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Low affinity NGF receptor expression in CMT1A nerve biopsies of different disease stages

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Summary
Duplication of the gene for the peripheral myelin protein 22 (PMP22) is the most common cause for Charcot–Marie–Tooth neuropathy type 1a (CMT1A) neuropathy. In early stages of the disease PMP22 is overexpressed in nerve biopsies from CMT1A patients. Recent studies with genetically modified Schwann cells have demonstrated that the altered expression of PMP22 modulates cell growth. Thus we hypothesized that elevated expression of PMP22 at the beginning of the disease might alter Schwann cell differentiation and phenotype. In this study we investigated Schwann cell phenotype in different stages of CMT1A neuropathy using antibodies to established Schwann cell markers. We found a pathological expression of low affinity nerve growth factor receptor (LNGF-R—also referred to in the literature as p75) in numerous Schwann cells of young CMT1A patients with almost normal myelin thickness and very few onion bulbs. During further progression of the disease, when Schwann cells began to form onion bulbs, we observed an intense LNGF-R immunoreactivity in all layers of the onion bulbs. In the most advanced stages of the disease, characterized by massive onion bulb formation, no LNGF-R immunoreactivity was shown. In age-matched control nerves LNGF-R staining was barely detectable. Furthermore, onion bulbs seen in patients with chronic idiopathic polyneuropathy (CIDP) were always negative for LNGF-R. In addition, at all CMT1A disease stages analysed, LNGF-R-positive Schwann cells were glial fibrillary acidic protein (GFAP) negative. Immunostaining with an antibody to the proliferation marker, proliferating cellular nuclear antigen (PCNA) indicated Schwann cell proliferation when onion bulb formation was well developed. In conclusion, we describe a disease stage-dependent altered Schwann cell phenotype in CMT1A neuropathy, which could be a direct consequence of the PMP22 overexpression on Schwann cell growth behaviour or, less likely, a secondary phenomenon related to myelin loss.

Keywords: cell differentiation; CMT1A neuropathy; immunohistochemistry; low affinity NGF receptor; PMP22; Schwann cells

Abbreviations: CIDP = chronic inflammatory demyelinating polyneuropathy; CMT1A = Charcot–Marie–Tooth neuropathy type 1a; GFAP = glial fibrillary acidic protein; LNGF-R = low affinity nerve growth factor receptor; MBP = myelin basic protein; PCNA = proliferating cellular nuclear antigen; PMP22 = peripheral myelin protein 22

Introduction
Hereditary demyelinating neuropathies are a genetically heterogeneous group of diseases, the majority of which are caused either by duplication or deletion of the gene for PMP22 or by point mutations in the genes PMP22, myelin protein P0 or Connexin 32 (Patel and Lupski, 1994; Harding, Kloepfer, 1979; Matsunami et al., 1992; Patel et al., 1992; Timmerman et al., 1992; Valentijn et al., 1992) a gene dosage mechanism has been suggested. However, analysis of PMP22 mRNA expression in sural nerves of CMT1A patients in several

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studies revealed that overexpression was found only in ~50% of the patients which seemed to be those in early stages of the disease (Hanemann et al., 1994, 1995; Yoshikawa et al., 1994). Since the PMP22 gene is homologous to the gas3 (growth arrest-specific) gene, a role for protein PMP22 in myelination and Schwann cell differentiation and growth has been postulated and demonstrated in vitro (Spreyer et al., 1991; Lemke, 1993; Zoidl et al., 1995). Based on this findings, we hypothesized (Hanemann et al., 1994) that elevated expression of PMP22 at the beginning of the disease alters Schwann cell differentiation and leads to a pathological phenotype.

Thus in this study, our investigations were designed to test whether the duplication of PMP22 in CMT1A patients is associated with an alteration of the Schwann cell phenotype during the course of the disease as compared with normal controls.

Various antibodies, such as those directed to the LNGFR-R, S100 protein, GFAP and myelin proteins, have been used in the past to characterize different Schwann cell phenotypes (Jessen and Mirsky, 1991; Fan and Gelman, 1992; Toews et al., 1992). Low affinity nerve growth factor receptor is expressed during proliferation of foetal Schwann cells but is downregulated when myelination is initiated (at ~18 weeks of foetal age) (Heumann et al., 1987; Yasuda et al., 1987; DiStefano and Johnson, 1988; Scarpini et al., 1988). In normal human nerves LNGFR-R is barely detectable (Sobue et al., 1988), but it is re-expressed when axon–Schwann cell contact is lost during Wallerian degeneration or in acute but not chronic demyelination in rats and humans (Taniuchi et al., 1986; Heumann et al., 1987; Fan and Gelman, 1992; Toews et al., 1992; Stoll et al., 1993). A similar regulation has been shown for GFAP expression in rats (Jessen et al., 1984, 1990; Toews et al., 1992).

In this study we show expression of LNGFR-R in Schwann cells of CMT1A nerve biopsies at early disease stages as well as changes of LNGFR-R staining during onion bulb formation. These LNGFR-R-positive Schwann cells were GFAP negative indicating an unique Schwann cell phenotype in CMT1A neuropathy that might result from the genetic defect.

### Material and methods

#### Nerve specimens

Diagnostic biopsies of the sural nerve were taken from patients diagnosed to suffer from CMT1A by clinical, electrophysiological and pathological criteria according to the guidelines of the European consortium to study hereditary motor sensory neuropathy (De Visser et al., 1993) prior to the availability of genetic testing after informed consent. Years later diagnosis of CMT1A neuropathy was genetically proven by testing for PMP22 duplication as previously described (Hoogendijk et al., 1992). Normal nerves were taken either from multiorgan donors without neuropathy after informed consent or from patients biopsied for diagnostic reasons and proved to have normal nerves during routine examination. Experiments have been approved by the local ethical committee.

#### Epon sections and morphometry

A whole sural nerve biopsy was performed and prepared for light and electron microscopic examination, using standard techniques (Gabreëls-Festen et al., 1992). Electron microscopic photographs (x2200) covering ~10% of the total transverse fascicular area were used for morphometric analysis. The density and diameter distribution of myelinated fibres were determined using a Zeiss TGZ 3 particle size analyser. On the same prints, the external and axonal diameters were measured and the g ratio (axon diameter to fibre diameter) was determined. ‘Myelinated axons’ were those presumed to belong to the myelinated fibre group, namely axons with myelin and those of larger diameters (>1.7 μm) without myelin but surrounded by one or more extra Schwann cell processes. The density of demyelinated axons was determined and scored as percentage of the density of the myelinated fibres per case. The number of onion bulbs was scored in randomly chosen areas comprising a total of 100 myelinated fibres. All structures composed of at least two extra Schwann cell processes were considered ‘onion bulbs’.

#### Immunohistochemistry

Nerves were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 5–10 μm thick serial sections. For immunohistochemical staining the following primary antibodies were used (dilution in parenthesis): mouse monoclonal anti-human LNGFR-R (1:100); mouse monoclonal anti-GFAP (1:10); mouse monoclonal anti-myelin basic protein (MBP) (1:500); mouse monoclonal PCNA (1:200) (all Boehringer Mannheim, Penzberg, Germany); rabbit monoclonal anti-S100 (1:200; Camon, Wiesbaden, Germany). As secondary antibodies biotinylated anti-rabbit resp. rat absorbed anti-mouse IgG (Vector, Burlingame, Calif., USA) diluted 1:50 were used. Staining was performed with the avidin–biotin–peroxidase complex method using Vectastain ABC kit (Vector) according to the manufacturer’s protocol.

#### Results

We have investigated a total of 13 CMT1A sural nerve biopsies. For 11 patients morphometric data are presented in Table 1. Morphometric results are ordered according to increase in percentage of onion bulbs and decrease in density of myelinated fibres. No such morphometric analysis was available for the two patients (Cases 12 and 13) with most advanced onion bulb formation, but routine histology on paraffin sections showed very large onion bulbs, and immunohistochemistry with anti-MBP antibody revealed severe loss of myelinated nerve fibres. Paraffin sections of sural nerve biopsies from patients in different age groups
Low affinity NGFR expression in CMT1A nerve biopsies

Table 1 Morphometric analysis of sural nerve biopsies from CMT1A patients

<table>
<thead>
<tr>
<th>Case</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>17</td>
<td>15</td>
<td>21</td>
<td>13</td>
<td>15</td>
<td>22</td>
<td>26</td>
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<td>TTFA % of normal for age</td>
<td>182</td>
<td>282</td>
<td>118</td>
<td>286</td>
<td>175</td>
<td>368</td>
<td>–</td>
<td>153</td>
<td>100</td>
<td>310</td>
<td>199</td>
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<td>Density of MF (mm(^{-2}))</td>
<td>7015</td>
<td>7030</td>
<td>9630</td>
<td>3900</td>
<td>1150</td>
<td>4430</td>
<td>1940</td>
<td>2200</td>
<td>2820</td>
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<td>2210</td>
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<td>% of normal for age</td>
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<td>50</td>
<td>68</td>
<td>30</td>
<td>11</td>
<td>42</td>
<td>20</td>
<td>21</td>
<td>15</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>% of demyelinated fibres</td>
<td>2.5</td>
<td>1.3</td>
<td>2.3</td>
<td>4.1</td>
<td>2.3</td>
<td>1.4</td>
<td>0.7</td>
<td>6.3</td>
<td>16</td>
<td>0</td>
<td>1.2</td>
</tr>
<tr>
<td>% of onion bulbs</td>
<td>27</td>
<td>35</td>
<td>37</td>
<td>72</td>
<td>78</td>
<td>83</td>
<td>94</td>
<td>96</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Mean g ratio(^a)</td>
<td>0.59</td>
<td>0.56</td>
<td>0.55</td>
<td>0.62</td>
<td>0.52</td>
<td>0.50</td>
<td>0.53</td>
<td>0.58</td>
<td>0.62</td>
<td>0.38</td>
<td>0.42</td>
</tr>
<tr>
<td>g ratio % of normal for age</td>
<td>80</td>
<td>77</td>
<td>75</td>
<td>93</td>
<td>78</td>
<td>75</td>
<td>80</td>
<td>91</td>
<td>93</td>
<td>88</td>
<td>64</td>
</tr>
</tbody>
</table>

TTFA = total transverse fascicular area; MF = myelinated fibres. *Mean of age-related controls: 14170 (2–5 years, n = 6); 13180 (6–10 years, n = 11); 10530 (11–20 years, n = 5); 9640 (21–30 years, n = 7). †Mean of age-related controls: 0.73 (2–5 years, n = 2); 0.67 (6–10 years, n = 2); 0.67 (11–20 years, n = 3); 0.66 (21–50 years, n = 3).

(patient age at biopsy: 5 years, n = 2; 15 years, n = 2) showed no abnormalities at routine morphological examination and endoneurial cells were only rarely LNGF-R or GFAP positive, as previously described (Sobue et al., 1988) (results not shown).

Immunostaining of sural nerves from patients with genetically proven CMT1A neuropathy showed a massive increase in LNGF-R expression that varied depending on the stage of myelin loss and onion bulb formation. The early stage of the disease was characterized by very few small onion bulbs (Ouvrier et al., 1987; Gabriëls-Festen et al., 1992), a moderate decrease in density of myelinated fibres and mostly normal myelin sheaths as revealed by morphometric analysis (see Fig. 1A and Case 1 in Table 1). In this group of patients, enhanced LNGF-R staining as compared with age-matched controls was observed (Fig. 1C and E). There were numerous LNGF-R-positive Schwann cells despite many normally myelinated nerve fibres (Fig. 1A and C). Figure 1B, D and F shows a more advanced disease stage showing an increased number of onion bulbs (Case 5, Table 1). The crescent-shaped staining adjacent to the myelin contour with LNGF-R antibody in transverse sections (Fig. 1E) and the immunostaining along the nerve fibres in longitudinal sections (Fig. 1D and F) suggest that myelin-forming Schwann cells in addition to nonmyelinating Schwann cells express LNGF-R (Fig. 1D–F). To analyse whether the increased expression of LNGF-R in CMT1A patients is related to enhanced Schwann cell proliferation, sections were stained with monoclonal antibody to the proliferation marker PCNA (Connolly et al., 1993). There was no nuclear staining detectable at the earliest disease stages (data not shown). At slightly more advanced disease stages (Case 5, see Table 1, Fig. 1B) only occasional single cells were PCNA positive (Fig. 1G). There was no PCNA immunoreaction in the endoneurium in any of the control nerves from the different age groups examined. Further characterization of Schwann cells at the early disease stages with an antibody to GFAP showed no GFAP staining (Fig. 1H), which is in contrast to findings in acute demyelination, where GFAP and LNGF-R are upregulated concomitantly (Toews et al., 1992).

When onion bulb formation and myelin fibre loss were more advanced (Fig. 2A, E and G; Case 10 in Table 1) LNGF-R staining was increased and was clearly seen associated with the onion bulbs (Fig. 2C); LNGF-R staining could be demonstrated in all lamellae of the onion bulbs including those adjacent to the myelin sheaths (Fig. 2C and E). Incubation of these nerves with an antibody against GFAP revealed a staining pattern very different to that of the LNGF-R. Glial fibrillary acidic protein staining was increased as compared with controls but onion bulbs were GFAP negative (arrows in Fig. 2G). The GFAP staining was presumably associated with non-myelinating Schwann cells (open arrows in Fig. 2G). At this more advanced disease stage, numerous PCNA-positive nuclei were detectable, indicating Schwann cell proliferation (Fig. 2H).

At most advanced stages of CMT1A neuropathy, we could no longer detect LNGF-R immunoreactivity (Fig. 2D) in accordance with a previous report (Sobue et al., 1988). In contrast to earlier disease stages, onion bulbs had become larger in diameter (Fig. 2B) as revealed by labelling with the Schwann cell marker S100. Only few fibres still contained small MBP-positive myelin sheaths (arrows in Fig. 2F). Most onion bulbs were devoid of myelinated nerve fibres (open arrows in Fig. 2F). PCNA staining revealed no proliferating cells at this late disease stage and staining with anti-GFAP was negative (data not shown). Despite the variable expression of LNGF-R at different stages of onion bulb formation, Schwann cells always remained S100 positive (Fig. 2B).

Staining of non-CMT onion bulbs in nerve biopsies from CIDP patients showed no LNGF-R staining (Fig. 3D) as previously described (Sobue et al., 1988). Few onion bulbs showed scarcely unmyelinated fibres in the outer layer as could be shown by ultrastructural analysis (Fig. 3A and B).
Discussion

In this study, we used different immunohistochemical markers to characterize Schwann cell phenotypes at various disease stages of CMT1A neuropathy as compared with age-matched normal controls. The principal finding of this study is that Schwann cells transiently and pathologically express LNGF-R in CMT1A neuropathy.

Schwann cells are the only endoneurial cells that are capable of expressing LNGF-R in peripheral nerves (Taniuchi et al., 1986; Jessen and Mirsky, 1991). In normal adult human nerves we and others found virtually no endoneurial LNGF-R staining (Sobue et al., 1988). In vitro non-myelinating Schwann cells normally express LNGF-R (Jessen et al., 1990; Jessen and Mirsky, 1991). In experimental demyelination, LNGF-R expression occurs only at acute stages (Fan and Gelman, 1992; Stoll et al., 1993) and is downregulated early during remyelination. Accordingly, in nerve biopsies of chronic demyelinating diseases, like CIDP, with thinly myelinated nerve fibres and onion bulb formation LNGF-R staining could not be observed (Fig. 3D) (Sobue et al., 1988).

In this paper we show abnormal LNGF-R staining already present in numerous Schwann cells at very early stages of the hereditary demyelinating neuropathy, CMT1A. The crescent-shaped staining adjacent to the myelin sheath suggests that myelin forming Schwann cells in CMT1A may also express LNGF-R in contrast to normal nerves where myelin forming Schwann cells are always LNGF-R negative. However, it is possible that a thin Schwann cell process is located next to the myelin sheath which does not express LNGF-R immunoreactivity. The biopsy material currently available for immunohistochemical studies does not allow a definite answer to this question at the light microscopic level. At later stages of onion bulb formation we were able to show that Schwann cells in all layers of the onion bulbs including those adjacent to the myelin sheath were LNGF-R positive. Again this is unusual, since nerve biopsies of CIDP patients with a similar content of onion bulb formation and thinly myelinated nerve fibres were always LNGF-R negative (Fig. 3D) (Sobue et al., 1988). Ultrastructural analysis showed few unmyelinated axons in the outer lamellae of some of the onion bulbs (Fig. 3A and B). Since they were only found in few onion bulbs and were restricted to the outer lamellae, these unmyelinated axons associated with cells in onion bulbs were far too few to explain staining of all lamellae in each onion bulb. Furthermore, morphometric analysis revealed evidence for demyelination in our series. In early disease stages we found some evidence for demyelination (Table 1) but at later disease stages, where LNGF-R staining could be found in all layers of onion bulbs (Case 10, Table 1; Fig. 2E) virtually no signs of demyelination could be detected. Thus there seems to be no correlation between LNGF-R expression and demyelination. It has also been postulated previously that LNGF-R is expressed in Schwann cells during proliferation (Fan and Gelman, 1992), while findings in Wallerian degeneration and acute demyelination provide evidence against the hypothesis that cell division is the only signal for upregulation of LNGF-R expression (Toews et al., 1992; Stoll et al., 1993). We found LNGF-R expression during early stages of CMT1A when no Schwann cell proliferation could be demonstrated by PCNA immunolabelling. Immunolabelling with antibody to PCNA, an auxiliary protein to DNA polymerase, gives an estimate of the cells in the late G1 and early S phase (Connolly et al., 1993). Thus, our findings provide evidence against a strict correlation between LNGF-R expression and Schwann cell proliferation in CMT1A. However, at later stages of the disease, when onion bulb formation is well underway, PCNA labelling was detectable.

Hence we describe an abnormal expression of LNGF-R in CMT1A. In accordance with previous studies (Sobue et al., 1988) LNGF-R was not found at the most advanced stages of the disease, when massive onion bulb formation had occurred and density of myelin fibres was further reduced. Whether this correlates with downregulation of PMP22 expression to normal levels in late stages of CMT1A (Hanemann et al., 1994) can only be speculated.

The finding of PCNA-positive Schwann cells, at advanced disease stage, could argue for Schwann cell proliferation in CMT1A being secondary to demyelination, since demyelination is pronounced at the early stages, and onion bulbs, as a result of Schwann cell proliferation, gradually appear later during the first decade of the disease (Gabreel-Festen et al., 1992). In addition, endoneurial fibroblasts could contribute to the pool of PCNA-positive proliferating cells.

To further characterize the Schwann cells in CMT1A, we
analysed expression of additional Schwann cell markers. Toews *et al.* (1992) reported that GFAP is upregulated together with LNGF-R if axon-Schwann cell contact is lost during acute toxin-induced demyelination, and Fan *et al.* (1992) concomitantly described downregulation of S100 expression. We found S100 labelling of all Schwann cells in all CMT1A biopsies (data not shown). Glial fibrillary acidic protein immunostaining could only be detected in disease stages with ongoing onion bulb formation and showed a pattern of cellular localization that is completely different from LNGF-R immunostaining in CMT1A neuropathy (see Fig. 2).

In conclusion, we describe an unique Schwann cell phenotype in CMT1A neuropathy that has not been reported before. Early in the course of the disease with mostly normal myelin sheaths, Schwann cells express LNGF-R and S100 but lack GFAP and PCNA staining. In more advanced disease stages we found LNGF-R-positive, S100-positive and PCNA-positive Schwann cells in the inner as well as outer lamellae of the onion bulbs, which were still GFAP-negative.

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**Fig. 2** Micrographs showing expression of LNGF-R and other markers at more advanced stages of CMT1A neuropathy. A shows that all myelinated fibres are surrounded by large onion bulbs of multiple Schwann cell lamellae (1 μm plastic section, Case 10 in Table 1). Some onion bulbs lack a central myelinated fibre (arrow). C, E, G and H are corresponding paraffin sections stained for LNGF-R (C and E), GFAP (G) and PCNA (H). Note that Schwann cells in onion bulbs strongly express LNGF-R (arrows in C, and larger magnification, E). G shows that Schwann cells in onion bulbs do not express GFAP (arrows), while Schwann cells probably associated with nonmyelinated nerve fibres are GFAP positive (open arrows). In contrast to Fig. 1G, many nuclei are labelled by anti-PCNA antibodies at this more advanced stage of the disease indicating Schwann cells proliferation (arrows in H). B, D and F are immunostained paraffin sections of a sural nerve at most advanced CMT1A neuropathy. Note the well-developed onion bulbs as assessed by staining of Schwann cells by S100 antibodies (B) and the dramatic reduction of myelinated nerve fibres within these onion bulbs as shown by MBP staining of remaining few myelin sheaths (arrows in F). Open arrows denote onion bulbs devoid of myelinated nerve fibres. Concomitantly, LNGF-R immunoreactivity is lost at this most advanced stage of onion bulb formation (D). Magnifications: A, ×500; B, C, D and F-H, ×330; E, ×825.
The unusual LNGF-R expression by Schwann cells in CMT1A neuropathy could either be a primary effect due to PMP22 gene duplication and the growth regulatory functions of PMP22 in the cell cycle, or a secondary effect caused by the disease process. It is an intriguing question whether or not Schwann cells pathologically remain LNGF-R positive in foetal development after commencement of myelination in CMT1A neuropathy, an issue that obviously cannot be addressed experimentally. Nevertheless, the present findings support our earlier hypothesis that PMP22 gene duplication and overexpression may directly or secondarily alter the Schwann cell phenotype early in the course of the disease. At present, the functional consequences of aberrant LNGF-R expression of Schwann cells in CMT1A neuropathy is unclear, since there are probably multiple roles of LNGF-R in the peripheral nervous system. Taniuchi et al. (1988) put forward the hypothesis that Schwann cells present their surface LNGF-Rs, saturated with NGF, to ingrowing axons. Anton et al. (1994) provided evidence for a role of LNGF-R in Schwann cell migration and Fan et al. (1992) hypothesized about a function for LNGF-R in the early phase of remyelination. Since both overexpression and point mutation of PMP22 lead to the same disease, future studies will have to be aimed to investigate whether point mutations of PMP22 lead to the same abnormal Schwann cell differentiation state. In addition human Schwann cell cultures from CMT1A patients are needed for the functional characterization of these abnormal Schwann cells.

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