Different proteolipid protein mutants exhibit unique metabolic defects

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ABSTRACT

PMD (Pelizaeus–Merzbacher disease), a CNS (central nervous system) disease characterized by shortened lifespan and severe neural dysfunction, is caused by mutations of the PLP1 (X-linked myelin proteolipid protein) gene. The majority of human PLP1 mutations are caused by duplications; almost all others are caused by missense mutations. The cellular events leading to the phenotype are unknown. The same mutations in non-humans make them ideal models to study the mechanisms that cause neurological sequelae. In the present study we show that mice with Plp1 duplications (Plp1tg) have major mitochondrial deficits with a 50% reduction in ATP, a drastically reduced mitochondrial membrane potential and increased numbers of mitochondria. In contrast, the jp (jimpy) mouse with a Plp1 missense mutation exhibits normal mitochondrial function. We show that PLP in the Plp1tg mice and in Plp1-transfected cells is targeted to mitochondria. PLP has motifs permissive for insertion into mitochondria and deletions near its N-terminus prevent its co-localization to mitochondria. These novel data show that Plp1 missense mutations and duplications of the native Plp1 gene initiate uniquely different cellular responses.

Key words: mitochondrion, oligodendrocyte, oxidative phosphorylation, Pelizaeus–Merzbacher disease, Plp1 mutant.

INTRODUCTION

PMD (Pelizaeus–Merzbacher disease) is caused by mutations in the CNS (central nervous system) PLP1 (X-linked proteolipid protein 1) gene (Hodes et al., 1993; Boespflug-Tanguy et al., 1994; Ellis and Malcolm, 1994). PLP1 mutations fall into four broad classes: (i) duplications of the native (wild-type) gene, (ii) point mutations, (iii) deletions, and (iv) frameshift mutations. Duplications account for nearly 70% of human PLP1 mutations (Garbern et al., 1999; Garbern, 2007). In many humans, the duplications are lethal, with death ensuing within the first decade. No treatments are available for PMD patients except for medications to counteract seizures and spasticity. The sequence of cellular events that cause neurological dysfunction and ultimately death is poorly understood in PMD patients. Because Plp1 mutations in animals are often identical with those in humans and both present with similar motor deficits, they are useful models to study PMD. An UPR (unfolded protein response) has been demonstrated in rodents and in cell lines with missense mutations (Southwood et al., 2002; McLaughlin et al., 2007) and in cell lines that overexpress mutant Plp1 (Dhaunchak and Nave, 2007). Not surprisingly, trafficking of mutant PLP to the plasma membrane is altered (Thomson et al., 1997). In animals with missense mutations, this UPR and aberrant protein trafficking is thought to cause Olg (oligodendrocyte) malfunction. However, in rodents with duplications of the Plp1 gene, investigation of cellular and molecular events is limited. In mice with low Plp1 gene copy number, abnormal accumulation of PLP in the ER (endoplasmic reticulum) and a subsequent UPR is barely detectable (Cerghet et al., 2001). Moreover, it is apparently lacking in Ols transfected with wild-type PLP (Kramer-Albers et al., 2006). We also observed major differences in expression of apoptotic markers between these two mutants. For example, we surprisingly found that AIF (apoptosis-inducing factor) was translocated into nuclei of Plp1tg (mice with Plp1 duplications) mice 4-fold more than in jp (jimpy) mice (Supplementary Figure S1 at http://www.asnneuro.org/an/001/an001e014.add.htm). We predicted the reverse results because apoptosis is approx.

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Abbreviations: AIF, apoptosis-inducing factor; CoO, cytochrome c oxidase; CK, creatine kinase; DMEM, Dulbecco’s modified Eagle’s medium; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IMM, inner mitochondrial membrane; jp, jimpy; MBP, myelin basic protein; mtCK, mitochondrial creatine kinase; Olg, oligodendrocyte; OMM, outer mitochondrial membrane; PLP1, X-linked proteolipid protein 1; Plp1tg, mice with Plp1 duplications; PMD, Pelizaeus–Merzbacher disease; TMS, transmembrane sequence; UPR, unfolded protein response; YFP, yellow fluorescent protein.

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3–4-fold less in Plp1tg than in jp mice (Cerghet et al., 2001). Translocation of AIF to nuclei is significant in understanding cell death pathways because this protein is highly upregulated in the caspase-independent or mitochondrially dependent pathway (Cregan et al., 2004; Haeberlein, 2004). Moreover, it is activated in response to PARP (poly(ADP-ribose) polymerase) activation which we also found elevated in the Plp1tg mice (Cerghet et al., 2001). These observations, taken together, suggest that different cell death pathways operate in the two major classes of Plp1 mutations. In the present study, we examine mitochondrial function in the two mutants and show, for the first time, that mice with duplications of the Plp1 gene exhibit major mitochondrial defects.

MATERIALS AND METHODS

Animals, phenotyping and genotyping
All animals were housed in the Division of Laboratory Animal Resources, a federally approved animal facility, and all procedures were approved by the Wayne State University Animal Investigation Committee. Plp1/jp jimpys/Tabby female carriers (jp+/jp+Ta) and males (+/Ta) were purchased from Jackson Laboratories. Jp mice were genotyped by PCR. Tails were cut from neonatal mice, DNA isolated and purified with an Extract-N-Amp Tissue PCR Kit (Sigma), and PCR was performed in the presence of 5 mM ADP or 5 mM ATP. ATP concentrations were determined in triplicate per animal using the ATP bioluminescence assay kit HS II (Roche) according to the manufacturer’s protocol, using an Optocomp 1 luminometer (MGM Instruments). Data were standardized to the protein concentration which was determined using the DC protein assay kit (Bio-Rad). Results were expressed as the means ± S.E.M. for three independent measurements per animal. The significance between multiple groups was determined using ANOVA.

ATP measurements
All mice were starved for 5–6 h, starting in the morning (water was provided ad libitum). Mice were sacrificed by cervical dislocation, brains immediately excised, frozen in liquid nitrogen, and stored at −80°C until measurement. In order to release cellular ATP, frozen tissue (25 mg) was boiled for 2 min after the addition of 300 μl of water containing 100 mM Tris/HCl (pH 7.75) and 4 mM EDTA. Samples were placed on ice and homogenized by sonification (micro tip, 1 × 10 s pulse). ATP concentrations were determined using the DC protein assay kit (Bio-Rad). Results were expressed as the means ± S.E.M. for three independent measurements per animal. The significance between multiple groups was determined using ANOVA.

CcO (cytochrome c oxidase) brain measurements
CcO activity was determined as previously described (Lee et al., 2005) with modifications. Brain plugs (25 mg) were solubilized in 500 μl of chilled measuring buffer [10 mM potassium Hepes (pH 7.4), 40 mM KCl, 1% Tween 20, 2 μM oligomycin, 1 mM PMSF, 10 mM potassium fluoride, 1 mM sodium vanadate and 2 mM EGTA] using a Teflon microtinite pestle applying five strokes followed by sonification (micro tip, 3 × 10 s pulses). Cell debris was removed by centrifugation (2 min at 16000 g) and the supernatant was used for respiration measurements. The protein concentration was determined using the DC protein assay kit. CcO activity was determined at 25°C in a closed 200 μl chamber containing a micro Clark-type oxygen electrode (Oxygraph system, Hansatech Instruments) by increasing the amount of substrate cytochrome c. Since CcO is regulated by adenine nucleotides (Napiwotzki et al., 1997), measurements were performed in the presence of 5 mM ADP or 5 mM ATP including an ATP-regenerating system (Lee et al., 2002). Oxygen consumption was recorded on a computer and
calculated using the Oxygraph software (Hansatech). TN (turnover number) is defined as consumed oxygen [μM/min per mg of total protein].

CcO histochemistry

CcO activity was measured on 15 μm frozen brain sections at the level of the striatum (Wong-Riley, 1979) with modifications (Hüttemann et al., 2008). Briefly, slides were transferred from −80°C to room temperature (20°C) and tissue sections were circled with a hydrophobic pen, allowing the application of small reaction buffer volumes. Then, 100 μl of freshly prepared reaction buffer [100 mM KH₂PO₄ (pH 7.4), 4% sucrose, 0.50 mg/ml DAB (diaminobenzidine), 200 μg/ml catalase and 0.15 μg/ml cow heart cytochrome c] was added to each section, and slides were incubated in a moist chamber for 20 min at 37°C in the dark. The reaction was terminated by washing the slides three times in 100 mM KH₂PO₄ (pH 7.4). Slides were rinsed once in distilled water, air-dried for 3 h and mounted with Permount. For semi-quantitative assessment of the intensity of the CcO histochemical reaction, images were captured with a SPOT Flex Camera (Diagnostic Instruments) attached to a Leitz Laborlux 12 Microscope equipped with a 25 × objective. The white balance was adjusted for the slide before taking images so the same background was used for both Plp1tg and controls. Images taken from the dorsal medial striatum, ventral lateral striatum, corpus callosum, hippocampus and cortex of both Plp1tg and control sections, both of which were on the same slide, were taken at 24 bits per pixel RGB. Images were opened in Adobe Photoshop, inverted and three histograms including an area of 300 × 300 pixels were obtained, averaged and compared between Plp1tg and controls. Brains from six Plp1tg and six controls were analysed. Statistical analysis (ANOVA) was used to determine differences between groups. The average of Plp1tg was reported as the percentage increase over the average histogram of control sections.

Mitochondrial membrane potential (ΔΨm)

We first measured ΔΨm with the probe JC-1 (Invitrogen) in transfected COS7 cells and secondly ex vivo in brain slices. Cells grown on 12 mm coverslips, were transiently transfected using LipofectamineTM (Invitrogen) with a N-terminal EGFP (enhanced green fluorescent protein)-linked full-length PLP cDNA when cells were approx. 70% confluent (see below). Cells were also transfected with P0–EGFP, Na⁺ channel β-subunit–EGFP, PMMP22–EGFP and LacZ–EGFP. At 8 or 24 h after transfection, JC-1 was added to the cells (10 μg/ml) in DMEM (Dulbecco’s modified Eagle’s medium; Gibco) supplemented with 10% (v/v) FBS (fetal bovine serum; Gibco) with antibiotics. Cells were incubated at 37°C with 5% CO₂ for 15 min. Medium was replaced with fresh medium, and cells were incubated for 15 min, washed three times in PBS, mounted in PBS and immediately imaged. Images were obtained on a Leica DMIRB Microscope equipped with a 20 × objective using a SPOT RT Slider camera, and images were compiled in Adobe Photoshop. The green monomeric form of JC-1 is excited at 485 nm and emits at 535 nm; the red aggregate form is excited at 550 nm and emits at 600 nm. Red and green can be imaged either simultaneously or individually using the appropriate filters. However, excitation with a FITC filter for green JC-1 also excites EGFP, making the two difficult to distinguish.

For in vivo measurements, unfixed brains were immediately removed, 100 μm sections were cut on a Vibratome in PBS at 37°C, collected in PBS at 37°C, first incubated in DMEM for 5 min at 37°C with 5% CO₂, next in DMEM containing 3.33 μg/ml JC-1 for 30 min, and lastly in DMEM without JC-1 for 20 min, rinsed twice in PBS and mounted on glass slides with a coverslip in PBS. Slices were photographed within several minutes of rinsing. Images were captured as described above.

Cellular fractionation and Western blot analysis

Mitochondrial/cytosolic and nuclear/cytosolic fractionation kits were used following the manufacturer’s instructions (Biovision). To confirm the purity of the mitochondrial fractions, Western blots were probed with markers for Golgi (Golgin 97; Molecular Probes), ER (anti-KDEL; StressGen), lysosomes (anti-LAMP2 (lysosome-associated membrane protein 2); ABR), mitochondria (anti-CcO; Molecular Probes) crude plasma membrane fractions (anti–NG2; Chemicon), pan-Na⁺ channel and β-actin (Sigma), and an anti-PLP antibody directed against the PLP-specific domain generated for the Skoff laboratory by ABR. Blots were lightly stripped and reprobed as many as six times. For antigens with higher molecular masses (above 180 kDa), the same homogenates were used, but run on an 8% gel rather than our standard 4–12% gel. Fractions of 100 μg were loaded into each lane, except for brain homogenates when 20 μg was loaded. Each gel was transferred on to a PVDF membrane, membranes were blocked with 8% (w/v) non-fat powdered milk, incubated in antibody overnight, washed, incubated with secondary antibody, washed and detected using the ChemiLucent Detection System (Chemicon). In a separate experiment, cytosolic and mitochondrial fractions were probed for MBP (myelin basic protein; Sternberger Monoclonals) and PLP/DM20 against the C-terminus (generated for the Skoff laboratory by ABR). A 100 μg aliquot of cytosolic and mitochondrial fractions along with 25 μg of whole brain homogenate were run on 4–12% gels and transferred on to a PVDF membrane. Membranes were processed as described above, and MBP was detected using the ChemiLucent Detection System (Chemicon), stripped and reprobed for C-terminal PLP and DM20, and again for β-actin.

PLP plasmid construction

Plasmid clone 68 of pDM100 (pDM100.68) contained a full-length cDNA for mouse Plp1 (kindly provided by Professor AT Campagnoni, University of California at Los Angeles, Los
The levels of ATP in whole brain homogenates of Plp1tg and jp mice were compared with their appropriate controls (Figure 1). Plp1tg were 30–35 days old; their phenotype was...
manifested by tremors and tonic-clonic seizures; their genotype was confirmed by the presence of a transgene and/or quantitative PCR of the \textit{Plp1} gene. Because \textit{jp} male mice die between 19 and 22 days, we studied them and their age-matched controls at 17–19 days. Both sets of mice were starved for 5–6 h to provide a well-defined metabolic state for controlled mitochondrial studies (Lee et al., 2005).

Strikingly, \textit{Plp1}tg mice have a 50% reduction in ATP levels, whereas \textit{jp} mice have normal levels. The ATP reduction in \textit{Plp1}tg mice demonstrates a severely compromised energetic state, suggesting major mitochondrial dysfunction. Overnight starvation, which has no apparent effect on wild-type mice, often resulted in death of \textit{Plp1}tg mice, indicating not only that these animals have a severely compromised energy homoeostasis, but also that food must constantly be provided to compensate for their acute energy deficit.

To determine whether the respiratory chain has intrinsic deficits, we examined a key mitochondrial enzyme, CcO. CcO is the terminal enzyme of the mitochondrial respiratory chain (complex IV) and consumes more than 90% of cellular oxygen. Overall mitochondrial respiration is tightly coupled to CcO, and CcO is the proposed pacemaker of oxidative metabolism in intact cells (Villani et al., 1998; Villani and Attardi, 2000). In order to determine the range of activities in which CcO operates, allosteric regulation through ATP (an allosteric inhibitor that operates under conditions of sufficient cellular energy) and ADP (an allosteric activator that operates under conditions when ATP is utilized and converted into ADP) has to be considered and analysed (Hütttemann et al., 2007). We measured CcO activity of \textit{Plp1}tg, \textit{jp} and control mice in the presence of 5 mM ATP and an ATP-regenerating system or 5 mM ADP. \textit{Ex vivo} CcO activities are similar in \textit{Plp1}tg and \textit{jp} mice when compared with their controls (Figure 2). This finding suggests that oxidative phosphorylation is not intrinsically altered in either mutant. This \textit{ex vivo} result does not mean that the mitochondrial defect is not detectable in brain homogenates owing to the fact that OlgS represent only a minor fraction of brain cells. \textit{In vivo} CcO activity should still be increased because its activity and oxygen consumption are allosterically regulated through the ratio of ATP (inhibitor) and ADP (activator). A 50% reduction in ATP and a concomitant increase in ADP should lead to full allosteric activation, assuming that the total adenine nucleotide concentration is not dramatically changed in the \textit{Plp1}tg animals. The resulting increased aerobic activity may explain increased substrate (food) demand and thus the striking effects on the overnight starved \textit{Plp1}tg mice.
CcO histochemistry

CcO activity is directly measurable on brain sections using a histochemical technique (Wong-Riley, 1979). This method assesses CcO function in a more physiological context since the cellular environment is present and mostly intact. Densitometric sampling of CcO reaction product in white and grey matter of mice starved for 5–6 h revealed an average increase of 10% throughout the cerebrum of Plp1tg mice (Table 1). Although the increase is greatest in white matter, the grey matter also shows increases, especially the hippocampus. The smaller than expected changes in CcO histochemical reactivity are in line with other studies that show 10–30% increases in reactivity in diseases with oxidative metabolic disorders (e.g. Wong-Riley et al., 1997). The modest changes found with this technique may also be due to the methodology that dilutes cellular components, including ATP and ADP (Hüttemann et al., 2008). These values may underestimate the role of allosteric regulation via the ATP/ADP ratio.

Histological density analysis of grey matter includes mainly neuronal components, suggesting that neurons are somehow involved. Interestingly, scattered neurons in Plp1tg striatum and hippocampus show noticeable increases in histochemical staining compared with the most intensely stained neurons in controls. This finding strongly suggests that metabolism is compromised not only in Olgps, but also in neurons. Given the 50% reduction in ATP in Plp1tg brains, oxidative phosphorylation deficiencies in many neural cell types might be predicted.

Mitochondrial membrane potential (ΔΨm)

ΔΨm is generated by the electron-transport chain complexes including CcO that pumps protons across the IMM (inner mitochondrial membrane). Depolarization of ΔΨm is detectable with voltage-dependent probes including JC-1. JC-1 exists as a green monomer at low membrane potentials and accumulates as a red aggregate in mitochondria at around normal membrane potentials (Lugli et al., 2007). We first examined ΔΨm in COS7 cells transiently transfected with a full-length cDNA PLP–EGFP, a full-length cDNA P0–EGFP, a full-length PMP22–EGFP, a full-length Na+ channel β-subunit–EGFP and a LacZ–EGFP. Cells with a normal ΔΨm exhibit many red punctate ellipsoids in their cytoplasm. With all constructs, mitochondria in COS7 cells are mainly perinuclear (Figure 3). At 8–12 h after transfection with PLP–EGFP, COS7 cells showed low levels of PLP and many red mitochondria (Figures 3A and 3B). At 24 h after transfection, COS7 cells exhibited higher levels of PLP and exhibited little or no red fluorescence in their cytoplasm. Non-transfected cells on the same coverslip produce red fluorescent mitochondrial signals indicating that their ΔΨm is normal. (In the PLP-transfected cells, EGFP and the JC-1 monomer in mitochondria are excited at nearly the same wavelength, making it difficult to distinguish between depolarized

Table 1 Histochemical analysis of CcO in cerebrum from control (n=6) and Plp1tg (n=6) mice shows a modest increased CcO density in Plp1tg mice (see the Results section)

| Location                      | Percentage increase in Plp1tg over control |
|-------------------------------|-------------------------------------------|
| Striatum (dorsal medial)      | 3.67                                       |
| Striatum (ventral lateral)    | 8.13                                       |
| Hippocampus                   | 11.31                                      |
| Corpus callosum               | 18.97                                      |
| Cortex                        | 6.13                                       |

Figure 3  JC-1 staining of transiently transfected COS7 cells

(A and E) Cells transfected with wild-type PLP fused to EGFP 8 h afterwards show abundant red-stained mitochondria in cells expressing moderate levels of PLP. (B and F) At 24 h after transfection with wild-type PLP–EGFP, most transfected cells express high levels of PLP and have abnormally low ΔΨm, indicated by loss of red-stained mitochondria. Untransfected cells (indicated by arrows) show abundant red-stained mitochondria, indicative of normal ΔΨm (F). (C and G) Cells transfected with myelin P0 protein (C) or PMP22 (G) fused to EGFP show many red-stained mitochondria, even in cells expressing high levels of EGFP. (D and H) Cells transfected with the β-subunit of the Na+ channel fused to EGFP (D) or with LacZ–EGFP (H) show many red-stained mitochondria. Arrows indicate non-transfected cells that have mostly red-stained mitochondria. Scale bar=10 μm.
mitochondria and PLP–EGFP). Cells transfected with P0, PMP22, Na$^+$ channel β-subunit or LacZ constructs (Figures 3C–3H) show red fluorescent mitochondria, similar to untransfected cells. The pictures shown in Figure 3 illustrate low- and high-level expression of these plasma membrane proteins to demonstrate that, even in cells expressing high levels of proteins, mitochondria stain red. Therefore overexpression of plasma membrane proteins that leads to intense GFP fluorescence in the cytoplasm does not necessarily cause mitochondrial depolarization.

We next compared ΔΨm in brain slices from age-matched Plp1tg, jp and wild-type mice (Figure 4). Transverse slices of cerebrum were incubated with JC-1 dye in buffer within 5 min of removing their brains, imaged within 2 min after rinsing the dye, and images were deconvolved at a later time. We quantified the ratio between red (mitochondria with normal ΔΨm) and green (mitochondria with lowered ΔΨm). Whereas mitochondria with lowered ΔΨm are abundant in all three preparations and probably reflect oxygen depletion due to a time lag in imaging, differences between Plp1tg mice with wild-type and jp mice are dramatic. The ratio of red-to-green fluorescent signal for 35-day Plp1tg mice is significantly reduced 2.5-fold. The ratio of red-to-green fluorescent signal for 15–17 day jp mice is slightly increased compared with 15–17 day control mice (Figure 4E). Thus mitochondria in jp exhibit values similar to controls, even though a larger percentage of Olg are dying in jp than in Plp1tg mice (Cerghet et al., 2001). The hypopolarization of Plp1tg mitochondria compared with the modest hyperpolarization of jp mitochondria is relevant to the extensive apoptosis in jp mice. Hyperpolarized mitochondria lead to production of increased free radicals, a crucial signal for apoptosis. Since Plp1tg mitochondria have depolarized membrane potentials, it is unlikely that they use the same apoptotic pathway as jp mitochondria.

Mitochondrial abnormalities in vivo
The functional in vivo studies described above suggest that Olg mitochondria in Plp1tg mice might differ morphologically from wild-type and jp mice. We quantified the number of mitochondria per mm$^2$ of cytoplasm in Plp1tg mice and compared their numbers with wild-type and jp mice (Figure 5A). Compared with wild-type mice and jp mice, the density of mitochondria in Plp1tg Olgs is more than two times greater than controls. In contrast, the density of mitochondria in jp Olgs follows the same linear regression as wild-type Olgs. When the total surface area of Olg mitochondria is plotted against their cytoplasmic surface area, Plp1tg Olg mitochondria occupy more than 2-fold the area of wild-type mice (Figure 5B). Interestingly, the surface area for Olg mitochondria in wild-type mice hovered at around 7% with little standard error, suggesting tight regulation of their numbers. In contrast, Olgs in Plp1tg mice exhibited extreme morphological variations. In many Olgs, the cytoplasm is filled with mitochondria (Figure 6). Other Olgs are morphologically similar to their normal counterparts, have a normal distribution of mitochondria and are often associated with thin myelin sheaths, suggesting that they are newly generated and express low levels of PLP. We have found increased generation of new glia throughout the lifespan of these animals, some of which we have confirmed as microglia (C Tatar and RP Skoff, unpublished data) and others, possibly Olgs. In addition to the differences in the density of mitochondria between Plp1tg and control mice, the mitochondria in Plp1tg mice appear to be structurally...
abnormal. High-magnification pictures of silver–gold sections of mitochondria show disruptions of the OMM (outer mitochondrial membrane). Often, rough ER appears fused to the OMM. Fission of mitochondria and OMM integrity is associated with apoptosis, loss of ΔΨm and AIF translocation (Frank et al., 2001).

Association of PLP with mitochondria

We transfected an N-terminal EGFP full-length PLP construct, a C-terminal GFP full-length PLP construct, an N-terminal EGFP full-length P0 construct, an N-terminal EGFP full-length PMP22 construct, an N–terminal EGFP full-length Na+ channel β-subunit, and EGFP by itself into COS7 cells, and then labelled mitochondria with Mitotracker Red 580. Cells were fixed at 12 h intervals up to 48 h after transfection. At 12 h after transfection, COS7 cells expressing GFP–PLP or PLP–EGFP showed little co-localization of PLP with mitochondria (Figure 7A). Between 24 and 48 h after transfection, extensive co-localization was present throughout the cytoplasm (Figures 7B and 7C).

Deconvolution of confocal images exhibits better resolution and reveals specificity of the co-localization. Both confocal and deconvolved images show only green fluorescence (PLP) in the plasma membrane, indicating that Mitotracker Red 580 dye does not bleed into the green fluorescent channel (Figures 7D–7G). Secondly, the green, orange/yellow and red profiles associated with mitochondria imaged are often composed of three distinct elements. An all-green EGFP crescent partially surrounds an all-yellow structure that sometimes encloses an all-red mitochondrial component. The green is most likely to be ER that contains PLP adjacent to mitochondria, the yellow component is most likely to be the OMM and/or IMM containing PLP, and the red staining is a portion of a mitochondrion that lacks PLP. Simultaneous co-transfection of PLP and mtCK (mitochondrial creatine kinase), an IMM-associated protein (Speer et al., 2005), followed by Mitotracker Red 580 staining strongly suggests the insertion of PLP into the IMM (Figures 7H–7M). Mitotracker Red 580 and mtCK fluorescence co-localizes, as predicted, only in the core of the mitochondria, whereas the outer mitochondrial envelope only stains red (Figures 7H–7J). PLP co-localizes with Mitotracker Red (Figures 7K and 7L) and mtCK (Figures 7L and 7M) to produce yellow variants. The co-localization of PLP to mitochondria is not restricted to COS7 cells, as studies performed with an immortalized Olg cell line (Ghandour et al., 2002) into which PLP–EGFP is transfected also shows that PLP co-localizes to mitochondria (Figures 7N–7P). In contrast with COS7 cells in which most mitochondria show co-localization with PLP, some mitochondria in these Olgs show strong co-localization, whereas others show no co-localization.

Because the N-terminus of PLP contains motifs for localization to the IMM (see the Discussion section below), we made deletions of the first 10 and 20 amino acids that retain the first amino acid methionine residue. These constructs show that PLP does not co-localize to mitochondria (Figures 8A–8F). Faint PLP–EGFP staining is detectable at the plasma membrane, indicating modest transport of these mutant constructs. Not surprisingly, live immunostaining with the O10 antibody (Jung et al., 1996) shows that the mutants are not correctly inserted into the plasma membrane. The specificity of the co-localization of PLP with mitochondria is confirmed by transfection of other plasma membrane markers and Mitotracker Red 580 staining. The myelin plasma membrane protein P0, when transfected into COS7 cells, does not co-localize with mitochondria (Figures 9A–9C); PMP22, another Schwann cell plasma membrane protein, does not co-localize with mitochondria (Figures 9D–9F). Cells transfected with EGFP alone exhibited very bright nuclear and cytoplasmic staining, but little co-localization with mitochondria (results not shown). The intensity of the EGFP signal in these P0- and PMP22-transfected COS7 cells is as intense as in the PLP–EGFP or
GFP–PLP cells, indicating that overexpression of myelin plasma membrane proteins does not necessarily lead to their co-localization in mitochondria.

Because ER and mitochondria are structurally in close proximity with each other, it might be argued that the yellow mitochondrial fluorescence observed with PLP–EGFP or GFP–PLP constructs and Mitotracker Red is due to fluorescence bleed through from the ER into mitochondria, or that the two organelles overlap each other in the z-axis. However, live Mitotracker Red 580 staining and anti-KDEL (an ER marker) antibody immunostaining show very little co-localization (Figures 9G–9I).

We next asked whether PLP co-localized with mitochondria in vivo using immunoblots and immunogold electron microscopy. We first prepared whole spinal cord and brain homogenates, cytosol and mitochondrial fractions from 60 day old wild-type and Plp1tg mice. Immunoblots were probed with an anti-PLP-specific antibody, stripped and re-probed with different organelle and plasma membrane markers. Pure mitochondrial fractions were more easily purified from spinal cord (Figure 10A) than from brain. Spinal cord homogenate containing myelin and cytosolic fractions from wild-type and Plp1tg mice predictably immunostained for PLP. Approx. 5-fold less homogenate was loaded compared with the other fractions because the homogenate contains myelin membranes. The intensity of the homogenate bands shows the abundance of myelin proteins in membranes. Mitochondrial fractions from wild-type mice did not show PLP bands, but they were easily detectable in Plp1tg mice. Its absence in wild-type mice is not a loading artefact because actin levels are approximately equal in both wild-type and Plp1tg mitochondrial fractions. The specificity of the mitochondrial fractions was shown with different antibodies. The subunit of CcO, which is coded by mitochondrial DNA and localized to the mitochondrial membrane is restricted to mitochondria and absent from the cytosolic fraction. Contamination of mitochondria with other cytosolic organelles was eliminated by probing for ER, Golgi and lysosomal markers. Two plasma membrane markers, a pan-Na\(^+\) channel and the NG2 antibody that recognizes a proteoglycan on glial progenitors, were also, predictably, absent from mitochondrial fractions. Because of the high molecular mass of the last two antibodies, they were run on a separate gel, but the same sample was used for both blots. We also used MBP as a potential control for the purity of the mitochondrial fractions, but, unfortunately, MBP is drastically reduced in the transgenics, making this protein a less than ideal control. In C57 mice, all four MBP isoforms were abundantly present in homogenates and faintly present in the cytosolic fraction, whereas only the 21.5 kDa form was detectable in the Plp1tg cytosolic fraction (Figure 10B). Drastic reductions of MBP, using immunostaining and Western blot analysis of total brain homogenates, have been observed by our and other laboratories (Readhead et al., 1994; Karim et al., 2007). Although all MBP isoforms were absent from the Plp1tg mitochondrial fraction (Figure 10B), PLP and DM20 were detected in the mitochondrial fraction of Plp1tg mice. Their molecular masses were slightly lower than in homogenates, but this result is predicted if these proteins are inserted into mitochondria (see the Discussion section below). [The molecular mass of PLP in the mitochondrial fraction in Figure 10(A) appears slightly lower than in the cytosolic fraction; the obvious difference in molecular masses in the lower compared with the upper blot may be due to the length of time the gel was run].

We next investigated whether PLP localizes to mitochondria in Plp1tg mice using pre- and post-embedding immunogold electron microscopy (Figure 11). With pre-embedding

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Figure 6 Electron micrographs of an Olg from a 35 day old wild-type mouse (A) and Olgs from a 35 day old Plp1tg mouse (B and C). Nuclei are outlined in green and mitochondria in pink. The distribution of mitochondria and other organelles from the wild-type mouse is typical of normal Ols. Although the cytoplasm and nuclei of Plp1tg Olgs are abnormally electron-dense, their nuclei and plasma membranes are intact, suggesting that they are still viable.

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Figure 7  PLP co-localizes with mitochondria in COS7 and immortalized Olg cells

(A–C) Confocal images of COS7 cells transiently transfected with a full-length PLP-EGFP construct and stained with Mitotracker Red. (A) At 12 h after transfection, most mitochondria in transfected cells stain red, but some show co-localization with PLP (arrow and inset). Mitochondria in non-transfected cells are all red. (B and C) By 36 h after transfection, co-localization is abundant (arrow and inset). (D–G) COS7 cells transfected with the GFP–PLP construct for 36 h and live-stained for Mitotracker Red. Single-channel confocal images (D and E), merged (F) and deconvolved (G) with Huygens Essential Software. PLP-positive plasma membranes and cytoplasmic organelles (arrowheads) remain green in the merged and deconvolved images; some mitochondria remain red (crossed arrowhead) in the merged and deconvolved images. Co-localization of PLP and mitochondria (arrows) is abundant in the merged confocal image. (G) In the deconvolved image, green staining (PLP) surrounds yellow structures (PLP and mitochondria) that often surrounds a red portion of mitochondria. (H–M) Confocal images of COS7 cells simultaneously transfected with CK–YFP and PLP–EGFP, and stained with Mitotracker Red 580. (H–J) Mitotracker Red and CK, visualized as canary yellow, co-localize as goldenrod (J). A thin rim of red, presumably OMM, often surrounds IMM-localized CK (arrows). (K and L) Co-localization of PLP and Mitotracker Red produces yellow/orange staining. PLP (green) often surrounds the matrix-localized CK staining (yellow; arrows). (M) Co-localization of PLP (green) and CK (canary yellow) produces a bright yellow staining. Green surrounds CK which has a worm-like configuration, typical of mitochondrial cristae. (N–P) Confocal pictures of Olg cell line 158N transfected with PLP-EGFP and stained with Mitotracker Red. Single-channel confocal images (N–O) and merged (P). Scale bar=10 µm.
electron microscopy immunocytochemistry, penetration was very limited, approx. 0.25 μm from the surface of the tissue block, so very few OlgS were located and detected. This technical problem prevented reliable quantification. Still, the few OlgS near the tissue surface had abundant gold particles overlying their mitochondria, whereas mitochondria in other cells lacked gold particles. With the post-embedding method, quantification of the number of gold particles overlying Olg mitochondria showed a severalfold increase compared with the number of gold particles in mitochondria of astrocytes and neurons immediately adjacent to the OlgS (percentage of non-Olg cytosolic structures with gold particles in mitochondria = 4; percentage of OlgS with gold particles in mitochondria = 16.7). The appropriate control for this study is to compare numbers of gold particles in mitochondria of OlgS with adjacent non-Olg cells in the same section. With the electron microscopy immunogold post-embedding method, the background (random gold particles) varied considerably from animal to animal and even, we found, one side of the grid to the other. This variability is due to several factors and includes differences in penetration of the primary antibody into the plastic. This in vivo electron microscopy data, combined with Western blotting and transfection experiments, indicates that native PLP co-localizes with mitochondria.

**DISCUSSION**

PMD is due to different types of mutations in the *Plp1* gene. Disabilities range from severe motor impairment and cognitive loss to mild forms in which patients ambulate and have near normal lifespans (Garbern et al., 1999; Woodward and Malcolm, 1999; Regis et al., 2005). Future therapies to treat PMD hinge upon understanding the molecular and cellular sequence of events that contribute to behavioural disabilities. *Plp1* transgenic mouse line 66 closely mimics PMD patients with duplications and triplications of the native gene because the mice we use have mainly duplications, triplications and quadruplications of the native *PLP1* gene. They both have modest increases in proteolipid protein (Supplementary Figure S2 at http://www.asnneuro.org/an/001/an001e014.add.htm) (Anderson et al., 1998; Karim et al., 2007), and exhibit a behavioural phenotype similar to PMD patients (see the Materials and methods section).

Hallmarks of all *Plp1* mutations at the histological level are varying degrees of dys-, hypo- and de-myelination accompanied by Olg death. The failure to form myelin is a direct consequence of Olg death rather than a feedback mechanism in which abnormal myelin formation triggers Olg death (Knapp et al., 1986; Skoff and Knapp, 1990; Skoff, 1995; Yang and Skoff, 1997). An UPR has been studied in rodents with missense/nonsense mutations and in OlgS cultured from *Plp1* mice with duplications (Southwood et al., 2002; Dhaunchak and Nave, 2007; Southwood et al., 2007) because an UPR is a likely candidate to activate cell death pathways (Harding et al., 2002). An UPR is easily demonstrated in mice with point mutations such as *jp*, myelin synthesis deficient and rumpshaker, but markers of an UPR are, at best, slightly increased in mice with duplications compared with wild-type mice (Cerghet et al., 2001). Also, markers of cell death pathways in these two groups of mutants were different; the results suggest that the caspase-dependent pathway was activated in mice with missense mutations and the caspase-independent pathway was activated in mice with *PLP1* duplications (see the Introduction section).

We first studied ATP levels in brains of *Plp1*tg and *jp* brains and found dramatic differences. ATP levels are drastically
reduced in *Plp1* tgs brains, but are normal in jp brains. Concomitant with ATP reduction in the *Plp1* tgs, ΔΨm is severely reduced in both white and grey matter compared with controls, but not in jp. Predictably, CcO, the terminal enzyme in oxidative phosphorylation, was modestly increased in *Plp1* tgs using a histochemical stain. A 50% reduction in ATP, dramatic decreases in ΔΨm and increases in CcO activity throughout the white and grey matter make it difficult to attribute these metabolic differences exclusively to Olgs. The reason is that Olgs constitute a small volume of brain tissue. However, energetic coupling between brain cells has been reported and may explain our observed global energy defects. Via the so-called astrocyte–neuron lactate shuttle, astrocytes metabolize glucose to lactate that is secreted and taken up by neurons to drive aerobic energy production (Kasischke et al., 2004). Such an energetic coupling could be a more generalized mechanism in the brain that involves additional participants including Olgs. Our ΔΨm measurements sampled grey matter, as well as white matter, making it highly likely that neurons were included in these measurements. Large cells, undoubtedly neurons, show stronger CcO staining in the striatum of *Plp1* tgs than in controls. The suggestion that neurons in *Plp1* tgs are metabolically abnormal may be paradoxical considering that the mutation is expressed in Olgs and not in neurons. However, axonal degeneration in these same *Plp1* tgs mice has been previously described (Anderson et al., 1998), indicating that the PLP mutation affects neuronal function.

Our previous studies and the present study now shed light upon the neuronal abnormalities. We found global, large

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**Figure 9** Other myelin proteins do not co-localize with mitochondria

(A–C) COS7 cells transiently transfected with P0–EGFP and stained with Mitotracker Red 580. (A) Mitochondria are tubular or round structures spread throughout the cytoplasm, but are often aggregated around the nucleus. (B) P0 forms punctate dots throughout the cytoplasm, large and intensely fluorescent aggregates, and it also outlines the plasma membrane. (C) In the merged image, mitochondria surrounding the nucleus are not co-labelled with P0 (left-hand arrow); a single mitochondrion near the P0 aggregate (right-hand arrow) is not co-labelled. (D–F) COS7 cells transiently transfected with PMP22–EGFP and stained with Mitotracker Red 580. PMP22 and P0 do not co-localize with mitochondria in these confocal images except for occasional particles. (G–I) Confocal, deconvolved images of COS7 cells stained for Mitotracker Red 580, fixed and immunostained for KDEL using a FITC-labelled secondary antibody. Reticulated pattern characteristic of ER (arrows) and tubular mitochondria (crossed arrows) often abut each other, but do not co-localize. Close apposition of mitochondria with ER form occasional small dots (arrowheads), but do not show extensive overlap of PLP with mitochondria. Scale bar=10 μm.
Figure 10 PLP is present in mitochondrial fractions from Plp1tg mice

(A) Immunoblots of cytosolic and mitochondrial fractions of spinal cord of 60 day old Plp1tg and wild-type C57 mice. Mitochondrial and cytosolic fractions were prepared using the Biovision Kit. The immunoblot was first probed with a PLP-specific antibody, stripped and subsequently reprobed for five other antibodies. The last two antibodies, because of their higher molecular masses, were run on a gradient gel using the same homogenate.

(B) Immunoblots of whole-brain homogenates, cytosolic and mitochondrial fractions probed for PLP and MBP from 60 day old C57 and Plp1tg mice. The antibody to the C-terminus of PLP recognizes both PLP and DM20. PLP and DM20 molecular masses in the mitochondrial fraction are slightly less than in whole brain or cytosolic fractions. The antibody to MBP recognizes all four MBP isoforms present in homogenate and cytosolic fractions in C57 mice, but are absent from mitochondrial fractions. Actin in the mitochondrial fraction is always less than in the cytosol [see part (A) above].

decreases in pH of Plp1tg brains (Skoff et al., 2004a) and in stably transfected PLP-expressing cells (Boucher et al., 2002). When PLP, but not DM20 or control cells, were co-cultured with dorsal root ganglion neurons, axonal and neuronal degeneration was striking in these co-culture experiments. These results are not a tissue culture phenomenon because a time-lapse proton-flux ex vivo assay revealed that the pH of buffer derived from Plp1tg brains was significantly decreased compared with wild-type brains (Skoff et al., 2004a). These studies suggest that PLP modulates neural dysfunction/death by regulation of pH in vitro and in vivo. The level of PLP regulates the number of Olg's in roughly inverse proportions. The number of Olg's cultured in the presence of antisense PLP was increased as much as 7-fold compared with Olg's grown in control conditions (Yang and Skoff, 1997). The number of Olg's cultured from Plp1-knockout mice was 2-fold greater than in wild-type mice (Skoff et al., 2004b). Overexpressing DM20, an isoform of PLP, knock-in mice (Stecca et al., 2000; Spörkel et al., 2002) have near-normal lifespans compared with shortened lifespans of different overexpressing PLP mouse lines (Mastronardi et al., 1993; Kagawa et al., 1994; Readhead et al., 1994; Bradl et al., 1999). Our studies demonstrate that transfection of native PLP causes PLP to intercalate into mitochondria, presumably into the IMM. Mitotracker Red 580 staining combined with transfection of CK, located in the IMM, shows oval-shaped mitochondria enclosing a classically shaped IMM/matrix. When PLP is transfected into the above combination, PLP co-localizes with CK. Importantly, the co-localization of PLP with mitochondria is not due to 'overexpression' of PLP in COS7 cells and immortalized Olg cells, such that the protein associates non-specifically with mitochondria. We have 'overexpressed' several different plasma membrane proteins such that their level of fluorescence was similar and as bright as that for the PLP-expressing cells, yet these proteins did not co-localize with mitochondria.

A genome-wide bioinformatics screen and localization prediction programs did not predict PLP to be a tail-anchored mitochondrial protein (Nakai and Horton, 1999; Guda et al., 2004; Kalbfleisch et al., 2007), but this may be due to the high-stringency parameters applied. The absence of a canonical mitochondrial-targeting sequence for PLP is not surprising because, if there were one, PLP would be exclusively targeted to mitochondria and not to myelin. Other analyses suggest that PLP is targeted to the OMM and/or IMM. It is important to discuss this information in some detail because it lays the theoretical foundation for our co-localization studies. For tail anchoring into the OMM a positively charged C-terminus is required (Horie et al., 2002; Habib et al., 2003) which is present in PLP (L167KLMOROTKF278). The requirements for insertion into the OMM are a relatively short C-terminus with a hydrophobic TMS (transmembrane sequence) that for PLP includes 21 of 28 amino acids in the fourth TMS. All myelin proteins do not exhibit these C-termini features; P0, for example, which has nearly 70 amino acids in its C-terminus, is an unlikely candidate and did not show co-localization to mitochondria. Similarly, PMP22 and a Na+ channel β-subunit did not co-localize with mitochondria in transfection studies, nor was MBP present in the mitochondrial fractions. Taken together, these studies indicate that the localization of PLP to mitochondria is specific to this myelin membrane protein.

For protein localization to the intermembrane space, a redox-coupled pathway was discovered that involves a redox chain consisting of Erv1, Mia40 and cytochrome c (Allen et al., 2005). Proteins imported by this pathway usually contain a twin Cx3C or Cx9C motif that are oxidized by Mia40 to form disulfide bonds that can trap proteins in the mitochondrial inter-membrane space. PLP contains single Cx3C and Cx9C motifs in the N-terminal region, in addition to a Cx8C sequence located toward the C-terminal part. Other proteins with disulfide bonds that localize to this
Submitochondrial compartment do not follow this strict rule (Gabriel et al., 2007; Herrmann and Kohl, 2007). Another recently described internal signal for protein localization involves the four amino acid core sequence AVPI together with a matrix-targeting signal that, if present, is sufficient to localize proteins to the inter-membrane space (Ozawa et al., 2007). PLP contains a similar four amino acid motif (A\textsuperscript{171}VPV\textsuperscript{174}) where the terminal isoleucine residue is replaced by valine, which only differs by the absence of a methyl group.

Another algorithm which predicts the probability of membrane protein insertion into the mitochondrial matrix, shows that PLP has an 18% probability for insertion into the mitochondrial matrix (Claros and Vincens, 1996). Insertion into the IMM requires, among other factors, an N-terminal cleavable pre-sequence which, for PLP, might be between the 19th and 20th amino acid. This N-terminal leader sequence usually forms an amphipathic helix that contains non-polar amino acids in addition to positively charged residues, clustered on one side. Interestingly, our Western blot analysis shows that the molecular mass of PLP/DM20 in the mitochondrial fractions is slightly less than in the homogenates and cytosolic fractions.

Partial localization or translocation to mitochondria of plasma membrane and cytoplasmic proteins has been described, providing a basis for PLP, a plasma membrane, insertion into mitochondria. The mammalian protein Slit3 localizes to both the cell surface and mitochondria (Little et al., 2001), and the tyrosine phosphatase Ship2 localizes to the cytoplasm and mitochondrial intermembrane space (Salvi et al., 2004). Proteins also translocate to the mitochondria under certain conditions that include the EGFR (epidermal growth factor receptor) where it interacts with the CcO subunit II (Boerner et al., 2004) and Akt after activation of the pathway (Bijur and Jope, 2003). Localization and insertion of PLP into the IMM could account for the observed depolarization and decreased energy levels. In this scenario, direct interaction with and inhibition of one or more oxidative phosphorylation complexes, or through generation of a pore would directly dissipate $\Delta\Psi$m similar to uncoupling protein 1 that operates in brown adipose tissue (Brand et al., 1999). The co-localization of PLP with CK strongly supports its association with the IMM.

The reduction in ATP levels and decreased $\Delta\Psi$m predicts, as we found, an increase in the numbers of mitochondria in Olgs of Plp1tg mice compared with wild-type and jp mice. An increase in mitochondria is characteristic of cells with reduced respiratory capacity, perhaps due to increased fission and decreased fusion (Detmer and Chan, 2007). The increase in native Plp1 gene dosage raises the question of whether it has a role in normal development when mRNA and protein levels are highly up-regulated. Plp1 mRNA and protein are expressed at high levels in interdigital webbing of normal mice, where it co-localizes with apoptotic cells (Skoff et al., 2004a). In the testes, when PLP/DM20 mRNA is abundant, apoptotic cells are increased in Plp1tg mice, but decreased in PLP-null mice, indicating that PLP modulates apoptosis in many cell types. Whether PLP associates with mitochondria under these conditions remains to be studied. Most importantly, our findings of ATP reduction are directly relevant to PMD patients with duplications. Drugs that operate through a variety of direct and indirect mechanisms to prevent reduction of ATP and/or development of gene therapy to reduce Plp1 expression levels should be feasible candidates to treat PMD patients.

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