Reduced neurofilament expression in cutaneous nerve fibers of patients with CMT2E

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Supplemental data at Neurology.org

ABSTRACT

Objective: To investigate the effects of NEFL Glu396Lys mutation on the expression and assembly of neurofilaments (NFs) in cutaneous nerve fibers of patients with Charcot-Marie-Tooth disease type 2E (CMT2E).

Methods: A large family with CMT2E underwent clinical, electrophysiologic, and skin biopsy studies. Biopsies were processed by indirect immunofluorescence (IF), electron microscopy (EM), and Western blot analysis.

Results: The clinical features demonstrated intrafamilial phenotypic variability, and the electrophysiologic findings revealed nerve conductions that were either slow or in the intermediate range. All patients had reduced or absent compound muscular action potential amplitudes. Skin biopsies showed axons labeled with the axonal markers protein gene product 9.5 and α-tubulin, but not with NFs. The results of Western blot analysis were consistent with those of IF, showing reduced or absent NFs and normal expression of α-tubulin. EM revealed clusters of regenerated fibers, in absence of myelin sheath abnormalities. Both IF and EM failed to show NF aggregates in dermal axons. The morphometric analysis showed a smaller axonal caliber in patients than in controls. The study of the nodal/paranodal architecture demonstrated that sodium channels and Caspr were correctly localized in patients with CMT2E.

Conclusions: Decrease in NF abundance may be a pathologic marker of CMT2E. The lack of NF aggregates, consistent with prior studies, suggests that they occur proximally leading to subsequent alterations in the axonal cytoskeleton. The small axonal caliber, along with the normal molecular architecture of nodes and paranodes, explain the reduced velocities detected in patients with CMT2E. Our results also demonstrate that skin biopsy can provide evidence of pathologic and pathogenic abnormalities in patients with CMT2E.

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GLOSSARY

CMT = Charcot-Marie-Tooth disease; CMT2E = Charcot-Marie-Tooth disease type 2E; CMTNSv2 = Charcot-Marie-Tooth Neuropathy Score version 2; EM = electron microscopy; IF = immunofluorescence; NEFL = neurofilament, light polypeptide; NF = neurofilament; PGP = protein gene product; WB = Western blot.

Charcot-Marie-Tooth disease (CMT) is a group of peripheral neuropathies associated with mutations in more than 80 distinct genes. CMT is divided into different forms based on the pattern of inheritance and neurophysiology. Electrophysiologic studies allow for classification of CMT into demyelinating (CMT1) and axonal (CMT2) forms. The classification of CMT has been further divided into subtypes, identified by letters, as defined by the mutated gene. CMT2E refers to autosomal dominant CMT2 caused by mutations in the neurofilament light chain (NEFL) gene. Some patients with NEFL mutations have slow conduction velocities, in the “demyelinating” range, and are characterized as having CMT1F. Patients with CMT2E and CMT1F are clinically heterogeneous and the age at onset ranges from infancy, typically with severe impairment, to adulthood with milder impairment. Usually those with slowed conduction present early either in infancy with delayed motor milestones or in late childhood/early adolescence.
Neurofilaments (NFs) are neuron-specific intermediate filaments that are necessary for maintaining the caliber of large-diameter axons in the peripheral nervous system. Axonal caliber is maintained by precise stoichiometric ratios of the 3 NF subunits: light (NF-L), medium (NF-M), and heavy (NF-H).\(^4\) NF-L mutations are distributed throughout the 3 functional domains of the NF-L (head, rod, and tail) and different mutations have been shown to lead to distinct pathologic effects.\(^5,6\) Previous studies in a transgenic model carrying a Glu396Lys have demonstrated accumulation of NFs in the neuronal cell body, with decreased NFs in axons.\(^6\) Sural nerve biopsies from patients with the Glu396Lys have shown the presence of axons with a variable density of NFs.\(^7,8\) We have evaluated a large 4-generation family with the Glu396Lys mutation and investigated the expression and assembly of NFs in cutaneous nerve fibers.

**METHODS** We evaluated 9 affected individuals from 2 generations of a large family with a clinical and genetic diagnosis of CMT2E (Glu396Lys) (figure 1). Clinical impairment was evaluated using the validated CMT Neuropathy Score version 2 (CMTNSv2).\(^9\) All patients but one underwent skin biopsies that were processed for indirect immunofluorescence (IF), electron microscopy (EM), or Western blot (WB) analysis. A control population of healthy age-matched controls was included for the morphologic and morphometric studies.

**Standard protocol approvals, registrations, and patient consents.** The Institutional Review Board (Ethics) Committee approved the study, and a written informed consent was obtained from all participants in the study.

**Skin biopsy: IF.** Three skin samples for IF were obtained by a 2.5-mm punch from the proximal phalanx of the II finger. Specimens were fixed in 4% paraformaldehyde for 30 minutes, washed in phosphate-buffered saline 3 times for 10 minutes, and then embedded in optimal cutting temperature medium in a plastic mold and gradually frozen. The tissues were then cut into 20-μm-thick sections and mounted on glass slides. These slides were then incubated with a panel of primary antibodies at 4°C overnight, reacted with secondary antibodies for 2 hours the following day, washed, dried, and coverslipped for examination with a Zeiss 710 confocal microscope. Primary and secondary antibody features, sources, and dilutions are listed in table e-1 on the Neurology® Web site at Neurology.org.

**Fluorescence quantification.** Images were obtained on a Zeiss 710 confocal microscope using a 40X objective. All image parameters including pinhole size, detector gain, amplifier offset, amplifier gain, and laser intensity were first set for normal control tissue, and the same setting used for all samples imaged on a given day.\(^10\) Both control and patient sample images were obtained at the same day after the staining. For each sample, 5 nonoverlapping sections were acquired. Image analysis was performed using Image J software (NIH, Bethesda, MD). Fluorescence intensity was measured for both NFs and protein gene product (PGP) 9.5 at the same time, choosing an intensity threshold in the PGP channel so that only the axons were highlighted, corresponding to our regions of interest on both PGP and NF channel. Then we calculate the NF/PGP ratio to obtain a relative value of NF intensity over the PGP-stained axons and rule out a possible role of nerve fiber loss in reduced NF staining. Alpha-tubulin fluorescence intensity was evaluated as well.

**Morphometric analysis.** Z-stack digital images of all myelinated fibers present in 5 PGP/myelin protein double-stained sections were acquired using confocal microscopy (Zeiss 710 confocal microscope). The axonal caliber was measured using Image J software (NIH). Four caliber measurements for each nerve fiber were obtained and a mean value was reported.\(^11,12\)

**Skin biopsy: EM.** Two skin samples for EM study were obtained by a 2.5-mm punch from the volar aspect of the forearm. Specimens were fixed in 2.5% glutaraldehyde solution overnight, then washed in phosphate-buffered saline 3 times for 10 minutes, osmicated for 1.5 hours, dehydrated, and embedded in Epon. The semithin sections (1-μm thickness) were stained with toluidine blue and examined under a light microscope. The ultrathin sections were contrasted with lead citrate and uranyl acetate and examined under EM (Transmission Electron Microscope, JEOL 1230, Peabody, MA).

**Skin biopsy: WB.** Three skin samples for WB analysis were obtained by a 3.5-mm punch from the shoulder and immediately frozen in liquid nitrogen. Snap-frozen samples were then pulverized, dissolved in lysis buffer (RIPA [radioimmunoprecipitation assay] buffer, Sigma R0278, and protease inhibitors), kept on ice for at least 30 minutes, and centrifuged at 14,000g for 10 minutes at 4°C. The protein content of the supernatant was determined using a bovine serum albumin standard curve. Equal amounts of the protein were loaded on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, and electroblotted onto polyvinyl difluoride membranes.\(^13\) The primary antibodies used are listed in table e-1. Horseradish peroxidase–conjugated secondary antibodies (1:5,000–1:20,000 dilution; Sigma) were detected using enhanced chemiluminescence (ECL) reagents (Amersham ECL Prime Western Blotting Detection Reagent, RPN 2232) with autoradiography film (Kodak Scientific Imaging Film, Blue XB).\(^13\) Results were normalized for α-tubulin and the control sample signal was used as the reference. Moreover, the signal density was calculated using Image J software (NIH), the ratio NF-L/α-tubulin was obtained for both patients and controls, and any value less than the control value was considered abnormal.

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**Figure 1** Pedigree of the family

![Pedigree of the family](image-url)
### Clinical Features

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at examination, y/sex</th>
<th>CMTNS (phenotype)</th>
<th>Ankle deep tendon reflexes</th>
<th>Distal/government weakness UL</th>
<th>Distal/government weakness LL</th>
<th>Vibration sense UL/LL</th>
<th>Pinprick UL/LL</th>
<th>Light touch UL/LL</th>
<th>Ulnar MNCV (CMAP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.1</td>
<td>57/F</td>
<td>Moderate</td>
<td>Normal/Normal</td>
<td>Absent</td>
<td>Absent</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>40 m/s (3.1 mV)</td>
</tr>
<tr>
<td>II.2</td>
<td>53/M</td>
<td>Severe</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>40 m/s (3.8 mV)</td>
</tr>
<tr>
<td>II.3</td>
<td>58/F</td>
<td>Moderate</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>40 m/s (3.6 mV)</td>
</tr>
<tr>
<td>II.4</td>
<td>56/M</td>
<td>Severe</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>27 m/s (2.5 mV)</td>
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<tr>
<td>II.5</td>
<td>25/M</td>
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<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>38 m/s (3.2 mV)</td>
</tr>
<tr>
<td>III.1</td>
<td>42/F</td>
<td>Moderate</td>
<td>Reduced</td>
<td>Absent</td>
<td>Absent</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>40 m/s (3.1 mV)</td>
</tr>
<tr>
<td>III.2</td>
<td>42/F</td>
<td>Moderate</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>40 m/s (3.1 mV)</td>
</tr>
<tr>
<td>III.3</td>
<td>42/M</td>
<td>Moderate</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>37.3 m/s (1.5 mV)</td>
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<tr>
<td>III.4</td>
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<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>Normal/Normal</td>
<td>41 m/s (3.3 mV)</td>
</tr>
</tbody>
</table>

Abbreviations: CMAP = compound muscular action potential; CMTNS = Charcot-Marie-Tooth Neuropathy Score; UL = upper limbs; LL = lower limbs; MNCV = motor nerve conduction velocity; NF = non-myelinated fiber; PGF = peripheral ganglia fraction.

### Statistical analysis

Statistical analysis was performed using SigmaStat 3.0 for Windows (StataCorp LP, College Station, TX). Student $t$ test for unpaired data and Mann–Whitney test (when analyzing nonparametric data) were used to compare skin biopsy findings from patients and controls. Spearman rank correlation test was used to correlate fluorescence intensity values with patients’ disease severity determined by the CMTNSv2. A $p$ value of $<0.01$ was considered significant.

### RESULTS

**Patients.** Disease severity, as determined by the CMTNSv2, ranged from mild to severe impairment (scores between 5 and 30). Electrophysiologic findings revealed either slow or intermediate motor nerve conduction velocities when a compound muscular action potential was recordable. Individual scores along with additional clinical information are provided in table 1.

**Skin biopsy.** Cutaneous nerve fibers show a decrease in NF abundance. We performed immunohistochemistry on myelinated dermal nerves from patients and controls. Results from controls demonstrated many axons labeled by the axonal marker PGP 9.5 and with antibodies to NF-L but not labeled by PGP (figure 2, A.a and A.b). Alternatively, axons from patients were not labeled with antibodies to NF-L, but were labeled by PGP (figure 2, A.d and A.e). To measure the difference between controls and patients, we quantified the fluorescence intensity in the 2 groups. The analysis demonstrated a lower NF/PGP ratio in patients than in controls ($p < 0.01$) (figure 2B). There was no correlation between NF-L levels and clinical severity as measured by the CMTNSv2 ($p = $ not significant). We then labeled axons with antibodies to α-tubulin to determine whether the transport of other molecules such as α-tubulin was affected by the mutation. Many axons in both patients and controls were labeled with α-tubulin and no difference in staining intensity was detectable between the 2 groups. In addition, many axons from patients but not controls were labeled with α-tubulin but not with NF-L (figure 2, A.c and A.f). We concluded that the decreased NF-L immunolabeling was not a consequence of axonal degeneration but rather was a specific consequence of the NF-L mutation. Results from WB analysis confirmed these data as NF-L expression was decreased in patients with a complete absence of NF-L band in 2 of them, while α-tubulin expression was normal (figure 2C). The ratio NF-L/α-tubulin was markedly reduced in patients (figure 2D).

**NF aggregates are not detected distally in the axon.** To investigate NF accumulation, we also evaluated dermal nerves from our patients by EM (figure 3A). No abnormalities of myelin sheathes were observed on either semithin or EM sections. Many Schwann cells without axons (bands of Büngner) were present, suggesting prior axonal degeneration. No aggregates...
Cutaneous nerve fibers from patients with CMT2E show a lack of NFs

(A) Confocal images of dermal axons stained with antibodies against PGP 9.5 (red), NF-L (green), and MBP (blue) in control (A.a, A.b) and in patient (A.d, A.e) samples. A reduced or absent expression of NFs is observed in the patient compared with the control sample. Confocal images of dermal nerve fibers stained with antibodies against α-tubulin (red), NF-L (green), and PGP 9.5 (blue) in control (A.c) and in patient (A.f) samples. No difference in the expression of α-tubulin is found but a reduced NF labeling is still present in the patient compared with the control sample. Scale bar: 20 μm. (B) NF fluorescence intensity (expressed as the percentage of the value of NFs over the PGP intensity: NF/PGP ratio) between patients and controls. The ratio is significantly lower in each patient than in the controls (p < 0.01). (C) Western blot analysis of NF-L from skin of patients and controls, normalized for α-tubulin. NF-L expression is decreased in patients with a complete absence of NF-L band in 2 of them, while α-tubulin expression is normal. (D) NF-L/α-tubulin ratio is markedly reduced in patients compared with controls. α-tub = α-tubulin; CMT2E = Charcot-Marie-Tooth disease type 2E; MBP = myelin basic protein; NF = neurofilament; NF-L = neurofilament light chain; PGP = protein gene product.
were observed. Our results were similar to those previously reported in sural nerve biopsies from patients with CMT2E and Glu396Lys.7,8

Because the aggregates were made up of phosphorylated NFs in the mutant mouse,6 we performed additional staining with antibodies to phosphorylated NFs at HIC, but again, we did not detect any aggregate formation (data not shown).

Furthermore, previous studies showed that the distribution of mitochondria was altered in cultured cells with aggregated NFs because of expression of CMT-causing NEFL mutants.14 In our patients, mitochondria were distributed normally into the axons and neither their shape nor their number was affected by NF disruption.

Patients with CMT2E have smaller axonal caliber than controls. Prior studies have demonstrated a close correlation between the number of NFs and the diameter of myelinated axons in the peripheral nervous system.15,16 To determine whether the reduction of NF-L affected the caliber of axons in our patients, we performed a morphometric analysis on the dermal nerves from our patients and controls. Our results demonstrated smaller axonal calibers in patients compared with controls (p < 0.01) (figure 3B), providing further evidence of the NF role in the maintenance of the axonal diameter and supporting the feasibility of evaluating dermal nerves to address this question.

DISCUSSION Our results demonstrated a reduced expression of NFs and smaller axonal diameter in cutaneous nerve fibers of patients with CMT2E. In addition, our findings did not show NF aggregates in the axons, suggesting that their proximal localization in the cell body may lead to NF disruption distally. The cytoskeletal perturbation is specific for NFs and does not affect other components involved in the axonal transport and scaffold, and in nodal/paranodal architecture.

NEFL-linked CMT2E mutations are distributed throughout the 3 functional domains and different mutations have been shown to cause distinct pathologic effect.5,6 The mutation carried by our patients that leads to a substitution of glutamic acid to lysine at position 396 (Glu396Lys) occurs in a highly conserved motive at the end of the rod domain, and several families with the same mutation as well as a transgenic mouse model have been reported in literature.5–7,18,19 The axons of the mutant mouse had fewer NFs than those of the wild-type, and accordingly, sural nerve biopsies from patients have shown the presence of axons either devoid of or with a variable density of NFs. Our results from both immunohistochemistry and WB analysis are in keeping with these data, demonstrating reduced levels of NFs in cutaneous nerve fibers. We think that a decrease in NF abundance in peripheral nerves might be a pathologic marker of CMT2E and independent from the axonal loss. Specifically, the Glu396Lys mutation, as occurs in a protein domain involved in the dimerization process, may affect the ability of NFs to assemble properly into filaments, disrupting the NF network and reducing the transport of NFs into the axons. The loss of axonal NFs distally may be a consequence of their accumulation proximally in both nerve fiber and cell body (as shown in transgenic models and iPSC-derived motor neurons from a patient with CMT2E20), leading to the formation of aggregates and to subsequent alterations in the axonal cytoskeleton. The lack of NF aggregates distally supports this hypothesis. Of note, Fabrizi et al.8...
reported the presence of giant axons with NF accumulation in sural nerve biopsies from 4 patients with CMT2E carrying 3 different *NEFL* mutations, but not in their patient with the Glu396Lys mutation. These findings confirm that different mutations behave differently as far as disruption of NF organization is concerned, and that abnormalities of NF organization may be a marker of CMT2E. The possible alteration of axonal transport due to NF disruption does not involve other molecules that have a role in this process. Indeed, we did not find either an increase or a defect in the expression of acetylated α-tubulin as respectively shown in *NEFL* knockout mice and in other models of axonal CMT.21,22

A linear relationship between conduction velocity and fiber diameter is well known.23 Reduction in axon diameter decreases velocity, largely because the longitudinal resistance is higher. The most essential function of NFs is regulating the axonal caliber and thereby the conduction velocity. NFs are therefore particularly abundant in neurons with large-diameter axons where fast impulse conduction velocities are crucial for proper functioning.24 Within the skin, NF is expressed by both Aδ and Aβ fibers, which are fast-conducting nerve fibers, but not by C fibers that conduct more slowly.25 In transgenic mice, the loss of NF-L resulted in axons with reduced diameters and slowed conduction velocities.21,26 Morphometric analysis of sural nerve biopsies from patients with CMT2E disclosed decreased axonal diameters in myelinated fibers.7,8 Our morphometric data demonstrated that reduced axonal diameters can also be detected in a less invasive way in cutaneous nerve fibers. These smaller axonal calibers can explain the slow conduction velocities observed in CMT2E, supporting the notion that conduction velocity can be slow even in the absence of demyelination or abnormalities of nodes and paranodes, since no significant myelin sheath, nodal, or paranodal abnormalities were detected in our patients. Although the decreased axonal caliber observed in our patient might be the consequence of the loss of large-diameter fibers. Indeed, mice in which NF-L has been deleted (*NF-L* ^−/−^) showed a unimodal distribution of axon calibers with the large-sized myelinated axons being shifted to the small-size axons, and a reduction in the large-diameter fibers with loss of the bimodal spectrum have been reported in patients with CMT2E as well.7,8,21 We believe that both phenomena occur in CMT2E and, along with the normal molecular architecture of nodes and paranodes, explain the reduced velocities detected in patients with CMT2E.

Our study extends the pathology of CMT2E and provides clues to the pathogenesis, supporting the
feasibility of skin biopsy to investigate its mechanisms. A better understanding of the pathomechanism underlying each single NEFL mutation can lead to identifying a clear genotype–phenotype correlation.

AUTHOR CONTRIBUTIONS
C. Pisciotta: study concept and design, data acquisition, data analysis, statistical analysis, drafting the manuscript. Y. Bai: data acquisition, data analysis. K. Brennan: data acquisition, data analysis. X. Wu: data acquisition, data analysis. T. Grider: data acquisition, data analysis. S. Feely: data acquisition, data analysis. S. Wang: data acquisition, data analysis. M. Gonzalez: data acquisition, data analysis. S. Zuchner: data acquisition, data analysis. M.E. Shy: study concept and design, study supervision, interpretation of the data, revising the manuscript.

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DISCLOSURE
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AUTHOR CONTRIBUTIONS
M.E. Shy: study concept and design, study supervision, interpretation of the data, revising the manuscript. S. Zuchner: data acquisition, data analysis. S. Moore: interpretation of the data. C. Siskind: data analysis. Y. Bai: data acquisition, data analysis. M. Gonzalez: data acquisition, data analysis. C. Pisciotta, Y. Bai, S. Moore, S. Wang: interpretation of the data, revising the manuscript.

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