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Modeling the natural history of Pelizaeus–Merzbacher disease

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Abstract

Major gaps in our understanding of the leukodystrophies result from their rarity and the lack of tissue for the interdisciplinary studies required to extend our knowledge of the pathophysiology of the diseases. This study details the natural evolution of changes in the CNS of the shaking pup (*shp*), a model of the classical form of the X-linked disorder Pelizaeus–Merzbacher disease, in particular in glia, myelin, and axons, which is likely representative of what occurs over time in the human disease. The mutation in the proteolipid protein gene, *PLP1*, leads to a delay in differentiation, increased cell death, and a marked distension of the rough endoplasmic reticulum in oligodendrocytes. However, over time, more oligodendrocytes differentiate and survive in the spinal cord leading to an almost total recovery of myelination. In contrast, the brain remains persistently hypomyelinated. These data suggest that *shp* oligodendrocytes may be more functional than previously realized and that their early recruitment could have therapeutic value.

Keywords

X-linked; Hypomyelination; *PLP1*; Oligodendrocyte; Axon

Introduction

Pelizaeus–Merzbacher disease (PMD) is a rare, X-linked recessive myelin disorder of young boys that results from mutations or duplications of the proteolipid protein gene (*PLP1*) (Hodes et al., 1993; Cailloux et al., 2000; Koeppen and Robitaille, 2002; Hudson, 2003; Garbern, 2007). The majority of boys have duplications of the gene, with the number of extra copies of the gene dictating the severity of the disease (Harding et al., 1995; Inoue et al., 1999; Mimault et al., 1999; Wolf et al., 2005). PMD presents a heterogeneous clinical picture. In the most severe, or connatal, form of the disease, death usually occurs in the first decade and sometimes as early as the first few weeks of life. The ‘classical’ form of PMD

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also presents early in life, but affected boys can live into the 2nd-6th decade with significant, though variable, neurological deficits (Koeppen and Robitaille, 2002). At the opposite end of the spectrum, spastic paraplegia Type 2 (SPG-2), an allelic form of the disorder, has a much milder phenotype and affected boys can live up to the 6–7th decade (Saugier-Verber et al., 1994; Cailloux et al., 2000).

As with many rare human neurologic diseases, gaps in our understanding of PMD exist because of a lack of numbers of patients to study, the phenotypic variation in the disease, and a dearth of tissue for microscopy, biochemical, and molecular studies. PMD, however, has a significant number of animal models that have known mutations in *Plp1* though most are short lived (Griffiths et al., 1998). These models are part of a larger group of animals known as the myelin mutants (Duncan, 1995; Lunn et al., 1995; Werner et al., 1998; Duncan et al., 2011). The first X-linked mutant which was proven to have a mutation of *Plp1* was the jimpy (*jp*) mouse (Nave et al., 1986). Jimpy and its alleles, *jp^{msd}* and *jp^{4J}* die early (21 to 25 days of age), while the *jp^{rsh}*, (rumpshaker) mouse has a much milder phenotype and a longer lifespan (Werner et al., 1998). The first three mice are similar in phenotype to connatal PMD (the *jp^{msd}* mutation has been described in a connatal PMD patient (Komaki et al., 1999)), while *jp^{rsh}*, given its mild myelin defect, resembles SPG-2 (the *jp^{rsh}* mutation has been found in a boy with SPG-2 (Kobayashi et al., 1994)). The myelin-deficient rat (*md*) is similar to *jp* and dies at 21–25 days, but is even more severely dysmyelinated (Dentinger et al., 1982; Jackson and Duncan, 1988). A mutant rabbit, known as paralytic tremor (*pt*), also has a mutation in *PLP1* but has a milder phenotype (Tosic et al., 1994; Sypecka and Domanska-Janik, 2005). However, perhaps the most useful model of PMD is the canine mutant known as the shaking pup (*shp*) (Griffiths et al., 1981a, 1981b; Duncan and Griffiths, 1983; Duncan, 1995). As a large animal model of PMD, the *shp* provides ideal opportunities to study the evolution of changes in the CNS of a naturally occurring mutant as it lives up to two years or more. Therefore, it is possible to study the long-term survival of oligodendrocytes (OLs), or their death and replacement, as well as the state of myelination. The study of the *shp* can also determine whether chronic non-myelination or dysmyelination of axons leads to axon death. As a large animal model, it is possible to follow many of these changes by serial MRI evaluation (Wu et al., 2011; Samsonov et al., 2012).

Here, we follow the myelin defect in the *shp* from fetal stages through the early post-natal period and into adulthood. Using multidisciplinary approaches, we have studied OL development and death, *PLP1* expression, and myelin formation. We also have evaluated axon survival in the CNS as well as the astrocyte response to these changes. These data provide a ‘window’ into the likely cellular, biochemical, and molecular events that occur and evolve over time in the CNS of those PMD patients with point mutations in the *PLP1* gene. This information will be important in devising future therapeutic strategies that are designed to promote myelin repair in PMD.

Materials and methods

Animals

The *shp* mutation arose in a line of Welsh Springer spaniels and has been maintained as a colony for 30 years. Affected male and female carrier pups were identified prior to onset of clinical signs by PCR of genomic DNA isolated from whole blood. The *shp* mutation at nucleotide 219 created an Avail restriction site in exon 2. (Nadon et al., 1990). This results in a His → Pro amino acid substitution.

Pups were euthanized with an overdose of pentobarbital at intervals from P0 (day of birth) to 2 3/4 years of age. Animals were perfused through the aorta with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) or modified Karnovsky's fixative. In some cases, a block of thoracic spinal cord was removed prior to perfusion and immediately frozen in liquid nitrogen for RNA and protein analysis. After perfusion, the brain, spinal cord, and optic nerves were removed and post-fixed in PFA for immunohisto-chemistry, Karnovsky's fixative, or 2.5% glutaraldehyde. The brain was weighed on collection or after fixation. Fetal tissue was collected by Cesarean section at a range of gestational ages beginning at E40 (canine gestation, 63 days).

All pups were handled and treated according to the guidelines and recommendations of the Research Animal Resources Center and the Animal Care and Use Committee at the University of Wisconsin (UW) – Madison.

Tissue sectioning

Tissue blocks post-fixed with Karnovsky's fixative or 2.5% glutaraldehyde were embedded in Epon plastic. Semithin sections (1 µm) were cut on a microtome and stained for myelin with 1% toluidine blue/1% sodium borate. Images were captured on a Nikon Eclipse microscope equipped with Elements software. Ultrathin sections were mounted on copper grids (Electron Microscopy Sciences) and stained with uranyl acetate followed by lead citrate. Images were captured on a Philips CM120 transmission electron microscope (UW School of Medicine and Public Health).

Immunohistochemistry

After overnight fixation in 4% PFA, tissue blocks were cryoprotected in 30% sucrose overnight. Tissue was snap frozen in powdered dry ice, cut on a cryostat to 20 µm, and used for free-floating or slide-mounted immunohistochemistry. Tissue was incubated with the following antibodies: mouse anti-GFAP (1:30,000, Millipore) and rabbit anti-Iba1 (1:5000, Wako).

In situ hybridization

Radioactive *in situ* hybridizations were performed as described previously (Griffiths et al., 1989). Non-radioactive *in situ* hybridizations were performed as described previously (Abler et al., 2011) with the exception that tissues were fixed during perfusion and cut to 20 µm thickness before being mounted onto slides.

Myelin quantitation

To examine the apparent increased myelination over time in the *shp* and how chronic non-myelination or dysmyelination affects axon survival, we quantified myelin and axons by measuring myelin sheath thickness and axonal diameters. From these measurements, g-ratios were determined. Axon diameters, axon density, and myelin sheath thickness were quantified in the ventral column using 1 μ m thoracic spinal cord sections stained with toluidine blue from WT and *shp* animals. For all time points except 2 years of age, 4–5 images each from 2 wild-type and 2 *shp* animals were quantified. At 2 years of age, images from 2 WT and 4 *shps* were measured. Axon diameters and myelin sheath thicknesses in captured images were measured using ImageJ software (NIH). To quantify the apparent increase in myelin content in the *shp*, the total area of myelin in each image was measured and divided by the total image area minus axonal area.

TUNEL

Five cervical spinal cord sections from 2 dogs per genotype per time point were slide-mounted and assayed for apoptosis using the TUNEL assay with the DeadEnd TUNEL kit (Promega) with modifications. For fluorometric detection, slides were incubated in AlexaFluor 488 streptavidin antibody (1:500), stained with DAPI, and coverslipped with SloFade (Invitrogen). Representative images were captured as described above and TUNEL⁺ cells were counted manually.

Western blots

Protein extraction and Western blotting were performed as described previously (Mayer et al., 2011). After a dry transfer and blocking, membranes were incubated with antibodies as follows: mouse anti- β -actin (1:8000, Sigma), rabbit anti-PLP (1:10,000, gift from Dr. 1. Griffiths), and rabbit anti-MBP (1:1000, Millipore). Visualization and image capture were performed as previously described. Expression was quantified using ImageJ.

Autoradiography

A select number of WT and *shp* dogs were injected intraperitoneally with (methyl-³H) thymidine (50–80 Ci/mM, New England Nuclear) at a dose of 2 μ Ci/kg. Ninety minutes later, animals were anesthetized and perfused as above. Tissue blocks from the optic nerve and cervical, thoracic, and lumbar spinal cord were processed for embedding in Epon sectioning and processing for autoradiography as previously described (Duncan and Hoffman, 1997). The number of dividing cells in the spinal cord and optic nerve were quantified in a small number of *shps* (one at 2, 5, 10, 13, 17, 19 weeks and two at 5 months) and WT dogs (one dog at 2, 4, and 18 weeks). Only those cells in the white matter (not gray matter) with more than five silver grains were counted; labeled endothelial and pericytes as well as dividing cells in the leptomeninges were excluded from the count.

Statistical analysis

WT and *shp* results were compared with the Student's two-tailed *T*-test using GraphPad Prism software. P-values < 0.05 were considered significant. Error bars represent SEM.

Results

Origin of the disease, genetic and clinical findings

The *shp* disorder was first diagnosed in the Border region of Scotland in 1980 in Welsh Springer Spaniel dogs. Since that time, we have studied the offspring of heterozygous females (genotyped by PCR) (Cuddon et al., 1998) and have recorded 90 litters; 48% of males were found to be affected hemizygous, while 43% females were found to be carriers. These numbers confirm that this is an X-linked recessive disorder.

A mild tremor can be detected in some affected males as early as 5–6 days-of-age. However, by 10–12 days, tremor involving the head, trunk, and limbs is obvious in all hemizygous males. Unlike WT littermates, they are unable to stand at 12–14 days and this persists throughout life. The tremor lessens if they are left undisturbed and is absent during sleep. A detailed neurological examination is difficult due to the tremor, nonetheless a number of observations can be made. In the early postnatal period, patellar reflexes, pain sensation, and withdrawal reflexes are intact. With the exception of the optic nerve, cranial nerves appear unaffected, and there is no nystagmus, a cardinal symptom in PMD. Pupillary light reflexes are intact but vision is likely absent throughout life as evaluated by the menace reflex and their lack of ability to follow objects moved in their visual field. In contrast, audition is intact.

At 4–5 months, seizures develop in all *shps*, though they are variable in severity ranging from minor to grand mal seizures. Treatment with phenobarbital either prevents or lessens further seizure activity. By 6 months, the tremor changes in character with the development of coarse, uncontrolled whole body movements and opisthotonus. At this stage, muscle tone increases in all four limbs with the development of hyperactive reflexes and clonus in the hind limbs (patellar reflexes). Affected dogs are hand-fed throughout life and gain weight, though usually less than WT littermates. In a single litter, certain *shps* occasionally appear to improve neurologically more than other affected littermates, developing the ability to move into sternal recumbency and appearing more alert.

Heterozygous females in the breeding colony were initially found to be clinically normal, but some years after the establishment of the colony it was noticed that many, if not all, of the carrier females developed a tremor of variable severity, ranging from mild to marked (variable within littermate carriers) (Cuddon et al., 1998). Unlike affected males, however, affected heterozygous females are able to ambulate. The tremor disappears over time, though this improvement varies from 8 to 18 weeks depending upon the initial tremor severity.

General brain pathology

Upon sectioning the brain, the putative white matter was thin, gray and gelatinous (Fig. 1A). This was in marked contrast to the cranial nerves (except the optic nerves), which were white and appeared well-myelinated. As the *shp* aged, hydrocephalus developed over time in all dogs but to a varying degree (Fig. 1A, Suppl. Fig. 1A).

Coronal whole mount, stained sections of *shp* brains revealed a number of pathological changes. The ventricles were dramatically increased in size, an enlargement that appeared to represent a hydrocephalus *ex vacuo*, since there were no obstacles to CSF circulation (Suppl. Fig. 1A). White matter tracts, including the corpus callosum, deep hemispheric white matter, and fornix were thin. In contrast, the cortical ribbon appeared normal. Microscopic examination of the white matter with hematoxylin and eosin (H&E) showed pallor and a decrease in the number of interfascicular OLs (Suppl. Fig. 1B). In addition, *shp* brains showed a prominent population of cells with elongated nuclei, representing microglia and astrocytes (see below). Weil stain for myelin revealed a marked paucity of myelin in the deep hemispheric white matter, subcortical white matter, and corpus callosum (not shown). Patches of myelin, equivalent to the ‘tigroid’ pattern seen in PMD patients (Koeppen and Robitaille, 2002), were seen in the sub-cortical white matter and internal capsule (Fig. 1B). Immunolabeling for GFAP was significantly increased in the *shp* brains, especially in the sub-pial zone, and processes appeared thicker than the normal, fine astrocyte processes in the WT brain (Suppl. Fig. 1C). Microglial processes, immunolabeled for Iba1, also appeared thicker than those of ramified microglia in the WT brain (Suppl. Fig. 1C). The brains of *shps* were slightly smaller than those from WT males and weighed less. At 2–4 years of age, the mean affected male brain weight was 72.5 g (63.7–76.4 g) compared to 83.1 g (78.8–90.0 g) for WT males (Fig. 1C).

Spinal cord pathology

Myelin—There was a clear delay in myelination of the *shp* spinal cord compared to WT and axons appeared thinly myelinated. However, by 2 years of age, many scattered large diameter axons had normal-thickness myelin sheaths (Fig. 2A). The most striking finding was the increase in the number of myelinated axons in all *shps* over 2 years of age, and in some, almost all axons in the dorsal column were myelinated (Fig. 2B). Inter-animal variation in the amount of myelin in *shps* of the same age was noted (Suppl. Fig. 2).

Quantitation of myelinated fiber density showed an overall increase from 4 weeks to 2 years with some variation between the four *shps* at 2 years (Fig. 2C). Larger diameter axons showed the greatest increase in myelination. In addition to the increase in the number of myelinated axons, the thickness of the myelin sheaths increased over time, especially large and medium diameter axons, resulting in decreased g-ratios (Fig. 2C).

Axon density—Microscopic evaluation of axon preservation in toluidine blue (1 μ m) and silver-stained (10 μ m) sections (Suppl. Fig. 3), showed little axon loss in the spinal cord. In rare instances in older *shps*, adjacent areas of white matter showed an apparent difference in axon survival suggesting some focal axon loss (Suppl. Fig. 4). Quantitation of myelinated and non-myelinated axons in the ventral column of *shp* confirmed the increase in the number of myelinated axons and the total axon density over time relative to WT (Fig. 2C). The latter is likely due to reduced myelin content in *shp* spinal cord compared to WT.

Cell death and cell division

The death of cells by apoptosis was determined in the spinal cord of *shps* and WT dogs by TUNEL assay at 6 weeks and 2 years of age. In addition, morphologic criteria was used to

define cell death in 1 μ m sections from embryonic time points (E40–48 and E50) to 28 days of age, (see below) (Fig. 3). TUNEL labeling showed clear evidence of apoptotic cells scattered diffusely throughout the white matter but also occasionally in the gray matter. Quantitation of TUNEL-positive cells showed significantly more dying cells in the *shp* than WT at 6 weeks of age. Cell death was also seen in both the *shp* and WT dogs at 2 years of age although there was no statistical difference (Fig. 3C).

In 1 μ m sections, cells in the early stages of apoptosis with margin-ation of nuclear chromatin were readily identified (Figs. 3D,E). More commonly, pyknotic nuclei were seen scattered throughout the white matter. Though double labeling to identify these cells was not performed, there was presumptive proof that the dying cells were OLs. Cells with marginated chromatin were seen to extend processes to axons, highly suggestive of OLs ensheathing axons. Further proof that the dying cells were OLs was seen where a pyknotic cell had extended a process to a myelinated axon (Fig. 3E). Ultrastructural evidence of the dying cell type was difficult to ascertain as most cells with pyknotic nuclei had degenerative changes of their cytoplasm. We were unable to find clear examples of cells with distended rough endoplasmic reticulum (RER – the hallmark of the defect in the *shp*) that had chromatin margination or a pyknotic nucleus. Quantitation of these pyknotic cells showed that there was cell death in both WT and *shp* dogs from E50 to P28, though there were more dying cells in the *shp* between P0 and P28 than WT (Fig. 3F). Although cell death was not quantified in 1 μ m sections in older dogs, pyknotic nuclei were only rarely seen in *shps* from 2 months to 2 years of age.

There were clearly more dividing cells in the spinal cord and optic nerve of the *shp* between 2 and 5 weeks than in the WT (Suppl. Fig. 5). Three *shps* examined at 5–6 months of age had no labeled cells in the spinal cord and optic nerves. However, the small intestine (WT tissue) was very heavily labeled (Suppl. Fig. 5G). In WT dogs evaluated at 4–5 months, no cells were labeled, confirming the lack of division within the cord. Numerous endothelial cells were heavily labeled in the white and gray matter in both *shps* and WTs as well as cells in the leptomeninges; pericytes were also labeled though less frequently than endothelia (Suppl. Figs. 5D,E). Labeled cells were also noted in the spinal cord gray matter but were less common than in white matter. These data suggest that there was a modest increase in cell division in the *shp* white matter compared to WT in the early post-natal period (Suppl. Fig. 5F).

Oligodendrocyte ultrastructure

OLs were identifiable on EM according to ultrastructural criteria (Mori and Leblond, 1970). In early development, OLs had prominent RER and Golgi apparatus (Fig. 4A). However during the first weeks of life, many OLs of *shp* developed swelling of the RER, which sometimes occupied almost the entire cell soma with hugely dilated cisterns (Fig. 4E). This dilated RER contained a floccular material (Fig. 4B,E). Frequently, the cytoplasm of cells with distended RER completely surrounded thinly myelinated axons (Figs. 4C – E). In some instances, cells with distended RER were seen extending processes around nonmyelinated axons, indicating their active myelinating status (Fig. 4B). The numbers of OLs with distended RER decreased over time, yet at 2 years of age, occasional OLs with mildly

dilated RER were still present. Despite this massive organelle disruption, no OLs with distended RER had either nuclear chromatin margination or other changes indicative of the cell undergoing apoptosis. Interestingly, even though the optic nerves were severely dysmyelinated, fewer OLs were seen with distended RER than in the spinal cord. In addition to the abnormality in RER, notable microtubule accumulations in 'collars' of cytoplasm surrounding non-myelinated and myelinated axons were frequently seen in both young and old affected dogs (Figs. 4F–H). Similar changes were seen in OLs in the spinal cord and brain.

In situ hybridization of PLP1

We investigated cell labeling for *PLP1/DM-20* mRNA in the *shp* from E50 to 2 years and in age-matched WT, using both radioactive and non-radioactive *in situ* hybridization in spinal cord tissue. In WT pups collected by C-section, the earliest time point that cells were labeled was E50 (Fig. 5A), whereas there was no labeling of presumptive OLs in the *shp* at this time (Fig. 5B). By E52, however, some cells were labeled in the *shp* and this increased by P0 (Figs. 5D,F). Quantitation of the numbers of cells labeled from E58 to 12 weeks of age showed an increase in number of positive cells in WT up to 6 weeks with a modest increase, in the number of labeled cells in the *shp* (Fig. 5I). At all time points examined, the number of grains per cell (*i.e.* level of *PLP1/DM-20* mRNA) was considerably less in the *shp* (Fig. 5I), similar to that reported in the *jp* mouse (Verity et al., 1990). This may result from decreased transcription or greater turnover of *PLP1* mRNA. However, a few cells in the *shp*, were labeled almost as intensely as WT, suggesting some heterogeneity in the *shp* cell population or a difference in their temporal development (Figs. 5G,H). Post-natal *in situ* hybridization in the dorsal column at 7 days, 6 weeks, and 2 years of age, showed a greater number of *PLP1*-positive cells in WT compared to *shp* at all time points (Fig. 6A). Quantitation of *PLP1*-positive cells showed an overall increase over time in WT and *shp*, although the density of labeled cells decreased in both due an increase in axon size and myelination over time in both (Fig. 6B).

Western blots

Myelination was further followed over time by analyzing the expression of major myelin proteins, including PLP, DM-20, and MBP in thoracic spinal cord tissue (Fig. 7). Western blots were performed with protein extracts from WT and *shp* animals at time points ranging from P1 to 2 years of age. Western blot analysis of WT and *shp* samples showed a peak in PLP expression at 2 months of age in WT samples and 1 year in *shp* samples. DM-20 expression significantly increased at 4 months of age in the *shp* and was greater than WT levels at 4 months and older. Analysis of MBP expression showed a peak at 1 year of age in WT samples and 2 years in *shp* samples.

Astrocyte reaction

A marked astrocyte reaction to the lack of myelinated axons was seen in the spinal cord of all *shps* as demonstrated in GFAP- immunolabeled sections, with a clear and progressive increase in GFAP labeling from 2 weeks in the neuropil and most notably in sub-pial areas (Figs. 8A–C). EM examination of the spinal cord from as early as four weeks of age showed

numerous instances of abnormal astrocyte/axon interaction, with multiple astrocytic processes interdigitating between the axon and a thin myelin sheath (Fig. 8D). Ultrastructural examination of white matter in older *shp* dogs (1 year and older) showed patchy gliosis of white matter with an increase in the number of astrocyte processes and glial filament density (Fig. 8E).

Biochemical quantitation of GFAP by ELISA from the spinal cord of WT and *shp* dogs confirmed the immunolabeling results with a significant increase in GFAP in *shps* at all time points (Fig. 8F).

Axonal change

1) Light microscopy—While the predominant global pathology throughout the CNS in the *shp* was the myelin defect, notable but variable changes in axons were seen, specifically in the spinal cord. Axonal pathology was most common in the dorsal column of the lumbar spinal cord, though abnormal axons were seen in all funiculi and at all levels of the cord. Although it was observed in only one case, the earliest axonal abnormality seen on light microscopy was at 4 weeks of age. Axonal pathology increased with aging, though it was not uniform and considerable variation was seen in *shps* of the same age. From approximately 6 months of age, all *shp* dogs demonstrated axon involvement. Three distinct axonal abnormalities were seen in the spinal cord and brain. Enlarged axons or spheroids were either dark and granular in appearance (Type 1) or light and amorphous (Type 2) (Fig. 9a). These light spheroids, which were either thinly myelinated or non-myelinated, were most frequently found in the base of the dorsal columns and also scattered throughout the lateral and ventral columns. Dark spheroids were seen in all columns of the spinal cord but were more frequent than the light spheroids in the lateral and ventral columns. They were also seen occasionally in spinal cord gray matter. Likewise, dark spheroids were noted in the brain stem, cerebellum, and corpus callosum. In many of the dark spheroids, a central, paler core could be seen (Figs. 9B, D) and in others, three different zones were present (Fig. 9C).

The third type of axonal pathology was axons that were often swollen and filled with multiple vacuoles (Type 3) (Fig. 9A inset) and distinct from the light and dark spheroids. In the dorsal nerve roots in some of the older *shps*, scattered swollen axons with thin myelin sheaths were noted which were similarly distended with vacuoles (Suppl. Fig. 6). Axons undergoing obvious degeneration were seen at all levels of the spinal cord and brain, though they were very scattered in distribution and not numerous. There was no evidence of a distal axonopathy in sensory or motor tracts of the spinal cord (Suppl. Fig. 7).

2) Electron microscopy—Ultrastructural examination confirmed the diversity of the abnormalities in axons seen on light microscopy and the broad distinction into the three classes of spheroids. EM also demonstrated that axons seen on light microscopy with different zones (Figs. 10B–D) had sequestration of organelles (Figs. 10E–H). However, the earliest abnormality noted was the accumulation of mitochondria in axons with a normal diameter (Fig. 10A). In some instances, these mitochondria were clearly enlarged (Fig. 10B). Increased mitochondria were frequently noted beneath the axolemma (Fig. 10A) and these mitochondria often became vacuolated (Fig. 10C) and could occupy the entire axon

(Fig. 10D). As mitochondria accumulation increased, so did the size of axons and accumulation of other organelles. These dark spheroids varied in size and appearance throughout the cord. Dark spheroids were often thinly myelinated, but massively swollen spheroids lacked a myelin sheath (Figs. 10F,G). The axoplasm of light spheroids ranged in constituents from light, watery axoplasm to those containing branched tubules (Figs. 10G,J). Some of the giant spheroids were 'mixed' in their organelle accumulation with both an increase in mitochondria and an amorphous axoplasm with microtubules (Figs. 10F,I). In contrast, few axons contained accumulations solely of neurofilaments. In rare instances obviously swollen, degenerated axons were seen (Figs. 10K,L).

Brain and optic nerves

One μm sections of the major white matter structures of the brain showed a severe paucity of myelination from early stages through 2 3/4 years of age. The corpus callosum showed variable, though severe myelin deficiency across the genu, body, and splenium. This deficit was more severe than in the spinal cord in the same animal where myelination had progressed (Fig. 11). Ultrastructural examination of the callosum showed that axonal loss was not a feature, as the structure consisted of densely packed non-myelinated axons interspersed between occasional myelinated axons (Fig. 12).

The severe lack of myelin seen in the brain was also noted in the early neonatal period in the optic nerve (Suppl. Fig. 8). Like the spinal cord, myelination increased variably over time across the whole nerve. However, in some dogs at 2 years of age and older, only the sub-pial zone of the nerve contained substantial myelin and the center of the nerve remained dysmyelinated. These areas showed obvious axonal loss and marked gliosis (Suppl. Fig. 9).

Discussion

Missense mutations in the *PLP1* gene result in variably severe deficits in CNS myelination in both humans and animals; is the latter a valid model for PMD? In both animals and man, the nature of the mutation determines the severity of the phenotype. For example, the *jp^{rsh}* mutation causes mild signs in mice and the less severe SPG2 disorder in humans, whereas the *jp^{msd}* mutation is responsible for severe disease in both man and mouse. Animal models offer the opportunity to evaluate the probable evolution of OL and axon changes in their human counterparts. While rodent models are invaluable, the much larger body size and longevity of the dog provide a greater relevance to man. We propose that the changes in the CNS of the *shp* reflect those seen in the classical form of PMD associated with missense mutations.

While the clinical presentation and neurological symptoms are not identical in the *shp* and PMD, this is not surprising given differences in neuroanatomy and myelin development between the species. The primary sign of shaking or tremor in the *shp* is not identical to boys with PMD, yet the later development of head bobbing/truncal ataxia in the *shp* may have similarities to that seen in PMD such as titubation. Nystagmus, which is a cardinal sign in PMD, is never seen in the *shp*. Seizures are always seen in the canine but are variable in PMD, being more common in congenital disease. Visual disturbance is common in both yet the pupillary light reflexes are usually intact suggesting some impulse conduction in the

visual system (Koeppen and Robitaille, 2002). Audition is intact in the *shp* while in PMD, although auditory testing may reveal discrete abnormalities, hearing *per se* is intact (Wang et al., 1995; Kuan et al., 2008; Coticchia et al., 2011). In the *shp*, brainstem auditory evoked responses are clearly abnormal however (Cuddon et al., 1998), as they are in PMD (Wang et al., 1995; Kuan et al., 2008). Over time, the tremor in the *shp* changes to a gross truncal ataxia with head bobbing and the development of hypertonia and increased reflexes and opisthotonus. While the increased tone and patellar reflexes may suggest upper motor neuron involvement, the apparent lack of significant axon loss in motor tracts might suggest another mechanism. Perhaps, this mechanism is a failure of conduction in chronically non-myelinated axons. Opisthotonus in dogs usually reflects a disturbance in the rostral lobe of the cerebellum, which is inhibitory to the stretch reflex mechanism of antigravity muscles, and lesions here frequently lead to extensor rigidity. It can also be seen in PMD. We have not studied dogs beyond 2 3/4 years of age; however, it is possible to note clinical improvement in those dogs over 2 years of age in which more myelin was seen microscopically in the spinal cord. While this lifespan may seem short compared to many PMD patients, it is generally accepted that each year of a dog's life is equivalent to seven years of a human. Thus we predict that our observations are equivalent to PMD patients of 20 to 25 years of age. Therefore, this model allows significantly longer periods of time to study changes in the CNS and over a longer period than is possible in the other severely affected animal *PLP1* mutants.

Perhaps the most surprising result from this study is the degree to which myelination of the spinal cord improves over time as determined by morphological quantitation and Western blot. Though variable between animals, in some cases almost all axons were myelinated in the dorsal column. Most of these axons had thin myelin sheaths, but some large diameter axons had sheaths of normal thickness. Western blot data showed increased expression of myelin proteins MBP, PLP, and DM-20 in the *shp* over time. PLP and MBP expression in the *shp* did not reach WT levels, but DM-20 expression in the *shp* was significantly greater than WT expression at 4 months and older. This observation of an increase in DM-20 in the *shp* is in agreement with our earlier studies showing a disproportional expression of DM-20 in *shp* at 10 weeks of age (Yanagisawa et al., 1987), and is also found in normal developing white matter of many species (Macklin, 1992). The brain and optic nerves, however, appear to remain poorly myelinated with only a modest increase in myelin content. This discrepancy between the brain and the spinal cord remains unexplained but potentially could relate to environmental differences between the two that result in greater cell death or delay in differentiation of OLs. This difference appears to be crucial to future therapeutic approaches. It also remains to be determined whether similar improvement in myelination occurs in the spinal cord in PMD as there have been no reports on the pathology of the cord (except SPG2). However, MRI studies have shown that the brainstem is more myelinated than the brain, hence this may extend to the spinal cord.

Ultrastructural observations of OLs during early stages of myelination showed two clear abnormalities that are likely crucial in the failure of the cell to myelinate axons normally. The first is the distension of RER which showed considerable variability in severity and extent, sometimes occupying almost the entire cytoplasm (Fig. 5) or extending into 'collars'

of cytoplasm surrounding hypomyelinated axons. Importantly, the RER contained a floccular material suggesting protein accumulation (Ghadially, 1982). As the *shp* aged, this RER dilation was seen less frequently in OLs. The RER defect is cell autonomous, as we have shown that OLs cultured from neonatal *shps* have distended RER that increases over time in culture (Suppl. Fig. 10). It is possible that distended RER may occur in some PMD patients, though convincing ultrastructural details are lacking (Adachi et al., 1970; Watanabe et al., 1973). Similar RER distension has been recorded in the *md* rat (Dentinger et al., 1982; Jackson and Duncan, 1988), but not in the other myelin mutants. The *shp* and *md* rat are both missense mutations in which the substituted amino acid, a proline, likely results in a misfolded protein. Interestingly the *shp* mutation is at the same residue (His36) as the paralytic tremor (*pt*) rabbit. In the *pt* rabbit, however, a His → Glu substitution results in a much milder phenotype (Tosic et al., 1994; Sypecka and Domanska-Janik, 2005) more akin to SPG2 with no obvious OL abnormality.

Dilation of the RER is a common finding in normal mammalian development and protein synthesis in certain tissue and cells as well as being seen in a wide variety of disorders. It has also been described during development in plant tissue (Bones et al., 1989). Cells such as fibroblasts, synovial cells, chondrocytes, and plasma cells frequently appear particularly susceptible to distension of RER. Chondrocytes in metaphyseal dystosis have marked distension of RER (Cooper and Pedrini-Mille, 1973), as do melanocytes in a mouse model of vitiligo (Boissy et al., 1991). It is thought that distension of RER, through overproduction or delayed transport of protein to the Golgi, results in an unfolded protein response and the expression of genes such as BiP, CHOP, and ATF6 (Lin and Popko, 2009) leading to the initiation of apoptosis (Southwood et al., 2002). A brief study of BiP expression in the spinal cord of the *Plp1/PLP1* mutants (*jp*, *jp^{msd}*, *md* rat, and *shp*) has shown that that mRNA expression of BiP is increased in *jp* and *jp^{msd}*, but not the *md* rat or the *shp* (Hudson and Nadon, 1992). While the reasons for this are speculative, these are the only two myelin mutants with clear RER distension. It has been suggested that this will likely trigger an unfolded protein response and cell death, but this differs between these two mutants. The expression of CHOP is in fact anti-apoptotic in OLs (Gow and Wrabetz, 2009). Thus, we lack a clear understanding here of the effect of RER swelling on the initiation of an unfolded protein response. We are currently determining the differential gene expression (using RNAseq) in *shp* white matter to define this further.

Here, we report a modest increase in the absolute number of dying cells (likely OLs) in the *shp*. This, however, cannot be directly compared to the incidence of cell death in the *jp* mouse (Knapp et al., 1986) and *md* rat (Lipsitz et al., 1998), where cell death was reported as a percentage of total glial cells. The size of the spinal cord and optic nerve in the *shp* precluded this calculation. Given the fact that it seems likely that the majority of OLs in the *shp* develop an RER distension at some stage of development, relatively few seem to die as a result of this. These cells likely have a resolution of the distension of RER and retain their myelin sheaths. A similar situation is seen in cells of the salivary glands in *Drosophila* where marked distension of RER occurs during development without cell death (Thomopoulos, 1987). While PLP/DM-20 transport in OLs appears disrupted in the *shp*, it may only be delayed or diminished as immunolabeling has shown that a reduced amount of

PLP is eventually incorporated in myelin (Yanagisawa et al., 1987). In contrast, COS-7 cells transfected with the *shp* mutation showed a failure of transport of PLP/DM-20 to the cell surface (Tosic et al., 1997), but this difference may relate to the short time of the *in vitro* study. A similar approach to study differences in the transport of mutated PLP/DM-20 in transfected COS-7 cells in connatal and classical PMD, showed that disease severity relates to the failure of trafficking of both PLP and DM-20 to the cell surface (connatal PMD), in contrast to a delay in DM-20 alone (classical PMD) (Gow and Lazzarini, 1996). Most recently, OLs derived from induced pluripotent cells from PMD patients were reported to have distended RER, but this was not marked (Numasawa-Kuroiwa et al., 2014). Challenging these cells *in vitro* with tunicamycin induced expression of genes associated with ER stress.

The second ultrastructural abnormality seen in OLs was a collar of cytoplasm that surrounded myelinated or non-myelinated axons containing densely packed microtubules. Although more common at early time points, it was also noted in a 2 year old *shp*. This abnormality has not been described in any of the other myelin mutants or in PMD. Microtubules are an important component of the OL and are critical for the transport of PLP protein and *MBP* RNA to the cell surface and developing myelin sheath (Song et al., 2003). In the *shp*, we would suggest that this increase in microtubules close to the myelin sheath is a compensatory response by the OL to increase the transport of misfolded PLP.

In addition to these changes in OLs, a notable reaction of astrocytes was seen from very early in development with frequent abnormal insertion of astrocyte processes between the myelin sheath and axon (Griffiths et al., 1981a). This may reflect abnormal paranodal and juxtanodal structures in the *shp*, but it is also seen in the *jp* mouse, hence it is non-specific (Omlin and Anders, 1983). ELISA quantification showed an increase in GFAP at the earliest time points with ongoing gliosis over time. Despite this notable astrocyte reaction, axons continue to be myelinated suggesting that gliosis is not an impediment to late-onset ensheathment and myelination.

A crucial issue in all myelin disorders is whether axons are lost as a result of the myelin defect, resulting in permanent disability. There is growing evidence that myelin and OLs play an essential role in axon health and survival (Nave, 2010). While axon loss clearly occurs in multiple sclerosis (Ferguson et al., 1997; Trapp et al., 1998), the situation in the leukodystrophies, and PMD specifically, is less clear. It is known that mice with deficiency in PLP or another myelin protein, CNP, show marked axon changes at late or early onset respectively (Lappe-Siefke et al., 2003; Edgar et al., 2009). Mice that overexpress PLP develop late-stage axon degeneration (Anderson et al., 1998). In PMD likewise, a deficiency of the protein leads to axon degeneration (Garbern et al., 2002). However, the overall consensus from neuropathological reports on PMD, describes little/no axon loss. Likewise, in an *MBP* mutation in a rat where axons are demyelinated for up to 9 months, there is no axon loss (Smith et al., 2013). There are clear axonal changes in the *shp*, especially in the spinal cord. In addition, although they are less common, axonal changes are also present in the brain and perhaps most notably in the optic nerve. In summary, abnormal axons in the *shp* are, 1) seen as early as 3 months of age, 2) increase over time 3) most numerous in the deep white matter of the lumbar cord dorsal column, and 4) vary in number in *shps* of the

same age. The axonal changes are very variable in their microscopic appearance. The first abnormality noted was the accumulation of mitochondria in otherwise healthy axons. As this progresses, axons became swollen and there is frequently a separation of different classes of organelles that are being transported by fast or slow transport in anterograde or retrograde directions. Such organelle separation has been previously reported in axons in the PLP knockout mouse (Edgar et al., 2004b) and also in non-related conditions such as the neuroaxonal dystrophies (Cork et al., 1983). Similarly, the lack of CNP leads to axon swelling and degeneration (Lappe-Siefke et al., 2003; Edgar et al., 2009). In the PLP null mouse, axon loss is length dependent and occurs in the dorsal column of the cervical cord, *i.e.* typical of a distal axonopathy (Edgar et al., 2004a). In the *shp*, swellings are primarily seen in the proximal parts of the sensory fibers. Accumulations of mitochondria and other organelles frequently occur at paranodes in PLP null mice (Edgar et al., 2004b), though we did not investigate this. Other swollen axons contained predominately tubular structures, in both non-myelinated axons and, less frequently, in myelinated axons. It is possible that the large, swollen axons previously were myelinated but the swellings led to myelin attenuation or loss over the swelling. The cause of these axonal abnormalities remains unexplained however. Recent studies have identified the critical role of glucose and lactate in OL development, myelination, and axon survival. Metabolic coupling between axons and OLs has been shown with OLs providing aerobic glycolysis products to axons under certain conditions (Funfschilling et al., 2012). The roles of monocarboxylate transporter 1 (MCT1) in OL development and myelination (Rinholm et al., 2011) and in lactate transfer to axons (Lee et al., 2012) and the fact that MCT-1 is enriched in OLs may contribute to axon damage in areas of the CNS where OLs are deficient.

The critical issue however for PMD patients is whether the changes we report here lead to significant loss of axons. Quantification of myelin and axons in the spinal cord revealed that the number of myelinated axons in the *shp* increases over time, with the most notable increase observed in the large diameter axons. A similar increase in axon density in the brain of a PLP1 transgenic mouse has been reported (Ruest et al., 2011). Although the extent of myelination is not comparable to WT, axons continue to be ensheathed over time in older *shps*. This is confirmed with g-ratios, which showed significant decreases (thus increasing myelin sheath thickness) in medium- and large-caliber axons in the *shp*. Myelinated axon density increased in *shps* from young (4 wk) to old (1 yr) before decreasing at 2 years, which is at least in part due to increased myelin content at that time point. Given the size of the canine CNS and overall number of myelinated fibers, it appears that the loss of axons may not reach a functionally significant level. Despite marked axon swelling in the lumbar cord dorsal column, there was no obvious loss of axons distally (in the cervical cord). It may be that many of the axonal changes are reactive (Lampert, 1967) and temporary and do not lead to axon loss as has been suggested in the CNP null mouse (Edgar et al., 2009), cuprizone toxicity (Xie et al., 2010), and experimental autoimmune encephalomyelitis (Nikic et al., 2011). Although *shps* develop spasticity with clonus, which is suggestive of upper motor neuron involvement, axon loss in these tracts appears minimal and only obvious in small areas of the spinal cord white matter. In the brain, the corpus callosum of affected animals at 2 years and older showed no loss of axons. In contrast to the rest of the CNS, significant axon loss can be seen in the optic nerves. In summary, while axons are at

risk in PMD, and there is scattered and sometimes focal loss, this is less important than the myelin deficit. Increased myelination over time may reduce the axonal pathology, although this needs further scrutiny.

Do the data presented here on the model of classical PMD provide ideas on new therapeutic approaches? Perhaps the most revealing information in the *shp*, is the ongoing myelination of the spinal cord which results from the slow increase and accumulation of mature OLs. In contrast, the brain seems to remain severely hypomyelinated, though there may be modest increases in myelin. Studies on PMD in general have focused on the brain, especially in imaging with MRI. Likewise, there is a paucity of pathologic information on the spinal cord of classical PMD patients. From the data presented here, it might be proposed that the generation of more endogenous OLs during the neonatal period could result in greater myelination of the brain, and earlier onset of myelination of the spinal cord. We know from the *shp* that, in a few cases, increased myelination of the spinal cord improved function as might be expected, and could equally have benefit in PMD. Therefore, early pharmacologic intervention using a drug or drugs that promote OL differentiation may be a straightforward approach to treat patients even without gene therapy to correct the mutation in the target cell. If achieved, the question could be asked on the likelihood of survival of axons myelinated by *shp* OLs and in which the myelin sheath contains less than the normal amount of PLP. While the murine transgenics have shown that a lack or overexpression of PLP can lead to late-stage axonal abnormalities, evidence in the *shp* does not suggest that this is inevitable. However, the question still remains as to whether this might occur in much older PMD patients. The idea, of course, that a genetic disease could be treated without gene therapy and with endogenous cells, is a challenging postulate.

In summary, these data show that there is a delay in maturation of OLs in the *shp*, but in the spinal cord this recovers over time with ongoing myelination. Death of OLs does occur, but most survive and distension of RER and an unfolded protein response is not of major significance. While axons clearly are affected by the myelin deficiency and a lack of PLP and axons are lost in the optic nerve, this may not limit the ability of the majority of the CNS to be myelinated over time, especially if this is enhanced early in life by therapeutic intervention.

Conclusions

These studies clearly demonstrate that the early failure of myelination in the CNS of this model of classical PMD recovers dramatically with time, yet the brain remains poorly myelinated. The recovery of myelination in the spinal cord relates to an apparent increase in mature oligodendrocytes. Oligodendrocytes have a marked distension of RER, most notable in early development, suggesting the accumulation of a misfolded protein. While axon changes were noted, appearing early in life and increasing with age, these did not lead to marked loss of axons in the brain or spinal cord. These data suggest that enhancing the differentiation of oligodendrocytes early in life, especially in the brain, could have therapeutic significance in PMD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PMD	Pelizaeus–Merzbacher Disease
<i>PLP1/Plp1</i>	proteolipid protein 1 gene
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling
OL	Oligodendrocyte
GFAP	Glial Fibrillary Acidic Protein

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Fig. 1. Myelination of the *shp* brain is severely retarded. Coronal and sagittal sections of the *shp* brain at 1 and 2 years of age show an obvious lack of myelin (A). This is confirmed in sections stained for myelin at 2 years of age (B). However, poorly stained 'patches' of myelin in sub-cortical regions are present in the *shp* (arrows). Measurement of wet brain weights shows that the *shp* brains are lighter from about 3 months of age and this difference is significant (* $P < 0.05$) at 2 years and older (C). (B) Heidenhain myelin stain.

Fig. 2.

The number of myelinated axons and myelin sheath thickness increase in the spinal cord of the *shp* over time and there is no significant loss of axons. Similar areas of the ventral column of WT and *shp* are shown from 1 day (1d) to 2 years (2y) (A). The *shp* has practically no myelin at 1 day of age compared to WT which appears well myelinated. However, in the *shp* there is a gradual increase and by 2 years of age, many axons are myelinated though the myelin sheaths are thinner than WT. This increase of myelination over time is even more apparent in the dorsal column (B). There is progressive myelination

in *shps* at 2 and 6 months (2 m, 6 m) and 2 years (2y), with almost all axons being myelinated at 2 years which is confirmed in higher power (insets). Histograms represent the quantification of myelin and axons in the ventral columns of WT and *shp* from 4 weeks to 2 years (C). Myelin density is significantly greater in WT than *shp*, though a general increasing trend was observed in the *shp* over time. At right, the variability of myelin density in four individual *shps* is evident. G-ratios were greater in the *shp* than WT, as expected with thicker myelin sheaths in the latter. Myelinated large axon density, sorted by diameter, significantly increased over time in the *shp* while small and medium caliber myelinated axon density did not approach WT levels over time. This is further represented by the myelinated axon density histogram, which also demonstrated the variability in the 2 year *shps*. Total axon area density showed an overall higher density of axons in *shp* compared with WT, likely due to lower myelin content Error bars represent SEM and $P < 0.05$ were deemed significant Scale bar = 10 μ m (A), 100 μ m (B), 10 μ m (B-inset).

Fig. 3.

Apoptotic cells are found in the spinal cord white matter of the *shp*. TUNEL assay on cervical spinal cord tissue from 6 week old WT (A) and *shp* (B) shows many more apoptotic cells in the *shp*. Quantitation of TUNEL-positive cells at 6 weeks (n = 3) and 2 years (n = 3) confirms the difference between WT and *shp* and demonstrates that reduced cell death is still seen in both the mature CNS but at a reduced level (C). One micron sections show clear evidence of dying cells undergoing apoptosis that have the appearance of OLs (C–D). A cell with margined chromatin has a process extending to an axon (D). Apyknotic cell has a process extending to a thinly myelinated axon suggesting that it is an OL (E). Pyknotic cells are more common in the *shp* from E50 through P14–28 (F). Scale bars = 10 μ m (A–C), 20 μ m (D and E).

Fig. 4.

Oligodendrocytes have organelle abnormalities suggesting accumulation of misfolded protein and problems with its transport to the developing myelin sheath. (A) At early time points, *shp* OLs have prominent Golgi apparatus and RER (arrow) as they associate with axons. (B) Over time, the RER begins to swell, containing electron-dense material (inset) yet OLs continue to send processes around axons (arrows). The distension of RER becomes more marked within the cell cytoplasm (C) and is seen in cytoplasmic collars around the myelinated axons (arrows in C and D), becoming more pronounced (D) and finally

occupying the entire cytoplasm (E). The cytoplasmic rings that surround myelinated axons contain densely packed microtubules (C, D). An enlarged view of the microtubule collar marked by an arrowhead in (D) is shown (F). In older dogs, microtubule-filled collars of cytoplasm can still be seen, but without the distended RER, around both non-myelinated (G) and myelinated axons (H). In the corpus callosum of older *shps* (over 2 years of age), numerous OLs were present without distended RER, but had myelinated adjacent axons and extended fine processes to other axons (arrows) (I). Scale bars = 2 μm (A–C, G and I), 3 μm (D–E), 500 nm (B inset and F).

Fig. 5.

Expression of *PLP1* identifies a delay in maturation of oligodendrocytes (OLs) in the *shp*. Radioactive *in situ* hybridization identifies a delay in the appearance of *PLP1*-positive cells in the *shp* at E50(B) with the first development of positive cells at E52(D). On the day of birth (P0), many more *PLP1*-positive cells were seen in WT(F) than in *shp* (G). In addition, the level of message expression was reduced in the *shp* (F, H) compared with WT (E, G). Two OLs (arrows) from each genotype (G, H) confirmed these differences. Error bars

represent SEM and $P < 0.05$ were deemed significant Scale bars = 30 μm (A–E), 10 μm (G–H).

Fig. 6.

PLPI *in situ* from older dogs shows an increase in mature OLs over time. Non-radioactive *in situ* hybridization shows an overall increase in the number of PLPI-positive OLs in WT and *shp* spinal cord tissue from P7 and 6 week (cervical) to 2 years of age (A). Density of PLPI-positive cells in the dorsal column decreases over time due to increases in axonal size and myelination in both WT and *shp*. Histogram depicting the total number of PLPI-positive cells (bars) and cell density (lines) (B). Error bars represent SEM and $P < 0.05$ were deemed significant Scale bar = 2 mm (A, whole spinal cord), 20 μ m (A, insets).

Fig. 7.

Myelin protein levels increase over time in the *shp*. Western blots were performed on the thoracic spinal cord for the major myelin proteins PLP, DM-20, and MBP with β -actin as a loading control (A). The normal increase in PLP levels is notably delayed in the *shp*, yet significantly increases in older animals at 1 and 2 years of age (B). DM-20 expression significantly increases in the *shp* from 4 months on (C). A similar increase to that of PLP in older animals was observed for another major myelin protein, MBP, with significantly increased levels at 1 and 2 years of age in the *shp* (D). Error bars represent SEM and $P < 0.05$ were deemed significant.

Fig. 8.

Astrocytosis is present early in development in the *shp* and is progressive. There is a notable increase in GFAP immunolabeling in the *shp* dorsal column spinal cord at 2 weeks of age (B) compared with WT (A). This increases over time as seen at 19 weeks of age (C). From 4 weeks of age, astrocyte processes are frequently seen in the *shp* spinal cord to interdigitate between axons and the myelin sheath (D). In this example, numerous astrocyte processes can be seen (arrows) and in a serial thick section immunolabeled for GFAP (inset), this 'collar' of processes is positive for GFAP (arrow). On EM, in an older *shp* (2 years of age), areas of the spinal cord are notably gliotic (E). Quantitation of GFAP by ELISA shows an increase at very early time points (1–2 days) with a progressive increase over time (F). Error bars represent SEM and $P < 0.05$ were deemed significant. Scale bars = 20 μm (A–C), 1 μm (D), 4 μm (D inset), 5 μm (E).

Fig. 9.

Axon changes are present in the spinal cord and most profuse in the lumbar spinal cord dorsal column of older *shps*. In a 2 year old *shp*, numerous swollen axons are seen in the dorsal column at the base of the lumbar cord (A). Three types of spheroids are seen, 1) axons containing dark axoplasm, 2) both myelinated and non-myelinated axons containing light axoplasm, and 3) axons containing multiple vesicles (see inset of area marked by asterisk). The dark axons (1) often contain different 'zones' that suggest accumulation of separate populations of organelles (B–D). On EM, those axons on light microscopy with a

light core (B, D) have accumulation of mitochondria in the periphery with granular cytoplasm and tubules in the core (E,G). Those with a dark core (C) have central mitochondrial accumulation (F,H). Scale bars = 1 μm (A), 2 μm (E–G), 5 μm (K–L), 500 nm (B and H–J).

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Fig. 10.

Ultrastructural changes in axons are heterogeneous in their axoplasmic pathology. Numerous axons that appear normal at any other level or tract of the spinal cord had an increase in mitochondria (A). In some instances these mitochondria were enlarged (B) and eventually they vacuolated (C), progressing to fill the entire axoplasm (D). Swollen myelinated (E) and non-myelinated (F, G) axons that were predominately seen in the lumbar cord dorsal column, varied in organelle accumulation. Many had predominately microtubules and some mitochondria (E) while others contained a mix of microtubules, mitochondria and dense bodies (F) and others had almost solely microtubules (G). High power of areas marked by asterisks in E, F and G are shown in H, I and J respectively. Occasional degenerating axons were also seen (K, L). Scale bars = 1 µm (A), 0.5 µm (B), 2 µm (C), 4 µm (D), 2 µm (E, F, G), 0.5 µm (H, I, J), 5 µm (K, L).

Fig. 11.

The myelin deficit is more severe in the brain than spinal cord which recovers over time. The corpus callosum of a 2 year old WT (A) and *shp* (B) shows a major lack of myelin in the *shp*. In the same *shp*, however, the cervical spinal cord (C) is has more myelin. Scale bar = 30 μ m.

Fig. 12.

Axons are preserved in the brain. In the genu of the corpus callosum of a 2 year *shp*, small diameter non-myelinated axons are tightly packed together, but the medium diameter axons are myelinated (A). In the age matched WT, practically all the axons are myelinated (B). Scale Bar = 2 μ m.