Abstract

Title: Axonopathy in Peripheral Myelin Protein 22 Insufficiency.

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The role that various myelin membrane proteins play during development and disease processes is not well understood. To better understand their role in vivo we have crossed transgenic mice possessing a single truncated \( pmp22 \) gene with mice expressing yellow fluorescent protein in the cytoplasm of their neurons. The resulting double transgenic mice were examined by a combination of confocal microscopy, transmission electron microscopy, and immunohistochemistry to determine if \( pmp22 \) insufficiency alters the structural integrity of myelin, glial cells, axons, or the subcellular milieu of these various components. Axons from mice with \( pmp22 \) insufficiency developed sprouts and debris localized to nodes with no signs of degeneration of a Wallerian type. Ultrastructurally, the nodes accumulated tubovesicular structures as well as disrupted cytoskeleton that did not appear to alter axon transport. Together, these results suggest that \( pmp22 \) insufficiency leads to a non-lethal axonopathy that is restricted to nodes.
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LIST OF ACRONYMS/ABBREVIATIONS:

AChR – acetylcholine receptor
ALS – amyotrophic lateral sclerosis
CIDP – chronic inflammatory demyelinating polyneuropathy
CMAP - compound muscle action potential
CMT - Charcot-Marie-Tooth disease
CMT1A - Charcot-Marie-Tooth disease type 1A
CNS – central nervous system
DRG – dorsal root ganglion
ER - endoplasmic reticulum
GBS – Guillain Barre’s syndrome
HNPP – hereditary neuropathy with liability to pressure palsies
MAG – myelin associated glycoprotein
MBP – myelin basic protein
MPZ – myelin protein zero
MS – multiple sclerosis
NCS – nerve conduction study
NF – neurofilament
NFH - heavy molecular weight neurofilament
NFHP – phosphorylated heavy molecular weight neurofilament
NFL – low molecular weight neurofilament
NFM - middle molecular weight neurofilament
NFMP – phosphorylated middle molecular weight neurofilament
NMJ – neuromuscular junction
PMP22 – peripheral myelin protein-22
PNS – peripheral nervous system
PLP - proteolipid protein
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Introduction

Glial-Axonal Relationship

Our ability to move, see, hear, smell, taste, and even our cognitive abilities rely upon the coordinated long distance electric signaling between neurons and their targets. Although neurons can vary in structure, they generally consist of a cell body (or soma), dendrites and a single axon. The cell body and dendrites function by receiving information from other neurons and pass an outgoing signal to the axon. This outgoing signal is unique to the axon and consists of a rapidly repeating oscillation of the membrane voltage (potential), called an action potential, which is due to the influx and efflux of ions thorough selectively permeable membrane channels.

Considering a single axon can be a meter in length, two mechanisms have evolved to ensure that action potentials are conducted quickly: increasing axonal diameter and myelin. While increasing the interior diameter of the axon improves conduction velocity by decreasing the longitudinal resistance, it is not efficient in the mammalian nervous system because of the large number of neurons. The combination of a large number of large diameter neurons would necessitate an extremely large nervous system. An alternative is the development of myelin. Myelin is a lipid rich multilamellar membrane of axons (Schweigreiter, Roots et al. 2006). In myelinated axons, action potentials are restricted to non-myelinated segments, known as nodes of Ranvier (Poliak and Peles
Current influx at a node can rapidly spread in the myelinated internode segment, due to the reduced membrane capacitance, resulting in action potential conduction velocities as fast as 150 meters/sec. The function of myelin is known to be identical in central and peripheral nervous systems; however, there are differences in the cell biology of myelination where oligodendrocytes surround as many as fifty axons in the CNS while Schwann cells associate with only one axon in the PNS (Stevens and Fields 2002).

The glial-axonal interaction is not only essential for speeding action potential conduction velocity, but also for the survival of the axons (Martini 2001). If the myelin is disrupted, axon degeneration can often ensue. For instance, demyelination is the hallmark of many neurological diseases including multiple sclerosis (MS), Guillain-Barre’s syndrome (GBS), and various peripheral neuropathies. However, how demyelination leads to axonal degeneration is unknown and there is currently no treatment to hinder this pathological process. One way to better understand this relationship is to understand the functional role of the proteins that hold the compact layers of myelin together.

Peripheral Myelin Protein 22 – Expression Levels and Neuropathies

Although there are many different proteins that hold layers of myelin together, one of the more abundant is peripheral myelin protein 22 (PMP22). The \textit{pmp22} gene encodes a 22kD tetraspan membrane glycoprotein that resides on the surface of myelinating Schwann cells localized to compact myelin (Suter and Forscher 1998). Newly-synthesized PMP22 in the myelinating Schwann cells rarely makes it to the membrane since >90% of it is degraded in the endoplasmic reticulum (ER) (Pareek,
Notterpek et al. 1997). This quality control mechanism appears to be functionally essential, since certain mutations in PMP22 may cause aberrant trafficking and accumulation of proteins within intracellular organelles, which has been proposed to be a pathogenic mechanism of certain neuropathies (Colby, Nicholson et al. 2000). In addition to simple mutations in PMP22, its expression levels also appear to be critical to axon function since alterations of pmp22 expression results in clinically distinct disorders: hereditary neuropathy with liability to pressure palsies (HNPP) and Charcot-Marie-Tooth disease type 1A (CMT1A). HNPP and CMT1A are among the most frequently inherited neurological diseases and are caused by deletions or duplication of chromosome 17p11.2-12 containing pmp22 gene (Lupski and Garcia 1992; Chance and Pleasure 1993).

HNPP results from insufficient levels of PMP22 and leads to symptoms that include transient, asymmetric, multifocal sensory motor deficits (Li, Zhang et al. 2004). These symptoms are usually triggered by physical activities involving compression, repetitive movement, or stretching suggesting that axons with insufficient amounts of PMP22 are vulnerable to mechanical forces. In human patients, electrophysiology demonstrates that compound action potential conduction velocity is indeed slowed at sites subject to mild mechanical compression (Li and Li 2002).

Charcot-Marie-Tooth disease (CMT) is a group of inherited neuropathies with a prevalence of one in 2500 people (Shy, Garbern et al. 2002). CMT1A is the most common form of CMT, affecting one-half of all CMT cases (Ionasescu, Ionasescu et al. 1993; Wise, Garcia et al. 1993). CMT1A is an autosomal dominant neuropathy caused by a 1.4 Mb duplication of chromosome 17p11.2-12 (Raeymaekers, Timmerman et al.
1991). CMT1A patients develop a slowly progressive, symmetrical, demyelinating neuropathy with secondary axonal loss (Krajewski, Turansky et al. 1999).

**Demyelination and Axon Loss**

Demyelination is a pathological process that takes place in many neurological disorders, such as MS, GBS, and chronic inflammatory demyelinating polyneuropathy (CIDP). Although demyelination leads to a slowed conduction velocity, it often does not correlate with clinical symptoms. Axon loss, on the other hand, does appear to correlate with a number of different demyelinating diseases (Krajewski, Lewis et al. 2000; Bjartmar and Trapp 2003). The cellular and molecular mechanisms responsible for demyelination mediated axon loss remains unclear. Further, whether or not the axon degeneration in these instances occurs through previously described mechanisms, such as Wallerian degeneration, is also unknown.

One way that demyelination could mediate axon loss is through direct connections to the underlying axonal cell membrane. Myelinated axons are composed of four discrete compartments: the node of Ranvier, the paranode, the juxtaparanode, and the internode. Each of these compartments contains a unique, non-overlapping set of protein constituents (Scherer 2002). The paranodal region, comprised of loops of Schwann cell membrane that interact with axonal membrane adjacent to the node of Ranvier, contain Schwann cell proteins myelin associated glycoprotein (MAG), Connexin 32 (Cx32), and axonal proteins Caspr and Contactin. These proteins participate in Schwann cell-axonal interaction and define the nodal region. The juxtaparanodal region, the portion of the Schwann cell and the interacting axonal membrane adjacent to
the paranode, contains voltage gated potassium channels that are expressed on the axolemma. The internodes, the remaining segment flanked by two nodes of Ranvier, contain the myelin structural proteins, myelin protein zero (MPZ), PMP22, and myelin basic protein (MBP), which participate in forming the tightly-compacted myelin sheath. When nerve fibers are demyelinated, a number of these proteins become redistributed. This redistribution may contribute to axonal degeneration. For example, conspicuous reorganization of molecular architecture has been found in Cx32 and pmp22 knockout mice, the two chronic demyelination animal models (Neuberg, Sancho et al. 1999).

A second possible mechanism that could explain demyelination mediated axon loss is through cytoskeletal changes in the axon. Myelination affects the phosphorylation of axonal neurofilaments (NF). NFs are the principal element of the axonal cytoskeleton and are composed of three subunits, referred to as low (NFL), medium (NFM), and heavy (NFH) molecular weight NF proteins (Grant and Pant 2000). They all share a similar protein structure that consists of an N-terminal head domain, a α-helical central domain, and a C-terminal tail domain. The C-terminal tail domain forms sidearms that protrude from the NF backbone and are thought to regulate both the rate of axonal transport and the caliber of axons. The best evidence for this comes from experiments in Trembler mice that have a chronic demyelinating neuropathy. These mice have decreased NF phosphorylation, increased NF density, along with decreased axonal transport (de Waegh, Lee et al. 1992). Transplantation of a segment of a Trembler nerve into a normal nerve also produces similar changes in axons that have regenerated through the nerve graft but not in the surrounding nerve, demonstrating that this effect is induced by contact with the
abnormal Schwann cells (Sahenk, Chen et al. 1999). Together, these results argue that glial contact can alter the underlying axonal cytoskeleton.

Finally, abnormalities with the Schwann cell itself may lead to axon degeneration independent of demyelination. Such uncoupling of axonal loss from segmental demyelination has been previously observed in several disorders. Human patients with proteolipid protein 1 (PLP1) deletion develop axonal loss without demyelination as well as PLP1 knockout mice (Griffiths, Klugmann et al. 1998; Garbern, Yool et al. 2002). Transgenic mice in which the glial-specific protein, 2’;3’-cyclic nucleotide phosphodiesterase (CNP1) was deleted also develop axonal degeneration without demyelination (Lappe-Siefke, Goebbels et al. 2003). Mice with deficient MAG develop a late-onset axonal degeneration with well preserved myelin (Hanemann, Gabreels-Festen et al. 2001).

**Ultrastructural changes during PMP22-Associated Neuropathies**

Perhaps not surprising is the notion that alternations in PMP22 levels can lead to disruption of myelin structure. For instance, focal hypermyelination in HNPP was first described by Behse and colleagues (Behse, Buchthal et al. 1972). Madrid and Bradley later described tomaculated myelin in various neuropathies due to its sausage shape (Bradley, Madrid et al. 1975). This pathological phenomenon was subsequently found in many other neuropathies, including anti-myelin associated glycoprotein (MAG) paraproteinemic neuropathy, CMT1B (due to P0 gene mutations), CMT4B (due to myotubularin-related 2 gene mutation), along with several animal models of peripheral nerve disorders (Sander, Ouvrier et al. 2000). In general, tomacula are distributed mainly
in the paranodal regions and occasionally in the internodal regions as a result of redundant myelin infoldings and outfoldings. Myelin in the tomacula appears to have reduced stability and tend to degenerate over time (Adlkofer, Naef et al. 1997).

Whether or not tomacula confer toxicity to the underlying axon remains unanswered. Axons encased by tomacula frequently appear constricted. Sometimes, this constriction can be so severe that axons can become difficult to discern at the ultrastructural level (Bradley, Madrid et al. 1975). Studies in MAG knockout mice suggest that redundant myelin folding in tomacula is a result of axonal shrinkage based on the observation of overall reduction of axonal diameters in mag/- mice (Yin, Crawford et al. 1998). However, tomacula were subsequently found in the early life of these mice (1-month-old) when axonal diameters had not yet been reduced (Cai, Sutton-Smith et al. 2002). Together, it is unclear how axons lose caliber and whether or not dysmyelination is its cause or consequence.

**Summary**

The role that various myelin proteins play in regulating the glial-axonal relationship remains unclear. Therefore, the primary focus of this work is to examine axons from mice with insufficient levels of PMP22 with the highest spatial resolution possible to determine if this reduction results in a structurally distinct axonopathy.
Materials and Methods

Animals. All animals were provided by Dr. Jun Li from Vanderbilt University School of Medicine. All animal handling was approved by the Vanderbilt University School of Medicine IACUC. Briefly, *pmp22* was inactivated through homologous recombination (Adlkofer, Martini et al. 1995). *Pmp22*+/− mice were crossed with *yfp*+/+ mice that expressed yellow fluorescent protein in the cytoplasm of their neurons under the influence of the *thy-1* promoter. After deep anesthesia, double transgenic mice were transcardially perfused with 4.0% paraformaldehyde. Both left and right gluteus maximus, soleus, omoyhoid, and sternomastoid muscles were carefully dissected from the animals. One of the paired muscles was postfixed in 4.0% paraformaldehyde while its corresponding contralateral limb was postfixed in 2.5% paraformaldehyde with 4% glutaraldehyde. The former muscles were used for confocal microscopy while the latter muscles were used for electron microscopy.

Confocal Microscopy and Analysis. Muscles fixed in 4.0% paraformaldehyde were placed in 35mm petri dishes (Corning Glass Works, Coming, NY) containing Sylgard Sillicon elastomer (Dow Coming Corporation, Midland, MI) are pinned with fine minutien pins (Fine Science Tools, Foster City, CA) for stabilization during confocal
imaging and photoconversion. First the tissue are cleaned of any debris and then washed with 3 changes of 0.1 M phosphate buffer saline (PBS). Acetylcholine receptors were labeled with α-bungarotoxin conjugated to Alexa-594 for one hour and then washed with 3 changes of 0.1 M PBS buffer. Muscles were transferred to a slide place in glycerol with 1.0% DABCO and sealed with #1.5 coverslips. YFP was imaged using a 488nm laser line and detected with a 505-530nm band pass filter. Alexa 543nm laser line and detected with a 560nm long pass filter. All images were collected with a 40x, 1.2NA objective lens and sampled at Nyquist limits. Image stacks were projected to 2D using a maximum pixel projection algorithm.

Preterminal axon length was determined by measuring the distance between the last heminode to as far as the axon could be traced proximally. The proximal measurements were always stopped at a node, so as to get a more accurate measurement of internode distance (see below). We avoided axons that traveled mostly in the z-dimension to get closer to Euclidean measurements of distance. Nodal sprouts were counted when a branch emanated from a node that did not innervate acetylcholine receptors. Small spheres of fluorescent debris that were not connected to an axon were counted. Nodal sprouts and debris were pooled and counted as nodal defects. Internode distance was determined by dividing preterminal distance by the number of nodes. Nodal defects and internode distance were compared between the experimental and control animals for statistical significance using a student’s t-test.

**Electron microscopy and Analysis.** Muscles destined for electron microscopy were first analyzed by confocal microscopy. After confocal microscopy, areas of interest were
targeted for serial section transmission electron microscopy by placing fiducial markers in adjacent muscle fibers. Crystals of 1, 1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine-5,5-disulfonic acid (Dil, Invitrogen, Carlsband, CA) were applied to surface of the selected muscle of the axon or NMJ of interest using iontophoresis. Briefly, a micropipette puller (Kopf, Tujunga, California) was used to pull a micropipette (World Precision Instrument, Sarasota, FL) to a resistance of 5-10 MΩ. A 1.0% solution of Dil in 100% dicholormethylene (Sigma-Aldrich, St. Louis, MO) was loaded into the micropipette. The pipette was next threaded with a silver chloride electrode to an SD9 stimulator (Grass Instruments, Quincy, MA). The microelectrode was inserted into the muscle near the axon and Dil was unloading with an electrical pulses of 10v. To render the Dil crystal electron dense, DiI was illuminated near its excitation peak with a 40x water-immersion lens (Ziess) in the presence of 3, 3-Diaminobenzidine (DAB, 5.0mg/ml, Sigma-Aldrich).

After photoconversion, the tissue of interest was then stained with 1.0% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) reduced in 1.5% potassium ferrocyanide (Sigma-Aldrich) for thirty minutes. Tissue was then washed with 4 changes of 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences) and dehydrated in an ascending ethanol series (25, 50, 75 and 100% ethanol) (AAPER Alcohol and Chemical Company, Shelbyville, KY). After dehydration steps, the tissue block was place in a final 2 changes of propylene oxide (Electron Microscopy Sciences) and infiltrated with Araldite 502/Embed812 resin and polymerized at 60 degrees C for 48 hours (Electron Microscopy Sciences). Next after 48 hours the polymerized tissue block was trimmed to appropriate size into a trapezoid shape and thick sectioned (0.5-1.0 µm)
with a glass knife on a Leica Ultracut R microtome (Leica, Wetzlar, Germany). Thick sections were carefully examined individually with an Axiovert 35M light microscope (Zeiss) to locate the fiducial markers. Once the markers were located the block was then trimmed down into a smaller trapezoid where only the area the tissue and area of interest was. The tissue was then sectioned (60-80nm) into hundreds of serial sections containing the axon or NMJ of interest. The sections were cut with a diamond knife (Diatome) and a Leica UC6 ultramicrotome (Leica). Thin sections were then picked up on 1.2% pioloform (Ted Pella, Inc. Redding, CA, in chloroform, Sigma-Aldrich) coated slot grids (Electron Microscopy Sciences). The grids were counterstained with 2.0% aqueous uranyl acetate and Reynold’s lead citrate. Sections were viewed at accelerating voltage of 75kV on a Hitachi H-600 TEM. Electron micrographs were captured at 5000X and scanned at a resolution of 1200dpi with an Epson Perfection V700 Photo Scanner.

Serial electron micrographs were imported into the Reconstruct software developed by Kristen Harris and John Fiala (http://synapse-web.org/tools/index.stm). Micrographs were aligned to each other and then manually traced to provide us with a 3D model of our area of interest. Section thickness was determined using the cylindrical diameters method (Fiala and Harris 2001). Nerve terminal volume was determined from the manual traces and section thickness. Synaptic vesicles were counted and normalized using the Ambrercrombie method for cell counting. A student’s t-test was used to determine if synaptic vesicle density was statistically significant between the experimental and control animals.
**Results**

We first crossed *yfp16* $^{+/+}$ mice with *pmp22* $^{+/c}$ mice to create double transgenic mice that express fluorescent protein in the cytoplasm of their neurons along with a deficiency in *pmp22* in their peripheral nerves. We then used confocal microscopy to examine peripheral nerves and neuromuscular junctions in these mice and fluorescent controls. Confocal microscopy revealed no evidence of denervated or partially innervated muscle fibers suggesting *pmp22* insufficiency is not immediately lethal to axons. However, we noticed in *pmp22* $^{+/c}$ mice that peripheral axons had a number of small branches that did not innervate muscle fibers (Figure 1). These sprouts emanated from nodes of Ranvier where myelin does not cover the axon. In other cases, we did not notice sprouts, but rather found accumulations of fluorescent debris near the nodes (Figure 1).

We compared the total number of nodal defects (i.e., nodal sprouts and debris) in *pmp22* $^{+/c}$ and control mice. We found a statistically significant difference ($p<0.001^*$) in nodal defects between that *pmp22* $^{+/c}$ axons (0.021 +/- 0.014 nodal defects/μm) compared to controls (0.006 +/- 0.008 nodal defects/μm) (see Figure 2). To determine if this result was simply due to a difference in the number of nodes per micron in *pmp22* $^{+/c}$ axons compared to control, we determined the average internode distance. There was no statistically significant difference in internode distance between *pmp22* $^{+/c}$ axons (36.0 +/-
17.1 μm) compared to controls (38.8 +/- 16.1 μm) (see Figure 3). Together, these results suggest \( pmp22^{+/−} \) axons may have a vulnerability restricted to their nodes.

To better understand what pathology may be underlying the nodal defect, we took advantage of the far greater resolution of the transmission electron microscope to examine the nodal and paranodal regions in \( pmp22^{+/−} \) axons. In the paranodal region, we noticed layers of compact myelin that surrounded the axon had begun to separate (Figure 4). This demyelination occurred on both the outer and inner layers suggesting the entire compact myelin had been compromised. At the node, we noticed an accumulation of tubulovesicular structures that we were unable to find in controls (Figure 5). Further, the cytoskeleton in regions of demyelination had lost its orderly parallel structure axial to the membrane. Since the cytoskeleton is essential for both anterograde and retrograde transport of organelles in axons, such a localized disruption could interfere with organelle delivery to axon terminals.

We decided to count the number of synaptic vesicles in serial electron micrographs of nerve terminals to see if there are fewer vesicles in potentially axon transport impaired \( pmp22^{+/−} \) mice (Figure 6). We examined eight nerve terminals from three \( pmp22^{+/−} \) and four nerve terminals from two control mice. In some nerve terminals, we found evidence of reduced vesicle density in \( pmp22^{+/−} \). However, this difference was not statistically significant different between the \( pmp22^{+/−} \) compared to controls (\( \bar{x} = 522.3 \) verses 617.3 vesicles/μm³, respectively) (Figure 7).
Discussion

Our results demonstrate that \textit{pmp22}^{+/−} axons exhibit nodal sprouting and debris at one month of age. This difference was not simply due to an increased number of nodes since the average internode distance was not significantly different between \textit{pmp22}^{+/−} axons and controls. We were interested in determining what consequences, if any, these defects might have upon the axon and nerve terminals. To this end, we took advantage of transmission electron microscopy to examine the subcellular milieu of \textit{pmp22}^{+/−} axons. At the paranodal regions of these axons we found evidence of dysmyelination/demyelination in compact myelin layers that surround the axon.

We also noticed that the cytoskeleton seemed to have lost its orderly parallel structure. The function and survival of neurons are dependent upon axonal transport, where newly synthesized proteins and organelles from the cell body located in the spinal cord are transported long distances along the axon. Cytoskeleton disruption, which has been known to impair axon transport in disease models such as the fALS model of amyotrophic lateral sclerosis (Dutta and Trapp 2006), can lead to a decrease in trophic support as well as decreased organelle density in synaptic terminals. Despite disruption of the cytoskeleton, we were unable to find a statistically significant difference in vesicle density between \textit{pmp22}^{+/−} axons compared to controls.
Our data suggests that even though there are nodal defects coupled with demyelination and cytoskeleton rearrangements, these changes do not lead to a Wallerian type degeneration resulting in denervation or partially innervated muscle fibers. In such a case, we would have expected that the membrane be severed at some point causing the distal axon to fragment, become engulfed by Schwann cells, and finally disappear entirely. That we were unable to find evidence of this Wallerian type of degeneration underscores the idea that \textit{pmp22} insufficiency does not appear to lead to a denervating axonopathy.

Instead, \textit{pmp22}\textsuperscript{+/-} insufficiency appears to result in an abnormality restricted to the nodes. In a normal myelinated axon, voltage gated sodium channels are highly concentrated at the nodes (Kazarinova-Noyes and Shrager 2002), resulting in ionic flux and action potential generation from node to node. The efficiency of action potential propagation, therefore, is dependent on the both myelin and nodal integrity. Any changes or damages in the myelin or node can alter the delicate balance of sodium channel density and/or local metabolism. For instance, in demyelinating diseases such as MS, myelin damage often results in the upregulation of sodium channels (Stys, Waxman et al. 1992). The increasing demand for ATP exceeds the production capabilities of existing mitochondria. Na\textsuperscript{+}/K\textsuperscript{+} ATPase pumps increase their activity in an attempt to maintain ionic gradients placing a further increase on the metabolic demands of oxidative metabolism. As a result, the localized accumulations of intracellular Na\textsuperscript{+} ions result in excess Ca\textsuperscript{2+} ions entering the cell through the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger resulting in a Ca\textsuperscript{2+}-mediated axon damage along the axons. Other genetic manipulations have similarly lead to axon loss possibly through other cellular mechanisms. For instance, overexpressing
P₀, a major structural myelin protein (Previtali, Quattrini et al. 2000), leads not only to demyelination in the peripheral nervous system in mice, but also axon terminal loss within six months (Bjartmar, Yin et al. 1999).

Observations of both MS and P₀ overexpression lead to the intriguing possibility that nearly any manipulation to myelin can result in axon loss. However pmp22 insufficiency appears to progress differently than both MS and overexpression of P₀. In MS, where autoimmune loss of myelin over the long term results in large scale denervation of CNS tracts, sclerotic areas of former myelinated axons serve as the hallmark of the disease. Considering we were unable to confirm axon loss in up to four-month old pmp22⁺/− animals, it appears any compromise induced by pmp22⁺/− insufficiency does not follow a similar course to MS or P₀ overexpression. Our results strengthen the notion that damage to myelin does not universally lead to axon loss.

One reason for differences in MS and pmp22⁺/− insufficiency may simply be related to differences between the glial cell type and the relationship they share with central and peripheral axons. Oligodendrocytes in the central nervous system myelinate many axons compared to Schwann cells which provide surround only one axon. It is this insulating and branching of oligodendrocytes to other axons that may confer its debilitating properties. A second factor may be related to the immune response generated in MS compared to pmp22⁺/− mice. We found no evidence of immune cell infiltration into the peripheral nerves of pmp22⁺/− mice indicating an immune response was not generated despite the cellular debris. This is in sharp contrast to MS where immune cell infiltration is a hallmark of the disease. Perhaps the immune cells confer the ultimate damage to axons that is not conferred in pmp22⁺/− insufficiency.
During the early phases of MS, a relapsing-remitting phase is characterized by slowed and even stopped action potential conduction. *Pmp22<sup>+/−</sup>* mice have recently been shown to suffer conduction block following short term mechanical occlusion (Bai, Zhang et al. 2010). Although Na<sup>+</sup> channel density did not change in these studies, conduction block can induced significantly quicker in *pmp22<sup>+/−</sup>* animals compared to controls. This effect could be due to biophysical properties of the axons since these animals also showed contractions in the paranodal regions of the sciatic nerve. Our studies could not confirm these severe constrictions in the distal axons of leg muscles perhaps suggesting the insufficiency primarily attacks the more proximal large caliber parts of the axon as compared to the smaller more distal terminations. This is in contrast to other disease models, such as fALS, that have been previously described as distal axonopathies that tend to progress more proximally over time (Fischer, Culver et al. 2004).

Transgenic *pmp22* knockout mice have been an accepted model of Charcot-Marie-Tooth disease where dysmyelination/demyelination often leads to axonal changes. Tomacula are well-described structures in these mice that we did not encounter in our study. Although recent reports (Bai, Zhang et al. 2010), show these structures in proximal portions of sciatic nerves, they are not nearly as abundant as in *pmp22<sup>+/−</sup>* mice. The ultrastructure of tomacula in *pmp22<sup>+/−</sup>* in these more proximal regions was not explored as in the detail of our study. An examination of more proximal portions of sciatic nerves in both *pmp22<sup>+/−</sup>* and *pmp22<sup>−/−</sup>* mice might better elucidate the role this protein plays in the glial axon relationship in the peripheral nervous system.
References


Figure 1. Nodal defects in pmp22 +/- axons. Panel (A) shows a confocal projection from a pmp22 +/- muscle where axons appear green, and acetylcholine receptors appear red. Higher magnification images indicated by the boxed regions appear in panels C, D and E. Panel (B) shows a confocal projection from a control animal. Panels C, D and E show evidence of nodal sprouts and debris (arrows). Scale bar = 20.0 µm in panels A and B, and 5.0 µm in panel C, D and E.
Figure 2. A statistically significant difference (p<0.001*) in nodal defects and sprouts of pmp22 +/- and controls. Nodal defects and sprouts in pmp22 +/- and controls were observed using confocal microscopy. A mean value of 0.006 +/- 0.008 SD defects/µm, and 0.021 +/- 0.014 SD defects/µm was observed for pmp22 +/- and controls respectively. Data was analyzed using a Student’s t-test.
Figure 3. No statistically significant difference (p=0.440) in nodes/μm of pmp22 +/- and controls. Nodal distance per micron in pmp22 +/- and controls was measured using confocal microscopy. A mean value of 36.0 +/- 17.1 SD μm, and 38.8 +/- 16.1 SD μm was measured for pmp22 +/- and controls respectively. Data was analyzed using a Student’s t-test.
Figure 4. Demyelination in the *pmp22* +/− axon. The top left panel shows a confocal projection from a *pmp22* +/− axon (green) that forms a synapse over acetylcholine receptors (red). A surface rendering from serial sections from the boxed region is expanded below. A single electron micrograph has been inserted into the rendering at its exact location. The electron micrograph is expanded in the right panel. Arrows show regions of demyelination. Scale bar = 1.0 μm.
**Figure 5. Subcellular defects in the nodes of the pmp22 \(^{+/-}\) axon.** The top left panel shows a confocal projection from a pmp22 \(^{+/-}\) axon (green) that forms a synapse over acetylcholine receptors (red). A surface rendering from serial sections from the boxed region is expanded below. A single electron micrograph has been inserted into the rendering at its exact location. The electron micrograph is expanded in the right panel and shows tubulovesicular structures within the unmyelinated node. The cytoskeleton through this region is disrupted as well. Scale bar = 1.0 \(\mu\)m.
Figure 6. Synaptic vesicle distribution in the pmp22−/− and control axons. Panel (A) shows an electron micrograph from a pmp22−/− axon through a single nerve terminal. Panel (B) shows an electron micrograph from a control axon through a nerve terminal. Scale bar = 1.0 μm.
Figure 7. No statistically significant difference ($p=0.620$) in synaptic vesicle density in nerve terminals of $pmp22^{+/+}$ and controls. Vesicle density of nerve terminals in $pmp22^{+/+}$ and controls were counted from serial section transmission electron micrographs. A mean value of $522 \pm 228$ SD vesicles/$\mu m^3$, and $617 \pm 430$ SD vesicles/$\mu m^3$ was counted for $pmp22^{+/+}$ and controls respectively. Data was analyzed using a Student’s t-test.