Experimental Neurology 308 (2018) 13–25

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

Myelinated axons fail to develop properly in a genetically authentic mouse model of Charcot-Marie-Tooth disease type 2E

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ARTICLE INFO

Keywords:
Neuropathy
Neurofilament
CMT
Axonal caliber
Nerve conduction
Behavioral testing

ABSTRACT

We have analyzed a mouse model of Charcot-Marie-Tooth disease 2E (CMT2E) harboring a heterozygous p.Asn98Ser (p.N98S) *Nefl* mutation, whose human counterpart results in a severe, early-onset neuropathy. Behavioral, electrophysiological, and pathological analyses were done on separate cohorts of *Nefl* $^{N98S/+}$ mutant mice and their wild type *Nefl* $^{+/+}$ littermates between 8 and 48 weeks of age. The motor performance of *Nefl* $^{N98S/+}$ mice, as evidenced by altered balance and gait measures, was impaired at every age examined (from 6 to 25 weeks of age). At all times examined, myelinated axons were smaller and contained markedly fewer neurofilaments in *Nefl* $^{N98S/+}$ mice, in all examined aspects of the PNS, from the nerve roots to the distal ends of the sciatic and caudal nerves. Similarly, the myelinated axons in the various tracts of the spinal cord and in the optic nerves were smaller and contained fewer neurofilaments in mutant mice. The myelinated axons in both the PNS and the CNS of mutant mice had relatively thicker myelin sheaths. The amplitude and the nerve conduction velocity of the caudal nerves were reduced in proportion with the diminished sizes of myelinated axons. Conspicuous aggregations of neurofilaments were only seen in primary sensory and motor neurons, and were largely confined to the cell bodies and proximal axons. There was evidence of axonal degeneration and regeneration of myelinated axons, mostly in distal nerves. In summary, the p.N98S mutation causes a profound reduction of neurofilaments in the myelinated axons of the PNS and CNS, resulting in substantially reduced axonal diameters, particularly of large myelinated axons, and distal axon loss in the PNS.

1. Introduction

Charcot-Marie Tooth disease (CMT) or hereditary motor and sensory neuropathy (HMSN) are the most common names for inherited neuropathies that are not part of a syndrome, with an estimated prevalence of 1 in 2500 (Bassam, 2014; Klein et al., 2013; Scherer et al., 2015). Mutations in > 80 different genes cause CMT, which is classified according to its mode of inheritance and whether demyelination or axonal loss appears to be the primary pathogenic event. The dominantly inherited axonal forms (CMT2) typically have normal or slightly reduced conduction velocities with reduced motor and sensory compound action potential amplitudes, and axonal loss is the chief finding in biopsied nerves.

CMT2E is caused by variety of dominant mutations in the *NEFL*, the gene that encodes neurofilament light (NFL) (Fridman et al., 2015; Horga et al., 2017). The p.N98S mutation is one of the most common mutations, and like many other *NEFL* mutations, causes a severe, early onset neuropathy, which are typically de novo mutations (http://hihg.med.miami.edu/code/http/cmt/public_html/index.html#/). Neurofilaments are intermediate filaments, and the main cytoskeletal proteins of myelinated axons (Leterrier et al., 2017; Pan and Chan, 2017), and are obligate heterotrimers of neurofilament light, medium, and heavy - NFL, NFM, and NFH, respectively (Ching and Liem, 1993; Lee et al., 1993; Yuan et al., 2012). NFL can form filaments on its own, but NFM and NFH require NFL to form filaments. When expressed by transfection in cultured cells, including neurons, most dominant *NEFL* mutations, including p.N98S, fail to form normal filaments, and often form aggregates (Brownlees et al., 2002; Perez-Olle et al., 2004; 2002; 2005). In addition, NFL accumulates in cultured neurons from the *Nefl* $^{N98S/+}$ mice (Saporta et al., 2015), the first knock-in mice of a dominant *NEFL*

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https://doi.org/10.1016/j.expneurol.2018.06.010
Received 17 April 2018; Received in revised form 14 June 2018; Accepted 19 June 2018
Available online 22 June 2018
0014-4886/

mutation (Adebola et al., 2015). Whether these, or other abnormalities (Tradewell et al., 2009; Wujek et al., 1986; Zhai et al., 2007), are the cause of neuropathy remains to be determined. As a first step to illuminate how the p.N98S mutation causes neuropathy, we examined *Nefl* $^{N98S/+}$ mice, and find that these mice have a phenotype that mirrors the human disease.

2. Materials and methods

2.1. Behavioral testing

A cohort of 15 male *Nefl* $^{N98S/+}$ mutant and 15 wild type (WT) *Nefl* $^{+/+}$ littermates were bred on a C57BL6J background at PsychoGenics, and maintained in a 12/12 h light/dark cycle at 22 ± 1°C and a relative humidity ~50%. Food and water was provided ad libitum. All animals were examined, handled, and weighed prior to initiation of the study to assure adequate health and to minimize the non-specific stress associated with testing. The experiments were conducted during the animals' light cycle phase. Sensory impairment was assessed by the hotplate plate test (Ankier, 1974). Deficits in motoric activity was assessed using the tapered balance beam test (Schallert and Woodlee, 2005; Strome et al., 2006). For grip strength (Meyer et al., 1979), mice were acclimated in the test room for 1 h, then held by the tail and lowered towards the mesh grip piece on a push-pull gauge (San Diego Instruments, San Diego, CA) until the animal grabbed the mesh grip piece with both fore limbs. The animal was then gently pulled backwards with consistent force until it released its grip. The forelimb grip force was recorded on the strain gauge. The animal was gently pulled backwards along the platform until the animal's hind paws grabbed the mesh grip piece on the push-pull gauge and then released its grip. The hind limb grip force was recorded on the strain gauge. Five consecutive grip trials separated by 1 min were recorded. The NeuroCube® System (Alexandrov et al., 2015) employs computer vision to detect changes in gait geometry and gait dynamics in rodents. Mice were placed in the NeuroCube® for a 5 min test. The most dominant of the features that separate WT from *Nefl* $^{N98S/+}$ mutant mice were identified and ranked. Bioinformatic algorithms were employed to calculate the discrimination probability between the mutant and WT mice. The outcomes differentiate a set of features that include average speed, body position, gait, paw positioning, paw contact imaging, and relative limb movement (rhythmicity).

2.2. Mixed nerve conduction studies of the tail (Maia et al., 2010)

Mice were anesthetized with ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively, *i.p.*). After full anesthesia was verified, tails were cleaned with 70% ethanol, and wire loop electrodes (Schuler Medizintechnik, Germany) were placed so that the distance between the recording electrode and stimulating cathode electrode was 30 mm apart – a pair of recording loops (the reference loop was 2 mm from the tail base and the active loop 5 mm more distal) and a pair for stimulation loops (separated by 5 mm), grounded by a pin electrode halfway between the active electrodes. Using this configuration, supramaximal stimulation was determined by averaging a series of 5 responses (to reduce noise and variability). Tail surface temperature was maintained at 30–32 °C using a heating pad and monitored using infrared non-contact thermometer during recording. Nerve conduction studies were performed with a Nicolet Viking Quest EMG/NCS machine (Natus Neurology, Middleton, WI, USA). The amplitude of compound action potentials (CAP) was measured by voltage difference between the baseline and the peak of CAP. Conduction velocity (CV) were calculated by dividing the distance between active stimulating electrode and recording electrode (30 mm) by the latency of CAP (time from the initiation of shock artifact and the initiation of CAP); this measures the fastest conducting myelinated axons. The half duration of CAP was measured at the half of the peak CAP amplitude.

2.3. Histology

Separate cohorts of mutant male *Nefl* $^{N98S/+}$ mice and their male WT littermates were analyzed by histology 8, 16, 24, 32, 40, and 48 weeks. They were euthanized with an intraperitoneal injection of ketamine (200 mg/kg) and xylazine (10 mg/kg), then transcardially perfused with 10 ml of 0.9% NaCl followed by 50 ml of 2% glutaraldehyde and freshly prepared 2% paraformaldehyde in 0.1 M phosphate buffer (PB; pH = 7.4). From each mouse, we dissected the ventral pair of caudal nerves from the tail, the cervical spinal cord, the optic, sciatic, and tibial nerves, as well as the femoral motor and sensory nerves and the L4 and/or L5 dorsal root ganglia (DRG) and attached nerve roots. The tissues were fixed for 3 h, rinsed in 0.1 M PB, transferred to 2% OsO4 in 0.1 M PB, for 1 h, then processed for embedding in Epon (Potter et al., 2011). Semi-thin sections were stained with alkaline toluidine blue, and visualized by light microscopy (Leica DMR) using interactive software (Leica Application Suite). Thin sections were stained with lead citrate and uranyl acetate and imaged in a Jeol-1010 transmission electron microscope. Selected images were processed with Photoshop to generate the figures.

We measured the distance from where we performed electrophysiology on the caudal nerve (37 mm from the base of the tail; this is 53 mm from the S2 DRG in adult mice) and the tibial nerve at the ankle (40 mm from the L4 DRG). We quantified axons in semi-thin sections of the ventral caudal nerve (at the site of electrophysiological stimulation) and the femoral motor nerve. The axons in each nerve were counted from photomontages that were assembled from 100× images of semi-thin sections, and measured by using Image J software by highlighting and quantifying the axoplasmic area of each axon. To quantify the number of neurofilaments per axon, we imaged 100 of the largest axons in one ventral caudal nerve per animal with the electron microscope, and counted the number of neurofilaments in each axon. To quantify the number of neurofilaments per axon in the optic nerve, we examined 6 mutant and 5 WT optic nerves of 8-week-old mice. From each image, we determined for axonal size for the 35 large axons, and the smallest myelinated axon in the same image, and counted the number of neurofilaments and microtubules in these individual large and small axons. The g-ratio was calculated by the square root of the ratio of inner to outer axonal area. For the femoral motor nerve, we analyzed all of the myelinated axons in a 100 μm by 100 μm square area from a light microscopic image from each animal (240 or more myelinated axons). For the optic nerve, we analyzed all of the myelinated axons in 15 electron microscopic images (120 or more myelinated axons) for each animal. The inner and outer axonal area were measured using Image J software. GraphPad Prism software was used to generate scatter plots and perform linear regression.

2.4. Experimental design and statistical analysis

For behavioral tests, data were analyzed by repeated measures ANOVA followed by Fisher PLSD post hoc analysis with significance established as $p < 0.05$. Data from NeuroCube were analyzed as previously published (Alexandrov et al., 2015). Nerve conduction studies were statistically analyzed with two-way ANOVA with repeated measure followed by multiple comparisons. To analyze the distribution of axonal size between WT and N98S/+ mice, their cumulative histograms were plotted and Kolmogorov-Smirnov test were performed using GraphPad Prism software. The number of myelinated axons in CN and FMN and NF and MT counts in ON were analyzed by unpaired *t*-test. The results of the statistical analysis are presented in the result, table, and figure legends.

3. Results

The generation and initial characterization of *Nefl* $^{N98S/+}$ mutant mice has been described (Adebola et al., 2015; Saporta et al., 2015;