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Composition and Biophysical Properties of Myelin Lipid Define the Neurological Defects in Galactocerebroside- and Sulfatide-Deficient Mice

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Abstract: Oligodendrocytes and Schwann cell-specific proteins are assembled with a highly ordered membrane lipid bilayer to the myelin sheath of axons, which functions as an insulator and allows rapid saltatory conduction. We approached the question of the function of the CNS and PNS myelin-specific galactospingolipids cerebrosides and sulfatides by generating a ceramide galactosyltransferase null allelic mouse line ($cgt^{-/-}$). Galactocerebroside- and sulfatide-deficient myelin loses its insulating properties and causes a severe dysmyelinosis that is incompatible with life. Here, we describe the biochemical and biophysical analysis of the myelin lipid bilayer of $cgt^{-/-}$ mice. The lipid composition of CNS and PNS myelin of $cgt^{-/-}$ mice is seriously perturbed and the sphingolipid biosynthetic pathway altered. Nonhydroxy and hydroxy fatty acid-substituted glycosylceramides (GlcC) are synthesized by oligodendrocytes and sulfated GlcC in addition in Schwann cells. The monogalactosyldiglyceride fraction is missing in the $cgt^{-/-}$ mouse. This new lipid composition can be correlated with the biophysical properties of the myelin sheath. The deficiency of galactocerebrosides and sulfatides leads to an increased fluidity, permeability, and impaired packing of the myelin lipid bilayer of the internodal membrane system. The loss of the two glycosphingolipid classes causes the breakdown of saltatory conductance of myelinated axons in the $cgt^{-/-}$ mouse. **Key Words:** Myelin lipid bilayer—Packing of acyl chains—Polar head group—Insulation and conductance—Ceramide galactosyltransferase deficiency.
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The myelin sheath of CNS and PNS axons consists of a multilayered membrane system. Its lipid bilayer is largely responsible for the properties of myelinated axons, saltatory conduction due to internodal insulation, energy conservation, and volume reduction of CNS and PNS structures. Beyond the high cholesterol content, galactocerebrosides (GalC) and GalC-derived sulfatides (sGalC) are major oligodendrocyte- and Schwann cell-specific constituents of the myelin lipid bilayer. Numerous studies on the properties of these lipids have been performed, to clarify the function, with a variety of methods including surface monolayers (Oldani et al., 1975; Maggio et al., 1978, 1981), spectroscopy (Bunow and Levin, 1980), scanning calorimetry (Bunow, 1979; Bunow and Levin, 1980; Haas and Shipley, 1995), and x-ray diffraction (Pascher et al., 1992; see, also, Curatolo, 1987). We have chosen an in vivo approach to unravel the function of the two oligodendrocyte- and Schwann cell-specific sphingolipids GalC and sGalC. We first isolated the key enzyme in their biosynthetic pathway, UDP-galactose-ceramide galactosyltransferase (CGT; EC 2.4.1.45), from rat brain, a 67-kDa glycoprotein residing in the membranes of endoplasmic reticulum, and cloned and expressed its cDNA (Schulte and Stoffel, 1993). Subsequently, we assigned the human *cgt* locus to chromosome 4q26 (Bosio et al., 1996*a*). Isolation of the mouse CGT gene (Bosio et al., 1996*b*) led to the establishment of a $cgt^{-/-}$ mouse line by homologous recombination (Bosio et al., 1996*c*). Myelin membranes of $cgt^{-/-}$ mice are GalC- and sGalC-deficient. The results of independent studies elaborated the essential contribution of GalC and sGalC to the insulator function of the myelin sheath (Bosio et al., 1996*c*; Coetzee et al., 1996). The differentiation of the oligodendrocytes and Schwann cells is not disturbed and no gross alteration

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Abbreviations used: CGT, UDP-galactose-ceramide galactosyltransferase; CHO, Chinese hamster ovary; FAB-MS, fast atom bombardment–mass spectroscopy; GalC, galactocerebrosides; sGalC, sulfatides; n-GalC (n-sGalC), galactocerebrosides (sulfatides) substituted with nonhydroxy fatty acids; OH-GalC (OH-sGalC), galactocerebrosides (sulfatides) substituted with hydroxy fatty acids; mono-GalDG, monogalactosyldiglyceride; GlcC, glucocerebrosides; sGlcC, sulfated glucocerebrosides; HPTLC, high-performance thin-layer chromatography; LPE, 1-alkyl- and 1-alkenylglycerophosphorylethanolamine; PLP, proteolipid protein; SPM, sphingomyelin; wt, wild type.

of the myelin compaction was observed. Nevertheless, $cgt^{-/-}$ mice revealed the phenotype of a dysmyelinosis, with constant body tremor and loss of locomotor activity. Most of the animals die at the end of myelination. The neuropathological phenotype was verified by measurements of the conduction velocity. The saltatory conductance of peripheral nerves (sciatic nerve) is reduced to that of unmyelinated axons. The reduced conduction velocity appears to be intimately related to the loss of structural properties of GalC and sGalC. The studies reported here describe the impact of the glycosphingolipid deficiency on the structural and biophysical properties of the myelin lipid bilayer of homozygous $cgt^{-/-}$ mice. The functional destruction gives insight into the function of polar head groups and the hydrophobic core of the galactosphingolipids for myelin membrane stability and function. Our data allow molecular interpretation of the dramatically altered conductance and the phenotype of dysmyelinosis.

MATERIALS AND METHODS

Experimental animals and tissue preparation

Heterozygous ($cgt^{+/-}$) and homozygous ($cgt^{-/-}$) mice were generated as described previously (Bosio et al., 1996*c*). Brains of 10 mice, 20–30 days of age, of each genotype were isolated, pooled, and used for myelin preparation by density gradient centrifugation (Norton, 1974). Sciatic nerves of 20 animals of the same age were pooled for lipid extraction.

Total lipid preparation

Density gradient-purified CNS myelin was extracted twice in 10 volumes of chloroform/methanol 2:1 (vol/vol) and centrifuged. Sciatic nerves were homogenized in 1 ml of chloroform/methanol 2:1 (vol/vol) with an Ultraturrax and centrifuged. The pellet of both preparations was reextracted twice with the same solvent mixture. The combined supernatants were dried in a stream of nitrogen. Lipids were dissolved in chloroform/methanol 2:1 (vol/vol) at a final concentration of 20 mg/ml.

Alkaline hydrolysis of lipids

Aliquots of the total lipid extract were saponified in 1 ml of 0.5 *M* methanolic KOH at 37°C for 1 h. Water (1 ml) was added and the mixture extracted three times with chloroform. Phases were separated by centrifugation. The combined chloroform extracts were evaporated to dryness under nitrogen.

Separation and quantitation of lipids

Total and alkali-stable lipids were separated on high-performance thin-layer chromatographic (HPTLC) plates using the solvent system chloroform/methanol/water 65:25:4 (by volume) or chloroform/methanol 15:1 (vol/vol). Lipid bands were visualized by staining for phospholipids (Dittmer and Lester, 1964), glycolipids (Ledeen and Yu, 1982), and sulfatides (Schnaar and Needham, 1994). Spraying with 50% H_2SO_4 and subsequent charring at 120°C for 15 min visualized all lipid classes.

For quantitation of lipids, chromatograms were photographed after charring (Agfapan negative film) with transmitting light. Positive images were digitalized with the GDS 7500 System (UVP Inc.) and integrated with Molecular Dynamics ImageQuaNT software version 4.2. Values of all lipids were normalized to the amount of alkenyl-lysophosphatidylethanolamine (LPE), which served as internal standard. The amount of phosphorus in total lipid extracts was determined as described by Bartlett (1959). Quantification of cholesterol was carried out with the cholesterol oxidase kit (Boehringer Mannheim).

Isolation and characterization of single lipids and derived fatty acids

Lipids were separated by HPTLC plates, visualized by spraying with water, and individual bands scraped from the plates. Lipids were extracted twice with chloroform/methanol 2:1 (vol/vol) and aliquots subjected to fast atom bombardment–mass spectrometry (FAB-MS).

Preparation of glycolipid- and monogalactosyldiglyceride (mono-GalDG)-derived free fatty acids and sugar moieties

Glycolipid and mono-GalDG were transferred into small Sovirel tubes, concentrated in a N_2 stream and dried under vacuum, redissolved in 0.6 ml of 1 *M* HCl, and heated at 100°C for 12 h in a sealed tube. Fatty acids were extracted three times with hexane and chloroform each and stored at 4°C. The aqueous phase containing the sugars was concentrated to dryness under nitrogen, then further dried in a desiccator over KOH under vacuum. The sugar of mono-GalDG was reduced in 0.2 ml of 53 m*M* $NaBH_4$ solution at room temperature for 2 h. The resulting alditol was dried under vacuum, dissolved in 30 μl pyridine, and acetylated with 30 μl anhydrous acetic acid at 80°C for 12 h. The peracetylated alditol was identified by GLC using an Erba 8000 GLC apparatus equipped with a DB-225 (J and W Scientific) capillary column (25 m), the carrier gas helium, with column temperature controlled isothermally at 220°C for 30 min. Synthetic peracetylated mannitol, galactitol, and glucitol were used as standards.

Fatty acid methyl esters were isolated from sulfatides, cerebrosides, mono-GalDG, and sphingomyelin (SPM) by direct transmethylation with 5% (weight/weight) methanolic HCl (1 ml) at 80°C for 3 h in a nitrogen atmosphere. Methyl esters were extracted three times with hexane and concentrated to dryness. Hydroxy fatty acid methyl esters were acetylated in 30 μl pyridine and 30 μl anhydrous acetic acid at room temperature for 12 h. The acetylated hydroxy and nonhydroxy fatty acid methyl esters were analyzed by GLC. A temperature program was run between 180 and 220°C at 4°C/min with an initial hold of 2 min at 180°C and a final hold of 30 min at 220°C or from 100 to 240°C at 10°C/min with a final hold at 240°C for 15 min. For identification, the peak retention time was compared with that of standard fatty acids. Analysis was also performed by GLC mass spectrometry. Fatty acids were quantitated by integration using a dedicated microprocessor.

Preparation of total lipid-derived free fatty acids

Aliquots of total lipids were transesterified as described above at 80°C for 3 h under nitrogen. The resulting fatty acid methyl esters were saponified in 1 ml of 0.5 *M* methanolic KOH at 37°C for 2 h. Water (1 ml) was added and the mixture extracted three times with chloroform. Phases were separated by centrifugation. The combined aqueous phases were acidified with 2 *M* HCl. Free fatty acids were again extracted three times with hexane and ether and evaporated to dryness under nitrogen. Aliquots of the free fatty

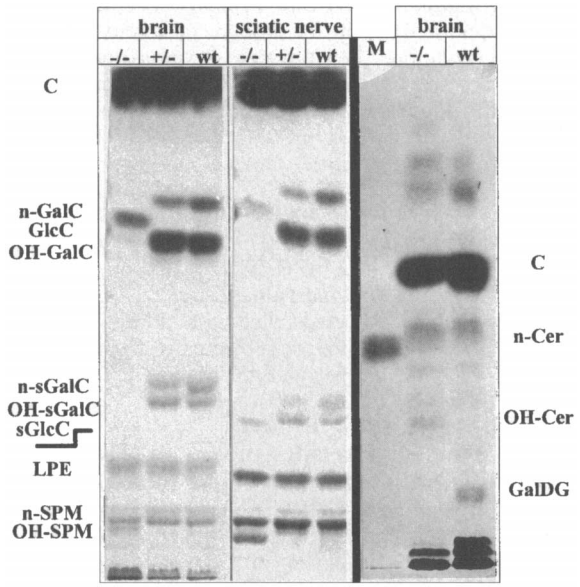

FIG. 1. HPTLC of total and alkali-stable lipids from wt, $cgt^{+/-}$, and $cgt^{-/-}$ mice. Myelin of CNS was purified by density gradient centrifugation. Myelin and sciatic nerve were extracted with chloroform/methanol 2:1 (vol/vol). Lipid bands were visualized by spraying with 50% H_2SO_4 and charring at 120°C for 15 min. Alkali-stable lipids from brain myelin (**left**) and sciatic nerve (**middle**) were separated by HPTLC in the solvent system chloroform/methanol/water 65:25:4 (by volume). The left and middle panels are reproduced from Bosio et al. (1996*c*). Total lipids of brain myelin were separated by HPTLC in the solvent system chloroform/methanol 15:1 (vol/vol; **right**). C, cholesterol; n-GalC, normal fatty acid-substituted galactocerebroside; GlcC, glucocerebroside; n-sGalC, normal fatty acid-substituted sulfated galactocerebroside; LPE, lysophosphatidylethanolamine (lysoplasmalogen of PE type); n-SPM: normal fatty acid-substituted sphingomyelin; OH-SPM, α-hydroxy fatty acid-substituted SPM; M, marker: *N*-stearoylsphingosine.

acid mixtures were used for the Langmuir technique. For GLC, the total lipid-derived free fatty acids were methylated and acetylated as described above.

Lipid monolayer measures

The monolayer properties of total lipids and derived free fatty acids of CNS myelin from wild-type (wt) and $cgt^{-/-}$ mutants were analyzed with a surface film balance of the Langmuir type (kindly provided by C. Sucker, Bayer Leverkusen). This instrument continuously recorded the *F/A* isotherm in correct mN × Å^2 × m^{-1} × molecule^{-1} scale. An average weight of 670 g/mol for the total lipids and of 300 g/mol for the free fatty acids was estimated. Measures were performed at 28°C and at a constant velocity of compression and expansion of V_c = 125 Å^2 × molecule^{-1} × min^{-1}.

RESULTS

Oligodendrocytes of $cgt^{-/-}$ mice partially substitute the deficiency of GalC by glucocerebrosides (GlcC) and Schwann cells by GlcC and sulfated GlcC (sGlcC)

We first analyzed the components of the lipid bilayer of density gradient-purified myelin of brain and sciatic nerves of wt and $cgt^{-/-}$ mice. Total and unsaponifiable lipids were separated by HPTLC (Fig. 1; the left and middle panel are reproduced from Bosio et al., 1996*c*, and introduced for clarity's sake). The analysis of total lipid extracts of CNS myelin and sciatic nerve lipids revealed no apparent differences in the pattern and amounts of phospholipids, cholesterol, and gangliosides (not shown). However, significant differences became apparent when the alkali-stable myelin lipids of homozygous $cgt^{-/-}$ and wt mice were compared. We and others reported previously that neither nonhydroxy fatty acid-substituted galactocerebrosides (n-GalC) and hydroxy fatty acid-substituted galactocerebrosides (OH-GalC) nor the derived sulfatides (n-sGalC and OH-sGalC) are present in $cgt^{-/-}$ mice myelin (Bosio et al., 1996*c*; Coetzee et al., 1996). Instead, significant amounts of GlcC are visible among myelin lipids with an R_f value between that of n-GalC and OH-GalC. An alkali-stable and Azur A-positive band is present among the unsaponifiable lipids of PNS but not of CNS myelin of $cgt^{-/-}$ mice with a similar R_f value as OH-sulfatides (OH-sGalC) of wt mice. This indicates the presence of sGlcC (Fig. 1). SPM of CNS and PNS myelin of $cgt^{-/-}$ mice is also altered. SPM of wt myelin appears as a single band but separates into two bands in the myelin of $cgt^{-/-}$ mouse. The lower band represents SPM substituted with α-hydroxy fatty acids. Ceramide, the precursor of all sphingolipids, is present as a single band in wt myelin lipids but separates into two bands in the HPTLC analysis of total $cgt^{-/-}$ mouse brain myelin lipids. FAB-MS analysis of these isolated lipids recognized the additional band in $cgt^{-/-}$ mice as ceramides substituted with α-hydroxy fatty acids (OH-Cer) (Table 1). Mono-GalDG, a GalC-related class of glycolipids, is present as a minor constituent in wt myelin but is completely missing in the myelin lipids of $cgt^{-/-}$ mice (Fig. 1). Mono-GalDG of wt mice was identified by FAB-MS (Table 1); the sugar head group was verified

TABLE 1. *FAB-MS analysis of ceramide and mono-GalDG of brain myelin from wt and* cgt$^{-/-}$ *mice*

Compound	*m/z*	Structure
n-ceramide (wt)	670	MNa^+ Cer 18/24:1
	616	MNa^+ Cer 18/20:0
	588	MNa^+ Cer 18/18:0
Mono-GalDG (wt)	807	MNa^+ GalDG 18:0/18:1
	779	MNa^+ GalDG 16:0/18:1
	753	MNa^+ GalDG 16:0/16:0
	725	MNa^+ GalDG 14:0/16:0
n-ceramide ($cgt^{-/-}$)	588	MNa^+ Cer 18/18:0
	566	MH^+ Cer 18/18:0
OH-ceramide ($cgt^{-/-}$)	688	MNa^+ Cer 18/24:0-OH
	686	MNa^+ Cer 18/24:1-OH
	660	MNa^+ Cer 18/18:0-OH

Peaks were detected with a thioglycerol matrix and sodium acetate. n-ceramide, nonhydroxy-substituted ceramide; OH-ceramide, α-hydroxy fatty acid-substituted ceramide.

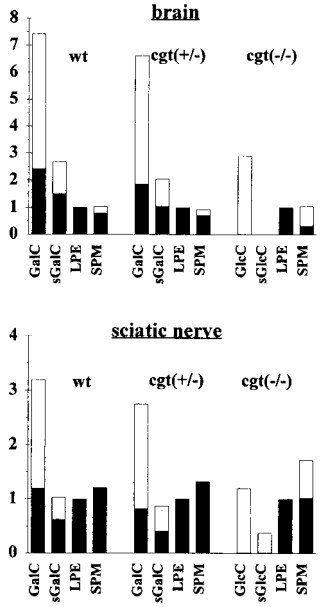

FIG. 2. Quantitation of alkali-stable sphingolipids. Optical densities of bands in Fig. 1 were measured. Densities of sphingolipids were normalized to alkenyl-lysophosphatidylethanolamine (LPE). Abbreviations, see Fig. 1. Filled columns represent lipids substituted with nonhydroxylated fatty acids; open columns represent lipids substituted with hydroxylated fatty acids.

by GLC as galactosyl derivative after acid hydrolysis, reduction, and peracetylation. These findings strongly suggest that CGT catalyzes not only the galactose transfer to ceramide to form GalC but also to diglyceride with mono-GalDG synthesis. A recent report shows the synthesis of [^{3}H]mono-GalDG in the presence of [^{3}H]galactose in Chinese hamster ovary (CHO) cells stably expressing CGT but not in CGT-negative CHO cells, which supports the observation in the *cgt*$^{-/-}$ mouse described here (van der Bijl et al., 1996).

Sphingolipid species of myelin of CNS and PNS are severely altered

Quantitation of sphingolipids is facilitated after alkali treatment of total lipids of myelin and sciatic nerve of wt, *cgt*$^{+/-}$, and *cgt*$^{-/-}$ mice and separation on HPTLC. Relative amounts of individual lipids were determined by measuring the optical density of corresponding bands (Fig. 1). The relative concentration of total and individual classes of phospholipids is not affected by CGT deficiency. Plasmalogens were hydrolyzed to 1-alkyl- and 1-alkenylglycerophosphorylethanolamine (LPE). We used this band as standard for the normalization of the optical density of the sphingolipid bands (Fig. 2). The ratio of sphingolipid to LPE is generally higher in CNS than in PNS.

The amount of total GalC and sGalC in heterozygous *cgt*$^{+/-}$ mice is reduced by 10–20% compared with wt littermates. This reduction is restricted to the glycosphingolipids substituted with α-hydroxylated fatty acids. The small reduction indicates that the monoallelic expression of *cgt* is sufficient for adequate GalC synthesis. OH-GalC and n-GalC of wt CNS and PNS are present in a ratio of 2 in agreement with previous reports (O'Brien and Rouser, 1964). The ratio of OH-sGalC/n-sGalC varies between 0.6 and 0.7 in CNS and PNS, respectively. GlcC in myelin of *cgt*$^{-/-}$ mice amounts to ~40% of total GalC of myelin in wt mice. As mentioned before, sGalC is completely missing in CNS myelin of *cgt*$^{-/-}$ mice. However, in myelin of PNS OH-sGlcC, a lipid with a similar R_f value and 40% intensity of the OH-sGalC of wt mice is visible. The concentration of SPM in wt, *cgt*$^{+/-}$ and *cgt*$^{-/-}$ CNS myelin is comparable; however, PNS myelin of *cgt*$^{-/-}$ mice contains 30% more sphingomyelin than wt mice.

The hydrophobic domain of the lipid bilayer of myelin is altered in the *cgt*$^{+/-}$ mouse

Fatty acids of total lipids as well as of the purified individual lipid classes n-GalC, OH-GalC, GlcC, and SPM of wt and *cgt*$^{-/-}$ CNS myelin were esterified and acetylated for GLC analysis. The composition of total lipid-derived fatty acids is given in Table 2. The fatty acid analysis revealed hydroxy and nonhydroxy fatty acids with chain lengths of 14–26 C atoms as well as plasmalogen-derived dimethylacetals. A minor shift of the group of very long chain (C18–C26) to that of long chain fatty acids (C14–C18), together with a slight increase in the amount of saturated fatty acids of the lipids of myelin of *cgt*$^{-/-}$ mice, is obvious. The

TABLE 2. *GLC-MS analysis of fatty acid constituents of total myelin lipids of wt and* cgt$^{-/-}$ *mice*

Fatty acid	Total fatty acids					
	wt			*cgt*$^{-/-}$		
	n-f.a.	OH-f.a.	ald.	n-f.a.	OH-f.a.	ald.
16:0	12.7			13.8		2.6
16:1				1.7		
18:0	15.8	0.9	2.5	18.6	0.7	4.2
18:1	21.6		3.4	21.6		
18:2	0.6			0.7		
20:0	1.0			1.7	0.6	
20:1	5.5			4.4		
20:4	5.4			5.7		
22:0	1.5	4.6		5	1.6	
22:1	0.6			0.5		
22:4	3.2			2.2		
23:0		0.9				
24:0	2.4	4.4		3.1	3	
24:1	6.2	3.0		1.3	1	
Others	3.6			5.4		
Σ	80.1	13.8	5.9	85.7	6.9	6.8
Σ_{total}		99.8			99.4	

Amounts of fatty acids were determined by peak integration (percentage of total fatty acids). The average of three different preparations was calculated. Values of <0.5% are not listed. ald., plasmalogen-derived aldehydes; n-f.a., normal fatty acids; OH-f.a., α-hydroxy fatty acids.

TABLE 3. *Fatty acid analysis of total CNS myelin lipid by GLC of methyl esters and dimethylacetals, respectively, of wt and* cgt$^{-/-}$ *mice (percentage of total fatty acids)*

	<C18	>C18	OH-f.a.	n-f.a.	Saturated	Unsaturated
wt	57.5	42.3	13.8	86	44.2	55.6
cgt$^{-/-}$	63.9	35.5	6.9	92.5	48.1	51.3

<C18, fatty acids with chain lengths of 18 and less; >C18, fatty acids with chain lengths exceeding 18; OH-f.a., α-hydroxy fatty acids; n-f.a., normal fatty acids.

most striking difference is the reduction of total α-hydroxy fatty acids by 50% in myelin sphingolipids of *cgt*$^{-/-}$ mice (Table 3). The analysis revealed that in the *cgt*$^{-/-}$ mouse α-hydroxy fatty acids accumulate in the ceramide moieties of GlcC (100%) and SPMs (70%). SPM of *cgt*$^{-/-}$ mouse separates into two distinct bands in HPTLC (Fig. 1), the fatty acids of which we analyzed separately. The upper band consists of SPMs substituted exclusively with long chain fatty acids of which ~20% are α-hydroxylated fatty acids (mainly 22:0, 24:0, and 24:1) and 80% are nonhydroxy fatty acids (mainly 16:0, 18:0, 18:1, 20:0, 22:0, 24:0, and 24:1). The band with the smaller R_f value contained SPMs with ~80% α-hydroxy fatty acids (mainly 18:0, 20:0, 22:0, 23:0, 24:0, and 24:1) and 20% nonhydroxy fatty acids (mainly 16:0, 18:0, and 18:1). SPM of wt myelin, although showing only one main band in HPTLC, is also substituted by α-hydroxylated fatty acids (27%), an observation that has not been made previously (Table 4) (O'Brien and Rouser, 1964; DeVries and Norton, 1973).

Spatial requirement, compressibility, phase transitions, and collapse pressure of myelin lipids of wt and *cgt*$^{-/-}$ mice

Monolayers of lipid molecules at the air–water interface provide a simple model to study physical–chemical properties and interactions of single lipid species and lipid mixtures, which helps to interpret the properties of membranes. We have studied parameters of the altered lipid composition of myelin and of their derived fatty acids from *cgt*$^{-/-}$ mice with the Langmuir technique (Gaines, 1966). This technique has been applied successfully to fatty acids as well as naturally occurring and synthetic phospholipids (Stoffel and Pruss, 1969; Stoffel et al., 1974) and to glycolipids (Oldani et al., 1975; Maggio et al., 1978, 1981).

We first analyzed the three predominant single purified GalC species, kerasin (1-galactosyl-*N*-tetracosanoylsphingosine), cerebron (1-galactosyl-*N*-D-2-hydroxytetracosanoylsphingosine), and nervon (1-galactosyl-*N*-Δ^{15}-tetracosanoylsphingosine). The pressure area (*F/A*) isotherms of their monomolecular films were recorded with a Langmuir balance at the air–water interphase and the minimum of film compressibility (C_M), average area (Å^2) per molecule (A_T), and film pressure (F_T) at the collapse point deduced (Table 5).

TABLE 4. *GLC analysis of fatty acid constituents of cerebrosides and SPM of wt and* cgt$^{-/-}$ *mice*

	Cerebroside				SPM			
	wt		*cgt*$^{-/-}$		wt		*cgt*$^{-/-}$	
Fatty acid	n-f.a.	OH-f.a.	n-f.a.	OH-f.a.	n-f.a.	OH-f.a.	n-f.a.	OH-f.a.
16:0					2.4		1.3	
18:0	2.5	3.4	0.8	4.7	14.6		5.9	7.4
18:1	0.9		0.9		2.0		1.9	
20:0	1.0	3.6		2.1	2.4		2.1	2.8
20:1					1			
21:0			0.5					
22:0	9.2	37.2		25.5	9.0	9.0	4.8	16.7
22:1	2.4	0.6			4.6		3.6	
23:0	2.9	5.5		5.6	4.6	2.0	4.3	3.3
24:0	22.1	30.0		51.2	11.9	7.5	1.1	19.5
24:1	57.3	19.1		4.1	14.9	1.7	3.0	1.4
24:2	1.4							
25:0				0.6		4.8		16
25:1						2.2		2.3
26:0								1.2
Σ			2.2	93.8	66.4	27.2	28	70.6
Σ_{total}	99.7	99.4	96		93.6		98.6	

Fatty acids were quantitated by peak integration and are given as percentages of the total mixture. The average of three different preparations was calculated. Values of <0.5% are not listed. wt *n*-GalC and OH-GalC fatty acids were determined from two separate samples. n-f.a., normal fatty acids; OH-f.a., α-hydroxy fatty acids.

With the exception of nervon, the monolayer of the other cerebrosides form only liquid-condensed films. Their minimal compressibility C_M varies between 1.3 and 7.5 × 10^{-3} m/mN. C_M expresses the compaction of the monolayer and generally correlates in reciprocal manner with A_T and F_T. Cerebron exhibits the lowest C_M and the highest F_T, indicating the highest compaction of the cerebroside species. Cerebron differs from kerasin by its α-hydroxy acyl substituents allowing additional hydrogen bonding, which adds to the further stabilization of the monolayer. The different C_M and F_T values support this interpretation. Nervon exhibits a phase transition from the liquid-expanded to the liquid-

TABLE 5. *Influence of CNS myelin lipid and derived fatty acid composition on* C_M *(minimum compressibility),* A_T *(average area (Å^2) per molecule at the collapse point), and* F_T *(pressure at the collapse point) of monolayer films*

	C_M (10^{-3} m/mN) (±0.5)	A_T (Å^2/molecule) (±0.5)	F_T (mN/m) (±1)
Kerasin	5.5	13	65
Nervon	7.5	11	64
Cerebron	1.3	13	74
Total lipids wt	5	29.5	48
Total lipids *cgt*$^{-/-}$	5	50	48
Fatty acids wt	11[a]	25[a]	27[a]
Fatty acids *cgt*$^{-/-}$	12[a]/14[b]	36[a]/8[b]	28[a]/53[b]

[a] Phase transition liquid-expanded to liquid-condensed state.
[b] Phase transition at collapse point.

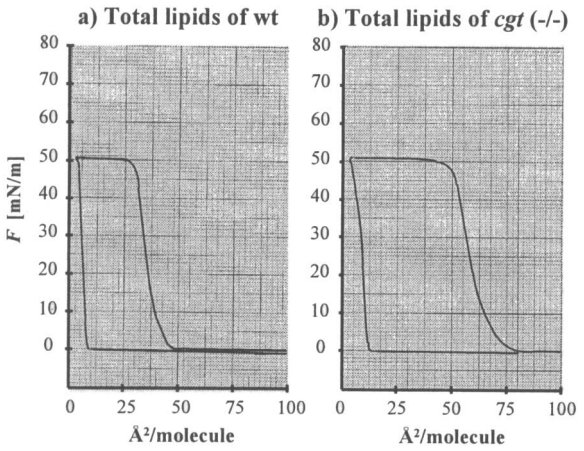

FIG. 3. Pressure (F)/area (A) isotherms of total lipids of density gradient-purified CNS myelin of wt (**a**) and $cgt^{-/-}$ (**b**) mice. Two different preparations of every sample were recorded three times each. Representative plots are shown. Isotherms were recorded at 28°C.

condensed state due to the *cis* double bond of nervonic acid, which interferes with the highly ordered state. The average molecular area (Å^2) per molecule of the GalC is predominantly determined by the space filling polar head group of the cerebrosides. The F/A isotherms of total lipid monolayers of wt and $cgt^{-/-}$ mice myelin are depicted in Fig. 3. Lipids form only a liquid-condensed state. Phospholipids and cholesterol form monolayer films less stable than those observed for pure cerebroside species, which is reflected in the lower F_T and higher A_T (Table 5). Compressibility C_M (5×10^{-3} m/mN) and collapse pressure F_T (48 mN/m) of lipid monolayers are almost identical, respectively. They differ, however, remarkably in the area per molecule at the point of tightest packing. The lipids of the $cgt^{-/-}$ mice require a much greater average molecular area at the collapse point as wt myelin lipids, i.e., 50 vs. 29.5 Å^2/molecule, respectively.

The F/A isotherms of total lipid-derived fatty acids are similar to those characterizing total lipids. Isotherms of wt fatty acids are missing a distinct collapse point. Therefore, the parameters C_M, F_T, and A_T at the phase transition point between the liquid-expanded and the liquid-condensed state of fatty acids from myelin lipids of wt and $cgt^{-/-}$ mice were compared (Table 5). Similar to total lipids, the compressibility C_M and the collapse pressure F_T of wt and $cgt^{-/-}$ fatty acids were almost identical, i.e., 11 and 12×10^{-3} m/mN, and 27 and 28 mN/m, respectively. However, the required molecular area A_T is much greater for the $cgt^{-/-}$ fatty acids, 36 Å^2/molecule, as for wt fatty acids, 25 Å^2/molecule.

DISCUSSION

Myelin is one of the most lipid-rich membranes of mammalian cells (~75% of dry weight). Major features of this lipid bilayer are the high cholesterol content (40 mol% of total lipids), the substitution of ester phosphatidylethanolamines by plasmalogens (1-alkenyl-2-acylglycerophosphorylethanolamine; 12 mol%), GalC (20 mol%), and sGalC (5 mol%) (DeWille and Horrocks, 1992). The latter two galactolipids are almost exclusively formed in oligodendrocyte-derived CNS myelin and Schwann cell-derived PNS myelin. We and others have recently shown in the unique $cgt^{-/-}$ mouse model that the myelin sheath of CNS and PNS axons is completely deficient of the galactolipids and that the structural features of these lipids are a prerequisite for the insulator function of the myelin sheath around axons. The loss of these structures and their function is incompatible with life (Bosio et al., 1996*c*; Coetzee et al., 1996).

The composition and structural properties of myelin lipids impose on the lipid bilayer a poor membrane fluidity with high phase transition temperatures (Bunow and Levin, 1980; Curatolo and Jungalwala, 1985; Haas and Shipley, 1995). The all-*trans* configuration of the alkane chains of sphingosine and its N-linked α-hydroxylated fatty acid substituents render cerebroside and sulfatide most suitable to form highly ordered and tightly packed bilayer structures either as clusters or continuously distributed. In addition, strong intermolecular interactions might occur at the interphase by multiple H bonds between hydroxy groups of the sugar moiety and the α-hydroxy groups of fatty acids, the amide group of ceramide backbones and the 3-hydroxy group of sphingosine, and cholesterol. This network of hydrogen bonds may not only stabilize the outer leaflet of the lipid bilayer but form a barrier for ion permeability. Preliminary studies (W. Stoffel, unpublished data) indicate that cholesterol and galactosphingolipids are asymmetrically distributed in the outer leaflet of the lipid bilayer associated with the main integral membrane protein, proteolipid protein (PLP). A similar asymmetrical distribution of glycosphingolipids into the apical part and of phospholipids in the basal part of polarized cells has been reported for kidney cells (Madin–Darby bovine kidney cells) (Dotti et al., 1991). GalC and sGalC are concentrated in the outer leaflet of the plasma membrane bilayer (Raff et al., 1978; Linnington and Rumsby, 1980). This asymmetric distribution is lost in the lipid extract of the myelin bilayer used for the biophysical behavior at the air–water interface. Consequently, the impact of GalC and sGalC on the properties of the monolayer film (C_M, A_T, and F_T) is by far weaker than in the genuine order of the bilayer.

The loss of saltatory conductance can only be explained by the ion permeabilization of the internodal myelin membrane system in $cgt^{-/-}$ CNS and PNS axons, the molecular basis of which is the deficiency in GalC and sGalC in the myelin lipid bilayer of $cgt^{-/-}$ mouse. Oligodendrocytes and Schwann cells synthesize GlcC. However, newly synthesized GlcC cannot compensate for the loss of GalC and sGalC, neither

by mass nor by specificity. No sulfation of GalC to sGalC occurs in CNS. This alters the charge pattern of the surface of the lipid bilayer of $cgt^{-/-}$ mice myelin dramatically. The membrane surface becomes depleted of multiple anionic charges that might be involved in intermolecular lipid–lipid and lipid–protein interactions. The GalC deficiency reduces the number of the long chain α-hydroxylated fatty acids to one-half, thereby reducing the number of potential proton donor and acceptor groups forming hydrogen bonds.

The GalC- and sGalC-depleted bilayer has lost its ordering structural elements in favor of the less ordered phospholipids. Langmuir isotherms of the $cgt^{-/-}$ myelin lipids reveal an area per molecule with twice the average spatial requirement of the wt lipids. This should render the lipid bilayer of myelin of the CGT-deficient mouse ion permeable in the internodal region and disrupt the saltatory conduction of the myelinated wt CNS and PNS axons.

The enhanced synthesis of glucosylceramide warrants some consideration. Ceramides substituted with α-hydroxy long chain fatty acids are the predominant substrates in cerebroside and sulfatide synthesis. During myelination, CGT expression parallels that of the oligodendrocyte-specific main myelin proteins PLP and myelin basic protein (Schulte and Stoffel, 1993). The massive myelin synthesis by the oligodendrocytes in the $cgt^{-/-}$ mouse produces nonhydroxy and hydroxy ceramides for the GalC synthesis, which in the absence of CGT, however, become acceptor molecules in the CGT reaction and SPM synthesis. The glucosylceramide formed in the $cgt^{-/-}$ mouse is completely and SPM two-thirds substituted by α-hydroxy fatty acids.

In summary, biochemical and biophysical analysis of the lipid bilayer of CNS and PNS myelin of the $cgt^{-/-}$ mutant mouse, which is depleted of the GalC and sGalC, has given insight into the molecular function of the glycolipids in the intact myelin sheath of CNS and PNS axons and provides a basis for the neuropathological phenotype of the associated dysmyelinosis. Use of the $cgt^{-/-}$ mouse is the first approach to gain insight into the function of a specific lipid class in a membrane bilayer by using the technique of gene targeting by homologous recombination. The conventional generation of $cgt^{-/-}$ mice leads to a mouse line with a limited life span. Conditioned, inducible gene ablation will provide access to study the function of these lipid classes in myelin membrane structure and function during development.

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