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Expression of a Myelin Proteolipid Protein (*Plp*)-*lacZ* Transgene is Reduced in both the CNS and PNS of *Plp^{jp}* Mice

Patricia A. Wight

Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

Cynthia S. Duchala, H. Elizabeth Shick, Tatyana I. Gudz, and Wendy B. Macklin

Department of Neurosciences, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA e-mail: mackliw@ccf.org

Abstract

Jimpy (*Plp^{jp}*) is an X-linked recessive mutation in mice that causes CNS dysmyelination and early death in affected males. It results from a point mutation in the acceptor splice site of myelin proteolipid protein (*Plp*) exon 5, producing transcripts that are missing exon 5, with a concomitant shift in the downstream reading frame. Expression of the mutant PLP product in *Plp^{jp}* males leads to hypomyelination and oligodendrocyte death. Expression of our *Plp-lacZ* fusion gene, PLP(+)*Z*, in transgenic mice is an excellent readout for endogenous *Plp* transcriptional activity. The current studies assess expression of the PLP(+)*Z* transgene in the *Plp^{jp}* background. These studies demonstrate that expression of the transgene is decreased in both the central and peripheral nervous systems of affected *Plp^{jp}* males. Thus, expression of mutated PLP protein downregulates *Plp* gene activity both in oligodendrocytes, which eventually die, and in Schwann cells, which are apparently unaffected in *Plp^{jp}* mice.

Keywords

Jimpy mouse; Gene regulation; Myelin proteolipid protein; Oligodendrocyte differentiation; Schwann cell; Transgenic mice

Introduction

Dysmyelinating disorders such as Pelizaeus–Merzbacher disease in humans or the *jimpy* *Plp* mutation in mice are characterized by dramatically altered oligodendrocyte differentiation and brain function, which result from mutations in the myelin proteolipid protein (*Plp*) gene [reviewed in 1,2]. A point mutation in the acceptor splice site of exon 5 in the *jimpy* *Plp* gene (*Plp^{jp}*) results in deletion of this exon from *Plp/Dm20* transcripts and a concomitant frameshift and truncation of the C-terminal amino acids [3,4]. In very young animals, the transcription rate of the *Plp^{jp}* gene is similar to that in mice containing the wild-type gene. However, as development proceeds, *Plp* gene transcription in the mutant does not increase as it does in wild-type mice [5]. In addition, the *Plp^{jp}* mutation exhibits pleiotropic effects, for example, reducing

Correspondence to: Wendy B. Macklin.

Patricia A. Wight and Cynthia S. Duchala contributed equally to this study.

Present Address: C. S. Duchala, Centocor, Inc., 145 King of Prussia Road, Radnor, PA 19087, USA

Present Address: T. I. Gudz, Department of Neurosciences, Medical University of South Carolina, 114 Doughty St., Rm 338, Charleston, SC 29425, USA

Special issue dedicated to Anthony Campagnoni.

the transcription rate of the myelin basic protein (*Mbp*) gene, which is expressed later in the oligodendrocyte differentiation pathway. Other point mutations in the *Plp* gene, resulting in both conservative and non-conservative amino acid substitutions, have been identified, which generally cause developmental disorders that are similar to that caused by the *Plp^{jp}* mutation [6–8]. On the whole, *Plp* gene mutations are far more devastating than mutations in other genes that cause dysmyelination. Mice and rats with *Plp* mutations generally die within 3–4 weeks after birth, while *shiverer*, *quaking* and *trembler* mice survive to adulthood. Mutant PLP proteins tend to accumulate in the endoplasmic reticulum of oligodendrocyte cell bodies [9], and it is proposed that the defective protein activates the unfolded protein response, culminating in oligodendrocyte cell death [10]. The greater lethality of these mutations, however, likely results from expression of mutated PLP in neurons in the brainstem, which leads to dramatic respiratory depression in response to hypoxia [11].

The current studies were designed to investigate the effects of the *Plp^{jp}* mutation on *Plp* gene expression in both the CNS and PNS, using a transgenic mouse model in which expression of the *lacZ* reporter gene is governed by *Plp* genomic sequences. The PLP(+)*Z* transgene contains the proximal 2.4 kb of 5'-flanking DNA, exon 1, intron 1 and the first 37 bp of exon 2 of the *Plp* gene, and encodes a fusion protein between the first 13 amino acids of PLP and β -galactosidase (β -gal), which is trafficked to the myelin membrane [12]. A series of transgenic animals have subsequently been derived that further corroborate the ability of these *Plp* sequences to promote transgene expression, and to serve as a reliable readout of endogenous *Plp* gene activity [13–18], with inclusion of *Plp* intron 1 sequences being important [19]. In the current studies, the PLP(+)*Z* transgene was crossed into the *Plp^{jp}* background and used to monitor the effects of the *Plp^{jp}* mutation on *Plp* promoter activity via *lacZ* reporter gene expression.

Methods

Animals

The studies were conducted on transgenic mice that express the *Plp-lacZ* fusion gene, PLP(+)*Z* [12]. The transgene encodes a protein containing the first 13 amino acids of PLP fused to β -gal. Male mice, homozygous for the PLP(+)*Z* transgene (line 26H), were bred to non-transgenic *Plp^{jp}* female carriers (heterozygous *Plp^{jp}* females). Thus, all of the resulting progeny were heterozygous for the PLP(+)*Z* transgene. Genomic DNA was isolated from tail biopsies as previously described [12] and the progeny genotyped by PCR for the presence/absence of the *jimpy* mutation (*Plp^{jp}*). PCR primers, derived from sequence flanking the *Plp^{jp}* point mutation in the acceptor splice site of *Plp* exon 5, were: *Plp* intron 4 sense primer [5'-CATGGCCTCTAGCCTTATGAAGTTTAC-3'] and *Plp* exon 5 antisense primer [5'-CCTCAGCTGTTT TGCAGATGGACAG-3']. The PCR product generated was 126-bp in length. Since the *Plp^{jp}* mutation results in loss of a DdeI recognition site, PCR products were digested with DdeI and analyzed by gel electrophoresis. The PCR product amplified from the wildtype *Plp* allele was cleaved by DdeI, while the *Plp^{jp}*-derived PCR product was not cleaved. Thus, affected *Plp^{jp}* males displayed a single band of 126-bp, while wild-type male littermates exhibited two bands migrating as a doublet (approximately 50 bp and 80 bp in size). Heterozygous *Plp^{jp}* females had both the 126-bp band and the shorter doublet.

β -Galactosidase histochemistry

Cells expressing the PLP(+)*Z* transgene were identified in tissue sections from mouse brain using β -gal chromogenic substrates (X-gal or Bluogal) as previously described [12], except that 1.0% glutaraldehyde in PBS (pH 7.3) was the fixative. The Bluogal substrate was used for analysis of cryostat sections at high magnification since the blue reaction product does not diffuse out of cells. Thus, better cell morphology can be assessed with this stain.

Immunohistochemistry

Mice at 21 days of age (P21) were perfused with 4% paraformaldehyde in PBS (pH 7.6) under halothane anesthesia. Tissues were removed, post-fixed for 24 h in 4% paraformaldehyde and cryoprotected in 20% glycerol. Tissue samples were sectioned on a sliding microtome. Sections (30 μ m) were immunostained according to the methods of Bö et al. [20]. Free-floating sections were pretreated in 0.1% OsO₄ for 30 s, rinsed well with PBS, and placed in a solution of 1% H₂O₂ containing 10% Triton X-100 for 30 min. Tissue sections were blocked with 3% normal goat serum in PBS for 30 min and incubated in the following series of solutions: primary antibody diluted in PBS, overnight at 4°C; biotinylated secondary antibodies (Vector Laboratories Inc., Burlingame, CA) diluted 1:2,000 in PBS for 30 min; enzyme (HRP)-linked biotin (1:1,000) and avidin (1:1,000) (Vector Laboratories) in PBS for 1 h; diaminobenzidine (DAB)/H₂O₂ for up to 8 min; 0.1% OsO₄ for 30 s. Sections were mounted in glycerol. For double-label immunofluorescence, fluorescein- and Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch, Laboratories, Inc., West Grove, PA) were used (diluted 1:1,500 in PBS for 1 h) and the sections mounted in Vectashield mounting media (Vector Laboratories). Primary antibodies included: rabbit anti-PLP (M2) [21] at 1:1,000; rabbit anti- β -gal (Cortex Biochem., San Leandro, CA) at 1:1,000; mouse anti-2',3'-cyclic nucleotide-3'-phosphodiesterase (CNP; Sternberger Monoclonals Inc., Lutherville, MD) at 1:1,000; mouse anti-myelin basic protein (MBP, Sternberger Monoclonals Inc.) at 1:5,000 and 1:10,000 for *Plp*^{flp} and wild-type mice, respectively. Primary antibodies used for double-label immunofluorescence were: rabbit anti-PLP (M2) at 1:1000 and mouse anti- β -gal (Roche, Indianapolis, IN) at 1:1,000.

Western blot analysis

Lysates for Western blot analysis comparing proteins from brain and spinal cord were prepared by homogenization of tissues in 4% SDS (1:20 wt:vol). Proteins were fractionated on a 10% polyacrylamide gel (SDS-PAGE) [22] and analyzed using the ECL kit (Amersham, Piscataway, NJ). Blots were incubated with PLP-specific (M2) and β -gal (Cortex) anti-rabbit polyclonal antibodies, overnight at 4°C. The blots were then washed and incubated with secondary antibody at a dilution of 1:5,000 for 2 h at room temperature.

Tissue lysates for Western blot analysis comparing proteins from brain and sciatic nerve were prepared in Lysis Buffer (0.15 M NaCl, 0.05 M Tris, 0.5 mM EDTA, 1% Triton X-100, 0.05% SDS, pH 7.5) supplemented with protease inhibitors (20 g/ml leupeptin, 100 g/ml aprotinin, 2 mM PMSF, 5 mM NEM). After 1 h on ice, samples were centrifuged at 15,000 $\times g$ for 10 min to remove insoluble material. Protein concentration in the supernatants was determined using the bicinchoninic acid method (Sigma, St. Louis, MO). Proteins were separated on a 10% polyacrylamide gel (SDS-PAGE) and transferred to a PVDF membrane. The blots were blocked with 5% non-fat dry milk in TBS-T buffer (10 mM Tris, 150 mM NaCl, 0.2% Tween 20, pH 8.0) overnight at 4°C and subsequently incubated with primary rabbit antibody against PLP, β -gal, MBP (Sternberger Monoclonals) or β -actin (Sigma,) for 1 h at room temperature. After washing, the blots were incubated with secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized using ECL Plus detection reagents (Amersham).

RT-PCR analysis

Total RNA was isolated by the procedure of Chomczynski and Sacchi [23]. Reverse transcription (RT) was performed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). PCR primer pairs were (1) *Plp/Dm20*: sense 5'-CCAAAACTACCAGGACTA-3' and antisense 5'-TCAGAGACGCGACTACGGT-3' generating products of 459-bp (*Plp*) and 354-bp (*Dm20*); (2) *lacZ*: sense 5'-GTGCGGATTGAAAATGGTCT-3' and antisense 5'-CACCGCATCAGCAAGTGTAT-3'

generating a 1,500-bp product; (3) *'-tubulin: sense 5'-CGTGAGTGCATCTCCATCCAT-3'* and antisense *5'-GCCCTCACCCACATACCAGTG-3'* generating a 1,233-bp product.

Results

PLP(+)Z transgene expression in the developing CNS in *Plp^{ip}* mice

Expression of the PLP(+)Z transgene was evaluated in the *Plp* dysmyelinating mouse mutant, *Plp^{ip}*. The transgene contains *Plp* genomic DNA extending from the proximal 2.4 kb of 5'-flanking DNA, downstream to the first 37 bp of exon 2, which were used to drive expression of a *lacZ* reporter gene cassette. Thus the transgene contains the *Plp* promoter as well as a tissue-specific enhancer located in *Plp* intron 1 DNA [24,25]. Expression of the PLP(+)Z transgene was evaluated in affected *Plp^{ip}* males, which express a mutated *Plp* gene (*Plp^{ip}*), and compared to that produced in wild-type male littermates containing the normal *Plp* gene. In some cases, transgene expression was also measured in heterozygous *Plp^{ip}* female carriers (*Plp^{ip/+}*). As shown in Fig. 1, expression of the transgene, as determined by X-gal staining, was markedly reduced in the brains of *Plp^{ip}* males at all ages, when compared to wild-type male littermates. In contrast, heterozygous *Plp^{ip}* females demonstrated only a slight reduction in β -gal activity when compared to wild-type male littermates, with the anterior cortical regions being affected the most. Throughout the peak period of myelinogenesis, transgene expression in the heterozygous *Plp^{ip}* females lagged behind that of wild-type littermates. These data are consistent with the known early reduction in *Plp* gene expression in *Plp^{ip}* female carriers [26–28], suggesting that the PLP(+)Z transgene is regulated, similarly, in the heterozygous *Plp^{ip}* background. Thus, the early reduction in *Plp* gene expression in *Plp^{ip}* females is likely caused by a reduction in *Plp* gene transcription.

In order to further assess the reduction of transgene expression in the *Plp^{ip}* background, Western blot analysis was performed with brain and spinal cord lysates. As shown in Fig. 2, the levels of PLP and β -gal were greatly reduced in the CNS of *Plp^{ip}* males, compared to those present in wild-type males and *Plp^{ip}* female carriers. Heterozygous *Plp^{ip}* females demonstrated diminished amounts of both proteins at P7 (particularly in the spinal cord), but by P14, the levels of PLP and β -gal were comparable to those in wild-type males. *Plp^{ip}* males expressed low, but detectable, amounts of β -gal protein, however PLP was not detected by Western blot analysis, presumably due to altered folding of the mutant protein and likely aggregation and/or degradation.

To better examine the morphology of cells expressing the transgene at high magnification, brain sections were stained with the β -gal substrate, Bluogal. Bluogal stained cells (oligodendrocytes) in the wild-type background had a stellate appearance at P5 (Fig. 3A) and exhibited large β -gal-expressing tracts, with little staining in cell bodies by P12 (Fig. 3B). Yet even at P21, β -gal expressing cells in the *Plp^{ip}* male were morphologically similar to immature oligodendrocytes present in wild-type littermates at P5, staining pre-dominantly the multiple short processes of mutant cells, with little staining in cell bodies (Fig. 3C).

Immunocytochemical analysis confirmed a decrease in PLP(+)Z expression in the *Plp^{ip}* background. When compared to age-matched wild-type males, P21 *Plp^{ip}* males exhibited a dramatic decrease in both PLP and β -gal immunoreactivity in all regions examined, and MBP and CNP immunostaining was also decreased in affected males, although not as severely as for PLP and β -gal (data not shown). As seen at high magnification, the little PLP remaining was localized almost exclusively in the cell body of *Plp^{ip}* oligodendrocytes (Fig. 4A), whereas in mature wild-type oligodendrocytes, localization of PLP to the cell body was rarely seen (data not shown). In sharp contrast, the β -gal transgene product in *Plp^{ip}* mice was found in short oligodendrocyte processes and fibers, and only occasionally in cell bodies (Fig. 4B). CNP staining was observed in both the cell body and short fibers of *Plp^{ip}* oligodendrocytes (Fig.

4C). Even though less CNP was expressed in oligodendrocytes from the *Plp^{jp}* mutant, the distribution of CNP within the cell was similar to that in wild-type animals (data not shown).

Double labeling for PLP and β -gal in *Plp^{jp}* oligodendrocytes demonstrated significant partitioning of the proteins (Fig. 5). The PLP protein appeared to be restricted to the *Plp^{jp}* oligodendrocyte cell body, while β -gal was detected predominantly along the processes and short fibers, with occasional cells having stain in the cell body. As seen in other studies [9, 10], it is likely that the mutant PLP protein is accumulating in the cell body. In contrast, the β -gal fusion protein, which contains the first 13 amino acids of PLP at its N-terminus and is targeted to compact myelin in wild-type mice [12], does not remain in the cell body and gets properly trafficked to the plasma membrane.

PLP(+)*Z* transgene expression in the PNS in *Plp^{jp}* mice

Transgene expression was also investigated in the PNS of *Plp^{jp}* and wild-type males. As shown in Fig. 6, the amounts of both PLP and β -gal in sciatic nerve were decreased in *Plp^{jp}* sciatic nerve, similar to that in brain. However unlike the CNS, the *Plp^{jp}* mutation did not display pleiotropic effects in the PNS, as evidenced by equivalent amounts of MBP in sciatic nerve from *Plp^{jp}* and wild-type males (Fig. 6).

Plp/Dm20 and *lacZ* mRNA levels in the CNS and PNS were also analyzed semi-quantitatively by RT-PCR, which permits analysis of both *Plp* and *Dm20* mRNAs (Fig. 7). In brain, the amounts of *Plp/Dm20* mRNA were higher than *lacZ* in both wild-type and *Plp^{jp}* males. Interestingly, *lacZ* mRNA levels in sciatic nerve were higher than either *Plp* or *Dm20* mRNA in both wild-type and *Plp^{jp}* males at P21. This may partially reflect the presence of two pools of alternative splice products (*Plp/Dm20*), while there is only one for PLP(+)*Z* transgene mRNA, although this does not impact their absolute expression in brain. Brain *Plp* mRNA levels were approximately 20-fold less in *Plp^{jp}* compared to wild-type males, e.g., comparable amounts of *Plp*-specific RT-PCR products were generated using cDNA reaction mixture inputs of 50 ng for wild-type and 1 μ g for *Plp^{jp}* (Fig. 7). The amount of *lacZ* mRNA in *Plp^{jp}* brain was decreased to an even greater extent. By contrast, in sciatic nerve, *Plp/Dm20* mRNA levels were decreased less than 4-fold in *Plp^{jp}* mice compared to wild-type (with *Dm20* mRNA as the major constituent), while PLP(+)*Z* transgene expression decreased approximately 20-fold.

Discussion

Downregulation of the *Plp* gene and the PLP(+)*Z* transgene in oligodendrocytes

The current studies demonstrate that *Plp* genomic sequences contained within PLP(+)*Z*, which regulate expression of the PLP(+)*Z* transgene comparably to the endogenous *Plp* gene, downregulate expression of the *Plp* gene in both the CNS and the PNS in *Plp^{jp}* mice, relative to wild-type mice. This is to be expected in the CNS. *Plp^{jp}* mice, which express a mutant form of PLP due to a point mutation in the acceptor splice site of exon 5, have significant alterations in the oligodendrocyte cell cycle, where a high mitotic rate is observed accompanied by significant cell death [29–32]. Campagnoni's group showed two decades ago that PLP protein and mRNA are dramatically downregulated in *Plp^{jp}* mice [33–35]. Additionally, it has been known for some time that both *Plp* and *Mbp* transcriptional activity is decreased in the brains of affected males [5,36]. While the obvious assumption has been that the mutant PLP protein is responsible for this change, it is possible that the point mutation itself could have had a *cis* effect on *Plp* gene activity. The current studies conclusively disprove this, since expression of the PLP(+)*Z* transgene, which does not contain the *Plp^{jp}* point mutation, is likewise decreased in the *Plp^{jp}* CNS relative to wild-type. Perhaps the reduction in transgene expression simply reflects the fact that *Plp^{jp}* oligodendrocytes are compromised, triggering higher levels of cell death, and thus reducing their overall number in affected animals. However, significant

numbers of GC+ oligodendrocytes are present in these animals [35,37], and transcription of glycerol phosphate dehydrogenase, an early marker of oligodendrocyte differentiation, is unaffected [5]. In contrast, genes such as *Plp*, *Mbp*, and *Mog* (myelin/oligodendrocyte glycoprotein), whose expression is upregulated late in the oligodendrocyte differentiation program, are expressed at lower levels in the *Plp^{jp}* CNS [5,36,38], much like the PLP(+)Z transgene. Thus it appears that it is the ability of the cells to fully differentiate, and hence express high levels of gene products found in mature oligodendrocytes that is compromised in the *Plp^{jp}* mutant. This concept is consistent with the observation that *Plp^{jp}* oligodendrocytes expressing the PLP(+)Z transgene were morphologically immature (Fig. 3). Previous studies [5] have shown that *Plp* gene transcription, in brain, is reduced in affected mice, with *Plp^{jp}* mice at P25 having similar levels of *Plp* transcription as wild-type mice at P7. Other studies [38] have shown that the pattern of myelin gene expression in *Plp^{jp}* oligodendrocytes is consistent with that found in immature wild-type cells. Thus, consistent with much data in the literature, expression of the PLP(+)Z transgene is reduced in the *Plp^{jp}* CNS due to the immature status of oligodendrocytes in these animals.

Downregulation of the *Plp* gene and the PLP(+)Z transgene in Schwann cells

Few studies have assessed *Plp* gene regulation in the PNS. In contrast to the CNS, the *Plp^{jp}* mutation in the PNS does not cause abnormal Schwann cell development or dysmyelination [39,40]. However, expression of the PLP(+)Z transgene in the PNS, as well as the endogenous *Plp* gene, were decreased in *Plp^{jp}* sciatic nerve relative to wild-type, while MBP levels were unaffected (Fig. 6). Since there are no overt pleiotropic effects of the *Plp^{jp}* mutation in the PNS, the decreased levels of β -gal and PLP in the *Plp^{jp}* PNS must be due to (*Plp*) regulatory sequences common to both the transgene and the endogenous gene, or to the shared nucleotide or amino acid sequence (13 N-terminal residues of PLP) between the mRNAs and proteins they encode. On the other hand, the mutated *Plp^{jp}* PLP protein could conceivably participate in a negative feedback loop, decreasing its own expression, as well as that of the transgene. Since the PLP/DM20 C-terminus portion is out of frame in the mutant, it is possible that a nuclear localization signal (NLS) might have inadvertently been created. However, neither *Plp^{jp}* PLP nor wild-type PLP contains an NLS when analyzed with a program that searches for NLS (<http://cubic.bioc.columbia.edu/predictNLS>) [41,42]. Furthermore Anderson et al. [40] demonstrated that expression of the *Plp^{jp}* PLP protein in Schwann cells did not prevent expression of wild type PLP protein. Thus, the decrease in the transcriptional activity of both the transgene and the endogenous *Plp* gene in Schwann cells is unlikely to be directly regulated by the mutated protein.

It is striking that there was a more precipitous drop in transgene mRNA than *Plp/Dm20* mRNA gene in *Plp^{jp}* mice, relative to wild-type. This was true in both the CNS and PNS, and may result from greater stability of *Plp/Dm20* transcripts than transgene mRNA. Previous studies indicate that the *Plp*-specific sequence, which is absent from *Dm20* mRNA, and the 3'-untranslated region of the *Plp/Dm20* mRNA are important for stability of *Plp* gene transcripts in glial cells [15,43,44]. Since neither of these sequences is present in the transgene mRNA, amounts of the PLP(+)Z transcript are a true reflection of the transcriptional activity. Thus, the greater loss of transgene mRNA than *Plp/Dm20* mRNAs in both the CNS and PNS in *Plp^{jp}* mice relative to wild type is probably due, entirely, to a decrease in PLP(+)Z transcription initiation.

The downregulation of *Plp/Dm20* mRNAs was less in Schwann cells than in oligodendrocytes. The stabilization elements that have been identified in the *Plp* mRNA particularly stabilize this mRNA in myelinating Schwann cells [43]. Thus, it is possible that since there is no loss of myelin in *Plp^{jp}* peripheral nerve, the stabilization signals actively maintain *Plp/Dm20* mRNAs.

Since, as noted above, those stabilization elements are not present in the transgene mRNA, the decrease in PLP(+)Z mRNA levels would solely reflect a decrease in transcriptional activity.

It is unclear whether the reduction in expression of the transgene and the endogenous *Plp* gene in *Plp^{ip}* mice results from expression of mutated PLP or DM20, or both. Unfortunately the only antibody available to detect DM20 protein by Western blot analysis recognizes a C-terminal epitope, which is altered in *Plp^{ip}* PLP/DM20 proteins. Thus, only the mutant PLP protein in *Plp^{ip}* mice could be examined due to the existence of PLP-specific antibodies [21]. Although the amounts, if any, of mutant DM20 in *Plp^{ip}* sciatic nerve could not be determined, the PCR data (Fig. 7B) demonstrate higher amounts of *Dm20* mRNA than *Plp* mRNA in both wild-type and *Plp^{ip}* sciatic nerve. Thus, it is likely that the *Plp^{ip}* DM20 protein also accumulates in Schwann cells of affected mice.

Intracellular translocation of PLP and β -galactosidase in *Plp^{ip}* oligodendrocytes

Interestingly, the transgene protein and other myelin proteins moved out into the processes of *Plp^{ip}* oligodendrocytes, while the *Plp^{ip}* PLP protein remained in the cell body (Figs. 4 and 5). PLP and DM20 proteins are processed through the endoplasmic reticulum (ER) and Golgi membranes. Thus, as reported in other studies, the mutant proteins encoded by the *Plp^{ip}* gene likely cannot fold correctly and are prevented from moving further through the intracellular transport pathway [9,45]. As mentioned earlier, the PLP(+)Z transgene encodes a protein that contains the 13 N-terminal amino acids of PLP/DM20. We have shown that the transgene fusion protein is trafficked to compact CNS myelin, presumably by these PLP/DM20 N-terminal residues [12]. Therefore, retention of *Plp^{ip}* PLP/DM20 in the ER is a dominant effect, likely mediated by amino acids other than those at the N-terminus, since the transgene protein containing those amino acids was able to move through the ER and Golgi and out into the processes of *Plp^{ip}* oligodendrocytes. Thus the movement of PLP/DM20 into the myelin membrane appears to be controlled substantially differently from that of other membrane proteins such as the acetylcholine receptor, in which N-terminal sequences are essential for the initial formation of a protein complex that then can be sorted to the appropriate membrane [46].

In summary, results presented here suggest that expression of the PLP(+)Z transgene is an excellent prognosticator of *Plp* promoter activity, even in *Plp^{ip}* mice. While expression of the transgene was decreased in both the CNS and PNS of *Plp^{ip}* mice relative to that in wild-type littermates, the decrease in Schwann cells occurred in the absence of any overt pleiotropic effects. MBP levels in the PNS were unaffected, unlike the CNS, where the levels dropped in *Plp^{ip}* mice. Thus, expression of *Plp^{ip}* PLP/DM20 protein must signal indirectly and feedback to control *Plp* transcriptional activity, decreasing expression of the *Plp^{ip}* gene as well as the transgene in the PNS, without affecting the expression of other myelin genes. Whether a similar signaling pathway exists in the CNS is obscured by the death of mature oligodendrocytes and the failure of immature oligodendrocytes to fully mature in the *Plp^{ip}* mutant, but may well also be responsible for their decreased expression in the *Plp^{ip}* CNS. The mechanisms mediating the feedback downregulation remain to be determined.

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Fig 1. PLP(+)Z transgene expression in brain. Sagittal sections from brains of transgenic mice at postnatal ages 7 days (**A, B, C**), 14 days (**D, E, F**), and 21 days (**G, H, I**) were stained with X-gal to localize β -gal activity. Staining was substantially reduced in *Plp^{ip}* males (**B, E, H**) compared to wild-type male littermates (**A, D, G**) or heterozygous *Plp^{ip}* females (**C, F, I**). Bar: 0.5 cm

Fig 2.

Western blot analysis of β -gal and PLP expression in the developing brain and spinal cord of PLP(+)*Z* transgenic mice. Proteins were isolated from wild-type (lanes 1–3) and *Plp^{ip}* (lanes 4–6) males and heterozygous *Plp^{ip}* females (lanes 7–9) at P7 (lanes 1, 4, 7), P14 (lanes 2, 5, 8), and P21 (lanes 3, 6, 9) of age. Twenty micrograms of total protein was loaded per lane. Blots were incubated with anti-PLP-specific M2 (1:2000) or anti- β -gal (1:1,000) antibodies

Fig 3.
Bluo-gal staining for β -gal activity in brains of PLP(+)Z transgenic mice. Cortical tissue was examined from male wild-type mice at P5 (**A**) and P12 (**B**) and a *Plp^{ip}* mouse at P21 (**C**). Bar: 10 μ m

Fig 4. Distribution of PLP (**A**), β -gal (**B**) and CNP (**C**) in cells from the brain of a *Plp^{ip}/PLP(+)*Z transgenic male, at P21. Bar: 10 μ m

Fig 5.
Confocal image of immunofluorescent colocalization of PLP (Fluorescein) and β -gal (Texas Red) in cells from the brain of a *Plp^{flp}* male, PLP(+)/Z transgenic mouse, at P21. Bar: 3.5 μ m

Fig 6.

Western blot analysis of proteins in brain and sciatic nerve from PLP(+)Z transgenic mice at P21 of age. **A:** Proteins isolated from brain (lanes 1, 2) or sciatic nerve (lanes 3, 4) of wild-type (lanes 1, 3) and *Plp^{ip}* (lanes 2, 4) males were analyzed for levels of β -gal and PLP. Significantly different amounts of protein from brain (7 μ g) and sciatic nerve (100 μ g) were loaded for analysis on the same blot. The blot was analyzed first for PLP using the M2 anti-PLP-specific antibody (1:500), then stripped and incubated with an antibody against β -gal (1:1,000). [The apparent increase in β -gal expression in normal sciatic nerve relative to normal brain simply reflects the significant increase in the amount of protein analyzed.] **B:** Proteins (30 μ g) isolated from brain (lanes 1, 2) or sciatic nerve (lanes 3, 4) of wild-type (lanes 1, 3) and *Plp^{ip}* (lanes 2, 4) males were analyzed for MBP and β -actin. The blot was analyzed first for MBP using an anti-MBP antibody (1:500), then stripped and incubated with an anti- β -actin (1:250) primary antibody.

Fig 7.

Relative levels of *Plp/Dm20* and *lacZ* mRNA in PLP(+)Z transgenic mice by RT-PCR analysis. RNA isolated from whole brain or sciatic nerve of wild-type and *Plp^{jp}* males at P21 was used for first strand cDNA synthesis. PCR reactions were performed with varying amounts of the cDNA mixture and primer pairs for *Plp/Dm20*, *lacZ* and α -tubulin (data not shown). **A:** RT-PCR results with whole brain from wild-type and *Plp^{jp}* males. PCR reactions were performed with cDNA mixture inputs of 1 µg (lane 1), 500 ng (lane 2), 250 ng (lane 3), 100 ng (lane 4), 50 ng (lane 5), 25 ng (lane 6) and 12.5 ng (lane 7). **B:** RT-PCR results with sciatic nerve from wild-type and *Plp^{jp}* males. PCR reactions were performed with cDNA mixture inputs of 4 µg (lane 1), 3 µg (lane 2), 2 µg (lane 3), 1 µg (lane 4), 500 ng (lane 5), 250 ng (lane 6), 100 ng (lane 7), 50 ng (lane 8), 25 ng (lane 9) and 12.5 ng (lane 10)