Myelination in the absence of UDP-galactose: ceramide galactosyl-transferase and fatty acid 2-hydroxylase

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Abstract

Background: The sphingolipids galactosylceramide (GalCer) and sulfatide are major myelin components and are thought to play important roles in myelin function. The importance of GalCer and sulfatide has been validated using UDP-galactose:ceramide galactosyltransferase-deficient (Cgt-/-) mice, which are impaired in myelin maintenance. These mice, however, are still able to form compact myelin. Loss of GalCer and sulfatide in these mice is accompanied by up-regulation of 2-hydroxylated fatty acid containing (HFA)-glucosylceramide in myelin. This was interpreted as a partial compensation of the loss of HFA-GalCer, which may prevent a more severe myelin phenotype. In order to test this hypothesis, we have generated Cgt-/- mice with an additional deletion of the fatty acid 2-hydroxylase (Fa2h) gene.

Results: Fa2h-/-/Cgt-/- double-deficient mice lack sulfatide, GalCer, and in addition HFA-GlcCer and sphingomyelin. Interestingly, compared to Cgt-/- mice the amount of GlcCer in CNS myelin was strongly reduced in Fa2h-/-/Cgt-/- mice by more than 80%. This was accompanied by a significant increase in sphingomyelin, which was the predominant sphingolipid in Fa2h-/-/Cgt-/- mice. Despite these significant changes in myelin sphingolipids, compact myelin was formed in Fa2h-/-/Cgt-/- mice, and g-ratios of myelinated axons in the spinal cord of 4-week-old Fa2h-/-/ Cgt-/- mice did not differ significantly from that of Cgt-/- mice, and there was no obvious phenotypic difference between Fa2h-/-/Cgt-/- and Cgt-/- mice.

Conclusions: These data show that compact myelin can be formed with non-hydroxylated sphingomyelin as the predominant sphingolipid and suggest that the presence of HFA-GlcCer and HFA-sphingomyelin in Cgt-/- mice does not functionally compensate the loss of HFA-GalCer.

Background

Galactosylceramide (GalCer) is the most abundant sphingolipid of mammalian myelin [1]. It is synthesized in the endoplasmic reticulum by UDP-galactose:ceramide galactosyltransferase (encoded by the Cgt gene). In the Golgi apparatus, part of the GalCer is sulfated by cerebroside sulfotransferase (encoded by the Gal3st1 gene), forming sulfatide [2] (see Figure 1). In the absence of a functional Cgt gene, compact myelin can be formed, which is, however, unstable and Cgt-/- mice develop tremors and ataxia at 3 to 4 weeks of age [3,4].

This phenotype could be explained by disturbed axon-glial contacts at the paranodes in the CNS caused by mistargeting of essential adhesion molecules, NF-155 and Caspr [5,6]. At least in part, these structural alterations are caused by the loss of sulfatide rather than GalCer, as demonstrated by a similar alteration of the paranodal region in Gal3st1-deficient mice, which lack sulfatide but have normal GalCer levels [7]. However, myelin appear to be more stable in Gal3st1-deficient mice, suggesting additional, yet less defined roles of GalCer in myelin [8].

In Cgt-/- mice, the loss of GalCer and sulfatide is accompanied by a significant upregulation of 2-hydroxylated fatty acid-containing (HFA) glucosylceramide (HFA-GlcCer) and HFA-sphingomyelin [3,4]. Interestingly, there are no indications for presence of HFA-gangliosides or...
other higher glycosylated HFA-sphingolipids in the brain of Cgt<sup>−/−</sup> mice [9]. The presence of HFA-GlcCer and HFA-sphingomyelin was interpreted as a compensatory upregulation, which may also (in part) functionally replace HFA-GalCer, enabling Cgt<sup>−/−</sup> mice to form compact myelin [3,10].

HFA-sphingolipids in CNS and PNS myelin are synthesized from 2-hydroxylated fatty acids, formed by the fatty acid 2-hydroxylase (encoded by the Fa2h gene) [11-14]. Although loss of 2-hydroxylated sphingolipids in myelin does not affect initial myelin formation, it causes late onset (in mice older than 6 months) axon and myelin sheath degeneration [15]. Fa2h-deficiency was identified as the cause of a new leukodystrophy with spastic paraparesis [16], and hereditary spastic paraplegia SPG35 [17], respectively.

In order to test the hypothesis that the presence of HFA-GlcCer and HFA-sphingomyelin in Cgt<sup>−/−</sup> mice is a functional important compensatory upregulation, preventing a more severe phenotype, we generated Fa2h<sup>−/−</sup>-deficiency mice with an additional deficiency in the Fa2h gene. Our analysis shows that the additional deletion of Fa2h does not obviously affect the phenotype of Cgt<sup>−/−</sup> mice. This suggests that HFA-GlcCer and HFA-sphingomyelin do not functionally compensate the loss of HFA-GalCer in Cgt<sup>−/−</sup> mice.

### Results

#### Generation of Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup> double deficient mice

In order to test the hypothesis that HFA-GlcCer partially compensates the loss of HFA-GalCer in Cgt<sup>−/−</sup> mice and thereby prevents a more severe phenotype, we generated Fa2h<sup>−/−</sup>-deficiency mice with an additional deficiency in Fa2h. As shown previously, young Fa2h<sup>−/−</sup>-deficiency mice form structural and functional normal myelin [15] and did not show behavioral abnormalities that would indicate myelin deficiency. Older Fa2h<sup>−/−</sup>-deficiency mice, however, developed a progressive axonal degeneration in peripheral nerves and brainstem, accompanied by myelin sheath degeneration [15]. As shown before [3,4], Cgt<sup>−/−</sup>-deficiency mice had a strongly reduced life span, whereas Fa2h<sup>−/−</sup>-deficiency mice did not show increased mortality (data not shown). Survival of Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup>-deficiency mice was not significant different from Cgt<sup>−/−</sup>-deficiency mice (around 50% survival at four weeks of age). There were no obvious behavioral differences between the two genotypes. However, because of the low amount of age-/weight- and gender-matched 4-week-old Cgt<sup>−/−</sup>-deficiency and Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup>-deficiency mice available, extensive behavioral testing could not be performed, and thus minor behavioral differences between Cgt<sup>−/−</sup>-deficiency and Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup>-deficiency mice cannot be ruled out. The following biochemical and morphological analyses were done with mice at 4 weeks of age.

#### GlcCer levels are reduced in CNS and PNS of Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup>-deficiency mice when compared to Cgt<sup>−/−</sup>-deficiency mice

TLC analysis of total brain lipids from wild-type, Fa2h<sup>−/−</sup>-deficiency, Cgt<sup>−/−</sup>-deficiency and Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup>-deficiency mice showed significant levels of HFA-GlcCer in Cgt<sup>−/−</sup>-deficiency mice, in line with earlier reports [3,4]. Unexpectedly, NFA-GlcCer levels in total brain of Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup>-deficiency mice were strongly reduced compared to HFA-GlcCer levels in Cgt<sup>−/−</sup>-deficiency mice (Figure 2A). Presence of NFA-GlcCer in Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup>-deficiency mice was better visible when the amount of lipids from these mice loaded was increased 4-fold compared to controls (Figure 2B). A similar reduction in GlcCer levels was seen when sphingolipids of purified CNS myelin were examined (Figure 3A). Densitometry revealed a reduction of NFA-GlcCer by more than 80% compared to HFA-GlcCer levels in CNS and PNS of Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup>-deficiency mice when compared to Cgt<sup>−/−</sup>-deficiency mice (Figure 2B). In CNS of 2-week-old Cgt<sup>−/−</sup>-deficiency mice, presence of HFA-GlcCer was strongly reduced compared to HFA-GlcCer levels available, extensive behavioral testing could not be performed, and thus minor behavioral differences between Cgt<sup>−/−</sup>-deficiency and Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup>-deficiency mice cannot be ruled out. The following biochemical and morphological analyses were done with mice at 4 weeks of age. 

![Figure 1 Pathway of sphingolipid biosynthesis in myelinating glia cells. Changes in sphingolipids observed in Cgt<sup>−/−</sup> mice (blue arrows) and expected possible changes in Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup> mice (red arrows). Shown are the genetic symbols of genes encoding the respective enzymes: CerS, ceramide galactosyltransferase; CenS, ceramide synthase; DesS, dihydroceramide desaturase; Fa2h, fatty acid 2-hydroxylase; Gal3str1, galactose-3-O-sulfotransferase 1; Sgms, sphingomyelin synthase; Ugcg, UDP-glucose ceramide glucosyltransferase.](#)
were not analyzed) (Figure 3E). The increase of sphingo-
myelin in the PNS was about 50% in Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup> mice (Figure 3F).

In line with data published by Bosio et al. [18], HFA-
ceramide was detectable in Cgt<sup>−/−</sup> mice but not in mice of other genotypes (Figure 4A). However, in contrast to the 18-day-old Cgt<sup>−/−</sup> mice analyzed by Saadat et al. [19], we did not observe a strong increase of ceramide in 4-
week-old Cgt<sup>−/−</sup> mice compared to wild-type controls. This might be due to the different ages examined.

To examine the possibility that GlcCer was replaced by gangliosides in myelin of Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup> mice, gangliosides were isolated and analyzed by TLC. Although the total amount of gangliosides in the myelin fraction of Fa2h<sup>−/−</sup>/
Cgt<sup>−/−</sup> and Cgt<sup>−/−</sup> mice was increased compared to wild-type, the major myelin ganglioside GM1 was unchanged and only gangliosides normally found in neuronal membranes (GD1a, GD1b, GT1b) were increased (Figure 4B). Most likely these gangliosides are derived from neuronal membranes and possibly reflect a higher proportion of neuronal membrane contaminations in the Cgt<sup>−/−</sup> and Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup> mice, with their significant reduced amounts of compact myelin. In line with this, Saadat et al. [19] showed a similar relative composition of major gangliosides in myelin preparation from mice deficient in Ugcg in myelinating cells. We therefore believe that mye-
lin ganglioside levels in Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup> mice were not increased compared to Cgt<sup>−/−</sup> mice or wild-type controls. In summary, we conclude that HFA-GlcCer present in Cgt<sup>−/−</sup> CNS myelin was replaced by only low amounts of NFA-GlcCer but larger amounts of NFA-sphingomyelin in Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup> mice. Taken together, these results indicate that myelin hexosylceramides were mainly replaced by sphingomyelin in the absence of Fa2h expression. It should be noted that the increase of sphingomyelin and the decrease of hexosylceramides were more pronounced in CNS myelin than in the PNS. This might indicate differen-
tes between oligodendrocytes and Schwann cells in the trafficking or metabolism of sphingolipids.

**Myelination in Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup> mice as compared to Cgt<sup>−/−</sup> mice**

Myelin content in the brain of Cgt<sup>−/−</sup>, Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup>, and wild-type mice was examined by Western blot analysis of myelin basic protein (MBP) and by gravimetry of purified compact myelin. At the examined time point (4 weeks of age), MBP levels were reduced in Cgt<sup>−/−</sup> mice compared to wild-type controls (Figure 5A), in agree-
ment with our previous observations [20]. A comparable reduction of MBP was found in Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup> mice. Accordingly, the amount of compact myelin isolated by sucrose gradient centrifugation was significant reduced in both, Fa2h<sup>−/−</sup>/Cgt<sup>−/−</sup> and Cgt<sup>−/−</sup> mice, compared to wild-
type controls (Figure 5B), but there was no significant difference between the first two genotypes.

**Absence of GalCer and HFA-sphingolipids does not affect stability of CHAPS-insoluble membrane fractions**

Compact myelin of wild-type mice is stable against extraction with the detergent CHAPS at low [21] and high temperature [15]. Data by Simons et al. [21] suggest that loss of HFA-GalCer in Cgt<sup>−/−</sup> mice affects the association of the myelin proteolipid protein (PLP) with CHAPS-insoluble membrane fractions (CIMF), suggest-
ing a role for (HFA-)GalCer in the formation or stabiliza-
tion of CIMF. We have recently shown that the absence of HFA-sphingolipids does not affect stability of myelin CIMF [15]. However, in order to examine a possible
Figure 3 Myelin lipid analysis of Fa2h<sup>-/-</sup>/Cgt<sup>-/-</sup> mice. (A) TLC analysis of lipids extracted from purified myelin confirmed the low concentration of NFA-GlcCer in Fa2h<sup>-/-</sup>/Cgt<sup>-/-</sup> mice and showed that myelin from Fa2h<sup>-/-</sup>/Cgt<sup>-/-</sup> mice contained larger amounts of NFA-sphingomyelin (lane 3) compared to wild-type (lane 1) or Cgt<sup>-/-</sup> mice (lane 2). Lipid samples were subjected to alkaline hydrolysis before TLC. LPE, lyso-phosphatidylethanolamine; SM, sphingomyelin. (B) Total lipids were isolated from sciatic nerves of 4-week-old mice of the indicated genotypes and separated by HPTLC. Shown are 2 representative chromatograms (Note that lanes 5 to 8 are from the same TLC plate, however, 2 lanes containing lipids from mice with other genotypes have been removed from the Figure). PE, phosphatidylethanolamine. Hexosylceramide (HexCer) (C, D) and sphingomyelin levels (E, F) in CNS myelin (C, E) and sciatic nerves (D, F) were determined by densitometric scanning of HPTLC plates. HexCer levels were normalized to cholesterol. Data were combined from three independent experiments, and lipids from 3-6 animals per genotype were analyzed. Shown are the mean ± SD (CNS myelin: n = 3-5; sciatic nerves: n = 4-6); asterisks indicate statistically significant differences (ANOVA with post hoc Fisher's LSD test, *p < 0.05).
The synergistic effect of Fa2h and Cgt deficiency, purified myelin of Fa2h\textsuperscript{-/-}/Cgt\textsuperscript{-/-} mice was subjected to CHAPS extraction at 37°C. Optiprep gradient centrifugations were performed and the fractions were examined for sphingolipid content, as described [15]. These experiments showed that myelin was resistant to CHAPS extraction irrespective of the genotype (Figure 6). Therefore, we conclude that neither GalCer nor HFA-sphingolipids are essential for stabilization of CIMF.

Comparable myelination in Fa2h\textsuperscript{-/-}/Cgt\textsuperscript{-/-} and Cgt\textsuperscript{-/-} mice

To evaluate the extent of myelin sheaths thickness, cross sections were obtained from the cervical spinal cord of 4-week-old mice. On each side of the midline, starting at the deep medial boundary of the ventral funiculus and extending ventrally and laterally, fibers of all calibers were included. In this unbiased sampling approach, the number of myelinated axons was unchanged in Cgt\textsuperscript{-/-} and Fa2h\textsuperscript{-/-}/Cgt\textsuperscript{-/-} mice (179 ± 19 versus 194 ± 30). Only few axons in both mouse mutants displayed no myelin at this postnatal stage (Figure 7A). The mean axon diameter did not differ significantly between Cgt\textsuperscript{-/-} and Fa2h\textsuperscript{-/-}/Cgt\textsuperscript{-/-} mice (3.88 μm versus 3.77 μm). The axons of Fa2h\textsuperscript{-/-}/Cgt\textsuperscript{-/-} mice recruit inappropriately thick myelin relative to their absolute calibers resulting in an average myelin thickness of 0.60 ± 0.02 μm versus 0.53 ± 0.01 μm in Cgt\textsuperscript{-/-} (p < 0.01, Chi-Square test, n = 3) (Figure 7B). However, due to the relative small difference in myelin thickness, the g-ratios did not differ significantly between the two genotypes (Figure 7C).

Furthermore, electron microscopy of myelinated axons revealed normal compact myelin in Fa2h\textsuperscript{-/-}/Cgt\textsuperscript{-/-} mice (Figure 7D). These results demonstrate that a compact myelin sheath can be generated in the absence of GalCer and any 2-hydroxylated sphingolipids.
No obvious structural differences between oligodendrocytes of Fa2h\(^{+/+}\)/Cgt\(^{+/+}\) and Cgt\(^{+/+}\) were observed by the histological or electron microscopic analyses, however, a detailed structural analysis was not performed, and therefore we cannot exclude subtle structural changes in Fa2h\(^{+/+}\)/Cgt\(^{+/+}\) when compared to Cgt\(^{+/+}\) mice (e.g. in the paranodal region).

**Discussion**

Although HFA-sphingolipids appear to be dispensable for the formation of compact myelin [15], they are essential for long-term myelin maintenance and may also play a role in glia-dependent axonal support. In the absence of GalCer in Cgt\(^{+/+}\) mice, compact myelin can be formed [3,4], which is, however, unstable, also suggesting a role of GalCer in myelin maintenance. We hypothesized that HFA-sphingolipids may also play a more subtle role in early postnatal development, which could be detectable on a Cgt\(^{+/+}\) background, where compact but less stable myelin is formed. However, we did not observe signs of a more severe phenotype in Fa2h\(^{+/+}\)/Cgt\(^{+/+}\) mice compared to Cgt\(^{-/}\) mice. At the behavioral level, Cgt\(^{-/}\) and Fa2h\(^{+/+}\)/Cgt\(^{-/}\) mice were indistinguishable. Though there was a shift towards thicker myelin in Fa2h\(^{+/+}\)/Cgt\(^{-/}\) compared to Cgt\(^{-/}\) mice, the g-ratios did not differ significantly between Cgt\(^{-/}\) and Fa2h\(^{+/+}\)/Cgt\(^{-/}\) mice. Furthermore, myelin from Fa2h\(^{+/+}\)/Cgt\(^{-/}\) and Cgt\(^{-/}\) mice was resistant towards extraction with CHAPS, as shown before for Fa2h\(^{+/+}\) and wild-type mice [15].

Unexpectedly, HFA-GlcCer present in Cgt\(^{-/}\) mice was replaced by only low amounts of NFA-GlcCer in Fa2h\(^{+/+}\)/Cgt\(^{-/}\) mice but higher levels of NFA-sphingomyelin. This suggests that the GlcCer concentration in myelin is not critical and furthermore that elevated GlcCer levels in Cgt\(^{-/}\) mice do not functionally compensate loss of GalCer. Accordingly, Saadat et al. [19] showed that deleting oligodendroglial glucosylceramide synthase (Ugcg) in Cgt\(^{-/}\) mice did not reinforce the myelin phenotype. This demonstrates that upregulation of (HFA)-GlcCer in Cgt\(^{-/}\) mice does not functionally compensate loss of GalCer in these mice. In Ugcg-deficient Cgt\(^{-/}\) mice, HFA-GlcCer was partially replaced by HFA-sphingomyelin [19]. Our results demonstrate that HFA-sphingomyelin can be replaced by NFA-sphingomyelin without any obvious effect on the phenotype of Cgt\(^{-/}\) mice. Thus, the significant up-regulation of HFA-sphingomyelin in Cgt\(^{-/}\) mice is not a functional compensation.

The reason for the much lower NFA-GlcCer level in CNS myelin of Fa2h\(^{+/+}\)/Cgt\(^{-/}\) mice compared to the HFA-GlcCer level in wild-type mice [15]. One possible explanation for the high HFA-GlcCer level and absence of HFA-gangliosides [9] in Cgt\(^{-/}\) mice could be the inability of the responsible glycosyltransferases to use HFA-GlcCer as a substrate. However, presence of a high amount of HFA-gangliosides in other tissues [22] and in tumor cells [23] argues against this. An alternative explanation could be differential sorting of HFA- and NFA-GlcCer, as shown for polarized
epithelial cell lines [24], or reduced half-life of HFA-sphingolipids.

Although structural changes at the paranodes of Cgt−/− mice are clearly caused by sulfatide rather than GalCer deficiency [7,25], myelin appear to be much more stable in young adult gal3st1-deficient mice, whereas myelin maintenance is already affected in young Cgt−/− mice [8], indicating additional function roles of HFA- and/or NFA-GalCer. Taken together, our results and those of Saadat et al. [19] strongly suggest that the upregulation

Figure 7 Normal lamellar spacing in Fa2h−/Cgt− myelin. (A) Typical cross sections of spinal cords from Cgt−/− and Fa2h−/Cgt− mice (two different magnifications are shown). Arrows indicate axons with thin or without myelin. Asterisks indicate demyelinated axons. Scale bar, 40 μm (upper panel) 10 μm (lower panel). Myelin thickness (B) and g-ratios (C) were determined in the spinal cord of three mice per genotype. Though there was a shift towards increased myelin thickness in Fa2h−/Cgt− compared to Cgt−/− mice (Data shown are the mean SD [n = 3 animals per genotype]; **p < 0.01, Chi-Square test), the g-ratios of myelinated axons were similar in Cgt−/− and Fa2h−/Cgt− mice. (D) Electron micrographs showing normal compact myelin in Fa2h−/Cgt− mice (Magnification: 16,000×). Scale bar, 50 nm.
of HFA-GlcCer and HFA-sphingomyelin in Cgt+/− mice does not functionally compensate the loss of HFA-GalCer in these mice. This, however, does not exclude the possibility that GalCer could be functionally replaced by GlcCer, if the latter would be present at higher concentrations than in Cgt+− mice. The HFA-GlcCer concentration in Cgt+/− mice is clearly below the concentration of HFA-GalCer in wild-type but also in heterozygous Cgt+/− mice (which show a wild-type phenotype). There might be a relatively high threshold of hexosylceramide concentration in myelin to efficiently fulfill its role in myelin maintenance. This possibility could be tested using transgenic mice overexpressing Ugcg under control of an oligodendrocyte specific promoter, which to our knowledge are not available yet. Alternatively, the functional role of HFA- and/or NFA-GalCer may not be taken over by GlcCer or other glycolipids.

Conclusions
Our data indicate that compact myelin can be formed with non-hydroxylated sphingomyelin as the predominant sphingolipid, though myelin maintenance is impaired. While the specific role of GalCer in myelin maintenance remains mysterious, our results suggest that the presence of HFA-GlcCer and HFA-sphingomyelin in Cgt−/− mice does not functionally compensate the loss of HFA-GalCer.

Methods
Mice
Fa2h+/− mice have been described previously [15]. Heterozygous Cgt+/− mice (kindly provided by Dr. Brian Popko, University of Chicago) were crossed with Fa2h−/− mice and double heterozygous Fa2h+/−/Cgt−/− mice were interbred or bred with Fa2h+/−/Cgt+/− mice to obtain mice of all possible nine genotypes. Genotyping was done using tail genomic DNA and the following oligonucleotides: 1045 bp for wild-type and 685 bp for the targeted allele; Cgt genotyping: 5′-GCTTTCTTCAAGAGCATCC-3′, 5′-GTGGCTGTACCTCAGCTGGTC-3′, 5′-TTGGCAGGCCATCGCTTTATC-3′, PCR products: 1045 bp for wild-type and 685 bp for the targeted allele; Fa2h genotyping: 5′-TACCAAGGATTTAGCAACC-3′, 5′-CCTCTCAGAAGGCAGACATTGC-3′, 5′-CTCGCACAGACATGTGACGC-3′, PCR products: 684 bp for wild-type and 820 bp for the targeted allele. All animal experiments followed internationally recognized guidelines and were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, Germany.

Lipid extraction and thin layer chromatography
Total lipid extracts were prepared from brains, sciatic nerves, or purified myelin (from 4-week-old animals) according to Bligh and Dyer [26]. In some experiments, phosphoglycerolipids were removed by mild alkaline hydrolysis as described [27]. In order to isolate gangliosides from purified myelin, lipids were extracted as described by Folch et al [28] and the ganglioside containing upper phase was desalted by reversed-phase chromatography using RP-18 columns (Merck, Darmstadt, Germany). Lipids were separated by thin layer chromatography (TLC) in one of the following solvent systems: (1) chloroform/methanol/water (65/25/4) for hexosylceramides and sphingomyelin, (2) chloroform/methanol/acetic acid (190/9/1) for ceramides, and (3) chloroform/methanol/0.22% CaCl2 (60/35/4) to separate gangliosides. HPTLC silica gel 60 plates (Merck) were used for all experiments. To visualize lipids, HPTLC plates were sprayed with cupric sulfate in aqueous phosphoric acid [27] followed by charring at 180°C for 5 min. Lipids were quantified by densitometry and the GalCer and GlcCer levels were normalized to cholesterol. Data are shown as the mean ± SD and were tested for statistically significant differences by ANOVA with post hoc Fisher’s least significant difference (LSD) test using the program STATISTICA.

Isolation of myelin
Compact myelin was isolated by sucrose gradient centrifugation as described by Norton and Poduslo [29], with minor modifications. Brains were homogenized in water using an Ultra-Turrax tissue homogenizer (IKA-Werke, Staufen, Germany). Aliquots of the homogenates were used for lipid extraction. The residual homogenates were adjusted to 10.5% (w/v) sucrose in 5 mM Tris-HCl (pH 7.4) and overlaid onto 10 ml of 30% (w/v) sucrose in 5 mM Tris-HCl (pH 7.4). After centrifugation (68,000×g, 50 min, 4°C) the enriched myelin fraction was recovered from the 10.5%/30% interphase, resuspended in 5 mM Tris-HCl (pH 7.4) and centrifuged at 68,000×g for 10 min. The resulting pellet was resuspended in a small volume of water, lyophilized and stored at -80°C.

Analysis of CHAPS insoluble membrane fractions
CHAPS insoluble membrane fractions (CIMF) of purified myelin were prepared by optiprep density gradient centrifugation of myelin samples treated with 20 mM CHAPS at 37°C, as described previously [15,21]. Six fractions of 350 μl each were removed from the top of the gradient and lipids were isolated from each fraction according to Bligh and Dyer [26], and analyzed by TLC as described above.
MALDI-TOF mass spectrometry
MALDI-TOF MS of hexosylceramides was done as described [13].

Western blotting
Brain samples were homogenized in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl (TBS), containing 5 mM EDTA and 1 mM PMSF, using an Ultra-Turrax tissue homogenizer (IKA-Werke, Staufen, Germany). Homogenates were centrifuged at 1,000xg for 5 min and the supernatant was mixed with SDS-PAGE sample buffer containing 2-mercaptoethanol. Proteins were separated in 12.5%-polyacrylamide gels and transferred to nitrocellulose membranes by semi-dry blotting. Membranes were stained with rabbit anti-myelin basic protein (MBP; dilution 1:10,000; Millipore, Schwalbach, Germany) and mouse anti-alpha-tubulin (Developmental Studies Hybridoma Bank, University of Iowa). Bound secondary antibodies were detected by enhanced chemiluminescence as described [30]. Protein concentrations were determined with the Bio-Rad DC protein assay (Bio-Rad Laboratories, München, Germany) using bovine serum albumin as standard.

Morphometrical analysis
Axon caliber and myelin thickness were measured on toluidine-stained semithin sections of the cervical spinal cord of 4-week-old mice. Axonal caliber was determined by the diameter of a circle of area equivalent to each axon. The g-ratio was determined by dividing the diameter of the axon by the diameter of the fiber (axon with myelin). Quantification of myelinated axons was performed with a semi-automatic program on the basis of AnalySIS using a light microscope (100x objective, BX60, Olympus). All morphometric measurements were conducted in a blinded manner using coded sections. Ultrathin sections (50 nm) were photomicrographed with an EM10 electron microscope (Zeiss, Germany) at a magnification of 16,000x.

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Authors’ contributions
MM carried out the animal work, performed lipid and Western blot analyses and CIMF experiments. JJ carried out the morphometrical analyses and participated in writing the draft manuscript. VG participated in the design and coordination of the study. ME conceived and designed the study, wrote the draft manuscript, and participated in the lipid and Western blot analyses. All authors read and approved the final manuscript.

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