mother had relapsing-remitting multiple sclerosis with mild hemiparesis and no lower motor neuron findings. His 2 siblings and offspring were normal. No persons had bony abnormalities (figure 1B).

Expression vectors. A human cDNA clone (IMAGE: 40125977) was used as a template. Two primers anchored with an *Xho*I (*TRPV4*-TP1:5'-ctgtctcagcaggcatggcggattccagcgaag-3') and *Bam*HI (*TRPV4*-TP2:5'-catcggatcctagacggggcgtcatcag-3') were used to amplify the coding sequence. The amplified fragment was cloned into vector pBluescript-M13. The TRPV4 sequence was verified by sequencing. The R232C mutation was introduced into the vector by site-directed mutagenesis using a primer *TRPV4*-R232C:5'-cattaactcgcctctctgtgacatctactatcg-3'. The R316H was introduced by *TRPV4*-R316H:5'-gaagcgggacatcgggaccaggactcggcagg-3'. The *Xho*I/*Bam*HI fragment containing wild-type TRPV4, TRPV4^{R232C}, and TRPV4^{R316H} was cloned into the *Xho*I and *Bam*HI sites of a dual expression vector pIRES2-ZsGreen1 (Clontech).

Expression of wild-type and mutant TRPV4. HEK293T or HeLa cells were grown on glass coverslips in DMEM containing 10% serum at 37°C. The cells were transfected with wild-type TRPV4, TRPV4^{R232C}, and TRPV4^{R316H} using Lipofectamine 2000 (Invitrogen).

Intracellular Ca²⁺ measurements. HEK293 were transfected, incubated with ruthenium red (10 μ M) for 24 hours, and loaded with 3 μ M Indo-1 AM for 45 minutes. Cultures were then rinsed and kept in the dark in HEPES buffer for 30 minutes to allow for dye de-esterification. Calcium flux was measured as a ratio of 405/510 fluorescence using a MoFlo cell sorter before and during treatment with 2 μ M of TRPV4-specific agonist 4 α -phorbol 12, 13-didecanoate (4 α PDD), and analyzed using Summit (DakoCytomation). The argon-ion (488 nm) and krypton (UV) lasers were used for excitation. The GFP and propidium iodide (PI) signals were collected using 530/40 nm and 670/20 nm bandpass filters. Indo-1 signals were collected using 405/30 nm and 510/21 nm filters. Measurements were calibrated using the Grynkiewicz equation.²⁰ Values for R_{min} and R_{max} were determined in Ca²⁺-free solution and high Ca²⁺-

Figure 2 Localization of wild-type and mutant TRPV4 on the plasma membrane in HEK293 cells



Confocal microscopy was performed using HEK293 cells transfected with plasmids pIRES2-ZsGreen1 containing wtTRPV4 (A–D), TRPV4^{R232C} (E–H), and TRPV4^{R316H} (I–L). Cells expressing exogenous TRPV4 were labeled by green fluorescent protein (GFP) (C, G, and K). TRPV4 is shown by blue (A, E, and I) and cadherin by red (B, F, and J). Merged images are shown on the right panels (D, H, and L). Arrows indicate TRPV4 signals on the plasma membrane.

containing solution in the presence of 5 μ M ionomycin. The dissociation constant (K_d) of 250 nM for Indo-1 AM was used for calculations. In all experiments, data were gated on cells that were PI-negative and showed high levels of GFP expression. Two-tailed unpaired Student t test ($p < 0.0005$) was used for statistical analysis.

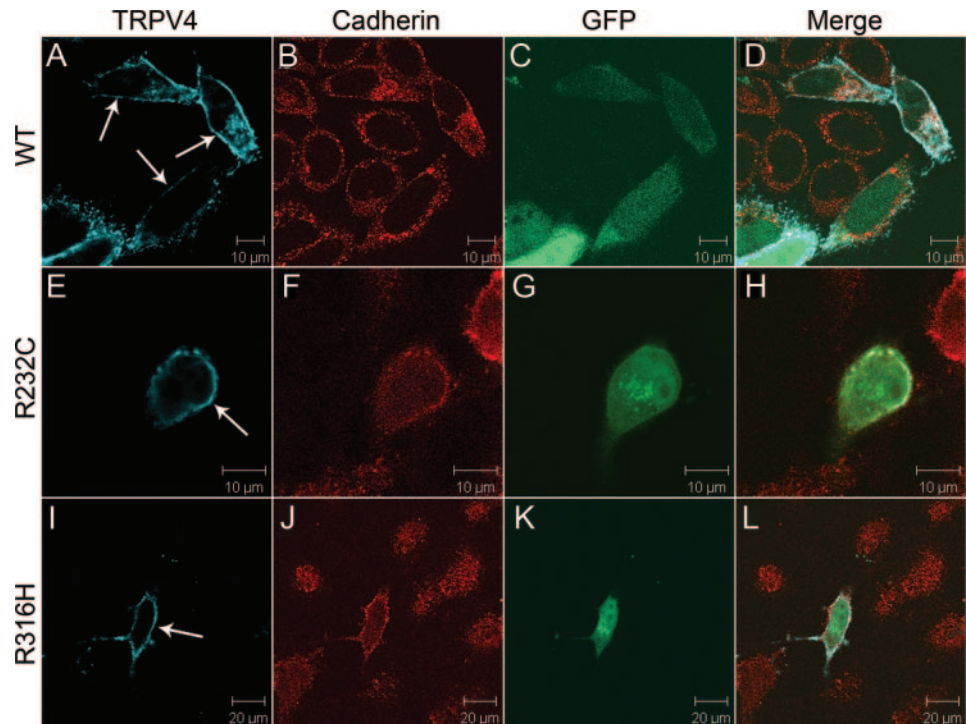
Cell viability assay. HEK293 or HeLa were transfected, incubated either with or without ruthenium red (20 μ M) for 48 hours, and harvested in PBS containing 25 μ g/mL PI (Calbiochem). All flow cytometric data were analyzed using a Dako Cytomation Cyan flow cytometer and Summit software (Dako-Cytomation) recording at least 50,000 events. The argon-ion 488 nm was used for excitation. The GFP and PI signals were collected using 530/40 nm and 680/30 nm filters. In all experiments, data were gated on cells showing high levels of GFP expression. Percent cell death was the percentage of GFP-positive cells that were also PI positive. Two-tailed unpaired Student t test ($p < 0.05$) was used for analysis.

RESULTS DNA sequencing of 15 exons of *TRPV4* in a total of 53 unrelated CMT2 probands with vocal cord paralysis including subjects with diaphragmatic weakness ($n = 17$) and undifferentiated CMT2 ($n = 36$) revealed 2 mutations. A c.694C>T mutation in exon 4, leading to p.R232C, was identified in kindred 1 (figure 1A). A c.947G>A mutation in exon 6, resulting in p.R316H, was found in a CMT2C individual (figure 1B). R316H has not been reported before and is a de novo mutation. The R232 and R316 are both highly

conserved amino acids (figure 1C). These mutations were absent in our 800 control samples.

TRPV4 is a cation channel located on the cytoplasmic membrane. The R232C and R316H mutations are in the ankyrin-repeat domain (figure e-1 on the *Neurology*[®] Web site at www.neurology.org). To examine the impact of the mutations on targeting TRPV4 to the cytoplasmic membrane, we analyzed the subcellular distribution of TRPV4 with R232C (TRPV4^{R232C}) and R316H (TRPV4^{R316H}) in transiently transfected HEK293 cells, using the human wild-type TRPV4 (wtTRPV4) as a control. We found these mutants had a similar pattern of subcellular localization comparing to the wtTRPV4 in the transfected HEK293 cells (figure 2), suggesting that these mutations may not interfere with channel assembly and intracellular trafficking. Previous studies have demonstrated that mutant TRPV4 channels have a physiologic distribution in HEK293 cells, but not in HeLa cells,⁷ suggesting a possibility of cell-type-specific distribution of the mutant TRPV4. To test this possibility, we further examined the localization of the mutant TRPV4 in HeLa cells and used wtTRPV4 as a control. We found that the mutants localized on the cytoplasmic membrane in HeLa cells as well (figure 3).

Figure 3 Localization of wild-type and mutant TRPV4 on the plasma membrane in HeLa cells



Confocal microscopy was performed using HeLa cells transfected with plasmids pIRES2-ZsGreen1 containing wtTRPV4 (A-D), TRPV4^{R232C} (E-H), and TRPV4^{R316H} (I-L). Cells expressing exogenous TRPV4 were labeled by green fluorescent protein (GFP) (C, G, and K). TRPV4 is shown by blue (A, E, and I) and cadherin by red (B, F, and J). Merged images are shown on the right panels (D, H, and L). Arrows indicate TRPV4 signals on the plasma membrane.

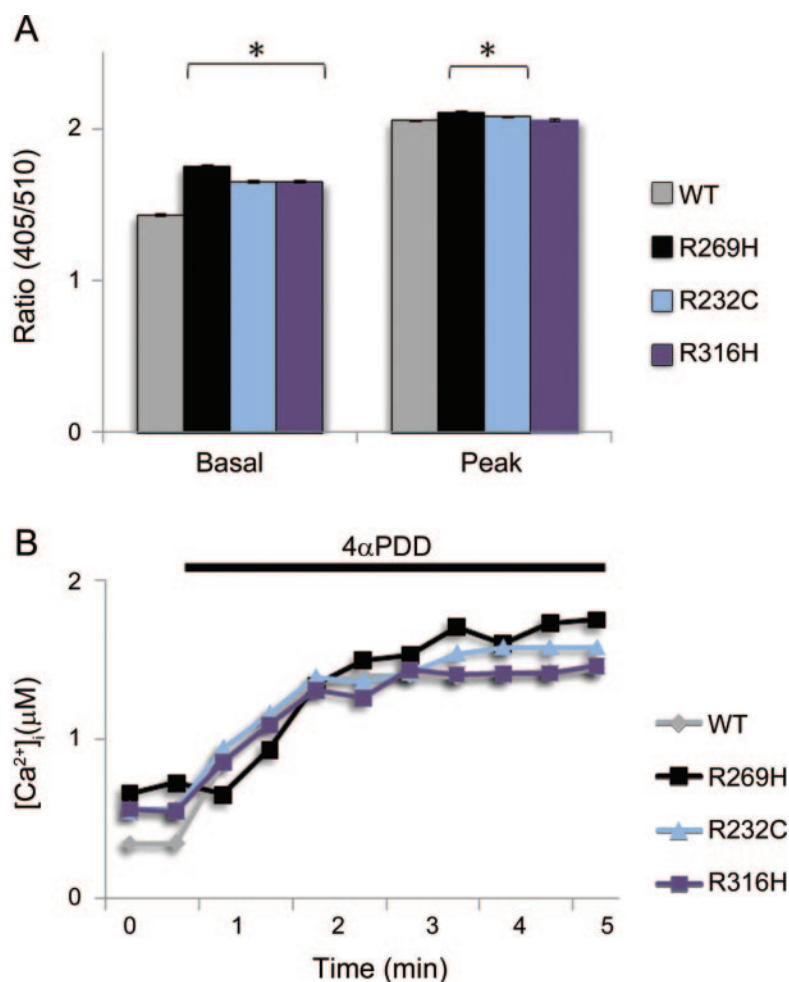
The TRPV4 channel responds to a large variety of stimuli. To test the effects of the mutations on calcium channel activity, we analyzed the channel activity in transiently transfected HEK293 cells. We examined the calcium channel activity of TRPV4 using Indo-1 AM Calcium Sensor Dye. Internal Indo-1 fluorescence ratio (405 nm/510 nm) was used as an indicator of the intracellular Ca²⁺ levels, which depend on Ca²⁺ influx. In addition to the 2 new mutations (TRPV4^{R232C}, TRPV4^{R316H}), wt-TRPV4 and previously identified neuropathy-linked mutation (TRPV4^{R269H}) were also included as controls. Similar to TRPV4^{R269H}, TRPV4^{R232C} and TRPV4^{R316H} showed increased basal intracellular calcium concentration vs the wtTRPV4 ($p < 0.0005$) (figure 4). The calcium channel activity was further analyzed using TRPV4-specific agonist 4 α -phorbol 12, 13-didecanoate (4 α PDD). The wild-type and all the mutant TRPV4 channels could be activated by 4 α PDD. Similar to TRPV4^{R269H}, the TRPV4^{R232C} mutation showed increased calcium channel activity compared to wtTRPV4 when activated by 4 α PDD ($p < 0.0005$) (figure 4).

We then analyzed the effect of the mutant TRPV4 channels on cell viability. HEK293 and HeLa cells were transfected with above dual expression vectors. After 48 hours of incubation with or

without ruthenium red (20 μ M), cytotoxicity in GFP-positive cells was analyzed by flow cytometry. We observed a trend toward increased cell death in mutant TRPV4-expressing HEK293 without reaching statistical significance (figure e-2). We then further tested cell viability in HeLa cells. We observed that HeLa cells expressing TRPV4^{R232C} and TRPV4^{R316H} had markedly increased cell death when compared to the wtTRPV4-expressing cells (figure 5). Cells expressing TRPV4^{R269H}, a previously identified mutation in our original CMT2C family, also led to increased cell death. All observed cell death could be suppressed by TRPV antagonist ruthenium red (figure 5).

DISCUSSION Through screening 36 unrelated individuals with nondistinct CMT2 and 17 unrelated families with CMT2C phenotype having vocal cord and diaphragmatic involvement, we identified 2 TRPV4 mutations R232C and R316H. R316H mutation has not been reported before and is de novo. R232C is from a CMT2C family, but only the proband has vocal cord involvement whereas other affected do not. This family was previously localized to 12q23–24³ and the variability of vocal cord involvement was discussed.¹⁹ The findings here emphasize the importance of considering TRPV4 mutation

Figure 4 Effects of mutations on calcium channel activity of TRPV4



(A) Internal fluorescence ratio in HEK293 cells transfected with WT, R269H, R232C, or R316H construct at rest or stimulated condition (2 μ M 4 α PDD) is given as basal (left) or peak (right) value ($n > 3,000$ cells for each condition). Mean calcium responses before and during 4 α PDD application are given. Significant difference was observed in TRPV4 mutants (R269H, R232C, or R316H) at basal level, and mutants (R269H or R232C) at activated condition when compared to WT-TRPV4 (2-tailed Student t test, * $p < 0.005$). (B) Application of 4 α PDD induced an increase in intracellular calcium ([Ca²⁺]_i). Error bars, mean \pm SEM.

when vocal cord paralysis feature is not present in inherited axonal neuropathy, i.e., nondistinct clinical CMT2. The previous and the current study suggest the location of *TRPV4* mutations does not account for this specific neurologic variability.⁵⁻⁸

This study highlights the genetic heterogeneity of dominantly inherited axonal neuropathies with vocal cord and diaphragmatic involvement. Specifically, only 2 out of 17 screened CMT2C families had identified mutations in *TRPV4* and no *TRPV4* mutation was found in 36 screened undifferentiated CMT2 families. Mutations in *GDAP1* have been linked to vocal cord paresis with peripheral neuropathy but are uncommon in dominant or axonal cases. Most affected cases caused by *GDAP1* mutation have recessive inheritance and demyelination.²¹ Also highlighted is that the lack of family history should

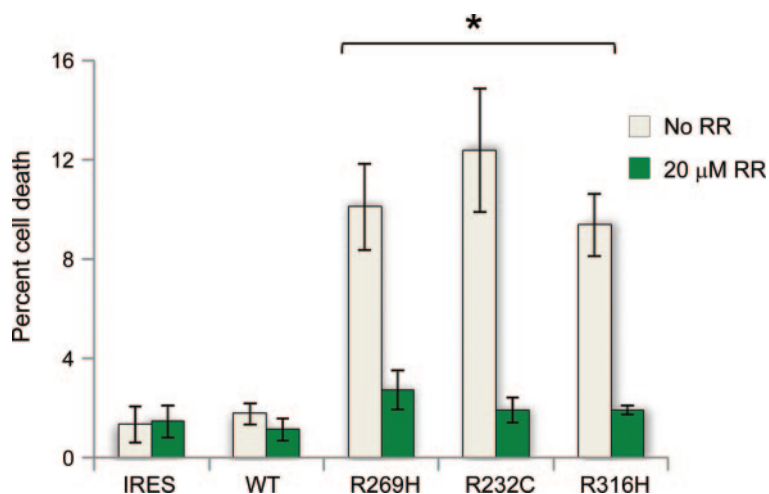
not dissuade from consideration of CMT2C with the proper phenotype as seen in the individual with a de novo mutation R316H. Furthermore, incomplete penetrance or markedly variable expression may also obscure the genetic cause as seen in our original kindred with R269H mutation where repeated clinical examinations over time including specialized nerve conductions were required to identify very mildly affected persons and hence the accurately diagnosed affected status.^{2,5,8} Therefore penetrance should only be determined close to the end of life as symptoms among some may not occur until the eighth decade of life.² Scoliosis and small hands were characteristic of our severely affected persons with R316C and R269H mutations⁵ but were not seen in the individuals described here with R316H and R232C mutations. The fact that TRPV4 can cause nondistinct CMT2 and CMT2C, SPSMA, and distal SMA as well as different bone dysplasias¹⁶ implies the existence of complex pathogenic mechanism related to TRPV4 mutation for varied phenotypes, and pathogenic effect of TRPV4 mutation may not exert through cytotoxic calcium influx alone.

To date, 7 mutations in *TRPV4* have been identified in 15 families with axonal neuropathies. Six mutations located in the ankyrin-repeat domains, 5 showed segregation with the disease, and 2 de novo mutations. Our newly reported de novo mutation R316H combined with previous studies suggests codon 269R and 316R are both hot spots for axonal neuropathy-linked *TRPV4* mutations. The substitutions of 269R or 316R by either cysteine or histidine cause CMT2C.

The R232C mutation was reported in 3 other families with axonal neuropathies⁸ in addition to the family described here. The *TRPV4* mutations in the 3 initial reports were in the third and fourth ankyrin-repeat domains⁵⁻⁷ while R232C is in the second domain (figure 2). The only neuropathy mutation located outside of the ankyrin repeat domain is V620I,⁸ which was previously reported in brachyolmia.¹⁶ Neurologic examination and testing is not reported among the previously reported TRPV4-associated bone dysplasias. The V620I neuropathy case was a de novo mutation, raising the possibility of an additional hot spot in axonal neuropathy. This person had mild skeletal dysplasia features that suggested a possible overlap syndrome.

The previously reported discrepancies in the functional studies are addressed with our current experiments. Two studies showed TRPV4 mutations R269H, R316C, and R269C have normal cellular localization and increased calcium channel activity while another study showed R269H, R315W, and R316C had decreased calcium channel activity and

Figure 5 Quantification of propidium iodide uptake in HeLa cells expressing wild-type and mutant TRPV4



Transfected HeLa cells indicated an increase in cytotoxicity in mutant-expressing cells at 48 hours that is blocked by the TRPV channel blocker ruthenium red (RR). Data are averaged from at least 3 independent experiments. * $p < 0.05$, indicating significant differences when compared to WT-TRPV4 (2-tailed Student t test). Error bars, mean \pm SEM.

abnormal cellular localization. Since different cell types were used, the cell-type-specific response could explain the conflicting data. To investigate whether gain of function or loss of function of TRPV4 mutations accounted for the abnormality, we performed functional analysis in both HEK 293 and HeLa cells. Using wtTRPV4 as control, we performed subcellular localization, channel activity, and cell viability assay for R232C, R316H, and R269H. All 3 mutations properly localize to the cell membrane and cause gain of function of TRPV4, resulting in increased intracellular calcium with decreased cell viability. The R269H mutant experiments reproduced the prior results. Our data indicate that an increased constitutive function, rather than increased response to agonist stimulation, is probably the key property gained. Significantly increased cell death was only observed in mutant TRPV4-expressing HeLa cells although cell viability was increased in both transfected HeLa and HEK293 cells after using TRPV antagonist ruthenium red to block the mutant channel. The data support that TRPV4 ankyrin repeat mutations exert their effects by dominant gain of function with normal cell membrane localization. Nevertheless we remain open to the possibility that other unidentified factors are involved in pathogenesis and ongoing work is required.

Intracellular hypercalcemia is a common pathway of diverse pathogenesis.^{10,22} Ruthenium red, the calcium channel blocker, has recently been shown to improve mitochondrial ATP levels and membrane potentials in neurons expressing mutant α -synuclein or PINK-1 associated with Parkinson.²³ Optimism of

a pharmacologic approach also comes from recent research where L-type calcium blockers have been suggested to reduce the chance of developing PD.¹⁴ Further work will be required to establish whether the observed hypercalcemia cytotoxicity manifests its effect in the cytoplasm or mitochondria. Calcium channel inhibitors provide a rational new approach for investigation of TRPV4-mediated axonal neuropathies.

AUTHOR CONTRIBUTIONS

C.J.K., P.J.D., T.S., and H.-X.D. conceived this project. C.J.K., Y.W., and L.H. performed sequencing analysis. Y.S., F.F., and H.X.D. performed functional analysis. C.J.K., M.D., G.N., M.E.M., A.C., and K.M.M. examined and collected family information and samples. C.J.K., H.-X.D., T.S., and P.J.D. analyzed the data and wrote the article.

ACKNOWLEDGMENT

The authors thank Dr. Eric J. Ahlskog for helpful discussion. We appreciate help provided by the staff of the Robert H. Lurie Comprehensive Cancer Center Flow Cytometry Core Facility.

DISCLOSURE

Dr. Klein, Dr. Shi, Dr. Fecto, Dr. Donaghy, Dr. Nicholson, Dr. McEntagart, Dr. Crosby, Dr. Wu, Dr. Lou, and Dr. McEvoy report no disclosures. Dr. Siddique serves on the scientific advisory board of NIH: Skeletal Muscle and Exercise Physiology (SMEP) Study Section; serves on the editorial boards of *Neurogenetics* and *Amyotrophic Lateral Sclerosis*; holds a patent re: Human α -tocopherol transport protein: compositions and methods; and receives research support from the NIH (NINDS and NIEHS), the Harold Post ALS Research Fund, the Les Turner ALS Foundation/Herbert C. Wenske Foundation, Vena Schaaf, Frank White ALS Research Fund, the Spastic Paraplegia Foundation, Inc., the Amyotrophic Lateral Sclerosis Association, the CVS/ALS Therapy Alliance, and the Blazeman Foundation for ALS. Dr. Deng reports no disclosures. Dr. Dyck receives research support from the NIH and the FDA.

Received July 7, 2010. Accepted in final form September 22, 2010.

REFERENCES

1. Dyck PJ, Litchy WJ, Minnerath S, et al. Hereditary motor and sensory neuropathy with diaphragm and vocal cord paresis. *Ann Neurol* 1994;35:608–615.
2. Klein CJ, Cunningham JM, Atkinson EJ, et al. The gene for HMSN2C maps to 12q23–24: a region of neuromuscular disorders. *Neurology* 2003;60:1151–1156.
3. McEntagart ME, Reid SL, Irrthum A, et al. Confirmation of a hereditary motor and sensory neuropathy IIC locus at chromosome 12q23–q24. *Ann Neurol* 2005;57:293–297.
4. DeLong R, Siddique T. A large New England kindred with autosomal dominant neurogenic scapuloperoneal amyotrophy with unique features. *Arch Neurol* 1992;49:905–908.
5. Deng HX, Klein CJ, Yan J, et al. Scapuloperoneal spinal muscular atrophy and CMT2C are allelic disorders caused by alterations in TRPV4. *Nat Genet* 2010;42:165–169.
6. Landoure G, Zdebik AA, Martinez TL, et al. Mutations in TRPV4 cause Charcot-Marie-Tooth disease type 2C. *Nat Genet* 2010;42:170–174.
7. Auer-Grumbach M, Olschewski A, Papic L, et al. Alterations in the ankyrin domain of TRPV4 cause congenital distal SMA, scapuloperoneal SMA and HMSN2C. *Nat Genet* 2010;42:160–164.
8. Zimon M, Baets J, Auer-Grumbach M, et al. Dominant mutations in the cation channel gene transient receptor

- potential vanilloid 4 cause an unusual spectrum of neuropathies. *Brain* 2010;133:1798–1809.
9. Liedtke W. Molecular mechanisms of TRPV4-mediated neural signaling. *Ann NY Acad Sci* 2008;1144:42–52.
 10. Mattson MP. Calcium and neurodegeneration. *Aging Cell* 2007;6:337–350.
 11. Yang L, Wang Z, Wang B, Justice NJ, Zheng H. Amyloid precursor protein regulates Cav1.2 L-type calcium channel levels and function to influence GABAergic short-term plasticity. *J Neurosci* 2009;29:15660–15668.
 12. Jiang M, Schuster JE, Fu R, Siddique T, Heckman CJ. Progressive changes in synaptic inputs to motoneurons in adult sacral spinal cord of a mouse model of amyotrophic lateral sclerosis. *J Neurosci* 2009;29:15031–15038.
 13. Gandhi S, Wood-Kaczmar A, Yao Z, et al. PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. *Mol Cell* 2009;33:627–638.
 14. Ritz B, Rhodes SL, Qian L, Schernhammer E, Olsen JH, Friis S. L-type calcium channel blockers and Parkinson disease in Denmark. *Ann Neurol* 2010;67:600–606.
 15. Nilius B, Owsianik G, Voets T. Transient receptor potential channels meet phosphoinositides. *Embo J* 2008;27:2809–2816.
 16. Rock MJ, Prenen J, Funari VA, et al. Gain-of-function mutations in TRPV4 cause autosomal dominant brachyolmia. *Nat Genet* 2008;40:999–1003.
 17. Krakow D, Vriens J, Camacho N, et al. Mutations in the gene encoding the calcium-permeable ion channel TRPV4 produce spondylometaphyseal dysplasia, Kozlowski type and metatropic dysplasia. *Am J Hum Genet* 2009;84:307–315.
 18. Nishimura G, Dai J, Lausch E, et al. Spondylo-epiphyseal dysplasia, Maroteaux type (pseudo-Morquio syndrome type 2), and parastremmatic dysplasia are caused by TRPV4 mutations. *Am J Med Genet A* 2010;152A:1443–1449.
 19. Donaghy M, Kennett R. Varying occurrence of vocal cord paralysis in a family with autosomal dominant hereditary motor and sensory neuropathy. *J Neurol* 1999;246:552–555.
 20. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;260:3440–3450.
 21. Claramunt R, Pedrola L, Sevilla T, et al. Genetics of Charcot-Marie-Tooth disease type 4A: mutations, inheritance, phenotypic variability, and founder effect. *J Med Genet* 2005;42:358–365.
 22. Farber JL. The role of calcium in cell death. *Life Sci* 1981;29:1289–1295.
 23. Marongiu R, Spencer B, Crews L, et al. Mutant Pink1 induces mitochondrial dysfunction in a neuronal cell model of Parkinson's disease by disturbing calcium flux. *J Neurochem* 2009;108:1561–1574.

Career Moves Begin at Neurology Career Center

Job seekers: The AAN's Neurology Career Center is a one-stop shop for qualified candidates looking to make a career move in neurology. Search for opportunities in your state and area of interest and create a profile that you can share with only those employers in whom you're interested.

Advertising for a position in neurology? Reach more than 22,500 Academy members online, in print, and at special career events.

Make your next career connection with the Neurology Career Center!

www.aan.com/careers