Progesterone Antagonist Therapy in a Pelizaeus-Merzbacher Mouse Model

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Pelizaeus-Merzbacher disease (PMD) is a severe hypomyelinating disease, characterized by ataxia, intellectual disability, epilepsy, and premature death. In the majority of cases, PMD is caused by duplication of *PLP1* that is expressed in myelinating oligodendrocytes. Despite detailed knowledge of *PLP1*, there is presently no curative therapy for PMD. We used a *Plp1* transgenic PMD mouse model to test the therapeutic effect of Lonaprisan, an antagonist of the nuclear progesterone receptor, in lowering *Plp1* mRNA overexpression. We applied placebo-controlled Lonaprisan therapy to PMD mice for 10 weeks and performed the grid slip analysis to assess the clinical phenotype. Additionally, mRNA expression and protein accumulation as well as histological analysis of the central nervous system were performed. Although *Plp1* mRNA levels are increased 1.8-fold in PMD mice compared to wild-type controls, daily Lonaprisan treatment reduced overexpression at the RNA level to about 1.5-fold, which was sufficient to significantly improve the poor motor phenotype. Electron microscopy confirmed a 25% increase in the number of myelinated axons in the corticospinal tract when compared to untreated PMD mice. Microarray analysis revealed the upregulation of proapoptotic genes in PMD mice that could be partially rescued by Lonaprisan treatment, which also reduced microgliosis, astrogliosis, and lymphocyte infiltration.

Introduction

Most individuals affected by Pelizaeus-Merzbacher disease (PMD [MIM 312080]) show a severe and progressive leukodystrophy of early onset. Infants develop nystagmus, poor head control, cerebellar dysfunction, spasticity of upper and lower extremities, and cognitive impairment.[1–5] Onset and progression of PMD is clinically variable, however, and depends on the specific *PLP1* mutation and, based on insight from bona fide PMD mouse models, on unknown modifier genes.[6,7] Despite the detailed knowledge that has accumulated on the molecular genetic basis of the PMD,[4,8] there is no curative therapy available.

Both proteolipid protein (PLP) and its smaller splice isoform DM20 are tetraspan membrane proteins in compacted myelin that account for nearly 20% of the total CNS myelin proteome.[9] Mutations or dosage alterations of X-linked *PLP1* cause PMD and spastic paraplegia type 2 (SPG2 [MIM 312920]) in human and clinically similar phenotypes in rodent models of PMD. Dysmyelination, secondary inflammation, and axonal damage contribute to severe motor impairment.[4,5,10–13] The most common cause of PMD, accounting for approximately 60% of all cases, is duplication of entire *PLP*.[8,14,15]

It is thought that both *Plp1* overexpression and point mutations in the gene exert a toxic gain-of-function effect in oligodendrocytes that is considerably more severe than *Plp1* loss-of-function resulting from null mutations.[5,16–21] In fact, gene deletions or other null mutations[4,22] underlie a less severe disease, originally classified as SPG2 with degenerative changes of long axonal tracts.[23–25] The ability of proteolipids to bind cholesterol is important for myelination,[26] but the stoichiometry of PLP1 and cholesterol appears critical. In mice with *Plp1* overexpression, PLP1/DM20 (lacking equivalent amounts of cholesterol) accumulates in the endo/lysosomal compartment.[19,27] Indeed, treatment of these *Plp1*-overexpressing mice with dietary cholesterol dramatically ameliorates the mutant phenotype.[28]

Several *Plp1* transgenic PMD models, defined by extra copies of wild-type *Plp1*, have been generated that exhibit a range of phenotypes, depending on gene dosage.[29–33] In the present study, we used *Plp1* transgenic homozygous mice in line #72, as described by Readhead et al.[30] (referred to hereafter as PMD mice). In the CNS, the elevated *Plp1* expression level causes dys- and demyelination, oligodendrocyte death, axonal loss in long fiber tracts, and microgliosis with lymphocyte infiltration. We note that transgenic mouse line #72 used here models the comparatively milder forms of PMD or SPG2, whereas line #66 models the severely affected "classical" PMD.[21,28,30,32,34–36]

Ligand-controlled transcription factors, such as the nuclear progesterone receptor, are promising targets for the therapy of myelin diseases caused by myelin gene overexpression.[37] In the peripheral nervous system (PNS), progesterone promotes the synthesis of at least two myelin proteins, MPZ and PMP22, possibly by stimulating accumulation of two other transcription factors, EGR2 and SOX-10, in Schwann cells.[38–43] For the CNS, there is only

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indirect evidence that progesterone serves a similar role as a "promyelinating" factor. In cultures of primary oligodendrocytes and organotypic slice cultures of cerebellum, progesterone increases the accumulation of MBP and 2′,3′-cyclic nucleotide 3′ phosphodiesterase (CNPase).[44,45] Moreover, progesterone positively modulates remyelination after toxin-induced lesions of the cerebellar peduncle in aging rats.[46] Additionally, progesterone contributes to oligodendrocyte precursor cell proliferation and differentiation.[47,48] More recently, in a spinal injury model[49] and in a Cuprizone model of demyelination,[50] progesterone increased *Plp1* mRNA expression. These observations provide proof of principle that the nuclear progesterone receptor acts upstream of *Plp1* transcription in vivo and is therefore a plausible target to pharmacologically lower *Plp1* expression in diseases such as PMD.

The present study aimed at testing the potential therapeutic effect of a newly developed progesterone antagonist, Lonaprisan (ZK230211), in a transgenic mouse model of PMD. We hypothesized that antagonists of steroid hormones can lower total *Plp1* transcription in oligodendrocytes (and thus correct abnormal *Plp1* mRNA and protein levels). This approach could lead to a rational therapy for the most common subgroup of PMD caused by *PLP1* duplications.

Material and Methods

All experiments were conducted according to the Lower Saxony State regulations for animal experimentation in Germany, and animal studies were approved by the appropriate authority.

Transgenic Mice

Male mice in line #72 homozygous for an autosomal murine *Plp1* transgene[30] and maintained on the C57BL/6 background were used as bona fide models for human PMD. *Plp1* overexpression was 1.8-fold (see Results) and motor symptoms in PMD mice were already measurable at study start at 3 weeks of age. Generally, untreated homozygous #72 mice fail to survive beyond 6 months of age.

Antiprogesterone Treatment

Lonaprisan (ZK230211), a nuclear progesterone antagonist, was kindly provided by Bayer.[51] Male mice were assigned to treatment arms at random with respect to phenotype, weight, and parents. Lonaprisan or vehicle was administered by daily subcutaneous injections. Lonaprisan was dosed at 125 mg/kg body weight and suspended in a mixture of 90% sesame oil and 10% benzylbenzoate. In one control group, wild-type mice received vehicle without Lonaprisan. Treatment started at 3 weeks of age (time of weaning) and was performed for 10 weeks.

Brain Level Analysis of Lonaprisan

To examine the blood-brain-barrier passage of Lonaprisan, male wild-type mice were treated with Lonaprisan at 125 gm/kg body weight by subcutaneous injections on a daily basis for 10 days. At 1, 3, 9, and 24 hr after the last dose, the mice were sacrificed and perfused with HBSS in order to avoid interference with intra-vasal Lonaprisan, and brains were isolated. Lonaprisan levels were measured by mass spectrometry at Bayer.

Phenotype Analyses

Phenotypic analyses were performed by the same investigator blinded to genotype and therapy group. Motor phenotype was assessed by a limb-slipping test (grid test) in which mice were placed on a metal grid with 1 cm spacing between bars (bar diameter: 3 mm). Over a total walking distance of 2 m, the number of slips of both hindlimbs and forelimbs were assessed by the examiner. Disease severity was independently assessed with a clinical ranking scale from 1 to 5, with the following criteria: 1, normal phenotype (healthy); 2, ataxic gait; 3, tremor; 4, seizures; 5, death. Phenotype analyses were carried out at 3, 7, 10, and 13 weeks of age. Body weight was continuously recorded.

Histologic Analyses

Cervical spinal cord and total brain was fixed by immersion in 4% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) for electron microscopy (EM) or in 4% paraformaldehyde for immunohistochemistry (IHC).

For IHC analyses, diaminobenzidine (DAB) stainings were used and the antibodies were diluted to the following concentrations: OLIG-2 (Dana-Farber Cancer Institute, rabbit polyclonal) 1:200; GFAP (Novocastra, murine monoclonal) 1:200; MAC-3 (PharMingen, rat monoclonal) 1:400; CD3 (Serotec, rat monoclonal) 1:150; BAX (Santa Cruz, mouse monoclonal) 1:100; Ki67 (DakoCytomation) 1:100; c-MYC (Santa Cruz, mouse monoclonal) 1:100. Immunostained cells were quantified in the corticospinal tract (CST, at cervical vertebral body 5) and in the ventral corpus callosus (CC).

For EM analyses, epoxy resin (Serva) embedding was performed after fixation in 1% OsO$_4$.[52] Ultrathin sections were contrasted by uranylacetate and lead citrate. Myelinated and unmyelinated axons were counted in the CST.

RNA Expression Analyses

RNA from total brain was isolated at study end from 13-week-old mice according to the manufacturer's instructions (RNeasy lipid tissue minikit; QIAGEN) with TRIzol reagent (Invitrogen). Real-time quantitative PCR reactions (Taqman and SYBR Green assay) were carried out with the Lightcycler 480 (Roche). Samples ran in triplicate, each within 10 μl total volume according to a two-step protocol. Quantitation of PCR product was performed with the comparative $\Delta\Delta Ct$ method as recommended by the manufacturer. Transgenic *Plp1* mRNA overexpression was normalized against *Ppia* mRNA. For quantification of Lonaprisan effect on *Plp1* expression, normalization was performed against the non-steroid-regulated, non-myelin-related, and ubiquitously expressed exon 1B transcript of *Pmp22*.[39,53,54,55] *Bax*, *Jun*, and *Casp7* mRNA expression was normalized against the mean of *Rplp0*, *Ppia*, and *Atp5b* mRNA expression. Primer sequences can be provided upon request.

Microarray Analysis

Total brain mRNA was taken from each three individual placebo- (n = 3) and Lonaprisan- (n = 3) treated PMD mice. Mouse Gene 1.0 ST tiling arrays (Affymetrix) were used for the measurements. Data analyses were performed by the integrated software packages Genomics Suite (Partek). The Gene Set Enrichment Analysis
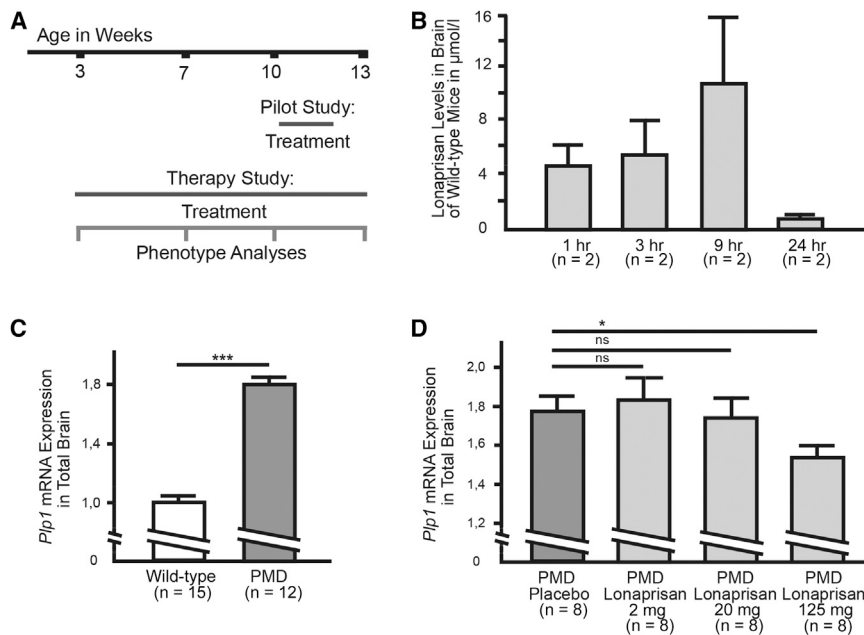
Figure 1. Pilot Studies in PMD Mice
(A) A short-term dosage study over 10 days and a long-term therapy study over 10 weeks were performed.
(B) Lonaprisan crossed the blood-brain barrier and was detectable 24 hr after the last subcutaneous dose.
(C) *Plp1* mRNA expression was increased 1.8-fold when compared to wild-type controls.
(D) Dose-response curve demonstrating downregulation of *Plp1* mRNA overexpression in a short-term pilot study.
ns indicates not significant, *p < 0.05, ***p < 0.001, shown mean ± SEM.

(GSEA) software was used to identify coordinated changes in a priori defined sets of functionally grouped genes.[56,57]

Sample Size Considerations

Sample sizes were calculated a priori for PMD mice by G*Power software taking an alpha error of 5% and a power (1 – beta error) of 80% into account. Effect sizes and standard deviations were prespecified as follows: d = 1.2 and SD = 15% for the reduction of *Plp1* mRNA fold-expression, d = 1.8 and SD = 10% for the preservation of myelinated axons/area, and d = 1.1 and SD = 20% for the reduction of number of slips.

Statistical Analysis

All values are expressed as mean ± SEM. We have tested all our data on Gaussian distribution and used parametric (Student's t test) or nonparametric (Mann-Whitney U-test) testing, where applicable. Correlation analyses were performed by the Spearman's rank correlation test. Statistica 7.0 (StatSoft) was used for statistical analyses.

Results

Lonaprisan Crosses the Blood-Brain Barrier and Downregulates *Plp1* Overexpression

A pilot study was performed to examine whether Lonaprisan crosses the blood-brain barrier (Figure 1A). Lonaprisan levels were determined by mass spectroscopy in total brain lysates from wild-type mice, prepared 1, 3, 9, and 24 hr after the last subcutaneous application. Drug levels were detectable at 1 hr, increased up to 9 hr after the last dose, and were still detectable after 24 hr (1 hr: mean = 4.8 μmol/l, SEM = 1.6; 3 hr: mean = 5.6 μmol/l, SEM = 2.6; 9 hr: 10.8 μmol/l, SEM = 4.9; 24 hr: mean = 0.8 μmol/l, SEM = 0.2) (Figure 1B). In an attempt to establish the optimal dosage for *Plp1* downregulation, we treated PMD mice over 10 days at three different dosages

and examined *Plp1* mRNA expression 24 hr after the last injection. Placebo-treated PMD mice showed a significant 1.8-fold *Plp1* overexpression (n = 12, mean = 1.8, SEM = 0.1) compared to wild-type controls (n = 15, mean = 1.0, SEM = 0.04, p < 0.001) (Figure 1C). Lonaprisan dosages of 2 mg/kg (n = 8, mean = 1.9, SEM = 0.1) and 20 mg/kg (n = 8, mean = 1.7, SEM = 0.1) were not sufficient to reduce the *Plp1* mRNA overexpression in PMD mice (n = 8, mean = 1.8, SEM = 0.1) after short-term treatment. However, Lonaprisan treatment in PMD mice at the dosage of 125 mg/kg significantly reduced *Plp1* mRNA to 1.5-fold expression (n = 8, mean = 1.5, SEM = 0.1, p < 0.05) when compared to placebo-treated PMD mice (Figure 1D). This decrease is similar in extent to the therapeutic effect of a progesterone antagonist (Onapristone) in a transgenic rat model of CMT1A.[37]

Lonaprisan Long-Term Treatment Ameliorates the Clinical Phenotype of PMD Mice

After identifying the optimal dosage for *Plp1* mRNA downregulation, Lonaprisan was subcutaneously applied to 3-week-old PMD mice for 10 consecutive weeks by daily injections at the dosage of 125 mg/kg, and placebo-treated wild-type and PMD mice served as controls (Figure 1A). Again, 10 weeks of Lonaprisan treatment reduced *Plp1* overexpression at the mRNA level to 1.6-fold expression (n = 11, mean overexpression level = 1.6-fold, SEM = 0.1, p < 0.01) when compared to PMD controls (n = 12, mean overexpression level = 1.8-fold, SEM = 0.1) (Figure 2A), confirming data from the pilot experiments (Figure 1D).

Three-week-old PMD mice showed minor but significant motor deficiencies (i.e., more slips in the grid test) at the beginning of the trial (n = 12, mean = 9.0 slips, SEM = 0.6, p < 0.05) compared to wild-type controls (n = 30, mean = 6.9, SEM = 0.4), but there was no difference compared to PMD mice in the Lonaprisan treatment group (n = 11, mean = 8.4, SEM = 0.6) (Figures 2B and 2C). This confirmed a balanced stratification between the placebo
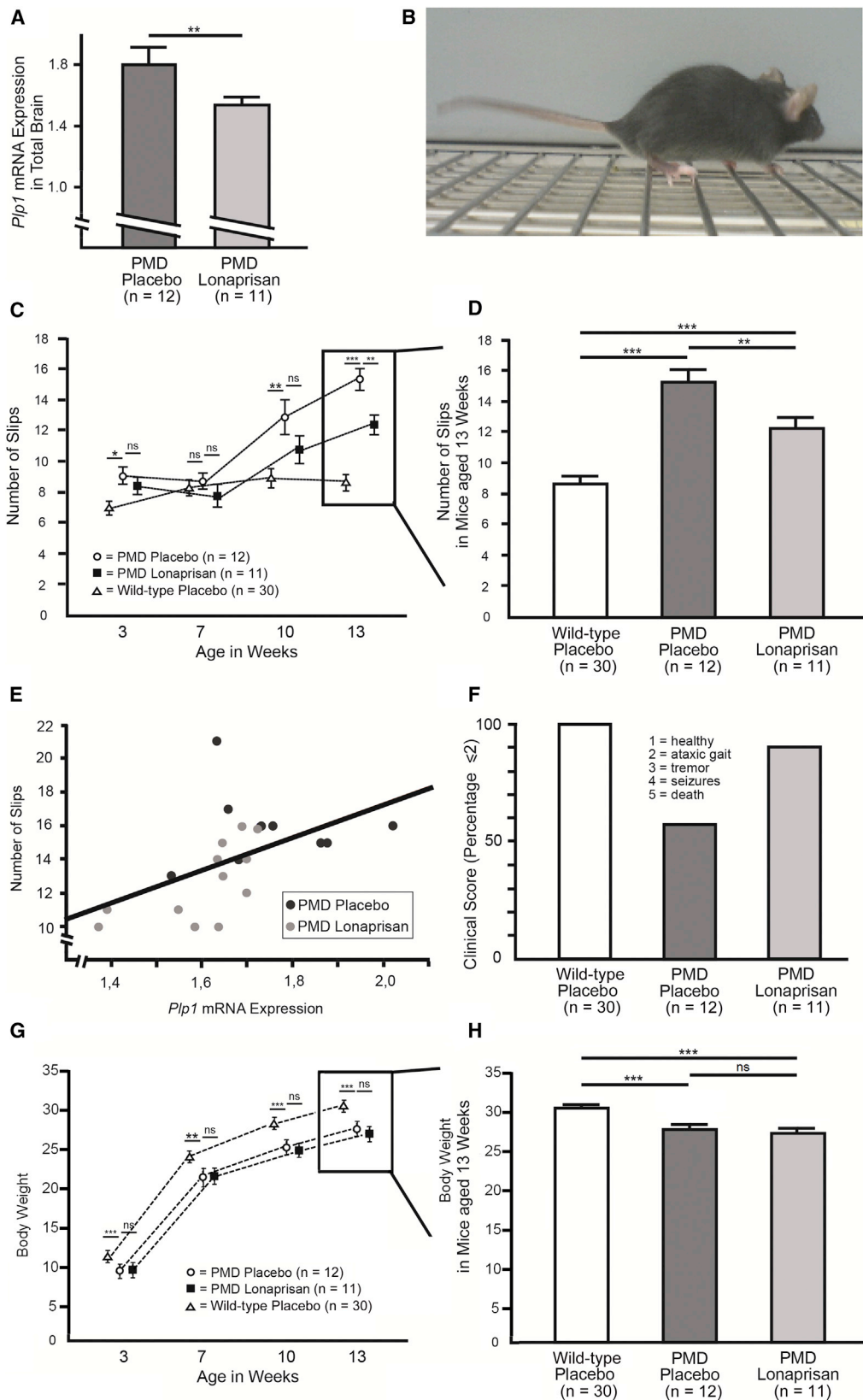
Figure 2. Behavioral Analysis of PMD Mice after Lonaprisan Therapy

(A) Lonaprisan long-term treatment downregulated *Plp1* mRNA overexpression.

(B) Regular grid test analyses counting the number of slips were performed at the beginning at 3 weeks of age, at 7 and 10 weeks, and finally at the end at 13 weeks of age.

(legend continued on next page)

and Lonaprisan PMD mice at therapy start. Seven weeks later, PMD groups had deteriorated in motor performance as expected. Moreover, Lonaprisan treatment (n = 11, mean = 10.7 slips, SEM = 0.9) did not reveal a significant difference in motor deficiencies in comparison to the performance of PMD mice receiving placebo (n = 12, mean = 12.8 slips, SEM = 1.1, not significant), although a clear trend was noticed (Figure 2C). However, after 10 weeks of Lonaprisan therapy, PMD mice showed significantly less progression (n = 11, mean = 12.4 slips, SEM = 0.6, p < 0.01) compared to placebo-treated PMD mice (n = 12, mean = 15.3 slips, SEM = 0.7) (Figure 2C), although the difference to wild-type mice remained obvious (n = 30, mean = 8.6 slips, SEM = 0.5) as shown in Figure 2D. Importantly, these grid test results could be directly correlated to the level of $Plp1$ mRNA overexpression in total brain (r^2 = 0.2676, p < 0.05), strongly suggesting a direct $Plp1$-dosage effect on the motor phenotype (Figure 2E).

Finally, we applied a clinical score at the end of the trial in order to independently quantify Lonaprisan's therapeutic effect. Here, Lonaprisan treatment increased the percentage of PMD mice with a low score of 2 (ataxic gait) or less (n = 11, 91%) when compared to placebo-treated PMD mice (n = 12, 58%). Wild-type controls always scored 1.0 (n = 30) (Figure 2F).

Body weight recordings revealed a significant body weight loss already present in 3-week-old PMD mice (n = 12, mean = 9.7 g, SEM = 0.2) compared to wild-type situation (n = 30, mean = 11.1 g, SEM = 0.2, p < 0.001). This difference remained present throughout the study at the ages of 7 (mean = 21.3 g, SEM = 0.8 and mean = 23.7 g, SEM = 0.3, p < 0.01), 10 (mean = 25.3 g, SEM = 0.5 and mean = 28.1 g, SEM = 0.3, p < 0.001), and 13 (mean = 27.8 g, SEM = 0.5 and mean = 30.4 g, SEM = 0.3, p < 0.001) weeks (Figure 2G). In contrast, PMD placebo controls and Lonaprisan-treated PMD mice did not show any body weight differences at study start (n = 11, mean = 10.2 g, SEM = 0.5). Also, Lonaprisan therapy in PMD mice did not alter the body weight in 7- (mean = 21.5 g, SEM = 0.6), 10- (mean = 24.7 g, SEM = 0.6), and 13- (mean = 26.8 g, SEM = 0.6) week-old mice at the study end (Figures 2G and 2H).

Preservation of Myelinated Axons

To investigate the therapeutic effect of Lonaprisan on dysmyelination and histopathology in the central nervous system, we examined a region that is severely affected in PMD mice (Figure 3A). The corticospinal tract (CST) of the cervical spinal cord was used for electron microscopy (EM) and morphometry. Whereas wild-type mice displayed on average 838 myelinated axons per 1,000 μm^2 cross sectional area in the CST (n = 3, SEM = 49.6), PMD mice with severe dysmyelination showed only 198 myelinated axons per 1,000 μm^2 (n = 10, SEM = 48.7, p < 0.001) (Figure 3A, quantification in 3B). Importantly, as judged by EM, the 10 week Lonaprisan therapy increased the number of myelinated axons by 25% to 254 in PMD mice (n = 11, SEM = 52.1, p < 0.05) (Figure 3B). Correspondingly, unmyelinated CST axons were numerous in PMD mice (n = 5, mean = 489/1,000 μm^2, SEM = 91.6) but rare in wild-type mice (n = 3, mean = 7/1,000 μm^2, SEM = 2.0, p < 0.01). However, Lonaprisan treatment did not alter the number of unmyelinated axons in PMD mice (n = 5, mean = 524/1,000 μm^2, SEM = 77.6) (Figure 3C).

As expected, g-ratio analysis also revealed a significant hypomyelination in PMD mice (n = 5, mean = 0.97, SEM = 0.01) compared to wild-type controls (n = 3, mean = 0.84, SEM = 0.01, p < 0.001). Again, Lonaprisan therapy over 10 weeks did not influence the extent of hypomyelination in PMD mice (n = 5, mean = 0.97, SEM = 0.01, not significant) (Figure 3D). The g-ratio distribution as a function of axon size was also not different between placebo- and Lonaprisan-treated PMD mice (Figure 3E). When combined with the results above, we conclude that Lonaprisan acts on axonal preservation without any effects on myelination.

Correlation of $Plp1$ mRNA Expression, Myelinated Axons, and Phenotype

$Plp1$ mRNA expression levels correlated with the motor phenotype as measured by the grid test analysis in treated and untreated PMD mice (Figure 2E). Histological analysis showed that the number of myelinated axons correlated inversely with the $Plp1$ mRNA expression (r^2 = 0.3693, p < 0.01) (Figure 3F), suggesting that $Plp1$ overexpression in oligodendrocytes (and abnormal gain of function) leads to the loss of myelinated axons. Likewise, the number of myelinated axons in the CST correlated with the motor phenotype (r^2 = 0.2729, p < 0.05) (Figure 3G), demonstrating a strong link between $Plp1$ dosage, number of myelinated axons, and clinical phenotype in PMD mice.


(C and D) Differences between Lonaprisan-treated PMD mice (n = 11) (filled squares) and placebo-treated control mice (n = 12) (circles) increased with age and became significant at 13 weeks of age at study end (C), which is illustrated in higher magnification of the final time point. Wild-type values (n = 30) are depicted for comparison (open bar) (D).

(E) $Plp1$ mRNA expression correlated with the grid test analysis, strongly suggesting a direct effect of $Plp1$ expression on the motor phenotype.

(F) By applying a clinical score in a descriptive analysis, PMD mice showed a pathologic phenotype and Lonaprisan increased the percentage of mice showing solely ataxic gait or even healthy condition.

(G and H) A significant body weight loss was already present in 3-week-old PMD mice (n = 12) compared to the wild-type situation (n = 30). This difference remained present throughout the study. In contrast, PMD placebo controls and Lonaprisan-treated PMD mice (n = 11) did not show any body weight differences at study start and Lonaprisan therapy did not alter the body weight by study end.

ns indicates not significant, *p < 0.05, **p < 0.01, ***p < 0.001, shown mean ± SEM.
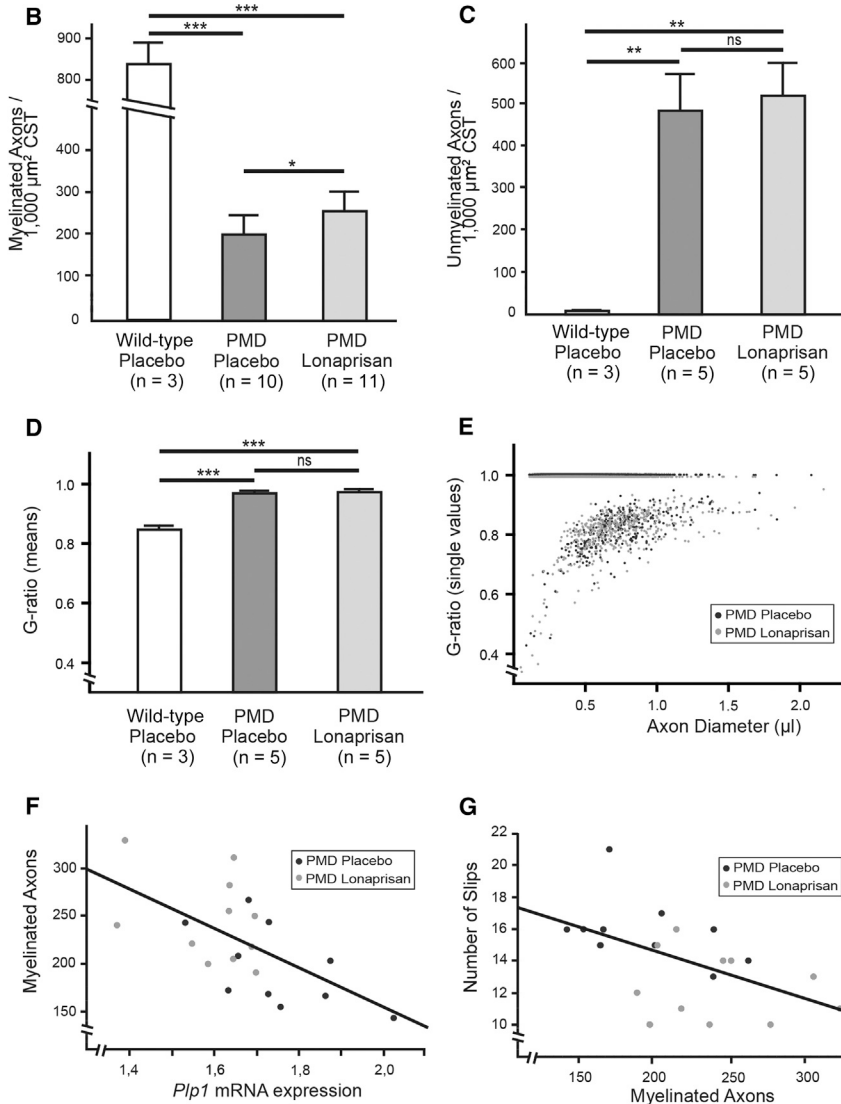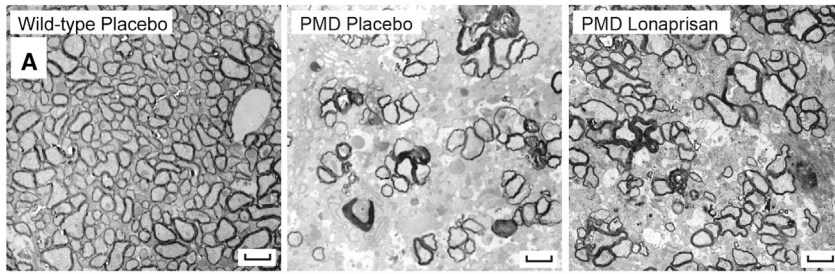
Figure 3. Electron Microscopic Analyses of the CNS after Lonaprisan Therapy in PMD Mice

(A) Electron microscopic graphs of the corticospinal tract showed preservation of myelinated axons after Lonaprisan treatment.

(B) Myelinated axons are reduced in PMD mice compared to wild-type controls, and after Lonaprisan therapy myelinated axons are increased.

(C) Nonmyelinated axon number was increased in the disease situation but was unaltered by Lonaprisan treatment.

(D and E) In PMD mice, Lonaprisan therapy did not increase the mean myelin sheath thickness (D) and corresponding myelin sheath thickness according to axonal size (E).

(F and G) Plp1 mRNA expression levels correlated to the myelinated axon number (F), and myelinated axon number correlated to the phenotype (G).
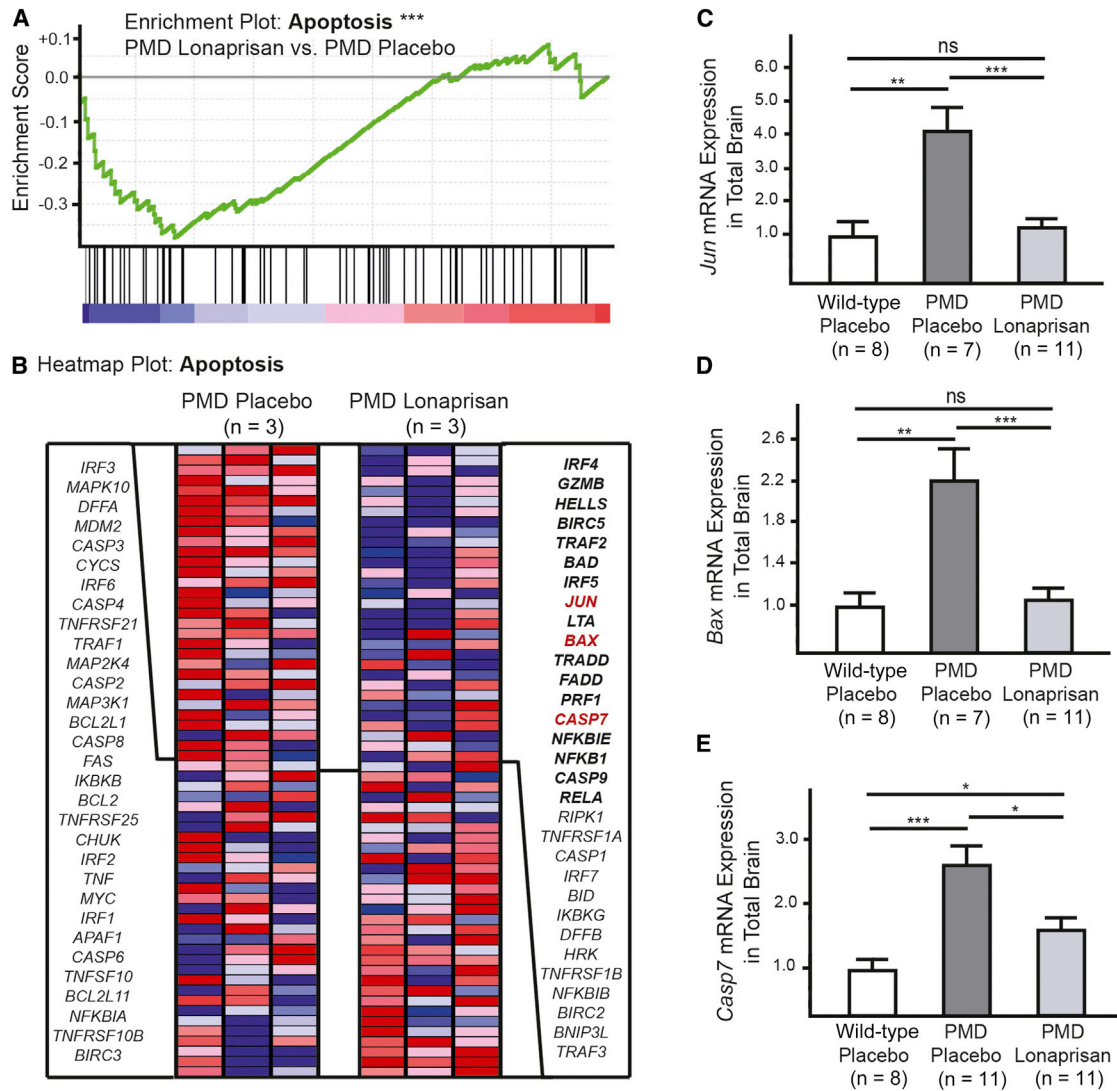
Scale bars represent 1.7 μm. CST indicates corticospinal tract, ns indicates not significant, *$p < 0.05$, ***$p < 0.001$, shown mean ± SEM.

Lonaprisan Therapy Modifies Expression of Apoptosis-Related Genes

To assess the molecular consequences of Lonaprisan treatment, we performed individual microarray analyses on brain cDNA preparations obtained from PMD mice treated with placebo (n = 3) and Lonaprisan (n = 3). When searching for functional groups, gene set enrichment analysis (GSEA) revealed a significant downregulation of genes involved in apoptosis in mouse brains after Lonaprisan therapy ($p < 0.001$, enrichment score = −0.38) (Figure 4A). The heatmap plot for apoptosis visualized 62 genes involved in the apoptosis pathway (Figure 4B). In order to validate the microarray data, we performed quantitative RT-PCR analyses on total brain cDNAs obtained from individual wild-type controls and PMD mice, treated either with placebo or Lonaprisan. For example, Bax mRNA expression was significantly increased in PMD mice (n = 7, mean = 2.2-fold, SEM = 0.3) compared to wild-type controls (n = 8, defined as 1.0, SEM = 0.2, $p < 0.01$). Here, Lonaprisan treatment significantly downregulated Bax overexpression when compared to placebo-treated PMD mice (n = 11, mean = 1.1-fold, SEM = 0.1, $p < 0.001$), reaching wild-type levels (Figure 4D). Jun mRNA expression was also increased in PMD mice (n = 7, mean = 4.2-fold, SEM = 0.7) compared to wild-type controls (n = 8, defined as 1.0, SEM = 0.5, $p < 0.01$) and Lonaprisan therapy normalized the mRNA overexpression to 1.3-fold (n = 11, mean = SEM = 0.3, $p < 0.001$), again approaching wild-type levels (Figure 4C). Finally, we measured Casp7 mRNA levels that were upregulated in PMD mice (n = 11, mean = 2.6-fold, SEM = 0.3) compared to wild-type controls (n = 8, defined as 1.0, SEM = 0.2, $p < 0.001$). Lonaprisan treatment over 10 weeks downregulated Casp7 overexpression in PMD mice to 1.6-fold (n = 11, SEM = 0.2,

Figure 4. Microarray Analysis in PMD Mice Treated with Lonaprisan

(A and B) Microarray analysis revealed a downregulation of genes involved in apoptosis after Lonaprisan therapy (core enriched genes in bold).

(C–E) *Jun*, *Casp7*, and *Bax* were validated by quantitative RT-PCR.

*p < 0.05, **p < 0.01, ***p < 0.001, shown mean ± SEM.

p < 0.05), but without reaching wild-type levels (p > 0.05) (Figure 4E).

Oligodendrocyte Loss, Microgliosis, and Infiltration of Lymphocytes

Next, we performed immunohistochemistry in the CST and corpus callosum (CC) in order to validate the effect of Lonaprisan treatment on the selected histopathological marker proteins OLIG-2, MAC-3, GFAP, and CD3 (Figure 5A). Counting OLIG-2-positive cells in the CST demonstrated more than 50% decrease of oligodendrocyte density in PMD mice (n = 6, mean = 7 cells per 30,000 μm^2, SEM = 0.7, p < 0.001) when compared to wild-type controls (n = 3, mean = 16 cells per 30,000 μm^2, SEM = 0.6). Here, 10 weeks of Lonaprisan treatment significantly prevented oligodendrocyte loss

(n = 6, mean = 11 cells per 30,000 μm^2, SEM = 1.4, p < 0.05), although wild-type values were not reached (Figure 5B). As expected, wild-type brains showed only a few microglia/macrophages within the CST as indicated by MAC-3-positive cells counts (n = 3, mean = 5 cells per 30,000 μm^2, SEM = 1.2). In PMD mice, MAC-3-positive cells were several-fold increased, indicating active microgliosis (n = 6, mean = 92 cells per 30,000 μm^2, SEM = 15.2, p < 0.01). Lonaprisan-treated PMD mice displayed fewer MAC-3-positive microglia and macrophages compared to untreated PMD mice (n = 6, mean = 31 cells per 30,000 μm^2, SEM = 4.2, p < 0.01) but without approaching wild-type values (Figure 5C). Finally, GFAP cell counts in the CST revealed that Lonaprisan treatment also reduced astrogliosis (n = 6, mean = 8 cells per 30,000 μm^2, SEM = 0.8) when compared to untreated
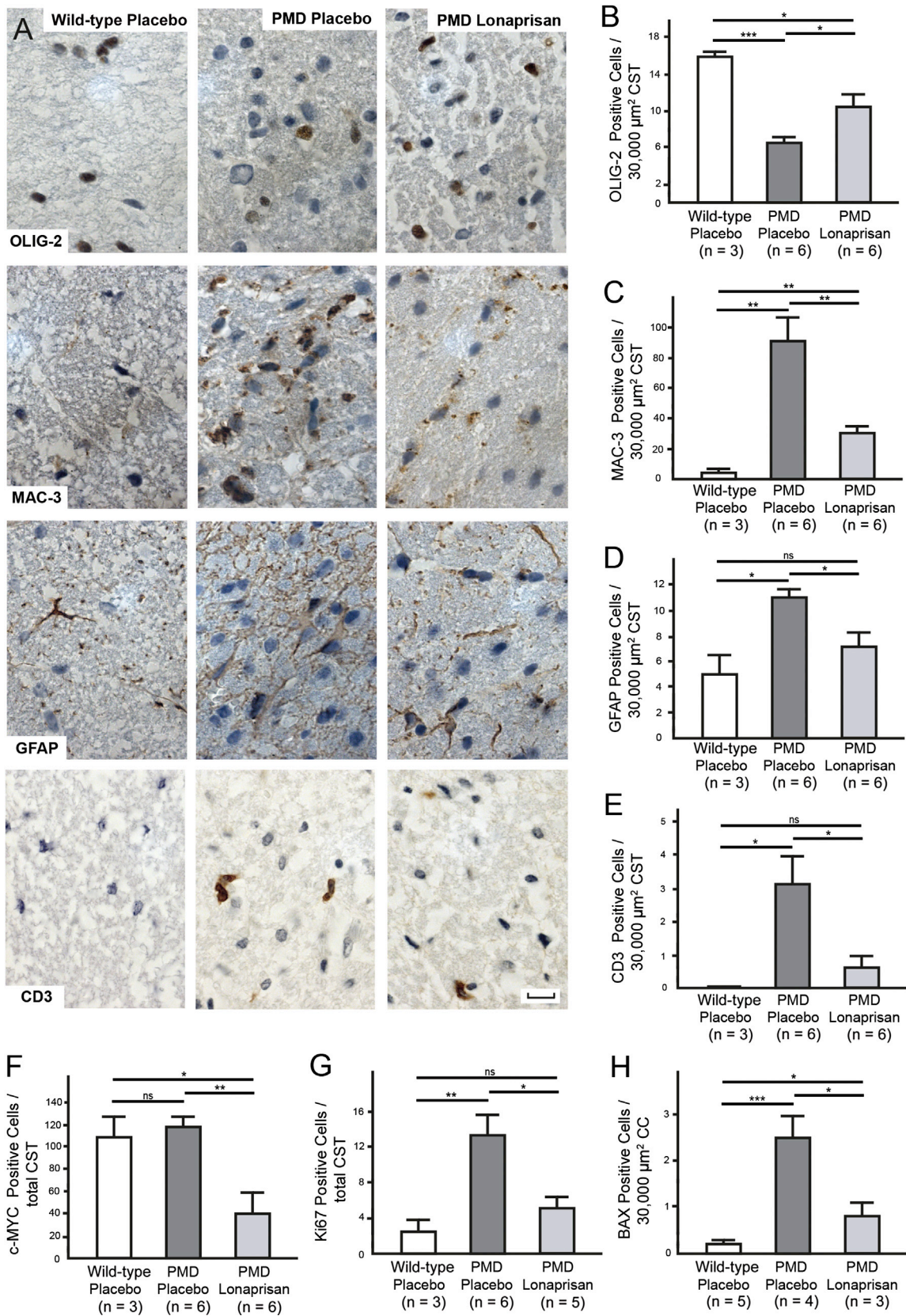
Figure 5. Immunohistochemistry in CNS after Treatment with Lonaprisan in PMD Mice

(A) Immunohistochemistry showing OLIG-2-, MAC-3-, GFAP-, and CD3-positive cells in wild-type and placebo- and Lonaprisan-treated PMD mice.

(B–E) Lonaprisan therapy reduced oligodendrocyte loss (B), microgliosis (C), astrogliosis (D), and lymphocyte infiltration (E).

(legend continued on next page)

PMD mice (n = 6, mean = 11 cells per 30,000 μm^2, SEM = 1.1, p < 0.05). Moreover, wild-type values were not reached (n = 3, mean = 5 cells per 30,000 μm^2, SEM = 1.5) (Figure 5D). In the CST area of wild-type mice, we never detected CD3-positive lymphocytes (n = 3, mean = 0 cells per 30,000 μm^2, SEM = 0). However, we detected CD3-positive T cells in untreated PMD mice, suggesting that lymphocyte infiltration is stimulated by the disease (n = 6, mean = 3 cells per 30,000 μm^2, SEM = 0.8, p < 0.05). In the Lonaprisan treatment group, the number of CD3-positive cells was significantly reduced (n = 6, mean = 0.7 cells per 30,000 μm^2, SEM = 0.3, p < 0.05), statistically approaching the values found in wild-type mice (Figure 5E).

Additionally, sections were analyzed for the expression of c-MYC (cell cycle regulator), Ki67 (proliferation marker), and BAX (apoptosis related marker). The density of c-MYC-positive cells in the CST (n = 6, mean = 119 cells per section, SEM = 9.5) was not different from wild-type controls (n = 3, mean 110 cells per section, SEM = 17.9). However, Lonaprisan therapy reduced this number to less than 50% in the CST of PMD mice (n = 6, mean = 41 cells per section, SEM = 19.4, p < 0.01) (Figure 5F). Further, Ki67-positive cells were found in significantly higher density in the CST of PMD mice (n = 6, mean = 14 cells per section, SEM = 2.3, p < 0.01) than in wild-type controls (n = 3, mean = 3 cells per section, SEM = 1.2). Ten weeks of Lonaprisan therapy reduced the density of Ki67-positive cells by more than 60% (n = 5, mean = 5 cells per section, SEM = 1.3, p < 0.05) without reaching wild-type values (Figure 5G). Interestingly and in line with transcriptional data (Figure 4D), the density of cells expressing the apoptosis-associated protein BAX in the corpus callosum was increased in PMD mice (n = 4, mean = 2.5 cells per 30,000 μm^2, SEM = 0.5, p < 0.001) when compared to wild-type mice (n = 5, mean = 0.2 cells per 30,000 μm^2, SEM = 0.1) and reduced after Lonaprisan therapy (n = 3, mean = 0.8 cells per 30,000 μm^2, SEM = 0.3, p < 0.05) (Figure 5H).

Discussion

We tested the hypothesis that lowering toxic *Plp1* overexpression in oligodendrocytes in a transgenic animal model of PMD via steroid antagonist would enable oligodendrocytes to better support axons. This may lead to a translatable therapy for a subgroup of individuals affected by PMD caused by *PLP1* duplications.

A number of factors regulating *Plp1* transcription in oligodendrocytes have been identified in vitro, such as MyT1,[58] microRNA *miR-20a*,[59] and the zinc finger protein

YY1.[60] Moreover, several potential transcription factor binding sites and enhancer elements have been identified in the 5′-flanking sequence[61–63] and within intron 1 of mouse *Plp1* (AP-1).[64,65] One widely expressed transcription factor that is present in oligodendrocytes[66] and can be pharmacologically targeted is the nuclear progesterone receptor, a ligand-activated Zinc-finger protein that is regulated by progesterone and inhibited by specific synthetic antagonists. The nuclear progesterone receptor is a member of a much larger steroid receptor superfamily that binds to palindromic response elements in the promoter regions of steroid-responsive genes.[67] Progesterone is synthesized in the brain by neurons and glial cells[68,69] and the progesterone receptor is expressed by neurons and oligodendrocytes.[49,66,70–72] Furthermore, steroid-responsive elements including progesterone receptor binding sites[73–75] can be predicted in *PLP1* promoter (data not shown) and daily progesterone injections upregulate *Plp1* mRNA in sciatic nerves of rats in vivo (Figure S1 available online).[37] In the PNS, the activating effect of progesterone on Schwann cell differentiation and the transcriptional activation of myelin genes *Pmp22* and *Mpz* are well known.[76,77] The progesterone receptor antagonists Mifepristone (RU486), Onapristone (ZK98299), and the newly developed Lonaprisan (ZK230211), which was used in the present study, specifically inhibit the effect of the progesterone receptor on transcriptional activation.[51] However, there are no data on the functional role of progesterone receptor on *Plp1* transcription.

In the present study, we have chosen Lonaprisan because of its pure progesterone antagonistic activity without partial agonistic potential such as demonstrated by Mifepristone.[78] Furthermore, Lonaprisan shows a more favorable safety profile than Onapristone, which may enable translation of our findings to humans.[79]

Lonaprisan was shown to cross the blood-brain barrier in wild-type mice and, in a dose-finding pilot study for 10 days in PMD mice, Lonaprisan daily treatment at 125 mg/kg body weight demonstrated potency to downregulate *Plp1* 1.8-fold mRNA overexpression to 1.5-fold expression. Importantly, after 10 weeks of daily Lonaprisan therapy, *Plp1* mRNA overexpression was still reduced to 1.6-fold expression. Because PLP1 is most abundantly expressed in oligodendrocytes and *Plp1* transgenic mice do not show ectopic PLP1 expression in the CNS,[30] the reduction of *Plp1* mRNA overexpression is predominantly restricted to oligodendrocytes. We have not quantified PLP1 protein because *Plp1* dosage interferes with myelination itself.[30] Therefore, any quantification of PLP1 proteins of PMD mice may yield a paradoxic result, because less (rather than more) PLP1 is detected, with PLP1 serving as a marker for myelin. Consequently, our attempts of

(F–H) Further, cells positive for c-MYC, Ki67, and BAX were reduced after Lonaprisan therapy, indicating changes in cell cycle regulation resulting in reduced proliferation and apoptosis in PMD mice.

Scale bars represent 10 μm. CST indicates corticospinal tract, CC indicates corpus callosum, ns indicates not significant, *p < 0.05, **p < 0.01, ***p < 0.001, shown mean ± SEM.

pharmacologically reducing *Plp1* transcription and rescuing myelination in the mouse model would consequently lead to more myelin and more myelin protein (including PLP1).

Lonaprisan therapy significantly prevented the loss of myelinated axons and improved the motor phenotype in PMD mice by approximately 50% toward wild-type level as shown with the grid test analysis. Applying a second phenotype measurement, the clinical score data revealed a higher percentage of PMD mice showing solely ataxic gait or even healthy condition after long-term therapy with Lonaprisan. Body weight loss as a sign for unspecific side effects was not observed in Lonaprisan-treated mice. However, Lonaprisan treatment did not show an effect on the number of unmyelinated axons nor on myelin sheath thickness. Importantly, *Plp1* mRNA levels correlated inversely with the number of myelinated axons and with the phenotype of PMD mice. Further, myelinated axon numbers correlated with the clinical phenotype. These observations confirm the importance of tight *Plp1* regulation and support the concept that the reduction of toxic *Plp1* mRNA overexpression poses the key mechanism for the progesterone antagonistic effect. However, it is not known to what degree the progesterone receptor contributes to *Plp1* mRNA expression.

In a randomized phase II study to investigate the efficacy, safety, and tolerability of Lonaprisan as second-line endocrine therapy for postmenopausal women with hormone receptor-positive metastatic breast cancer, maximum oral dosage of 100 mg was applied (ClinicalTrials.gov identifier NCT00555919). Therefore, when translating the current findings to human use, careful dose finding targeting *Plp1* expression in individuals suffering from PMD is necessary.

PMD mice suffer from a lymphocyte infiltration into the CNS,[36,80] microglia activation, and astrogliosis as well as described features.[30,32,34,35] Lonaprisan therapy significantly decreased the number of lymphocytes present in the CNS, suggesting that there is reduced inflammation after long-term treatment with the progesterone antagonist. Because loss of progesterone receptor function mutants show proinflammatory effects,[81] we interpret the reduced inflammation as secondary effect resulting from improved PMD pathology. Further effects of the long-term therapy with Lonaprisan included the prevention of OLIG-2-stained oligodendrocyte lineage cells and the reduction of MAC-3-stained microglia and GFAP-stained astrogliosis.

In order to address the mechanism by which Lonaprisan treatment can prevent axonal loss, we performed microarray analysis and applied the gene set enrichment analysis (GSEA). We found a significant downregulation of genes involved in apoptosis and validated this finding by quantitative RT-PCR at the level of single animals (n = 8–11) for three highly downregulated apoptotic genes: *Jun*, *Casp7*, and *Bax*. At the transcriptional level, the expression of all three genes was upregulated in PMD mice and downregu-

lated after Lonaprisan therapy. By immunohistochemistry, PMD pathology was directly associated with higher apoptosis and proliferation marker expression when compared to wild-type littermates (BAX[82] and Ki67,[83] respectively). These observations are in line with an increase in apoptotic oligodendrocytes in PMD mice.[32,35] Importantly, Lonaprisan therapy significantly reduced the number of cells overexpressing these apoptosis and proliferation markers. Furthermore, cell counts for c-MYC, a key regulator of cell cycle with proapoptotic and proproliferative actions,[84] showed less c-MYC expression after Lonaprisan treatment.

Saher et al. recently reported a therapeutic strategy with high-cholesterol diet in PMD mice.[28] This effect is mediated by the amelioration of intracellular PLP1 accumulation and increased PLP1 incorporation into myelin membranes that was accompanied by an upregulation of cholesterol-synthesizing enzymes (*Hmgcr* and *Fdft1* mRNA) without regulatory effects on *Plp1* mRNA overexpression itself. In contrast, Lonaprisan in PMD mice did not affect cholesterol-synthesizing-enzyme expression of *Hmgcr*, *Fdft1*, *Mvd*, or *Lss* mRNA (Figures S2A–S2D). Because cholesterol and Lonaprisan experimental therapies act independently, the combination may lead to synergistic effects.

Further more, a strong argument for therapeutic regulation of toxic overexpression of myelin proteins in the CNS is derived from a former therapy trial performed in a transgenic rat model for the most common inherited neuropathy, Charcot-Marie-Tooth disease ("CMT1A" rats).[37,85,86] CMT1A is caused by an intrachromosomal duplication on chromosomal region 17p11.2 that leads to an increased gene dosage of the peripheral myelin protein of 22 kDa and consecutive demyelination of peripheral nerve axons.[87] PMP22 is a small hydrophobic tetraspan protein expressed in myelinating glia of the PNS and shows a similar structure to PLP1. Daily administration of the pure progesterone antagonist Onapristone reduced *Pmp22* overexpression, ameliorated axonal loss, and improved the clinical phenotype in *Pmp22* transgenic CMT rats.[37,88] In contrast to the correlations in the present study, CMT rats did not demonstrate stringent correlations of *Pmp22* mRNA expression, axonal numbers, and the clinical phenotype upon antiprogesterone treatment, which may be explained by the missing consensus regions for the progesterone receptor in the *Pmp22* promoter region. However, the observation of preserved axonal loss without any effects on myelin sheath thickness in the present study is consistent with the former progesterone therapy trial in CMT rats. In that study, we hypothesized that *Pmp22* overexpression was reduced to a degree at which the axonal support function of Schwann cells was better maintained than myelination.[89] We note that *Plp1* loss-of-function mutants show no crude myelin abnormalities but demonstrate axonal pathology,[5,22] implying that PLP1 is responsible for axonal support rather than myelination in the CNS. Because we do not assume that overexpression linearly contributes to PMD pathology and that oligodendrocytes tolerate

increasing *Plp1* mRNA levels only to a certain degree, the initial correction toward wild-type level may be considered as most important for axonal support.

In summary, we demonstrate proof of principle that lowering toxic *Plp1* overexpression via a progesterone antagonist ameliorates axonal loss and the disease phenotype in *Plp1* transgenic mice.

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.cell.com/ajhg/.

Web Resources

The URLs for data presented herein are as follows:

ClinicalTrials.gov, http://clinicaltrials.gov
Gene Expression Omnibus (GEO), http://www.ncbi.nlm.nih.gov/geo/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/

Accession Numbers

Microarray data are available in the Gene Expression Omnibus database, accession number GSE55315.

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