Charcot-Marie-Tooth type 1A: natural course, pathophysiology and treatment

Verhamme, C.

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Myelin and axon pathology in a long-term study of *PMP22* over-expressing mice

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C. Verhamme
R.H.M. King
A.L.M.A. ten Asbroek
J.R. Muddle
M. Nourallah
R. Wolterman
F. Baas
I.N. van Schaik

Abstract

The aims of this study were to determine the course of the disease in two peripheral myelin protein-22 (*PMP22*) over-expressing mouse models over 1.5 years and to compare this course with the natural history in human CMT1A. The C22 mice have 7 and the C3-PMP mice have 3 to 4 copies of the human *PMP22* gene. The C3-PMP mice showed no overt clinical signs at 3 weeks and developed mild neuromuscular impairment whereas the C22 mice already showed signs at 3 weeks which progressed to severe impairment. Adult C3-PMP mice had stable, low nerve conduction velocities similar to adult patients with CMT1A; the velocities were much lower in C22 mice. In both models myelination was delayed and normal myelination was never reached. The degree of dysmyelination in C3-PMP mice was considerably less than in C22 mice; myelination was stable in adult mice of both models. Myelinated fibres - consisting of normally myelinated, inappropriately thinly myelinated and amyelinated fibres - were already reduced in number at 3 weeks of age in both models, suggesting that a normal number of myelinated fibres is never reached during development in both models. During adulthood, there was no detectable loss of myelinated fibres in C3-PMP mice and wild-type mice, whereas there was clear loss of myelinated fibres in C22 mice. Similar to human CMT1A, the results in C3-PMP mice indicate that a balance is reached between myelination status and axonal function early in life, while in C22 mice the early reduction of axons is more severe with major loss of axons in adult mice. Thus, we conclude that C3-PMP mice may be an appropriate model for the majority of CMT1A patients and C22 mice may be a useful model for the more severe for the more severely affected patients in the CMT1 spectrum.

Introduction

Charcot-Marie-Tooth disease type 1A (CMT1A), also called hereditary motor and sensory neuropathy type Ia (HMSN Ia), is the most prevalent hereditary demyelinating neuropathy (Boerkoel *et al.*, 2002). This autosomal, dominantly inherited disease is related to a 1.5 Mb duplication on chromosome 17p that includes the peripheral myelin protein 22 (*PMP22*) gene (Lupski *et al.*, 1991; Raeymaekers *et al.*, 1991). PMP22 is a tetraspan glycoprotein contained in compact myelin of the peripheral nervous system. There is also evidence that PMP22 is involved in the interaction of the Schwann cells with the extracellular environment (Amici *et al.*, 2006). The molecular mechanisms underlying CMT1A are not well understood, but impaired intracellular protein trafficking, inefficient degradation, and formation of aggregates may play a role (Fortun *et al.*, 2006; Fortun *et al.*, 2007).

In humans, symptoms and signs of predominantly distal loss of strength and sensation, more in the legs than in the arms, often develop in the first two decades of life (Berciano *et al.*, 2003). Over the years, there has been debate whether, and to what extent, adult patients deteriorate. We and others hypothesized that clinical disease progression could be due to increased axonal dysfunction, secondary to a fairly stable myelination status (Krajewski *et al.*, 2000; Verhamme *et al.*, 2004; Shy *et al.*, 2008). Longitudinal studies with a follow-up of 2 to 5 years showed clinical progression in adults (Padua *et al.*, 2008; Shy *et al.*, 2008; Verhamme *et al.*, 2009), with indications for increased axonal dysfunction over time as assessed indirectly with compound muscle action potential (CMAP) amplitudes, recorded with surface electrodes (Shy *et al.*, 2008; Verhamme *et al.*, 2009). The decline in surface CMAP amplitudes and in muscle strength in adult patients was, however, in the same range as in healthy people, so we postulated that this decline reflects to a considerable extent a process of normal ageing (Verhamme *et al.*, 2009).This finding is supported by a study that used a motor unit number estimation technique in a large group of adult CMT1A patients, showing that the age-dependent decrease in the number of motor units did not differ between patients with CMT1A and controls, indicating that loss of motor units in adult patients is limited (van Dijk *et al.*, 2010).

A reliable mouse model for CMT1A is important not only in order to clarify the molecular mechanisms underlying CMT1A but also for experiments with disease-modifying treatments before testing them in patients. Several transgenic mouse models for dys/demyelinating peripheral hereditary neuropathies have been generated that resemble human CMT1A (Huxley *et al.*, 1996; Magyar *et al.*, 1996; Perea *et al.*, 2001; Robertson *et al.*, 2002), although none are perfect in this regard (Robertson *et al.*, 2002). Several experiments involving animal models for CMT1A and using nerve grafts from patients with CMT1A showed different degrees of abnormal myelination, as well as axon pathology. Trembler nerve transplants produced local biochemical changes in axons that have regenerated through them, including alterations in neurofilament

phosphorylation, increased neurofilament density and decreased axonal transport (de Waegh and Brady, 1990; de Waegh *et al.*, 1992). Mice carrying approximately 16 and 30 *Pmp22* copies showed a reduced number of axons at 12 months, but other time points were not documented (Magyar *et al.*, 1996; Sancho *et al.*, 1999). Mouse axons that regenerated through nerve grafts from patients with CMT1A had a reduced calibre compared with surrounding nerves (Sahenk *et al.*, 1999). As detailed follow-up studies are scarce, it is unclear whether disease progression in these mouse models is similar to the progression in humans with CMT1A.
The aims of this study were to determine the course of the disease in two *PMP22* over-expressing mouse models, clinically, electrophysiologically and morphometrically over 1.5 years and compare the changes with wild-type (WT) mice. These findings were then compared with data obtained during a recently completed and published natural history study in CMT1A patients (Verhamme *et al.*, 2009) to clarify whether or not these *PMP22* over-expressing mice are useful models.

Materials and Methods

Animals and determination of genotypes

The Animal experimental committee of the Academic Medical Centre, Amsterdam, approved the experiments. C22 mice in a C57BL/6J x CBA/Ca background, carrying 7 copies of a YAC containing the human *PMP22* gene (Huxley *et al.*, 1996), were backcrossed to a C57BL/6J wild-type strain for more than ten generations. Genotyping of WT and C22 mice were determined by conventional PCR after extraction of mouse DNA from a piece of ear tissue. Primers used for amplification of the human *PMP22* transgene were CTTCAGGCCCTGCACCTC and CATTCCGCAGACTTGGATG.
During breeding, three mice (one male and two females) in one litter of eight were observed to show a milder phenotype. These mice were found to have a reduced *PMP22* transgene copy number as assessed by quantitative real-time PCR on a LightCycler 480 (Roche Diagnostics, Germany) using Universal Probes according to the manufacturer's protocol for monocolor hydrolysis probe. Samples were assayed in triplicate on 4 dilutions (2.5, 5, 10, and 20 ng DNA input). Quantification was performed using the Roche LightCycler software for crossing point calculation with an assumed PCR efficiency of 2. The endogenous mouse *Pmp22* and the 7 copies of the *PMP22* transgene in C22 mice were used as reference. Primer sequences used for LC480 reactions were (gene, forward primer, reverse primer, Universal probe number, respectively): human *PMP22*: TCAGGAAATGTCCACCACTG, CAAGCTCATGGAGCACAAAA, #9; mouse *Pmp22*: GGGAATGGCTTGCTGATCT, TGGTATTCCCCAGCCTCAT, #51. The mice had 3 to 4 copies of the human *PMP22* gene. These mice, which we named C3-PMP, were bred separately and proved to have a stable genotype with respect to human *PMP22* copy number.

Neuromuscular SHIRPA

A modified version of the SHIRPA (Smith Kline Beecham, MRC Harwell, Imperial College, the Royal London Hospital phenotype assessment) protocol (Rogers *et al.*, 1997) was used to give an overall assessment of neuromuscular function. In this were 19 items of the original SHIRPA protocol and one extra item. With the mouse in the viewing jar, body position, spontaneous activity, tremor, and, the extra item, letting the limbs slip through the grid, were scored. The scores in the arena were for transfer arousal, gait, pelvic and tail elevation, and touch escape. Above the arena, positional passivity, trunk curl, limb grasping, grip strength, body tone and toe pinch were scored. Limb tone and abdominal tone were scored during supine restraint. The wire manoeuvre, negative geotaxis and contact righting reflex were scored with a wire, a tube and a grid, respectively. A sum score was calculated, ranging from 0 indicating no signs and 43 being maximally affected. Locomotor activity in arena during 30 sec was scored separately.

Electrophysiological examination

Mice were anesthetized with intraperitoneal injection of FFM (Hypnorm (Janssen, fentanyl citrate 0.15mg/ml; fluanisone 10mg/ml):midazolam:water=1:1:2). Temperature was maintained above 34 degrees Celsius. The hind legs and the tail were strapped to a polystyrene board. The sciatic nerve and caudal nerve were studied on one side. For motor conduction studies of the sciatic nerve monopolar needle electrodes were inserted as follows: the stimulating cathodes were placed at the medial ankle and at the sciatic notch; the stimulating anode was inserted 3 mm distal to the stimulating cathode; the recording cathode was placed intramuscularly between the hallux and the 2nd digit and the anode was inserted laterally at the 5th digit; an earth electrode was attached halfway between the distal stimulating and recording electrodes. Supramaximal square-wave pulses of 0.1 ms duration were delivered. Compound muscle action potential (CMAP) amplitudes (peak to peak) were recorded. Motor nerve conduction velocities (MNCV) over the segment between the ankle and the sciatic notch were calculated. For studies of the caudal nerve, monopolar needle electrodes were inserted as follows: the stimulating cathode was placed laterally in the tail at exactly 2 cm from the base of the tail; the stimulating anode was inserted 3 mm distal to the stimulating cathode; the recording cathode and anode were inserted at the base of the tail just 3 mm apart; the earth electrode was attached halfway between the stimulating and recording electrodes. Supramaximal square-wave pulses of 0.5 ms duration were delivered. A maximum of 30 responses were averaged for each recording. Compound nerve action potentials (CNAPs) amplitudes (peak to peak) were recorded. The nerve conduction velocity (NCV) of the tail nerve was calculated from the latency of the stimulus artifact to the

onset of the negative peak of the action potential elicited and the distance between the stimulating and the recording cathodes.

Histology and morphometry

In deeply anaesthetized mice, the chest was opened and blood flushed out with saline via a canula inserted into the left ventricle of the heart followed by fixative (4% paraformaldehyde in PBS). The nerves were removed and further fixed in 1% paraformaldehyde plus 1% glutaraldehyde in 0.1 M PIPES buffer overnight. Samples were processed conventionally into Durcupan resin for light and electron microscopy (Robertson *et al.*, 1997). The sections examined were taken from the peroneal nerve, the motor branch of the femoral nerve and the lumbar ventral and dorsal roots. For light microscopy and morphometry, 0.5 µm sections were cut and stained with thionine and acridine orange. For electron microscopy, ultrathin sections (70 nm thick) were counterstained with methanolic uranyl acetate and lead citrate. Histology assessment was undertaken with a combination of high magnification light microscopy using x100 oil immersion lens, and electron microscopy. Sections were examined for pathological changes including signs of active de- and remyelination, abnormally thin myelin, onion bulbs, active axonal degeneration and regenerative clusters and the presence of uncompacted myelin. Morphometry was performed using a Zeiss Axioplan microscope fitted with a Märtzhäuser motor driven stage connected to computer running Zeiss KS400 software. The entire cross-sectional areas of the nerves were analysed using a x100 oil immersion lens. Myelinated fibres were defined as having axons which had a diameter large enough to be myelinated, which was defined as ≥ 2 µm. These fibres were quantified and divided in normally myelinated, thinly myelinated, and amyelinated fibres based on their g-ratios (ratio of the axon diameter to the total fibre - axon plus myelin - diameter). A g-ratio of 0.7 was considered as normal. A reference image showing a series of annuli from 2 µm to 14 µm in diameter with a wall thickness indicating a g-ratio of 0.7 was incorporated into the measurement program. Normally myelinated fibres were defined as fibres with a myelin sheath thickness which was appropriate for axon diameter (g-ratio ≤ 0.7). Thinly myelinated fibres were defined as fibres with a myelin sheath thinner than appropriate (g-ratio > 0.7). Amyelinated fibres were defined as fibres large enough to be myelinated but having no myelin sheath resolvable by light microscopy. These three categories (normal, thinly myelinated and amyelinated) when summed are together referred to as myelinated fibres for brevity. Unmyelinated fibres, which had a diameter < 2 µm and no visible myelin sheath, were not analysed in this study. The software used for morphometry automatically segmented the myelinated fibres and allowed for manual modifications, such as the addition of fibres with no myelin sheath resolvable by light microscopy. Axonal diameters

were derived from myelinated fibre diameters by subtracting the myelin thickness (calculated from the g-ratio).

Statistical analysis

Mouse characteristics, data from the modified SHIRPA, electrophysiological examination, and morphometry were analysed using descriptive statistics. In WT, C3-PMP and C22 mice, changes between 3, 24, 48 and 72 weeks were evaluated with analysis of variance (ANOVA), with a post-hoc Bonferroni correction for multiple comparisons. Differences between young (3 weeks of age) WT, C3-PMP and C22 mice and differences between adult (≥ 24 weeks of age) WT, C3-PMP and C22 mice were evaluated with unpaired *t*-tests. For adult mice (≥ 24 weeks of age) associations were studied between the neuromuscular SHIRPA, electrophysiological and morphometric parameters (Spearman's correlation coefficient). Due to the difficulty of the electrophysiological measurements this was not successful on all mice. All analyses were done with the Statistical Package for the Social Sciences software (SPSS Inc. version 15.0, Chicago, IL, USA).

Results

Neuromuscular SHIRPA

The results of the neuromuscular SHIRPA sum score are presented in figure 6-1 and table 6-1. SHIRPA scores in both the C3-PMP and C22 mice increased significantly from week 3 to week 24 reflecting a decrease in performance. Adult C22 mice (≥ 24 weeks of age) were most severely affected ($n = 38$, 15.1 ± 3.8 (mean ± SD), $P < 0.001$), while C3-PMP mice had intermediate scores ($n = 27$, 5.1 ± 2.9, $P < 0.001$) as compared to WT mice ($n = 31$, 1.5 ± 1.0). Values of the separate items are given in a table 6-1. Items contributing most to the overall score (mean ≥ 1.0 for C22) were tremor, gait, tail elevation, touch escape, grip strength, and wire manoeuvre. Locomotor activity was less in adult C3-PMP mice ($n = 27$, 9.0 ± 3.8 fields, $P = 0.02$) and C22 mice ($n = 38$, 8.2 ± 4.8 fields, $P = 0.001$) as compared to WT mice ($n = 31$, 13.9 ± 8.9 fields), with no difference between C22 and C3-PMP mice.

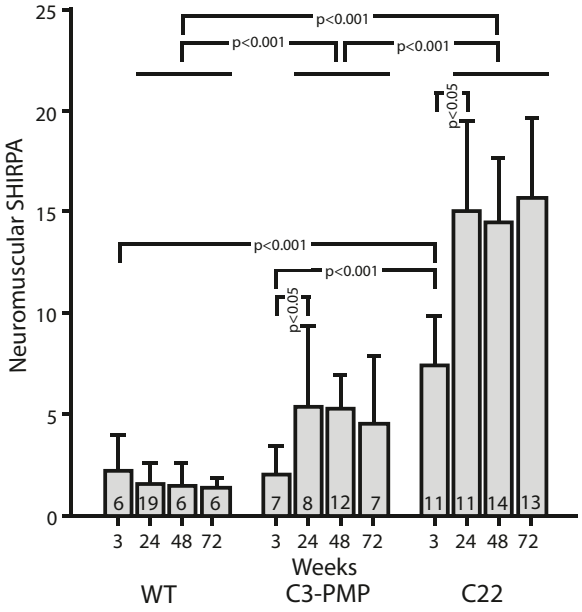


Figure 6-1. Neuromuscular SHIRPA sum score as a composite measure of impairment during 72 weeks in WT mice, the affected C3-PMP mice and the more affected C22 mice; bars indicate means, error bars indicate SDs, number of mice per group in the bars. The sum score ranges from 0 to 43, with 0 indicating normal performance and 43 maximally affected. Young C22 mice (3 weeks of age) performed significantly worse on the SHIRPA than young WT and C3-PMP mice; those in the WT and C3-PMP did not differ significantly (unpaired *t*-tests). The sum scores increased significantly between 3 and 24 weeks in the C3-PMP and C22 mice indicating a decreased performance on the SHIRPA, but not in the WT mice (ANOVA, post-hoc Bonferroni). The mean sum scores in the adult C3-PMP and C22 mice (≥ 24 weeks of age) were significantly higher than those in the WT mice; those in the C22 mice were higher than in the C3-PMP mice (unpaired *t*-tests).

Electrophysiological examination

Electrophysiological parameters of the tibial division of the sciatic nerve and the caudal nerve are summarized in table 6-2. Mean (M)NCVs were lower in C3-PMP and in C22 mice than in WT mice at all ages, and also lower in C22 than in C3-PMP mice at all ages. All mouse strains showed an increase in mean (M)NCVs from 3 to 24 weeks, after which there was no further change. The MNCV of the sciatic nerve (tibial division) was 21.5 ± 5.4 in adult C3-PMP mice (n = 24), 4.6 ± 0.7 in adult C22 mice (n = 35) and 41.1 ± 4.9 in adult WT mice (n = 26). Young C3-PMP and WT mice had needle CMAPs of the intrinsic foot muscles similar in mean amplitude; adult C3-PMP mice had lower mean CMAP amplitudes than WT mice. C22 mice had lower mean CMAP amplitudes than C3-PMP and WT mice, at all ages tested. Mean CNAP amplitudes over the tail were lower in C3-PMP and C22 mice than in WT mice, regardless of age. Mean CNAP amplitudes were lowest in C22 mice. Mean CNAP amplitudes decreased significantly from 3 to 48 and 72 weeks in the C22 mice, but not in the C3-PMP and WT mice.

Table 6-1. Scores for the neuromuscular SHIRPA for adult mice

	WT (*n* = 31)		C3-PMP (*n* =27)		C22 (*n* = 38)	
	Mean ± SD	Median (range)	Mean ± SD	Median (range)	Mean ± SD	Median (range)
Body position	0.0 ± 0.0	0 (0)	0.0 ± 0.2	0 (0-1)	0.0 ± 0.0	0 (0)
Spontaneous activity	0.1 ± 0.4	0 (0-2)	0.0 ± 0.2	0 (0-1)	0.1 ± 0.3	0 (0-1)
Tremor	0.0 ± 0.0	0 (0)	0.5 ± 0.5	0 (0-1)	1.6 ± 0.5	2 (1-2)
Letting limbs	0.0 ± 0.0	0 (0)	0.3 ± 0.5	0 (0-1)	0.9 ± 0.3	1 (0-1)
Transfer arousal	0.1 ± 0.3	0 (0-1)	0.2 ± 0.4	0 (0-1)	0.5 ± 0.6	1 (0-2)
Gait	0.0 ± 0.0	0 (0)	0.6 ± 0.5	1 (0-1)	1.1 ± 0.3	1 (1-2)
Pelvic elevation	0.0 ± 0.0	0 (0)	0.4 ± 0.6	0 (0-2)	0.7 ± 0.9	0 (0-2)
Tail elevation	0.0 ± 0.0	0 (0)	0.3 ± 0.5	0 (0-1)	1.0 ± 0.2	1 (0-1)
Touch escape	0.6 ± 0.5	1 (0-1)	0.7 ± 0.5	1 (0-1)	1.0 ± 0.4	1 (0-2)
Positional passivity	0.0 ± 0.0	0 (0)	0.0 ± 0.0	0 (0)	0.0 ± 0.0	0 (0)
Trunk curl	0.0 ± 0.2	0 (0-1)	0.0 ± 0.0	0 (0)	0.0 ± 0.0	0 (0)
Limb grasping	0.0 ± 0.0	0 (0)	0.0 ± 0.2	0 (0-1)	0.8 ± 0.4	1 (0-1)
Grip strength	0.0 ± 0.0	0 (0)	0.8 ± 0.6	1 (0-2)	1.4 ± 0.5	1 (1-2)
Body tone	0.0 ± 0.0	0 (0)	0.0 ± 0.2	0 (0-1)	0.7 ± 0.5	1 (0-1)
Toe pinch	0.0 ± 0.2	0 (0-1)	0.0 ± 0.0	0 (0)	0.6 ± 1.0	0 (0-3)
Limb tone	0.2 ± 0.4	0 (0-1)	0.6 ± 0.8	0 (0-2)	0.9 ± 0.8	1 (0-2)
Abdominal tone	0.0 ± 0.0	0 (0)	0.0 ± 0.0	0 (0)	0.6 ± 0.5	1 (0-1)
Wire manoeuvre	0.3 ± 0.5	0 (0-2)	0.7 ± 0.9	0 (0-3)	2.7 ± 1.0	3 (1-4)
Negative geotaxis	0.1 ± 0.2	0 (0-1)	0.0 ± 0.2	0 (0-1)	0.6 ± 1.4	0 (0-4)
Contact righting	0.0 ± 0.0	0 (0)	0.0 ± 0.2	0 (0-1)	0.0 ± 0.0	0 (0)
Neuromuscular SHIRPA sum score	1.5 ± 1.0	1 (0-4)	5.1 ± 2.9	5 (1-11)	15.1 ± 3.8	15 (9-23)

The neuromuscular SHIRPA sum score ranges from 0 to 43, with 0 indicating normal performance and 43 maximally affected. Mean ± SD and median scores (range) are presented for the separate items. An explanation of the separate items is supplied at the end of chapter 6.

Histology and morphometry

The peroneal nerve, the motor branch of the femoral nerve and the lumbar ventral and the dorsal roots were examined by light microscopy and, additionally, sections of 21 C22, 5 C3-PMP and 10 WT mice were examined by electron microscopy. Representative transverse sections are shown in figures 6-2A-O. In C3-PMP and C22 mice there were numerous inappropriately thinly myelinated and amyelinated fibres in the peroneal nerve at 3 weeks as compared to WT mice; this was most marked in the C22 mice and to a lesser degree in the C3-PMP mice (figures 6-2A-C). The numbers of normally myelinated fibres in the C3-PMP and C22 mice increased from 3 weeks of age to adulthood, although this never became the same as in WT mice. In adulthood the C22 mice remained more severely affected than the C3-PMP mice (figures 6-2D-F). The incidence of inappropriately

Table 6-2. Electrophysiological measurements of the sciatic nerve (tibial division) and the caudal nerve

		Scores					Changes within model	Differences between models	
		Young	Adult			Total adult		Young	Total adult
Weeks		3	24	48	72	≥ 24	3, 24, 48, 72	3	≥ 24
WT	*n*	6	16	5	5	26			
Sciatic	CMAP	3.9 ± 1.9	8.7 ± 2.9	9.6 ± 4.1	8.2 ± 1.8	8.8 ± 2.6	3 and 24, 48	C22	C3-PMP, C22
(tibial div.)	MNCV	29.1 ± 5.4	40.9 ± 5.4	43.3 ± 2.2	39.8 ± 5.1	41.1 ± 4.9	3 and 24, 48, 72	C3-PMP, C22	C3-PMP, C22
	n	6	17	5	5	27			
Caudal	CNAP	90.6 ± 53.1	196.3 ± 48.0	164.6 ± 45.0	194.4 ± 53.8	190.1 ± 48.3	3 and 24, 72	C3-PMP, C22	C3-PMP, C22
	NCV	18.6 ± 2.5	28.2 ± 2.6	28.6 ± 0.0	27.1 ± 2.0	28.1 ± 2.2	3 and 24, 48, 72	C3-PMP, C22	C3-PMP, C22
C3-PMP	*n*	7	6	12	7	24			
Sciatic	CMAP	3.0 ± 1.5	4.3 ± 1.5	4.4 ± 1.9	4.6 ± 1.8	4.4 ± 1.7		C22	WT, C22
(tibial div.)	MNCV	11.9 ± 2.5	22.8 ± 3.3	21.0 ± 4.9	21.2 ± 8.2	21.5 ± 5.4	3 and 24, 48, 72	WT, C22	WT, C22
	n	7	6	11	7	24			
Caudal	CNAP	11.9 ± 3.9	80.9 ± 74.2	46.0 ± 43.3	67.0 ± 44.8	60.9 ± 52.5		WT	WT, C22
	NCV	6.8 ± 1.7	12.1 ± 5.4	11.2 ± 3.1	12.4 ± 6.7	11.8 ± 4.7		WT, C22	WT, C22
C22	*n*	11	10	13	12	35			
Sciatic	CMAP	1.3 ± 0.7	2.1 ± 0.8	2.0 ± 0.9	1.7 ± 0.9	1.9 ± 0.9		WT, C3-PMP	WT, C3-PMP
(tibial div.)	MNCV	2.3 ± 0.2	4.3 ± 0.9	4.5 ± 0.7	4.8 ± 0.5	4.6 ± 0.7	3 and 24, 48, 72	WT, C3-PMP	WT, C3-PMP
	n	11	11	13	12	36			
Caudal	CNAP	9.5 ± 3.5	8.1 ± 5.5	4.5 ± 2.2	4.3 ± 2.9	5.5 ± 4.0	3 and 48, 72	WT	WT, C3-PMP
	NCV	1.9 ± 0.3	2.9 ± 1.6	3.0 ± 0.8	3.2 ± 1.0	3.0 ± 1.2		WT, C3-PMP	WT, C3-PMP

Electrophysiological measurements of the sciatic nerve (tibial division) and the caudal nerve for WT, C3-PMP and C22 mice (mean ± SD)). Changes over time between 3, 24, 48 and 72 weeks were tested separately for WT, C3-PMP and C22 mice (ANOVA, post-hoc Bonferroni). Significant changes are shown ($P < 0.05$). Differences between the models at 3 weeks of age ('Young') and at ≥ 24 weeks of age ('Total adult') were tested (unpaired *t*-tests). Significant differences are shown ($P < 0.05$). CMAP = Compound muscle action potential amplitude (mV), CNAP = compound nerve action potential amplitude (μV); (M)NCV = (motor) nerve conduction velocity (m/s)

thinly myelinated and amyelinated fibres was more variable in the C3-PMP mice than in the C22 mice, both between C3-PMP mice of the same age and between different nerves in individual C3-PMP mice. In the peripheral nerves, there appeared to be a greater proportion of inappropriately thinly myelinated and amyelinated fibres in the motor branch of the femoral nerve (figures 6-2G-I) compared to the peroneal nerve (figures 6-2D-F). In both models, the lumbar ventral roots had an even higher proportion of these fibres (figures 6-2J-L) compared to the dorsal roots (figures 6-2M-O) and the peripheral nerves. The most important electron microscopy findings are shown in figure 6-3. Many amyelinated and very thinly myelinated fibres were found in both strains as well as many small diameter fibres which were hypermyelinated (figure 6-3A, B). Partially or completely uncompacted myelin sheaths were often present in the peripheral nerves (figure 6-3B), but not in the roots in C22 mice; this abnormality was not found in C3-PMP mice. Occasionally, demyelinated axons accompanied by myelin debris in the associated Schwann cell, suggesting active myelin breakdown, were identified in nerves and roots at all ages in C22 mice but only rarely in C3-PMP mice (figure 6-3C). Small onion bulb formations indicating repeated episodes of de- and remyelination were only rarely seen in C22 (figure 6-3D, E) but not in C3-PMP mice. Some fibres were remyelinated in both models.

There were occasional atrophic fibres (figure 6-3A), signs of early Wallerian degeneration (figure 6-3F) and bands of Büngner (figure 6-3G) indicating fibre loss at all ages. These were much more frequently seen in C22 mice than in the C3-PMP mice. Debris-containing macrophages were sometimes seen in C22 mice (figure 6-3C) but were very rarely found in C3-PMP mice. Some sequestration of axon organelles by Schwann cells was occasionally found, implying axonal damage (figure 6-3H) (Spencer and Thomas, 1974). Regenerative clusters of myelinated fibres were not identified in either model. Remak fibres were often abnormal with an increase in membranous structures in both strains. Occasionally, even in older animals, some had abnormally large numbers of small axons and also contained larger axons suggesting a block at the promyelination stage (figure 6-3I). None of these abnormalities were found in WT mice apart from some indication of axonal sprouting in one 72 week old WT mouse and 3 demyelinated fibres in another 72 week old WT mouse.

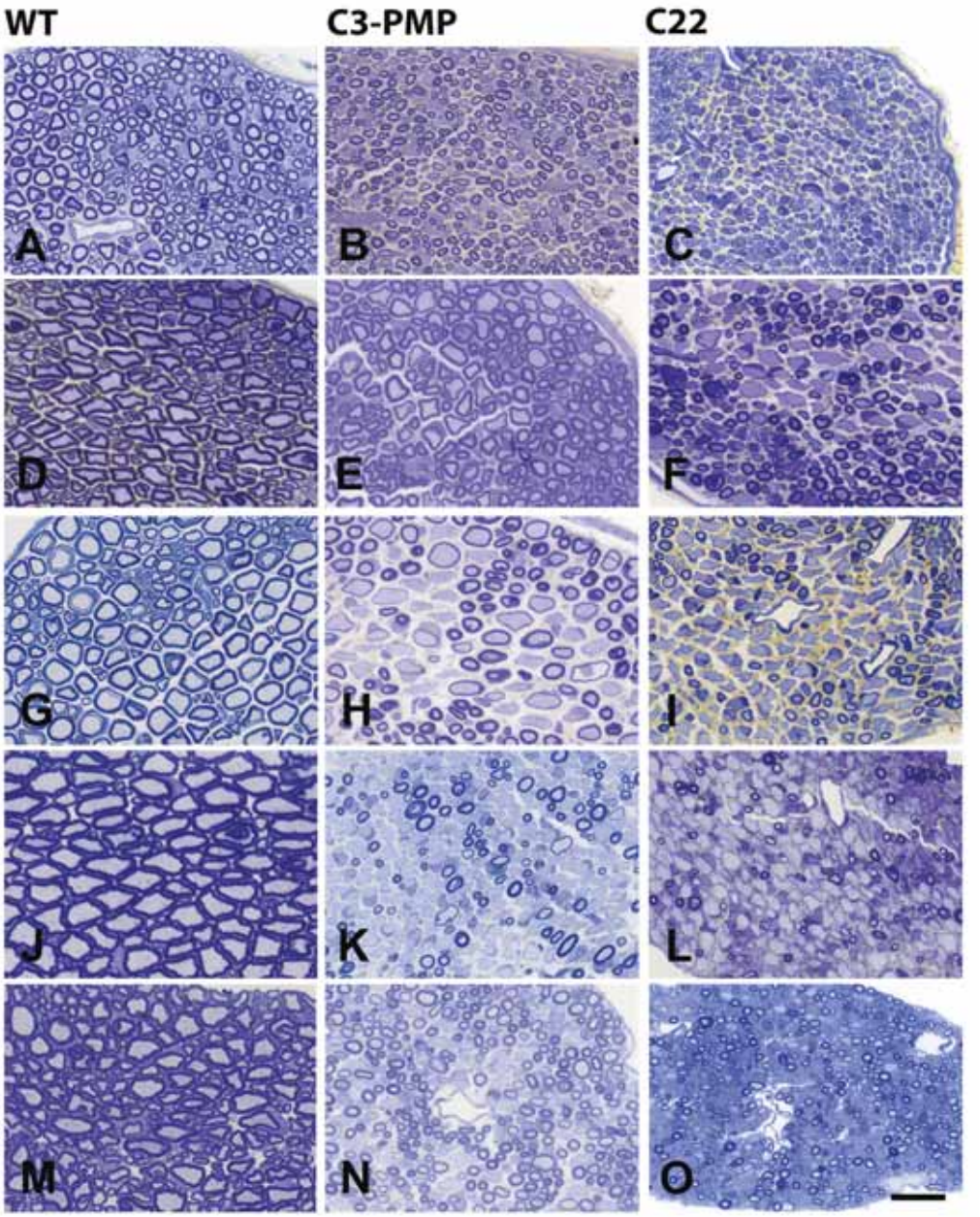
The peroneal nerve and the motor branch of the femoral nerve were used for morphometric analysis. Morphometry of the peroneal nerve was performed in 3, 24, 48 and 72 week old mice (figure 6-4A, table 6-3). In WT mice of all ages the number of myelinated fibres in the peroneal nerve remained stable around 1066 ± 61 (n = 37, 3-72 weeks). By 3 weeks of age, virtually all these fibres were normally myelinated in WT mice (n = 6). In C3-PMP mice the number of myelinated fibres (including normally myelinated, inappropriately thinly and amyelinated fibres) was 986 ± 76 (n = 34, 3-72 weeks), which was slightly lower than in WT mice (P = 0.04) and was fairly stable up to 72 weeks of age. The percentage of fibres that were normally myelinated in

C3-PMP mice was 74% at 3 weeks, increased to around 84% at 24 weeks of age and then remained stable. In C22 mice, due to the small diameter of most fibres at 3 weeks of age (see below) and the lack of collagen separating them, it was hard to discriminate between myelinated fibres (with a diameter ≥ 2 µm but no myelin sheath) and unmyelinated fibres. With this limitation, the number of myelinated fibres was low in 3 week old C22 mice (n = 11, 767 ± 114). By 24 weeks this number had increased slightly (n = 11, 873 ± 79, P = 0.07) but then showed a decline at 48 weeks (n = 14, 720 ± 104, P = 0.001) and at 72 weeks (n = 13, 572 ± 72, P = 0.001). The percentage of normally myelinated fibres was 40% at 3 weeks, increased up to approximately 64% in adult mice and then remained stable.

Morphometry of the motor branch of the femoral nerve was performed in 48 week old animals (figure 6-4B). The number of myelinated fibres in the motor branch of the femoral nerve did not differ between C3-PMP mice (n = 11, 554 ± 46) and WT mice (n = 6, 571 ± 23, P = 0.42). The C22 mice had a lower number of myelinated fibres (n = 6, 365 ± 63) as compared to the WT (P < 0.001) and C3-PMP (P < 0.001) mice. The percentages of normally myelinated fibres were approximately 75% in the C3-PMP mice and 40% in the C22 mice, which is lower than in the peroneal nerve in both models.

Median axon diameters were calculated by subtracting the calculated myelin thickness from the measured fibre diameter. The mean median axon diameters in the peroneal nerve in WT mice increased from 3.9 ± 0.1 µm at 3 weeks (n = 4) to 6.0 ± 0.4 µm at 24 weeks (n = 4) and 6.4 ± 0.4 µm at 48 weeks (n = 4). The mean median axon diameters in C22 mice increased from 2.6 ± 0.3 µm at 3 weeks (n = 4) to 3.6 ± 0.1 µm at 24 weeks (n = 4) and 4.3 ± 0.1 µm at 48 weeks (n = 4). At all these time points the differences between WT and C22 were significant (P < 0.001). The mean median axon diameters in the motor femoral nerve in WT mice was 7.3 ± 0.2 µm (n = 4) and in C22 mice 3.7 ± 0.7 µm (n = 4, P < 0.001) at 48 weeks.

Figure 6-2. Representative microphotographs of thionine and acridine orange stained 0.5 µm thick sections demonstrate differences in myelination in the genotypes at different ages and at different sites in the peripheral nerves. The genotypes are presented in the columns (WT, C3-PMP and C22). Peroneal nerve sections at 3 weeks (A-C) and at 48 weeks (D-F); motor branch of the femoral nerve at 48 weeks (G-I); ventral roots at 48 weeks (J-L), dorsal roots at 48 weeks (M-O). In C3-PMP and more severely in C22 mice the degree of myelination in the peroneal nerve was reduced at 3 weeks as compared to WT mice (A-C). The degree of myelination improved over time, although it never became the same in the C3-PMP and C22 mice as in WT mice. The C22 mice remained more severely affected than the C3-PMP mice (D-F). The myelin of the motor branch of the femoral nerve (G-I) was more abnormal than the myelin of the peroneal nerve (D-F). In both models abnormal myelination was most prominent in the lumbar ventral roots (J-L) as compared to the myelination of the dorsal roots (M-O) and the peripheral nerves. Scale bar = 20 µm.


WT
C3-PMP
C22
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

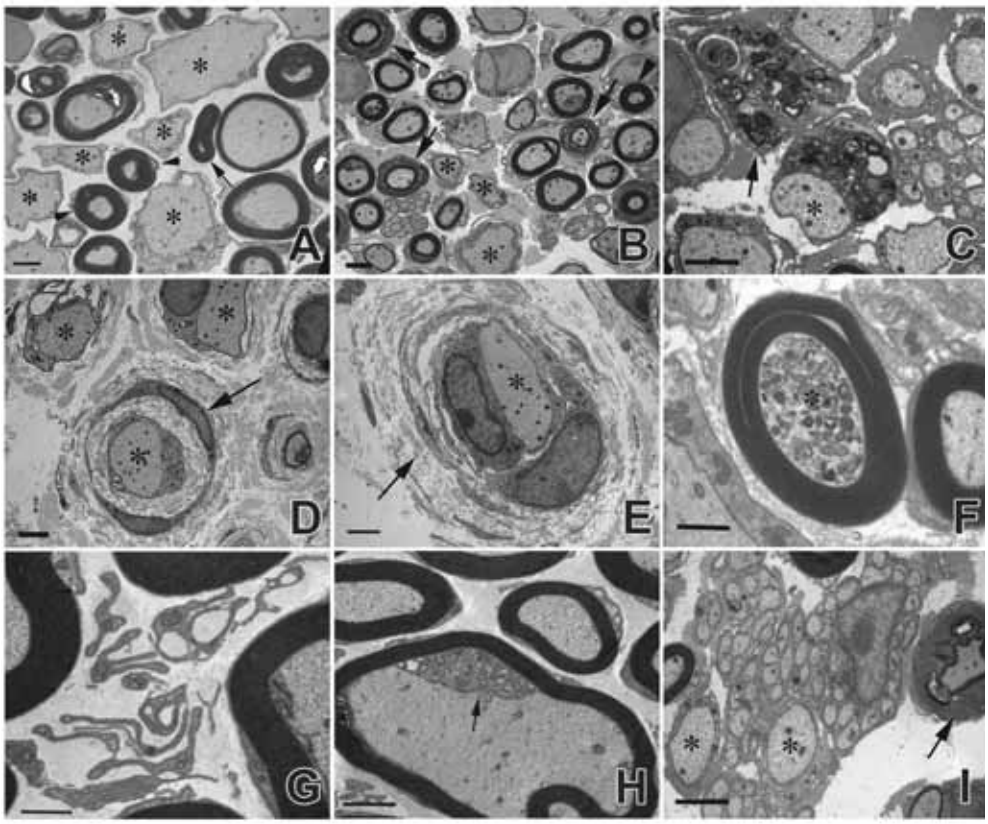


Figure 6-3. Neuropathological aspects; electron microscopy. (A) Lumbar ventral root, 48 weeks old C3-PMP mouse. There are several amyelinated and very thinly myelinated fibres (*), some fibres have abnormally thick myelin sheaths for axon diameter (arrowheads), and there is an atrophic axon (arrow). (B) Peroneal nerve, 24 weeks old C22 mouse. There are amyelinated axons and very thinly myelinated fibres (*), one fibre has abnormally thick myelin sheaths for axon diameter (arrowhead) and there are numerous fibres with partially uncompacted myelin sheaths (arrows). (C) Peroneal nerve, 48 weeks old C22 mouse. There are several large amyelinated axons, one (*) with myelin debris in the associated Schwann cell. A macrophage containing myelin debris is lying alongside (arrow). (D) Lumbar ventral root, 24 weeks old C22 mouse. There are several fibres lacking myelin sheaths (*), one is encircled by Schwann cell processes (arrow); others have some basal laminal and Schwann cell encirclement. (E) Lumbar ventral root, 24 weeks old C22 mouse. Onion bulb consisting of small Schwann cell processes and redundant basal laminal fragments (arrow) around a central demyelinated axon (*). (F) Lumbar dorsal root, 24 weeks old C22 mouse. Wallerian degeneration; collection of organelles in an axon (*). (G) Peroneal nerve, 48 weeks old C3-PMP mouse. Band of Büngner, consisting of attenuated Schwann cell processes, indicating axonal loss. (H) Peroneal nerve, 48 weeks old C3-PMP mouse. Sequestration of axon organelles by a Schwann cell (arrow). (I) Peroneal nerve, 48 weeks old C22 mouse. A Remak fibre containing unusually large numbers of small, unmyelinated axons. There are two embedded axons that are large enough to be myelinated. An adjacent fibre has a partially uncompacted myelin sheath (arrow). Scale bar = 2 µm, except for G, H and I for which scale bar = 1 µm.

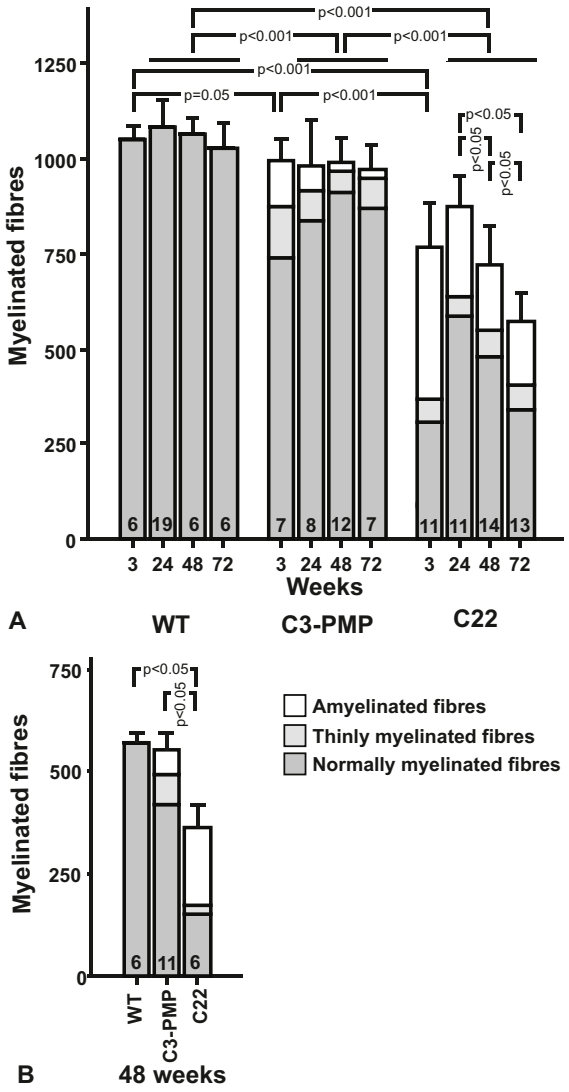


Figure 6-4. Morphometry of (A) the peroneal and (B) the motor branch of the femoral nerve in control mice (WT), the affected C3-PMP mice and the more affected C22 mice. The total bars represent the mean number of myelinated fibres, consisting of fibres which have a diameter large enough to be myelinated (≥ 2 µm), with stacks for the mean numbers of normally myelinated, thinly myelinated, and amyelinated fibres. The error bars show the SDs for the numbers of myelinated fibres. The numbers of mice per group are indicated in the bars. (A) The mean numbers of myelinated fibres in the peroneal nerve in young (3 weeks of age) and in adult (≥ 24 weeks of age) C3-PMP and C22 mice were significantly lower than those in the WT mice; those in the C22 mice were lower than in the C3-PMP mice (unpaired t-tests). In the C22 mice the mean number of myelinated fibres decreased significantly between 24, 48 and 72 weeks (ANOVA, post-hoc Bonferroni). (B) The number of myelinated fibres in the motor branch of the femoral nerve did not differ between C3-PMP mice and WT mice 48 weeks of age. The C22 mice had a lower number of myelinated fibres as compared to WT and C3-PMP mice (unpaired *t*-tests).

Table 6-3. Morphometry of the peroneal nerve

		Scores					Changes within model	Differences between models	
		Young	Adult			Total adult		Young	Total adult
Weeks		3	24	48	72	≥ 24	3, 24, 48, 72	3	≥ 24
WT	*n*	6	19	6	6	31			
	Myelinated	1050 ± 33	1083 ± 68	1066 ± 42	1030 ± 65	1070 ± 65	-	C3-PMP[1], C22	C3-PMP, C22
	Normally myelinated	1050 ± 33	1082 ± 68	1065 ± 41	1029 ± 65	1068 ± 65	-	C3-PMP, C22	C3-PMP, C22
	Normally myelinated %	100 ± 0.1	100 ± 0.2	100 ± 0.1	100 ± 0.1	100 ± 0.2	-	C3-PMP, C22	C3-PMP, C22
C3-PMP	*n*	7	8	12	7	27			
	Myelinated	995 ± 55	981 ± 120	991 ± 67	975 ± 58	984 ± 81	-	WT[1], C22	WT, C22
	Normally myelinated	741 ± 130	837 ± 178	909 ± 74	872 ± 113	878 ± 122	3 and 48	WT, C22	WT, C22
	Normally myelinated %	74 ± 10	84 ± 9	92 ± 3	89 ±7	89 ± 7	3 and 48, 72	WT, C22	WT, C22
C22	*n*	11	11	14	13	38			
	Myelinated	767 ± 114	873 ± 79	720 ±105	572 ±72	714 ± 148	3 and 72; 24 and 48, 72 48 and 24, 72 72 and 3, 24, 48	WT, C3-PMP	WT, C3-PMP
	Normally myelinated	305 ± 49	586 ± 55	480 ±143	340 ± 78	463 ± 141	3 and 24, 48 24 and 3, 48, 72 48 and 3, 24, 72 78 and 24, 48	WT, C3-PMP	WT, C3-PMP
	Normally myelinated %	40 ± 8	67 ± 2	66 ± 9	59 ± 8	64 ± 8	3 and 24, 48, 72	WT, C3-PMP	WT, C3-PMP

Morphometry of the peroneal nerve for WT, C3-PMP and C22 mice (mean ± SD). Changes over time between 3, 24, 48 and 72 weeks were tested separately for WT, C3-PMP and C22 mice (ANOVA, post-hoc Bonferroni). Significant changes are shown ($P < 0.05$). Differences between the models at 3 weeks of age ('Young') and at ≥ 24 weeks of age ('Total adult') were tested (unpaired *t*-tests). Significant differences are shown ($P < 0.05$). [1] $P = 0.05$.

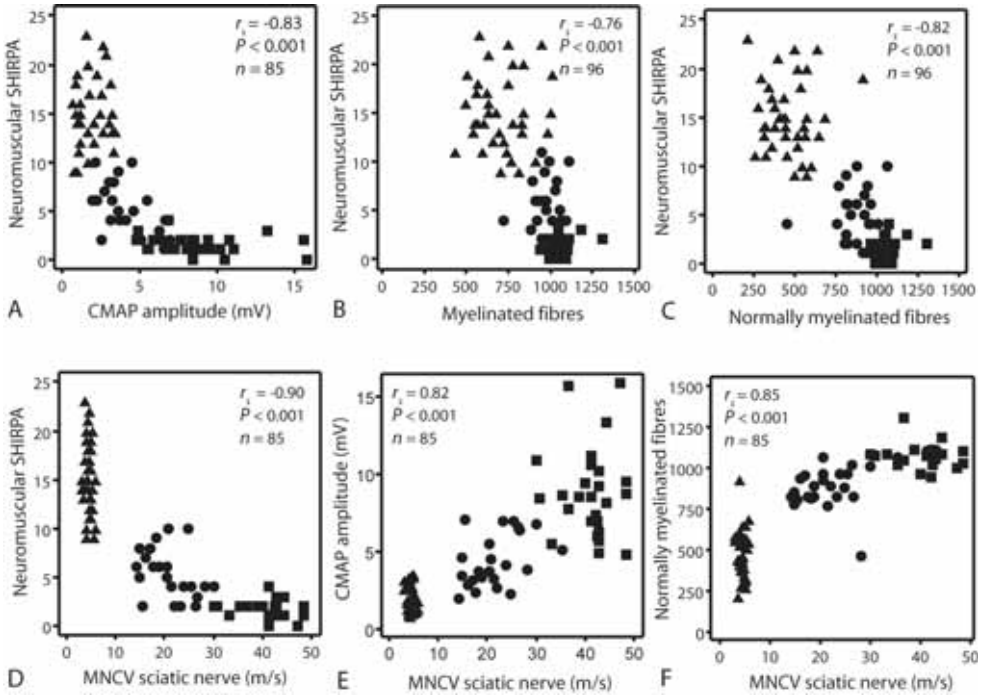


Figure 6-5. Scatter plots including data of adult ■ WT, ● C3-PMP and ▲ C22 mice (≥ 24 weeks of age). Higher neuromuscular SHIRPA sum scores were associated with (A) lower CMAP amplitudes, (B) lower numbers of axons with diameter in the myelinating range and (C) lower numbers of myelinated axons. Lower sciatic nerve, tibial division, MNCV was associated with (D) higher neuromuscular SHIRPA sum scores, (E) lower CMAP amplitudes and (F) lower numbers of myelinated axons. CMAP = Compound muscle action potential, MNCV = motor nerve conduction velocity. r_s= Spearman's correlation coefficient

Associations between clinical disease severity, electrophysiological and morphometric parameters

Correlations between the neuromuscular SHIRPA sum score, electrophysiological and morphometric parameters for adult mice (≥ 24 weeks of age) are presented in figure 6-5. Higher neuromuscular SHIRPA sum scores were associated with lower numbers of myelinated fibres, lower numbers of normally myelinated fibres and lower needle CMAP amplitudes. Lower sciatic nerve MNCV was associated with higher neuromuscular SHIRPA sum scores, lower numbers of myelinated axons and lower needle CMAP amplitudes. All these associations were significant (P < 0.001).

Follow-up in CMT1A patients

In the 5-year follow-up study the MNCVs in 44 CMT1A adult patients (age range 17-69 years) were lower than in controls (range 25-65 years), but stable over time (Verhamme

et al., 2009). The MNCVs for the median nerve at baseline were 23 ± 5.2 m/s in the patients and 58 ± 3.0 m/s in the controls ($P < 0.01$). The MNCVs for the tibial nerve at baseline were 21 ± 3.9 m/s and 46 ± 3.5 m/s, respectively ($P < 0.01$). Muscle strength and surface CMAP amplitudes at baseline were lower in patients than in controls, but the decrease was similar for adult patients and controls alike. Three-point grip, a measure of strength of distal arm muscles, was lower at baseline in patients (72 ± 32 N) than in controls (117 ± 30 N) ($P < 0.01$), but the decrease was not different over time ($P = 0.51$). A summated CMAP amplitude of distal arm and distal and more proximal leg muscles was lower at baseline in patients (12.2 ± 4.8 mV) than in controls (36.9 ± 7.3 mV) ($P < 0.01$), but the decrease was not different over time ($P = 0.48$). Table 6-4 shows a further comparison between the *PMP22* over-expressing mouse models and CMT1A patients.

Table 6-4. Summary of *PMP22* over-expressing mouse models and human CMT1A

	C3-PMP	C22	WT	CMT1A patients	Human controls
Nerve conduction studies					
- Nerve conduction velocities					
Over time during development	Never reach normal adult NCVs	Never reach normal adult NCVs	Increase to adult NCVs	Never reach normal adult NCVs (Killian *et al.*, 1996; Roy *et al.*, 1989; Yiu *et al.*, 2008)	Increase to adult NCVs (Garcia *et al.*, 2000)
Adult NCV (m/s) - tibial nerve	21.5 ± 5.4	4.6 ± 0.7	41 ± 4.9	21 ± 3.9 (Verhamme *et al.*, 2009)	46 ± 3.5 m/s (Verhamme *et al.*, 2009)
Over time in adults	Stable NCVs	Stable NCVs	Stable NCVs	Stable NCVs (Verhamme *et al.*, 2009)	Stable NCVs (Verhamme *et al.*, 2009)
- CMAP amplitude					
Over time during development	Never reach normal adult CMAP ampl.	Never reach normal adult CMAP ampl.	Increase to adult CMAP ampl.	Decrease or never reach normal adult CMAP ampl. (Berciano *et al.*, 2000; Yiu *et al.*, 2008)	Increase to adult adult CMAP ampl. (Garcia *et al.*, 2000)
Adult CMAP amplitude	Low	Very low	Normal	Low (Verhamme *et al.*, 2009)	Normal (Verhamme *et al.*, 2009)
Over time in adults	Stable	Stable	Stable	Minor reduction (Verhamme *et al.*, 2009)	Minor reduction (Verhamme *et al.*, 2009)

Table 6-4. Continued.

	C3-PMP	C22	WT	CMT1A patients	Human controls
Pathology					
- Abnormal myelination					
Dysmyelination	Moderate	Severe	No	Moderate (Gabreëls-Festen *et al.*, 1995; Saporta *et al.*, 2009)	No (Jacobs and Love, 1985)
Active demyelination	Rare	Rare	Not up to 1.5 years of age	Sporadic up to about 6 years of age (Gabreëls-Festen *et al.*, 1992)	No
Onion bulbs	No	Rare	Not up to 1.5 years of age	Reported in sural nerve biopsies (Gabreëls-Festen *et al.*, 1995; Hattori *et al.*, 2003), absent in skin biopsies (Saporta *et al.*, 2009)	Some signs of de- and remyelination after 60 years of age (Jacobs and Love, 1985)
- Myelinated fibres					
Over time during development	Never reach normal adult numbers	Never reach normal adult numbers	Increase to adult numbers	Indications that normal adult numbers are never reached (Gabreëls-Festen *et al.*, 1995)	Increase to adult numbers (Jacobs and Love, 1985)
Adult number of myelinated fibres	Low	Very low	Normal	Low (Gabreëls-Festen *et al.*, 1995)	Normal (Jacobs and Love, 1985)
Over time in adults	Stable	Loss	Stable	Unknown	Unknown

NCV = nerve conduction velocity; values of the of the sciatic nerve, tibial division, for mice and tibial nerve for CMT1A patients. CMAP amplitude = compound muscle action potential amplitude recorded with needle electrodes for mice and surface electrodes for CMT1A patients.

Discussion

We studied the disease course over 1.5 years in two different *PMP22* over-expressing mouse models. The C3-PMP mice with 3 to 4 copies of the human *PMP22* gene showed no overt clinical signs at 3 weeks and developed mild neuromuscular impairment whereas the C22 mice with 7 copies of the human *PMP22* already showed signs at 3 weeks which progressed to severe impairment. We have summarized similarities and differences between these mouse models and human CMT1A with regard to myelin and axonal pathology in table 6-4, and discuss these below.

One of the hallmarks of human CMT1A is low NCVs, the electrophysiological surrogate marker for myelin status. NCVs in young child patients never reach normal adult values (Roy *et al.*, 1989; Killian *et al.*, 1996; Yiu *et al.*, 2008), as in the *PMP22* over-expressing models. In adult CMT1A patients NCVs are stable and do not deteriorate with age (Shy *et al.*, 2008; Verhamme *et al.*, 2009), as in the *PMP22* over-expressing models.

The NCVs in adult C3-PMP mice are in the same range as in adult CMT1A patients. This indicates that in both humans and C3-PMP mice axons are surrounded by functional myelin over a considerable portion of nerve length. The very low NCVs in the C22 mice, that have been reported already (Huxley *et al.*, 1996), are in the range of NCVs found in unmyelinated axons (Gasser, 1950; Gasser, 1958). As the velocities of the fastest conducting axons are measured, this indicates that in the C22 mice the myelin must be lacking over most of the internodes along the length of the nerve to explain the very low NCVs.

The limited pathological data in human CMT1A suggest that dysmyelination may be of major importance, as in the *PMP22* over-expressing models. Dysmyelination implies that myelination is delayed during development and that normally myelinated nerve fibres are never reached. To the best of our knowledge, nerve pathology studies investigating the disease course before the age of 2 years in humans do not exist. In young children after the age of 2 years, but still in the phase of myelin development, abnormal myelination has been described in biopsy studies of the sural nerve (Gabreëls-Festen *et al.*, 1992). Histological studies in CMT1A in adults consist of biopsy studies of the sural nerve, the sensory branch of the superficial peroneal nerve and the skin (Gabreëls-Festen *et al.*, 1995; Hattori *et al.*, 2003; Carvalho *et al.*, 2005; Koike *et al.*, 2007; Saporta *et al.*, 2009) and some post mortem case studies, most probably in CMT1A patients, although carried out before CMT1A could be genetically proved (Smith *et al.*, 1980). These studies show that the myelinated fibres are predominantly affected and abnormal myelination probably extends over the whole nerve length. The most severe pathological changes were seen distally in nerves, but there were also changes in the proximal nerves and in the roots (Smith *et al.*, 1980). Recently, it was shown that myelinated internodes are uniformly shortened in human skin biopsies, which suggests that CMT1A is a dysmyelinating disorder (Saporta *et al.*, 2009). In both *PMP22* over-expressing mouse models myelination was delayed and normal myelination was never reached. Consistent with the differences in NCVs between the C3-PMP and C22 mice, the degree of dysmyelination in C3-PMP mice was considerably less in the C3-PMP than in C22 mice. Severe dysmyelination was reported previously in C22 mice (Robaglia-Schlupp *et al.*, 2002). In both mouse models, amyelinated or thinly myelinated fibres were most abundant in the ventral roots, less so in the motor femoral quadriceps nerve and even less in the dorsal roots and the mixed motor-sensory peroneal nerve (figure 6-3). In WT mice the ventral root consists almost exclusively of large myelinated axons whereas, the motor femoral and peroneal nerves are more mixed with larger numbers of small fibres in the peroneal than in the motor femoral nerve. Hence the variation in degree of dysmyelination in these mouse models could be due to the different fibre size distributions at the different sites, indicating that dysmyelination predominantly affects larger diameter fibres, rather than whether

they are motor or sensory in origin. Uncompacted myelin sheaths were not seen in human CMT1A or in the C3-PMP mice, but this abnormality was often present in the peripheral nerves of C22 mice (figure 6-3B and I).

A difference between the mouse models and human CMT1A is that demyelination is more prominent in human CMT1A. Active demyelination was identified in early childhood in CMT1 patients with infantile onset (Gabreëls-Festen *et al.*, 1992), but not further specified in a later article specifically addressing CMT1A (Gabreëls-Festen *et al.*, 1995). The frequency of active demyelination was low and the same at all ages examined in the mouse models; a proportion of the thinly myelinated fibres were probably the result of de- and remyelination. The paucity of onion bulb formations in these, but also other mouse models (Robertson *et al.*, 2002) compared to patients with CMT1A (Gabreëls-Festen *et al.*, 1995; Hattori *et al.*, 2003) may reflect a higher degree of active de- and remyelination during the first years of life in humans (Gabreëls-Festen *et al.*, 1995), be related to the shorter lifespan of a mouse, or some other difference between mice and men. It is of interest that onion bulbs were not found in dermal myelinated fibres in adult CMT1A patients (Saporta *et al.*, 2009) in contrast to the sural nerve.

The most extensive data on axonal function in humans are derived from surface CMAP amplitude studies, being an easily measurable indirect surrogate marker for axonal function. In young CMT1A patients distal arm surface CMAP amplitudes are often lower from early age onwards, without any indication of the normal physiologic increase with ageing (Yiu *et al.*, 2008), suggesting that normal axonal function is not reached at any time during development. The situation seems to be different for the distal leg surface CMAP amplitudes, which show a decrease over time (Garcia *et al.*, 1998; Berciano *et al.*, 2000; Berciano *et al.*, 2003). This can not be explained just by an impaired development, but suggests a deterioration of axonal function over time at young age in the longer leg nerves. Distal leg and arm surface CMAP amplitudes and distal arm motor unit number estimates in adult CMT1A patients are lower than in healthy controls and show a small decrease over time in patients and controls (Verhamme *et al.*, 2009; van Dijk *et al.*, 2010). This small decrease in patients is similar to that found in controls, indicating that the decline in axonal function is limited in adult CMT1A patients. We used needle electrodes instead of surface electrodes for the recording of CMAPs to study the mouse models because of the small size of the mice, especially at 3 weeks. It is of note that the needle CMAP amplitudes are not directly comparable to surface CMAP amplitudes: needle CMAPs represent a relatively small area of the recorded muscle, and thus may not readily detect small changes in axonal function over time. Nonetheless, the needle CMAP amplitudes were less reduced in C3-PMP than in C22 mice at all ages, while WT, C3-PMP nor C22 mice did not show reductions in needle CMAP amplitudes over time.

Morphometric studies showed that axon numbers in both CMT1A patients and in the *PMP22* over-expressing mouse models are lower than in healthy controls. Studies in humans are scarce and limited to sensory nerves. Morphometry studies of the sural nerve early in life, the youngest child studied being 2 years of age, suggest a lower number of myelinated fibre number (Gabreëls-Festen *et al.*, 1995). These data have to be interpreted with caution as studies were performed in a small number of young patients and did not assess the total myelinated fibre population in all fascicles; the total myelinated fibre number was calculated from fibre densities (which are affected by the increase in Schwann cell processes and in fibrous endoneurial collagen) and areas (Gabreëls-Festen *et al.*, 1995). Nonetheless, this would be in accordance with the early lower number of myelinated fibres as shown in the mouse models. Similarly to the developmental studies, detailed investigations of the sural nerve in adult CMT1A patients comprise only small numbers so it is difficult to draw conclusions about axonal loss over time (Gabreëls-Festen *et al.*, 1995). This caveat also applies to morphometric studies in the sural nerve in healthy controls (Jacobs and Love, 1985): a wide variation in myelinated fibre density and total fibre numbers in a limited number of healthy controls makes a proper estimate of the decline over time very difficult. In a recent study, the number of dermal myelinated fibres was quantified by measuring the density of Meissner corpuscles and showed lower densities in adult CMT1A patients than in controls (Saporta *et al.*, 2009). As early as 3 weeks of age, C3-PMP mice showed reduced numbers of (normally) myelinated fibre in the peroneal nerve as compared to WT. These numbers were even lower in C22 mice. During adulthood, there was no detectable loss of myelinated fibres in the peroneal nerve in C3-PMP mice and WT mice, indicating that axons are preserved to a similar extent in both adult WT and C3-PMP mice, whereas there was a clear loss in C22 mice in this nerve. Changes in axon diameter over time might be informative as another indicator of the long-term axonal integrity. Median axon diameters in the peroneal nerve were smaller at all ages in C22 mice as compared to WT mice, but there were no indications for a reduction in median diameter over time in adult mice. It is therefore unlikely that there would be a reduction over time in axon diameter in the less affected C3-PMP mice.

Data in human CMT1A support the hypothesis that the severity of the disturbance of the myelination process is one of the factors that eventually determines the extent of axonal dysfunction during the first two decades of life (Verhamme *et al.*, 2009). After the nerves have reached their final length, a balance may be reached between myelination status and axonal function. Studies showed the intimate interaction between developing axons and myelin (Mirsky *et al.*, 2002; Woodhoo and Sommer, 2008; Feltri *et al.*, 2008; Kaplan *et al.*, 2009). It might be hypothesized that the abnormal axon-Schwann cell interaction during development early in life reduces the number of axons that become large enough to be myelinated, meaning that normal numbers of myelinated fibres are never reached. Our results in the mouse models suggest that a

more severely abnormal myelination leads to more axonal dysfunction, which is also dependent on the length of the nerve. Early degeneration and loss of dysmyelinated axons may be another explanation, although no pathological evidence for this was seen in this study. The results in adult mice may still reflect the amount of axonal dysfunction that has arisen earlier in life due to the abnormal myelination: clinically more affected adult mice, with higher neuromuscular SHIRPA sum scores, showed lower needle CMAP amplitudes, lower numbers of (normally) myelinated fibres and lower MNCVs. As in human CMT1A, the results in C3-PMP mice indicate that a balance is reached between myelination status and axonal function early in life, while in C22 mice the number of axons is lower during maturation with major loss of axons in adult mice.

The strengths of this study are its duration, the investigation of different disease severities and the relatively large groups of mice. Ideally, longitudinal assessment of the same mice would have been an optimal design to trace changes over time. However, as the morphometric analysis is invasive, we had to study different mice at several time points. Despite this, due to the stable genetic background, we do not believe that we may have missed relevant changes. The limitations of needle CMAP recordings we already discussed above. The duration of the study does not span the life of a mouse. However, 1.5 years extends well into adult mouse life and in this time period changes over time in numbers of axons were detectable in the C22 mice. We do not expect that a longer duration of the study would change our conclusions. The endogenous mouse *Pmp22* as well as the transgenic human *PMP22* are expressed in the mouse models. Previous studies determined the level of expression of the human *PMP22* transgene relative to the level of mouse *Pmp22* mRNA and found that higher ratios were associated with a more severe polyneuropathy. The ratio was 1.6 for the C22 heterozygote mice with 7 copies of the human *PMP22* gene, and 1 for C61 heterozygotes with 4 copies of the transgene (Huxley *et al.*, 1998). However, these measurements were performed on affected nerves containing a relative high proportion of non-myelinating cells. Therefore we think that the previous measurements are underestimations of the over-expression of *PMP22* mRNA. It is difficult to extrapolate these ratios to the situation in human CMT1A, since absolute PMP22 amounts cannot be deduced from the mRNA ratios, non-myelinating Schwann cells also produce *PMP22* mRNA, and a major part of PMP22 is degraded normally (Pareek *et al.*, 1997). Theoretically, the human *PMP22* duplication will lead to a 1.5 times increase in *PMP22* mRNA expression. The density of PMP22 in compact myelin from CMT1A nerves in human skin biopsies was elevated around two times on average, but highly variable between patients (Katona *et al.*, 2009). In our opinion the resultant polyneuropathy in terms of clinical symptoms and signs, together with the electrophysiology and pathology, should be the major criterion to judge the usefulness of the model.

To the best of our knowledge, this is the first study that quantifies the course of the disease clinically, electrophysiologically and morphometrically in two mouse models

over a long period of 1.5 years and that compares this course with the natural history in human CMT1A. Taken together, we conclude that C3-PMP mice may be more appropriate model for CMT1A than C22 mice. The C3-PMP mice may be very useful to investigate the abnormalities in initial nerve maturation, taking into account the myelin and the axon maturation and the Schwann cell-axon interaction. This might give opportunities to improve myelination early in life, but the main goal should be to reach normal numbers of axons and normal axonal function during nerve maturation. Furthermore, the model is useful to investigate whether improving myelination in adult mice still might be able to restore axonal function. The C22 mice may still be a useful model for the severe part of the CMT1 spectrum, including Dejerine-Sottas syndrome and hereditary hypomyelinating neuropathy.

Neuromuscular SHIRPA: a modified version of the SHIRPA protocol (Rogers *et al.,* 1997) with 19 original items and one extra item. A sum score of the modified scores (in square brackets) is calculated, ranging from 0 indicating no signs and 43 being maximally affected.

Behaviour recorded in viewing jar

Body position
[3] 0 = Completely flat
[2] 1 = Lying on side
[1] 2 = Lying prone
[0] 3 = Sitting or standing
[0] 4 = Rearing on hind legs
[0] 5 = Repeated vertical leaping

Spontaneous activity
[2] 0 = None, resting
[1] 1 = Casual scratch, groom, slow movement
[0] 2 = Regular scratch, groom, moderate movement
[0] 3 = Vigorous, rapid/dart movement
[0] 4 = Extremely vigorous, rapid/dart movement

Tremor
0 = None
1 = Mild
2 = Marked

Letting the limbs slip through grid (added item)
0 = Absent
1 = Present

Behaviour recorded in the arena

Tansfer arousal
[3] 0 = Coma
[2] 1 = Prolonged freeze, then slight movement
[1] 2 = Extended freeze, then moderate movement
[0] 3 = Brief freeze (few seconds), then active movement
[0] 4 = Momentary freeze, then swift movement
[0] 5 = No freeze, immediate movement
[0] 6 = Extremely excited ("manic")

Gait
0 = Normal
1 = Fluid but abnormal
2 = Limited movement only
3 = Incapacity

Pelvic elevation
[2] 0 = Markedly flattened
[1] 1 = Barely touches
[0] 2 = Normal (3 mm elevation)
[0] 3 = Elevated (more than 3 mm elevation)

Tail elevation
[1] 0 = Dragging
[0] 1 = Horizontal extension
[1] 2 = Elevated/straub tail

Touch escape finger stroke from above
[2] 0 = No response
[1] 1 = Mild (escape response to firm stroke)
[0] 2 = Moderate (rapid response to light stroke)
[0] 3 = Vigorous (escape response to approach)

Behaviour recorded above the arena

Positional passivity
[0] 0 = Struggles when held by tail
[0] 1 = Struggles when held by the neck (finger grip, not scruffed)
[1] 2 = Struggles when laid supine (on back)
[2] 3 = Struggles when held by hind legs
[3] 4 = No struggle

Trunk curl
[1] 0 = Absent
[0] 1 = Present

Limb grasping
[0] 0 = Absent
[1] 1 = Present

Grip strength
Lower the animal and allow it to grip the grid, then apply a gentle horizontal backwards pull.
[3] 0 = None
[2] 1 = Slight grip, semi-effective
[1] 2 = Moderate grip, effective
[0] 3 = Active grip, effective
[0] 4 = Unusually effective

Body tone
Compress sides of animal between thumb and index finger.
[1] 0 = Flaccid, no return of cavity to normal
[0] 1 = Slight resistance
[0] 2 = Extreme resistance, board like

Toe pinch
Gentle lateral compression of mid digit of hind foot with fine forceps. During the procedure the hind limbs are lifted clear of the grid.
[3] 0 = None
[2] 1 = Slight withdrawal
[1] 2 = Moderate withdrawal, not brisk
[0] 3 = Brisk, rapid withdrawal
[0] 4 = Very brisk repeated extension and flexion

Wire manoeuvre
Animal is held above the wire by tail suspension and lowered to allow the forelimbs to grip the horizontal wire. The mouse is held in extension and rotated around to the horizontal and released.
0 = Active grip with hind legs
1 = Difficulty to grasp with hind legs
2 = Unable to grasp with hind legs
3 = Unable to lift hind legs, falls within seconds
4 = Falls immediately

Behaviour recorded during supine restraint

Limb tone
Resistance to gentle finger tip pressure on plantar surface of left and right hind paw.
[2] 0 = No resistance
[1] 1 = Slight resistance
[0] 2 = Moderate resistance
[0] 3 = Marked resistance
[0] 4 = Extreme resistance

Abdominal tone
Palpation of the abdomen
[1] 0 = Flaccid, no return of cavity to normal
[0] 1 = Slight resistance
[0] 2 = Extreme resistance, board like

Contact righting reflex
Place the animal into the plastic tube and turn mouse upside down.
0 = Present
1 = Absent

Negative geotaxis
Place the animal on horizontal grid. This is then raised to the vertical with the animal facing the floor.
0 = Turns and climbs the grid
1 = Turns and then freezes
2 = Moves, but fails to turn
3 = Does not move within 30 seconds
4 = Falls off