Peripheral Nervous System Manifestations in a Sandhoff Disease Mouse Model: Nerve Conduction, Myelin Structure, Lipid Analysis

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Accessibility
Peripheral nervous system manifestations in a Sandhoff disease mouse model: nerve conduction, myelin structure, lipid analysis
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Abstract

Background: Sandhoff disease is an inherited lysosomal storage disease caused by a mutation in the gene for the β-subunit (Hexb gene) of β-hexosaminidase A (αβ) and B (ββ). The β-subunit together with the GM2 activator protein catabolize ganglioside GM2. This enzyme deficiency results in GM2 accumulation primarily in the central nervous system. To investigate how abnormal GM2 catabolism affects the peripheral nervous system in a mouse model of Sandhoff disease (Hexb⁻/⁻), we examined the electrophysiology of dissected sciatic nerves, structure of central and peripheral myelin, and lipid composition of the peripheral nervous system.

Results: We detected no significant difference in signal impulse conduction velocity or any consistent change in the frequency-dependent conduction slowing and failure between freshly dissected sciatic nerves from the Hexb⁺/⁻ and Hexb⁻/⁻ mice. The low-angle x-ray diffraction patterns from freshly dissected sciatic and optic nerves of Hexb⁺/⁻ and Hexb⁻/⁻ mice showed normal myelin periods; however, Hexb⁻/⁻ mice displayed a ~10% decrease in the relative amount of compact optic nerve myelin, which is consistent with the previously established reduction in myelin-enriched lipids (cerebrosides and sulfatides) in brains of Hexb⁻/⁻ mice. Finally, analysis of lipid composition revealed that GM2 content was present in the sciatic nerve of the Hexb⁻/⁻ mice (undetectable in Hexb⁺/⁻).

Conclusion: Our findings demonstrate the absence of significant functional, structural, or compositional abnormalities in the peripheral nervous system of the murine model for Sandhoff disease, but do show the potential value of integrating multiple techniques to evaluate myelin structure and function in nervous system disorders.

Background

Gangliosides are a diverse class of glycosphingolipids (GSL) involved in cell-to-cell interactions, regulation of cell growth, apoptosis, neuritogenesis, and differentiation of cells [1]. Gangliosidoses, like Tay-Sachs, occur when these lipids are incompletely catabolized due to an inherited enzyme deficiency; GM2 gangliosidoses are characterized by incomplete GM2 catabolism due to the absence of β-hexosaminidase activity. The α- and β-subunits of β-hexosaminidase are encoded by the HEXA and HEXB
genes. In non-pathogenic conditions, ganglioside GM2 is degraded to GM3 in the lysosome by the HexA isoenzyme combined with the GM2 activator protein. Without the activity of the HexA isoenzyme, massive lysosomal GM2 accumulation is observed which disrupts the normal cytoarchitecture of the neuronal cells [2]. Sandhoff disease (SD) is an inherited GM2 gangliosidosis that occurs in 1 of every 384,000 live births [3]. Both HexA and HexB are non-functional. Curative therapy for SD and other GSL storage disorders has not yet been elucidated; however, some treatments that have shown promise managing these diseases are enzyme replacement therapy, gene therapy, bone marrow transplant, stem cell therapy, substrate reduction therapy, and caloric restriction [4-8].

The SD mouse model (Hexb−/−) shows rapid GM2 accumulation characteristic of early onset SD in patients. By contrast, heterozygotes (Hexb+/−) do not display any of these symptoms, express normal ganglioside distribution, and live a normal life span around 2 years [9]. By postnatal day 5, the Hexb−/− mice exhibit GM2 and, its asialo derivative, GA2 accumulation in the brain [10]. This accumulation of GM2 parallels neurochemical features of the infantile form of SD. After 3 months, Hexb−/− mice begin a steady progression to near complete loss of hind limb movement, excess muscle wasting, especially in the hind limbs, and abnormal motor function. After 4.5 months, Hexb−/− mice are unable to move, eat, or drink, and there is a 300% increase of GM2 in the brains of these animals [6]. In the Hexb−/− mice, extensive neuronal storage is observed throughout the cerebrum, cerebellum, spinal cord, trigeminal ganglion, retina, and myenteric plexus [9].

Abnormalities in the PNS as part of the pathology of the GM2 gangliosidoses have also been found. Specifically, studies have shown a motor neuron disease phenotype, loss of large diameter myelinated fibers in the peroneal nerve, and abnormal sympathetic nervous skin responses in patients with chronic GM2 gangliosidosis [11-13]. In addition, GM2 accumulation has been detected in anterior horn motor neurons and in the Schwann cells of the dorsal root ganglion in a mouse model of SD [9,14,15]. This mouse model also demonstrates apparent hind-limb paralysis and extensive hypotonia [9]. Despite these studies, SD is commonly considered a disease of the central nervous system (CNS) and elucidation of the peripheral nervous system (PNS) in patients and animal models remains incomplete. To illuminate our understanding of SD as pertaining to the integrity of PNS myelin in the mouse model of SD (Hexb−/−), we used electrophysiological methods for function, low-angle x-ray diffraction (XRD) for structure, and high-performance thin-layer chromatography for lipids. Our working hypothesis for the present study was: if the lipid composition of the neuronal or myelin membranes in the PNS was altered due to faulty catabolism of GM2, then changes in the myelin and in nerve electrophysiology would be observed. Classically, XRD is used for periodicity measurements of internodal myelin; here, we also used it to quantitate the relative amount of myelin in whole nerves [16,17]. The results demonstrate the value of integrating multiple techniques to evaluate myelin structure and function and offer a potential strategy that will be useful for future investigations into nervous system disorders that could involve demyelination.

Results

Electrophysiological measurements were normal

Sciatic nerves from 5 Hexb+/− and 7 Hexb−/− mice were used for electrophysiological experiments. Compound nerve conduction velocity (CNCV) values of the two groups were not different (Table 1). The sciatic CNCVs of the Hexb+/− and Hexb−/− mice were 23.6 m/s ± 0.6 and 25.1 m/s ± 0.9, respectively (mean ± SEM). The data show that the CNCV falls significantly more in the Hexb−/− mice than the Hexb+/− mice when stimulated at 100 sec−1 for 1 second (p < 0.05, two-tailed, unpaired t-test). However, this difference was not observed at higher stimulation frequencies (400 sec−1 and 600 sec−1), at which the CNCV values of both groups of nerves decreased by much larger percentages, with no difference between them. The Wedensky ratios (see Materials and Methods) and large (L) and small (S) amplitude decreases were analyzed at different stimulation frequencies to monitor the frequency-dependent conduction failure. At 100 sec−1 and 600 sec−1 stimulation, no significant difference between the Wedensky ratios for the Hexb+/− and Hexb−/− mice was detected. In addition, the data show that the L and S signals dispersed at similar rates in the Hexb+/− and Hexb−/− nerves at 400 sec−1 and 600 sec−1 stimulation. However, at 400 sec−1, the Wedensky ratio was significantly higher for the Hexb+/− nerves than the Hexb−/− nerves (p < 0.05, two-tailed, unpaired t-test). To analyze the effects of stimulation frequency on the Wedensky ratio, the two values were plotted against one another (Figure 1). The slopes of the linear regressions for the Hexb−/− and Hexb+/− data did not differ significantly within 95% confidence limits.

CNS myelin was hypomyelinated, PNS myelin was normal

XRD analysis (Figure 2) revealed that the myelin period of optic nerves (CNS) for the Hexb+/− and Hexb−/− mice were 156.2 Å ± 0.2 (n = 3) and 156.0 Å ± 0.1 (n = 4), respectively (mean ± SEM). Myelin period of sciatic nerves (PNS) for the Hexb+/− and Hexb−/− mice were 175.3 Å ± 0.4 (n = 8) and 175.0 Å ± 0.3 (n = 8), respectively. Based on the relative strengths of the diffraction patterns [16,17], the relative amounts of myelin in the optic nerves of the Hexb+/− and Hexb−/− mice were 0.24 ± 0.01 (n = 3) and 0.22 ± <0.00 (n = 4), respectively. This suggests slightly...
less relative amounts of myelin in the optic nerve of the Hexb-/- mice (p < 0.02; two-tailed, unpaired \( t \)-test). By contrast, the relative amounts of myelin in the sciatic nerves of the Hexb+/- and Hexb-/- mice were indistinguishable (0.34 ± 0.02 (n = 8) and 0.35 ± 0.03 (n = 8), respectively).

The widths (\( w \)) of the x-ray peaks provide information about the relative number of myelin layers in a diffracting region of the sheath and the regularity of the membrane packing [16]. When the squares of the integral widths (\( w^2 \)) are plotted against the fourth power of the Bragg order (\( h^4 \)), the \( y \)-intercept of the trend-line is inversely proportional to the number of the repeating units (i.e., myelin membrane pairs) and the slope is proportional to the membrane packing disorder [18] (Figure 3). In the CNS, the slope for the Hexb+/- samples was 0.85 ± 0.12 with a \( y \)-intercept of 334 ± 20, and for the Hexb-/- samples the slope was 0.87 ± 0.15 with a \( y \)-intercept of 335 ± 35. In the PNS, the slope for the Hexb+/- samples was 0.11 ± 0.01 with a \( y \)-intercept of 188 ± 6, and for the Hexb-/- samples was 0.09 ± 0.01 with a \( y \)-intercept of 187 ± 8. These differences in the myelin packing and thickness between the Hexb+/- and Hexb-/- mice were not statistically significant.

In accordance with recently published data [16], the steeper slope and higher \( y \)-intercepts for the optic nerve indicate that its myelin sheaths are thinner and have more packing disorder than myelin in the sciatic nerves.

### GM2 present in PNS

The total ganglioside content of the sciatic nerves in the Hexb+/- and Hexb-/- mice was analyzed and the results are expressed as \( \mu g \) sialic acid/100 mg dry weight (mean ± SEM) (Table 2). No significant difference in total gangliosides was detected between the Hexb+/- and Hexb-/- samples. The ganglioside distribution of the sciatic nerves was determined from densitometric scanning of the HPTLC plate (Figure 4).

The most noticeable difference was the presence of GM2 in the Hexb-/- compared to Hexb+/- mice (Table 2). The Hexb-/- samples contained 1.0 and 0.9 \( \mu g \) sialic acid/100 mg dry weight of GM2 and neither Hexb+/- sample had any detectable levels of GM2. The presence of GM2 is apparent in the Hexb-/- sample lanes (Figure 4). No statistically significant differences were detected among the distribution of the other gangliosides, neutral lipids, and acidic lipids (Table 2).

### Discussion

Brain dysmyelinogenesis is suspected as a secondary symptom of GM2 gangliosidoses [6,19-21]. Supporting this hypothesis, the present XRD results indicated hypomyelination in the amount of compact myelin in the optic nerve of Hexb-/- mice. According to these results, future lipid analysis of myelin isolated from optic nerves from Hexb-/- mice would be expected to show a slight reduction in their relative amounts of myelin.
in cerebrosides and sulfatides, myelin markers. In response to these XRD results and the growing literature supporting myelin abnormalities in the CNS, the present study examined a number of PNS characteristics that would be affected if abnormal PNS myelin is present in the Hexb-/- mice.

The present electrophysiological studies indicated only slight variations in frequency-dependent conduction failure of excised sciatic nerve tissue between the Hexb+/- and Hexb-/- mice, and these changes were not observed consistently under the different stimulation conditions. In addition, there was no significant difference in the CNCV values. This is consistent with past case studies reporting normal motor conduction velocities in patients with chronic GM2 gangliosidosis [13] and with the results of the present study obtained from the PNS using XRD. These findings suggest that the structure and function of the nodal and paranodal regions are normal.

We used XRD here as a sensitive and quantitative probe of the relative amount of myelin and its periodicity in a large volume of unfixed tissue (i.e., whole sciatic and optic nerves) rather than from just a thin-section, as for electron microscopy. Previous measurements demonstrate the consistency of XRD findings with those from microscopy [16,17,22]. No significant differences between the Hexb+/- - and Hexb-/- mice were found for the breadths of the x-ray
reflections, which informs about average myelin thickness and membrane packing disorder. Together with the decrease of the relative amount of compact myelin detected in the optic nerve, these results suggest that the axon fiber density (number of axon fibers per cross-sectional area) in the optic nerves of the Hexb−/- mice may be less than in the Hexb+/− mice. A decrease in the axon fiber density in the Hexb−/- mice may be due to the neurodegeneration observed at late stages of disease progression in mouse models of SD [23,24]. Electron microscopy of optic nerve cross-sections would be required to test this hypothesis. Unlike the CNS findings, no reduction of compact myelin was detected in the PNS. In accordance with this finding, no change in the amount of cerebrosides or sulfatides was detected in the PNS tissue. Past studies have shown that LM1 is found to be mainly in rat PNS nerve myelin and that it deposits like cerebrosides and sulfatides. Therefore, relative amounts of LM1 could possibly be used as a marker for the amount of myelin in the PNS tissue if the ganglioside distribution in mouse PNS tissue is similar to that in rat PNS tissue [25]. In the future, lipid analysis of LM1 in myelin isolated from sciatic nerve samples could provide further verification of the present XRD results. Whether or not myelination was delayed in the CNS, as previously suggested [21], or in the PNS cannot be resolved from the present experiments. XRD analysis would be required at various age points during the progression of the disease to detect delayed myelination.

Our XRD findings indicating no decrease in the amount of compact myelin in the PNS seem inconsistent with the case study of an adult with GM2 gangliosidosis in which nerve biopsy of the peroneal nerve showed severe loss of myelinated fibers, especially those with the largest diameter [13]. One might expect that this would have a significant impact on the relative amount of compact myelin detected by XRD if similar loss of myelinated fibers in the PNS was present in the Hexb−/- mice. The discrepancy may be explained by the phenotypic differences between the

| Lipids             | Hexb+/−       | Hexb−/-       |
|--------------------|---------------|---------------|
| Total Gangliosides | 44.2 ± 1.2 (n= 5) | 39.7 ± 3.8 (n = 5) |
| Individual Gangliosides (n = 2) | | |
| GM3                | 2.0, 2.5      | 2.8, 1.9      |
| GM2                | n.d.         | 1.1, 1.0      |
| LM1                | 1.5, 3.5      | 1.4, 0.8      |
| GM1                | 0.7, 1.4      | 1.8, 1.0      |
| GD3                | 0.6, 0.6      | 0.6, 0.8      |
| GD1a               | 23.1, 23.9    | 23.8, 19.0    |
| GT1a               | 0.3, 0.3      | 1.0, 0.4      |
| GD1b               | 1.4, 1.8      | 1.6, 1.5      |
| GT1b               | 8.7, 7.7      | 8.6, 7.0      |
| GQ1b               | 6.5, 5.1      | 5.1, 4.5      |

*Values are expressed as mean ± SEM in mg/100 mg dry weight (neutral, acidic) or μg sialic acid/100 mg dry weight (ganglioside).  
* n, the number of independent samples analyzed. (6–8 sciatic nerves were pooled per independent sample)  
* due to small amounts of gangliosides present in tissue, only two samples were obtained for analysis  
* n.d., not detected

Table 2: Lipid Distribution of Sciatic Nerve in Hexb Mice

Figure 3
Myelin Membrane Packing in Optic and Sciatic Nerves from Hexb+/- and Hexb−/- Mice. The integral widths w² are plotted as a function of h⁴ to determine the relative amount of myelin packing disorder according to the theory of paracrystalline diffraction [18]. The projected intercept on the ordinate axis is inversely related to the number of repeating units N (the coherent domain size), and the slope is proportional to the fluctuation in period, ∆ (lattice or stacking disorder). There were no differences within 95% confidence levels between the Hexb+/− (open symbols, dashed line) and Hexb−/- (filled symbols, solid line) slopes of the optic (circles) or sciatic (squares) nerves (n = 3–8) indicating no change in the membrane packing of the internodal compact myelin for the sciatic nerves (PNS) and for the optic nerves (CNS).
necessary to confirm this. This accumulation may be partly
associated with the Schwann cells. Lipid analysis of myelin iso-
lation from sciatic nerve of the
ensheathing Schwann cells. Lipid analysis of myelin iso-
lated from sciatic nerve of the

differs from previously reported ganglioside composition in the
mouse brain of the Hexb-/- mice [6]. GM3 was found in
small amounts and GD1a was the major ganglioside in the
Hexb/- and Hexb+/- samples. These results also differ from
previously reported ganglioside distribution for
mouse sciatic nerve [25]. A difference in the mouse strain
and age may account for the discrepancies.

Conclusion
In summary, these experiments offer evidence for dysmy-
elination in the CNS in SD models. PNS findings suggest
that peripheral symptoms observed in SD models stem
from abnormalities in the CNS. Further studies will be
necessary to elucidate the extent to which the PNS is
involved in the pathology of SD and to determine the use-
fulness of targeting this system during treatment design.

Methods
Transgenic mice
Sandhoff mice (Hexb-/-), derived by homologous recom-
bination and embryonic stem cell technology [26], were
obtained from Dr. Richard Proia (National Institutes of
Health, Bethesda, MD, USA). The heterozygous (Hexb+/+)
and knockout (Hexb-/-) mice that were used during these
experiments were bred at the Boston College Animal
Facility by crossing Hexb+/- females with Hexb-/- males.
Hexb+/- animals exhibit identical lipid profiles as Hexb+/
animals, show no phenotype, and live a normal mouse
life span [9]. To ensure the genotype of the mice, the hex-
osaminidase specific activity was measured from tail tis-
sue using a modified Galjaard procedure [27,28]. All mice
were kept in individual plastic cages with filter tops con-
taining Sani-Chip bedding and cotton nesting pads. The
room was kept at 22°C on a 12 h light and 12 h dark cycle
and were fed Prolab RMH 3000 chow (LabDiet, Rich-
mond, IN, USA). All animal experiments were carried out
in accordance with the Boston College Institutional Ani-
mal Care and Use Guidelines.

Electrophysiology
Mice were sacrificed around 4 months of age (120 – 142
days) by cervical dislocation and decapitation. Sciatic
nerves were immediately dissected from the ankle to the
spinal column (1.6 – 2.5 cm) and placed in Locke solu-
tion (154 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl2, 5 mM
dxystrose, 2 mM HEPES, pH 7.2) at room temperature. The
nerve chamber contained circulating Locke solution that
was equilibrated to 28°C using a Peltier device. This tem-
perature was chosen instead of body temperature in order
to slow conduction and thereby maximize separation
between the stimulus artifact and the CAP. In addition,
28°C is a temperature where metabolism is sufficient to

Lipid analysis indicated no significant change in the total
ganglioside content of the sciatic nerve tissue in the
Hexb/- mice; however, GM2 was increased. Previously, GM2
accumulation has been reported in the anterior horn
motor neurons and Schwann cells in the dorsal root gan-
glion [9,14,15], regions that were not isolated with the
peripheral tissue samples examined here. Therefore, the
slight GM2 elevation we observed suggests GM2 storage
throughout the PNS, and perhaps localized to the
ensheathing Schwann cells. Lipid analysis of myelin iso-
lated from sciatic nerve of the Hexb-/- mice would be nec-
essary to confirm this. This accumulation may be partly

Figure 4
HPTLC of Ganglioside Distribution in Hexb+/- and Hexb-/- Mice.
HPTLC of two Hexb+/- and two Hexb-/- samples show the
ganglioside distribution of sciatic nerve tissue. For each sam-
ple, gangliosides having approximately 1.3 μg of sialic acid
were spotted on the HPTLC plates. The plates were devel-
oped by a single ascending run with chloroform:metha-
nol:water (55:45:10, v:v) containing 0.02% CaCl2·2H2O. GM2
is present in the Hexb/- lanes (arrows) and undetectable in
the Hexb+/- lanes. The identity of the GM2 band was con-
firmed using an external standard (Hexb/- brain tissue, neural
tube).

two systems. The case study is an adult-onset variation of
GM2 gangliosidosis, whereas the Hexb/- mice resemble
the infantile variant most closely [9]. Regarding electroph-
ysiology, our recording set-up may not have been able
to detect the behavior of the largest diameter myelinated
fibers (fastest conducting fibers). We measured peak
amplitudes and latencies that are not representative of the
fastest fibers. Analysis of the behavior of these fibers was
hindered by the overlap with the falling phase of the stim-
ulus artifact. In future experiments, electron microscopy
on cross-sectioned sciatic nerve could elucidate the rela-
tive ratios of large and small diameter nerve fibers in the
sciatic nerves of the Hexb/- mice.

responsible for the phenotypic symptoms observed in the
Hexb/- mice. Lipid analysis also revealed that the gangli-
oside composition in the mouse PNS is very different from
the previously reported ganglioside composition in the
mouse brain of the Hexb+/- mice [6]. GM3 was found in
small amounts and GD1a was the major ganglioside in the
Hexb/- and Hexb+/- samples. These results also differ from
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spinal column (1.6 – 2.5 cm) and placed in Locke solu-
tion (154 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl2, 5 mM
dxystrose, 2 mM HEPES, pH 7.2) at room temperature. The
nerve chamber contained circulating Locke solution that
was equilibrated to 28°C using a Peltier device. This tem-
perature was chosen instead of body temperature in order
to slow conduction and thereby maximize separation
between the stimulus artifact and the CAP. In addition,
28°C is a temperature where metabolism is sufficient to

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maintain ion gradients for several hours. At higher tempera-
tures, the stimulus artifact and action potential over-
lapped owing to the small length of the nerve. After
immersing the nerve in the 28°C solution, the proximal
end was laid across a pair of stimulating Ag/AgCl elec-
trodes above the solution. The distal end was then drawn
into a suction electrode containing a Ag/AgCl wire and
also lifted above the solution. To minimize the size of
the stimulus artifact and maximize the size of the CAP signal,
the diameter of the suction electrode matched the diame-
ter of the nerve where the two made contact. Between the
stimulating and recording electrodes, the nerve remained
immersed in the circulating, 28°C Locke solution.
Cathodal stimulation was employed for all of the experi-
ments. Stimulus duration was set to 0.035 ms and the
supramaximal stimulus (Grass Instruments, Quincy, MA,
USA) was determined by monitoring the height of the
CAP on an oscilloscope (Tektronix, Beaverton, OR, USA).
The stimulus voltage was increased until the height of the
CAP no longer increased. Then, raising the stimulus by
25%, the supramaximal stimulus was obtained. Through-
out the experiment, one minute of resting activity was
maintained between each recording. Recordings at high
frequency stimulation were taken for 1 second. Wave-
forms were captured using model 1401 A/D converter
(Cambridge Electronic Design, Cambridge, UK). Data
were analyzed off-line using Spike 2 software (Cambridge
Electronic Design, Cambridge, UK). Frequency-dependent
procedures were organized as follows: 3 single CAPs,
3 at 100 sec^-1, 3 single CAPs, 3 at 400 sec^-1, 3 at 600 sec^-1,
and 3 single CAPs.

Compound nerve conduction velocity values were deter-
mined for each nerve by dividing the length of the nerve
by the latency between the stimulus and the highest point
of the earliest CAP peak (Tl; see Figure 1), corresponding
by the latency between the stimulus and the highest point
mined for each nerve by dividing the length of the nerve
Compound nerve conduction velocity values were deter-
mined between small and large axons. This latter phenomenon, known as
Wedensky inhibition (in which the CAP amplitudes al-
nated between small and large), was quantitated by com-
paring the amplitude above the pre-stimulus baseline of
the first CAP signal in a train of impulses to those of the
last 6 CAP signals in the train. At 400 sec^-1 and 600 sec^-1
stimulation, when such Wedensky inhibition was
observed, the small (S) and large (L) amplitudes at the
end of the train were analyzed separately to monitor the
behavior of the conduction failure (Figure 1). Ratios for
the S to the first CAP amplitude, L to the first CAP ampi-
itude, and S to L ("Wedensky ratio") were calculated. Two-
tailed, unpaired t-tests were employed to determine the p
values between the Hexb/- and the Hexb+/- mice for these
different conduction parameters. For all results, sample
values that were greater or less than the mean value by 6
standard errors were not included in the analysis.

X-ray diffraction and myelin structure analysis
Nerve tissue samples were prepared for XRD as described
[16]. Mice were sacrificed around four months of age by
cervical dislocation and the sciatic and optic nerves were
immediately dissected by tying them off at both ends with
silk suture. The nerves were continually rinsed with phys-
iological saline (154 mM NaCl, 5 mM Tris buffer, pH 7.4)
during the dissection. The nerves were slightly extended in
0.7-mm (sciatic nerves) or 0.5-mm (optic nerves) quartz
capillary tubes (Charles Supper Co., Natick, MA, USA)
containing saline. The capillaries were then sealed at both
ends with wax.

XRD experiments utilized nickel-filtered, single-mirror-
focused CuKα radiation from a fine-line source on a 3.0
kW Rigaku x-ray generator (Rigaku/MSC Inc., The Wood-
lands, TX, USA) operated at 40 kV by 14 mA. In accord-
ance with our established protocol [16], XRD patterns for
each sample were recorded for 1 h using a linear, position-
sensitive detector (Molecular Metrology, Inc., Northamp-
ton, MA, USA). The diffracted intensity was then input
into Excel, and the corresponding intensities from each
side of the beam stop were averaged to obtain a more
accurate measurement of the myelin periodicity, which is
calculated from the positions of the peaks. The intensity
data was subsequently input into PeakFit (Jandel Scien-
tific, Inc.) and the background was subtracted. The inten-
sity of the resulting peaks was integrated to obtain integral
areas I(h) and integral widths w(h) for each reflection of
order h. To determine the relative amounts of myelin
packing disorder, the integral widths w^2 were plotted as a
function of h^4, in which the intercept on the ordinate axis
is inversely related to the number of repeating units N (the
coherent domain size), and the slope is proportional to the
fluctuation in period Δ (lattice or stacking disorder)
[18]. Lastly, the relative amount of compact myelin in the
whole nerve was estimated by summing the integrated
intensity for myelin (M) after background (B) subtraction
(excluding the small-angle region around the beam stop
and the wide-angle region of the pattern). A scatterplot of
the fraction of total, integrated intensity that is a result of
myelin (M/(M+B)) versus myelin period (d) [16] was used
to determine whether there are differences in the myelin
period and/or the relative amount of compact myelin
between the two groups of transgenic mice.
Lipid isolation, purification, and quantification

Total lipids were isolated from mouse brain standards and sciatic peripheral nerve tissue for analysis using established protocols [29]. To prepare the samples, 40 Hexb+/− and 38 Hexb−/− mice were sacrificed around 4 months of age. Due to insufficient amount of tissue, lipid analysis of optic nerve was not conducted. Each sciatic nerve sample contained nerves from 6–8 mice. After storage at -80°C, the samples were lyophilized overnight and the lipids were prepared as previously described [29]. Briefly, total lipids were extracted using chloroform:methanol (1:1, v:v) and dH2O, then resuspended in chloroform:methanol:water (30:60:8, v:v), and applied over a DEAE-Sephadex A-25 Column (Pharmacia Biotech, Uppsala, Sweden). The eluant was collected as the F1 fraction, which contains the neutral lipids cholesterol, phosphatidylcholine, phosphatidylethanolamine, plasmalogens, ceramide, sphingomyelin, and cerebrosides. The F2 fraction, which contains the gangliosides and the acidic lipids, was then eluted from the column with chloroform:methanol:0.8 M sodium acetate (30:60:8, v:v). To further purify the F2 fraction, the samples were subjected to the Folch procedure, which separated the gangliosides and salts (upper aqueous phase) from the acidic lipids (lower organic phase) [30,31]. The ganglioside fraction was then further purified by base treatment with sodium hydroxide followed by desalting using a C18 reverse-phase Bond Elute column (Varian, Harbor City, CA). Total gangliosides were quantified using the resorcinol assay previously described [29]. Svennerholm nomenclature for gangliosides is used [32].

All lipids were analyzed qualitatively by high-performance thin-layer chromatography (HPTLC) using previously described methods [29]. Briefly, for gangliosides, 1.5 μg sialic acid was spotted per lane. Due to the small amount of gangliosides present in the sciatic nerve, ganglioside samples were pooled to obtain an N of 2. The plates were developed by a single ascending run with chloroform:methanol:dH2O (55:45:10, v:v) containing 0.02% CaCl2·2H2O. Gangliosides were visualized using a resorcinol-HCl reagent and heating at 105°C for 30 min. For acidic lipids, 100–200 μg dry weight of each sample was spotted, and for neutral lipids, 35–70 μg dry weight of each sample was spotted. An internal standard (oleoyl alcohol) was added to both the lipid standards and to the samples as previously described [33]. The neutral and acidic lipid plates were developed with chloroform:methanol:acetic acid:formic acid:water (35:15:6:2:1, v:v) to a height of 4.5 cm or 6.0 cm, respectively, and then developed completely with hexanes:diisopropyl ether:acetic acid (65:35:2, v:v). The plates were subsequently charred with 3% cupric acetate in 8% phosphoric acid solution followed by heating at 160°C for 7 min for visualization.

To quantify the ganglioside results, the percentage distribution and density of the individual bands were determined by scanning the plates on a Personal Densitometer SI with ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The total ganglioside distribution was normalized to 100%, and the percentage distribution values were used to calculate sialic acid concentration (μg of sialic acid per 100 mg dry weight) of individual gangliosides [34]. The results for both neutral and acidic lipids were quantified using the same technique described for the gangliosides except the density values for the lipids were fit to a standard curve of the respective lipid and used to calculate individual concentrations (mg per 100 mg dry weight).

Abbreviations

SD = Sandhoff disease
PNS = peripheral nervous system
CNS = central nervous system
GSL = glycosphingolipid
XRD = low-angle x-ray diffraction
CNCV = compound nerve conduction velocity
w = integral width
h = Bragg order
M = integrated intensity for myelin
B = background intensity
d = myelin period

Competing interests

The author(s) declare that they have no competing interests.

Authors’ contributions

MAM conceived the study, conducted x-ray experiments, electrophysiological experiments, lipid analysis, and drafted the manuscript. RCB ran parallel lipid analysis (data included). RLA established x-ray diffraction protocol. TNS participated in the design of the study. GRS developed the electrophysiology experiment and analysis protocol. DAK established the analysis protocol for x-ray diffraction, participated in the design of the study, and helped draft the manuscript. All authors read and approved the final manuscript.
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References
1. Taylor C, Marta C, Bansal R, Pfeiffer S: The transport, assembly, and function of myelin lipids. In Myelin Biology and Disorders 1 Volume I. Edited by: Lazzarini RA, Griffin JW, Lassman H, Nave K-A, Miller RH. Trapp BD. Amsterdam: Elsevier/Academic Press; 2004:57-88.
2. Gravel RA, Kableck MM, Proia RL, Sandhoff K, Suzuki K, Suzuki K: The GM2 gangliosidoses. In The Metabolic and Molecular Bases of Inherited Disease Volume I. Edited by: Scriver CR, Beaudet AL, Valle D, Sly WS. New York: McGraw-Hill; 1995:3827-3876.
3. Meikle PJ, Hopwood J, Clague AE, Carey WF: Prevalence of lysosomal storage disorders. JAMA 1999, 281(3):249-254.
4. Jeyskumar M, Butters TD, Dwek RA, Platt FM: Glycosphingolipid lysosomal storage diseases: therapy and pathogenesis. Neurophathol Appl Neurobiol 2002, 28(5):343-357.
5. Platt FM, Butters TD: New therapeutic prospects for the glycosphingolipid lysosomal storage diseases. Biochem Pharmacol 1998, 56(4):421-430.
6. Denny CA, Kasperzyk JL, Gorham KN, Bronson RT, Seyfried TN: Influence of caloric restriction on motor behavior, longevity, and brain lipid composition in Sandhoff disease mice. J Neurosci Res 2006, 83(6):1028-1038.
7. Norflus F, Tifft CJ, McDonald MP, Goldstein G, Crawley JN, Hoffmann A, Sandhoff K, Suzuki K, Proia RL: Bone marrow transplantation prolongs life span and ameliorates neurologic manifestations in Sandhoff disease mice. J Clin Invest 1998, 101(9):1881-1888.
8. Andersson U, Smith D, Jeyskumar M, Butters TD, Borja MC, Dwek RA, Platt FM: Improved outcome of N-butyldeoxycorticosteroid-imycin-mediated substrate reduction therapy in a mouse model of Sandhoff disease. Neurobiol Dis 2004, 16(3):506-515.
9. Sango K, Yamanaoka S, Hoffmann A, Okuda Y, Grinberg A, Westphal H, McDonald MP, Crawley JN, Sandhoff K, Suzuki K, Proia RL: Neuronal and oligodendroglial cell isolation and purification of total lipids from animal tissues. J Neurosci Methods 1994, 51(2):170-176.
10. Baek RC, Kasperzyk J, Platt F, Seyfried T: N-butyldeoxycorticosteroid-imycin reduces brain ganglioside and GM2 content in neonatal Sandhoff disease mice. J Neurochem 2004, 90(Suppl 1):89.
11. Salmon MS, Clarke JT, Midroni G, Waxman MB: Peripheral and autonomic nervous system involvement in chronic GM2 gangliosidosis. J Inherit Metab Dis 2001, 24(1):65-71.
12. Federico A, Palmieri S, Malandrini A, Fabrizi G, Mondelli M, Guazzi GC: The clinical aspects of adult hexosaminidase deficiencies. Dev Neurosci 1991, 13(4-5):280-287.
13. Mondelli M, Rossi A, Palmieri S, Rizzuto N, Federico A: Neurophysiological study in chronic GM2 gangliosidosis (hexosaminidase A and B deficiency), with motor neuron disease phenotype. Ital J Neurol Sci 1989, 10(4):433-439.
14. Sango K, Yamanaoka S, Ajiki K, Tokashiki A, Watabe K: Lysosomal storage results in impaired survival but normal neurite outgrowth in dorsal root ganglion neurons from a mouse model of Sandhoff disease. Neuropathol Appl Neurobiol 2002, 28(1):23-34.
15. Ofusa S, Kotani M, Tajima Y, Tsuji D, Ishibashi Y, Kuroki A, Itoh K, Watabe K, Sango K, Yamanaoka S, et al.: Establishment of immortalized Schwann cells from Sandhoff mice and corrective effect of recombinant human beta-hexosaminidase A on the accumulated GM2 ganglioside. J Hum Genet 2005, 50(9):460-467.
16. Avila RL, Inouye H, Baek R, Yin X, Trapp BD, Feltis ML, Wraatz L, Kirschner DA: Structure and stability of interstitial myelin in mouse models of hereditary neuropathy. J Neuropathol Exp Neurol 2005, 64:976-990.