

# Mutations in the neurofilament light chain gene (*NEFL*) cause early onset severe Charcot–Marie–Tooth disease

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## Summary

Neurofilament light chain polypeptide (NEFL) is one of the most abundant cytoskeletal components of the neuron. Mutations in the *NEFL* gene were recently reported as a cause for autosomal dominant Charcot–Marie–Tooth type 2E (CMT2E) linked to chromosome 8p21. In order to investigate the frequency and phenotypic consequences of *NEFL* mutations, we screened 323 patients with CMT or related peripheral neuropathies. We detected six disease associated missense mutations and one 3-bp in-frame deletion clustered in functionally defined domains of the NEFL protein. Patients have an early onset and often a severe clinical phenotype.

Electrophysiological examination shows moderately to severely slowed nerve conduction velocities. We report the first nerve biopsy of a CMT patient with a *de novo* missense mutation in *NEFL*, and found an axonal pathology with axonal regeneration clusters and onion bulb formations. Our findings provide further evidence that the clinical variation observed in CMT depends on the gene mutated and the specific type of mutation, and we also suggest that *NEFL* mutations need to be considered in the molecular evaluation of patients with sporadic or dominantly inherited CMT.

**Keywords:** peripheral neuropathy; neurofilament light chain; mutation analysis

**Abbreviations:** c. = codon; CMT = Charot-Marie-Tooth; DHPLC = denaturing high performance liquid chromatography; MRC = Medical Research Council; NCV = nerve conduction velocity; NEFL = neurofilament light chain polypeptide

## Introduction

Charcot–Marie–Tooth (CMT) disease represents a group of inherited peripheral neuropathies characterized by progressive weakness and atrophy of the distal limb muscles, sensory

abnormalities and absent tendon reflexes (Dyck *et al.*, 1993). CMT is divided into two major types on the basis of electrophysiological criteria. CMT type 1 (CMT1), the

demyelinating form, is characterized by motor median nerve conduction velocities (NCVs) <38 m/s. CMT type 2 (CMT2), the axonal form, is characterized by normal or slightly reduced motor NCVs, i.e. >38 m/s (Harding and Thomas, 1980). However, in some families, patients have been difficult to classify using strict electrophysiological criteria, leading to the concept of 'intermediate CMT' with motor NCVs between 25 and 45 m/s (Davis *et al.*, 1978).

Inherited peripheral neuropathies show an extensive genetic heterogeneity and mutations have been reported in at least 17 genes (Inherited Peripheral Neuropathies Mutation Database, <http://molgen-www.uia.ac.be/CMTMutations/>). Patients with identical phenotypes may harbour mutations in different genes. Alternatively, mutations in one particular gene may lead to a variety of clinical phenotypes (Boerkoel *et al.*, 2002).

Recently, two missense mutations in the neurofilament light chain polypeptide (*NEFL*) gene causing different CMT phenotypes have been described. A Gln333Pro amino acid substitution in *NEFL* was reported in a multigeneration Russian CMT2E family linked to chromosome 8p21 (Mersiyanova *et al.*, 2000) (OMIM, Online Mendelian Inheritance in Man reference no 162280, <http://www3.ncbi.nlm.nih.gov/omim>). Subsequently, a Pro8Arg mutation was found in a Belgian family in which all 10 affected members were evaluated as having a classical, although severe CMT phenotype. Based on the electrophysiological criteria, most patients in the Belgian family could be diagnosed as having CMT1, while the family as a whole could be classified as intermediate CMT (De Jonghe *et al.*, 2001).

*NEFL* encodes a 62 kDa structural protein, which is one of the most abundant cytoskeletal components of neuronal cells (Friede and Samorajski, 1970). *NEFL* assembles with neurofilaments of higher molecular mass, medium (*NEFM*) and heavy (*NEFH*) chain polypeptides, into intermediate filaments and forms an extensive fibrous network in the cytoplasm of the neuron. Neurofilament accumulation tightly correlates with radial growth of axons during myelination. Neurofilaments determine the axonal diameter and, hence, the conduction velocity of peripheral nerves (Lee and Cleveland, 1996; Arbuthnott *et al.*, 1980).

We investigated the frequency and phenotypic consequences of *NEFL* mutations in a cohort of 323 patients with CMT disease or a related peripheral neuropathy. We detected six novel disease-causing mutations and report the patients' clinical and electrophysiological features. In addition, we present the first histological study of a nerve biopsy of a patient with a *NEFL* mutation.

## Patients and methods

The study included a total of 323 unrelated CMT patients. Based on electrophysiological criteria, 69 patients were diagnosed with CMT1 (motor median NCVs <38 m/s), 104 had CMT2 (motor median NCVs >38 m/s) and 150 patients

had an unspecified peripheral neuropathy based on clinical records. The detailed results of the electrophysiological investigations were not available in the latter group. Informed consent was obtained according to the Declaration of Helsinki and approved by the Institutional Review Board of each laboratory that contributed to this study. In addition, 65 healthy Caucasians were screened as controls for sequence variations found in the present work. Following detection of the mutation in an index patient, additional family members (if available) were analysed in order to determine cosegregation of the disease with the observed sequence variation. Paternity was confirmed for patients BAB1119, BAB1345 and BAB863, who had *de novo* mutations using an Identigene kit (Identigene, Houston, TX, USA).

Standard procedures were used to extract DNA from peripheral blood samples. In all patients, mutations in the most common CMT causing genes (*PMP22* duplication/deletion, and mutations in *PMP22*, *MPZ* and *Cx32*) were excluded prior to this study. The *NEFL* mutation screening was performed using denaturing high performance liquid chromatography (DHPLC). The four exons of *NEFL* were amplified as 10 polymerase chain reaction (PCR) fragments. To improve the sensitivity of the method, primers were designed to amplify overlapping fragments in exons 1 and 3, and were placed at least 15 bases from the exon/intron boundaries. The oligonucleotide primer sequences and analytical conditions are listed in Table 1. All fragments were amplified using HotGoldstar™ DNA polymerase (Eurogentec, Seraing, Belgium) according to the manufacturer's protocol. The products were denatured at 94°C for 3 min, cooled slowly to room temperature and analysed by DHPLC on a WAVE® automated instrument (TRANSGENOMIC, Omaha, USA). Optimal DHPLC conditions were determined using WAVEMAKER™ software version 3 (TRANSGENOMIC) and the chromatograms were analysed using the WAVE™ System Manager Software (TRANSGENOMIC). PCR products with abnormal DHPLC patterns were sequenced on an ABI Prism® 3700 DNA analyser (Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). DNA sequence data were collected and analysed using the ABI DNA Sequencing Analysis 3.6 software and the LASERGENE package (DNASTAR, Madison, USA). Mutations that create or disrupt endonuclease restriction sites were analysed in control individuals using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) (see Table 1), while the other mutations were analysed by direct DNA sequencing.

The numbering of the *NEFL* codons was based on the published amino acid sequence, using the first methionine as a start codon (National Center for Biotechnology Information accession numbers P07196 for the protein sequence and X05608 for the genomic sequence). The *NEFL* mutations were defined according to the guidelines described by den Dunnen and Antonarakis (2000).

**Table 1** Sequence of the oligonucleotide primers and analytical conditions for mutation screening of the *NEFL* gene

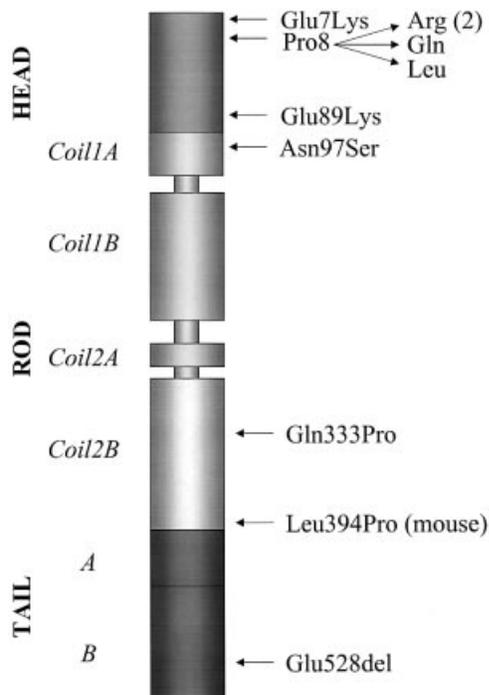
Exon	Segment	Primer sequence (5'→3')		Fragment size (bp)	Annealing temperature (°C)	DHPLC conditions <sup>2</sup>	
		Forward	Reverse			Column temperature (°C)	Eluent B (%)
1	1	CAGAAATCTCGCCTTGG	GATCCAGAGCTGGAGGAGTA	397	57	66/65	69/70
2	2	GCITTAAGCTACTCGGC	GCTCGTACAGCCCGGAAGC <sup>1</sup>	307	60	65	68
3	3	CAGAAACGACCTCAAGTCCATC	CCTCGGGTCTCGGGGTC <sup>1</sup>	338	60	65/66	69/68
4	4	GGAGGAGACCTGGCAACC <sup>1</sup>	GTTCCTGCATGTTCTTGGCGG	325	60	67	64
5	5	CAGATCCAGTACGGCAGAT	GATTTCCAGGGTCTTGGCCT	249	60	65/67	66/63
6	6	GAGAGGCCGCCAAGAACC <sup>1</sup>	ACCCCTGGAAACGCTTCTG	263	53	65	66
2	1	CTAGGCCCTTGCAACTACACTAC	CCTAAGGTTTAAATGGCTGCTG	254	60	56/59	65/61
3	1	GGTACTCAGAGCAAGTTGTG	TTCGGTCTGCTCCTTGGAC <sup>1</sup>	255	57	62	64
3	2	CAGTCCTATCTGATGTCACC <sup>1</sup>	CACCCAGTTTACACTTGAAGTTGC <sup>1</sup>	249	58	63	61
4	4	ACTGGACTTACCCTGGATTTC <sup>1</sup>	CCTGATTTCCGGGAGAAATTATCC <sup>1</sup>	241	56	57/60	64/60

<sup>1</sup>Primer sequence reported previously (Mersyanova et al., 2000). <sup>2</sup>For some PCR amplicons, two different DHPLC conditions were used. Eluent B (0.1 M triethylammonium acetate, 25% acetonitrile) is part of the running buffer system described in the WAVE<sup>®</sup> instrument's manual.

**Table 2** Sequence variations found in the coding region of the *NEFL* gene

Nucleotide change	<i>NEFL</i> exon	Amino acid change	<i>NEFL</i> domain	Index patient	Mode of transmission	Allele frequency	Restriction site change
<b>Disease associated mutations</b>							
c.19G>A	1	Glu7Lys	Head	BAB1795	ND	0/130	No
c.23C>G	1	Pro8Arg	Head	BAB1795	Inherited	0/130	+MbiI
c.23C>T	1	Pro8Leu	Head	BAB1119	<i>De novo</i>	0/130	+AluI
c.23C>A	1	Pro8Gln	Head	BAB1226	Inherited	0/130	<b>+ScaI</b>
c.265G>A	1	Glu89Lys	Head	BAB863	<i>De novo</i>	0/130	<b>+MbolI,</b>
c.290A>G	1	Asn97Ser	Rod	BAB1345	<i>De novo</i>	0/130	<b>+DdeI, -BseMII</b>
c.158_1583delGAG	4	Glu528del	Tail	BUL40-1	Inherited	0/130	No
<b>Polymorphisms</b>							
c.120A>T	1	Ser40Ser	Head	BUL60-1	ND	ND	-Bce83I, +MaeI
c.189G>A	1	Leu63Leu	Head	V122-2	ND	ND	-HaeIV, +MseI
c.420G>A	1	Gln140Gln	Rod	F12A	ND	ND	No
c.670C>T	1	Leu224Leu	Rod	D11B	ND	ND	-Eco57I, -Hyp188I
c.723C>T	1	Tyr241Tyr	Rod	BUL2-1	ND	ND	+MstI, -RsaI
c.1213C>T	3	Ser405Ser	Tail	BUL12-1	ND	ND	+BsrI, -MwoI
c.1405G>A	3	Asp469Asn	Tail	BAB1575	ND	0/130	-FokI, -SecI
c.1459C>T	3	Ala487Ala	Tail	D9B	ND	ND	No

Nucleotide positions are relative to the translation initiation site of the *NEFL* cDNA. Restriction endonucleases used in the study to test for the presence or absence of mutation are highlighted in bold. + creation and - abolition of restriction endonuclease site; ND = not determined.



**Fig. 1** Localization of disease-causing mutations in the NEFL protein in human and mouse. The size of the NEFL protein domains corresponds to the number of amino acids involved. The data are obtained from a public database (NCBI accession number P07196). The Pro8Arg missense mutation in the NEFL head domain resulted from different nucleotide substitutions [c.23C>G and c.22C>A+c.23C>G (De Jonghe *et al.*, 2001)]. The Glu333Pro mutation in the NEFL rod domain (Coil2B) was reported previously (Mersyanova *et al.*, 2000).

The sural nerve biopsy of patient BAB863, performed at the age of 7 years, was analysed using standard morphological procedures for light and electron microscopy.

## Results

### Molecular analyses

We performed mutation screening of the entire coding region, exon/intron boundaries and promoter region of *NEFL* in a cohort of 323 unrelated patients with different CMT types. Using DHPLC and direct DNA cycle sequencing, we found 14 single nucleotide changes, and one trinucleotide deletion in the coding region of *NEFL* in 14 different samples. Seven sequence variations result in silent mutations and seven base substitutions are missense mutations leading to an amino acid change. One deletion of three base pairs causes an in-frame deletion of one amino acid. All sequence variations are summarized in Table 2 and the locations of pathogenic mutations in the NEFL protein are shown in Fig. 1.

Six different missense mutations were detected in *NEFL* exon 1. Three of these occur in the same CpG dinucleotide changing the cytosine at cDNA position 23 (c.23c) to another nucleotide (G, T or A) and generate the amino acid alterations of proline to arginine (Pro8Arg), leucine (Pro8Leu) or

glutamine (Pro8Gln), respectively. The Pro8Arg mutation was found in an American family with one affected parent (BAB1795) and three affected children (BAB1796, 1797 and 1455). Co-segregation of the heterozygous C>G mutation at cDNA position 23 (c.23>G) with the disease phenotype was confirmed by DNA sequencing. In addition, the affected parent (BAB1795) had a second transition (c.19G>A) leading to a Glu7Lys mutation. This mutation was not transmitted to any of the children (data not shown). Since the patient (BAB1795) was adopted, no DNA samples from her parents were available to trace the origin of the second mutation. The Pro8Leu missense mutation was found in an American patient (BAB1119) of Northern European descent. The heterozygous mutation c.23C>T was not detected in either parent (BAB1848 and BAB1849), and thus occurred *de novo* in the proband. Another mutation in codon 8 (Pro8Gln) was observed in the index patient (BAB1226) of a family with affected relatives in three generations and autosomal dominant inheritance. However, no DNA samples from additional family members were available for segregation analysis. The heterozygous c.265G>A transition creates a Glu89Lys missense mutation. It was found in the affected person (BAB863) of a Mexican–American family and no CMT history. The presence of this mutation was tested in both parents and five asymptomatic siblings by DNA sequencing and PCR–RFLP. The Glu89Lys was not found in the other family members, suggesting a *de novo* mutation. A heterozygous c.290A>G substitution causes Asn97Ser in an American patient (BAB1345) with a Vietnamese ethnic background. There were no other affected individuals in the family. Exclusion of this mutation in the proband’s parents by PCR–RFLP and direct DNA sequencing suggests a *de novo* mutation.

In *NEFL* exon 3, we found a missense mutation (c.1405G>A) leading to the Asp469Asn change. It was found in patient BAB1575 belonging to an American family with affected members in several generations. No additional family members were available for DNA analysis.

Finally, a heterozygous 3-bp deletion in exon 4 (c.1581\_1583delGAG) causing an in-frame deletion of glutamic acid at codon 528 (Glu528del) was found in a Bulgarian family (BUL40). DNA sequencing demonstrated co-segregation with the disease phenotype in all four affected members in three generations of the pedigree.

All disease-causing mutations were detected only in CMT patients and in none of 65 unrelated healthy control persons (130 unaffected chromosomes) tested.

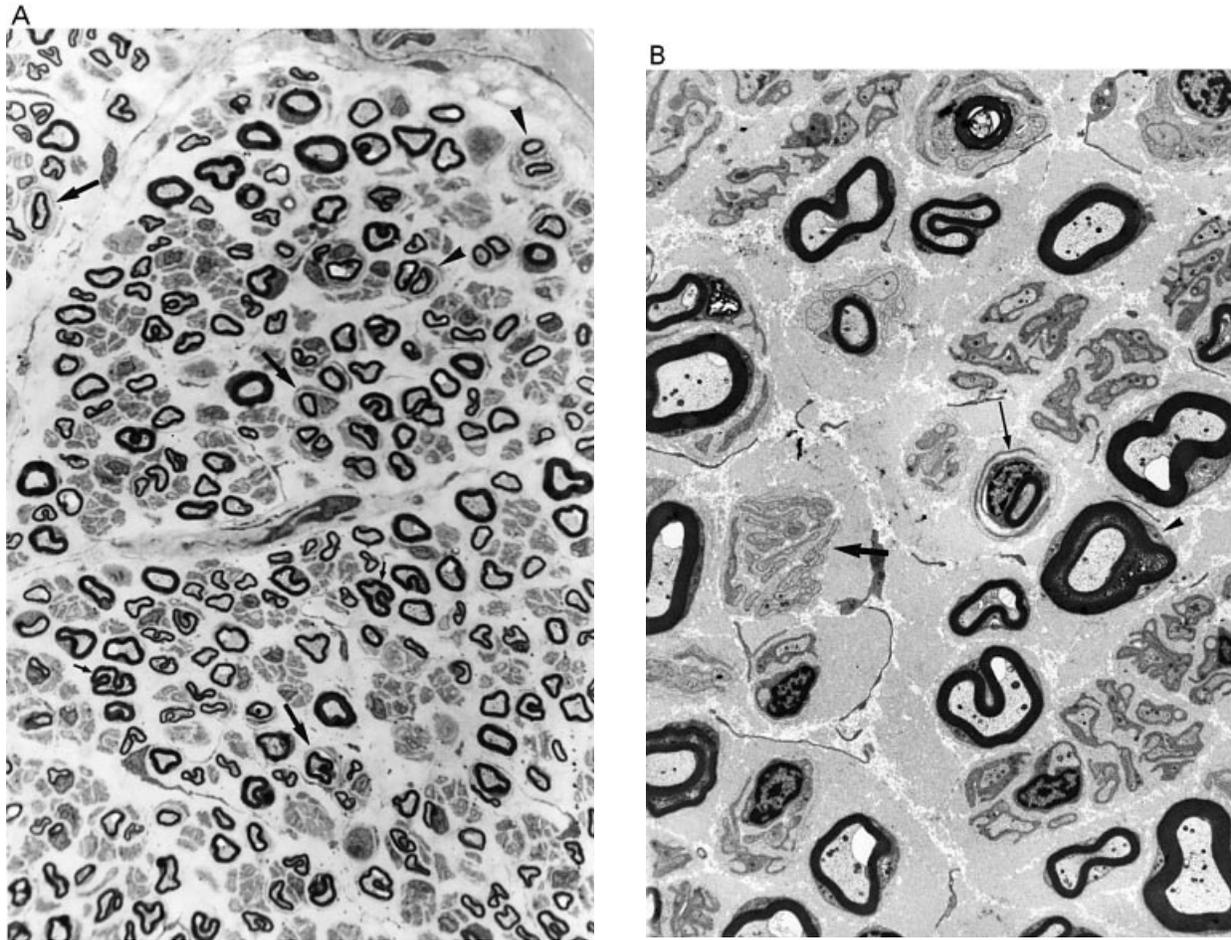
### Clinical and electrophysiological features of the patients with NEFL mutations

Clinical assessment was scored according to the MRC (Medical Research Council) scale. The clinical and electrophysiological features are summarized in Table 3. In all patients, the disease started with gait problems before the age

**Table 3** Clinical and electrophysiological data of patients with newly described NEFL mutations

Mutation	Patient	Present age (years)	Age at onset (years)	Initial symptoms	Muscle weakness		Atrophy		Sensory loss	Reflexes	Pes cavus	Additional symptoms	Motor NCV (m/s)		
					Lower limbs	Upper limbs	Lower limbs	Upper limbs					Median	Ulnar	Peroneal
Pro8Arg	BAB1795	65	11	Gait problems	+++	ND	Yes	Yes	Yes	D	Yes	Tremor, dysphagia	ND	ND	ND
	BAB1797	42	12	ND	++	ND	Yes	Yes	Yes	D	Yes	-	ND	ND	ND
	BAB1796	39	13	ND	++	ND	Yes	Yes	Yes	N	Yes	-	ND	ND	ND
	BAB1455	35	7	ND	++	+	Yes	Yes	Yes	A	Yes	-	38 (4.9)	47 (0.9)	17 (0.1)
	BAB1226	30	<5	ND	+++	+	Yes	Yes	Yes	D	Yes	Contractures of lower limbs	21 (1.0)	33 (1.1)	-
Pro8Leu	BAB1119	30	<2	Delayed walking (18 months)	++	++	Yes	Yes	No	A	Yes	Tremor	15 (2.0)/13 (2.0)	19 (3.0)	23 (0.7)
Glu89Lys	BAB863	15	<2	ND	+	+	ND	ND	Yes	A	Yes	-	27 (3.9)	28 (4.6)	20 (0.5)
	Asn97Ser	BAB1345	16	<1	Delayed motor milestones	+	+	Yes	Yes	D	Yes	Global developmental delay	26 (7.4)	28 (4.0)	20 (0.3)
Glu528del	BUL40	27	12	Gait problems	+	-	Yes	No	Yes	N	Yes	-	30 (11.5)/28 (8.2)	31 (12.8)	3 1(1.1)

Muscle weakness in lower limbs: + = ankle dorsiflexion 4/5 on MRC scale; ++ = ankle dorsiflexion <4/5 on MRC scale; +++ = proximal weakness and wheelchair dependent. Muscle weakness in upper limbs: + = intrinsic hand weakness 4/5 on MRC scale; ++ = intrinsic hand weakness <4/5 on MRC scale; - = no symptoms. Amplitudes of evoked responses, in mV, are in parentheses. If the same nerve was measured on both the right and the left side, the two values are separated by a forward slash. Normal NCV values: motor median and ulnar nerves, >49 m/s; motor peroneal nerve, >41 m/s. Normal amplitude values: motor median and ulnar nerves, >6 mV. ND = not determined. Reflexes: N = normal; D = diminished; A = absent.



**Fig. 2** Neuropathological examination of a sural nerve biopsy performed in patient BAB863 (A) Transverse semithin section showing several thinly myelinated fibres, onion bulb formations (arrows) and axonal regeneration clusters surrounded by an onion bulb (arrowheads). Note few irregularly folded myelin sheaths (small arrows). Magnification:  $\times 799$ . (B) Electron microscopy. General view showing small myelinated fibres. A small onion bulb (arrow) as well as Schwann cell bands (B and big arrow) with or without myelin debris are seen. Note widened Schmidt–Lanterman incisures (arrowhead) and increased collagen. Magnification:  $\times 2464$ .

of 13 years. Some patients had a very early onset and presented with delayed motor milestones. Paresis in the distal parts of the lower limbs varied from mild weakness to a complete paralysis of the distal muscle groups. Many patients used ankle-foot orthoses and the eldest patient (BAB1795), who has a mutation on each allele, was wheelchair dependent. Weakness and atrophy started and predominated in the legs, and sensory loss—a common finding in these patients—usually included all sensory modalities. Tendon reflexes were decreased or absent. All patients had a *pes cavus* deformity. Of the additional symptoms, only tremor was present in more than one patient. Patient BAB1345 had a complex phenotype with poor somatic growth, chronic vomiting, delayed social, language and intelligence milestones, deafness necessitating the use of hearing aids, nystagmus and facial weakness.

The electrophysiological investigations demonstrated that motor NCVs were moderately to severely reduced with the exception of the ulnar nerve in patient BAB1455, which was only slightly slowed (see Table 3). The motor NCVs ranged from 13–38 m/s for the median nerve (normal  $>49$  m/s) and

from 19–47 m/s for the ulnar nerve (normal  $>49$  m/s). The amplitudes of the compound motor action potentials were usually severely reduced. Sensory nerve action potentials were often unrecordable. Concentric needle EMG showed chronic neurogenic alterations.

### ***Histopathological features of the patient with the NEFL Glu89Lys mutation***

Only one sural nerve biopsy was available for histopathologic studies, i.e. patient BAB863 carrying the Glu89Lys mutation. In toluidine blue-stained sections (Fig. 2A), onion bulbs as well as few axonal regeneration clusters sometimes associated with an onion bulb were recognized. There was paucity of large myelinated fibres and a few large fibres had thin myelin sheaths. Irregular foldings of myelin sheaths were observed. Rare linear myelin ovoids were seen in longitudinal sections.

Electron microscopy confirmed the loss of large myelinated fibres ( $>5 \mu\text{m}$ ) and the finding of several clusters of regenerating myelinated fibres. Sporadic demyelinated axons

were found. Onion bulbs (Fig. 2B) were composed of circular or nearly closed layers of extended Schwann cell processes with rare unmyelinated axons and redundant basal lamina. Some myelinated axons were surrounded by a single layer of disrupted basal lamina. There were few irregularly folded myelin sheaths, but no tomacula formation was observed. Denervated Schwann cell complexes contained myelin debris (Fig. 2B). Membranous whorls within some widened Schmidt–Lanterman incisures and several paranodal myelin debris were seen in longitudinally oriented fibres showing normal nodes of Ranvier. Although no morphometric analysis was performed, the number of unmyelinated fibres was obviously decreased. Miniature unmyelinated axons (not illustrated) were seen occasionally and numerous collagen pockets were found. There was also a few retracting Schwann cell processes with remaining basal lamina.

## Discussion

CMT diseases are genetically and clinically heterogeneous. A large number of disease-causing mutations in several genes have been described over the last decade (Inherited Peripheral Neuropathies Mutation Database at <http://molgen-www.uia.ac.be/CMTMutations/>). In this study, we found six pathogenic missense mutations and one 3-bp in-frame deletion in the *NEFL* gene in 323 patients with different CMT phenotypes (i.e. 2%). The disease-causing mutations occurred in patients with different ethnic background, and three patients represent *de novo* cases. Genotype–phenotype correlations in distinct CMT types have often shown an overlap between the demyelinating CMT1 and the axonal CMT2. Mutations predominantly resulting in demyelination may cause concomitant axonal loss, and mutations primarily leading to axonal loss may be associated with demyelination (Boerkoel *et al.*, 2002). Mutations in the *NEFL* gene also result in CMT neuropathies with variable clinical and electrophysiological expression; the Gln333Pro mutation causes CMT2E in a Russian kindred (Mersiyanova *et al.*, 2000), while the Pro8Arg mutation leads to a classical, although rather severe, CMT phenotype in a Belgian family (De Jonghe *et al.*, 2001). The nine patients with *NEFL* mutations described in this report have an early disease onset and are usually severely affected. Based on these clinical characteristics, some of them were diagnosed with Dejerine–Sottas syndrome, a severe variant of inherited peripheral neuropathy (Dyck *et al.*, 1993). The fact that the majority of these mutations are detected as *de novo* mutations in the more severely affected patients suggests that the patients' chances for reproduction are compromised.

Combined evidence from this study and the previously reported Belgian family with the Pro8Arg mutation (De Jonghe *et al.*, 2001) indicates that, in persons with *NEFL* mutations, NCVs are usually within the CMT1 range. Occasionally, patients may have NCVs that approach the normal range. However, the Russian family with the Gln333Pro mutation (Mersiyanova *et al.*, 2000) was reported

as a CMT2 family with limited clinical or electrophysiological data. It remains to be established whether these electrophysiological differences are genuine, and are related to the site of the *NEFL* mutations. However, our genotype–phenotype correlation study suggests that the yield of the *NEFL* mutation screening could be increased markedly by preferentially screening patients with an early onset, severe CMT or Dejerine–Sottas syndrome phenotype with moderately to severely slowed NCVs.

In conclusion, our analyses indicate that *NEFL* mutations can convey a severe peripheral neuropathy phenotype that includes markedly reduced motor NCV. Such mutations may occur *de novo* in sporadic neuropathy patients or segregate with autosomal dominant CMT. *NEFL* mutations should be considered in the evaluation of patients with CMT or related neuropathy.

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