Genetic dissection of oligodendroglial and neuronal Plp1 function in a novel mouse model of spastic paraplegia type 2

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1 INTRODUCTION

Spastic paraplegia (SPG) comprises a genetically heterogeneous group of rare disorders characterized by progressive degeneration of the long descending axons of upper motor neurons in the central nervous system (CNS). Causative mutations affect at least 41 genes (Fink, 2013). For the vast majority of SPG types, neuronal dysfunction is thought to be the primary pathologic cause in patients and respective mouse models. However, oligodendroglial dysfunction has also been suggested as a primary cause, at least in SPG2, SPG35, and SPG44, which are defined by mutations of the genes for proteolipid protein (PLP), fatty acid 2-hydroxylase (FA2H), and connexin 47 (GJC2/CX47), respectively. Expression of these genes is strongly enriched in oligodendrocytes compared with other neural cell types.

For PLP1, the clinical spectrum associated with mutations of the X-chromosomal gene ranges from spastic paraplegia (in SPG2) to the connatal form of Pelizaeus-Merzbacher Disease (PMD), a lethal leukodystrophy caused by missense mutations (Dimou et al., 1999; Duncan, 2005; Saugier-Veber et al., 1994; Vanderver et al., 2015; Woodward, 2008). Although PLP and its splice isoform DM20 are abundant myelin proteins (de Monasterio-Schrader et al., 2012; Jahn, Tenzer, & Werner, 2009), they appear largely dispensable for myelination in mice (Klugmann et al., 1997). However, PLP-deficiency results in subtle changes in glutamate-dependent migration of oligodendrocyte progenitor cells (Harlow, Saul, Komuro, & Macklin, 2015), the rate of myelin biogenesis (de Monasterio-Schrader et al., 2013; Yool et al., 2001), and in myelin ultrastructure (Duncan, Hammang, & Trapp, 1987; Klugmann et al., 1997; Möbius, Patzig, Nave, & Werner, 2008; Patzig, Kusch, et al., 2016). Moreover, PLP is required for the incorporation into CNS myelin of cholesterol (Werner et al., 2013) and the deacetylase sirtuin 2 (Werner et al., 2007).

The most prominent neuropathological feature of PLP-deficient (Plp1<sup>−/−</sup>) mice is the progressive degeneration of axons particularly in long myelinated tracts, which makes Plp1<sup>−/−</sup> mice a genuine model of SPG2 caused by loss-of-function mutations (Garbern et al., 2002; Griffiths et al., 1998). A similar mouse model with significant residual CNS myelination is the Plp<sup>−<sup>−/−</sup></sup> mouse (Edgar, McLaughlin, Barrie, et al., 2004; Schneider et al., 1992), defined by the same I186T
missense mutation as the first human family diagnosed with SPG2 (Saugier-Veber et al., 1994). It is generally assumed that neuropathological impairments in SPG2 and PMD, including axonal pathology, are caused by the lack of PLP from oligodendrocytes. Yet, direct proof has been difficult because all previous *in vivo* analyses were performed in mice lacking expression of PLP from all cells. More importantly, low but significant neuronal expression of PLP has been documented, for example by using reporter gene expression in transgenic mice, *Plp*-mRNA *in situ* hybridization, PLP/DM20 immunolabeling, and by transcriptional and translational profiling (see overview in Table 1 and references therein). Together, these and other publications (Campagnoni & Skoff, 2001) showing that PLP-expression is not exclusive to oligodendrocytes have led to the alternative hypothesis that the absence of neuronal PLP-expression causes the axonal degeneration phenotype in SPG2 patients and the respective mouse models (Fulton et al., 2011; Jacobs, Bongarzone, Campagnoni, & Campagnoni, 2003; Sarret et al., 2010).

In this study, we have directly addressed the relevance of neuronal *Plp1* gene expression and its possible contribution to the axonal pathology in PLP-deficient mice. We generated a novel mouse model that allows cell-type specific recombination of a floxed *Plp* allele in neurons and oligodendrocytes. The results of their neuropathological analysis cannot support the hypothesis that neuronal dysfunction of *Plp1* underlies an axonal pathology but confirms a PLP-dependent role of oligodendrocytes in sustaining axonal function and preservation.

### Materials and Methods

#### 2.1 Mouse models

Embryonic stem cells (ES) harboring an engineered allele of the *Plp* gene were acquired from the European Conditional Mouse Mutagenesis Program (Eucomm). ES were microinjected into blastocysts derived from FVB mice, and embryos were transferred to pseudo-pregnant foster mothers, yielding 13 chimeric males. For ES clone EPD0160–2-D10, germline transmission was achieved upon breeding with C57BL/6N females, yielding mice harboring the *Plp*<sup>lacZ-neo</sup> allele. The lacZ-neomycin resistance cassette was excised in vivo upon interbreeding with mice expressing FLIP recombinase (129S4/SvJaeSor-Gt(ROSA)26Sortm1(Fpl1)Dym/J; backcrossed into C57BL/6N), yielding mice carrying a *Plp<sup>lox<sub>neo</sub></sup>* allele in which exon 3 of the *Plp* gene is flanked by loxp-sites. Routine genotyping of the *Plp<sup>lox<sub>neo</sub></sup>* allele was performed by genomic polymerase chain reaction (PCR) using primers 24460 (labeled P1 in Figure 1c) (5'-GACATAGCCC TCAGTGTTCAGG), 24461 (P2) (5'-

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**Table 1** Previous studies reporting the detection of PLP/*Plp* gene products in neurons.

<table>
<thead>
<tr>
<th>Detection level</th>
<th>Technique</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA</td>
<td>PCR on neuronal cell lines</td>
<td>Rat, mouse</td>
<td>Ikenaka, Kagawa, &amp; Mikoshiba (1992)</td>
</tr>
<tr>
<td>mRNA, protein</td>
<td>RPA, RT-PCR, ISH, IHC</td>
<td>Mouse</td>
<td>Bongarzone et al. (1999)</td>
</tr>
<tr>
<td>Protein, gene activity</td>
<td><em>Plp</em>&lt;sup&gt;EGFP&lt;/sup&gt; reporter line</td>
<td>Myelin deficient (md) rat, mouse</td>
<td>Miller et al. (2003)</td>
</tr>
<tr>
<td>Protein</td>
<td>IHC</td>
<td>Mouse</td>
<td>Jacobs et al. (2003)</td>
</tr>
<tr>
<td>Gene activity</td>
<td><em>Plp</em>&lt;sup&gt;EGFP&lt;/sup&gt; BAC (gensat.org)</td>
<td>Mouse</td>
<td>Gong et al., (2003)</td>
</tr>
<tr>
<td>Protein</td>
<td>IHC</td>
<td>Mouse</td>
<td>Jacobs, Bongarzone, Campagnoni, &amp; Campagnoni (2004)</td>
</tr>
<tr>
<td>Protein</td>
<td>IHC ('PLP-like epitopes')</td>
<td>Mouse, cow, owl, frog, cichlid, shark</td>
<td>Greenfield et al. (2006)</td>
</tr>
<tr>
<td>mRNA</td>
<td>ISH (mouse.brain-map.org)</td>
<td>Mouse</td>
<td>Lein et al. (2007)</td>
</tr>
<tr>
<td>Translated mRNA</td>
<td>Various neuronal BAC tg lines, TRAP</td>
<td>Mouse</td>
<td>Doyle et al. (2008)</td>
</tr>
<tr>
<td>Enhancer activity</td>
<td><em>Plp</em>&lt;sup&gt;EGFP-lacZ&lt;/sup&gt; reporter line</td>
<td>Mouse</td>
<td>Tuason et al. (2008)</td>
</tr>
<tr>
<td>Gene activity, mRNA, protein</td>
<td><em>Plp</em>&lt;sup&gt;EGFP&lt;/sup&gt; reporter line, ISH, IHC</td>
<td>Mouse</td>
<td>Miller, Kangas, &amp; Macklin (2009)</td>
</tr>
<tr>
<td>mRNA, protein</td>
<td>ISH, IHC</td>
<td>Human</td>
<td>Sarret et al. (2010)</td>
</tr>
<tr>
<td>Gene activity, protein</td>
<td><em>Plp</em>&lt;sup&gt;EGFP&lt;/sup&gt; reporter line, IHC, Patch clamp</td>
<td>Mouse</td>
<td>Fulton et al. (2011)</td>
</tr>
<tr>
<td>Gene activity</td>
<td><em>Plp</em>&lt;sup&gt;Cre&lt;/sup&gt; crossed with reporter lines, IHC, X-Gal</td>
<td>Mouse</td>
<td>Michalski, Anderson, Beauvais, De Repentigny, &amp; Kothary (2011)</td>
</tr>
<tr>
<td>mRNA</td>
<td>Immunopanning, RNA-Seq (web.stanford.edu/group/barres_lab/brain_rnaseq.html)</td>
<td>Mouse</td>
<td>Zhang et al. (2014)</td>
</tr>
<tr>
<td>mRNA</td>
<td>Single cell RNA-Seq (linnarssonlab.org/cortex)</td>
<td>Mouse</td>
<td>Zeisel et al. (2015)</td>
</tr>
</tbody>
</table>

Given are detection level, method and species. BAC, bacterial artificial chromosome; FACS, Fluorescence-activated cell sorting; IHC, immunohistochemistry; ISH, *in situ* hybridization; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; RNA-Seq, whole-transcriptome shotgun sequencing; RPA, ribonuclease protection assay; TRAP, translating ribosome affinity purification.
To inactivate expression of PLP in excitatory projection neurons, exon 3 was excised in vivo upon appropriate interbreedings of \textit{Plpflox} mice with mice expressing Cre recombinase under control of the \textit{Nex/NeuroD6} promoter (Goebbels et al., 2006). Genotyping of the \textit{Nex/NeuroD6} allele was with primers 3131 (5'-GAGTCCTGGA ATCAGTCTTTC), 3132 (5'-AGAATGTGGAGTAGGGTGAC), and 2409 (5'-CCGCATAACCGTGAAACAG). To inactivate expression of PLP in myelinating cells, exon 3 was excised in vivo upon interbreeding \textit{Plpflox} mice with mice expressing Cre recombinase under control of the \textit{Cnp} promoter (Lappe-Siefke et al., 2003). Genotyping of the \textit{Cnp} allele was with primers 2016 (5'-GCCTTAAACCTGTCCATCTC), 7315 (5'-GACATGCGA), and 2017 (5'-AGGATATGTATGACACAGG).

**FIGURE 1** Detection of \textit{Plp} mRNA in neurons imposes cell type-specific analysis. (a) By RNA-Seq of cells immunopanned from mouse cortex, \textit{Plp} mRNA (the SPG2 disease gene) is detected in neurons at low but significant level. The cell-type specific abundance was extracted from Zhang et al. (2014). Mean with SEM; \( n = 2 \); stippled line indicates a threshold of 0.8 FPKM. For comparison mRNA-abundance of \textit{Fa2h} and \textit{Gjc} (mutated in SPG35 and SPG44, respectively) is below threshold. Note that neuronal \textit{Plp} expression was previously reported in several publications (see Table 1). (b) Expectedly, the same dataset shows that \textit{Plp}, \textit{Fa2h}, and \textit{Gjc} mRNAs are highly abundant in oligodendrocytes. Mean with SEM; \( n = 2 \). (c) Targeting scheme for the conditional inactivation of the \textit{Plp} gene. Exon 3 of the \textit{Plp}\textsuperscript{loxP} allele is flanked by loxP-sites for Cre-mediated recombination. Positions of PCR primers (P1, P2, P4) are indicated. (d, e) Genomic PCR of DNA isolated from mouse brains at postnatal day 21 detects the \textit{Plp}\textsuperscript{WT} allele (591 bp product of P1 and P2), the non-recombined \textit{Plp}\textsuperscript{loxP} allele (683 bp product of P1 and P2) as well as the recombined \textit{Plp}\textsuperscript{loxP} allele (426 bp product of P1 and P4). Note that recombination of the \textit{Plp}\textsuperscript{loxP} allele is observed after expression of Cre under control of the oligodendroglial \textit{Cnp} promoter (d) or the neuronal \textit{Nex} promoter (e). (d, e): Genomic PCR of brain DNA detecting the \textit{Cnp}\textsuperscript{Cre} (d') or \textit{Nex}\textsuperscript{Cre} (e') allele. (f): qRT-PCR to determine the abundance of \textit{Plp} transcripts in mouse brains at P75. Note that \textit{Plp} mRNA is more abundant in \textit{Plp}\textsuperscript{loxP/loxP}\textit{Cnp}\textsuperscript{Cre/WT} compared with \textit{Plp}\textsuperscript{null/WT} mice, probably reflecting nonoligodendroglial expression. Mean with SEM; \( n = 4–6 \); two-tailed unpaired \( t \) test; \( p < .001 \) (**). (g): Immunoblot of mouse brains homogenized at postnatal day 21. PLP/DM20 was virtually undetectable in \textit{Plp}\textsuperscript{loxP/loxP}\textit{Cnp}\textsuperscript{Cre/WT} brains. Actin was detected as a loading control. (h): Confocal microscopic analysis of mouse brains immunolabeled at age 26 weeks detects intense MBP labeling (red) as exemplified for a single internode in the cortex. PLP-immunolabeling (green) essentially co-distributed with MBP in \textit{Plp}\textsuperscript{loxP/Y} mice but was virtually undetectable in \textit{Plp}\textsuperscript{loxP/Y}\textit{Cnp}\textsuperscript{Cre/WT} mice [Color figure can be viewed at wileyonlinelibrary.com]
CCAGCCCTT TTATTACCAC), 4193 (5'–CTGGGAAAT GCTTCT GTCCG), and 4192 (5'–CAGGGTGTTA TAAGCAATCC). Plp<sup>−/−</sup> mice lacking expression of PLP in all cells were described previously (Klugmann et al., 1997). Genotyping of the Plp allele was with primers 1864 (5'–TTGGCGCGCA ATGCGTGAC), 2729 (5'–GGAGAGGAGG AGGGAACAG), and 2731 (5'–TACGGTTTTC GGTGACTTGTG). Mice were bred and kept in the animal facility of the Max Planck Institute of Experimental Medicine with a 12 hr light/dark cycle and 2–5 animals per cage. All experiments were performed in accordance with the German animal protection law.

2.2 | Quantitative reverse transcriptase PCR

Quantitative reverse transcriptase PCR (qRT-PCR) was essentially as described (de Monasterio-Schrader et al., 2013). Half brains of P75 old male mice were homogenized in 0.32 M sucrose with protease inhibitor (Complete Mini, Roche; Basel, Switzerland) using an Ultraturrax homogenizer (IKA T10 Basic; IKA; Staufen, Germany). 200 μl of the homogenate was directly transferred to lysis buffer (RLT; RNeasy Miniprep kit; Qiagen; Hilden, Germany) with β-mercaptoethanol. RNA extraction and purification was performed using the RNeasy Miniprep kit (Qiagen; Hilden; Hilden, Germany) by a method described (de Monasterio-Schrader et al., 2013). Half brains of P75 old male mice were homogenized in 0.32 M sucrose with protease inhibitor (Complete Mini, Roche; Basel, Switzerland) using an Ultraturrax homogenizer (IKA T10 Basic; IKA; Staufen, Germany). 200 μl of the homogenate was directly transferred to lysis buffer (RLT; RNeasy Miniprep kit; Qiagen; Hilden, Germany) with β-mercaptoethanol. RNA extraction and purification was performed using the RNeasy Miniprep kit (Qiagen; Hilden; Hilden, Germany). cDNA was synthesized using random nonamer primers and Superscript II (Invitrogen). The pipetting robot ePMotion 5075 (Eppendorf; Hamburg, Germany) was used for pipetting, and the qRT-PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems; Warrington, UK) and the Light Cycler 480II (Roche). mRNA abundance was analyzed in relation to the mean of the duplicates, using the 2<sup>−ΔΔCT</sup> method.
For quantification of relative size of area occupied by MAC3-immunopositive microglia in Figure 3l, p values are as follows: Plpflox/Y vs. Plp flox/Y*NexCre/WT p > .9999, Plpflox/Y vs. Plp flox/Y*CnpCre/WT p < .0001, Plpflox/Y vs. Plp null/Y p = .0105, PlpxY*NexCre/WT vs. PlpxY*CnpCre/WT p = .0004, PlpxY*NexCre/WT vs. PlpxY*CnpCre/WT p < .0001, PlpxY*NexCre/WT vs. Plp null/Y p = .0450, PlpxY*NexCre/WT vs. PlpxY*CnpCre/WT p = .7092, PlpxY*CnpCre/WT vs. PlpxY*CnpCre/WT p = .1226, PlpxY*CnpCre/WT vs. PlpxY*CnpCre/WT p = .0108.

For quantification of relative size of area occupied by GFAP-immunopositive astrocytes in Figure 4f, p values are as follows: Plpflox/Y vs. Plp flox/Y*NexCre/WT p = .9972, Plpflox/Y vs. Plp flox/Y*CnpCre/WT p < .0001, Plpflox/Y vs. Plp null/Y p = .2115, PlpxY*NexCre/WT vs. PlpxY*CnpCre/WT p < .0001, PlpxY*NexCre/WT vs. Plp null/Y p = .0213, PlpxY*CnpCre/WT vs. PlpxY*CnpCre/WT p < .0001, PlpxY*NexCre/WT vs. Plp null/Y p = .0684, PlpxY*CnpCre/WT vs. PlpxY*CnpCre/WT p = .8297, PlpxY*NexCre/WT vs. Plp null/Y p = .0004, PlpxY*CnpCre/WT vs. PlpxY*CnpCre/WT p < .0001, PlpxY*CnpCre/WT vs. PlpxY*CnpCre/WT p = .0001.

For quantification of CD3-immunopositive T-cells in Figure 5f, p values are as follows: Plpflox/Y vs. Plp flox/Y*NexCre/WT p > .9999, Plpflox/Y vs. Plp flox/Y*CnpCre/WT p < .0001, Plpflox/Y vs. Plp null/Y p = .2115, PlpxY*NexCre/WT vs. PlpxY*CnpCre/WT p < .0001, PlpxY*NexCre/WT vs. Plp null/Y p = .0213, PlpxY*CnpCre/WT vs. PlpxY*CnpCre/WT p < .0001, PlpxY*NexCre/WT vs. Plp null/Y p = .0684, PlpxY*CnpCre/WT vs. PlpxY*CnpCre/WT p = .8297, PlpxY*NexCre/WT vs. Plp null/Y p = .0004, PlpxY*CnpCre/WT vs. PlpxY*CnpCre/WT p < .0001, PlpxY*CnpCre/WT vs. PlpxY*CnpCre/WT p = .0001.

For quantification of APP-immunopositive axonal spheroids in Figure 6f, p values are as follows: Plpflox/Y vs. Plp flox/Y*NexCre/WT p > .9999, Plpflox/Y vs. Plp flox/Y*CnpCre/WT p < .0001, Plpflox/Y vs. Plp null/Y p = .0213, PlpxY*NexCre/WT vs. PlpxY*CnpCre/WT p < .0001, PlpxY*NexCre/WT vs. Plp null/Y p = .0684, PlpxY*CnpCre/WT vs. PlpxY*CnpCre/WT p = .8297, PlpxY*NexCre/WT vs. Plp null/Y p = .0004, PlpxY*CnpCre/WT vs. PlpxY*CnpCre/WT p < .0001, PlpxY*CnpCre/WT vs. PlpxY*CnpCre/WT p = .0001.

2.5 | Confocal microscopy

Confocal microscopic analysis in Figure 1h was essentially performed as described (Patzig, Erwig, et al., 2016). In brief, immunolabeling was
FIGURE 3  Microglial activation after Plp-deletion in oligodendrocytes but not neurons. Representative light microscopic images of the hippocampal fimbria of 26-weeks-old mice immunolabeled for the microglial markers allograft-inflammatory factor (AIF1/IBA1) (a–e) and lysosomal-associated membrane protein 2 (LAMP2/MAC3) (g–k) and genotype-dependent quantification (f, l). Note that Plp$^{Rox/Y}$ (a) and Plp$^{Rox/Y}$Nex$^{Cre/WT}$ (b) brains do not display activated microglia while oligodendrogial (c, i) or systemic (d, e, j, k) deletion of Plp causes microgliosis. Microgliosis is enhanced in mice heterozygous for Cnp additional to PLP-deficiency (c, d, i, j). Scale bar = 10 μm. (f, l) Genotype-dependent quantification of the relative size of area occupied by IBA1-immunopositive (f) or MAC3-immunopositive (l) microglia in the fimbria. Mean with SEM; n = 3–7; one-way ANOVA with Tukey’s multiple comparison test; p < .05 (*), p < .01 (**), and p < .001 (***). [Color figure can be viewed at wileyonlinelibrary.com]
performed on 5 µm paraffin-embedded cross-sections of mouse brains. Brain slices were deparaffinized in xylene and rehydrated in a graded alcohol series. Tissue was permeabilized in citrate buffer. Blocking solution contained 10% goat serum and 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Primary antibodies were directed against PLP (clone aa3; 1:100; (Yamamura, Konola, Wekerle, & Lees, 1991)) and MBP (mouse; 1:1000, DAKO). Secondary antibodies were α-rat-Alexa488 and α-mouse-Alexa555 (dianova). Images were taken with a confocal microscope (Leica SP5) using the objective HCX PL APO CS 63.0×1.30 GLYC 21°C UV. An argon laser with the excitation of 488 nm was used to excite the Alexa488 fluorophore, and the emission was set to 500–526 nm. The laser DPSS 561 was used to excite the Alexa555 fluorophore, and the emission was set to 575–630 nm. LAS AF lite software was used to export images as TIF files.

3 | RESULTS

Considering that Plp expression was reported in neurons additional to oligodendrocytes (Table 1), we asked if neuronal expression is also reflected in an RNA-Seq database of brain cell types sorted from the cortex of mice (GEO dataset GSE52564). In the original publication of that dataset (Zhang et al., 2014), FPKM (fragments per kilobase of exon per million fragments mapped) values of 0.04 and 0.1 were chosen as minimum and conservative thresholds for a gene to be accepted as expressed in a particular cell type, respectively. Indeed, Plp mRNA exceeds the more rigorous threshold FPKM value of 0.8 in cortical neurons, astrocytes, and microglia, while that of Fa2h and Gjc2/Cx47 does not (Figure 1a). Expectedly, Plp, Fa2h, and Gjc2/Cx47 mRNAs are strongly enriched in newly formed and mature oligodendrocytes (Figure 1b). Together, Plp mRNA is enriched in but not exclusive to oligodendrocytes. Indeed, low-level expression of Plp has also been reported in neurons.

To investigate the possible pathobiological relevance of neuronal Plp expression in vivo, we have established a novel mouse model that allows cell type-dependent Cre-mediated recombination of the Plp gene. After germline transmission of the Plplox-neo allele and subsequent in vivo recombination of flipase recognition target (FRT) sites, we obtained mice harboring the Plplox allele in which exon 3 of the Plp gene is flanked by loxP-sites (Figure 1c). Male Plplox/Y and female Plplox/lox mice were born at expected frequencies, as determined by genotyping PCR (Figure 1d,e). By interbreeding female Plplox/lox with male Plplox/Y*CnpCre/WT mice expressing Cre in myelinating cells
(Lappe-Siefke et al., 2003), we generated males of the genotype $Plp^{lox/Y}Cnp^{Cre/WT}$ and $Plp^{lox/Y}$ control mice. We note that in $Cnp^{Cre}$ mice the integration of the open reading frame for Cre into the $Cnp$ locus at the same time results in a $Cnp$-deficient allele, and that $Cnp$ heterozygosity in $Cnp^{Cre/WT}$ mice per se causes late-onset neuropathology (Hagemeyer et al., 2012). Importantly, however, neuropathology is not yet measurable in $Cnp^{Cre/WT}$ mice at 8 months of age (Hagemeyer et al., 2012), that is, 2 months older than the age analyzed in this work. By interbreeding female $Plp^{lox/lox}$ with male $Plp^{lox/Y}Nex^{Cre/WT}$ mice (also termed NeuroD6Cre mice) (Goebbels et al., 2006), we obtained male mice of the genotype $Plp^{lox/Y}Nex^{Cre/WT}$ and control mice. NeuroD6 is a member of the NeuroD family of bHLH transcription factors; in accordance with its cellular expression pattern the $Nex$ promoter drives expression of Cre in most neocortical and hippocampal excitatory projection neurons and their immediate progenitors but not in glial cells (Belvindrah, Graus-Porta, Goebbels, Nave, & Muller, 2007; Bormuth et al., 2013; Brinkmann et al., 2008; Goebbels et al., 2006; Li, Fang, Fernandez, & Pleasure, 2013; Schwab et al., 1998; Wu et al., 2005). Importantly, Cre-mediated recombination of the $Plp$-allele was readily confirmed by genotyping PCR (Figure 1d,e), implying efficient Cre-mediated recombination of the $Plp^{lox}$ allele. By qRT-PCR, the abundance of $Plp$ mRNA was higher in the brains of $Plp^{lox/Y}Cnp^{Cre/WT}$ compared with $Plp^{null/Y}$ mice (Figure 1f), probably reflecting low but significant nonoligodendroglial expression of the $Plp$ gene. Yet by immunoblotting, PLP was virtually undetectable in the brains of $Plp^{lox/Y}Nex^{Cre/WT}$ and control mice. PLP labeling was readily detectable in the brains of $Plp^{null/Y}$ mice, where it colabeled with MBP (Figure 1h), a marker of compact myelin (Nawaz, Schweitzer, Jahn, & Werner, 2013). Conversely, PLP was undetectable in the brains of $Plp^{lox/Y}Cnp^{Cre/WT}$ mice (Figure 1h). As $Plp^{null/Y}$ mice represent a model of SPG2 with axonal pathology, microglial activation, astrogliosis, and the presence of T-lymphocytes (de Monasterio-Schrader et al., 2013), the brains of $Plp^{lox/Y}Nex^{Cre/WT}$, $Plp^{lox/Y}Cnp^{Cre/WT}$ and control mice were subjected to neuropathological

**FIGURE 5** Increased density of T-lymphocytes after $Plp$-deletion in oligodendrocytes but not neurons. (a–e) Representative light microscopic images of the hippocampal fimbria of 26-weeks-old mice immunolabeled for CD3 to detect T-lymphocytes. Note that $Plp^{lox/Y}$ (a) and $Plp^{lox/Y}Nex^{Cre/WT}$ (b) brains do not display T-lymphocytes, while oligodendroglial (c) or systemic (d, e) deletion of $Plp$. The apparently increased density of T-cells in $Plp^{null/Y}$ mice did not yield significance compared with $Plp^{lox/Y}$ mice at the analyzed age. Scale bar = 10 µm. (f) Genotype-dependent quantification of the number of CD3-immunopositive T-lymphocytes in the fimbria. Mean with SEM; n = 3–8; one-way ANOVA with Tukey’s multiple comparison test; $p < .001$ (***) [Color figure can be viewed at wileyonlinelibrary.com]
investigation by immunohistochemistry. For quantification, we analyzed
the hippocampal fimbria, a relatively uniform white matter tract through
which the long descending axons of Nex/NeuroD6-positive excitatory
neurons project (Fernandez-Miranda et al., 2008; Goebbels et al., 2006;
Li et al., 2013). To measure the extent of axonal pathology, we immuno-
labeled and quantified APP-immunopositive (APP\textsuperscript{1}) axonal spheroids
(or swellings) in the fimbria at the age of 26 weeks (Figure 2). In agree-
ment with previous reports, a significant number of axonal spheroids
were present in \textit{Plpnull/Y} mice (Griffiths et al., 1998; Werner et al., 2013),
and their density was further elevated by additional \textit{Cnp} heterozygosity in
\textit{Plpnull/Y*CnpCre/WT} mice (Edgar et al., 2009). Importantly, their density
was similar when comparing \textit{Plpnull/Y*CnpCre/WT} and \textit{Plpflox/Y*}
\textit{CnpCre/WT} mice. Strikingly, no APP\textsuperscript{1} axonal spheroids were found in \textit{Plpflox/Y*}
\textit{NexCre/WT} mice, as in \textit{Plpflox/Y} control mice. Together this implies that axonal
pathology is entirely caused by oligodendroglial but not by neuronal
recombination of \textit{Plp}.

Axonal damage is commonly associated with microgliosis, that is,
an increased density and “activation” of microglial cells (Sasaki, 2016).
We therefore immunolabeled microglia using antibodies against
allograft-inflammatory factor (AIF1/IBA1) (Figure 3a–e) and lysosomal-
associated membrane protein 2 (LAMP2/MAC3) (Figure 3g–k) and
quantified the relative area occupied by immunostaining (Figure 3f,l).
As quantified in the fimbria at the age of 26 weeks, the signal was
enhanced in \textit{Plpnull/Y} compared with \textit{Plpflox/Y} control mice and further
elevated by additional \textit{Cnp} heterozygosity in \textit{Plpnull/Y*CnpCre/WT} mice.
Importantly, the area of immunopositivity was not increased in \textit{Plpflox/Y*}
\textit{NexCre/WT} compared with \textit{Plpflox/Y} control mice. Thus, axonal damage and microglial activa-
tion correlate well as a consequence of oligodendroglial but not neuro-
nal recombination of \textit{Plp}. Interestingly, there was a good correlation
between IBA1 immunolabeling indicating microglial cell number and
MAC3 immunolabeling reflecting microglial activation.

The intercellular cross-talk in the diseased brain may include
microglia-dependent activation of astrocytes (Liddelow et al., 2017) as
well as astrocyte-dependent recruitment or activation of microglia (Jo
et al., 2017; Skripuletz et al., 2013). Indeed, both microgliosis and astro-
gliosis occur in various SPG/PMD patients and mouse models
enhanced axonal pathology, microgliosis, and presence of T-lymphocytes was striking that additional recombination in excitatory projection neurons does not. It is likely that the observed neuropathology affects all myelinated axons of long-projecting excitatory neurons in the CNS. To test this concept in an independent white matter tract, we quantified APP+ axonal spheroids in the corpus callosum at the age of 26 weeks (Figure 6). Indeed, similar to the hippocampal fimbria (Figure 2) a significant number of axonal spheroids were observed in the corpus callosum of PlpR137W mice; their density was further elevated by additional Cnp heterozygosity in PlpR137W/CnpCre/WT mice and similar when comparing PlpR137W/CnpCre/WT and PlpR137W*NexCre/WT mice (Figure 6). Importantly, no APP+ axonal spheroids were found in PlpR137W*NexCre/WT mice, as in PlpR137W control mice.

Taken together, recombination of Plp in oligodendrocytes affects axonal integrity and causes a general neuropathological reaction whereas recombination in excitatory projection neurons does not. It was striking that additional Cnp heterozygosity was accompanied by enhanced axonal pathology, microgliosis, and presence of T-lymphocytes but not by enhanced astrogliosis when compared with PlpR137W mice.

4 | DISCUSSION

The main consequence of PLP-deficiency is axonal degeneration, as observed in SPG2 patients and PlpR137W mice (de Monasterio-Schrader et al., 2013; Edgar, McLaughlin, Yool, et al., 2004; Garbern et al., 2002; Griffiths et al., 1998; Gruenenfelder, Thomson, Penderis, & Edgar, 2011; Petit et al., 2014). Here, we have genetically tested whether neuronal or oligodendrogial dysfunction is the primary cause of axonal impairment caused by loss-of-function mutations affecting the Plp gene. Indeed, PlpR137W mice provide a novel mouse model of SPG2 in which cell type-specific Cre-mediated recombination of the Plp gene in oligodendrocytes causes axonal pathology while recombination in excitatory projection neurons does not. These results provide genetic support for the concept that oligodendrogial Plp expression is required for the normal role of oligodendrocytes in supporting axonal integrity. Conversely, neuronal Plp expression appears dispensable for a healthy nervous system.

We note that the cascade of events leading from Plp gene mutations to axonal pathology has been resolved only in part. In oligodendroglial cells, PLP-deficiency impairs glutamate-dependent progenitor cell migration (Harlow et al., 2015) as well as biogenesis, ultrastructure and the molecular composition of myelin (de Monasterio-Schrader et al., 2013; Duncan et al., 1987; Klugmann et al., 1997; Möbius et al., 2008; Patzig, Kusch, et al., 2016; Werner et al., 2007, 2013; Yool et al., 2001). However, it is speculative which (if any) of these features actually cause axonal pathology. In addition, products of the Plp gene were hypothesized to modulate oligodendrocyte-dependent axonal preservation directly (Boucher, Cypher, Carlock, & Skoff, 2002).

Most recently, cryopreparation of nervous tissue by high-pressure freezing revealed the presence of noncompacted cytosolic channels in the otherwise largely compacted CNS myelin of PlpR137W mice (Möbius, Nave, & Werner, 2016). Diminished developmental closure of cytosolic channels through PLP-deficient myelin is probably due to the lack of the adhesive properties of PLP (Bakhti et al., 2013; Bizzozero, Bixler, Davis, Espinosa, & Messier, 2001). It is likely that the presence of cytosolic channels affects the intracellular transport routes between oligodendroglial cell body and the inner tongue of myelin and thus the transport of small metabolites that are exchanged between oligodendrocytes and axons, thereby providing trophic support (Frühbeis et al., 2013; Fünfschilling et al., 2012; Lee et al., 2012; Nave, 2010; Nave & Werner, 2014; Snidero et al., 2017). Indeed, impaired trophic support by oligodendrocytes may cause the pathology of myelinated axons, including reduced ATP levels (Trevisiol et al., 2017), impaired fast retrograde and anterograde transport and mitochondria (Edgar, McLaughlin, Yool, et al., 2004; Yin et al., 2016) and ultimately axonal spheroids and degeneration (Garbern et al., 2002; Griffiths et al., 1998; Gruenenfelder et al., 2011).

In SPG2/PMD spectrum disorders, the consequences of PLP/Plp gene loss-of-function mutations in SPG2 are milder compared with those of gene duplications or missense mutations in PMD. At the cellular level, increased PLP/Plp dosage causes accumulation of excess PLP in the endo/lysosomal system (Simons et al., 2002) while missense mutations cause conformation changes, accumulation of misfolded PLP in the endoplasmic reticulum, and activation of the unfolded protein response (Duncan, 2005; Garbern, 2007; Inoue, 2017; McLaughlin et al., 2007; Werner et al., 1998; Woodward, 2008). As a consequence,
the neuropathology observed in SPG2/PMD is of considerable heterogeneity. Indeed, the main consequence of loss-of-function mutations and particular missense mutations of the PLP/Plp gene is axonal pathology in SPG2 and its mouse models PLpnull/Y (Garbern et al., 2002; Griffths et al., 1998) and Plptransgenic-overexpressor (Edgar, McLaughlin, Barrie, et al., 2004). In addition to axonal pathology, increased PLP/Plp dosage and most of missense mutations cause dys-/demyelination and oligodendrocyte death in classical and connatal PMD and its mouse models, Plptransgenic-overexpressor (Cerghet, Bessert, Nave, & Skoff, 2001; Edgar et al., 2010; Gotow et al., 1999; Kagawa et al., 1994; Readhead, Schneider, Griffths, & Nave, 1994; Rosenfeld & Freidrich, 1983; Schneider et al., 1992; Tanaka et al., 2009), Plpdisjoy (Cerghet et al., 2001; Gotow et al., 1999; Rosenfeld & Freidrich, 1983; Schneider et al., 1992) and PlpL30R and PlpR137W (Groh et al., 2016), as well as in the longer-lived Plpshaking-pup/Y spaniel dog model (Mayer et al., 2015). In the myelin deficient (md) rat model of PMD carrying a missense mutation in the Plp gene (Plpmtm), the accumulation of misfolded PLP protein in neurons was suggested to cause respiratory dysfunctions and ultimately premature death (Miller et al., 2003), probably by a dominant-negative effect on calcium-activated potassium channels and thus reduced outward currents (Mayer et al., 2009).

It is striking that PLP/Plp gene mutations beyond axonal pathology cause microgliosis, astrogliosis, and presence of T-lymphocytes and thus the full spectrum of neuropathological changes observed in many neurodegenerative disorders (Gonzalez & Pacheco, 2014). The precise molecular trigger of gliosis and neuroinflammation is difficult to define in the present model and more generally in SPG2/PMD spectrum disorders owing to the complex interplay of cell types in the CNS. For example, gliosis may occur independent of evident axonal pathology and vice versa low-grade gliosis is not necessarily detrimental to axons or myelin, as seen in mice lacking the myelin protein tetraspanin-2 (de Monasterio-Schrader et al., 2013). Indeed, immune cells may be modulated by axonal pathology as well as by oligodendroglial molecules (Zeis, Enz, & Scharen-Wiemers, 2016). On the other hand, we are not aware of in vivo evidence indicating a direct role for activated microglia or astrocytes in enhancing axonal pathology in SPG2/PMD spectrum disorders.

The remarkable variety of the cellular pathogenesis indicates that no single therapy concept may work for all patients with SPG2 or PMD. Consequently, the therapeutic approaches to SPG2/PMD vary widely, including downregulating the toxic PLP/Plp overexpression (Prukop et al., 2014), cholesterol-dependent modulation of intracellular PLP trafficking (Sahe et al., 2012), neuroprotective dietary supplementation with Turmeric (Epen et al., 2015; Yu et al., 2012), stem cell therapy (Gupta et al., 2012; Marteyn et al., 2016; Osorio et al., 2017), manipulating the unfolded protein response (Southwood, Garbern, Jiang, & Gow, 2002) or microglial proteases and protease inhibitors (Shimizu et al., 2017), and ablating the activation of T-lymphocytes (Groh et al., 2016; Ip et al., 2006; Ip et al., 2012). Considering the presence of cytotoxic T-lymphocytes in the CNS of a wide range of SPG2/PMD models, T-lymphocytes are probably involved in the progression of axonal pathology in all or most SPG2/PMD spectrum disorders; yet they probably do not explain the axonal pathology to its full extent.

In conclusion, our analysis provides genetic support for the concept that oligodendroglial dysfunction is the primary cause of axonal pathology in SPG2. The same may hold true for SPG35 and SPG44 when considering that the expression of the causative genes FA2H and GJC2/CX47 within the CNS is restricted to the oligodendroglial lineage. It is more speculative which is the primarily affected cell type in other SPG types in which expression of the causative gene is not enriched in oligodendrocytes. Indeed, oligodendroglial dysfunction may contribute to the emergence and progression of more SPG-types than previously assumed, also in consideration of the recent implication of oligodendrocytes in classical neurodegenerative diseases such as Rett syndrome (Nguyen et al., 2013), amyotrophic lateral sclerosis (Kang et al., 2013), and Down syndrome (Olmos-Serrano et al., 2016). Yet, analysis of cell type-specific mutant models will be required to determine the contributions of individual cell types to the pathobiology of any neurodegenerative disorder.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they do not have a conflict of interest

AUTHOR CONTRIBUTIONS

J.P. and H.B.W. established the Plplox mouse line; K.A.L. performed all experiments and statistical assessments; K.A.L., M.S., K.-A.N., and H.B.W. designed and interpreted experiments; H.B.W. conceived the study and wrote the manuscript; all authors contributed to writing and approved the manuscript.

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