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RESEARCH ARTICLE

Curcumin therapy in a *Plp1* transgenic mouse model of Pelizaeus-Merzbacher disease

Dirk B. Epplen[1,a], Thomas Prukop[1,2,a], Tobias Nientiedt[1], Philipp Albrecht[3], Friederike A. Arlt[1], Ruth M. Stassart[1,4], Celia M. Kassmann[1], Axel Methner[5], Klaus-Armin Nave[1], Hauke B. Werner[1] & Michael W. Sereda[1,6]

This article is dedicated to the memory of Dr. Ajit S. Dhaunchak.

[1]Department of Neurogenetics, Max-Planck-Institute of Experimental Medicine, Göttingen, Germany
[2]Institute of Clinical Pharmacology, University Medical Center Göttingen (UMG), Göttingen, Germany
[3]Department of Neurology, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany
[4]Institute of Neuropathology, University Medical Center Göttingen (UMG), Göttingen, Germany
[5]Focus Program Translational Neuroscience (FTN), Rhine Main Neuroscience Network (rmn2), Department of Neurology, Johannes Gutenberg University Medical Center Mainz, Mainz, Germany
[6]Department of Clinical Neurophysiology, University Medical Center Göttingen (UMG), Göttingen, Germany

Correspondence
Hauke B. Werner (or) Michael W. Sereda, Research Group "Molecular and Translational Neurology", Department of Neurogenetics, Max-Planck-Institute of Experimental Medicine, Hermann-Rein-Str. 3, D-37075 Göttingen, Germany. Tel: +49-551-3899759 (or) +49 551 3899 764; Fax: +49-551-3899758 (or) +49 551 3899 753; E-mail: hauke@em.mpg.de (or) sereda@em.mpg.de

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[a]Equal contribution.

Abstract

Objective: Pelizaeus–Merzbacher disease (PMD) is a progressive and lethal leukodystrophy caused by mutations affecting the proteolipid protein (*PLP1*) gene. The most common cause of PMD is a duplication of *PLP1* and at present there is no curative therapy available. **Methods**: By using transgenic mice carrying additional copies of *Plp1*, we investigated whether curcumin diet ameliorates PMD symptoms. The diet of *Plp1* transgenic mice was supplemented with curcumin for 10 consecutive weeks followed by phenotypical, histological and immunohistochemical analyses of the central nervous system. *Plp1* transgenic and wild-type mice fed with normal chow served as controls. **Results**: Curcumin improved the motor phenotype performance of *Plp1* transgenic mice by 50% toward wild-type level and preserved myelinated axons by 35% when compared to *Plp1* transgenic controls. Furthermore, curcumin reduced astrocytosis, microgliosis and lymphocyte infiltration in *Plp1* transgenic mice. Curcumin diet did not affect the pathologically increased *Plp1* mRNA abundance. However, high glutathione levels indicating an oxidative misbalance in the white matter of *Plp1* transgenic mice were restored by curcumin treatment. **Interpretation**: Curcumin may potentially serve as an antioxidant therapy of PMD caused by *PLP1* gene duplication.

Introduction

Pelizaeus-Merzbacher disease (PMD) is an early-onset lethal leukodystrophy without curative therapy. PMD symptoms are clinically variable. Patients suffer from ataxia, cognitive impairment, seizures, and spasticity. The broad spectrum of neurological symptoms is reflected by various mutations within the proteolipid protein gene (*PLP1*) encoding for proteolipid protein (PLP1) and its smaller isoform DM20.[1] Mutations or dosage alterations of the X-linked *PLP1* gene cause PMD and the nonlethal spastic paraplegia type 2 (SPG2).[2] However, increased *PLP1* gene dosage is the most common cause for PMD which accounts for 60–70% of all cases.[3–6]

Transgenic mouse lines carrying additional copies of the wild-type *Plp1* gene have been generated (line #66 and #72)[7] and provide well-established mouse models for the most common form of PMD. *Plp1* transgenic mice show elevated *Plp1* mRNA levels, dysmyelination, loss of oligodendrocytes and axons, ataxia, tremor, epilepsy, and premature death beyond 6 months of age. Additionally, micro- and astrocytosis and lymphocyte infiltration were observed.[7–12] Intracellulary, PLP1 accumulates in late endosomes and lysosomes.[13] We note that the transgenic line #72 used here models the comparatively milder forms of PMD.

Two experimental strategies applying a cholesterol-enriched diet or the progesterone antagonist Lonaprisan to *Plp1* transgenic mice led to promising results.[14,15] Cholesterol promoted normal intracellular trafficking of the cholesterol-associated[16] PLP1 and its incorporation into myelin,[14] while Lonaprisan reduced *Plp1* mRNA overexpression to a degree at which oligodendrocytes better supported axonal integrity.[15] Together this indicates that the pathology of *Plp1* transgenic mice can be considerably improved by therapeutic intervention.

In *myelin synthesis deficient (msd)* mice, a PMD model carrying a spontaneous missense mutation in the *Plp1* gene, curcumin treatment resulted in 25% longer survival and inhibited the loss of oligodendrocytes. We note that curcumin treatment has not shown any significant effects on body weight, motor phenotype, number of myelinated axons, myelin amount, apoptosis, and unfolded protein response in wild-type controls.[17] Although the molecular mechanisms underlying the beneficial effect in *msd* mice remained undetermined, this indicated that curcumin may be a promising therapeutic compound for PMD, at least when caused by *PLP1* point mutations. Curcumin exhibits curative effects also in animal models of numerous other neurological diseases.[18–20] Curcumin is an ingredient of turmeric – a natural component of normal human diet commonly considered safe – and has been tested worldwide in more than 100 registered clinical trials, mainly investigating its effects on neurodegenerative, inflammatory and cancer diseases (http://clinicaltrials.gov, search for "curcumin," April 2015).

In the present study, we have tested whether curcumin diet ameliorates PMD-related symptoms of *Plp1* overexpressing transgenic mice.

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.

Plp1 transgenic mice

We used male homozygous *Plp1* transgenic mice in line #72[7] maintained on the C57Bl/6N background, which exhibits a 1.8-fold *Plp1* mRNA overexpression.[15]

Study design and treatment

Based on previous pilot experiments (data not shown), curcumin was applied as a 1:6 mixture of turmeric powder in normal chow available ad libitum. Curcumin is the major active compound in turmeric powder and the proportion of curcumin is about 3% (corresponding to 0.5% curcumin in the given chow).[21] Experimental treatment started in 3-week-old mice and was continued for 10 weeks. We additionally tested Meriva® (Indena S.p.A., Milan, Italy) curcumin which is formulated with phosphatidylcholine enabling a fivefold increased bioavailability compared to unformulated curcumin regarding Cmax and AUC in rodents and man,[22,23] which is more favorable for future preclinical studies in PMD models and any translational approaches in patients as well. Therefore, for glutathione (GSH) measurements (shown in Fig. 4C and D) curcumin was applied ad libitum as a 1:1000 mixture of Meriva® curcumin in standard chow (corresponding to 0.1% Meriva® in the given chow) starting in 3-week-old mice and continued for 7.5 weeks aiming at similar curcumin blood levels as when turmeric was applied chronically. The treatment start point is most critical for comparable effects rather than the exact duration.[14,24] *Plp1* transgenic and wild-type mice treated with normal chow served as controls for the present curcumin and recently published progesterone receptor antagonist treatment in *Plp1* transgenic mice[15] which were both tested in parallel within one common trial to minimize required animal numbers. All transgenic mice were assigned to treatment arms at random with respect to phenotype, weight, and litter. All analyses were performed

in a blinded manner regarding genotype and treatment and each analysis was performed by the same investigator. Tissue samples for analysis were taken at the end of dietary supplementation.

Motor phenotype

The grid test was performed to measure the motor symptoms as previously described.[15] This test measures the number of limb slips over a total walking distance of 2 m on a grid. Independently, the presence of involuntary body shaking as a typical mid- to late-onset feature in *Plp1* transgenic mice was assessed individually per animal and documented for analysis.

Histology

Spinal cord was isolated at the fifth cervical vertebra and the corticospinal tract (CST) was used for electron microscopic (EM) and immunohistochemical (IHC) analyses. For EM, tissue was fixed in 4% PFA/2.5% glutaraldehyde in cacodylate buffer followed by epoxy resin (Serva) embedding for ultrathin cross sections. Myelinated and nonmyelinated axons were quantified at 4400× and 12000× magnifications. Samples for IHC were fixed in 4% PFA and paraffin-embedded for diaminobenzidine (DAB) or fluorescent stainings. The following antibody dilutions were used: OLIG2 1:200 (Dana-Farber Cancer Institute, Boston, MA, USA); CC1 1:50 (Oncogene, La Jolla, CA, USA); Glial Fibrillary Acidic Protein (GFAP) 1:5000 (Dako, Glostrup, Denmark); CD107b (MAC3) 1:400 (BD Pharmingen, San Diego, CA, USA); CD3 1:150 (Serotec, Bicester, United Kingdom).

mRNA quantification

Plp1 mRNA was measured in total brains upon isolation according to the manufacturer's instructions (Qiagen, Venlo, Netherlands). Quantitative RT-PCRs were designed as SYBR Green approaches and carried out using the Lightcycler 480 system (Roche Diagnostics, Pleasanton, CA, USA). Samples ran in triplicates, each within 10 μL total volume according to a two-step protocol. The comparative $\Delta\Delta Ct$ method was applied for quantitation of PCR product and *Plp1* mRNA expression was normalized against *Ppia* mRNA. Primer sequences are available upon request.

GSH measurements

Total cellular GSH levels were measured enzymatically as previously described[25,26] and normalized to cellular protein measured by the bicinchoninic acid-based method (Thermo Fisher Scientific, Waltham, MA, USA). Tissues from cerebral cortex and corpus callosum were isolated from fresh transversal 1 mm brain sections and GSH levels were measured individually in each sample. Primary oligodendrocytes were prepared from newborn mice (P0). After decapitation of the animals and removing the meninges, brains were washed twice with phosphate buffered saline (PBS) (with 200 U/mL penicillin and 200 μg/mL streptomycin). Using fine scissors, brains were minced into small pieces and incubated for 1 h at 37°C in suspension consisting of one part PBS and one part Hank's buffered saline solution with 25 mmol/L HEPES buffer, pH 7.2 containing 0.25% trypsin (Invitrogen, Waltham, MA, USA), and 40 μg/mL DNase (Sigma-Aldrich, St. Louis, MO, USA). Tissue was dissociated by shear forces using a 10 mL pipette and filtered through a tube-top cell strainer (pore size, 100 μm; BD Biosciences, San Jose, CA, USA). The resulting cell suspension was diluted four times with growth medium (Dulbecco's modified Eagle medium with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL fungizone, and 10% fetal bovine serum, Invitrogen). Samples were centrifuged (15 min at 280 g), resuspended in growth medium, filtered through a tube-top cell strainer (pore size, 70 μm), again centrifuged (15 min at 280 g), and resuspended in growth medium. Cells were plated onto tissue culture flasks (Falcon, 75 cm^2) coated with poly-ornithine (1 mg/mL). Each 75 cm^2 flask contained cells obtained from one brain in a final volume of 12 mL growth medium. The medium was changed every other day.

Ten days after plating oligodendrocyte precursors were isolated from these primary cultures. First, the loosely adherent cells were removed by preliminary shake off (for 2 h at 225 rpm) and subsequent medium replacement. Flasks were then shaken for additional 16–18 h at 300 rpm at 37°C. The medium containing the shaken-off cells was filtered through a tube-top cell strainer (pore size, 40 μm), added to a culture dish, and incubated at 37°C for 1 h to allow astrocytes, microglia, macrophages, and fibroblasts to adhere to the plastic. The majority of oligodendrocyte precursor cells remained unattached. Cells in suspension were centrifuged at 280 g for 10 min, resuspended in fresh Sato medium (supplemented with 2% B27, Thermo Fischer Scientific (Waltham, MA, USA); 1% horse serum; Penicillin/Streptavidin solution, Thermo Fischer Scientific (Waltham, MA, USA); Glutamax, Thermo Fischer Scientific (Waltham, MA, USA); Glucose; pyruvate; tri-iodo-thyronine; L-thyroxine), and plated onto poly-L-lysine precoated plastic petri dishes at a density of 550,000 cells per 35 mm petri dish.

For GSH measurements at day 5 after oligodendrocyte precursor isolation cells were washed twice with PBS. Then 200 μL of precooled PBS (4°C) was added, and the

adherent cells were harvested by scraping them off using a cell scraper. The cell suspension was transferred into a 1.5 mL tube, 100 μL 10% sulfosalicylic acid (SSA) was added, and incubated for 10 min on ice. Cells were pelleted by centrifugation at 11,300 g for 10 min. The supernatant was transferred to a fresh 1.5 mL tube containing 24 μL TEA solution. GSH levels were measured in three technical replicates of a pool of $n = 7$ wildtype and $n = 10$ *Plp1* transgenic mice.

Statistics

Statistical analysis was performed by using GraphPad Prism software (GraphPad Software, Inc.: La Jolla, CA, USA). Statistical differences between two groups were determined using the two-tailed Student's *t*-test. Only for statistical analysis of body shaking, Fischer′s exact test was used.

Results

The present study was performed in *Plp1* transgenic mice applying a curcumin-containing diet ad libitum for 10 consecutive weeks. *Plp1* transgenic and wild-type mice receiving normal chow served as controls (Fig. 1A). At study start, 3-week-old *Plp1* transgenic mice already showed an increased number of limb slips over a walking distance of 2 m compared to wild-type controls (9.0 ± 2.0 and 6.9 ± 2.4, $P < 0.05$) (Fig. 1B). Ten weeks later, the difference between *Plp1* transgenic mice and wild-type controls was further enhanced (15.3 ± 2.5 and 8.6 ± 3.0, $P < 0.001$). However, *Plp1* transgenic mice treated with curcumin for 10 weeks had improved motor capabilities compared to *Plp1* transgenic controls (11.3 ± 2.6, $P < 0.01$) although wild-type levels were not reached ($P < 0.05$; Fig. 1C). Involuntary body shaking was generally not observed at the start of the study in all investigated groups including *Plp1* transgenic and wild-type mice (data not shown) but was present at the end of the study in 42% of *Plp1* transgenic controls. At this time point, wild-type mice still did not show any body shaking (0%, $P < 0.001$). Curcumin diet for 10 weeks successfully diminished the body shaking within the *Plp1* transgenic group to 11% so that the body shaking was statistically indistinguishable from wild-type mice (Fig. 1D).

Representative electron micrographs of the CST indicated that considerably fewer axons were myelinated in *Plp1* transgenic controls when compared to wild-type mice (Fig. 2A and B) while the number appeared increased in *Plp1* transgenic mice treated with curcumin diet for 10 weeks (Fig. 2C). The quantification confirmed the loss of myelinated axons in *Plp1* transgenic controls compared to wild-type mice (198 ± 49 and 839 ± 50, $P < 0.001$), and importantly curcumin diet increased the number of myelinated axons in *Plp1* transgenic mice (272 ± 55, $P < 0.01$; Fig. 2D). Interestingly, the number of nonmyelinated axons in *Plp1* transgenic mice was statistically not altered by curcumin diet (489 ± 92 and 740 ± 108 not significant; Fig. 2B1, C1 and E). Wild-type controls displayed almost no ummyelinated axons (7 ± 2, $P < 0.01$; Fig. 2E). Also, curcumin diet had no effects on the g-ratio as a measure for myelin sheath thickness when compared to *Plp1* transgenic controls (0.98 ± 0.01 and 0.97 ± 0.01, not significant). However and as expected, *Plp1* transgenic controls showed higher g-ratios indicating hypomyelinated axons compared to wild-type mice (0.84 ± 0.02, $P < 0.05$; Fig. 2F). When all *Plp1* transgenic mice (curcumin and control group) were taken into consideration, we observed an inverse correlation between the number of myelinated axons and the number of limb slips ($P < 0.001$; Fig. 2G). In the optic nerve, one of the most severely affected central nervous system (CNS) regions in *Plp1* transgenic mice characterized by almost complete amyelination of axons at adult ages, we did not observe any obvious curcumin effects (data not shown).

Oligodendrocytes (identified by their immunopositivity for the marker OLIG2 staining both, mature myelinating oligodendrocytes and progenitors or CC1 which stains only mature myelinating oligodendrocytes), activated microglia (MAC3), lymphocytes (CD3), and astrocytes (GFAP) were quantified in the CST area or the CST including the dorsal tracts (dorsal white matter); representative images are shown in Figure 3A and F. *Plp1* transgenic mice displayed fewer OLIG2-positive cells when compared to wild-type controls (7 ± 2 and 16 ± 1, $P < 0.001$), which were restored to wild-type levels by the curcumin diet (13 ± 4, $P < 0.01$) (Fig. 3B). Similarly, CC1-positive cells were reduced in *Plp1* transgenic mice compared to wild-type controls (22 ± 2 and 28 ± 2, $P < 0.01$), which were also restored to the wild-type level after chronic curcumin diet in *Plp1* transgenic mice (30 ± 6, $P < 0.01$) (Fig. 3C). The number of MAC3-immunopositive cells was increased in *Plp1* transgenic mice compared to wild-types (92 ± 37 and 5 ± 2, $P < 0.01$). Curcumin diet reduced the number of activated macrophages (34 ± 16, $P < 0.01$) although not reaching wild-type levels ($P < 0.05$; Fig. 3D). CD3-immunopositive cells were never observed in wild-type mice but were present in *Plp1* transgenic controls (0 ± 0 and 3 ± 2, $P < 0.05$). Curcumin diet in *Plp1* transgenic mice also reduced the number of CD3-immunopositive cells (0.6 ± 0.2, $P < 0.01$) to wild-type levels (not significant; Fig. 3E). Finally, curcumin diet in *Plp1* transgenic mice reduced the number of GFAP-positive cells when compared to the *Plp1* transgenic controls (38 ± 2 and

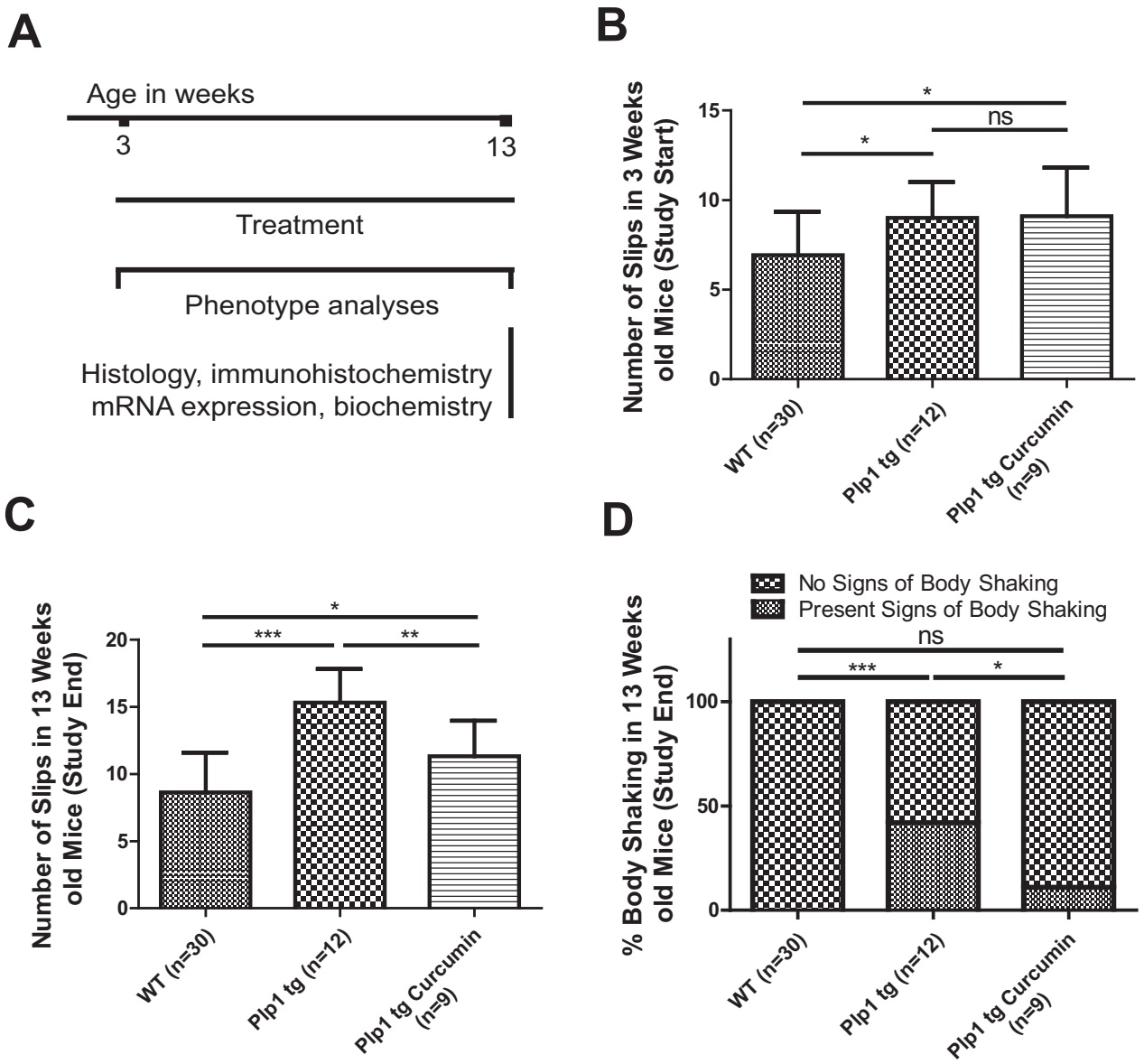
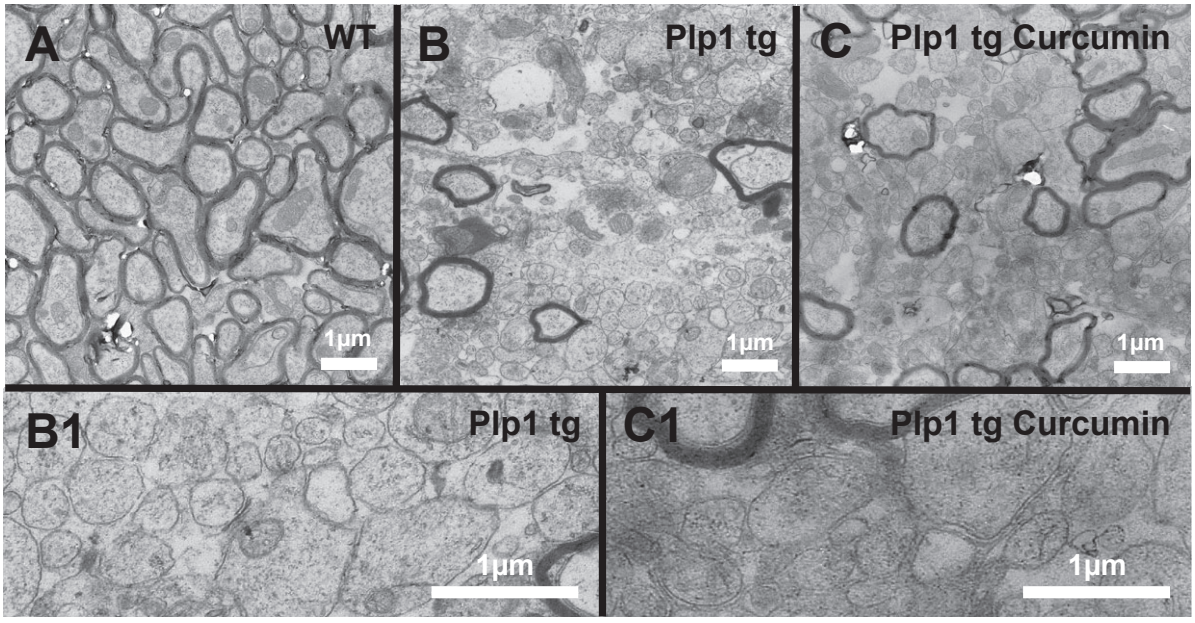
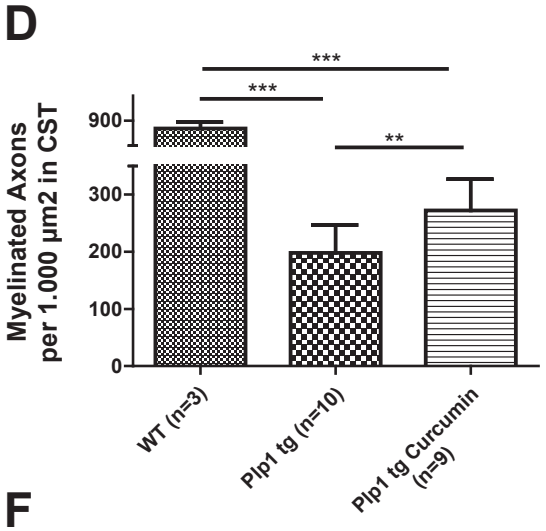


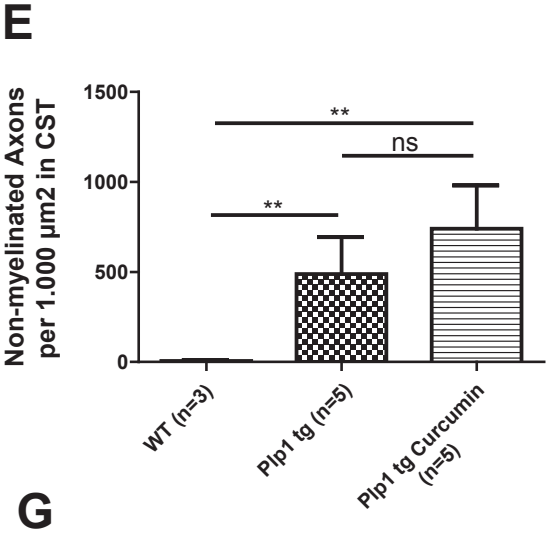
Figure 1. Overview on the experimental treatment in *Plp1* transgenic mice (A). Number of slips over a 2 m walking distance was increased at study start in both *Plp1* transgenic groups compared to wild-type controls (B) and was decreased in *Plp1* transgenic mice after chronic curcumin treatment (C). Also, curcumin dietary reduced present signs of body shaking in *Plp1* transgenic mice (D). WT, wildt-ype; *Plp1* tg, *Plp1* transgenic homozygous line #72; mean ± SD; ns, not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

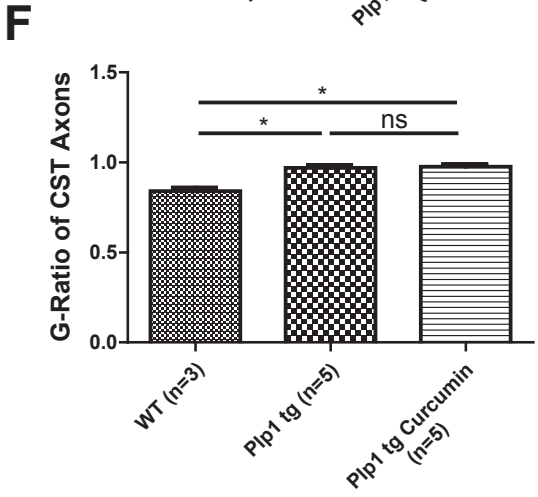
53 ± 6, $P < 0.05$) which still showed astrocytosis exceeding wild-type levels (26 ± 4, $P < 0.05$; Fig. 3G).

As expected, curcumin diet for 10 consecutive weeks had no influence on the 1.8-fold overexpression of *Plp1* mRNA in the CNS of *Plp1* transgenic mice (curcumin treated 1.8 ± 0.18 and *Plp1* transgenic controls 1.8 ± 0.2, not significant; Fig. 4A). Interestingly, primary oligodendrocytes from *Plp1* transgenic mice displayed elevated levels of cellular GSH as a potential indicator of oxidative misbalance when compared to wild-type controls (0.076 ± 0.005 and 0.063 ± 0.003, $P < 0.05$; each three technical replicates of $n = 10$ and 7 pools, respectively; Fig. 4B). Elevated GSH levels were also measured in the corpus callosum of *Plp1* transgenic mice compared to wild-type controls (0.027 ± 0.008 and 0.014 ± 0.003, $P < 0.01$). Strikingly, curcumin diet for 7 weeks reduced the increased GSH levels in the corpus callosum (white matter) of *Plp1* transgenic mice (0.018 ± 0.002, $P < 0.05$)









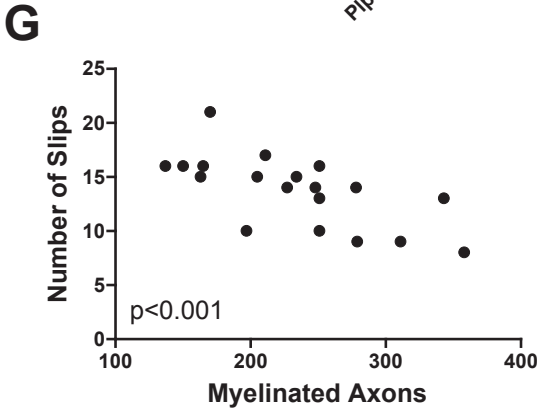


Figure 2. Electronmicrographs of wild-type (A) and *Plp1* transgenic controls (B) and curcumin-treated *Plp1* transgenic mice showing myelinated axons at 4400× (C) and pathologically amyelinated axons at 12000× magnification in the spinal cord (B1 and C1). Chronic curcumin-enriched diet preserved myelinated axons (D) without effects on the number of amyelinated axons (E) and the myelin sheath thickness in *Plp1* transgenic mice (F). Myelinated axons correlated inversely with the number of slips over a 2 m walking distance (G). WT, wildtype; *Plp1* tg, *Plp1* transgenic homozygous line #72; mean ± SD; ns, not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, scale bar = 1 μm.

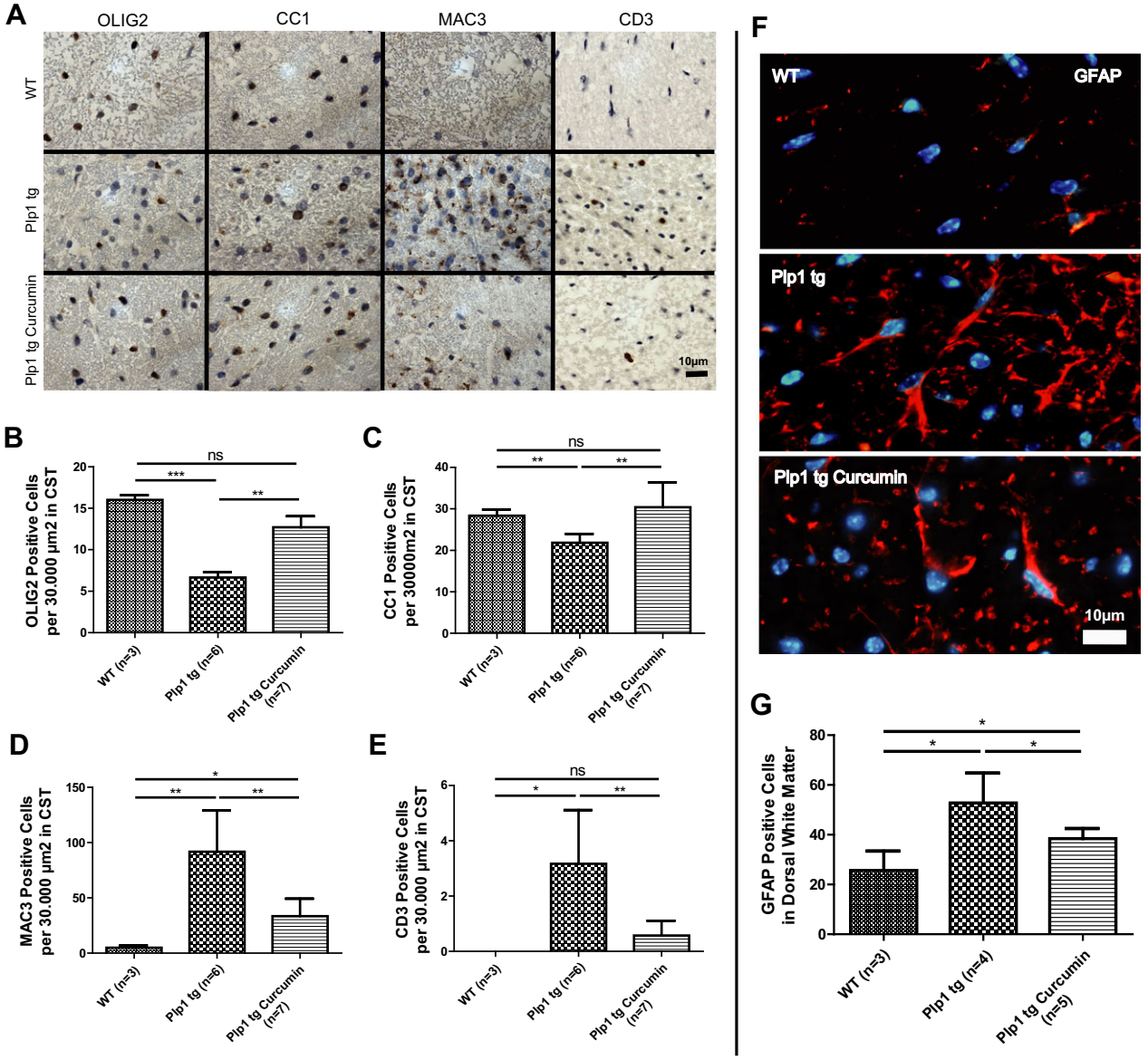


Figure 3. Lightmicroscopic images of wild-type and *Plp1* transgenic controls and curcumin-treated *Plp1* transgenic mice showing OLIG2 and CC1 (oligodendrocytes), MAC3 (microglia) and CD3 (lymphocytes) (A; DAB staining) as well as GFAP-immunopositive cells (astrocytes) in the spinal cord (F; fluorescent staining). Curcumin diet preserved the number of oligodendrocytes (B and C), reduced the microgliosis (D), the lymphocyte infiltration (E) and astrocytosis in *Plp1* transgenic mice (G). WT, wildtype; *Plp1* tg, *Plp1* transgenic homozygous line #72; mean ± SD; ns, not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, scale bar = 10 μm.

although not reaching wild-type values ($P < 0.05$; Fig. 4C). In contrast, GSH levels in the cerebral cortex (gray matter) did not differ between wild-type, untreated, and curcumin-treated *Plp1* transgenic mice (0.023 ± 0.005, 0.027 ± 0.010, and 0.021 ± 0.003, not significant; Fig. 4D).

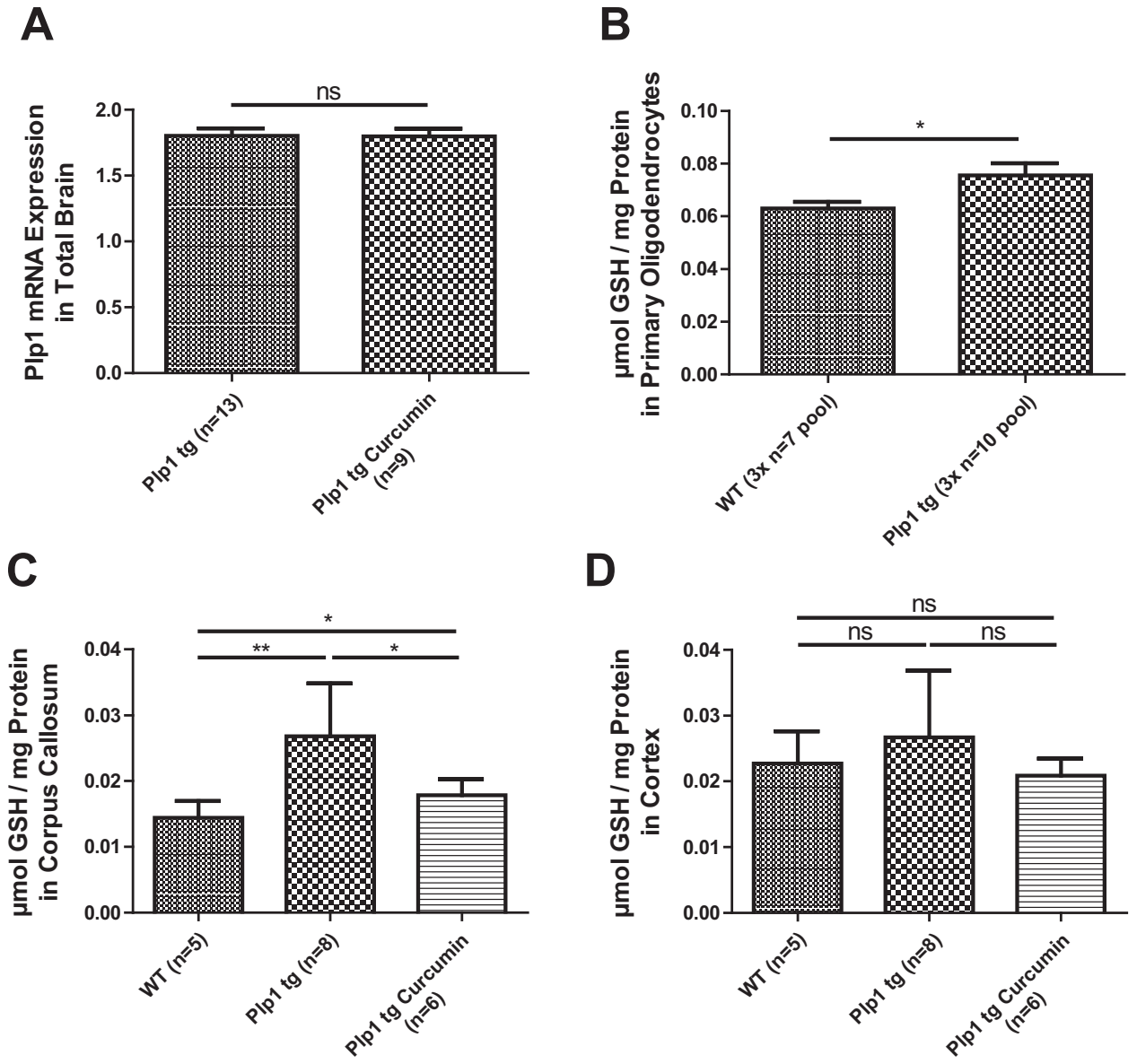


Figure 4. Curcumin treatment did not reduce the 1.8-fold *Plp1* mRNA overexpression in *Plp1* transgenic mice (A). However, we observed elevated glutathione (GSH) levels in primary oligodendrocytes of *Plp1* transgenic mice (B) and in the white matter of *Plp1* transgenic controls which were corrected by chronic curcumin diet (C). GSH levels were not increased in gray matter tissue of *Plp1* transgenic mice and not altered by chronic curcumin treatment. WT, wildtype; *Plp1* tg, *Plp1* transgenic homozygous line #72; mean ± SD; ns, not significant; $*P < 0.05$, $**P < 0.01$.

Discussion

In the present study, we tested whether the dietary supplement curcumin ameliorates the phenotype of *Plp1* transgenic mice, an established animal model for the most common form of PMD caused by duplications of the *PLP1* gene. Curcumin diet improved the motor phenotype and preserved myelinated axons in moderately affected CNS of *Plp1* transgenic mice without having significant effects on the number of pathologically unmyelinated axons. Importantly, the motor phenotype significantly correlated with the number of myelinated axons which strongly suggests the preservation of the myelinated axons as the major cause for the functional improvement. Curcumin diet also reduced astrocytosis, microgliosis, and lymphocyte infiltration as well-known disease hallmarks in *Plp1* transgenic mice. However, the reduced myelin sheath thickness was not corrected by

curcumin diet and thus may not contribute to the improvement of the motor capabilities. Curcumin did also not reduce *Plp1* mRNA overexpression. However, we observed increased GSH levels in primary *Plp1* overexpressing oligodendrocytes and its normalization in the white matter (corpus callosum) of *Plp1* transgenic mice. In contrast, the GSH levels in gray matter (cerebral cortex) which contains comparatively fewer oligodendrocytes were not different in *Plp1* transgenic controls from wild-type levels and also not affected by curcumin diet. This well reflects the white matter pathology in PMD. GSH is the most important cellular antioxidant in the brain.[27] The increased GSH levels may suggest that *Plp1* overexpressing oligodendrocytes in the white matter of the CNS undergo an oxidative misbalance. This may be compensated by an upregulation of the antioxidant response machinery giving rise to the observed concentration of GSH. Alternatively, alterations in the metabolic activity of the white matter may reduce the demand for GSH. Despite the usage of differently formulated curcumin formulations and analysis of different regions in the CNS, this may explain why we observed a higher number of mature myelinating oligodendrocytes after curcumin diet. In summary, we demonstrated that curcumin diet successfully improved the functional phenotype of *Plp1* transgenic mice by preservation of myelinated axons. We hypothesize that overexpression of *Plp1* in oligodendrocytes may cause an oxidative misbalance in the white matter which may be counteracted by curcumin – a known antioxidant substance.[28] This may cause the observed rescue of the loss of oligodendrocytes and enable the remaining oligodendrocytes to better maintain the integrity of myelinated axons, thereby alleviating the severity of motor capability impairment.[29] The reduced astrocytosis, microgliosis, and lymphocyte infiltration may be best explained as a secondary effect since they were previously observed in two different experimental strategies in *Plp1* transgenic mice either by promoting normal intracellular trafficking of the cholesterol-associated PLP-protein or by reducing *Plp1* mRNA overexpression.[14,15]

Curcumin may serve as a potential antioxidant therapy of PMD caused by *PLP1* gene duplication. However, despite numerous encouraging preclinical results using curcumin in animal models for neurodegenerative diseases, the proof-of-concept in controlled randomized clinical studies by the application of this molecule to human patients has still to be provided.

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Author Contributions

M. W. S. and H. B. W. designed the project. D. B. E. and T. P. equally contributed to the experiments. T. P., H. B. W., K.-A. N., A. M., and M. W. S. wrote the manuscript. T. N. measured myelin sheath thickness, C. M. K. supervised the primary oligodendrocyte experiments, P. A. and A. M. contributed to the glutathione and R. M. S. to the electron microscopic analysis.

Conflict of Interest

None.

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