Rare Variants in *MME*, Encoding Metalloprotease Neprilysin, Are Linked to Late-Onset Autosomal-Dominant Axonal Polyneuropathies

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Axonal polyneuropathies are a frequent cause of progressive disability in the elderly. Common etiologies comprise diabetes mellitus, paraproteinaemia, and inflammatory disorders, but often the underlying causes remain elusive. Late-onset axonal Charcot-Marie-Tooth neuropathy (CMT2) is an autosomal-dominantly inherited condition that manifests in the second half of life and is genetically largely unexplained. We assumed age-dependent penetrance of mutations in a so far unknown gene causing late-onset CMT2. We screened 51 index case subjects with late-onset CMT2 for mutations by whole-exome (WES) and Sanger sequencing and subsequently queried WES repositories for further case subjects carrying mutations in the identified candidate gene. We studied nerve pathology and tissue levels and function of the abnormal protein in order to explore consequences of the mutations. Altogether, we observed heterozygous rare loss-of-function and missense mutations in *MME* encoding the metalloprotease neprilysin in 19 index case subjects diagnosed with axonal polyneuropathies or neurodegenerative conditions involving the peripheral nervous system. *MME* mutations segregated in an autosomal-dominant fashion with age-related incomplete penetrance and some affected individuals were isolated case subjects. We also found that *MME* mutations resulted in strongly decreased tissue availability of neprilysin and impaired enzymatic activity. Although neprilysin is known to degrade β-amyloid, we observed no increased amyloid deposition or increased incidence of dementia in individuals with *MME* mutations. Detection of *MME* mutations is expected to increase the diagnostic yield in late-onset polyneuropathies, and it will be tempting to explore whether substances that can elevate neprilysin activity could be a rational option for treatment.

Introduction

Inherited polyneuropathies constitute a clinically and genetically heterogeneous group of neuromuscular disorders. The most common form, Charcot-Marie-Tooth disease (CMT, also known as motor and sensory neuropathy [HMSN]; for a phenotypic description and a discussion of genetic heterogeneity of CMT, see CMT1B [MIM: 118200] and CMT2A1 [MIM: 18210]), is among the most frequent hereditary causes of neurological disability. Onset is usually in childhood, adolescence, or young adulthood. Hallmarks include symmetric foot deformities, slowly progressive weakness and wasting in the distal parts of upper and lower limbs, and length-dependent sensory loss. Positive symptoms such as paraesthesias and pain may occur. The underlying pathological event is demyelinating (primarily affecting Schwann cells, CMT1) or axonal (primarily affecting axons, CMT2) nerve damage.[1] Research into the molecular causes of inherited neuropathies has led to the identification of more than 80 genes harboring disease-causing mutations.[2,3]

Despite the notable progress of gene discovery studies, some forms of CMT are still largely genetically undetermined. The most conspicuous example is late-onset

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CMT2, which starts after the age of 35 years (mean age at onset 57 years) but can be rapidly progressive and lead to severe gait disturbances and wheelchair dependence within few years.[4] Family history is often inconclusive due to late onset of symptoms and affected individuals might be considered as sporadic cases. Due to unremarkable family history, late onset, and rapid progression, delineation from acquired neuropathies such as chronic inflammatory demyelinating polyneuropathy (CIDP) and motor neuron disease is often challenging and correct diagnosis and therapeutic management may be delayed or never established. Although in occasional cases the disease can be explained by mutations in *MPZ* (MIM: 159440), *MFN2* (MIM: 608507), *MARS* (MIM: 156560), or *HSPB1* (MIM: 602195),[5–8] the genetic causes of late-onset CMT still remain unknown in the majority of case subjects.

In order to find molecular causes of late-onset inherited neuropathies, we clinically, genetically, and pathologically studied index case subjects and affected families from Europe and the United States and identified heterozygous mutations in the *membrane metalloendopeptidase* gene (*MME* [MIM: 1205201]) as strong candidates for late-onset CMT2. Intriguingly, a concurrent paper has highlighted bi-allelic *MME* mutations in a series of Japanese families with the rare autosomal-recessive variant of late-onset CMT2.[9] *MME* encodes a zinc-dependent metalloprotease, which is also known as neprilysin, neutral endopeptidase (NEP), cluster of differentiation 10 (CD10), common acute lymphoblastic leukemia antigen (CALLA), and endopeptidase-24.11. Neprilysin is located on the surface of cells in many tissues including the peripheral nervous system, but can also circulate as a soluble form.[10,11] It cleaves and inactivates various neuropeptides and peptide hormones, including glucagon, enkephalins, substance P, neurotensin, oxytocin, and bradykinin.[12] In addition, neprilysin has proteolytic activity toward β-amyloid, suggesting a possible association with Alzheimer disease.[13]

Material and Methods

Study Participants

We enrolled 51 unrelated index case subjects with late-onset peripheral neuropathies (31 of Austrian and 20 of German descent; 34 with a family history and 17 isolated case subjects). They were selected based on the following criteria: progressive peripheral motor and sensory neuropathy with disease onset after age 35 years, axonal or mixed pattern of nerve conduction studies in the lower limbs, exclusion of common acquired risk factors and causes of neuropathies in the elderly (e.g., diabetes mellitus and monoclonal gammopathy), no response to immunosuppressive treatment in those individuals with a tentative diagnosis of a CIDP variant, and exclusion of *GJB1* (MIM: 304040), *MPZ*, and *MFN2* mutations. After identification of *MME* mutations in this screening cohort, we queried whole-exome sequencing (WES) repositories covering genetic data of individuals with neuromuscular disorders and other inherited or acquired conditions and identified additional individuals carrying *MME* mutations. Beyond the index case subjects, we included additional affected and unaffected family members for segregation analysis whenever available.

All individuals were examined by experienced neurologists at their primary care centers. To assess cognitive function, Mini Mental State Examination (MMSE)[14] was performed in selected individuals. For detailed phenotyping of individuals with identified *MME* mutations, we re-assessed diagnostic sural nerve biopsies and studied skin biopsies for evaluation of intra-epidermal free nerve endings using established protocols.[15–17] Subcutaneous adipose tissue biopsies and blood samples were obtained from seven individuals with *MME* mutations and from 25 control individuals without known neuropathies (mean age 67 years, range 49 to 87 years). The study was approved by the ethics committee of the Medical University of Vienna. Written informed consent was obtained from all study participants.

Genetic Studies

For WES, we selected five familial case subjects, three of which were from families AT1, AT2, and AT3 (Figure 1A) and one isolated case of the 51 unrelated index case subjects with late-onset peripheral neuropathies. WES and data analysis were performed with standard methods as previously described.[15] We used Sanger sequencing to screen for *MME* mutations in further individuals. For primer sequences see Table S1. Screening for *MME* copy-number mutations was based on a self-developed multiplex ligation-dependent probe amplification (MLPA) probe set.[18]

Data Retrieval from Exome-Sequencing Databases

Repositories collecting clinical and genetic information from individuals with neurological disorders and other conditions were queried for *MME* variants. The GENESIS database of the University of Miami, Hussman Institute for Human Genomics, covers WES results from more than 3,000 individuals with neuromuscular or neurodegenerative diseases. The WES database of the Helmholtz Centre Munich, Institute of Human Genetics is a collection of more than 7,000 individuals with diverse genetic and acquired diseases. The WES database of the National Hospital for Neurology, London covers sequencing results of 46 individuals with neuromuscular and neurodegenerative disorders. Data were accessed, retrieved, and processed in accordance with institutional data protection standards.

Detection of Amyloid in Subcutaneous Adipose Tissue

Biopsies of subcutaneous adipose tissue from the abdominal wall of six individuals (AT1/III-5, AT1/IV-9, AT2/III-6, AT3/IV-2, AT8/III-2, and AT8/IV-3) with diagnosed polyneuropathy and *MME* mutations and six age-matched controls without known polyneuropathy were fixed in 10% neutral-buffered formalin and embedded in paraffin. After deparaffinization and hydration, 10-μm-thick sections were processed for hematoxylin and eosin, Congo red, and thioflavin (Sigma-Aldrich) staining. For detection of β-amyloid and transthyretin (TTR) type amyloid, we used mouse anti-β-amyloid antibody (6F/3D; Dako) and rabbit anti-transthyretin antibody (EPR3219; LSBio). Immunoreactivity was measured by incubation with a universal biotinylated secondary antibody, streptavidin-conjugated alkaline phosphatase, and Fast Red chromogen (Discovery RedMap Kit; Ventana). Slides were examined by light microscopy, including analysis in polarized light (for Congo red) and with fluorescence microscopy (for thioflavin).

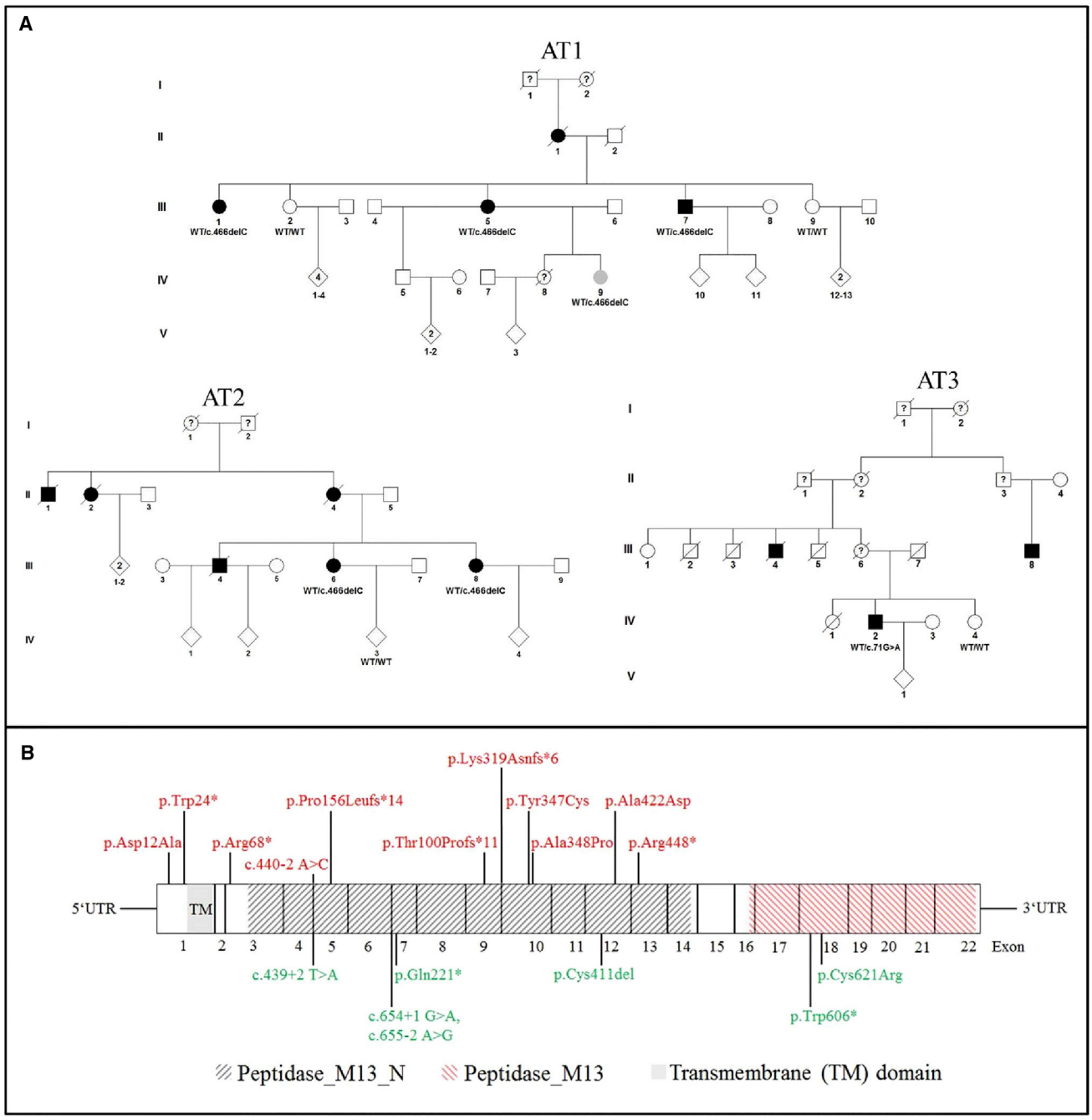

Figure 1. *MME* Mutations Associated with Autosomal-Dominant Late-Onset Peripheral Neuropathies
(A) Families with *MME* mutations identified by WES. Women are represented by circles, men by squares. Individuals with anonymized gender data are represented by diamonds. The symbols of affected individuals are filled (black, clinically affected; gray, subclinically affected), and those of unaffected individuals are empty. Members with unknown clinical status contain a question mark. There was no history of disease in obligate mutation carrier AT3/III-6 until her death at age 80 but neurological examination has not been documented. Individuals' *MME* genotypes are shown below the pedigree symbols.
(B) Schematic representation of neprilysin and distribution of mutations identified in this study and in the recent study of Higuchi et al.[9] Functionally relevant protein domains are shown. Exon numbering is given below the diagram. Variants shown in red color have been associated with late-onset peripheral neuropathies in this study. Variants shown in green color have been reported in individuals with autosomal-recessive late-onset CMT2.[9]

Quantification of Neprilysin Levels

Human subcutaneous adipose tissue (300 mg) from individuals AT1/III-5, AT1/IV-9, AT2/III-6, AT3/IV-2, AT8/III-2, and AT8/IV-3 and six age-matched control subjects without known polyneuropathy was homogenized in lysis buffer (10 mM Tris HCl [pH 7.4], 250 mM sucrose, protease inhibitors [Complete Mini; Roche Diagnostics]) using a Precellys24 homogenizer and the CK14 lysis kit (Bertin Technologies). After centrifugation, the protein

fractions were collected. Plasma was obtained from centrifugation of whole blood anticoagulated with EDTA or citrate. Neprilysin levels in plasma and protein extracts from adipose tissue were determined in duplicate with DuoSet ELISA Development System and DuoSet Ancillary Reagent Kit 2 (R&D Systems), using a FLUOstar OPTIMA microplate reader (BMG Labtech). To normalize neprilysin concentrations in adipose tissue samples, total protein contents were determined with the Micro BCA Protein Assay Kit (Thermo Scientific). Neprilysin concentrations were statistically analyzed with Shapiro-Wilk test, t test, and Mann-Whitney U test.

In Vitro Neprilysin Activity Measurements

The plasmids pCSC-SP-PW-Nep (Addgene 12338) and pCSC-SP-PW-NepX (Addgene 12340) were a gift from Inder Verma.[19] The cDNA encoding human wild-type neprilysin and the inactive mutant c.1754A>T (p.Glu585Val) were amplified by PCR and subcloned via the BamHI and ApaI sites into pcDNA3.1 (Invitrogen). The plasmids encoding the neuropathy-associated mutants c.1040A>G (p.Tyr347Cys) and c.1265C>A (p.Ala422Asp) were generated by site-directed mutagenesis. HEK293 cells were cultured in DMEM containing 4.5 g/L D-glucose, 0.58 g/L L-glutamine, 1 mM sodium pyruvate, and 10% (v/v) fetal bovine serum (FBS) (all materials from Life Technologies). Cells were seeded into 24-well plates and transiently transfected using PolyFect Transfection Reagent (QIAGEN) according to the manufacturer's procedure. After 24 hr, cells were incubated with 50 mM Tris buffer (pH 7.4) containing 5 μM of the neprilysin substrate Abz-GGfL-Agp-RV-EDA-Dnp (Biosyntan) for 15 min. Fluorescence intensity of the cell supernatant was measured using a SpectraMax M5 microplate reader (Molecular Devices; λexc = 320 nm, λem = 420 nm). Neprilysin activity measurements were performed twice in triplicate and results are presented as mean ± standard deviation. For statistical analysis, one-way ANOVA with Bonferroni post hoc test was used.

Animal Studies

Generation of *Mme* knockout mice has been reported earlier.[20] All experiments with animals followed approved protocols by the local authority (Government of Lower Franconia, Germany). Genotypes of mice were determined by PCR on genomic DNA derived from ear biopsies using appropriate primer pairs. For electrophysiological measurements, three 13- to 14-month-old $Mme^{-/-}$ mice and corresponding controls were anesthetized by an intraperitoneal injection of a mixture of ketavet and xylavet (10 μL per g body weight). Body temperature was controlled during the measurements (34°C–36°C). Neurophysiological properties of the left sciatic nerve were measured as described previously.[21] Femoral quadriceps nerves and cutaneous saphenus nerves were dissected from euthanized mutants and age-matched wild-type mice and processed for light and electron microscopy as described earlier.[22] G ratios were determined by dividing the diameter of the axon by the fiber diameter (axon including its myelin sheath). At least 150 fibers per animal were randomly selected and measured. Axons were separated into three different groups according to their respective caliber: small (<3 μm), medium (3–6 μm), and large (>6 μm). For assessment of non-myelinating Remak fibers, at least ten randomly selected non-overlapping visual fields (magnification ×4,100) of the femoral saphenous nerve were inspected in each of the three *Mme*-deficient and WT animals. Quantification of endoneurial macrophages was performed by immunofluorescence on femoral nerve cross-sections according to previously published protocols.[23,24]

Results

Identification of *MME* Mutations in Late-Onset CMT

Comparing WES results of six individuals selected from our cohort of 51 unrelated index case subjects with late-onset polyneuropathy revealed heterozygous truncating mutations in *MME* (GenBank: NM_000902.3) in three individuals: the c.466delC (p.Pro156Leufs*14) was present in individuals AT1/III-1 and AT2/III-6 and the c.71G>A (p.Trp24*) was found in AT3/IV-2 (Figure 1A and Table 1). Segregation was tested and confirmed in all three families (Figure 1A). Although variant c.71G>A (p.Trp24*) is not present in any database (Exome Variant Server [EVS], NCBI dbSNP build 142, 1000 Genomes Project, Exome Aggregation Consortium [ExAC] Browser), the c.466delC (p.Pro156Leufs*14) deletion is found in 5 of 6,251 individuals in the EVS database and in 20 of 60,245 individuals in the ExAC Browser. This variant has previously been observed in neprilysin alloimmunization resulting in neonatal glomerulopathies.[25,26] In these studies, none of the individuals carrying the c.466delC (p.Pro156Leufs*14) mutation was reported with symptoms of polyneuropathy; however, most of them obviously had not yet reached age at onset observed in our late-onset polyneuropathy cohort and neurological and neurophysiological examination had not been part of the study protocol. We sought additional evidence for the relevance of this variant by systematically excluding other potential causes. We made use of the pedigree structure and availability of DNA samples in family AT1 and extended WES to two additional affected individuals (AT1/III-5 and AT1/III-7). In addition to the *MME* mutation c.466delC (p.Pro156Leufs*14), we detected 35 non-synonymous variants shared by the three affected sibs. We excluded segregation of ten of these variants in additional family members by Sanger sequencing. Moreover, none of the additional five index case subjects for whom WES data were available carried a non-synonymous variant in any of these genes. Based on these results, we regarded *MME* as a strong candidate gene for late-onset CMT and continued with targeted Sanger sequencing of *MME* in the 45 additional index case subjects from our series. We found one splice-acceptor site mutation at c.440−2A>C (family DE1) and four rare missense variations at positions c.35A>C (p.Asp12Ala), c.1040A>G (p.Tyr347Cys) (two families), c.1042G>C (p.Ala348Pro), and c.1265C>A (p.Ala422Asp) (families AT4, AT5, AT6, AT7, AT8) (Table 1). All mutations affected highly conserved amino acid residues and were predicted to have deleterious effects on the protein by 4–5/5 in silico prediction scores and 2/2 conservation scores (Table S2). Whenever tested, mutations segregated with the phenotype in the families (Figure S1). MLPA

analysis of the index case subjects of families AT1 and AT2 with identified *MME* mutations and 27 additional individuals with late-onset polyneuropathy not carrying *MME* mutations did not detect *MME* copy-number variations.

MME Mutations Retrieved from WES Databases

Repositories collecting WES data from more than 10,000 individuals with neurological disorders and other conditions (University of Miami, Hussman Institute for Human Genomics; Helmholtz Centre Munich, Institute of Human Genetics; National Hospital for Neurology, Queen Square Hospital, London) contained 16 truncating and 89 missense variants. We retrieved clinical information concerning disease status and family history of 12 individuals carrying truncating *MME* mutations. Ten of these were diagnosed as either polyneuropathy, motor neuron disorder, or sensory ataxia (DE2, SE1, UK1, UK2, US1, US2, US3, US4, US5, US6) (Table 1). Again the mutations segregated in the families (Figure S1) with the exception of family US3 in which two mutation carriers (US3/IV-4, age 55 and US3/V-4, age 36 years) were unaffected by history. However, no detailed information on neurological and electrophysiological examination was available on these individuals. Two individuals carrying truncating *MME* mutations were young children who had an early-onset complicated form of hereditary neuropathy and neurodegeneration with brain iron accumulation, respectively. Clinical data have been published elsewhere.[27,28] No information on further family members was available.

Counts for Allele Carriers in Case and Control Datasets

MME loss-of-function mutations identified in our study were absent or rare (minor allele frequencies [MAF] < 0.02%) in in-house (Helmholtz Zentrum München [HZM] and Hussman Institute for Human Genomics [HIHG]) and public (ExAC and Wellderly) control datasets (Table S3). As predicted for variants associated with late-onset disease, *MME* variants showed lowest frequency in the Wellderly dataset, a series composed of healthy individuals who were older than 80 years. For statistical evaluation, we used two-sided Fisher's exact test to compare cumulative frequencies of *MME* loss-of-function variants between the initial screening cohort consisting of 51 individuals with late-onset CMT2 and control datasets (Table 2). Considering the approximately 20,000 protein-coding genes in the human genome, a Bonferroni-adjusted threshold for testing was set at $p = 0.05/20{,}000 = 2.5 \times 10^{-6}$. *MME* loss-of-function variants were strongly overrepresented among case subjects with late-onset CMT2 ($p = 2.86 \times 10^{-7}$ [odds ratio (OR) 82.55, 95% confidence interval (CI) 29.43–231.54] for the ExAC dataset, $p = 1.1 \times 10^{-6}$ [OR 73.33, 95% CI 21.72–247.52] for HZM, and $p = 9.92 \times 10^{-7}$ [OR 103.17, 95% CI 22.79–467.11] for HIHG samples). In a larger cohort of CMT-affected case subjects (n = 590) from the GENESIS project, not stratified for age at onset or axonal/demyelinating type of neuropathy, we confirmed enrichment of *MME* loss-of-function alleles among case subjects ($p = 2.33 \times 10^{-8}$ [OR 15.54, 95% CI 7.70–31.40] for the ExAC dataset, $p = 1.12 \times 10^{-6}$ [OR 13.81, 95% CI 5.32–35.85] for HZM, and $p = 2.12 \times 10^{-6}$ [OR 19.42, 95% CI 5.25–71.86] for HIHG samples). *MME* missense mutations observed in the late-onset CMT2 cohort were private variants or rare (MAF < 0.01%) in in-house and public control datasets, with the exception of the variant c.1040A>G (p.Tyr347Cys), which appears to be particularly enriched in the German population (Table S3). Again, variants showed lowest frequency in the Wellderly cohort. The statistical analysis of cumulative allele counts for missense and in-frame indel variants (MAF < 0.175% in ExAC samples) did not yield p values below the Bonferroni-adjusted threshold.

Clinical Manifestations of *MME* Mutations

In total, detailed clinical, electrophysiological, and genetic data were available from 19 families and 28 individuals carrying *MME* mutations. A summary is provided in Table 1. Pedigrees of families carrying *MME* mutations are shown in Figures 1A and S1.

Out of 28 case subjects, 22 were diagnosed with CMT or an unspecified polyneuropathy. The phenotype was rather uniform with symptoms starting in the lower limbs. Median age at disease onset was 55 years (range: 30 to 80 years, Table 1). Some individuals reported foot deformity from early childhood, but gait abnormalities, muscle weakness, and sensory disturbances were not noted before the 4th decade. Initially, the majority of affected individuals complained about distal sensory loss and dys- and paraesthesia that started in the toes, later spread out to the feet and then up to the knees. Subsequently, or sometimes as an initial sign, affected individuals noticed weakness in the toes. Atrophy and paresis of foot and toe extensors led to severe gait disturbances after a few years in many subjects (Figure 2A). Deep tendon reflexes became weak and disappeared in the course of the disease. At a higher age, several individuals with *MME* mutations even became wheelchair dependent and muscle weakness and wasting affected the small hand muscles. Neuropathic pain or muscle cramps were a frequent complaint, sometimes from the beginning. Electrophysiological studies showed severe axonal nerve damage of motor and sensory nerves and chronic neurogenic disturbances on EMG (Table 1). Notably, due to the late age of onset and the progressive course of the disease, some individuals were initially diagnosed as CIDP but anti-inflammatory treatment did not result in any improvement of the symptoms.

Individual UK2/II-1, initially diagnosed with sensory ataxia, had a complex phenotype consisting of axonal sensorimotor neuropathy, dysarthria, abnormal smooth pursuit eye movement, and gait and limb ataxia. The three affected brothers of family SE1 (SE1/II-1, SE1/II-3, SE1/II-5) were diagnosed as having hereditary motor neuropathy (HMN). Mild sensory disturbances became evident later in the course of the disease and deep tendon

Table 1. Phenotype of Families with Mutations in *MME*

Family/ Individual	AO (Years)	Clinical Diagnosis	Initial Symptoms/ Disease Course	Additional Symptoms/ Remarks	MMSE (Age in Years)	PTR	PeronealNCV (m/s) - CMAP (mV)	SuralNCV (m/s) - SNAP (μV)	*MME* Mutation
AT1/III-1	65	CMT2	unstable gait, sensory loss in feet/progressive weakness and sensory disturbances in LL, mild weakness in hands, wheelchair bound	–	ND	absent	ND	no response	c.466delC (p.Pro156Leufs*14)
AT1/III-5	80	CMT2	unstable gait, cramps in LL/ rapid progression of distal weakness and sensory loss in LL, later weakness in hands, wheelchair bound	hearing loss since age 30	28/30 (90)	absent	no response	no response	c.466delC (p.Pro156Leufs*14)
AT1/III-7	58	CMT2	problems lifting the toes, distal muscle atrophy, pins, needles, and numbness in toes/progressive weakness in LL	hearing loss at higher age	ND	reduced	no response	31.0–3.0	c.466delC (p.Pro156Leufs*14)
AT1/IV-9	NK	PNP	frequent cramps in feet, unable to walk on heels	subclinically affected	29/30 (66)	preserved	39.9–7.0	43.3–10.2	c.466delC (p.Pro156Leufs*14)
AT2/III-6	62	CMT2	sensory loss and weakness in toes/progressive weakness in LL, muscle cramps in UL and LL	–	27/30 (78)	absent	40.0–0.8	ND	c.466delC (p.Pro156Leufs*14)
AT2/III-8	55	CMT2	sensory loss and weakness in toes/progressive weakness and sensory loss in LL, mild weakness in hands, prominent burning pain	mild hearing loss	ND	absent	no response	ND	c.466delC (p.Pro156Leufs*14)
AT3/IV-2	60	CMT2	unstable gait/progressive weakness and sensory disturbances in LL, mild weakness in hand muscles, noticed pes cavus years before onset	asymptomatic mother died at age 80	29/30 (83)	absent	no response	ND	c.71G>A (p.Trp24*)
AT4/II-2	69	CMT2	unstable gait/paraesthesia and hyperaesthesia in LL, progressive gait disturbances, severe paresis of foot and toe extensors at age 76	mild pes cavus since early adulthood	29/30 (74)	absent	no response	34.9–5.4	c.35A>C (p.Asp12Ala)
AT5/III-2	45	CMT2	hypaesthesia and dysaesthesia in LL/ progressive gait disturbances, weakness of foot and toe extensors and to milder of plantar flexors	mild bilateral pes cavus	ND	ND	no response	ND	c.1040A>G (p.Tyr347Cys)

(*Continued on next page*)

Table 1. *Continued*

Family/ Individual	AO (Years)	Clinical Diagnosis	Initial Symptoms/ Disease Course	Additional Symptoms/ Remarks	MMSE (Age in Years)	PTR	PeronealNCV (m/s) - CMAP (mV)	SuralNCV (m/s) - SNAP (μV)	*MME* Mutation
AT6/III-2	55	CMT2	pain and cramps in LL, sensory disturbances/ progressive gait disturbances, weakness of foot and toe extensors	mild pes cavus, mild diabetes	ND	reduced	no response	no response	c.1040A>G (p.Tyr347Cys)
AT7/III-2	60	CMT2	unsteady gait/progressive weakness of foot and toe extensors, complete paresis at age 78, paraesthesia and hypaesthesia in LL, moderate weakness in small hand muscles	–	ND	absent	no response	no response	c.1042G>C (p.Ala348Pro)
AT7/IV-1	50	CMT2	hypaesthesia in feet/ weakness in foot and toe extensors, mild weakness in hands	–	ND	absent	no response	37.0 – 6.1	c.1042G>C (p.Ala348Pro)
AT8/III-2	40	CMT2	hypaesthesia in feet/ progressive sensory loss in LL, weakness of distal LL, complete paresis at age 84, mild atrophy in hand muscles	diabetes mellitus since age 50, mild hearing loss	30/30 (84)	absent	no response	ND	c.1265C>A (p.Ala422Asp)
AT8/IV-3	37	PNP	hypaesthesia in feet/ progressive sensory disturbances, atrophy of small foot muscles, no weakness at age 45	–	30/30 (45)	reduced	42.0–5.0	51.0–10.0	c.1265C>A (p.Ala422Asp)
DE1/III-1	57	CMT2	paraesthesia and pain in feet/distal weakness in LL, marked progression from age 60, walking aids from age 65, no weakness in UL	–	ND	absent	43.7–1.5	32.9–5.6	c.440−2A>C
DE2/II-1	33	ALS	fasciculations and weakness in left UL/ progression of weakness in both arms, stiffness in LL, frequent falls	bulbar signs, individual still alive after > 10 years	ND	brisk	ND	ND	c.298_298delA (p.Thr100Profs*11)
SE1/II-1	46	HMN	painful muscle cramps/ progressive symmetrical paresis in distal LL from age 54, mild distal sensory loss in LL, wheelchair bound	dysphagia and dysarthria, gynecomastia	ND	ND	greatly reduced	normal	c.466delC (p.Pro156Leufs*14)

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Table 1. ***Continued***

Family/ Individual	AO (Years)	Clinical Diagnosis	Initial Symptoms/ Disease Course	Additional Symptoms/ Remarks	MMSE (Age in Years)	PTR	PeronealNCV (m/s) - CMAP (mV)	SuralNCV (m/s) - SNAP (μV)	*MME* Mutation
SE1/II-3	47	HMN	muscle cramps in legs/ subsequently, paresis of dorsiflexion of both feet, fasciculations, progressive walking difficulties	gynecomastia, elevated CK, no sensory loss	ND	ND	reduced	NCV slightly reduced	c.466delC (p.Pro156Leufs*14)
SE1/II-5	46	HMN	insidious paresis of the left leg/after 2 years bilateral foot drop, progression of distal LL weakness, frequent falls, walks with a cane	gynecomastia, reduced vibration sense	ND	absent	ND	ND	c.466delC (p.Pro156Leufs*14)
UK1/II-1	70	CMT2	difficulty walking, thin ankles since childhood, good at sports until age 70/ progressive weakness in UL and LL	myeloproliferative disease, RLS	ND	absent	24.0–0.6	30.0–1.0	c.466delC (p.Pro156Leufs*14)
UK1/III-1	39	CMT2	toe weakness and numbness, foot drop, slight tremor in hands/ progression of distal weakness and wasting in LL, later also in UL	–	ND	absent	33.0–0.1	no response	c.466delC (p.Pro156Leufs*14)
UK2/II-1	68	sensory ataxia	unsteadiness, numbness in the feet/progression of gait unsteadiness due to ataxia but no muscle weakness, UL ataxia, dystonia, and tremor	dysarthria, abnormal pursuit eye movement, mild cognitive deficit	ND	absent	ND–1.1	no response	c.466delC (p.Pro156Leufs*14)
US1/III-1	65	CMT2	sensory disturbances in LL, back pain/considerable progression of foot drop within 3 years, burning pain, jerking in legs	initial diagnosis CIDP	ND	absent	LL: axonal	LL: axonal	c.466delC (p.Pro156Leufs*14)
US2/II-1	late 60s	ALS	sporadic bulbar-onset ALS with frontotemporal dementia	negative test for *C9ORF72* expansion	ND	NK	NK	NK	c.466delC (p.Pro156Leufs*14)
US3/IV-2	40	CMT2	weakness and sensory loss/ progressive distal muscle weakness in UL and LL	–	ND	absent	no response	no response	c.957delG (p.Lys319Asnfs*6)
US4/III-1	53	CMT2	numbness, tingling, and pain in legs/progressive weakness of ankle extensors and flexors and mild weakness in hand at age 68	high-arched feet	ND	preserved	no response	no response	c.202C>T (p.Arg68*)

(*Continued on next page*)

Table 1. *Continued*

Family/ Individual	AO (Years)	Clinical Diagnosis	Initial Symptoms/ Disease Course	Additional Symptoms/ Remarks	MMSE (Age in Years)	PTR	PeronealNCV (m/s) - CMAP (mV)	SuralNCV (m/s) - SNAP (μV)	*MME* Mutation
US5/III-2	30	CMT2	progressive weakness and imbalance, paraesthesia, and numbness in legs/ progressive pain in feet, atrophy of intrinsic hand muscles at age 62	possible history of polio-myelitis at age 6	ND	absent	diminished amplitudes	no response	c.202C>T (p.Arg68*)
US6/II-1	49	CMT2	pain mainly in feet, numbness, bilateral foot drop/progressive weakness in legs, sensitivity of pain in hands	history of tinnitus, heart disease	ND	reduced	35.0–1.0	no response	c.1342 C>T (p.Arg448*)

Abbreviations are as follows: ALS, amyotrophic lateral sclerosis; AO, age at onset; CMAP, compound motor action potential; CMT2, Charcot-Marie-Tooth disease type 2; HMN, hereditary motor neuropathy; LL, lower limbs; MMSE, mini mental state examination; NCV, nerve conduction velocity; ND, not done or not documented; NK, not known; PNP, peripheral neuropathy; PTR, patellar tendon reflex; RLS, restless legs syndrome; SNAP, sensory nerve action potential; UL, upper limbs. Information on ethnic origin can be deduced from the family pedigree identifiers: AT, Austria; DE, Germany; SE, Sweden; UK, United Kingdom; US, United States of America.

reflexes disappeared over time. One individual had mild bulbar signs and all of them showed gynecomastia. An expansion of the CAG triplet repeat in the *androgen receptor* gene (AR [MIM: 313700]) was excluded. Individual DE2/II-1 was initially diagnosed with ALS at the age of 33 years, but subsequently disease progression was slow and he was still alive 13 years after the diagnosis of ALS had been made. Individual US2/II-1 was reported as a case of ALS with bulbar disease onset and frontotemporal dementia. We were not able to retrieve further information on this case subject.

Sural Nerve and Skin Biopsies

On semithin sections, a sural nerve biopsy specimen of individual US3/IV-2 showed a prominent reduction of large and medium myelinated axons (Figures 2B and 2C). Several clusters of axonal regeneration were present. In general, myelin sheaths of remaining myelinated axons were of normal thickness. No myelin or axonal debris was present. There were no inflammatory infiltrates. Electron microscopy was carried out on two blocks. In addition to the loss of large, myelinated axons and clusters of regenerated axons that were seen by light microscopy, one could find examples of myelinated axons that were partly surrounded by crescents of Schwann cell processes (rudimentary "onion bulbs"). In addition, unmyelinated axons were typically found as single axons associated with an ensheathing Schwann cell, and some Schwann cells were not associated with any axons. Some denervated Schwann cells formed collagen pockets (Figure 2D).

Immunostaining for intraepidermal free nerve endings in skin biopsies from the abdominal wall revealed no qualitative or quantitative abnormalities (Figure S2). There were no detectable differences between age-matched control samples and individuals with truncating and missense *MME* variants. The subepidermal plexus appeared normal as well.

Assessment of Amyloid Deposits in Tissues

Congo red, thioflavin, and β-amyloid staining of sections of subcutaneous adipose tissue biopsies from the abdominal wall of five individuals with *MME* truncating and missense mutations (AT1/III-5, AT1/IV-9, AT2/III-6, AT3/IV-2, and AT8/IV-3) revealed no formation of amyloid deposits. A tissue specimen from individual AT8/III-2 contained several Congo red- and thioflavin-positive protein deposits that stained negative for β-amyloid but were strongly positive for transthyretin. Because of the absence of amyloid deposition in the subcutaneous adipose tissue biopsy specimen of his son (individual AT8/IV-3) and a negative test result for *TTR* (MIM: 176300) mutations, familial transthyretin amyloidosis (MIM: 105210)[29] was regarded as unlikely. Individual AT8/III-2 (aged 84 years) is probably best considered a case of senile systemic amyloidosis, which is frequent in the elderly population (up to 25% in individuals aged 80 years or older) and is caused by the deposition of wild-type transthyretin and not

Table 2. Cumulative Allele Frequencies of *MME* Variants in Case and Control Subjects

	Number of Samples	Loss-of-Function	Missense and Inframe Indel
Case Subjects			
Late-onset CMT2	51	4 (3.922%)	5 (4.902%)
GENESIS all CMT	590	9 (0.763%)	13 (1.102%)
Control Subjects			
In-house German HZM	823	0	11 (0.668%)
In-house international HZM	7,190	8 (0.056%)	89 (0.619%)
In-house international HIHG	3,793	3 (0.040%)	50 (0.659%)
ExAC international	60,706	60 (0.049%)	984 (0.810%)
Wellderly	534	0	3 (0.281%)

Definitions are as follows: late-onset CMT2, initial screening cohort used in this study (51 Austrian and German index case subjects); GENESIS all CMT, unselected case subjects with assumed inherited polyneuropathies contained in the GENESIS project; in-house German HZM, individuals of German origin in the WES repository of the Helmholtz Zentrum München; in-house international HZM, individuals with various genetic and acquired disease (except polyneuropathies) contained in the WES repository of the Helmholtz Zentrum München; in-house international HIHG, individuals with various genetic and acquired disease (except polyneuropathies) contained in the WES repository of the Hussman Institute for Human Genomics; ExAC international, all individuals in ExAC; Wellderly, healthy elderly individuals of European extraction. For missense changes, variants with a minor allele frequency (MAF) < 0.175% in ExAC international were included.

commonly associated with sensorimotor polyneuropathy.[30] Paraffin sections of a sural nerve biopsy of individual AT2/III-6 were negative for amyloid deposition.

Neprilysin Levels in Adipose Tissue and Blood Plasma Samples

Subcutaneous adipose tissue biopsies obtained from six individuals with truncating and missense *MME* mutations (AT1/III-5, AT1/IV-9, AT2/III-6, AT3/IV-2, AT8/III-2, and AT8/IV-3) contained significantly lower concentrations of neprilysin (2.36 ± 0.68 ng/mg total protein; n = 6) than those obtained from control individuals (6.76 ± 2.30 ng/mg total protein; n = 6; p = 0.001; Figure 3A). As reported earlier,[31,32] measurement of soluble neprilysin levels in blood plasma yielded wide interindividual variability and several samples had neprilysin levels below the lower limit of the analytical range of the assay (125 pg/mL). Neprilysin levels in EDTA plasma from individuals with *MME* mutations (AT1/III-5, AT1/IV-9, AT2/III-6, AT3/IV-2, AT4/II-2, AT8/III-2, and AT8/IV-3) were significantly reduced (median: 0 ng/mL; n = 7; 5/7 samples below the detection limit) as compared to the respective control subjects (median: 1.3 ng/mL; n = 24; 3/24 samples below the detection limit; p = 0.003) (Figure 3B). Citrate plasma of individuals with *MME* mutations also contained significantly lower neprilysin levels (median: 0 ng/mL; n = 7; 4/7 samples below the detection limit) than control plasma (median: 1.2 ng/mL; n = 25; 3/25 samples below the detection limit; p = 0.009) (Figure 3C).

Measurement of Neprilysin Activity In Vitro

Neprilysin activity was determined in HEK293 cells transiently transfected with plasmids encoding wild-type human neprilysin and mutants c.1040A>G (p.Tyr347Cys), c.1265C>A (p.Ala422Asp) (found in individuals with neuropathy), and c.1754A>T (p.Glu585Val) (artificial catalytically inactive mutant). As expected, the artificial control mutant completely abolished catalytic activity toward the substrate Abz-GGfL-Agp-RV-EDA-Dnp above the background level (empty vector control). The neuropathy-associated neprilysin mutants showed variable consequences: c.1265C>A (p.Ala422Asp) resulted in drastic reduction of neprilysin activity, whereas c.1040A>G (p.Tyr347Cys) had a less prominent but statistically significant effect (Figure 3D).

Examination of *Mme*-Deficient Mice

13- to 14-month-old homozygous $Mme^{-/-}$ mice were overtly normal in appearance and size. Mutant mice did not show obvious abnormalities in motor performance or coordination. Nerve conduction studies revealed no significant differences between mutant and control animals (Figure S3). Femoral quadriceps nerves and cutaneous saphenous nerves of three 13- to 14-month-old mutants appeared grossly normal when compared with nerves from age-matched wild-type animals. Examination of semithin sections revealed normal myelinated nerve fiber density and nerve fiber size distribution. However, myelinated fibers appeared more densely packed with smaller extracellular spaces. This observation was correlated to unusually thick myelin sheaths, particularly of large and medium-sized calibre axons. The occurrence of more prominent myelin sheaths in mutant mice was supported by smaller g-ratios in the group of mutant medium-sized calibre axons measuring 3–6 μm (WT: 0.72 ± 0.01; $Mme^{-/-}$: 0.70 ± 0.01). In addition, smaller internodal, non-compact myelin domains, like Schmidt-Lantermann incisures, were obvious. These mild but recognizable abnormalities could be confirmed by transmission electron microscopic investigations (Figure 4A). Moreover, an investigator (R.M.) unaware of the respective genotype (WT or $Mme^{-/-}$) could unequivocally identify the *Mme*-deficient mice by electron

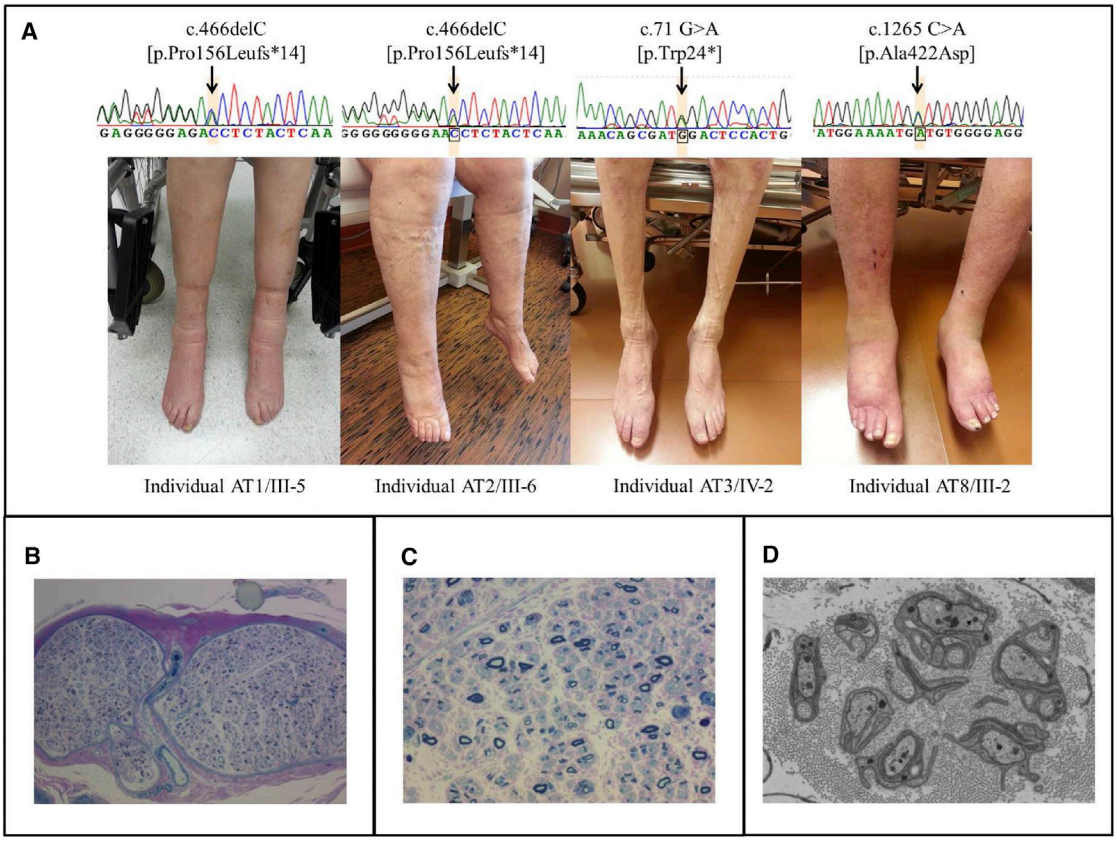

Figure 2. Clinical and Neuropathological Findings in Individuals with *MME* Mutations
(A) Distal muscle wasting and foot drop in individuals carrying heterozygous mutations in *MME*. Shown are individuals AT1/III-5 and AT2/III-6 (c.466delC [p.Pro156Leufs*14]), individual AT3/IV-2 (c.71G>A [p.Trp24*]), and individual AT8/III-2 (c.1265C>A [p.Ala422Asp]). Sequencing electropherograms displaying genotypes are shown above the clinical photographs. Note that reverse complement sequences are provided for the c.466delC (p.Pro156Leufs*14) single-nucleotide deletion.
(B–D) Severe axonal neuropathy in the sural nerve biopsy of individual US3/IV-2.
(B and C) The density of large-caliber myelinated axons is severely reduced while small myelinated fibers (Aδ) look largely normal in number. Semithin sections, toluidine blue staining.
(D) Electron microscopy revealed denervation of non-myelinating Schwann cells with collagen pockets and empty Schwann cell units.

microscopy due to the presence of abnormally shaped bundles of unmyelinated fibers. In wild-type mice, unmyelinated small calibre axons were surrounded and separated from each other by cytoplasmic protrusions of Schwann cells (Remak cells). In Mme-deficient mice, such protrusions were often missing and axonal profiles appeared irregularly shaped (Figure 4B). The axons often showed direct contacts with neighboring axons. Profiles indicative of degenerative features (e.g., onion bulb cells, denervated Schwann cells, myelin degeneration), as seen in other models for inherited polyneuropathies, were not found. Immunocytochemistry using F4/80 antibodies as a marker for macrophages showed cell numbers within normal limits and slim cell shapes indicating a resident rather than an activated state, comparable to wild-type nerves.

Discussion

In this study, we show that heterozygous mutations in *MME* predispose individuals to late-onset axonal neuropathies. We identified 11 different *MME* mutations: 7 of these were loss-of-function alleles (nonsense, frameshift, splice site) and 4 were missense variants (Figure 1B). Family studies showed that *MME* variants typically segregated with disease according to an autosomal-dominant model with age-dependent or occasionally incomplete penetrance, which has been previously documented in other types of inherited neuropathies as well.[33–36]

Identified *MME* mutations were unique to case subjects or contained in in-house and public databases with minor allele frequencies $< 0.02\%$, except for the more prevalent c.1040A>G (p.Tyr347Cys). We observed overrepresentation of each single *MME* variant among individuals with late-onset inherited neuropathies compared to any control dataset (Table S3). Moreover, the gene-based burden analysis demonstrated a significant association of *MME* loss-of-function variants with late-onset neuropathies compared to control subjects (Table 2). Notably, no loss-of-function variants were observed in a series of >800 in-house healthy German control subjects (largely excluding selection bias in our Austrian and German screening

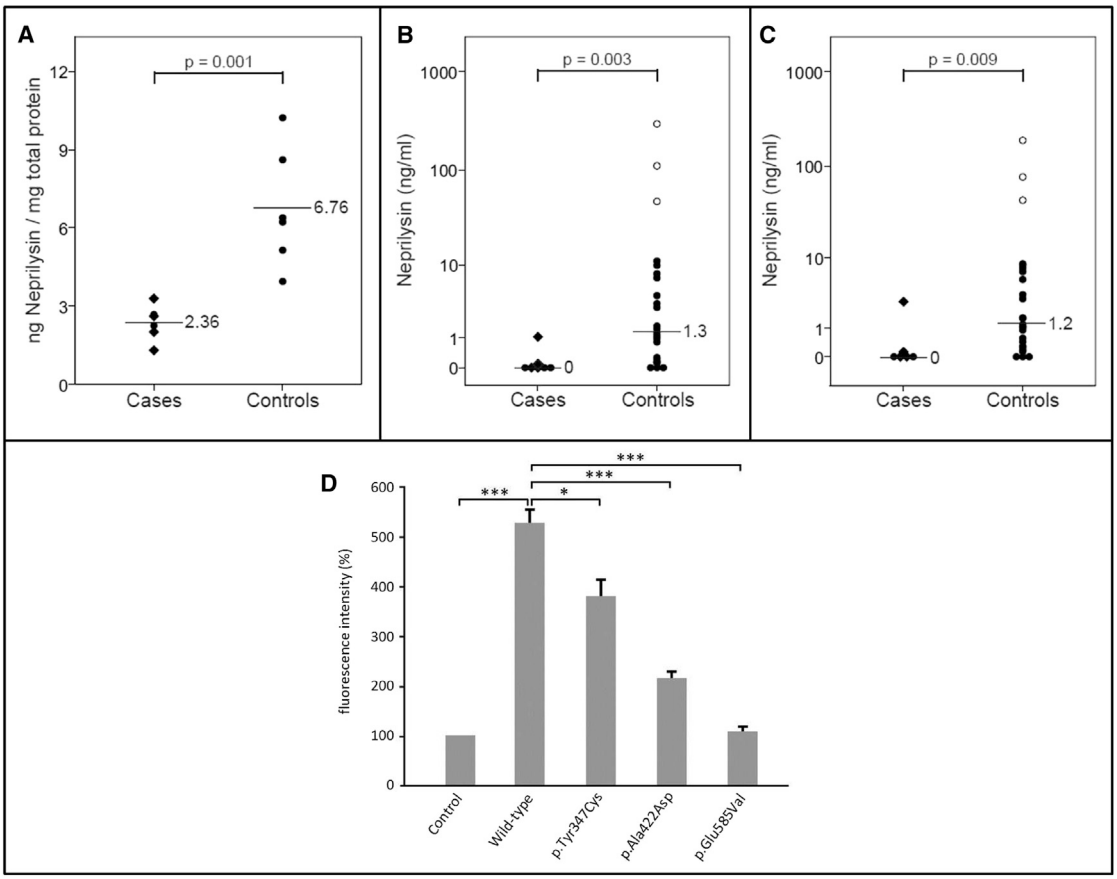

Figure 3. Neprilysin Levels in Biological Samples and Measurement of Neprilysin Activity In Vitro

(A–C) Neprilysin levels in affected individuals with *MME* mutations and control subjects.

(A) Dot plots comparing neprilysin levels in subcutaneous adipose tissue obtained from individuals with *MME* mutations (n = 6) and from healthy age-matched control subjects (n = 6). The symbols represent neprilysin concentrations for the individuals in each group and the bars indicate the respective mean values. In the cases group, dots represent individuals with missense mutations and diamonds represent individuals with loss-of-function mutations. After verification of normal distribution (Shapiro-Wilk test), the data were analyzed with an unpaired two-sided t test. The exact p value is given.

(B and C) Dot plots comparing neprilysin levels in blood plasma obtained from affected individuals with *MME* mutations and from healthy age-matched control subjects.

(B) EDTA plasma. Case subjects, n = 7; control subjects, n = 24.

(C) Citrate plasma. Case subjects, n = 7; control subjects, n = 25.

The symbols represent neprilysin concentrations for the individuals in each group and the bars indicate the respective median values. Empty dots were considered outliers and were not used for statistical analysis. In the cases group, dots represent individuals with missense mutations and diamonds represent individuals with loss-of-function mutations. Since measured values were not normally distributed (Shapiro-Wilk tests), the data were analyzed with a Mann-Whitney U test. The exact p values are given.

(D) Measurements of in vitro neprilysin activity using HEK293 cells transiently transfected with plasmids encoding human wild-type neprilysin, the neuropathy-related mutants c.1040A>G (p.Tyr347Cys) and c.1265C>A (p.Ala422Asp), and the artificial catalytically inactive neprilysin mutant c.1754A>T (p.Glu585Val). Fluorescence intensity was measured at 420 nm (λexc = 320 nm) after cells were incubated with 50 mM Tris buffer (pH 7.4) containing 5 μM of the neprilysin substrate Abz-GGfL-Agp-RV-EDA-Dnp. HEK293 cells transfected with an empty pcDNA3.1 vector served as control. Measurements were performed twice in triplicate and results are presented relatively to control cells as mean ± standard deviation (*$p < 0.05$, ***$p < 0.001$).

cohort) and in the Wellderly dataset, a series composed of >500 healthy Europeans who were older than 80 years (Tables 2 and S3). Some *MME* variants identified in our study emerge with detectable frequency in control datasets; this probably reflects age-related incomplete penetrance. In particular, the status of c.1040A>G (p.Tyr347Cys) remains controversial despite 8-fold overrepresentation among case subjects and will require replication or reclassification as a benign variant in future studies. On the other hand, it is conceivable that *MME* variants may also contribute to the etiology of conditions beyond CMT2, most plausibly seemingly "sporadic" late-onset peripheral neuropathies (see below) or motor neuron diseases. However, in keeping with the broad expression of this gene and the pleiotropy of proteolytic targets of neprilysin, other pathologies might be considered as well. Moreover, we cannot exclude that the penetrance of *MME* mutations has been overestimated in our family-based study due to ascertainment bias inherent in a familial sample (preferential recruitment of families with multiple affected members). All this could

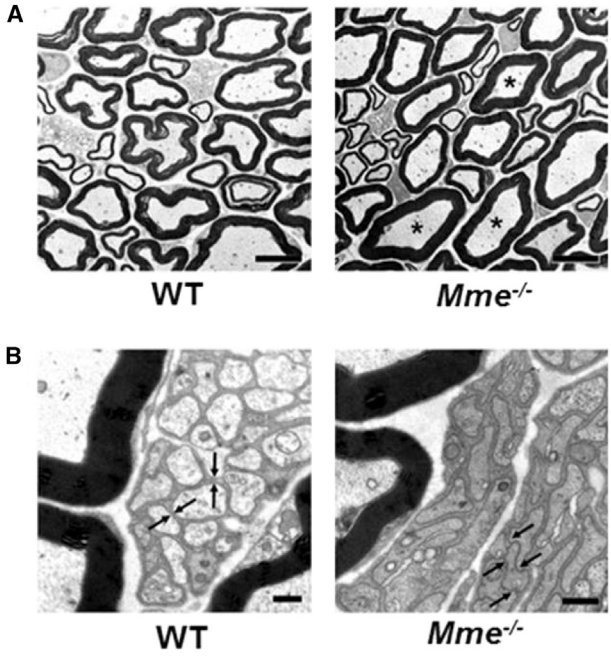

Figure 4. Electron Microscopy of Femoral Nerves of 13- to 14-Month-Old Wild-Type and *Mme*$^{-/-}$ Mice
(A) Some large-caliber myelinated axons in *Mme*$^{-/-}$ mice (right, asterisks) show a thicker myelin sheath than usually found in wild-type mice (left). Scale bars represent 5 μm.
(B) Axons of Remak fibers in wild-type mice (left) are usually separated by Schwann cell processes (arrows). Such separating Schwann cell protrusions are rare in *Mme*$^{-/-}$ mice (right) and axons often show direct contacts with neighboring axons (arrows). Scale bars represent 0.5 μm.

explain why minor allele frequencies are higher in control datasets than anticipated from the assumed prevalence of *MME*-associated CMT2 and CMT neuropathies in general. Notably, beyond *MME*, there are other examples of over-representation of variants causing adult-onset conditions in such databases: the pathogenic *TTR* mutation c.148G>A (p.Val50Met), causing familial transthyretin amyloidosis, a late-onset autosomal-dominant disorder, has a frequency of 18/60,000 in ExAC. However, the European prevalence of amyloidosis including more common secondary, non-hereditary forms was estimated at only 47/100,000[37] and it is unlikely that more than half of all cases of amyloidosis are related to a single *TTR* mutation as one may erroneously conclude from ExAC. Pathogenic *C9ORF72* (MIM: 614260) repeat expansions causing frontotemporal dementia and amyotrophic lateral sclerosis (FTDALS1 [MIM: 105550]) have been observed in 11/7,579 control samples in a recent study from the UK.[38] The authors of this study calculated a lifetime risk of *C9ORF72*-associated FTDALS of about 1 in 2,000 which is considerably lower than the prevalence of large *C9ORF72* expansions in the UK population.

Assessment of subcutaneous adipose tissue and blood samples of individuals with *MME* loss-of-function and missense mutations revealed markedly reduced tissue availability of neprilysin compared to control subjects. In addition, we show that at least two disease-related *MME* missense mutations, c.1040A>G (p.Tyr347Cys) and c.1265C>A (p.Ala422Asp), resulted in variably decreased enzymatic activity for a test substrate in vitro. These data suggest that mutations identified in our cohort result in haploinsufficiency for *MME* which contrasts with autosomal-recessive inheritance of *MME*-related neuropathies in Japanese families where obligatory heterozygous carriers of loss-of-function mutations were reportedly healthy.[9] Therefore, haploinsufficiency alone may not comprehensively explain emergence of the neurological phenotype in our families and as yet unknown genetic and environmental factors are expected to be involved. However, by WES and MLPA of *MME*, we did not find evidence for pseudo-dominant inheritance of *MME* variants or intralocus modifiers in index case subjects of several of our families. Digenic inheritance is also not supported by our WES data as affected individuals from different families did not share variants in a second gene that may precipitate the disease if combined with an *MME* mutation. These studies are naturally not exhaustive and further, probably omics-based approaches may ultimately pinpoint determinants of disease manifestation in heterozygous *MME* mutation carriers. On the other hand, heterozygous and bi-allelic loss-of-function mutations may also have similar outcomes as has been suggested for *MFN2* and *HSPB1* mutations causing other forms of hereditary neuropathy.[39–42] In such a scenario, the earlier median age at onset in Japanese individuals with bi-allelic mutations (49 years[9] versus 55 years in our cohort) may reflect a gene dosage effect with individuals carrying bi-allelic mutations being more strongly affected. Finally, we cannot exclude that the mutant proteins are synthesized at a low level in our case subjects and have acquired characteristics of a dominant-negative nature or that the readout for neprilysin function used here is not the (most) relevant aspect leading to disease.

Although degeneration of myelin sheaths or axons is usually considered a hallmark feature in peripheral neuropathies and was also observed in the nerve biopsy of an individual with an *MME* mutation (Figures 2B–2D), mice deficient in neprilysin showed no obvious degenerative changes in peripheral nerves. Such discrepancies between human and mouse are not restricted to *MME* mutations[43–45] and might relate to species differences, e.g., the short life-span of mice and reduced susceptibility of small organisms with relatively short nerves to disturbed peripheral nerve structure and function. These limitations might be particularly relevant for late-onset polyneuropathies probably arising from the effects of repetitive nerve damage (e.g., microtrauma, toxins, inflammation, metabolic disease), which accumulate during the long life-span of humans and are not efficiently alleviated by counteracting repair mechanisms. Notably, neprilysin is upregulated after nerve injury in adult rats[46] suggesting that neprilysin may act in pathways ensuring life-long proper function of peripheral nerves. Therefore, it is still possible that a

phenotype can be precipitated in neprilysin-deficient mice when challenging repair mechanisms by accelerating the normal aging process in nerve damage paradigms.[44]

The most notable change in nerves of *Mme*$^{-/-}$ mice (Figure 4) was a developmental defect of ensheathment of Remak bundles. These abnormalities are reminiscent of what is seen in nerves of several mouse mutants for extracellular matrix components, surface receptors, Rho GTPases, and signaling molecules.[47] Although we are not aware of a direct link between neprilysin and these molecules and their related pathways, it is conceivable that neprilysin may act upstream of such signaling networks (e.g., by processing of ligands) or downstream (e.g., as a transcriptional target gene). Interestingly, many of these molecules are also part of the pro-regenerative responses of the peripheral nervous system,[48] which might be compromised in *MME*-associated late-onset polyneuropathies. Alternatively, neprilysin might directly degrade peptides that have negative effects on neurons and peripheral nerves. We note that involvement of one well-known potential candidate is not supported by our study: although neprilysin's capacity to degrade β-amyloid proteins[13] is probably reduced in individuals with *MME* mutations and although β-amyloid also precipitates in tissues other than brain in Alzheimer disease,[49] we found β-amyloid deposition neither in subcutaneous adipose tissue biopsies nor in a sural nerve biopsy of individuals with *MME* mutations.

A major role for neprilysin deficiency in Alzheimer disease has been concluded from the results obtained in mouse models.[50,51] However, cognitive impairment was documented in only 2 out of 28 individuals with rare *MME* mutations identified in our study, which is not unexpected in an elderly aged cohort.[52] Mental state in other individuals remained essentially normal even at a high age (>80 years) (Table 1) as was the case in the families with autosomal-recessive *MME*-associated polyneuropathy.[9] This observation strongly argues against an increased risk for Alzheimer disease in individuals carrying *MME* mutations associated with low neprilysin levels.

Because inhibition of neprilysin increases the levels of endogenous cardiovascular protective peptides, combined angiotensin receptor blockade and neprilysin inhibition has recently been introduced as a new treatment of heart failure with reduced ejection fraction.[53] Our findings imply that individuals receiving this neprilysin inhibitor therapy could be at increased risk of developing polyneuropathy. Although this risk seems rather theoretical as life expectancy of most individuals with heart failure is usually short relative to the timescale on which neuropathy would be expected to develop, regular neurological and neurophysiological examination of these individuals might be warranted to ensure early detection of peripheral neuropathy. Finally, particular caution should be considered in individuals with pre-existing neuropathies.

Several individuals with *MME* mutations experienced painful sensations as presenting or early symptoms. C-fiber abnormalities in *Mme*-knockout mice and pathology in a human nerve biopsy were in line with malfunctioning pain-sensing neurons. This is not necessarily in contradiction with normal appearance of the terminals of these neurons in abdominal wall skin biopsy samples of individuals with *MME* mutations, because neuronal degeneration in a length-dependent neuropathy might be missed in proximal nerve endings. The pathological process may involve both C-fiber degeneration (such as in painful small fiber neuropathy[54]) and decreased neprilysin-dependent processing of mediators of nociceptive signals. Earlier studies suggested that lack of neprilysin in mice led to increased pain behavior in the chronic constriction injury model.[55] Notably, substance P, which is at the same time a neurotransmitter used by nociceptive neurons and a neprilysin substrate,[12] was elevated in this experimental setting.

The results from our study may have two wider implications: First, our data suggest that diminished neprilysin activity damages the peripheral nervous system, probably by insufficient turnover of molecules that are critical for the well-being of peripheral nerves. This aspect could be important from a medical standpoint because substances known to elevate neprilysin activity (e.g., green tea extract[56]) may hold promise for treatment of peripheral neuropathies and are ready for clinical trials. Second, although *MME* mutations are inherited as Mendelian disorders, the genetic cause of the disease might be easily overlooked. Owing to reduced penetrance, often small family sizes, and late age of onset, affected individuals may appear as isolated cases as did a number of case subjects in our series and one case subject in the paper by Higuchi et al.[9] Late-onset axonal neuropathies are notoriously difficult to unravel etiologically and neurologists have noted a particular group of neuropathy-affected case subjects of 50 years and older in whom no causes of neuropathy can be identified.[57,58] *MME* mutation screening is hoped to improve diagnosis in these individuals, and pooling cohorts from different centers world-wide should enable testing of this hypothesis by highly parallel sequencing technologies that are now available.

Supplemental Data

Supplemental Data include three figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.07.008.

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Web Resources

1000 Genomes, http://www.1000genomes.org
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
ExAC Browser, http://exac.broadinstitute.org/
GenBank, http://www.ncbi.nlm.nih.gov/genbank/
GENESIS, http://thegenesisprojectfoundation.org/
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
OMIM, http://www.omim.org/
Wellderly, https://genomics.scripps.edu/browser/files/wellderly/vcf

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