Neurological deficits and glycosphingolipid accumulation in saposin B deficient mice

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Saposin B derives from the multi-functional precursor, prosaposin, and functions as an activity enhancer for several glycosphingolipid (GSL) hydrolases. Mutations in saposin B present in humans with phenotypes resembling metachromatic leukodystrophy. To gain insight into saposin B’s physiological functions, a specific deficiency was created in mice by a knock-in mutation of an essential cysteine in exon 7 of the prosaposin locus. No saposin B protein was detected in the homozygotes (B−/−) mice, whereas prosaposin, and saposins A, C and D were at normal levels. B−/− mice exhibited slowly progressive neuromotor deterioration and minor head tremor by 15 months. Excess hydroxy and non-hydroxy fatty acid sulfatide levels were present in brain and kidney. Alcian blue positive (sulfatide) storage cells were found in the brain, spinal cord and kidney. Ultrastructural analyses showed lamellar inclusion material in the kidney, sciatic nerve, brain and spinal cord tissues. Lactosylceramide (LacCer) and globotriaosylceramide (TriCer) were increased in various tissues of B−/− mice supporting the in vivo role of saposin B in the degradation of these lipids. CD68 positive microglial cells and activated GFAP positive astrocytes showed a proinflammatory response in the brains of B−/− mice. These findings delineate the roles of saposin B for the in vivo degradation of several GSLs and its primary function in maintenance of CNS function. B−/− provide a useful model for understanding the contributions of this saposin to GSL metabolism and homeostasis.

INTRODUCTION

The prosaposin locus encodes in tandem four related ~80 amino acid proteins, the saposins, with activation effects on specific glycosphingolipid (GSL) hydrolases (1–4). Saposins are proteolytically cleaved from the protein precursor, prosaposin, in the late endosomes and lysosomes (5–7). The stability of each saposin is maintained by three conserved disulfide bridges (8). The genetic deficiencies of individual saposins or prosaposin highlight their physiological importance in GSL degradation (9–12). Saposin A is essential to the metabolism of β-galactosylceramide (GalCer) (13) and its deficiency leads to a late-onset form of globoid cell leukodystrophy or Krabbe disease (14). Saposin B has lipid transfer properties (15) and stimulates the degradation of sulfatide and other glycolipids (16). Saposin B deficiency in humans leads to sulfatide accumulation and a metachromatic leukodystrophy (MLD)-like disease similar to that observed with arylsulfatase A (ASA) deficiency, its cognate enzyme (17). Saposin C activates acid β-glucosidase (EC 3.2.1.45, GCase) and deficiency of saposin C leads to Gaucher-like diseases due to diminished GCase activity (10,18,19). Saposin D

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enhances acid ceramidase activity (20). In mice, saposin D deficiency is associated with ceramide accumulation, partial loss of Purkinje cells and impaired urinary system function (21). This phenotype does not mimic the embryonic lethality exhibited by mice with complete deficiency of acid ceramidase, saposin D’s cognate enzyme (22). A complete deficiency of human prosaposin results in the storage of multiple GSLs and early lethality (11,12). Targeted disruption of prosaposin in the mouse causes a complex neurodegenerative phenotype resulting in a severe leukodystrophy with accumulation of GSLs in both neurons and microglial cells (23).

Saposin B facilitates partial extraction of GSLs from membranes and presents sulfatides and globotriaosylceramide (TriCer or Gb3) to ASA and α-galactosidase A, respectively, for cleavage (24–26). The dimeric shell structure of saposin B is critical for its binding to these hydrophobic lipids (26). Both saposin B and C have in vitro activation effects on GalCer-β-galactosidase, and GM1-β-galactosidase, as well as on the β-galactosidase for hydrolysis of lactosylceramide (LacCer) (27). Saposin B may also function as a transporter of lipid substrates to these enzymes (15). In a recent study, saposins A–D have been reported to promote lipid binding to the CD1d, a molecule important for NKT cell development (28). Saposin B is found to be most efficient at facilitating this interaction (29).

MLD is an autosomal recessive neurodegenerative lysosomal disease caused by defective ASA activity (30). The MLD variants are characterized by accumulation of sulfatides, progressive demyelination and extensive white matter damage (30). A few cases of MLD-like disease are caused by saposin B deficiency. The saposin B defects arise from various mutations in the saposin B domain of prosaposin that produce substitutions for critical cysteine residues, insertions, transversions or obliteration of a glycosylation site (31,32). Human patients with saposin B deficiency have elevated sulfatide levels in kidney and brain, and variable neurological phenotypes (31).

Deficiency of ASA enzymatic activity caused by targeted disruption of ASA in mice results in diminished in vivo enzyme activity, accumulation of sulfatide in white matter and a neurological phenotype that manifests by 24 months of age. There is no apparent demyelination observed in these mice (33). One specific abnormality includes total absence of auditory brain stem potentials. ASA−/− mice resemble MLD patients biochemically, however, the pathological processes are much slower and milder.

To explore the in vivo effects and interactions of the saposins, a series of saposin mutant mice have been created with single (14,21) or double saposin deficiencies (34). Here, mice with deficiency of saposin B were characterized for GSL metabolism and phenotypic development. This model provides insight into the essential roles of saposin B in GSL metabolism.

RESULTS

Generation and verification of saposin B targeting

Saposin B deficient mice (B−/−) were created by a knock-in of cysteine 4 to create a phenylalanine substitution in exon 7 of the prosaposin locus that corresponds to the saposin B domain (Fig. 1A). The mutation (Cys→Phe) was expected to destroy one of the three essential disulfide bonds of saposin B as had been the previous experience with other saposins (14,21,34). The recombinant ES clones were screened by PCR and confirmed by Southern blot analyses (Fig. 1B). The mutation in the ES clone was verified by PCR and DNA sequencing. The resulting F1 heterozygous mice were bred with Zp3-Cre transgenic mice to remove the floxed neo in the female germline (see Materials and Methods). The Zp3-Cre was subsequently outbred by crosses into wild-type (WT) C57BL/6J mice and confirmed by PCR (Fig. 1C).

RT–PCR of brain RNA verified that prosaposin mRNA was expressed at WT levels in saposin B−/− mice (Fig. 1D). Immunoblotting using antibodies specific to each saposin demonstrated the absence of saposin B protein, whereas prosaposin and saposins A and D were expressed at WT levels in B−/− mice (data not shown). Confocal microscopy revealed a diminished saposin B signal in B−/− fibroblast cells compared with WT levels.

Phenotype of saposin B−/− mice

The onset of neurological impairment in B−/− mice was at ~12 months of age as manifested by an unsteady gait. When suspended inverted by the tail, the B−/− mice reflexively grasped and tucked their hind-limbs to their body, whereas WT mice spread their hind-limbs and remained hanging downward (Fig. 3A). In addition, B−/− mice exhibited agitation and head tremor while standing (Fig. 3B). Ataxia and intermittent shivering was observed by 15 months. Adult B−/− mice had a body weight of 86% of WT littermates and survived up to 23 months. Male and female B−/− mice had normal fertility and no sex-specific differences were observed in phenotype or biochemistry.

Neurobehavioral assessment in saposin B−/− mice

The narrow bridges test evaluates balance and motor performance. To avoid possible sex variation, only male mice were used in the study. B−/− mice showed progressive increases in latency to cross the beams and also in hind-limb slips on 5 and 12 mm square beams. At 2 months, increases in slips reached statistical significance on the 12 mm square beam. A significant increase in latency was shown by 12 months that was consistent with the decline of motor function in B−/− mice at this age. By 15 months, the ability to cross the beam was greatly diminished (Fig. 3C). On the 5 mm square beam, 90% of the B−/− mice at 15 months failed to maintain their balance and fell off the beam. The tests on the 25 mm square, and the 11 and 17 mm round beams showed significant increases in slips or latency to cross in 12 and 15-month-old B−/− mice (data not shown). Locomotor activity in the same mice was tested for general exploration and adaptation. There were no significant differences in locomotor activity between B−/− and WT mice at any of the ages tested (data not shown). Long-term potentiation (LTP) was measured to assess hippocampal plasticity, and no significant differences were observed in B−/− mice in hippocampal LTP compared with WT at 15 month of age.
These results demonstrated that $B^{-/-}$ mice had progressive decline in balance and motor coordination on the narrow bridges test, but general exploration and hippocampal LTP were similar to WT.

Histological and ultrastructural analyses

Hematoxylin and eosin (H&E) stained sections of 15-month-old $B^{-/-}$ mice showed inclusions in a few neurons of spinal cord, brain stem (Fig. 5) and white matter of cerebellum (data not shown). Neuronal cells in dorsal root ganglion contained foamy inclusion material (Fig. 5). To assess the activation of macrophage/microglial cells, anti-CD68 antibody was used to stain the sections. CD68 is an intracellular membrane glycoprotein expressed in quiescent and activated tissue macrophages (35). Positive CD68 staining cells were observed in spinal cord, sciatic nerve, kidney and various regions of brain including thalamus, cortex, cerebellum and brain stem (Fig. 6). Liver, spleen and lung in $B^{-/-}$ mice had background levels of CD68 signal comparable to WT mice. GFAP is an astrocyte marker and enhanced GFAP signal indicates astrogliosis or activated astrocytes. Using anti-GFAP antibody, strong signals were present in the brain stem, corpus callosum, thalamus (Fig. 6), spinal cord and cerebellum (data not shown) of $B^{-/-}$ mice relative to age-matched WT sections. Activation of microglial cells and astrocytes demonstrates proinflammatory responses in the CNS of $B^{-/-}$ mice.

In 15-month-old $B^{-/-}$ mice, strong alcinian blue staining was observed in the brain stem, cortex, cerebellum, thalamus and spinal cord, indicating the presence of acidic sulfated substances (Fig. 7A). Kidney tubules also stained positive for alcinian blue (Fig. 7A). Liver, lung, pancreas, thymus and spleen were negative for alcinian blue staining. Observable sulfatide accumulation began in the kidney at 7 weeks, in the spinal cord at 12 weeks and in the brain at 24 weeks (data not shown). To characterize the cell types containing sulfatide, consecutive spinal cord sections were stained with alcinian blue and cell-specific markers, including: CD68, a microglial cell marker, myelin basic protein (MBP), an oligodendrocyte marker, and anti-NeuN antibody, a neuronal nuclei marker (Fig. 7B). Alcinian blue staining cells were in the MBP positive region. Few $CD68$ stained microglia cells contained alcinian blue staining materials. Neuronal soma was free of alcinian blue, but such staining was detected on the neuronal processes.

These results provide evidence for excess acidic sulfated substances primarily in oligodendrocytes.

Ultrastructural analyses revealed large amounts of storage material in the kidney proximal and distal tubular epithelia.
Neurons in B-/- brains were found in other regions of brain and spinal cord. Materials in oligodendrocytes and neuronal processes also surrounded more electron lucent material (Fig. 8D). Storage oligodendrocytes in brain stem, next to myelin process, variable sized granular material and multivesicular bodies. The storage materials were heterogeneous aggregates of cells (Fig. 8A–C), while mesangial cells appeared normal. The storage materials were heterogeneous aggregates of variable sized granular material and multivesicular bodies. Oligodendrocytes in brain stem, next to myelin process, were filled with heterogeneous membranous material surrounding more electron lucent material (Fig. 8D). Storage materials in oligodendrocytes and neuronal processes also were found in other regions of brain and spinal cord. Neurons in B-/- brain stem and spinal cord contained a few scattered inclusion bodies (Fig. 8E). The Schwann cells in sciatic nerve had inclusion bodies composed of large vacuoles mixed with electron dense amorphous material (Fig. 8F). Myelin layering was normal in B-/- mice (Fig. 8F). Consistent with these results, the level of MBP, a component of myelin sheath, was not altered in B-/- mice as assessed by immunoblottting (data not shown). This result suggests that sulfatide accumulation has no apparent adverse effects on myelin sheath integrity.

**GSL analyses**

Resolution of lipids on thin-layer chromatography (TLC) showed a slight increase of LacCer in B-/- liver relative to the elevated levels in the prosaposin knock-out (PS-/-) mice (Fig. 9A). Elevated sulfatide was detected in the kidney, lung (Fig. 9B) and brain (not shown). A moderate increase of TriCer or Gb3 was observed in the kidney (Fig. 9B). The cerebral cortex displayed levels of gangliosides (GM1, GM2, GM3, GD 1a and 1b, GT1b) comparable to WT controls (Fig. 9C). In addition, increased amounts of sulfatide were in the urine of B-/- mice (data not shown).

The composition of accumulated sulfatide was analyzed by Electrospray ionization tandem mass spectrometry (ESI/MS). Increases in non-hydroxy fatty acid (NFA) and hydroxyl fatty acid (HFA) sulfatides were detected in kidney and brain of B-/- mice. At 48 weeks, B-/- mice kidney contained 5.1- and 2.3-fold increases of C20–C24 NFA and HFA sulfatide, respectively (Fig. 10A). To reduce background levels of sulfatide in white matter, the cerebral cortex and hippocampus were dissected and analyzed for sulfatide accumulation (Fig. 10B). Most of the HFA sulfatides (C18–C24) showed marked increases. At 14.5 months of age the major accumulated NFA sulfatide species were C18 and C24 in the cortex and hippocampus. Overall, ~2-fold increases in total HFA and NFA sulfatides were present in B-/- mouse brain samples. In the brain, C18 sulfatide was the major species affected, whereas long fatty acid acyl chain sulfatides predominated in the kidney. In contrast to the kidney, the NFA sulfatide levels in brain were higher than HFA sulfatide. Ceramide levels were unchanged in the brain and kidney of B-/- mice.

**DISCUSSION**

Genetic deficiencies of individual saposins (A, B, C and D) lead to lysosomal storage diseases (9,11,14,17,21). About 10 cases of saposin B deficiency states have been described in humans (31,32). To understand the in vivo functions of saposin B, mice were generated by introducing a point mutation into the saposin B domain of the prosaposin gene. This mutation disrupted a conserved disulfide bond that led to an unstable/undetectable saposin B protein, but preserved prosaposin, and saposin A, C and D processing and function. This strategy was also used to generate the saposin A, D and CD deficient mice (14,21,34), and facilitated the in vivo assessment of isolated saposin deficiency states in various organs.

The B-/- mouse mimicked the biochemistry and phenotype of the human disease. Similar to human patients (31), B-/- mice developed neurological impairment including ataxia, head tremor and impaired neuromotor coordination. The early onset severe and late onset variants of saposin B deficiency have phenotypes resembling MLD with normal ASA activity (31). Furthermore, the point substitution and aberrant splicing mutations described in humans lead to a deficiency of mature saposin B with no effect on precursor transport and processing (36), but manifest excesses of Gb3 (TriCer), LacCer and GM3 in cultured fibroblasts from affected patients (31,37). Urine from these patients also contains elevated levels of sulfatide, Gb3 and digalactosyldi-Ceramide (37). These findings are similar to those of the B-/- mouse. Electron microscopic findings in B-/- mice showed similar inclusion bodies as reported in humans (32). However, the disease course in mice was more slowly progressive than humans, suggesting substrate turnover differences between mice and humans as has been observed in Gaucher disease point mutation models in mice (38).

The predominant function of saposin B was in sulfatide degradation. However, the accumulation of LacCer and Gb3 (~10–20%) in B-/- mice demonstrated that saposin B also is involved in degradation of these lipids. This finding indicates that saposin B does have in vivo functions in assisting α-galactosidase A and the β-galactosidase(s) involved in Gb3 and LacCer degradation, respectively, but these roles are minor in most tissues. LacCer is increased >2-fold and is the major lipid accumulated in the PS-/- mice (23), and is likely a major contributor to the pathogenesis of the prosaposin deficiency. The effects of the increases in LacCer and
Gb3 in the B−/− mice are unknown, but may be minor, as suggested by the increases in Gb3 in the Fabry mouse model (39,40). The lipid transport functions of saposin B could be compensated by saposin C that also is involved in the degradation of the LacCer (27). In fact, slight increases in LacCer were detected in our newly generated saposin C deficient mice (Y. Sun et al., unpublished data). Such mouse models demonstrate overlapping functions of saposins in GSL metabolism in vivo.

B−/− mice had progressive accumulation of multiple GSLs, but predominantly sulfatide, in the CNS and PNS. In the brain and spinal cord, sulfatide was detected in microglial cells, oligodendrocytes and neuronal processes. A proinflammatory response was demonstrated by the presence of activated microglial cells or astrogliosis in tissues that accumulated sulfatide. This suggests that the initiating event for proinflammation in these tissues was the presence of excess sulfatide. The oligodendrocytes were the major cell types in B−/− mice brains filled with storage materials although no apparent changes were observed in myelin sheets. Both NFA and HFA sulfatide accumulated in B−/− mice. The ratio of NFA/HFA galactosylceramide has been linked to developmental programming (41) and myelin stability (42). However, there was no effect of sulfatide accumulation on myelin integrity in B−/− mice. The normal myelin structure in B−/− mice could be due to maintenance of unchanged NFA/HFA ratios despite an increase in total sulfatide.

The neuronal inclusions were observed in both ASA deficient and B−/− mice. In particular, storage inclusions were found in their acoustic neurons. Neuronal storage of sulfatide has been reported to cause hyperexcitability and axonal degeneration in mice (43). The acoustic startle response was tested in the B−/− mice, but neither WT nor B−/− mice showed adequate responses in the C57BL/6J strain. This is due to the development of deafness in this stain of mice by the age of testing. The C57BL/6J background strain is homozygous for the age-related hearing loss mutation Cdh23ahl that causes progressive hearing loss with onset after 10 months of age (JAX mice data base).

Saposin B participates in the degradation of sulfatide by enhancing ASA activity (37). ASA null mice have slow accumulation of sulfatide and develop CNS abnormalities (33). The B−/− mouse phenotype closely resembled this phenotype and biochemistry. Both models exhibit head tremor caused by accumulation of sulfatide in CNS and normal myelin ultrastructure. Like ASA null mice, B−/− kidney had remarkable sulfatide storage in the tubule epithelial cells that was detectable as early as 7 weeks. Lung had only a slight increase in sulfatide. Other visceral organs showed no

Figure 3. Phenotypes of saposin B−/− mice. (A) Saposin B−/− mice exhibited hind-limb clumping during tail hanging by 15 months and (B) head tremor was evident at 22 months. (C) Narrow bridges test. B−/− mice were tested on 12 mm2 square beam. The latency (left) and foot slips (right) in B−/− became progressively different from WT with increasing age. Male mice were used in the tests. WT, n = 17; B−/−, n = 26. *P < 0.01; **P < 0.001; ***P = 0.0001 (Student’s t-test).
A unique feature of Sap B−/− mice compared with ASA deficient mice was the accumulation of LacCer and Gb3. In both mouse models, the disease is much slower developing than the human analogues. Alternative sulfatide metabolism in mice may account for these differences between humans and mice (44). Differences between mouse models and the human diseases have been observed for several lysosomal storage diseases, including acid β-glucosidase knock-out mice (45), acid β-glucosidase N370S mutant mice (38) and β-hexosaminidase A (Tay-Sach disease) mice (46).

Saposins are essential lysosomal proteins in the GSLs degradation pathway. The saposin B−/− mouse model, as well as those for other saposins (saposin A, D and CD), should provide useful tools for investigating the pathogenesis of sphingolipid storage diseases and for understanding how saposins and their cognate enzymes interact and to maintain the homeostasis of GSLs in the cell/tissues specific manner.

**MATERIALS AND METHODS**

**Materials**

The following were from commercial sources: NuPAGE 4–12% Bis–Tris gel, NuPAGE MES SDS running buffer, mouse anti-GFAP monoclonal antibody, TopoTA cloning vector (Invitrogen, Carlsband, CA); Rat anti-mouse CD68 monoclonal antibodies (Serotec, Oxford, UK); M-PER (Mammalian Protein Extraction Reagent) and BCA protein assay reagent (Pierce, Rockford, IL). Molecular Dynamics Storm 860 scanner (GE

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**Figure 4.** Long-term potentiation (LTP) analyses of saposin B−/− hippocampal regions: LTP was recorded as the slope of resulting EPSPs from the parasagittal sections (350 μm) of hippocampal CA1 region. The slopes of the EPSP (LTP) were not significantly different between 15-month-old B−/− (black circles) and WT (open circles) male mice after recording for 90 min following stimulation. WT, n = 6; B−/−, n = 8.

**Figure 5.** Storage inclusions in neural tissues from B−/− mice: H&E staining of B−/− mice neural tissue sections showed that the inclusion materials (arrows) were present in neurons of spinal cord, brain stem, acoustic ganglion of inner ear and dorsal root ganglion. WT mice did not show inclusions in matched regions. The tissues sections were from 17-month-old WT and B−/− mice.

**Figure 6.** CD-68 and GFAP staining of CNS from B−/− mice. (Upper panel) Activated microglial cells were present in the CNS of B−/− mice. Microglial cells staining with anti-CD68 antibody (brown) in (A) WT and (B) B−/− cerebella, (C) B−/− spinal cord and (D) B−/− brain stem. The sections were counter stained with hematoxylin. (Lower panel) B−/− mice showed increased anti-GFAP (green) antibody positive cells indicating astrogliosis in several CNS regions. (E) WT and (F) B−/− thalami, (G) B−/− corpus colosum and (H) B−/− brain stem. The nuclei were stained with DAPI (blue). The tissues sections were from 17-month-old mice.
Healthcare, Chicago, IL) was used, and Hybond™-ECL™ nitrocellulose membrane and ECL detection reagent (Amersham Biosciences, Piscataway, NJ). Anti-fade/DAPI, methyl green, ABC Vectastain and Alkaline phosphatase kit II (Black) were used (Vector Laboratory, Burlingame, CA). DIG Easy Hyb, Anti-digoxigenin-AP, CDP-Star, positively charged nylon membrane and Lumi-film chemiluminescent detection film were used (Roche Applied Science, Indianapolis, IN) as well as the Qiagen Quick-change II XL kit (Qiagen Inc., Valencia, CA). Restriction enzymes were from New England BioLabs (Beverly, MA). TOTALLY RNA (Ambion Inc., Austin, TX) and High capacity cDNA archive kit (Applied Biosystems, Foster City, CA) was used. FITC-conjugated goat anti-rabbit antibody and rhodamine-conjugated goat anti-rat antibody was used (ICN/CAPPEL, Aurora, OH), as was Alcian blue (Poly Scientific, Bay Shore, NY).

Construction of the targeting vector and generation of B/− mice

The mouse prosaposin genomic DNA clones and targeting vector (Osdupdel) were kindly provided by Dr Kunihiko Suzuki. A subclone 6-1 in pBluescript vector containing exons 2-7 and subclone 4-1 with exons 4-7 was used to generate the saposin B targeting vector. Substitution of a conserved cysteine (Cys) breaks one of the three disulfide bridges in the selected saposin and results in the deficiency of that individual saposin (14,21). This strategy was adapted to produce saposin B deficiency by introducing a Cys→Phe substitution in saposin B. This was accomplished using Qiagen Quick-change II XL kit with mismatched oligonucleotide primers: B4F (5'-TGGTCTCTGCAAGTTTAAACGTG-G-3'), B4R (5'-CGTGTCTTAAATTCAAGACAG-3') using the subclone 4-1 as a template. The mismatched nucleotides are underlined. The short arm BamH I-Kpn I 1.6 kb fragment containing the saposin B mutation was generated by PCR and subcloned into the TopoTA cloning vector. The long arm Xho I-Cla I 4.0 kb fragments were generated by PCR using subclone 6-1 as a template. The product was cloned into the TA cloning vector. The saposin B targeting construct was assembled in Osdupdel containing the Polymer enhancer/ herpes simplex virus thymidine kinase (MC1) promoter with the neomycin (neo) gene flanked by two loxP sites and the thymidine kinase (TK) gene driven by the 3' phosphoglycerate kinase (PGK) promoter (23). The short arm (1.6 kb) containing the mutation was cloned into BamH I/Kpn I sites downstream of MC1-neo in the vector, thereby forming the 3' homologous region. The long arm (4.0 kb) released with EcoR I from the TA cloning vector was blunt ligated into Nhe I sites upstream of MC1-neo to form the 5' homologous region. The sequences of long arm and short arm were validated by direct sequencing.

The targeting vector was linearized with NotI and introduced into the 129/SvEv ES cell line. Recombinant ES cells were obtained by using standard strategies and transferred into 129/SvEvBd mouse embryos. Chimeras were identified by Southern blotting using a probe spanning the first intron and first exon of the saposin B gene..wav

Figure 7. Alcian blue (sulfatide) staining in CNS of B/− mice: (A) Alcian blue staining of B/− tissues showed sulfatide (blue) storage in: spinal cord (A), kidney tubules (B), brain stem (E) and thalamus (F). WT spinal cord (C) and kidney (D) were free of alcian blue staining. (B) Consecutive spinal cord sections from B/− mice were stained with alcian blue (A, C and E) and MBP (B), CD68 (D) and NeuN (F). (A and B) Alcian blue staining was localized to the MBP positive region (star). (C and D) CD68 positive microglial cells contained alcian blue positive material (arrows). (E and F) Alcian blue staining was on a neuronal process (arrow) and not in the neuronal soma. The tissues sections were from 15-month-old mice.

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clones were screened by PCR analysis using the primers inside of the short arm: ABR1 (5'-GCC AGA CCT GTC AGT TTG TGA TGA A-3') or outside of the short arm: ABR2 (5'-CTG GAG GAG TTC TAT ACG TGC CCA-3'), together with the primer in the 3' region of neo, NEO3'F2 (5'-GGT GTT GGG TCG TTT GTT CGG ATC A-3'). The recombinant and WT clones generated 1.4 and 1.6 kb PCR products, respectively. The recombinant ES clones were confirmed with Southern blot analyses. The clones were digested by BamHI / BglII and probed with the 5'0 probe. The mutations in the recombinant clone were verified by PCR and sequencing. The correctly targeted clones were used to generate chimeric mice by microinjection into C57BL/6J blastocysts. Electroporation, ES cell culture, selection and chimera breeding were carried out by the mouse Gene-targeting service core at the University of Cincinnati. To remove the neo gene, the heterozygous F1 mice were cross bred to Zp3-Cre mice [C57BL/6-TgN(Zp3-Cre)93Knw; The Jackson Laboratory]. The resulting females carrying the Zp3-Cre transgene were not changed compared with WT cortex. WT and B-/- samples were from 15-month-old mice and PS-/- is from 4-week-old mice.
Prosaposin Knock-out (PS−/−) mice (23) were used in the GSL analysis. The mice were maintained in microisolators in accordance with institutional guidelines under IACUC approval at Cincinnati Children’s Research Foundation.

**PCR genotyping and Southern blot analyses**

PCR reactions and Southern blotting were conducted as described (34). The genotyping primers were B-F (5′-GGA GGA AGC ATT TCA CCA GCC CAC T-3′) and B-R (5′-GGA GTA ATG ACT AGT AGG GAG TGA TCA-3′). The PCR product from WT was 200 bp, and from B−/− with neo was 1.4 kb and without neo was 307 bp. Using a 5′ probe in Southern blotting, Bgl II/BamH I digestion generated 10.8 kb WT fragment and 6.8 kb recombinant fragment.

**RT–PCR**

Total RNA was extracted from mouse tissues using TOTALLY RNA kit (Ambion Inc.). Reverse transcription of total RNA (10 μg) for each tissue was carried out using the High Capacity cDNA Archive Kit (Applied Biosystems) containing random hexamers primers. The reaction was for 10 min at 25°C and 120 min at 37°C. The resulting cDNA was amplified by PCR using the 10 pmol of primers PS-Left (5′-TGCAGCTGCGAAGTGAGC-3′) and PSm-Reverse...
The mice were tested on two different shapes of wood beams. Behavioral studies (narrow bridges) were visualized with primulin spray (100 mg/l in 80% ethanol) and blue fluorescence scanning (Storm 860, GE Healthcare). GSL analyses were determined by TLC with chloroform/methanol/0.22% CaCl$_2$ (60:35:8, v/v/v). GSLs in tissue samples (~100 mg wet weight) were extracted as described (47,48). Relative proportions of lipids from the tissue samples were determined by TLC with chloroform/methanol/water (65:25:4, v/v/v). The gangliosides were resolved in 60, 200 m$^2$ cross sections of 25, 12 and 5 m$m^2$, and round beams (1 m) with diameters of 28, 17 and 11 mm were used. The horizontal beams were 50 cm above the floor. Both ends of the beam were mounted to narrow supports. The starting point of the beam was illuminated with a 125 W floodlight and two halogen lights. The end of the beam was attached to an enclosed 20 cm$^2$ dark box. For the training phase, the mice traversed the 12 mm$^2$ beam for three consecutive days, four trials per day. The first trial lasted a maximum of 2 min and each subsequent trial was a maximum of 1 min. A successful beam cross was recorded when an animal passed the second support closest to the goal box. Animals were considered to be trained when they crossed the 12 mm$^2$ beam in <20 s. If a mouse failed to traverse the beam on the first day, then on the second day the mouse was allowed to start trials in the middle of the beam. Once the mouse successively traversed the beam, the start position was then moved back to the original start position. The test phase began on the fourth day and each mouse in turn received two consecutive trials (up to 60 s/trial) on each square and round beam in a progression from widest to narrowest. Latency to traverse each beam and the number of times the hind feet slipped off the beam were recorded. A total of 26 B$^+/-$ male and 17 age-matched WT male mice were included in the test. The tests were started when mice were 2-month-old and repeated at 12 and 15 month.

Tail hanging reflex observations
Mice were lifted by tail to observe the legs reflex. The legs of normal mice were in ‘V’ position. The affected mice exhibited inward trend with legs and a clasped position.

Locomotor activity
Individual male mice (26 B$^+/-$ and 17 WT) were placed in activity chambers for 60 min. Locomotor activity was measured in a 41 x 41 x 30 cm Accuscan activity monitor equipped with 16 pairs of photodetector-LED beams along the x and y axes (Accuscan Electronics with VersaMax software, Columbus, OH). The apparatus was cleaned with 70% ethanol between animals. Horizontal activity was recorded in 5 min intervals. B$^+/-$ and WT mice were alternated between each chamber in the test to minimize time of day effects. The test was conducted under normal fluorescent light conditions. The mice were tested at 2, 12 and 15 month.

Electrophysiology
Electrophysiology was performed using the MED64 multi-electrode array system (Alpha Med Sciences, Kadoma, Japan) (51). Fifteen-month-old mice (8 B$^+/-$ male and 6 WT male) were decapitated and the brains were placed in cold artificial cerebral spinal fluid (aCSF: 124 mM NaCl, 26 mM NaHCO$_3$, 10 mM dextrose 3 mM KCl, 1.25 mM Na$_2$HPO$_4$, 2 mM CaCl$_2$ and 1 mM MgSO$_4$). Parasagittal sections (350 mm) were taken from the hippocampus and transferred to a warm (34°C) oxygenated aCSF bath for 1 h. A section was placed in the recording chamber on an 8 x 8 electrode array with the hippocampus oriented so that the CA1...
region was on the array to determine LTP in the Schaffer collateral pathway. The recording chamber was perfused with warm (37°C) aCSF at a rate of 0.5 ml/min and a humidified oxygen (95%)/CO2 (5%) mix was added to the chamber at a rate of 2 ml/min. Pair-pulse stimuli were delivered at CA1 and excitatory post-synaptic potentials (EPSPs) were recorded until stable baseline slopes were achieved for a minimum of 10 min. Following baseline recording, a theta burst stimulation (5 Hz for 2 s) was applied to the section. The slope of resulting EPSPs was recorded for 90 min following stimulation. Brain sections were recorded in duplicate for each animal and the data were averaged per animal. Data were analyzed using Performer 2.0 software (Alpha Med Sciences).

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