Proteolipid Protein Cannot Replace P_0 Protein as the Major Structural Protein of Peripheral Nervous System Myelin

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The central nervous system (CNS) of terrestrial vertebrates underwent a prominent molecular change when proteolipid protein (PLP) replaced P_0 protein as the most abundant protein of CNS myelin. However, PLP did not replace P_0 in peripheral nervous system (PNS) myelin. To investigate the possible consequences of a PLP to P_0 shift in PNS myelin, we engineered mice to express PLP instead of P_0 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 P_0 and PLP had normal PNS myelination and lifespans similar to wild-type (WT) mice. When PLP was overexpressed with one copy of the P_0 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 P_0 in PNS myelin, PLP cannot replace P_0 as the major structural protein of PNS myelin.

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Key words: Schwann cell, hypomyelination, myelin compaction, myelin evolution, axon

Introduction

Myelin is a multilamellar, 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; Nave, 2010; Yin et al., 1998). Many other aspects of CNS and PNS myelin, however, differ (for review, see Trapp and Kidd, 2004). Schwann cells 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 P_0 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 (Macklin et al., 1987; Milner et al., 1985). Since the extracellular domain of P_0 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

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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 (Kirschner et al., 1989; Saavedra et al., 1989; Waehneldt, 1990; Waehneldt et al., 1986; Yoshida and Colman, 1996). Primitive fish CNS myelin contained both P_0 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 et al., 1987; Nave et al., 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 P_0 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 P_0. In reptiles/aves, the function of PLP became established, allowing the silent dropout of P_0 from CNS myelin (Waehneldt, 1990; Yoshida and Colman, 1996). From reptiles/aves, PLP was the major protein of CNS myelin, while P_0 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 (Hudson, 2004; Kirschner et al., 2004; Kurihara et al., 1997).

An interesting question regarding myelin evolution is why CNS myelin, but not PNS myelin, underwent the molecular switch from P_0 to PLP. We previously investigated the possible benefits of the P_0 to PLP shift in CNS myelin by reversing the process in mice (Yin et al., 2006). Specifically we generated mice that expressed P_0 instead of PLP in CNS myelin (P_0-CNS mice). When PLP was replaced by P_0 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, P_0-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 with wild type (WT) or PLP null mice (Yin et al., 2006, 2008). Mice with equal amounts of P_0 and PLP in CNS myelin had normal lifespans and no axonal degeneration.

The data summarized above support the hypothesis that the P_0 to PLP switch during myelin evolution provided a vital neuroprotective function to CNS myelin. In mouse CNS myelin, P_0 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 P_0 protein in PNS myelin? To address this question we generated mice whose Schwann cells expressed high levels of PLP under the control of the P_0 promoter in the presence or absence of native P_0 protein. In the presence of P_0 protein, PLP can be incorporated into compact PNS myelin that has the periodicity of CNS myelin. In the absence of P_0 protein, PLP cannot support normal Schwann cell myelination or survival of mice beyond 3–6 months of age. PLP therefore cannot replace P_0 protein as the major structural protein of PNS myelin.

Materials and Methods

PLP-P_0 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 mP_0TOT transgene, containing 6 kb of proximal promoter and all exons and introns (Feltri et al., 1999). The start site of translation for P_0 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/P_0 mice) and they displayed no obvious behavior abnormalities. P_0-null mice (Martini et al., 1995) were crossed with homozygous mice carrying the mP_0TOTA-PLP transgene. These mice were interbred for three generations to obtain mice that were P_0-null and homozygous for the mP_0TOTA-PLP transgene (PLP-PNS mice), as determined by genomic DNA analysis and outbreeding. Thus several mouse lines were used for this study: WT, P_0-null, PLP/P_0, PLP/$P_0^{+/-}$ (PLP transgene homozygous and P_0 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 *P_0*gene, respectively.

Primers for the mP_0TOTA-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 CC A T C A T G T T C T T G A G G-3′. Primers for the P_0 gene used were 5′-T C A G T T C C T T G T C CCCC 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/P_0, PLP-PNS, and P_0-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 P_0 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/P_0, PLP/$P_0^{+/-}$, 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/P_0, PLP/$P_0^{+/-}$, 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. Three-month-old sciatic nerves were cut at 10 μm in a cryostat (Leica Microsystems, Exton, PA) and cross sections were double-immunostained for the myelin proteins PLP (AA3, gift from Dr. Wendy Macklin) and P_0 (Trapp et al., 1981). Secondary antibodies were conjugated with Alex 488 or Alex 594 (Life Technologies, Grand Island, NY). Confocal images were collected using a Leica SP5 confocal microscope (Leica Microsystems).

Transmission Electron Microscopy (EM)

WT, P_0-null, PLP/P_0, PLP/$P_0^{+/-}$, 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; 1 μm sections was 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 (Anderson et al., 1997; Puckett et al., 1987), 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 (Fig. 2A,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 on 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

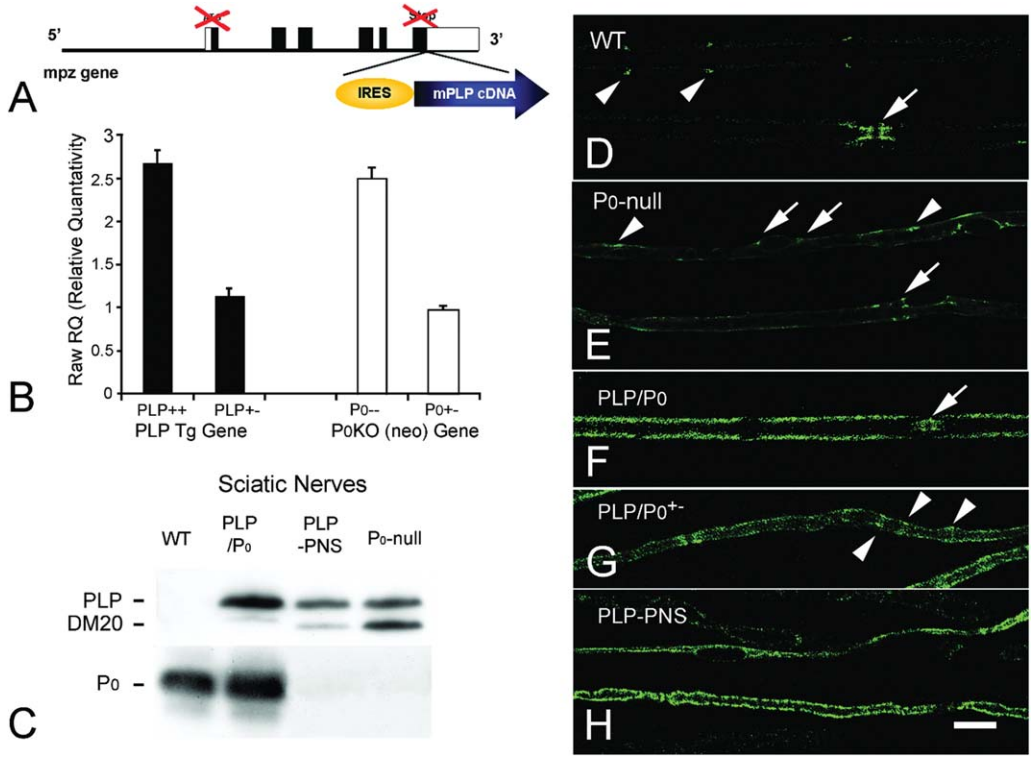

FIGURE 1: Diagram of the mP$_0$TOTA-PLP promoter and analysis of PLP-PNS transgenic founders. (A) In mP$_0$TOTA, the P$_0$ start site of translation is ablated (Red X, left) and here the P$_0$ stop codon was substituted with a restriction site (Red X, right). An IRES-mPLPcDNA fusion was inserted into the restriction site to make the mP$_0$TOTA-PLP transgene. (B) Identification of PLP-PNS homozygous mice using RT-PCR. PLP-PNS mice had two copies of both the mP$_0$TOTA-PLP transgene and the P$_0$ knock-out (NEO) gene. (C) Western blot analysis of PLP and P$_0$ protein. PLP and DM20 were detected in PLP/P$_0$, PLP-PNS, and P$_0$-null sciatic nerves and were not detected in WT sciatic nerves. P$_0$ was detected in WT and PLP/P$_0$ sciatic nerves, but not in PLP-PNS and P$_0$-null sciatic nerves. (D–H) Confocal images of PLP immunofluorescent staining in teased fibers of 1-month-old WT, P$_0$-null, PLP/P$_0$, PLP/P$_0^{+/-}$, 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 P$_0$-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/P$_0$ mice (F), PLP was located in the compact myelin sheath as well as in paranodal loops (F, arrow). In PLP/P$_0^{+/-}$ 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.

nerves that overexpress PLP (PLP/P$_0^{+/-}$), PLP was targeted to compact myelin but the resultant myelin sheaths appeared to be hypomyelinated (Fig. 2E,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 1-μm thick and ultrathin sections. In 1 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 1-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

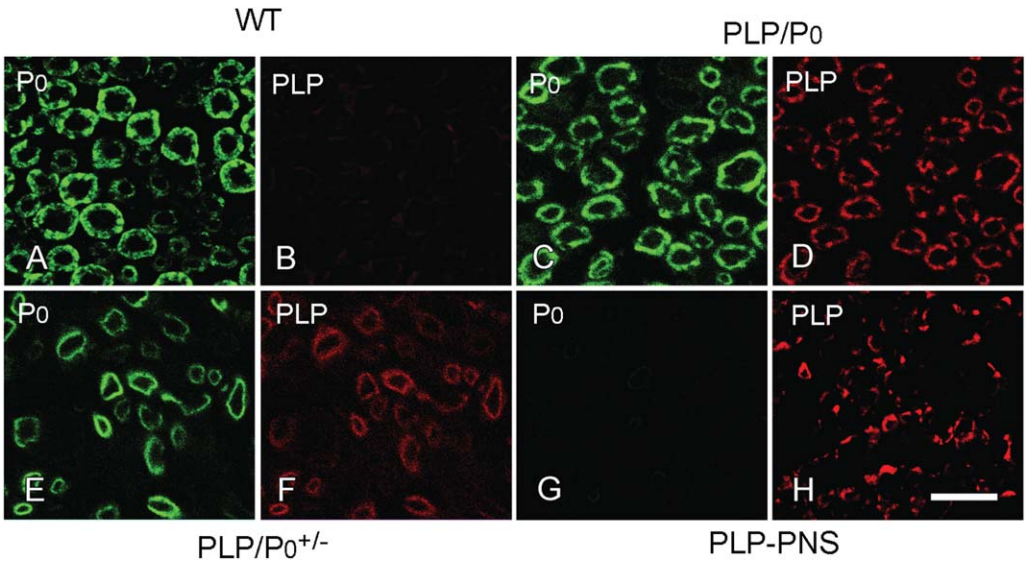

FIGURE 2: Immunofluorescent staining of 3-months old WT (A, B), PLP/P_0 (C, D), PLP/$P_0^{+/-}$ (E, F), and PLP-PNS (G, H) sciatic nerves was performed to determine the expression and location of P_0 (A, C, E and G, green) and overexpressed PLP (B, D, F, and H, red). Expression of P_0 in WT sciatic nerves (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 μm.

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, the 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.

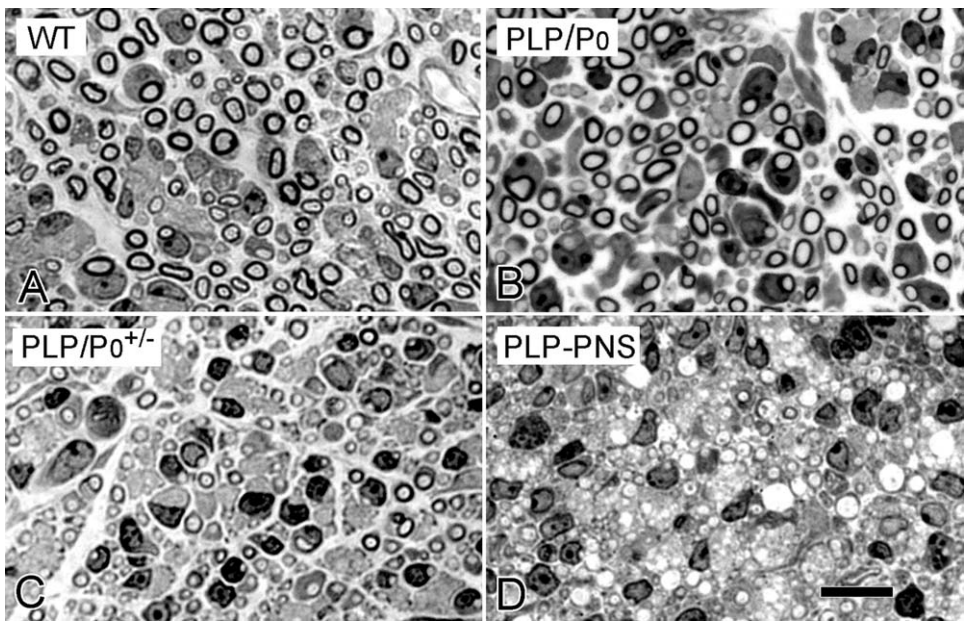

FIGURE 3: A 1-μm thick epon sections from 4-day-old WT (A), PLP/P_0 (B), PLP/$P_0^{+/-}$ (C), and PLP-PNS sciatic nerves. Myelinating Schwann cells formed one-to-one associations with axons and formed compact myelin sheaths in WT (A) and PLP/P_0 (B) sciatic nerves. Myelination of PLP/P_0^{+-} (C) sciatic 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 μm.

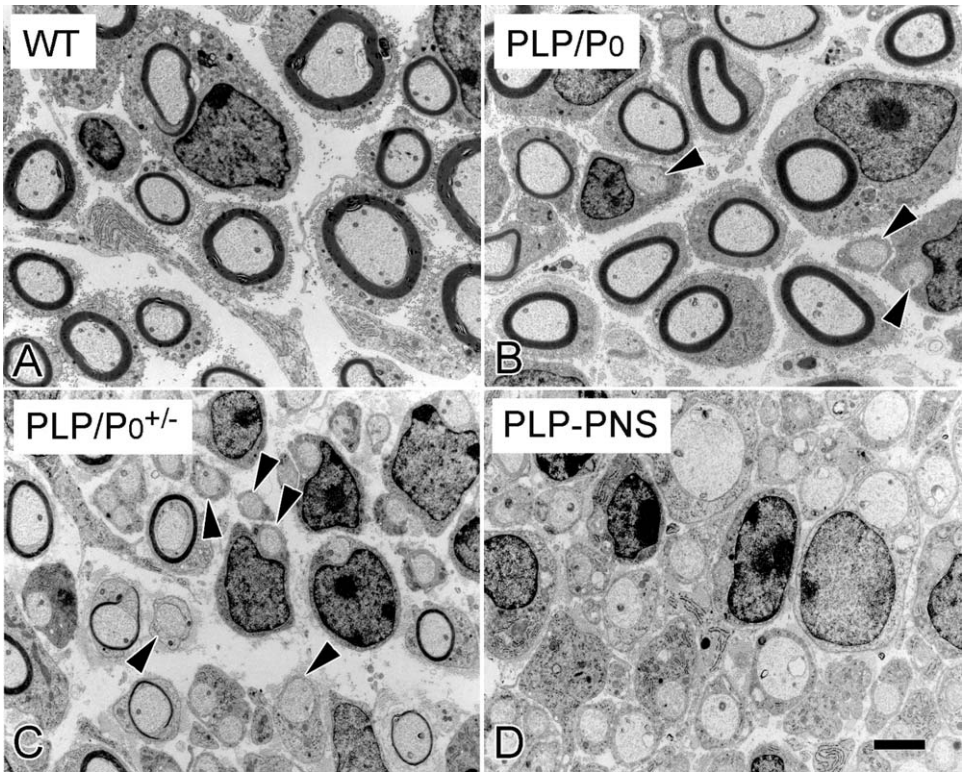

FIGURE 4: Electron micrographs of WT (A), PLP/P_0 (B), PLP/$P_0^{+/-}$ (C), and PLP-PNS (D) sciatic nerves from 4-day-old mice. All Schwann cells attained one-to-one relationships with axons larger than one micron diameter in all four mouse lines. Axons in WT sciatic nerves were appropriately myelinated (A). Some axons in PLP/P_0 sciatic nerves (B, arrowheads) and many axons in PLP/$P_0^{+/-}$ 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 μm.

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 1 μm thick sections (Fig. 5A,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/P_0 (Fig. 6B) sciatic nerves, myelin sheath thickness appeared to be thinner in the PLP/P_0 nerves. Thinner myelin was a striking feature in electron micrographs of PLP/$P_0^{+/-}$ 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/P_0 were similar (0.72, not significantly different). In contrast, PLP/$P_0^{+/-}$ were hypomyelinated with an average *g* ratio of 0.84 ($P < 0.005$ different from WT and PLP/P_0, *t* test with Bonferroni's multiple comparisons test). In the

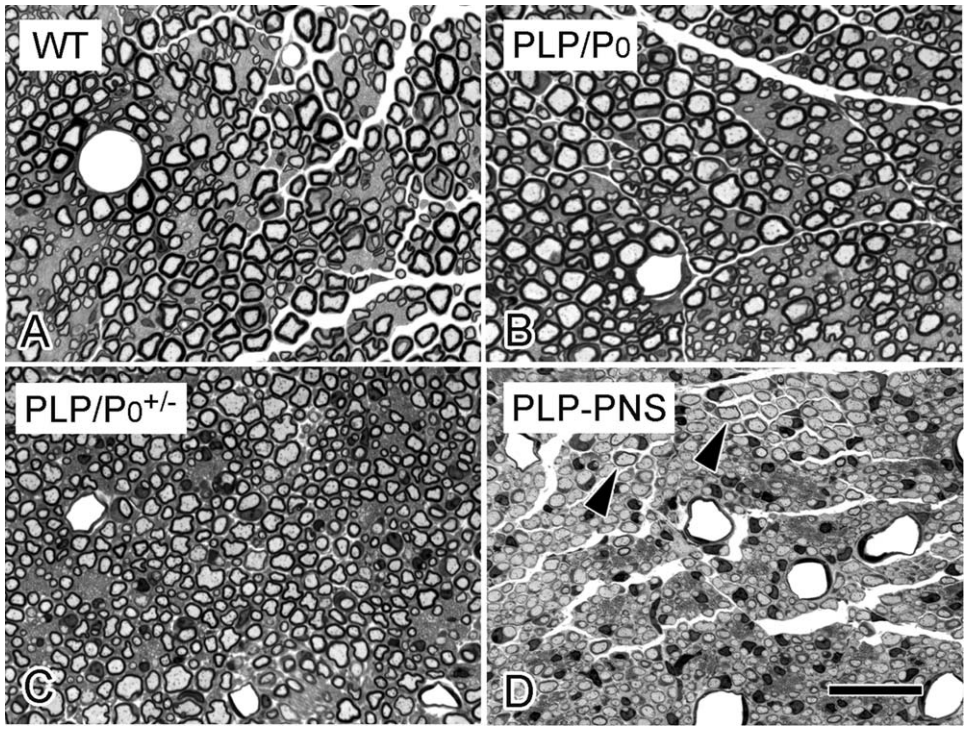

FIGURE 5: A 1-μm thick epon sections from 10-week-old WT (A), PLP/P_0 (B), PLP/$P_0^{+/-}$ (C), and PLP-PNS (D) sciatic nerves. The thickness of compact myelin of WT (A) and PLP/P_0 (B) sciatic nerves was identical, but there was an obvious hypomyelination in PLP/$P_0^{+/-}$ (C) sciatic nerves. Compact myelin was rare in PLP-PNS (D, arrowheads) sciatic nerves. Scale bar = 25 μm.

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/P_0, *t* test with Bonferroni's multiple comparisons test). These data indicate that PLP does not affect myelin thickness when P_0 is present, but does not compensate fully for loss of P_0 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 P_0 and PLP. The extracellular domain of P_0 is larger and more heavily charged that 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). P_0 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 P_0 protein. When P_0 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 P_0 in trans and/or in cis. When PLP was removed from P_0-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 P_0 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 P_0 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

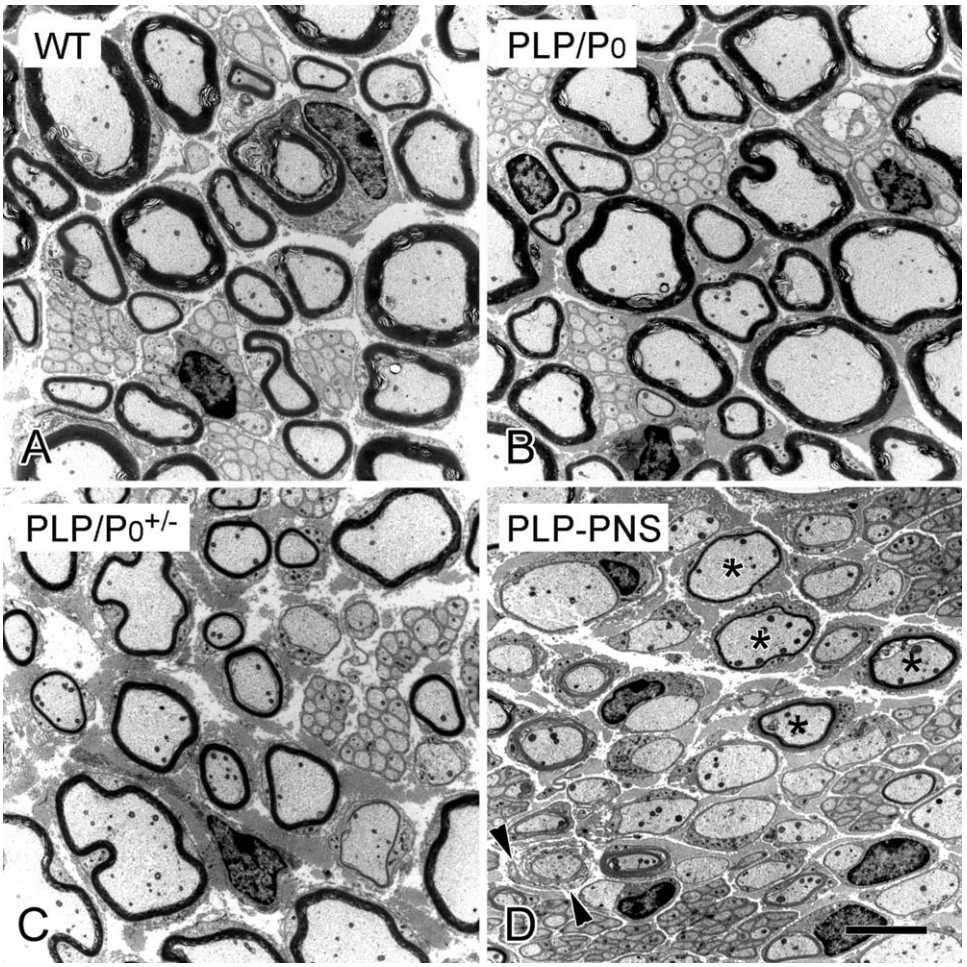

FIGURE 6: Electron micrographs of 10-week-old WT (A), PLP/P_0 (B), PLP/$P_0^{+/-}$ (C), and PLP-PNS (D) sciatic nerves. The PLP/$P_0^{+/-}$ sciatic nerves (C) had thinner myelin, compared to the myelin sheath from WT (A) and PLP/P_0 (B) sciatic nerves. A majority of the large axons in PLP-PNS (D) sciatic nerves were not myelinated; occasionally axons were surrounded by thin myelin sheaths (D, asterisks) or redundant basil lamina (D, arrowheads). Scale bar = 4 μm.

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. A 3-month-old PLP-PNS mice were did not stay on the treadmill for more than 5 s. 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 five 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

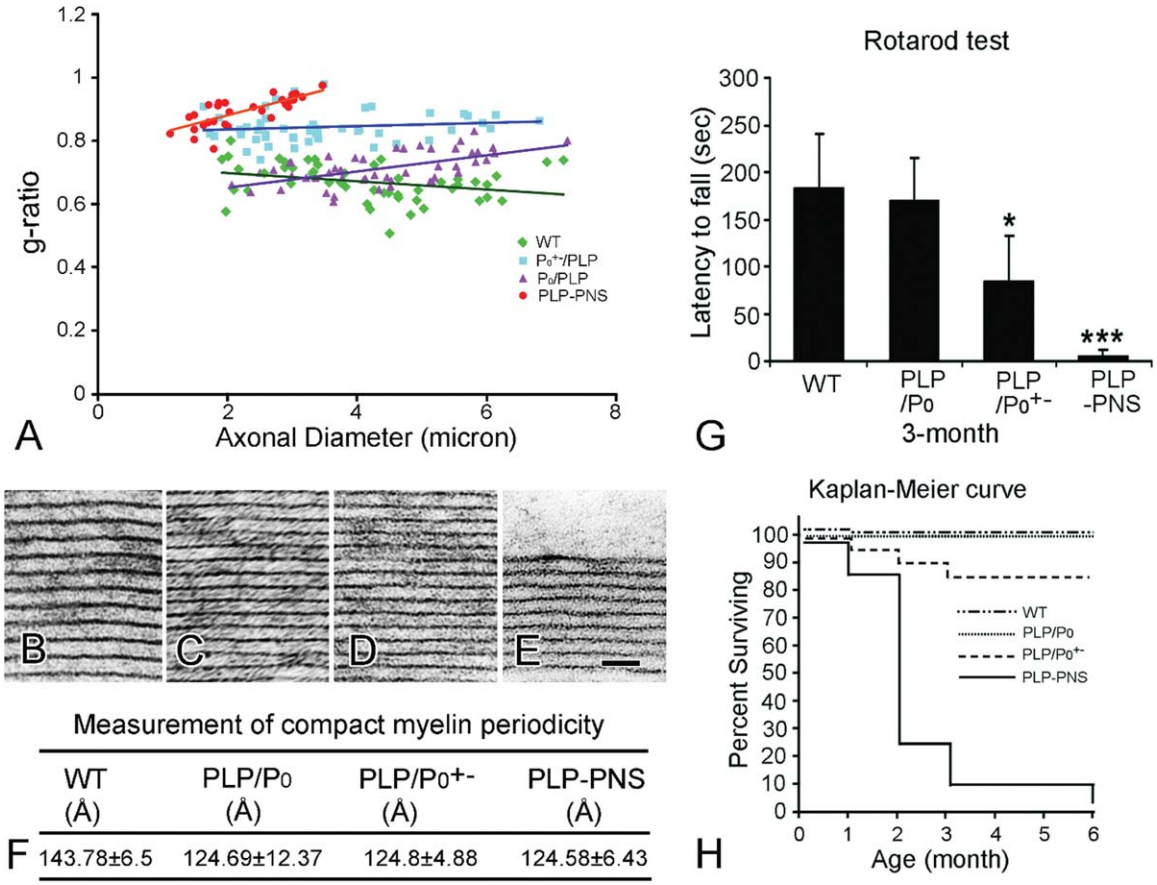

FIGURE 7: (A) Myelinated fiber *g* ratios compared with axonal diameters for sciatic nerve axons that express PLP in the presence or absence of P_0 ($n = 50$ axons pooled from 3 to 4 animals per group). WT and mice with PLP and normal P_0 expression had consistent *g* ratios of 0.6–0.7, but reduction 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's 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 s. (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).

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 P_0 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 P_0 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; Nave, 2010; Nave and Trapp, 2008; Sporkel et al., 2002; Yin et al., 2006). 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 (Dhaunchak et al., 2011; Hodes et al., 2000). 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 (Dhaunchak et al., 2011; Gow and Lazzarini, 1996). Synthesis and transport of PLP in rough ER and Golgi are significantly affected by specific gene mutations and mutations that increased PLP dosage (Gow and Lazzarini, 1996; Karim et al., 2007, 2010; Nave and Boespflug-Tanguy, 1996). 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). P_0 cannot rescue the axonal degeneration caused by loss of PLP from CNS myelin (Yin et al., 2006). In contrast, P_0 increases the axonal degeneration caused by loss of PLP. P_0-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.

P_0 and PLP Gene Dosage in PNS Myelin

Schwann cells are also affected by altered P_0 and PLP gene dosages. P_0 overexpression in mice dramatically inhibits PNS myelination and causes retraction of axonal terminals from neuromuscular junctions (Yin et al., 2000, 2004). These mice have severe neurological disability and shortened life spans. To date, P_0 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). P_0-null mutations cause the peripheral neuropathy Charcot-Marie-Tooth (CMT) disease in humans (Martini, 1999). P_0-null mice have also been generated (Giese et al., 1992). P_0-null Schwann cells spirally wrap axons with noncompacted membranes. Phenotypes in P_0-null humans can be moderate to severe and loss of P_0 in mice causes minimal neurological disability (Martini, 1999; Martini et al., 1995). Similar to PLP, P_0 gene duplication in mice has a greater negative impact on peripheral nerves than P_0 elimination (Wrabetz et al., 2000; Yin et al., 2000). In contrast to oligodendrocytes where P_0 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; Inouye et al., 1999; Shapiro et al., 1996; 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 (Heath et al., 1991; Rasband et al., 2005; Scherer et al., 1995; Trapp and Quarles, 1982; Trapp et al., 1989) 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,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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