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Proteolipid protein cannot replace P₀ protein as the major structural protein of peripheral nervous system myelin

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Abstract

The central nervous system (CNS) of terrestrial vertebrates underwent a prominent molecular change when proteolipid protein (PLP) replaced P₀ protein as the most abundant protein of CNS myelin. However, PLP did not replace P₀ in peripheral nervous system (PNS) myelin. To investigate the possible consequences of a PLP to P₀ shift in PNS myelin, we engineered mice to express PLP instead of P₀ in PNS myelin (PLP-PNS mice). PLP-PNS mice had severe neurological disabilities and died between 3 and 6 months of age. Schwann cells in sciatic nerves from PLP-PNS mice sorted axons into one-to-one relationships but failed to form myelin internodes. Mice with equal amounts of P₀ and PLP had normal PNS myelination and lifespans similar to wild-type (WT) mice. When PLP was overexpressed with one copy of the P₀ gene, sciatic nerves were hypomyelinated; mice displayed motor deficits but had normal lifespans. These data support the hypothesis that while PLP can co-exist with P₀ in PNS myelin, PLP cannot replace P₀ as the major structural protein of PNS myelin.

Keywords

Schwann cell; hypomyelination; myelin compaction; myelin evolution; axon

Introduction

Myelin is a multi-lamellar, tightly compacted membrane that surrounds many axons in the central (CNS) and peripheral nervous systems (PNS). The major functions of CNS and PNS myelin are identical. Both concentrate Na⁺ channels at nodes of Ranvier and thereby promote rapid nerve transmission via saltatory conduction (Pedraza et al., 2001). CNS and PNS myelin also provide trophic support to axons that is essential for long-term axonal survival (Griffiths et al., 1998; Yin et al., 1998; Nave, 2010). Many other aspects of CNS and PNS myelin, however, differ (for review, see Trapp and Kidd, 2004). Schwann cells

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form single myelin internodes in the PNS. PNS myelin internodes are surrounded by a basal lamina and have a series of cytoplasmic channels called Schmidt-Lanterman (S-L) incisures that transverse compact myelin and connect the outer and inner margins of the myelin internode (for review see (Salzer et al., 2008)). S-L incisures facilitate transport molecules and nutrients to compact PNS myelin (Balice-Gordon et al., 1998). Oligodendrocytes form multiple myelin internodes in the CNS. CNS myelin internodes do not have basal lamina or S-L incisures. There are also differences in the molecular composition of mammalian PNS and CNS myelin. The major structural protein of PNS myelin is P₀ protein, a type I transmembrane glycoprotein (Lemke and Axel, 1985). Proteolipid protein (PLP), a four-transmembrane-domain protein, is the major structural protein of CNS myelin (Milner et al., 1985; Macklin et al., 1987). Since the extracellular domain of P₀ protein is larger than that of PLP, the periodicity, or spacing between compact myelin lamella, is slightly larger in PNS myelin than in CNS myelin (Trapp and Kidd, 2004; Yin et al., 2006). Finally, myelin-associated glycoprotein (MAG) is present in periaxonal membranes of both CNS and PNS myelin internodes, but is present in paranodal and S-L incisure membranes of only PNS internodes (Sternberger et al., 1979; Trapp and Quarles, 1982; Trapp et al., 1989).

Myelin is a nervous system-specific organelle that first appeared ~440 million years ago in cartilaginous fish (Waehneldt et al., 1986; Kirschner et al., 1989; Saavedra et al., 1989; Waehneldt, 1990; Yoshida and Colman, 1996). Primitive fish CNS myelin contained both P₀ protein and DM20, a spliced variant of PLP that was derived from an ancestral M6/DM/ proteolipid gene that originated before the divergence of protostomia and deuterostomia, as evidenced by the identification of respective homologs in drosophila (Stecca et al., 2000) and a large number of other bilaterian species. PLP first appeared in CNS myelin about 400 million years ago in bony fish and differs from DM20 by the addition of 35 amino acids to DM20 (Macklin, Campagnoni, Deininger, and Gardinier, 1987; Nave, Lai, Bloom, and Milner, 1987). The PLP-specific 35 amino acids were probably generated by the emergence of an alternative splice donor site within intron 3 of the DM20 gene. Both P₀ and PLP had relatively high mutation rates until 300 million years ago. It appears that as PLP function was evolving in CNS myelin, it required co-expression of P₀. In Reptiles/Aves, the function of PLP became established, allowing the silent dropout of P₀ from CNS myelin (Waehneldt, 1990; Yoshida and Colman, 1996). From Reptiles/Aves, PLP was the major protein of CNS myelin, while P₀ was exclusively expressed in PNS myelin. Once this segregation was established, the mutation rates of both proteins dropped dramatically and both proteins are highly conserved (almost 100%) across all mammalian species analyzed (Kurihara et al., 1997; Hudson, 2004; Kirschner et al., 2004).

An interesting question regarding myelin evolution is why CNS myelin, but not PNS myelin, underwent the molecular switch from P₀ to PLP. We previously investigated the possible benefits of the P₀ to PLP shift in CNS myelin by reversing the process in mice (Yin et al., 2006). Specifically we generated mice that expressed P₀ instead of PLP in CNS myelin (P₀-CNS mice). When PLP was replaced by P₀ protein, compact CNS myelin had an identical periodicity to that of compact PNS myelin. Despite rescuing the altered myelin compaction that occurred in PLP-null CNS myelin, P₀-CNS mice had shorter CNS myelin internodes, S-L incisures, increased degeneration of myelinated axons, increased neurological disability and a 50% reduction in lifespan when compared to wild-type (WT) or

PLP-null mice (Yin et al., 2006; Yin et al., 2008). Mice with equal amounts of P₀ and PLP in CNS myelin had normal lifespans and no axonal degeneration.

The data summarized above support the hypothesis that the P₀ to PLP switch during myelin evolution provided a vital neuroprotective function to CNS myelin. In mouse CNS myelin, P₀ could replace the structural role of PLP in compact myelin, but could not provide the PLP-associated trophic support that is essential for long-term axonal survival. Another interesting question of myelin evolution is why PLP did not replace P₀ protein in PNS myelin? To address this question we generated mice whose Schwann cells expressed high levels of PLP under the control of the P₀ promoter in the presence or absence of native P₀ protein. In the presence of P₀ protein, PLP can be incorporated into compact PNS myelin that has the periodicity of CNS myelin. In the absence of P₀ protein, PLP cannot support normal Schwann cell myelination or survival of mice beyond 3–6 months of age. PLP therefore cannot replace P₀ protein as the major structural protein of PNS myelin.

Material and Methods

PLP-P₀ Transgenic Mouse Creation

We generated transgenic mice that expressed the mouse PLP cDNA under the control the *Mpz* promoter and intron 1 enhancer (Leblanc et al., 2006). The transgene was engineered by inserting the PLP cDNA into the mP₀TOT transgene, containing 6 kb of proximal promoter and all exons and introns (Feltri et al., 1999). The start site of translation for P₀ was eliminated and the PLP cDNA was fused downstream of an IRES and inserted into exon 6 of *Mpz*, leaving the PLP ATG start of translation and stop codon intact. These mice were maintained in the animal colony as homozygous (termed PLP/P₀ mice) and they displayed no obvious behavior abnormalities. P₀-null mice (Martini et al., 1995) were crossed with homozygous mice carrying the mP₀TOTA-PLP transgene. These mice were interbred for three generations to obtain mice that were P₀-null and homozygous for the mP₀TOTA-PLP transgene (PLP-PNS mice), as determined by genomic DNA analysis and outbreeding. Thus several mouse lines were used for this study: WT, P₀-null, PLP/P₀, PLP/P₀^{+/−} (PLP transgene homozygous and P₀ heterozygous), and PLP-PNS.

Genomic DNA Analysis

DNA was prepared from tail clips using standard protocols (Invitrogen, Carlsbad, CA). Samples were analyzed by double quantitative real-time PCR (RT-PCR). RT-PCR was performed with a 7300 RT-PCR system and Power SYBR® Green PCR Master Mix (A&B Applied Biosystems, Foster City, CA) to track the PLP transgene and the P₀ gene, respectively.

Primers for the mP₀TOTA-PLP transgene used were 5'-T C A T G A T T G C T G C C A C T T A C A A C T T C G -3' and 5'-G A G T C C A G G C C C A T C A T G T T C T T G A G G -3'. Primers for the P₀ gene used were 5'-T C A G T T C C T T G T C C C C C G C T C T C -3' and 5'-C T G T T A G C A T G G A T C T G A G G A G T -3'. CD47 SYBR Green was used as a control (A&B Applied Biosystems). Primers are: CD47F: 5'-T G G C A T T

G C C T C T T T G A A A A T G G -3'. CD47R: 5'-C C T T C T C T G G A T A A C T C T G T C A C T T -3'.

Protein Expression

Western blotting was applied to WT (C57BL/6), PLP/P₀, PLP-PNS and P₀-null 1-month-old sciatic nerve nerves homogenized in lysis buffer. Protein levels in homogenates were determined using a BioRad (Hercules, CA, USA) protein assay. Samples were run on 4–12% NuPAGE MES gels. Primary antibodies for Western blots included PLP/DM20 (clone AA3) antibody and P₀ antibody (Trapp et al., 1981).

Motor Function and Mortality

To assess motor function, we used the rota-rod test. Five mice from each of the WT, PLP/P₀, PLP/P₀^{+/-} and PLP-PNS groups were tested at 3 months of age. The time each mouse remained on the rotating cylinder was recorded. Additionally, a Kaplan-Meier analysis of mortality was conducted for a minimum of 20 mice from each group.

Immunohistochemistry

WT, PLP/P₀, PLP/P₀^{+/-} and PLP-PNS mice at 1 and 3 months of age were perfused with 4% paraformaldehyde. Samples of 1-month-old sciatic nerves were teased for immunohistochemical PLP (AA3) staining. 3-month-old sciatic nerves were cut at 10 µm in a cryostat (Leica Microsystems, Exton, PA, USA) and cross sections were double-immunostained for the myelin proteins PLP (AA3, gift from Dr. Wendy Macklin) and P₀ (Trapp et al., 1981). Secondary antibodies were conjugated with Alex 488 or Alex 594 (Life Technologies, Grand Island, NY, USA). Confocal images were collected using a Leica SP5 confocal microscope (Leica Microsystems).

Transmission Electron Microscopy (EM)

WT, P₀-null, PLP/P₀, PLP/P₀^{+/-} and PLP-PNS mice were perfused with 2.5% glutaraldehyde, 4% paraformaldehyde, and 0.08 M Sorensen's phosphate at postnatal day 4 (P4) as well as 10 weeks of age. Sciatic nerves were removed, placed in fixative overnight, osmicated, dehydrated, and processed with Epon 812. One µm sections were cut with glass knives in an Ultracut E and stained with toluidine blue. Ultrathin sections were cut on a diamond knife in an Ultracut E, placed on 300-mesh copper grids, stained with uranyl acetate and lead citrate, and examined in a Philips CM100 transmission electron microscope.

G-Ratios and Myelin Periodicity

G-ratios were calculated for 50 axons per group from sciatic nerves of 3–4 animals total from each group. From transmission EM images in transverse orientation, the diameter of the entire fiber (axon and myelin) and of the axon alone was determined, as well as the ratio between the two. Myelin spacing was determined by measuring the repeat distances of major dense lines for electron micrographs. G-ratios were compared using pairwise t-tests incorporating Bonferroni's corrections for multiple comparisons (R-statistical software).

Results

Generation and molecular characterization of mice

We generated transgenic mice in which mouse PLP cDNA was driven by the mouse P_0 6Kb promoter and intron 1 enhancer (Fig. 1A). These mice were bred to mice null for P_0 protein (Giese et al., 1992). The heterozygous F1 pups were interbred and the resultant pups were genotyped for the P_0 protein knock-out (NEO) gene and the PLP transgene. At the F3 generation, mice were identified as WT, P_0 -null, PLP/ P_0 , PLP/ $P_0^{+/-}$, and PLP-PNS mice. These five lines of mice were maintained and are included in the present study. Phenotypes were identified by quantitative RT-PCR of the PLP transgene and P_0 knock-out (NEO) gene (Fig. 1B). Genotypes were confirmed by Western blot analysis (Fig. 1C) and immunocytochemistry (Fig. 2) of sciatic nerves. In Western blots, WT sciatic nerves contained P_0 protein but no detectable PLP. Both P_0 and PLP were abundant in PLP/ P_0 sciatic nerves. P_0 was not detected in P_0 -null or PLP-PNS sciatic nerves. PLP however was abundant in PLP-PNS and P_0 -null sciatic nerves. The apparent high level of PLP in P_0 -null nerves is the result of the overall low protein content of myelin and possibly a modest up-regulation of PLP/DM20 expression. The P_0 -null nerves contained a higher percentage of DM20 than PLP-PNS nerves (Fig. 2B). We next compared the distribution of PLP in teased PNS fibers from WT, P_0 -null, PLP/ P_0 , PLP/ $P_0^{+/-}$ and PLP-PNS sciatic nerves. As described previously (Puckett, Hudson, Ono, Friedrich, Benecke, Dubois-Dalcq, and Lazzarini, 1987; Anderson, Montague, Nadon, Nave, and Griffiths, 1997), PLP immunoreactivity was detectable in paranodal regions of WT peripheral nerves but was not detectable in compact PNS myelin (Fig. 1D). In P_0 -null nerves, PLP was detected in perinuclear Schwann cell cytoplasm and occasionally at the outer margins of myelin internodes, but was not targeted to spirally wrapped myelin membranes (Fig. 1E). In addition to paranodal regions, PLP immunoreactivity was abundant in compact PNS myelin in PLP/ P_0 nerves (Fig. 1F). PLP was detected in compact myelin, paranodal loops and S-L incisures in PLP/ $P_0^{+/-}$ teased fibers (Fig. 1G). In PLP-PNS fibers, PLP was present in compact myelin (Fig. 1H) but these myelinated fibers were rare and much thinner than those in WT or PLP/ P_0 nerves. In addition, it appeared that PLP was present in Schwann cells that ensheathed, but did not myelinate, axons in PLP-PNS nerves (Fig. 1H, upper fiber).

The distributions of P_0 and PLP were compared in cross-sections of 3-month-old sciatic nerves. As expected, P_0 protein was abundant and PLP was not detected in compact myelin in WT sciatic nerves (Figs. 2A and B). In sciatic nerves that expressed equal amounts of P_0 and PLP (PLP/ P_0 nerves), both P_0 (Fig. 2C) and PLP (Fig. 2D) were detected in compact myelin. Based upon P_0 staining, the size of myelinated fibers appeared to be similar in WT and PLP/ P_0 nerves. Thus, overexpressed PLP is targeted to compact PNS myelin that has normal levels of P_0 protein and this addition of PLP does not appear to have a major effect on myelination. The levels of P_0 in peripheral myelin have a significant influence on the consequences of overexpressing PLP in PNS myelin. When one copy of the P_0 gene was removed from peripheral nerves that overexpress PLP (PLP/ $P_0^{+/-}$), PLP was targeted to compact myelin but the resultant myelin sheaths appeared to be hypomyelinated (Figs. 2E and F). When both copies of P_0 were removed (PLP-PNS; Fig. 2G), PLP overexpression was apparent by the abundance of PLP immunoreactivity in Schwann cells that appeared to

be in one-to-one associations with axons (Fig. 2H) but forming few compact myelin internodes.

Effects of PLP overexpression on early Schwann cell myelination

We next investigated the associations between Schwann cells and axons in P4 sciatic nerves in one μm -thick and ultrathin sections. In one μm -thick epon sections from P4 WT sciatic nerve, myelinating Schwann cells formed one-to-one associations with axons and compact myelin sheaths (Fig. 3A). PLP/ P_0 sciatic nerves were similar to WT nerves in one μm -thick epon sections (Fig. 3B). In contrast, Schwann cells expressing PLP and one copy of the P_0 gene (PLP/ P_0^{+-}) had a significant delay in PNS myelination (Fig. 3C). While most Schwann cells appeared to be in one-to-one relationships with large diameter axons, compact myelin was less frequent and appeared much thinner than in WT nerves. In PLP-PNS sciatic nerves, there was a dramatic delay in myelination with little or no compact myelin evident at P4 (Fig. 3D).

To extend these light microscopic observations, we analyzed P4 nerves by electron microscopy. As expected, almost all large diameter axons were in one-to-one relationships with myelinating Schwann cells in both WT (Fig. 4A) and PLP/ P_0 (Fig. 4B) sciatic nerves. Adding PLP to sciatic nerve Schwann cells appeared to have little effect on the early stages of Schwann cell myelination. Adding PLP to Schwann cells with one copy of the P_0 gene (PLP/ P_0^{+-}) had no effect on radial sorting of axons but did induce a significant delay in myelination (Fig. 4C). There was a significant decrease in the number of axons surrounded by compact myelin and a significant decrease in the thickness of the myelin that was formed. When P_0 was removed from PNS myelin, PLP overexpression (PLP-PNS) did not affect radial sorting of axons, but none of the Schwann cells formed myelin (Fig. 4D). Thus, expression of PLP in Schwann cells has no major effect on PNS myelination as long as the expression of P_0 is normal. Reducing P_0 expression in PLP overexpressing nerves delays myelination in a dose-dependent manner but has no effect on the radial sorting of axons. The number and appearance of unmyelinated axonal bundles or Remark fibers was not altered by any of the genetic manipulations of PLP or P_0 expression.

Effects of PLP overexpression on mature PNS myelin

As genetic manipulations of PLP and P_0 protein levels in peripheral nerve can delay myelination, we next investigated the long-term effects of these myelin protein changes by examining 10-week-old sciatic nerves. This is a time point when the majority of PNS myelination is complete. There was little difference between the appearance of WT and PLP/ P_0 sciatic nerves in one μm -thick sections (Figs. 5A and B). The number of myelinated axons appeared to be similar in PLP/ P_0^{+-} nerves, but there was significant hypomyelination (Fig. 5C). Most Schwann cells in a one-to-one relationship with axons appeared to form some compact myelin. Overexpression of PLP in nerves with one copy of P_0 delayed, but did not inhibit, PNS myelination. In contrast, there was little myelination in PLP-PNS sciatic nerves (Fig. 5D). As found at P4, most large axons appeared to be in a one-to-one relationship with Schwann cells in the 10-week-old PLP-PNS sciatic nerves, but myelination was rare. Expression of PLP in the absence of P_0 inhibits myelination in the sciatic nerve.

Electron microscopy of the 10-week-old sciatic nerves confirmed our light microscopic observations. While the number of myelinated fibers was similar in WT (Fig. 6A) and PLP/P₀ (Fig. 6B) sciatic nerves, myelin sheath thickness appeared to be thinner in the PLP/P₀ nerves. Thinner myelin was a striking feature in electron micrographs of PLP/P₀^{+/−} nerves (Fig. 6C). Compact myelin was a rare finding in PLP-PNS nerves and when present was restricted to a few wraps (Fig. 6D, asterisks). Occasional fibers were surrounded by redundant basal lamina (Fig. 6D, arrowheads), indicating some Schwann cell turnover. There were no obvious signs of axonal/Wallerian degeneration or immune cell accumulation in electron micrographs from 4-day-old or 10-week-old PLP-PNS sciatic nerves. In addition, the numbers of axons in one-to-one relationships with Schwann cells were similar in 4-day-old and 10-week-old WT and PLP-PNS sciatic nerves.

With the availability of electron micrographs we were able to perform two additional analyses: g-ratios and myelin periodicity. The g-ratio is defined as the diameter of the axon divided by the diameter of a myelinated fiber. The average g-ratio for myelinated WT sciatic nerve fibers is relatively constant (Fig 7A), averaging 0.67 (n=3 mice), and those for PLP/P₀ were similar (0.72, not significantly different). In contrast, PLP/P₀^{+/−} were hypomyelinated with an average g-ratio of 0.84 (p<0.005 different from WT and PLP/P₀, t-test with Bonferroni multiple comparisons test). In the PLP-PNS group, myelinated internodes were short and thin (Fig 7A), with an average g-ratio of 0.88 (p<0.001 compared to WT and PLP/P₀, t-test with Bonferroni multiple comparisons test). These data indicate that PLP does not affect myelin thickness when P₀ is present, but does not compensate fully for loss of P₀ in PNS myelin. The range of axonal diameters among the sampled axons was similar, except that no PLP-PNS axons larger than 4 μm were found.

The periodicities of compact CNS and PNS myelin differ and reflect the relative size of the extracellular domains of P₀ and PLP. The extracellular domain of P₀ is larger and more heavily charged than the extracellular domain(s) of PLP. The major difference therefore in PNS and CNS myelin periodicities is the spacing of the extracellular leaflets, which is about 20 Å greater in compact PNS myelin than in compact CNS myelin (Kirschner and Blaurock, 1992). P₀ is an obligate homophilic binding protein that is thought to dictate the spacing between extracellular leaflets of compact PNS myelin by trans-binding between cis-linked tetramers (Shapiro et al., 1996). The extracellular spacing of compact PNS myelin, however, is not exclusively based on the presence of P₀ protein. When P₀ was expressed equally with PLP in CNS myelin, the periodicity of the compact myelin was identical to CNS myelin (Yin et al., 2006). The presence of PLP apparently interferes with adhesion of the extracellular domains of P₀ in trans and/or in cis. When PLP was removed from P₀-containing CNS compact myelin, its periodicity was identical to WT PNS myelin. It was of interest, therefore, to determine whether PLP-PNS myelin had a CNS or a PNS periodicity (Fig. 7B–F). Similar to CNS myelin that expresses P₀ protein, PNS myelin that expresses PLP has a periodicity of compact CNS myelin (Fig. 7C). CNS myelin periodicities were also found in myelin that contained one copy of P₀ and two copies of PLP (Fig. 7D). While compact myelin was rare in PLP-PNS sciatic nerves, the compact myelin formed had the periodicity of WT CNS myelin (Fig. 7E).

Molecular control of Schmidt-Lanterman incisures

S-L incisures are a common feature of PNS myelin internodes. A surprising finding in the optic nerve of mice where P_0 replaced PLP in CNS myelin was the presence of S-L incisures (Yin et al., 2008). This was surprising because P_0 protein is not present in incisure membranes. We proposed, therefore, that P_0 is required for and can induce S-L incisures in myelin internodes. If P_0 is required for S-L incisure formation, S-L incisures should not be present in PLP-PNS peripheral nerves. While the numbers of myelin internodes were dramatically reduced, we identified several internodes with compact myelin in 3- and 6-month-old PLP-PNS sciatic nerves. S-L incisures were not found in electron micrographs or immunostained teased fibers (Fig. 1H) of PLP-PNS sciatic nerves. S-L incisures were present in PLP/ P_0 and PLP/ P_0^{+-} myelinated fibers.

Neurological disability and mortality

To investigate the phenotypic effects of replacing P_0 with PLP, we compared the motor performance of PLP-PNS mice with the other strains using a standard rota-rod treadmill. Three-month-old PLP-PNS mice did not stay on the treadmill for more than 5 seconds. Motor performance was similar in WT and PLP/ P_0 mice, but was significantly decreased ($> 50\%$; $p < 0.05$) in PLP/ P_0^{+-} mice (Fig. 7G). The lifespans of the 5 lines of mice were compared and all but PLP-PNS mice survived well beyond 6 months of age. Kaplan-Meier analysis showed that over 85% of PLP-PNS mice died by 3 months of age (Fig. 7H). These results demonstrate that replacing P_0 with PLP in mouse PNS myelin induces severe neurological disability and significant death by 3 months of age. Attempts to identify the cause of death have been unsuccessful. PLP/ P_0 mice occasionally display seizure-like symptoms; however the PLP transgene is not expressed in the CNS, microglia are not activated in the CNS and neuromuscular junctions appear to be intact. Further studies will be required to identify the mechanisms responsible for nerve dysfunction in and early death of PLP/ P_0 mice.

Discussion

The purpose of this study was to investigate whether PLP protein, the major structural protein of CNS myelin, could replace P_0 protein as the major structural protein of PNS myelin. Genetic switching of the dominant protein of PNS myelin to PLP had severe consequences on the viability of mice, with significant death prior to 3 months of age. When P_0 and PLP are similarly co-expressed in PNS myelin, it has the periodicity of CNS myelin. In the presence of P_0 , PLP is transported to compact PNS myelin and becomes the dominant protein in establishing the spacing of the extracellular leaflets of compact myelin. In the absence of P_0 protein, PLP inhibits spiral wrapping of Schwann cell membranes around axons and PNS myelination. Schwann cells in PLP-PNS mice appropriately sort axons into one-to-one relationships, indicating that in the absence of P_0 , PLP inhibits myelin membrane expansion. These data support fundamental differences in the mechanisms by which oligodendrocytes and Schwann cells expand myelin membranes. Oligodendrocytes can form myelin internodes with either PLP or P_0 as the major protein. Schwann cells require P_0 to make normal myelin internodes and PLP cannot substitute for P_0 .

PLP and P₀ gene dosage in CNS myelin

Myelin protein content can have major influences on the maturation of oligodendrocytes, Schwann cells and the axons they myelinate. Manipulation of PLP and P₀ gene dosages does not arrest CNS myelination, but can decrease CNS axonal viability and oligodendrocyte survival (Klugmann et al., 1997; Yin et al., 2006). PLP has a dual role in CNS myelin; it is responsible for proper myelin compaction and it provides trophic support essential for long-term axonal survival (Griffiths et al., 1998; Sporkel et al., 2002; Yin et al., 2006; Nave and Trapp, 2008; Nave, 2010). PLP gene duplication causes a more severe phenotype than PLP deletions. PLP duplications in humans cause Pelizaeus-Merzbacher Disease (PMD), a severe and often fatal leukodystrophy (Hodes et al., 2000; Dhaunchak et al., 2011). PLP-null mutations cause Hereditary Spastic Paraplegia (HSP), a less severe neurological condition that causes a “dying back” axonopathy of long CNS fiber tracts (Nave, 2010). Both of these human diseases have been modeled in rodents. PLP overexpression causes oligodendrocyte death due to activation of the endoplasmic reticulum (ER) Unfolded Protein Response (Gow and Lazzarini, 1996; Dhaunchak et al., 2011). Synthesis and transport of PLP in rough ER and Golgi are significantly affected by specific gene mutations and mutations that increased PLP dosage (Nave and Boespflug-Tanguy, 1996; Gow and Lazzarini, 1996; Karim et al., 2007; Karim et al., 2010). PLP-null mutations on the other hand appear to have little effect on oligodendrocyte survival but induce a late onset axonal degeneration (Griffiths et al., 1998). This axonal loss is preceded by axonal ovoids that are caused by altered axonal transport at paranodal regions of the PLP-deficient myelin internodes (Edgar et al., 2004). P₀ cannot rescue the axonal degeneration caused by loss of PLP from CNS myelin (Yin et al., 2006). In contrast, P₀ increases the axonal degeneration caused by loss of PLP. P₀-CNS mice have shorter myelin internodes, increased paranodal regions, more axonal ovoids, increased axonal degeneration, accelerated neurological disability and less than half the lifespan of PLP-null mice.

P₀ and PLP gene dosage in PNS myelin

Schwann cells are also affected by altered P₀ and PLP gene dosages. P₀ overexpression in mice dramatically inhibits PNS myelination and causes retraction of axonal terminals from neuromuscular junctions (Yin et al., 2000; Yin et al., 2004). These mice have severe neurological disability and shortened life spans. To date, P₀ gene duplication has not been identified in humans, but copy number variations in *MPZ* have been associated with dys- or demyelinating neuropathies (Hoyer et al., 2011; Maeda et al., 2012). P₀-null mutations cause the peripheral neuropathy Charcot-Marie-Tooth (CMT) disease in humans (Martini, 1999). P₀-null mice have also been generated (Giese et al., 1992). P₀-null Schwann cells spirally wrap axons with non-compacted membranes. Phenotypes in P₀-null humans can be moderate to severe and loss of P₀ in mice causes minimal neurological disability (Martini et al., 1995; Martini, 1999). Similar to PLP, P₀ gene duplication in mice has a greater negative impact on peripheral nerves than P₀ elimination (Wrabetz et al., 2000; Yin et al., 2000). In contrast to oligodendrocytes where P₀ protein can support CNS myelination, PLP cannot support PNS myelination. PLP-PNS Schwann cells segregate axons into one-to-one relationships and occasionally spiral mesaxon membranes, but rarely form compact myelin. Thus, PLP has a gain-of-function effect that inhibits Schwann cell myelination in the

absence of P_0 protein. The negative PLP effect on Schwann cell myelination is inhibited by P_0 in a dose-dependent manner. One copy of the P_0 gene and two copies of the PLP gene ($PLP/P_0^{+/+}$) results in hypomyelination and less severe neurological disability. Two copies of the P_0 gene eliminate the negative effect that PLP overexpression has on PNS myelination (PLP/P_0). Since PLP is expressed by ensheathing Schwann cells, it is feasible that PLP could provide trophic support that would maintain axonal viability in PLP-PNS mice. This does not appear to be the case, as PLP-PNS mice have significantly increased neurological disability and reduced lifespans compared to P_0 -null and PLP-null mice.

P_0 , PLP and myelin compaction

A major difference between CNS and PNS myelin is the periodicity of compact myelin, which reflects the molecular features of PLP and P_0 proteins. The extracellular domain of P_0 is larger than that of PLP and thus the spacing between the extracellular leaflets and overall periodicity of compact myelin is 20 Å greater in PNS than in CNS myelin (Kirschner and Blaurock, 1992). When PLP and P_0 are equally expressed in CNS myelin, both are targeted to compact myelin that has the periodicity of CNS myelin (Yin et al., 2006). We have now compared the effect of expressing equal amounts of P_0 and PLP in PNS myelin. Immunocytochemistry established that PLP is targeted to compact PNS myelin that expresses similar amounts of P_0 . Similar to CNS myelin, PLP determines the periodicity of compact PNS myelin when expressed at similar amounts as P_0 . This result is somewhat surprising if only considering the binding capacities of P_0 and PLP. P_0 is an obligate homophilic adhesion molecule (Filbin et al., 1990), whereas PLP binds in trans to negatively charged lipids (Horvath et al., 1988; ter Beest et al., 1994). One possible explanation may involve additional requirements for P_0 trans binding. P_0 binding occurs by trans binding of cis-linked P_0 tetramers (D'Urso et al., 1990; Shapiro et al., 1996; Inouye et al., 1999; Thompson et al., 2002). We proposed previously that PLP may dictate CNS myelin periodicity by inhibiting the formation of P_0 tetramers in cis. This hypothesis is supported by the present study, where PLP dictated the periodicity of PNS myelin containing equal amounts of P_0 and PLP. CNS compact myelin periodicities in both CNS and PNS myelin that express equal amounts of PLP and P_0 make it unlikely that differential expression of lipids or other myelin proteins influence the role of P_0 and PLP in compaction of PNS or CNS myelin. Identical to PLP/ P_0 myelin, compact myelin in PLP-PNS and PLP/ $P_0^{+/+}$ sciatic nerves had the periodicity of compact CNS myelin.

P_0 is required for S-L incisure formation

Schwann cells form single myelin internodes, which contain a series of cytoplasmic channels that connect the outer and inner margins of the myelin internode. These S-L incisures facilitate transport and diffusion of molecules and ions throughout PNS myelin internodes (Balice-Gordon et al., 1998). Oligodendrocytes form multiple myelin internodes that do not contain S-L incisures. Membranes of the S-L incisures are enriched in the myelin-associated glycoprotein, connexin-32, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), and E-cadherin (Trapp and Quarles, 1982; Trapp et al., 1989; Heath et al., 1991; Scherer et al., 1995; Rasband et al., 2005) but do not contain detectable levels of P_0 protein (Trapp and Quarles, 1982; Trapp et al., 1989). When P_0 replaced PLP in rodent CNS myelin, the resultant myelin internodes contained S-L incisures (Yin et al., 2008). The CNS

incisures appeared functional as they transversed the entire width of the myelin internode and contained microtubules. These CNS incisures, however, appeared detrimental to axonal viability as they induced axoplasmic pathology (Yin et al., 2008). It was somewhat surprising that P_0 could induce S-L incisures because P_0 is not expressed in incisure membranes. S-L incisures are present in MAG-null peripheral nerves (Yin et al., 2008), indicating that MAG is not required for incisure formation. The lack of S-L incisures in P_0 -null sciatic nerve myelin internodes also supports the essential role of P_0 in incisure formation. The current study provides additional evidence that the presence of P_0 in compact myelin is essential for S-L incisure formation as S-L incisures were present in peripheral nerves that contained PLP and one or two copies of P_0 (Fig 1F and G). In myelin internodes that contained PLP but no P_0 , S-L incisures were not present.

Conclusions

A major catalyst in performing the present study was the observation that CNS myelin underwent an evolutionary shift when PLP replaced P_0 as the major structural protein of CNS myelin in terrestrial vertebrates (Waehneldt, 1990; Yoshida and Colman, 1996). Reversing the P_0 to PLP shift in mice supported the concept that the P_0 to PLP shift provided a vital neuroprotective function to CNS myelin (Yin et al., 2006). Since PLP did not replace P_0 in PNS myelin, the present study investigated the possible consequences of replacing P_0 with PLP in rodent PNS myelin. When expressed equally, PLP and P_0 can co-exist in compact PNS myelin and facilitate normal peripheral nerve function. Expressing P_0 at half the level of PLP in peripheral nerve caused hypomyelination and neurological disability by 3 months of age. Expressing PLP and no P_0 essentially stopped PNS myelination and induced a severe neurological phenotype and early death. These studies highlight significant differences in 1) the ability of oligodendrocytes and Schwann cells to utilize P_0 and PLP protein, and 2) the function of PLP and P_0 in CNS and PNS myelin. This helps explain why PLP replaced P_0 in CNS myelin, but not in PNS myelin. Additional questions remain to be addressed, including how PLP loss of function causes axonal dysfunction/degeneration in the CNS and how PLP induces neurological disability in rodent peripheral nerves that do not contain P_0 .

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References

- Anderson TJ, Montague P, Nadon N, Nave KA, Griffiths IR. Modification of Schwann cell phenotype with Plp transgenes: evidence that the PLP and DM20 isoproteins are targeted to different cellular domains. *J Neurosci Res.* 1997; 50:13–22. [PubMed: 9379489]
- Balicer-Gordon RJ, Bone LJ, Scherer SS. Functional gap junctions in the Schwann cell myelin sheath. *J Cell Biol.* 1998; 142:1095–1104. [PubMed: 9722620]
- D'Urso D, Brophy PJ, Staugaitis SM, Gillespie CS, Frey AB, Stempak JG, Colman DR. Protein zero of peripheral nerve myelin: Biosynthesis, membrane insertion, and evidence for homotypic interaction. *Neuron.* 1990; 2:449–460. [PubMed: 1690568]

- Dhaunchak AS, Colman DR, Nave KA. Misalignment of PLP/DM20 transmembrane domains determines protein misfolding in Pelizaeus-Merzbacher disease. *J Neurosci.* 2011; 31:14961–14971. [PubMed: 22016529]
- Edgar JM, McLaughlin M, Yool D, Zhang SC, Fowler JH, Montague P, Barrie JA, McCulloch MC, Duncan ID, Garbern J, Nave KA, Griffiths IR. Oligodendroglial modulation of fast axonal transport in a mouse model of hereditary spastic paraplegia. *J Cell Biol.* 2004; 166:121–131. [PubMed: 15226307]
- Feltri ML, D'antonio M, Quattrini A, Numerato R, Arona M, Previtali S, Chiu SY, Messing A, Wrabetz L. A novel P0 glycoprotein transgene activates expression of lacZ in myelin-forming Schwann cells. *Eur J Neurosci.* 1999; 11:1577–1586. [PubMed: 10215910]
- Filbin MT, Walsh FS, Trapp BD, Pizzey JA, Tennekoon GI. The role of myelin P₀ protein as a homophilic adhesion molecule. *Nature.* 1990; 344:871–872. [PubMed: 1691824]
- Giese KP, Martini R, Lemke G, Soriano P, Schachner M. Mouse P₀ gene disruption leads to hypomyelination, abnormal expression of recognition molecules, and degeneration of myelin and axons. *Cell.* 1992; 71:565–576. [PubMed: 1384988]
- Gow A, Lazzarini RA. A cellular mechanism governing the severity of Pelizaeus-Merzbacher disease. *Nat Genet.* 1996; 13:422–428. [PubMed: 8696336]
- Griffiths I, Klugmann M, Anderson T, Yool D, Thomson C, Schwab MH, Schneider A, Zimmermann F, McCulloch M, Nadon N, Nave K-A. Axonal swellings and degeneration in mice lacking the major proteolipid of myelin. *Science.* 1998; 280:1610–1613. [PubMed: 9616125]
- Heath JW, Inuzuka T, Quarles RH, Trapp BD. Distribution of P₀ protein and the myelin-associated glycoprotein in peripheral nerves from Trembler mice. *J Neurocytol.* 1991; 20:439–449. [PubMed: 1714491]
- Hodes ME, Woodward K, Spinner NB, Emanuel BS, Enrico-Simon A, Kamholz J, Stambolian D, Zackai EH, Pratt VM, Thomas IT, Crandall K, Dlouhy SR, Malcolm S. Additional copies of the proteolipid protein gene causing Pelizaeus-Merzbacher disease arise by separate integration into the X chromosome. *Am J Hum Genet.* 2000; 67:14–22. [PubMed: 10827108]
- Horvath LI, Brophy PJ, Marsh D. Exchange rates at the lipid-protein interface of myelin proteolipid protein studied by spin-label electron spin resonance. *Biochemistry.* 1988; 27:46–52. [PubMed: 2450570]
- Hoyer H, Braathen GJ, Eek AK, Skjelbred CF, Russel MB. Charcot-Marie-Tooth caused by a copy number variation in myelin protein zero. *Eur J Med Genet.* 2011; 54:e580–583. [PubMed: 21787890]
- Hudson, LD. Proteolipid Protein Gene. In: Lazzarini, RA., editor. *Myelin Biology and Disorders*. San Diego: Elsevier Academic Press; 2004. p. 401–420.
- Inouye H, Tsuruta H, Sedzik J, Uyemura K, Kirschner DA. Tetrameric assembly of full-sequence protein zero myelin glycoprotein by synchrotron x-ray scattering. *Biophys J.* 1999; 76:423–437. [PubMed: 9876154]
- Karim SA, Barrie JA, McCulloch MC, Montague P, Edgar JM, Iden DL, Anderson TJ, Nave KA, Griffiths IR, McLaughlin M. PLP/DM20 expression and turnover in a transgenic mouse model of Pelizaeus-Merzbacher disease. *Glia.* 2010; 58:1727–1738. [PubMed: 20629189]
- Karim SA, Barrie JA, McCulloch MC, Montague P, Edgar JM, Kirkham D, Anderson TJ, Nave KA, Griffiths IR, McLaughlin M. PLP overexpression perturbs myelin protein composition and myelination in a mouse model of Pelizaeus-Merzbacher disease. *Glia.* 2007; 55:341–351. [PubMed: 17133418]
- Kirschner, DA.; Blaurock, AE. Organization, phylogenetic variations and dynamic transitions of myelin structure. In: Martenson, RE., editor. *Myelin: Biology and Chemistry*. Boca Raton, FL: CRC Press; 1992. p. 3–78.
- Kirschner DA, Inouye H, Ganser AL, Mann V. Myelin membrane structure and composition correlated: a phylogenetic study. *J Neurochem.* 1989; 53:1599–1609. [PubMed: 2795020]
- Kirschner, DA.; Wrabetz, L.; Feltri, ML. *Myelin Biology and Disorders*. San Diego: Elsevier (USA); 2004. The P0 Gene; p. 523–545.

- Klugmann M, Schwab MH, Puhlhofer A, Schneider A, Zimmermann F, Griffiths IR, Nave KA. Assembly of CNS myelin in the absence of proteolipid protein. *Neuron*. 1997; 18:59–70. [PubMed: 9010205]
- Kurihara T, Sakuma M, Gojobori T. Molecular evolution of myelin proteolipid protein. *Biochem Biophys Res Commun*. 1997; 237:559–561. [PubMed: 9299403]
- Lemke G, Axel R. Isolation and sequence of a cDNA encoding the major structural protein of peripheral myelin. *Cell*. 1985; 40:501–508. [PubMed: 2578885]
- Macklin WB, Campagnoni CW, Deininger PL, Gardinier MV. Structure and expression of the mouse myelin proteolipid gene. *J Neurosci Res*. 1987; 18:383–394. [PubMed: 2449535]
- Maeda MH, Mitsui J, Soong BW, Takahashi Y, Ishiura H, Hayashi S, Shirota Y, Ichikawa Y, Matsumoto H, Arai M, Okamoto T, Miyama S, Shimizu J, Inazawa J, Goto J, Tsuji S. Increased gene dosage of myelin protein zero causes Charcot-Marie-Tooth disease. *Ann Neurol*. 2012; 71:84–92. [PubMed: 22275255]
- Martini R. P₀-deficient knockout mice as tools to understand pathomechanisms in Charcot-Marie-Tooth 1B and P₀-related Dejerine-Sottas syndrome. *Ann N Y Acad Sci*. 1999; 883:273–280. [PubMed: 10586252]
- Martini R, Zielasek J, Toyka KV, Giese P, Schachner M. Protein zero (P₀)-deficient mice show myelin degeneration in peripheral nerves characteristic of inherited human neuropathies. *Nat Genet*. 1995; 11:281–286. [PubMed: 7581451]
- Milner RJ, Lai C, Nave K-A, Lenoir D, Ogata J, Sutcliffe JG. Nucleotide sequences of two mRNAs for rat brain myelin proteolipid protein. *Cell*. 1985; 42:931–939. [PubMed: 2414013]
- Nave KA. Myelination and the trophic support of long axons. *Nat Rev Neurosci*. 2010; 11:275–283. [PubMed: 20216548]
- Nave K-A, Boespflug-Tanguy O. X-linked developmental defects in myelination: from mouse mutants to human genetic diseases. *Neuroscientist*. 1996:33–43.
- Nave K-A, Lai C, Bloom F, Milner RJ. Splice site selection in the proteolipid protein (PLP) gene transcript and primary structure of the DM20 protein of central nervous system myelin. *Proc Natl Acad Sci USA*. 1987; 84:5665–5669. [PubMed: 2441390]
- Nave K-A, Trapp BD. Axon-glia signaling and the glial support of axon function. *Annual Review of Neuroscience*. 2008; 31:535–561.
- Pedraza L, Huang JK, Colman DR. Organizing principles of the axoglia apparatus. *Neuron*. 2001; 30:335–344. [PubMed: 11394997]
- Puckett C, Hudson L, Ono K, Friedrich V, Benecke J, Dubois-Dalcq M, Lazzarini RA. Myelin-specific proteolipid protein is expressed in myelinating Schwann cells but is not incorporated into myelin sheaths. *J Neurosci Res*. 1987; 18:511–518. [PubMed: 2449540]
- Rasband MN, Tayler J, Kaga Y, Yang Y, Lappe-Siefke C, Nave KA, Bansal R. CNP is required for maintenance of axon-glia interactions at nodes of Ranvier in the CNS. *Glia*. 2005; 50:86–90. [PubMed: 15657937]
- Saavedra RA, Fors L, Aebersold RH, Arden B, Horvath S, Sanders J, Hood L. The myelin proteins of the shark brain are similar to the myelin proteins of the mammalian peripheral nervous system. *J Mol Evol*. 1989; 29:149–156. [PubMed: 2478717]
- Salzer JL, Brophy PJ, Peles E. Molecular domains of myelinated axons in the peripheral nervous system. *Glia*. 2008; 56:1532–1540. [PubMed: 18803321]
- Scherer SS, Deschenes SM, Xu YT, Grinspan JB, Fischbeck KH, Paul DL. Connexin32 is a myelin-related protein in the PNS and CNS. *J Neurosci*. 1995; 15:8281–8294. [PubMed: 8613761]
- Shapiro L, Doyle JP, Hensley P, Colman DR, Hendrickson WA. Crystal structure of the extracellular domain from P₀, the major structural protein of peripheral nerve myelin. *Neuron*. 1996; 17:435–449. [PubMed: 8816707]
- Sporkel O, Uschkureit T, Bussow H, Stoffel W. Oligodendrocytes expressing exclusively the DM20 isoform of the proteolipid protein gene: myelination and development. *Glia*. 2002; 37:19–30. [PubMed: 11746780]
- Sternberger NH, Quarles RH, Itoyama Y, Webster Hd. Myelin-associated glycoprotein demonstrated immunocytochemically in myelin and myelin-forming cells of developing rats. *Proc Natl Acad Sci USA*. 1979; 76:1510–1514. [PubMed: 375239]

- ter Beest MB, Hoekstra K, Sein A, Hoekstra D. Reconstitution of proteolipid protein: some properties and its role in interlamellar attachment. *Biochem J.* 1994; 300 (Pt 2):545–552. [PubMed: 7516154]
- Thompson AJ, Cronin MS, Kirschner DA. Myelin protein zero exists as dimers and tetramers in native membranes of *Xenopus laevis* peripheral nerve. *J Neurosci Res.* 2002; 67:766–771. [PubMed: 11891790]
- Trapp BD, Andrews SB, Cootauco C, Quarles RH. The myelin-associated glycoprotein is enriched in multivesicular bodies and periaxonal membranes of actively myelinating oligodendrocytes. *J Cell Biol.* 1989; 109:2417–2426. [PubMed: 2478568]
- Trapp BD, Itoyama Y, Sternberger NH, Quarles RH, Webster H. Immunocytochemical localization of P₀ protein in Golgi complex membranes and myelin of developing rat Schwann cells. *J Cell Biol.* 1981; 90:1–6. [PubMed: 6166623]
- Trapp BD; Kidd, GJ. Structure of the Myelinated Axon. In: Lazzarini, R., editor. *Myelin Biology and Disorders*. Elsevier; 2004. p. 3-25.
- Trapp BD, Quarles RH. Presence of the myelin-associated glycoprotein correlates with alterations in the periodicity of peripheral myelin. *J Cell Biol.* 1982; 92:877–882. [PubMed: 6177705]
- Waehneldt TV. Phylogeny of myelin proteins. *Ann N Y Acad Sci.* 1990; 605:15–28. [PubMed: 2268116]
- Waehneldt TV, Matthieu JM, Jeserich G. Appearance of myelin proteins during vertebrate evolution. *Neurochem Int.* 1986; 9:463–474. [PubMed: 20493148]
- Wrabetz L, Feltri ML, Quattrini A, Imperiale D, Previtali S, D'antonio M, Martini R, Yin X, Trapp BD, Zhou L, Chiu SY, Messing A. P(0) glycoprotein overexpression causes congenital hypomyelination of peripheral nerves. *J Cell Biol.* 2000; 148:1021–1034. [PubMed: 10704451]
- Yin X, Baek RC, Kirschner DA, Peterson A, Fujii Y, Nave KA, Macklin WB, Trapp BD. Evolution of a neuroprotective function of central nervous system myelin. *J Cell Biol.* 2006; 172:469–478. [PubMed: 16449196]
- Yin X, Crawford TO, Griffin JW, Tu P-H, Lee VMY, Li C, Roder J, Trapp BD. Myelin-associated glycoprotein is a myelin signal that modulates the caliber of myelinated axons. *J Neurosci.* 1998; 18:1953–1962. [PubMed: 9482781]
- Yin X, Kidd GJ, Nave KA, Trapp BD. P0 protein is required for and can induce formation of Schmidt-Lantermann incisures in myelin internodes. *J Neurosci.* 2008; 28:7068–7073. [PubMed: 18614675]
- Yin X, Kidd GJ, Pioro EP, McDonough J, Dutta R, Feltri ML, Wrabetz L, Messing A, Wyatt RM, Balice-Gordon RJ, Trapp BD. Dysmyelinated lower motor neurons retract and regenerate dysfunctional synaptic terminals. *J Neurosci.* 2004; 24:3890–3898. [PubMed: 15084670]
- Yin X, Kidd GJ, Wrabetz L, Feltri ML, Messing A, Trapp BD. Schwann cell myelination requires timely and precise targeting of P(0) protein. *J Cell Biol.* 2000; 148:1009–1020. [PubMed: 10704450]
- Yoshida M, Colman DR. Parallel evolution and coexpression of the proteolipid proteins and protein zero in vertebrate myelin. *Neuron.* 1996; 16:1115–1126. [PubMed: 8663988]

Main Points

1. We engineered mice to express PLP instead of P_0 in PNS myelin (PLP-PNS mice), and these mice had severe neurological disabilities and died between 3 and 6 months of age.
2. Schwann cells in sciatic nerves from PLP-PNS mice sorted axons into one-to-one relationships but failed to form myelin internodes.
3. When PLP was overexpressed with one copy of the P_0 gene, sciatic nerves were hypomyelinated, and mice displayed motor deficits but had normal lifespans.

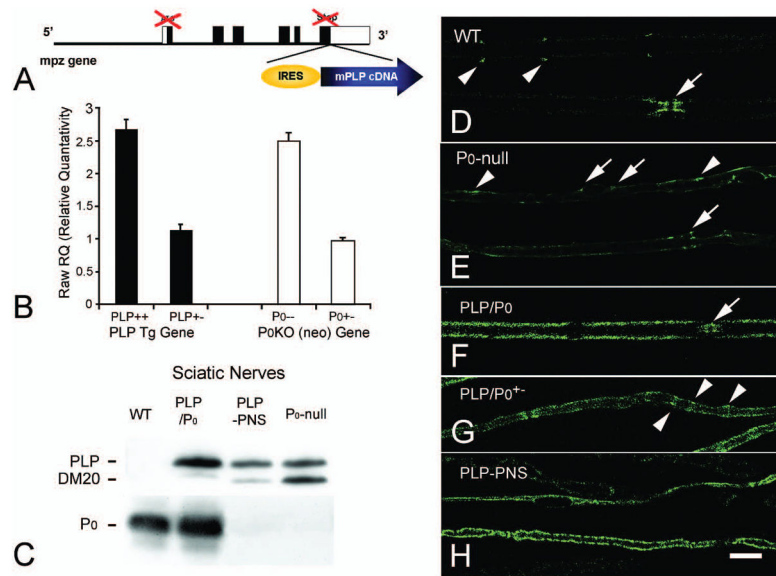


Figure 1.

Diagram of the mP0TOTA-PLP promoter and analysis of PLP-PNS transgenic founders. (A) In mP0TOTA, the P0 start site of translation is ablated (Red X, left) and here the P0 stop codon was substituted with a restriction site (Red X, right). An IRES-mPLPcDNA fusion was inserted into the restriction site to make the mP0TOTA-PLP transgene. (B) Identification of PLP-PNS homozygous mice using RT-PCR. PLP-PNS mice had 2 copies of both the mP0TOTA-PLP transgene and the P0 knock-out (NEO) gene. (C) Western blot analysis of PLP and P0 protein. PLP and DM20 were detected in PLP/P0, PLP-PNS and P0-null sciatic nerves and were not detected in WT sciatic nerves. P0 was detected in WT and PLP/P0 sciatic nerves, but not in PLP-PNS and P0-null sciatic nerves. (D-H) Confocal images of PLP immunofluorescent staining in teased fibers of 1-month-old WT, P0-null, PLP/P0, PLP/P0⁺ and PLP-PNS sciatic nerves. In WT mice (D), PLP was detected in paranodal myelin loops (D, arrow) and S-L incisures (D, arrowheads). In P0-null mice (E), PLP was observed in perinuclear Schwann cell cytoplasm (E, arrows) and sporadically at the outer surface of myelin internodes (E, arrowheads). In PLP/P0 mice (F), PLP was located in the compact myelin sheath as well as in paranodal loops (F, arrow). In PLP/P0⁺ mice (G), PLP was also detected in the compact myelin and S-L incisures (G, arrowheads). In PLP-PNS sciatic nerves (H), PLP was also located in compact myelin (G, lower fiber) and was expressed by Schwann cells that ensheathed but did not myelinate axons (H, upper fiber). Scale Bar = 10 μm.

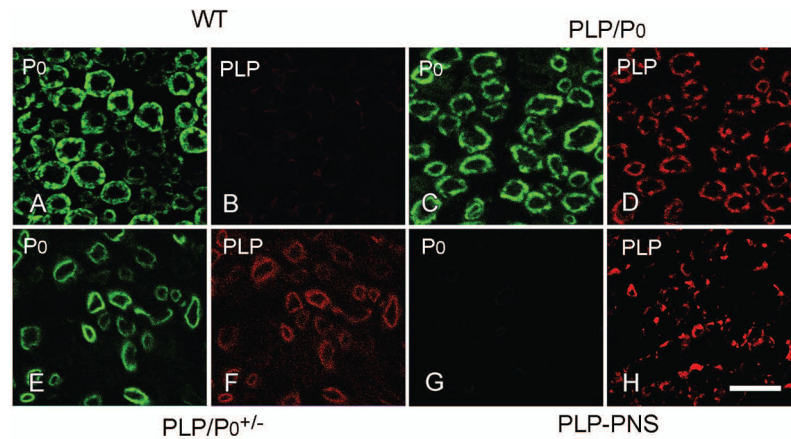


Figure 2.

Immunofluorescent staining of 3-month-old WT (A–B), PLP/ P_0 (C–D), PLP/ $P_0^{+/-}$ (E–F), and PLP-PNS (G–H) sciatic nerves was performed to determine the expression and location of P_0 (A, C, E and G, green) and overexpressed PLP (B, D, F and H, red). Expression of P_0 in WT sciatic nerves (A) was detected in myelin, while PLP was not detected in compact myelin. Overexpressed PLP (red) co-localized with P_0 (green) in PLP/ P_0 (C, D) and PLP/ $P_0^{+/-}$ (E, F) myelin. Most Schwann cells in PLP-PNS (G, H) sciatic nerve did not form myelin and PLP was detected in the cytoplasm of Schwann cells. Scale Bar = 5 μ m.

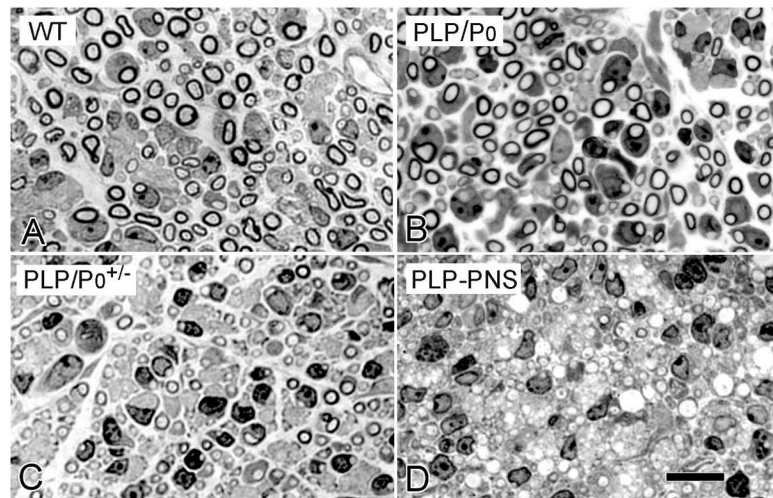


Figure 3.

One- μ m thick epon sections from 4-day-old WT (A), PLP/P₀ (B), PLP/P₀^{+/−} (C) and PLP-PNS sciatic nerves. Myelinating Schwann cells formed one-to-one associations with axons and formed compact myelin sheaths in WT (A) and PLP/P₀ (B) sciatic nerves. Myelination of PLP/P₀^{+/−} (C) sciatic nerves was delayed. Only some larger diameter axons were myelinated, but most axons remained in one-to-one associations with a Schwann cell. There was no obvious compact myelin in P4 PLP-PNS (D) sciatic nerves. Scale Bar = 10 μ m.

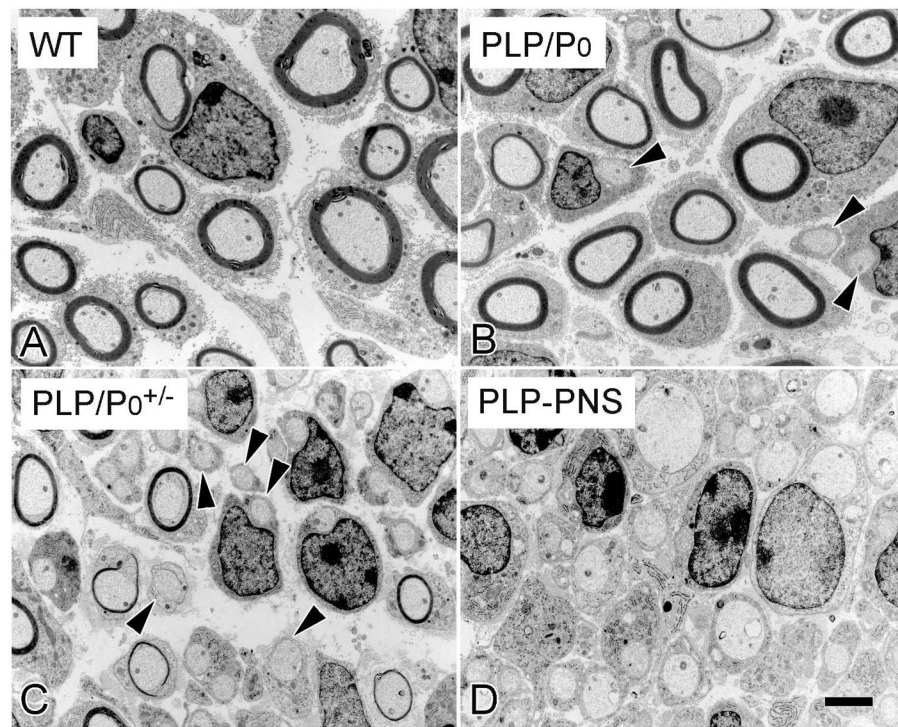


Figure 4.

Electron micrographs of WT (A), PLP/P₀ (B), PLP/P₀^{+/-} (C) and PLP-PNS (D) sciatic nerves from 4-day-old mice. All Schwann cells attained one-to-one relationships with axons larger than one micron diameter in all four mouse lines. Axons in WT sciatic nerves were appropriately myelinated (A). Some axons in PLP/P₀ sciatic nerves (B, arrowheads) and many axons in PLP/P₀^{+/-} sciatic nerves (C, arrowheads) were not myelinated. PLP-PNS Schwann cells attained one-to-one relationships with axons but failed to form compact myelin (D). Scale Bar = 2 μ m.

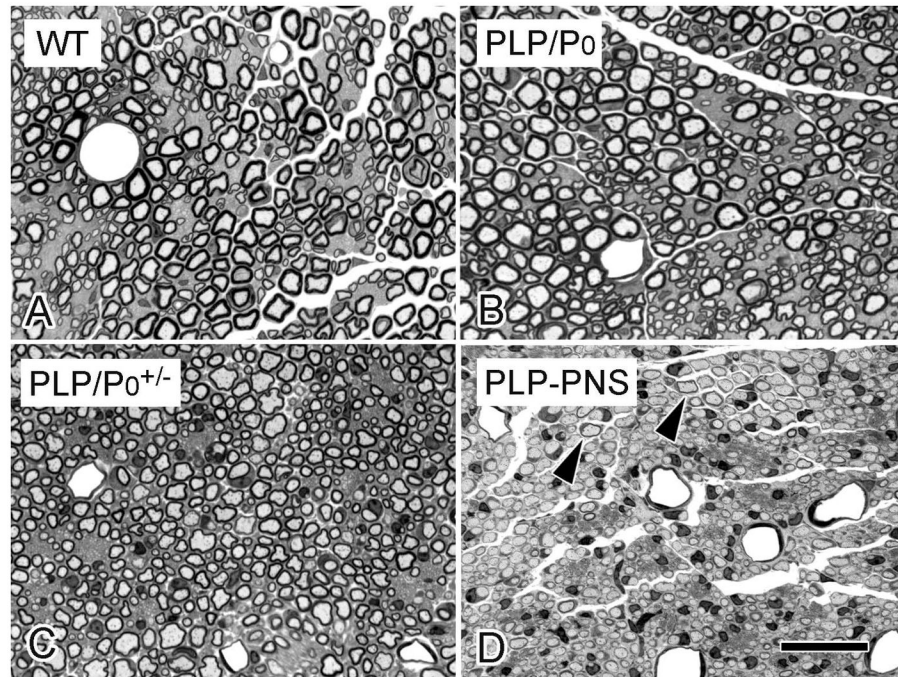


Figure 5.

One- μ m thick epon sections from 10-week-old WT (A), PLP/P₀ (B), PLP/P₀^{+/-} (C) and PLP-PNS (D) sciatic nerves. The thickness of compact myelin of WT (A) and PLP/P₀ (B) sciatic nerves was identical, but there was an obvious hypomyelination in PLP/P₀^{+/-} (C) sciatic nerves. Compact myelin was rare in PLP-PNS (D, arrowheads) sciatic nerves. Scale Bar = 25 μ m.

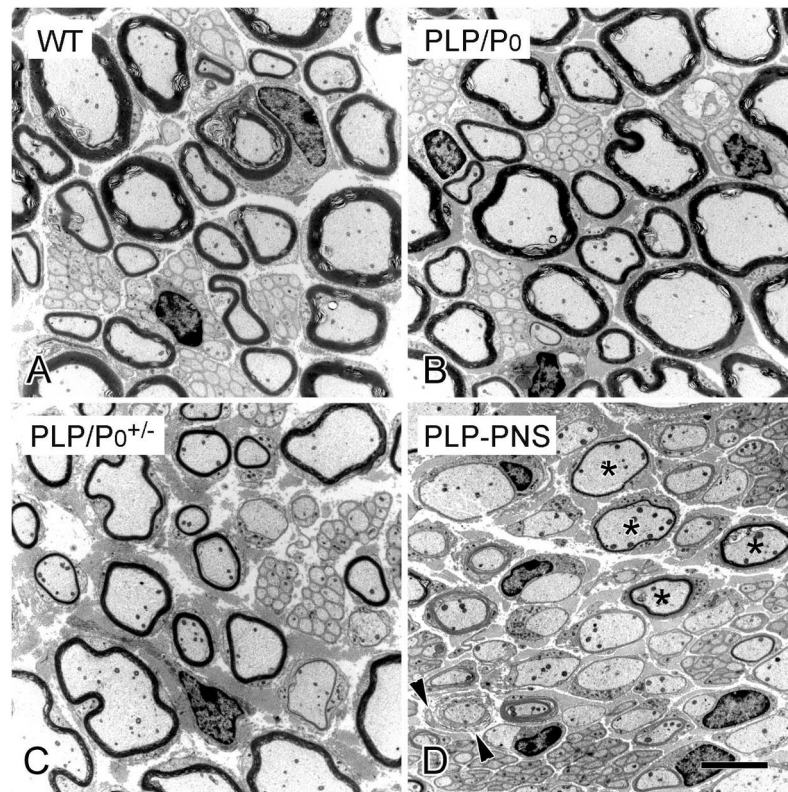
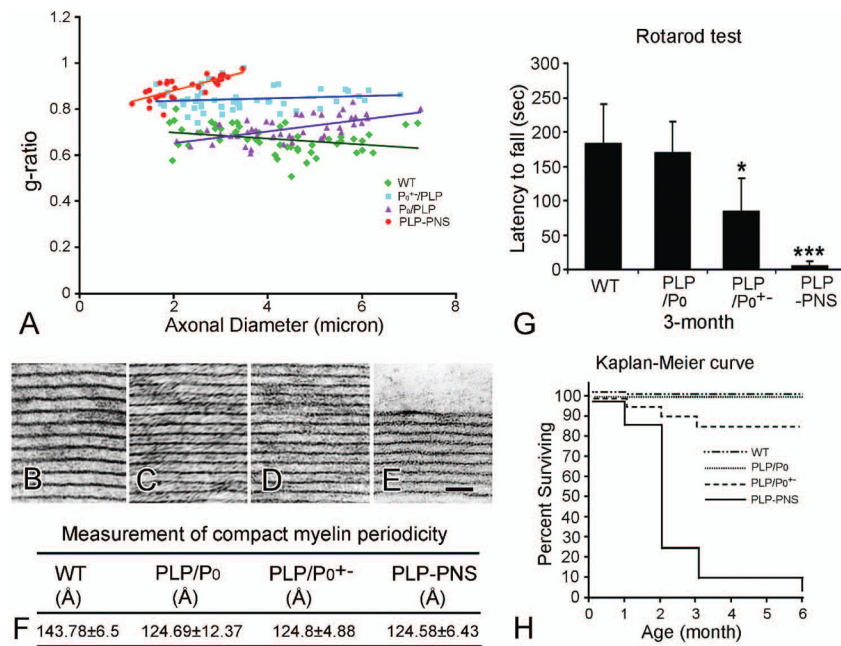


Figure 6.

Electron micrographs of 10-week-old WT (A), PLP/P₀ (B), PLP/P₀^{+/-} (C), and PLP-PNS (D) sciatic nerves. The PLP/P₀^{+/-} sciatic nerves (C) had thinner myelin, compared to the myelin sheath from WT (A) and PLP/P₀ (B) sciatic nerves. A majority of the large axons in PLP-PNS (D) sciatic nerves were not myelinated; occasionally axons were surrounded by thin myelin sheaths (D, asterisks) or redundant basal lamina (D, arrowheads). Scale Bar = 4 μm.

**Figure 7.**

(A) Myelinated fiber g-ratios compared with axonal diameters for sciatic nerve axons that express PLP in the presence or absence of P_0 ($n = 50$ axons pooled from 3–4 animals per group). WT and mice with PLP and normal P_0 expression had consistent g-ratios of 0.6–0.7, but reduction and removal of P_0 resulted in higher g-ratios (PLP/ P_0 and PLP-PNS both significantly differed from WT and PLP/ $P_0^{+/-}$, $p < 0.005$; pairwise t-tests with Bonferroni correction). (B–F) Periodicity of compact myelin in 10-week-old WT (B), PLP/ P_0 (C), PLP/ $P_0^{+/-}$ (D) and PLP-PNS (E) sciatic nerves. In transmission electron micrographs, the periodicities of PLP/ P_0 (C), PLP/ $P_0^{+/-}$ (D) and PLP-PNS (E) sciatic nerve myelin were similar to WT CNS myelin and smaller than that of WT (A) sciatic nerve compact myelin by 13.3% (F). Scale Bar = 50 nm.

Impaired motor performance and increased mortality in PLP-PNS mice. (G) Analysis of standard rota-rod testing of mice at 3 months of age. Rota-rod performance was similar for WT and PLP/ P_0 mice at 3 months of age, but motor performance was impaired in PLP/ $P_0^{+/-}$ mice. PLP-PNS mice were severely impaired and stayed on the rotating platform for less than 5 seconds. (H) Kaplan-Meier curve assessing survival as a function of age for the four lines of mice. The mean lifespan of PLP-PNS mice was reduced by 75% compared to the other lines of mice, which had mortality rates similar to those of WT ($n = 20$ WT, 20 PLP/ P_0 , 20 PLP/ $P_0^{+/-}$, and 20 PLP-PNS mice).