Distal axonopathy in peripheral nerves of PMP22-mutant mice

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Summary

A partial duplication of chromosome 17 is associated with Charcot–Marie–Tooth disease type 1A (CMT1A), a demyelinating peripheral neuropathy that causes progressive distal muscle atrophy and sensory impairment. Trisomic expression of peripheral myelin protein 22 (PMP22) whose gene is contained within the duplicated region is considered to be responsible for the disease. By using recombinant gene technology in rodents, we had demonstrated previously that PMP22 is sensitive to gene dosage. Homozygous PMP22 knockout (PMP22 0/0) mice and transgenic animals carrying additional copies of the PMP22 gene develop distinct peripheral polyneuropathies. We have now performed a detailed morphometrical analysis of the L3 roots, quadriceps and saphenous nerves of these PMP22-mutant mice to study whether the myelin and potential axonal deficits are evenly distributed. The L3 roots and the peripheral nerves were chosen as representatives of the proximal and distal segments of the peripheral nervous system. When the roots were compared with the peripheral nerves, myelin deficiencies appeared more severe at the radicular levels, in particular the ventral roots. Decreased numbers of large calibre axons were a prominent feature in the motor branches of both strains of PMP22-mutant mice, and these axonal deficits were more severe distally. Active axonal damage was only observed in the nerves of PMP22 0/0 mice. Despite the distinct effects on myelination and the Schwann cell phenotype that characterize the neuropathies of PMP22-mutant mice, both strains develop a distally accentuated axonopathy as a common disease mechanism which is likely to be responsible for the neurological deficits.

Keywords: Charcot–Marie–Tooth disease; peripheral myelin protein 22; knockout mice; transgenic mice; axonal atrophy

Abbreviations: CMT = Charcot–Marie–Tooth disease; Cx32 = connexin32; HMSN = hereditary motor and sensory neuropathy; HNPP = hereditary neuropathy with liability to pressure palsies; NF = neurofilaments; PLP = proteolipid protein; PMP22 = peripheral myelin protein 22; P0 = protein zero; Tr = Trembler

Introduction

The syndrome of peroneal muscular atrophy or Charcot–Marie–Tooth (CMT) disease is one of the most frequent inherited causes of neurological disability. Although initially considered to be a single clinical entity, Dyck and Lambert (Dyck and Lambert 1968a, b) established the heterogeneity of the syndrome on the basis of combined clinical, genetic, electrophysiological and nerve biopsy findings. Later, genetic studies substantiated that CMT disease encompasses several distinct peripheral neuropathies (reviewed by Patel and Lupski, 1994; Harding, 1995; Suter and Snipes, 1995; De Jonghe et al., 1997). The demyelinating form of CMT, also known as hereditary motor and sensory neuropathy (HMSN) type I, can be inherited as an autosomal dominant, X-linked or autosomal recessive trait (Dyck et al., 1993), and the most commonly found autosomal dominant CMT1 loci have been mapped to chromosome 17 (CMT1A) and chromosome 1 (CMT1B).

By far the most frequent cause of HMSN I (~70%) is a 1.5 Mb duplication in the chromosomal region 17p11.2 (Wise et al., 1993; Nelis et al., 1996) containing the peripheral myelin protein 22 (PMP22) gene which encodes a 22 kDa membrane glycoprotein located in compact myelin of peripheral nerves (reviewed by Naef and Suter, 1998). Mutations in the murine PMP22 gene are responsible for the naturally occurring CMT mouse models Trembler (Tr) and Trembler-Jackson (Tr-J) which initially implicated PMP22 as the critical gene within the CMT1A duplication (Suter et al., 1992a, b). Furthermore, PMP22 point mutations have also been found in some rare cases of familial CMT1A without the usual chromosomal duplication and in the severe
focal hypermyelination (tomacula). Tomacula in typical of a myelin disorder. In the early post-natal period, polyneuropathy with electrophysiological abnormalities pathological features reminiscent of HNPP in agreement copies of the (Lupski et al., 1991; Kaku et al., 1993; LeGuern et al., 1997). Additional evidence for the dosage sensitivity of PMP22 was obtained when the corresponding 1.5 Mb deletion was identified in patients with hereditary neuropathy with liability to pressure palsy (HNPP) (Chance et al., 1993), consistent with the finding of three PMP22 mutations leading to presumed null alleles in familial HNPP (Nicholson et al., 1994; Bort et al., 1997; Young et al., 1997).

Clinically, CMT1A due to increased PMP22 gene dosage is usually a relatively benign condition, most commonly with an onset in the first or second decade, and shows progression throughout life. Although variability is observed, even within the same family, most patients have classical CMT, alone or associated with additional symptoms (Birouk et al., 1997). The clinical phenotype includes a distal nerve length-related neuropathy affecting the lower limbs to a greater extent than the upper limbs, and motor function to a greater extent than sensory function. All sensory modalities, but in particular proprioception, can be affected. A high proportion of patients develop foot deformities, such as pes cavus and equinovarus. With disease progression, more proximal muscles also get involved. However, neurological deficits remain generally more prominent distally. Reduced nerve conduction velocity of both motor and sensory fibres is a regular finding. Histologically, a progressive demyelination of peripheral nerves is associated with Schwann cell hyperplasia, leading to the characteristic formation of onion bulb structures (Thomas et al., 1997).

To prove that indeed PMP22 is the dosage-sensitive gene in the CMT1A duplication and to provide accurate animal models, transgenic mice and rats have been generated (Adlkofer et al., 1995, 1997; Huxley et al., 1996, 1998; Magyar et al., 1996; Sereda et al., 1996). Homozygous PMP22 knockout (PMP22<sup>0/0</sup>) mice develop a severe polyneuropathy with electrophysiological abnormalities typical of a myelin disorder. In the early post-natal period, the lack of PMP22 results in delayed myelin formation and focal hypermyelination (tomacula). Tomacula in PMP22<sup>0/0</sup> nerves have been considered unstable structures because myelin degeneration rapidly ensues and Schwann cell onion bulbs develop resulting in a demyelinating neuropathy resembling CMT1 (Adlkofer et al., 1995). In contrast, heterozygous PMP22 knockout (PMP22<sup>+/0</sup>) mice develop pathological features reminiscent of HNPP in agreement with the corresponding genotypes (Adlkofer et al., 1997).

Material and methods

Animals

Homozygous PMP22<sup>0/0</sup> (Agouti SV129EV/C57BL/6) and heterozygous PMP22-transgenic (B6C3F1) mice were obtained from our own breeding colonies. Wild-type mice from the same genetic backgrounds were used as controls. The genotypes were assessed by Southern blot analysis of genomic DNA isolated from tail biopsies as described (Adlkofer et al., 1995; Magyar et al., 1996). Experiments were performed in accordance with the legal requirements of the Eidgenössische Technische Hochschule and Kanton Zürich (Switzerland).

Light and electron microscopy

Ten- to 13-month-old PMP22<sup>0/0</sup> mice, PMP22-transgenic mice with additional copies of the PMP22 gene and wild-type controls were deeply anaesthetized with sodium pentobarbital (100–150 µg/g of body weight) and transcardially perfused with 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3. Four to six animals from each group were studied. To be able to compare proximal versus distal levels of the same peripheral nerve, L3 ventral and mimics the pathological features of CMT1A patients. The nerves of these animals show features of a demyelinating neuropathy with remyelination and the formation of onion bulb structures (Huxley et al., 1996, 1998; Sereda et al., 1996). Severely increased PMP22 gene dosage results in a strongly dysmyelinated disorder resembling congenital hypomyelination both in rats and mice (Huxley et al., 1996, 1998; Magyar et al., 1996; Sereda et al., 1996). Thus, these transgenic animals together with the natural mouse mutants Tr, Tr-J and Trembler PMP22<sup>Ncnp</sup> (Tr-Ncnp) (Suh et al., 1997) have revealed that PMP22 is involved in early steps of myelogenesis, the determination of myelin thickness and the maintenance of myelin and axons of the PNS (reviewed by Naef and Suter, 1998).

In this report, we performed a systematic morphometric analysis of peripheral nerves of PMP22-mutant mice either lacking PMP22 or carrying elevated copy numbers of the PMP22 gene, in order to evaluate the mechanisms resulting in the observed neuropathies. In particular, given the distally more pronounced symptoms in CMT1 patients, we wanted to compare the pathological lesions affecting axons and myelin between proximal and distal segments of a given nerve. In addition, we were interested in assessing whether the pathological process affects motor and sensory branches evenly or whether motor axons are preferentially damaged. It was anticipated that this study would help to define potential targets of future therapies (e.g. axons and/or Schwann cells) and to determine which nerves and morphological parameters should be analysed to evaluate the success of such interventions.

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dorsal spinal roots (as representatives of the most proximal region of the femoral nerve) and the quadriceps and saphenous nerves (the two main distal branches of the femoral nerve) were dissected. The specimens were post-fixed in the same fixative overnight at 4°C, osmicated and, after dehydration, were dissected. The specimens were post-fixed in the same region of the femoral nerve) and the quadriceps and saphenous dorsal spinal roots (as representatives of the most proximal and examined with a JEOL 100C electron microscope.

sections were contrasted with uranyl acetate and lead citrate alkaline toluidine blue. Special care was taken to obtain transverse semithin sections were obtained and stained with PMP22

Thus, our morphometrical analysis of the sensory branches remained limited to the myelinated fibres.

Morphometrical analysis Morphometrical data were collected using the National Institute of Health Image 1.60 software program and a Power Apple-Macintosh 7300/166 computer. A special macro was designed to meet the specific requirements of this type of analysis. Briefly, a 1 µm semithin section of a nerve was visualized in a Zeiss Axiohot microscope with a 630 final magnification, and a series of partially overlapping fields covering the cross-sectional area of the nerve were captured with the help of a KAPPA CCD camera. The overlapping images were transferred to the computer and pasted together to obtain a composite image of the nerve. Next, the nerve was filled up with frames of identical size and nerve fibre profiles within these frames were measured. Frames to be analysed were chosen at random. Depending on the number of myelinated fibres and the size of the nerve, we analysed between 30% and 100% of the fascicular area and, if possible, 200–300 axons per nerve.

The cross-sectional area of the endoneurium, axons and myelinated fibres (fibre = axon plus myelin) were measured for each nerve. To obtain the area of axons and myelinated fibres, a threshold grey-scale level for axons and myelin profiles was defined and used to trace the border of axons and the outer edge of myelin. The diameter of axons and myelinated fibres was mathematically deduced from a circle of an equivalent area. Paranodal regions and Schmidt–Lanterman incisures were included in the analysis because these two specific domains of myelinated fibres could not be unequivocally identified in PMP22-mutant nerves. Information obtained from control animals showed that the axonal diameter of the smallest myelinated fibres measured at least 1 µm. Therefore, we measured myelinated axons irrespective of their size, and unmyelinated axons with a diameter equal or superior to 1 µm in the L3 ventral roots and quadriceps nerves of the PMP22-mutant mice. In the sensory branches of the mutant animals, unmyelinated axons with a diameter between 1 and 1.5 µm could not be quantitatively identified, since the axons were densely packed without enough threshold differences for individualization. Thus, our morphometrical analysis of the sensory branches remained limited to the myelinated fibres.

Quantification and statistical analysis The following data were obtained and analysed using Microsoft Excel 5 software: number of axons with a diameter ≥1 µm per nerve; number of myelinated fibres per nerve; distribution of the diameter of axons and myelinated fibres; g-ratio (axonal diameter/myelinated fibre diameter) as a function of the axonal diameter. Based on the distribution of the axonal diameters, we classified the axons into three categories according to their diameter: 1–2.99 µm, small; 3–6.99 µm, medium; ≥7 µm, large. Data were subjected to statistical analysis by the non-parametric Mann–Whitney U test using Statview, version 4.0 software. P values ≤ 0.05 were considered to be statistically significant.

Results To assess quantitatively the degree of myelin and axonal deficiencies due to altered PMP22 gene dosage, we performed a morphometrical analysis of the roots and peripheral nerves of ~1-year-old PMP22<sup>0/0</sup> mice, transgenic mice carrying additional copies of the PMP22 gene and age-matched wild-type control animals. We selected aged animals because we reasoned that some changes, like axonal loss, might be more pronounced at advanced stages of the disease. The L3 roots were taken as representatives of the most proximal segment of the PNS, containing axons which are close to the neuronal bodies and are either purely motor (L3 ventral root) or sensory (L3 dorsal root). As the distal counterpart, we chose the quadriceps and saphenous nerves, the two main branches of the femoral nerve. The femoral nerve is the major nerve trunk originating from the lumbar plexus and contains axons from the L3 roots. This analysis also allowed us to obtain information about the deficits in motor branches (ventral roots and quadriceps nerves) compared with sensory branches (dorsal roots and saphenous nerves).

Myelinated fibres The frequency distribution of the diameter of myelinated fibres of individual control mice showed a bimodal profile for L3 ventral roots and quadriceps nerves, and a unimodal distribution with a predominance of small calibre myelinated fibres for the L3 dorsal roots and saphenous nerves (data not shown). The total number of myelinated fibres in the L3 ventral roots (mean ± SD: 640 ± 118) and quadriceps nerves (557 ± 57) of the wild-type mice was roughly similar, and there was only slight variability between animals. In contrast, the total number of myelinated fibres in the L3 dorsal root (1683 ± 342) was almost double the number of myelinated fibres in the saphenous nerves (838 ± 79). In addition, the number of myelinated fibres in the L3 dorsal root showed the highest variability among individual controls (Fig. 1).

The roots and peripheral nerves of PMP22<sup>0/0</sup> mice displayed a severe reduction of myelinated fibres (Fig. 1) with a more uniform distribution of sizes when compared...
with controls. All nerve segments showed a unimodal distribution with the main peak usually found between 4 and 7 \( \mu m \) of diameter (data not shown). Myelinated fibres in the L3 ventral roots (110 \( \pm 17 \)) and quadriceps nerves (188 \( \pm 59 \)) were less numerous than in the L3 dorsal roots (347 \( \pm 93 \)) and saphenous nerves (222 \( \pm 64 \)), suggesting that demyelination might affect the motor axons more severely (Fig. 1). To address this issue more closely and because the number of myelinated fibres in control animals was higher in the sensory branches (L3 dorsal roots and saphenous nerves), we calculated the ratio of myelinated fibres between the L3 ventral and dorsal roots (Table 1, VR : DR) and between quadriceps and saphenous nerves (Table 1, QN : SN). The comparison of the values obtained for PMP22\( ^{0/0} \) mice with wild-type animals showed no statistically significant differences (Table 1). Similar results were obtained when we compared the mean number of myelinated fibres of PMP22\( ^{0/0} \) with wild-type nerves (Table 2).
Table 1  Ratios of myelinated fibres and myelin-competent axons (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>VR : DR myelinated fibres</th>
<th>QN : SN myelinated fibres</th>
<th>QN : VR myelinated fibres</th>
<th>SN : DR myelinated fibres</th>
<th>(QN : VR) axons &gt; 3 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.385 ± 0.062 (n = 6)</td>
<td>0.663 ± 0.091 (n = 4)</td>
<td>0.907 ± 0.123 (n = 5)</td>
<td>0.537 ± 0.099 (n = 5)</td>
<td>0.986 ± 0.102 (n = 5)</td>
</tr>
<tr>
<td>PMP22&lt;sup&gt;0/0&lt;/sup&gt;</td>
<td>0.335 ± 0.103 (n = 4, ns)</td>
<td>0.873 ± 0.306 (n = 4, ns)</td>
<td>1.731 ± 0.579 (n = 4, P &lt; 0.05)</td>
<td>0.635 ± 0.095 (n = 4, ns)</td>
<td>0.535 ± 0.114 (n = 4, P &lt; 0.05)</td>
</tr>
<tr>
<td>PMP22&lt;sup&gt;Tg&lt;/sup&gt;</td>
<td>0.064 ± 0.054 (n = 5, P &lt; 0.05)</td>
<td>0.390 ± 0.262 (n = 5, ns)</td>
<td>4.938 ± 6.911 (n = 6, ns)</td>
<td>1.009 ± 0.790 (n = 4, ns)</td>
<td>0.500 ± 0.038 (n = 6, P &lt; 0.05)</td>
</tr>
</tbody>
</table>

VR = L3 ventral roots; DR = L3 dorsal roots; QN = quadriceps nerves; SN = saphenous nerves; ns = not significant; Tg = transgenic.

8-Ratios

The scatter diagram of the 8-ratio against axon diameter correlates changes of myelin thickness in relation to axonal size (Fig. 2). In control nerves, most axons had 8-ratios between 0.6 and 0.8. Values below 0.6 were usually due to the presence of Schmidt–Laterman incisures and paranodal regions. The nerves of PMP22<sup>0/0</sup> mice had an increased scatter of 8-ratios with many unusually low values. Very low 8-ratios were invariably associated with small calibre of axons. In PMP22<sup>Tg</sup>-transgenic mice, there were more axons with 8-ratios below 0.6 compared to control nerves. The number of axons with low 8-ratios increased with the severity of the demyelination, indicating that the myelin content of axons in transgenic mice was more affected by the demyelinating process.

Table 2  Comparison of myelinated fibres and myelin-competent axons in PMP22-mutant with wild-type mice

<table>
<thead>
<tr>
<th></th>
<th>Myelinated fibres</th>
<th>Axons &gt; 3 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>VR : DR</td>
<td>0.17</td>
<td>10</td>
</tr>
<tr>
<td>QN : SN</td>
<td>0.14</td>
<td>20</td>
</tr>
<tr>
<td>VR : DR</td>
<td>0.16</td>
<td>26</td>
</tr>
<tr>
<td>SN : DR</td>
<td>0.09</td>
<td>89</td>
</tr>
<tr>
<td>VR : DR</td>
<td>0.15</td>
<td>35</td>
</tr>
<tr>
<td>SN : DR</td>
<td>0.08</td>
<td>51</td>
</tr>
<tr>
<td>VR : DR</td>
<td>0.13</td>
<td>28</td>
</tr>
<tr>
<td>SN : DR</td>
<td>0.07</td>
<td>96</td>
</tr>
<tr>
<td>VR : DR</td>
<td>0.12</td>
<td>31</td>
</tr>
<tr>
<td>SN : DR</td>
<td>0.06</td>
<td>46</td>
</tr>
</tbody>
</table>

8-Ratios were calculated between the mean values for PMP22<sup>0/0</sup> and wild-type nerves. VR = L3 ventral roots; QN = quadriceps nerves; SN = saphenous nerves.
Fig. 2 Scatter diagrams of g-ratios as function of axon diameters of the quadriceps nerve (left panel) and saphenous nerve (right panel) of representative wild-type (WT) and PMP22-mutant mice. Fibres with low g-ratio values in WT nerves reflect uncompacted myelin regions (Schmidt–Laterman incisures and paranodal regions). The increased dispersion of g-ratios in PMP22<sup>0/0</sup> nerves are due to the presence of tomacula and remyelinated fibres.

Table 3 Percentage of g-ratio values &gt;0.8 in PMP22<sup>0/0</sup> nerves (mean ± SD)

<table>
<thead>
<tr>
<th>VR</th>
<th>DR</th>
<th>QN</th>
<th>SN</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 ± 12</td>
<td>1.6 ± 1.2</td>
<td>12 ± 3</td>
<td>0.17 ± 0.34</td>
</tr>
</tbody>
</table>

n = 4; VR = L3 ventral roots; DR = L3 dorsal roots; QN = quadriceps nerves; SN = saphenous nerves.

probably atrophic axons. Only few large calibre axons were myelinated. They usually had high g-ratios suggesting remyelinated axons because congenital hypomyelination is not a feature of PMP22<sup>0/0</sup> nerves during post-natal development (Adlkofer et al., 1995). Axons with high g-ratios were more frequent in the motor branches than in sensory nerves (Fig. 2 and Table 3).

In PMP22-transgenic mice whose nerves contained myelinated fibres, the g-ratio distribution revealed that mainly the small calibre axons were myelinated. Axons either had a myelin sheath of appropriate thickness or were hypomyelinated (Fig. 2).

**Axonal deficiency**

Since the nerves of the examined PMP22-mutant animals contain many large non-myelinated axons in a 1 : 1 relationship with their Schwann cells (which are normally myelinating), we estimated the diameter size and distribution of myelin-competent axons. This analysis was designed to give an indication of the fate of such an axonal population, regardless of their myelination status. The examination of the axonal diameter of myelinated fibres from control nerves showed that over 99.8% of myelinated axons have a diameter ≥1 μm. Thus, we included all axons with a diameter of 1 μm or above as part of the myelin-competent axonal population in the analysis of PMP22-mutant nerves.

The frequency distribution of the diameter of myelinated...
Axonopathy in PMP22-mutant mice

Fig. 3 Histograms showing the distribution of axons with a diameter \( \geq 1 \mu m \) in the L3 ventral roots of two representative mice from each group (wild-type, WT; PMP22\(^{0/0}\) and PMP22-transgenic mice). Next to the histograms a summary of the statistical analysis is given. For this analysis, axons were divided in three categories: small (1–3 \( \mu m \)), medium (3–7 \( \mu m \)) and large (\( \geq 7 \mu m \)). \( P \) values were calculated using the non-parametric Mann–Whitney \( U \) test (comparisons with WT mice). n.s. = not significant; Tg = transgenic.

Axonopathy in PMP22-mutant mice

Axons from control L3 ventral roots showed a bimodal distribution with a first peak between 1 and 3 \( \mu m \) and a second peak between 7 and 11 \( \mu m \) (Fig. 3). This bimodal distribution was usually lost in the L3 ventral roots of PMP22\(^{0/0}\) or transgenic mice. Axons with a diameter \( > 7 \mu m \) were scarce in both PMP22-mutants, although an increase of the population of medium size axons (between 3 and 7 \( \mu m \)) was apparent (Fig. 3). In addition, the number of small calibre axons (between 1 and 3 \( \mu m \)) was also reduced in PMP22\(^{0/0}\) mice. Moreover, the total number of axons of a diameter \( \geq 1 \mu m \) in the L3 ventral roots of two PMP22\(^{0/0}\) mice was slightly decreased compared with controls and PMP22-transgenic mice (Fig. 4).

The frequency distribution of the diameter of myelinated axons from the quadriceps nerves of wild-type mice showed a peak at the low calibre range (2–3 \( \mu m \)), whereas the medium and large calibre axons were more uniformly distributed. The quadriceps nerves of PMP22\(^{0/0}\) and transgenic mice showed a severe reduction of axons with a diameter \( > 7 \mu m \) and a significant decrease of axons of medium size (between 3 and 7 \( \mu m \); Fig. 5). Furthermore, the total number of axons with a diameter \( \geq 1 \mu m \) in the quadriceps of both mutant mice was reduced compared with controls (Fig. 4). This reduction was more pronounced in PMP22\(^{0/0}\) mice than in PMP22-transgenic animals. To evaluate whether the reduced number of axons was more severe in distal segments, we determined the ratio of axons with a diameter \( \geq 1 \mu m \) between the quadriceps nerves and L3 ventral roots. The ratios of both groups of PMP22-mutant mice compared with the ratios of wild-type animals did not reach statistical significance (data not shown). However, when only the middle and large size axonal populations (\( > 3 \mu m \)) were considered, axon deficiency was much more severe in the quadriceps nerves of both strains of PMP22-mutant mice (Table 1, QN : VR, and Table 2).

Electron microscopy

The morphometrical data indicated a pronounced reduction of large calibre axons in the nerves of the PMP22-mutant mice. To evaluate whether axonal degeneration contributes to this observation, we analysed the ultrastructure of the L3 spinal roots and peripheral nerves of two PMP22\(^{0/0}\) and two PMP22-transgenic mice in comparison with control animals. The L3 roots and peripheral nerves of both types of PMP22-mutant mice showed an obvious deficiency of large calibre axons when compared with wild-type controls (Fig. 6). In the L3 ventral roots and quadriceps nerves of PMP22-
transgenic mice, large and medium size axons were amyelinated and only very few small myelinated fibres were seen (Fig. 6E). In contrast, the L3 dorsal roots and saphenous nerves contained some myelinated fibres (Fig. 6F) which had either a myelin sheath of normal thickness or were thinly myelinated. Some axons were only surrounded by a few wraps of myelin and in some, the myelin sheaths appeared not fully compacted. Occasionally, myelin debris was found in macrophages or Schwann cells. Schwann cell onion bulb formation was not a prominent feature in the nerves of the PMP22-transgenic mice although remains of basal laminae were often seen around axons. Evident signs of axonal damage were not observed.

Most large calibre axons were not myelinated in the L3 ventral roots and quadriceps nerves of PMP220/0 mice (Fig. 6C). Few of them, however, were associated with a thin myelin sheath. Hypermyelination with formation of redundant myelin loops, most probably reflecting tomacula, was a common feature in all nerves of PMP220/0 mice (Adikofer et al., 1995), but this pathology was more frequent in the L3 dorsal roots and saphenous nerves (Fig. 6D). Tomacula usually displaced and compressed the associated axons. Most axons in peripheral nerves and L3 roots were surrounded by onion bulbs consisting of slender cytoplasmic Schwann cell processes and basal lamina. However, these onion bulbs were not as well developed as in human CMT1 nerves (Thomas et al., 1997).

Indicative changes of axonal damage were found only in the nerves of PMP220/0 mice. Structural abnormalities were characterized by an accumulation of dense bodies and vesicles, vacuolization of axonal organelles, disruption of the axolemma and disorganization of cytoskeletal elements (Fig. 7A–C). In the saphenous nerves, Schwann cells surrounding myelin debris without any axonal profile in their cytoplasm were frequently observed, suggesting that the axon had degenerated (Fig. 7D). Occasionally, Schwann cell lamellae of onion bulbs contained a small non-myelinated axon, most probably representing axonal sprouting. Although some of these morphological changes observed in PMP22W0/0 nerves might be age-related, the results are consistent with the morphometric analysis indicating pronounced axonal deficits in these mouse mutants.

### Discussion

Despite the qualitative differences in myelination that characterize PMP220/0 and PMP22-transgenic mice, both mouse strains display a similar spatial distribution of myelin abnormalities. Quantitative analysis of myelinated fibres in the L3 roots and the quadriceps and saphenous nerves of both PMP22-mutant animals revealed that myelin deficits affect the ventral roots more severely than peripheral nerves. While the ratios between motor and sensory nerves in PMP220/0 mice showed similar preservation of myelinated fibres, g-ratios indicated that the demyelination process is more advanced in the motor branches. Similarly, sensory branches of some PMP22-transgenic mice contained a significant number of myelinated small calibre axons. Somatic transgene inactivation was suggested as a plausible hypothesis to account for the presence of a few thinly myelinated axons in the nerves of young PMP22-transgenic mice (Magyar

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**Fig. 4** Number of axons with a diameter $\geq 1 \mu m$ per total fascicular area of the L3 ventral roots (A) and quadriceps nerves (B) of control and PMP22-mutant mice as analysed using toluidine blue-stained transverse semithin sections. Below each diagram, a summary of the statistical analysis is shown. $P$ values were calculated using the non-parametric Mann–Whitney $U$ test (comparisons with wild-type, WT, mice). n.s. = not significant; Tg = transgenic.

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Axonopathy in PMP22-mutant mice

Fig. 5 Histograms demonstrating the distribution of axons with a diameter ≥1 μm in the quadriceps nerves of two representative mice from each group (wild-type, WT; PMP22\(^{0/0}\) and PMP22-transgenic mice). Next to the histograms, a summary of the statistical analysis is shown for each group of mice. For this analysis axons were divided in three categories: small (1–3 μm), medium (3–7 μm) and large (≥7 μm). \(P\) values were calculated using the non-parametric Mann–Whitney \(U\) test (comparisons with WT mice). n.s. = not significant; Tg = transgenic.

et al., 1996). Considering the number of myelinated fibres present in old PMP22-transgenic nerves, such a mechanism seems unlikely unless Schwann cells which have inactivated the transgene have a proliferation or survival advantage. Alternatively, Schwann cells associated with small calibre sensory axons might be less vulnerable to PMP22 gene dosage.

The predilection of myelin defects for motor nerves has previously been noticed in PMP22-mutant animals (Adkofe et al., 1995; Sereda et al., 1996), heterozygous protein zero (P\(_0\)) knockout mice (Martini et al., 1995; Shy et al., 1997), and connexin 32 (Cx32) knockout mice (Anzini et al., 1997; Scherer et al., 1998). Such a distribution is intriguing for a genetic defect which should affect myelinating Schwann cells evenly. Since the available evidence supports a common developmental origin for all types of Schwann cells found in mature peripheral nerves, other factors, such as specific axon–glia interactions, might be responsible for this peculiar distribution. With the exception of neurons responsible for proprioception, motor neurons have larger calibre axons than sensory neurons and, as a consequence, motor nerves contain a higher percentage of large fibres. Consistently, most myelinated axons in PMP22-mutant nerves were of small calibre. Furthermore, ventral roots which only contain the axons of motor neurons display the most severe myelination defect in both PMP22-mutant strains. Thus, our data support the hypothesis that Schwann cells myelinating large caliber axons, such as motor neurons and proprioception sensory neurons, might be more vulnerable to myelin gene defects. However, the increased susceptibility of large axons to abnormal myelination might also be partially attributed to PMP22 expression by motor neurons (Parmantier et al., 1995). Furthermore, the possibility that the thicker myelin sheath of large axons is more susceptible to disturbances of myelin stability remains open.

Morphometrical analysis of axonal calibre demonstrated a striking deficit of large axons with a diameter superior to 7 μm in the motor branches (L3 ventral roots and quadriceps nerves) of both PMP22\(^{0/0}\) and transgenic mice. This axonal deficiency might be explained by reduced axonal calibre
Fig. 6 Electron micrographs comparing the ultrastructure of the L3 ventral roots (A, C, E) and L3 dorsal roots (B, D and F) of wild-type (A and B), PMP220/0 (C and D) and PMP22-transgenic (E and F) mice. (A and B) Wild-type roots showing normally myelinated fibres (A). Several non-myelinating Schwann cells are seen in the dorsal root in association with small calibre axons (s). Comparison between the ventral (C) and dorsal (D) root of a PMP220/0 mouse: in the ventral root many axons are demyelinated (a) and only few myelinated fibres are present (A). Myelinated fibres are more abundant in the dorsal root, where hypermyelination and tomacula (t) are seen. Onion bulbs consisting of thin Schwann cells cytoplasmic lamellae (arrowheads) and basal lamina (arrows) are present around many axons. Comparison between the ventral (E) and the dorsal (F) root of a PMP22-transgenic mouse, showing many amyelinated axons (a) in the ventral root and abundant myelinated fibres (A), some of them thinly myelinated (asterisks), in the dorsal root. Note the increased cytoskeletal density of the axoplasm and the absence of large calibre axons in the roots of PMP22-mutant mice. M = compact myelin; Tg = transgenic. Bar = 3 µm.
Axonopathy in PMP22-mutant mice

Fig. 7 Ultrastructure of selected abnormal axons in the nerves of PMP22<sup>0/0</sup> mice. Transverse sections from the L3 ventral root (A) and saphenous nerve (B–D). (A) The axoplasm (A) of this myelinated fibre contains a large vacuole filled with granular and filamentous material and a dense osmophilic body of complex structure, besides many membrane-bound vesicles. (B) Myelinated fibre with partially folded, degenerating myelin. The axoplasm (A) is highly distorted by multiple inclusion bodies and disorganized neurofilaments. The adaxonal Schwann cell cytoplasm (asterisk) has a granular appearance. (C) Hypermyelinated fibre with a small, very dense axon (A) containing several membrane-bound vacuoles and mitochondria. (D) Schwann cell cytoplasmic processes (arrowheads) devoid of discernible associated axon profiles surround a membranous myelin-like debris (m). M = compact myelin; a = unmyelinated axons. Bar = 1 μm.

because a shift of axonal diameter to a smaller size with a concomitant relative increase of medium or small calibre axons was observed. Most hypermyelinated axons in the PMP22<sup>0/0</sup> mice were of small diameter, suggesting a direct relationship between hypermyelination and reduced axonal calibre. However, the mechanisms leading to the observed pathology are likely to be multifactorial. During development of the PNS, axons attain their final calibre progressively in two well-defined phases. After axons have contacted their targets and synapses are formed, axonal growth best correlates with the amount of microtubules. A further increase of axonal calibre, known as radial growth, is tightly coupled with myelination and is associated with increased neurofilament (NF) content (reviewed by Hoffman and Griffin, 1993). If myelination is necessary for normal radial growth during axonal maturation, defective radial growth might be expected to occur in PMP22-transgenic mice since their nerves remain largely amylinated (Magyar et al., 1996). Similarly, PMP22<sup>0/0</sup> mice have a dysmyelinating phenotype with highly perturbed myelination. Thus, radial growth may also be altered in these animals. Although myelination per se is not required for the radial growth of axons in the optic nerve (Sanchez et al., 1996), the situation may differ in the PNS. Large calibre axons do not develop in the peripheral nerves of the Tr mouse suggesting that a competent myelinating Schwann cell is required for normal radial growth in the PNS (Ayers and Anderson, 1976). An alternative or additional mechanism to account for the reduced axonal calibre present in PMP22-mutant mice is axonal atrophy which could develop or increase over time. Such a process is likely to be responsible for the reduction of axonal calibre observed in MAG (myelin-associated glycoprotein) knockout mice. Axonal radial growth proceeds normally in these mutant mice but they exhibit a reduction of the mean diameter of myelinated axons when adult (Yin et al., 1998).

NF number and NF spacing are two important intrinsic factors that determine axon calibre (reviewed by Hoffman and Griffin, 1993; Xu et al., 1996; Zhu et al., 1997; Elder et al., 1998). A progressive increase of NF synthesis together with a concomitant decline of NF transport modulates NF.
number during the radial growth of developing axons, and similar changes occur during regeneration of peripheral axons (reviewed by Hoffman and Griffin, 1993). In addition, alterations in NF spacing contribute to local variations of calibre along axons (Hsieh et al., 1994). Most probably myelinating Schwann cells locally regulate NF spacing by modulating the phosphorylation of NF-H and NF-M subunits (de Waegh et al., 1992). It is conceivable that any of these factors, alone or in combination, may be responsible for the reduced axonal calibre observed in PMP22-mutant mice. Indeed, reduced axonal caliber in the dysmyelinated nerves of Tr mice correlate with increased density of axonal cytoskeletal elements (Low, 1976), reduced NF spacing and reduced NF phosphorylation (de Waegh and Brady, 1991; de Waegh et al., 1992). Similarly, the reduction of axon calibre in MAG knockout mice is associated with a decrease of NF spacing and reduced phosphorylation of the NF-high molecular weight and NF-medium molecular weight subunits (Yin et al., 1998). Although we did not quantify NF density in our mutants, axonal cytoskeleton appeared more closely packed in PMP22-mutant nerves compared with controls.

In our study, the population of myelin-competent axons was decreased in the L3 ventral roots and quadriceps nerves of PMP220/0 mice and, to a lesser extent, in the quadriceps nerves of PMP22-transgenic mice suggesting that additional factors besides decreased axonal caliber might contribute to axonal loss. Additionally, morphological evidence for axonal damage and degeneration was found in the nerves of PMP220/0 mice. Axonal degeneration is being recognized as a quite common finding in both the PNS (Giese et al., 1992; Fruttiger et al., 1995; Anzini et al., 1997; Yin et al., 1998) and CNS (Anderson et al., 1998; Griffiths et al., 1998) of mice with mutations in myelin genes, but the responsible cellular and molecular mechanisms are largely unknown. The presence of axonal spheroids in the CNS of proteolipid protein (PLP) knockout and transgenic mice hints at altered axonal transport as a possible cause of fibre degeneration in these animals (Anderson et al., 1998; Griffiths et al., 1998). Axonal atrophy was considered to predispose to axonal degeneration in P0 and MAG knockout mice (Giese et al., 1992; Yin et al., 1998). However, decreased axonal calibre per se may not be sufficient to cause axonal degeneration because mice with targeted null mutations for NF-L and NF-M subunits behave normally and do not show signs of axonal degeneration despite a severe impairment of axonal radial growth (Zhu et al., 1997; Elder et al., 1998). Chronic deprivation of neurotrophic factors might also contribute to axonal degeneration since myelinating Schwann cells express a different repertoire of neurotrophic factors compared with non-myelinating Schwann cells (Friedman et al., 1996). Interestingly, we could not identify morphological signs of degeneration in the amylated axons of PMP22-transgenic mice, whereas degenerating axons in PMP220/0 mice were always associated with a myelin sheath. These findings may suggest that axonal degeneration is triggered by factors associated with myelinating Schwann cells, but it cannot be excluded that amylated/demyelinated axons indeed degenerate, but morphological changes of degeneration remained unrecognized (Anderson et al., 1998).

Similarly to CMT1 disease in humans (Dyck et al., 1993), PMP220/0 and transgenic mice develop a distally related axonopathy since the reduction of medium and large calibre axons is more severe in the quadriceps nerves than in the L3 ventral roots. Axons depend on the neuronal body for the supply of some vital organelles, such as mitochondria, and also newly synthesized proteins must be transported to their final destination for local assembly or secretion. Thus, it appears likely, that neurons with large and long axons have higher demands to maintain the integrity of their peripheral processes and they are probably more sensitive to factors perturbing their function. It is also to be expected that long axons would be more vulnerable than short axons if axonal transport is impaired. There is evidence that Schwann cells strategically positioned along the axon might help to regulate the transport or delivery of proteins to their specific localization along the axon. Myelinated axons show local variation of NF number with more NF in the internode than in the node of Ranvier (Hsieh et al., 1994). Furthermore, hypomyelinated Tr nerves have a slower rate of transport of NF subunits as well as a fraction of tubulins, and graft experiments suggest that Schwann cells locally modulate slow axonal transport (de Waegh and Brady, 1990; de Waegh et al., 1992).

Although CMT1 is primarily regarded as a demyelinating neuropathy, a long-standing point of view has been entertained that a distal axonopathy may contribute to the clinical progression of the disease. In particular, the distribution of muscle weakness and atrophy, and the pattern of sensory loss in CMT1 patients implies that distal nerves are more vulnerable. Additional evidence for this distal involvement came from the analysis of various levels of CMT1 saphenous nerves. The study showed that the frequency of demyelination and remyelination was higher in the distal segment compared with more proximal segments. Moreover, the quantification of myelinated fibres clearly indicated that loss of myelinated fibres was more severe distally and these data have been interpreted as a sign of axonal atrophy (Dyck et al., 1993). Reduced axonal size in CMT1 nerves has been shown to correlate with decreased NF number (Nukada and Dyck, 1984), hypophosphorylation of NF subunits and abnormal isofrom composition of b-tubulin (Watson et al., 1994). Moreover, myelin deficits are already present very early in CMT1 patients (Gabreels-Festen et al., 1995) and nerve conduction velocity remains quite stable during the course of the disease (Dyck et al., 1989). Thus, axonopathy is a likely explanation for the progressive muscle atrophy and clinical deficits in CMT1 patients. It remains to be determined, however, whether muscle weakness and wasting is more severe distally in PMP220/0 and transgenic mice.

In conclusion, a strong agreement exists between the data obtained from CMT1 nerves and animal models for CMT1 disease, and the combined evidence indicates that axonal...
involvement is a crucial pathogenic factor. The distally accentuated axonopathy probably develops gradually and contributes to the disease progression. It remains a challenge for future research to elucidate the molecular basis of the abnormal Schwann cell–axon interactions leading to axonal pathology. 

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