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3-*O*-acetyl-sphingosine-series myelin glycolipids: characterization of novel 3-*O*-acetyl-sphingosine galactosylceramide

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Abstract Several glycosphingolipids, less polar than galactosylceramide (GalCer), have been purified from rat brain and designated as fast migrating cerebrosides (FMCs). They co-appear with GalCer during myelinogenesis, reach a peak concentration at postnatal day 25–30 and are derivatives of GalCer. Extensive structural analysis of the partially methylated alditol acetates, mass-spectrometry, and ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy unequivocally established the structure of two of these FMCs as 3-*O*-acetyl-sphingosine GalCer with non-hydroxy and hydroxy fatty *N*-acylation respectively. That is, an acetyl group is linked at the C3-OH of the sphingosine base of GalCer. In addition, NMR spectroscopy of all of the purified FMCs indicates that they contain a 3-*O*-acetyl group linked with sphingosine and thus delineates a novel series. Several lines of evidence indicate that FMCs are myelin constituents. FMCs, enriched in both central nervous system (CNS) and peripheral nervous system (PNS) myelin, are concentrated in spinal cord and white matter that are composed of myelinated nerve fibers. There is *N*-acylation with α-hydroxy and C18 and C24 fatty acids, all characteristic of myelin components. They disappear along with GalCer in the murine genetic dysmyelinating disorders, jimpy and quaking, and in a knockout mutant which is devoid of GalCer. In addition, a decrease in FMC and GalCer concentration has been found in Krabbé's disease, a human genetic dysmyelinating disorder.—Dasgupta, S., S. B. Levery, and E. L. Hogan. **3-*O*-acetyl-sphingosine-series myelin glycolipids: characterization of novel 3-*O*-acetyl-sphingosine galactosylceramide. *J. Lipid Res.* 2002.** 43: **751–761.**

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Galactosylceramide (GalCer) and sulfatide (3-sulfo-GalCer) are the two major monoglycosylceramides (MGCs) found, along with the ganglioside GM4 (NeuAc-GalCer), in vertebrate central nervous systems (CNS). *O*-Acyl-GalCer (cerebroside esters) (1–3), galactosyldiglycerides (1, 4), plasmalopsychosines (5), and plasmalocerebrosides (6, 7) have also been characterized in brain though in much lower concentrations. All of these compounds except plasmalopsychosine have a higher TLC-Rf than GalCer and are designated here as fast migrating cerebrosides (FMCs). Extensive studies of the ester-type FMC components have characterized them as *O*-acyl-GalCer with fatty acids linked at different hydroxyls of the galactose residue (4, 8–12), or the fatty *N*-acyl moiety (13), or at the C3-OH of sphingosine (8). Other minor FMC components have been proposed as derivatives of GalCer (14), and two of these have respectively either a long chain alkyl ether-linked or a fatty aldehyde alkenyl-linked (designated as sphingoplasmalogen) to the C3-OH of sphingosine (15). Subsequently, these postulated structures could not be confirmed, and the components were characterized instead as either cerebroside esters (1–4, 8–12) or plasmalocerebrosides (GalCer derivatives having a fatty aldehyde acetal linked to the vicinal hydroxyls of the galactose residue) (6). The examination of MGC in developing brain, assayed after purification (16), indicates that there is a change from GlcCer to GalCer in the course of ontogeny from embryonic (E) to postnatal (P) rat brain with a concomitant shift from dominant ceramide: UDP-hexose glucosyltransferase to galactosyltransferase activities (17). Several non-polar MGC bands, identified at P10 and manifesting an increasing concentration with age, were found at the highest tissue concentration in brain at P25–P30,

Abbreviations: dqCOSY, double quantum filtered correlation spectroscopy; ESI-MS, electrospray ionization mass spectrometry; FMC, fast migrating cerebroside; GalCer, Galβ1-1Cer; GlcCer, Glcβ1-1Cer; GM4, NeuAcα2-3GalCer; GC-MS, gas chromatography mass spectrometry; GSL, glycosphingolipid; gHMBC, gradient heteronuclear multiple bond correlation; gHSQC, gradient heteronuclear single quantum correlation; HFA, hydroxy fatty acid; jp, jimpy; KO, knockout; NFA, nonhydroxy fatty acid; PMAA, partially methylated alditol acetate; qk, quaking; sulfatide, I^{3}-sulfate GalCer; TMS, tetramethylsilane; TOCSY, total correlation spectroscopy.

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the peak age of myelinogenesis (17). We have designated these compounds as fast migrating cerebrosides or FMCs. A purified rat brain FMC (FMC-1) was labile to both acid and alkali and its hydrolyzed product co-migrated with GalCer and stained with O1 antibody, which is specific for GalCer (17). The purified FMC was permethylated employing a neutral method, which has been modified in our laboratory and after hydrolysis, reduction, and peracetylation, the resulting partially methylated alditol acetates (PMAAs) were analyzed by gas chromatography mass spectrometry (GC-MS). Our data indicated that a terminal galactose was the only carbohydrate, thus suggesting a substitution of the ceramide moiety (a ceramide derivative) (17). Here, we describe the characterization of two purified rat brain FMCs that contain a novel long-chain lipid base structure. Several other FMCs, also purified from rat brain, have been tentatively characterized and have an identical core structure revealing a novel series of GSLs that has not been previously reported. Additional experimental data supports the interpretation that these FMCs are integral to myelin, and may play critical roles in myelin structure and function.

MATERIALS AND METHODS

Adult Sprague-Dawley rats were purchased from Harlan Laboratory (Indianapolis, IN). Silicic acid was purchased from Sigma (St. Louis, MO). Pre-coated TLC plates (E. Merck) were obtained from Fisher Scientific (Pittsburgh, PA). Jimpy (jp) and quaking (qk) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and GalT1 knockout (KO) brains were a generous gift of Brian Popko, University of North Carolina (Chapel Hill, NC). Rat brain tissue was collected from individual animals by decapitation after administering Metafane anesthesia. All reagents and chemicals obtained were of analytical grade. Human Krabbé's brain along with normal human brain (control) were received from the National Neurological Research Specimen Bank, UCLA (Los Angeles, CA). White matter (30–50 mg) from Krabbé's brain was carefully dissected along the marked area (following the enclosed diagram) and tissue from an identical region of normal brain was used as the control.

Purification of FMCs

FMCs were purified from CNS tissue as described previously (16). Briefly, lipids were extracted twice from tissue (10 g) with chloroform-methanol-water (2:4:1, v/v/v) and then once with chloroform-methanol (2:1, v/v). The three extracts were pooled, dried, and applied on a silicic acid column (1 × 50 cm) in chloroform-acetone (9:1, v/v). After thoroughly washing the column (20 v) with the same solvent, MGCs including the FMCs were eluted as a single fraction using chloroform-methanol 23:2 (v/v). FMCs were further purified into individual homogeneous components using a silicic acid column (0.8 × 20 cm) and eluting with various solvent compositions starting with chloroform-methanol-ammonia (150:3:0.15, v/v/v) and gradually increasing the concentration of methanol and ammonia to proportions of 150:8:0.6. An aliquot of approximately 3.5 ml was collected in each tube and 20 μl from each alternate tube was assayed by TLC, developed using chloroform-methanol-water (85:15:0.5, v/v/v), and bands were visualized by diphenylamine-aniline spray (16). Purified fractions were pooled, dried, and stored at 4°C until use. The purity of the FMC fraction was determined by TLC using three different solvent systems.

Acid and alkaline methanolysis

A defined amount (10 μg) of the purified FMC was transferred into three separate screw cap tubes and 0.5 ml of the following mixtures were added, sonicated, and incubated at 40°C for 30 min: *1*) 0.1 N NaOH/methanol, *2*) 0.1 N NaOH/water, and *3*) 0.3 N HCl/methanol. The reaction was terminated by adding 1 ml of chloroform in tubes 1 and 3 followed by 0.375 ml of water and in tube 2, 2 ml of chloroform-methanol (2:1, v/v) was added. The upper phase was discarded and the lower phase was washed with an equal volume of theoretical upper phase, dried under nitrogen and examined by TLC (13). FMCs were examined using aqueous (0.1 N NaOH in water) and methanolic alkali in order to compare their stability with sphingoplasmalogens, which were resistant to aqueous alkali (0.5 N NaOH in water) due to the acetal linkage (15).

Characterization of PMAAs of FMCs by GC-MS

FMCs are susceptible to both alkali and acid (17), and therefore cannot be permethylated using conventional methods (18, 19). A permethylation procedure at neutral pH has been modified in our laboratory using methyl trifluoromethanesulfonate, and 2,6-di-(tert)-butylpyridine in a neutral medium, trimethyl phosphate (17, 20). Briefly, 20–50 μg of FMCs were dissolved in trimethyl phosphate (200 μl) and 50 μl of methyl trifluoromethanesulfonate and 2,6-di-(tert)-butylpyridine were added. The reagents were mixed vigorously at 40°C for 2 h and the reaction was terminated by adding 1 ml of chloroform. The chloroform layer was washed twice with 1 ml of distilled water, aspirated carefully, and the methylated FMCs recovered in the chloroform layer and examined by TLC using chloroform-methanol (49:1, v/v) as the developing solvent. The methylated FMCs were hydrolyzed and acetylated as described previously (21). PMAAs were analyzed on a DB-1 column in a Hewlett Packard GC-MS (GC 5980, MS 5972).

Fatty acid and base composition of FMC-1 and FMC-2

Approximately 20–50 μg of the FMC was hydrolyzed with methanol-water-concentrated. HCl (29:4:3, v/v/v) in a sealed tube at 80°C for 18 h (22). Fatty acids were removed by partitioning with hexane and analyzed as methylester derivatives as described (23). The lower phase was neutralized with 1 N NaOH, the pH was raised to 10–12, and the solution was partitioned by the addition of 1 ml of petroleum ether. The ether layer was dried and analyzed for the sphingosine base by GC as the trimethylsilyl derivative.

One- and two-dimensional nuclear magnetic resonance spectroscopy of FMCs

Purified glycosphingolipids [FMCs and bovine brain GalCer Types I and II (Sigma)] were each dissolved in 0.6 ml DMSO-d_6 for nuclear magnetic resonance (NMR) spectroscopic analysis. One dimensional (1-D) ^{1}H-NMR spectra, two dimensional (2-D) ^{1}H-^{1}H-dq-COSY (24, 25), and ^{1}H-^{1}H-TOCSY (26, 27), ^{1}H-detected, ^{13}C-decoupled, phase sensitive, gradient ^{13}C-^{1}H-heteronuclear single quantum correlation (HSQC) and -heteronuclear multiple bond correlation (HMBC) spectra (28–31) were acquired at either 600 or 800 MHz (308°K) on Varian Unity Inova spectrometers using standard acquisition software available in the Varian VNMR software package and, where necessary, with suppression of residual HOD signal by a presaturation pulse during the relaxation delay. 1-D direct-detected ^{13}C NMR spectra were acquired on a 500 MHz Varian Unity Inova spectrometer. Proton chemical shifts are referenced to internal TMS (0.000), and ^{13}C chemical

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shifts to the centerline of the solvent DMSO signal (set at 39.96 ppm). Some interpretations of NMR spectra were checked by comparison to published data on mammalian galactosylceramides (32).

Electrospray ionization mass spectrometry

ESI-MS was performed in the positive ion mode on a PE-Sciex (Concord, Ontario, Canada) API-III spectrometer, with a standard ion spray source, using direct infusion (3–5 μl/min) of FMC samples dissolved (~20 ng/ml) in 100% MeOH [orifice-to-skimmer voltage (OR), 60 V; Ionspray voltage, 5 kV; interface temperature, 45°C]. Lithium adduction was achieved by addition of a solution of lithium iodide (LiI) (10 mM) in MeOH, as described previously (the final concentration of LiI was generally 2–3 mM) (33).

MGCs from murine and human dysmyelinating disorder and GalT1 KO mice

Brain samples of 30–50 mg were carefully dissected from the white matter of qk and jp mice, from the white matter of GalT1 KO mice, and from the white matter of a Krabbé's patient and a normal individual who died as a result of an automobile accident. For mutant mice, animals of normal behavioral phenotype, and animals of the wild-type strain from the same colony were used as controls. MGCs were purified using a silicic acid column as described above. The purified fractions were dissolved in a defined volume (4 ml/g, wet weight) of chloroform-methanol (2:1, v/v) and 10 μl of sample were examined by TLC (16). GalCer, purified from bovine spinal cord myelin, was used as a reference standard. The plates were visualized by diphenylamine-aniline and each lane was scanned (UMAX Power Look scanner and Adobe Photoshop software) and individual components were estimated as a percentage of total.

For GalT1 KO brain, the TLC was performed using a borate-impregnated high performance thin-layer chromatography (HPTLC) plate, resolved with chloroform:methanol-0.1 M boric acid (65:25:3, v/v/v) (17, 34) and visualized with diphenylamine-aniline spray. Both GalCer and GlcCer were used as standards.

FMCs in CNS and peripheral nervous system myelin

Myelin was purified from brain (CNS) and dorsal nerve roots [peripheral nervous system (PNS)] from bovine, human, and rat according to Norton and Poduslo (35) as described previously (36). Briefly, the tissue was homogenized in 0.32 M sucrose and overlaid carefully onto 0.8 M sucrose and centrifuged at 100,000 × *g* for 60 min. The interphase was collected, osmotically shocked with ice-cold water (10 v), and myelin was recovered after centrifugation at 17,000 × *g*. The pellet was re-suspended and re-washed in cold water and the myelin was recovered in cold water and lyophilized.

Lipids were extracted from the myelin (10–15 mg) and MGCs were separated using a silicic acid column as described (16). The purified fractions were dissolved in chloroform-methanol (2:1, v/v) and resolved by TLC using chloroform-methanol-water (85:15:0.5, v/v/v) and visualized by diphenylamine-aniline spray (17).

RESULTS

Purification and chemical characterization of FMCs

Five FMCs have been purified to homogeneity (two are described here and are designated as FMC-1 and FMC-2) and examined by TLC using three different solvent systems, *1*) chloroform-methanol-water, *2*) chloroform-methanol-0.1 N acetic acid and, *3*) chloroform-methanol-0.1 N ammonia at a proportion of 85:15:0.5 (v/v/v). In all three systems both FMC-1 and FMC-2 had a higher TLC-Rf than standard GalCer (**Fig. 1A, B, C**) suggesting that these FMCs are less polar than GalCer. The faster migration of FMC-1 than FMC-2 indicates that there is a difference in chemical composition between these two FMCs, and that FMC-1 is less polar than FMC-2. Relative concentrations of purified FMC-1 and FMC-2 were 110 μg/g and 150 μg/g of freshly collected tissue respectively.

GC-MS spectra of the PMAAs of the two FMCs and GalCer prepared under neutral conditions revealed that all three GSLs contain a terminal galactose (2,3,4,6-OMe$_4$Gal) as the only carbohydrate (electron ionization mass spectra) ions, 118, 161, 205, and an identical peak retention time). The GC-MS data along with the TLC migration indicated that, compared with GalCer, FMCs are chemically modified; an interpretation that is also supported by their susceptibility to acid/alkaline hydrolysis. Both FMC-1 and -2 were hydrolyzed by 0.1 N methanolic NaOH and 0.3 N methanolic HCl (**Fig. 2**, lanes 2, 4, 7, and 9, respectively) and the hydrolyzed products comigrated with non-hydroxy fatty acyl (NFA) and hydroxy fatty acyl (HFA) GalCer, respectively, while the migration of the alkali (0.1 N methanolic NaOH) treated GalCer (Fig. 2, lane 5) was not altered.

1- and 2-D NMR spectroscopy of FMC-1 and FMC-2, determination of primary structure as 3-*O*-acetyl-sphingosine-GalCer

For chemical shift and connectivity assignment of all proton and carbon resonances in FMC-1 and FMC-2, high resolution 1-D ^{1}H, 2-D ^{1}H-^{1}H COSY and TOCSY, and ^{1}H-detected ^{13}C-^{1}H gradient HSQC NMR spectra were acquired at 800 MHz in pure DMSO-d_6 (allowing assignment of exchangeable hydroxyl and amide protons); similar sets of spectra were also acquired for two standard GalCer preparations from bovine brain having either non-hydroxy- or 2-hydroxy-fatty-*N*-acylation (GalCer types II and I, respectively). For more accurate carbon shift assign-

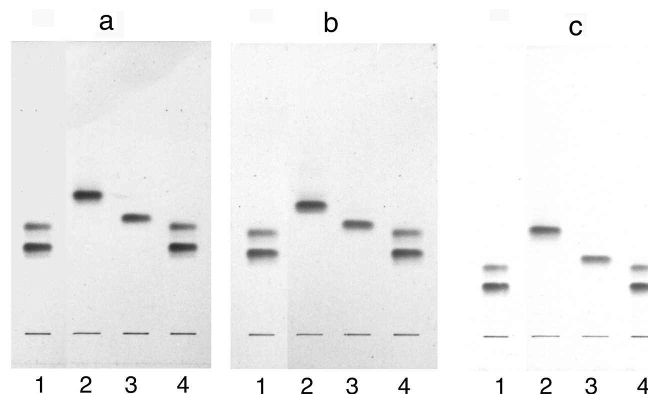

Fig. 1. Thin-layer chromatogram of purified fast migrating cerebroside (FMC)-1 and FMC-2. Plates were resolved in (a) chloroform-methanol-water, (b) chloroform-methanol-0.1 N ammonia, and (c) chloroform-methanol-0.1 N acetic acid, 85:15:0.5 (v/v/v), and the bands were visualized with diphenylamine-aniline spray. Ten micrograms of samples were applied in each lane. Lanes 1 and 4, standard Galβ1-1Cer (GalCer); Lane 2, FMC-1; Lane 3, FMC-2.

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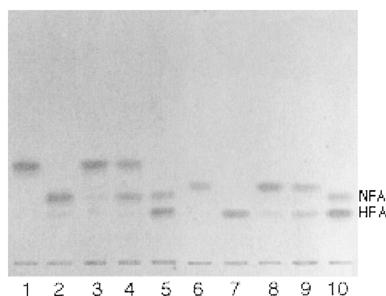

Fig. 2. Acid/Alkaline methanolysis of FMC-1 and FMC-2. FMCs and GalCer (10 μg) were treated with methanolic NaOH, methanolic HCl, and aqueous NaOH as described in the text and the products were examined by TLC using chloroform-methanol-water, 85:15:0.5 (v/v/v), and the bands were visualized by dipenylamine-aniline spray. Lane 1: FMC-1; Lane 2: FMC-1 + 0.1 N NaOH/MeOH; Lane 3: FMC-1 + 0.1 N NaOH; Lane 4: FMC-1 + 0.3 N HCl/MeOH; Lane 5: standard GalCer + 0.1 N NaOH/MeOH; Lane 6: FMC-2; Lane 7: FMC-2 + 0.1 N NaOH/MeOH; Lane 8: FMC-2 +0.1 N NaOH; Lane 9: FMC-2 + 0.3 N HCl/MeOH; Lane 10: standard GalCer (NFA and HFA).

ment, direct-detected 1-D ^{13}C NMR were also acquired for all compounds except FMC-2, which was not available in sufficient quantity. Chemical shift assignments for these compounds are summarized in **Tables 1–4**. Compared with the ^{1}H NMR spectrum of GalCer Type II, that of FMC-1 (**Fig. 3** and Table 1) clearly exhibits all features expected for a β-galactopyranosylceramide having non-hydroxy fatty-*N*-acylation, [specifying acquisition at a somewhat higher temperature (32)], with a number of differences: *1*) the absence of the exchangeable ^{1}H resonance assigned as HO-3 of sphing-4-enine; *2*) the presence of a 3-proton singlet in the CH_3-(−C=O)O-region at 1.956 ppm; *3*) significant shift changes for sphing-4-enine H-1a/

TABLE 1. ^{1}H chemical shifts of protons of FMC-1 and bovine brain Gal-Cer Type II

	FMC-1		Bovine Brain GalCer II	
	H-C	H-O/N	H-C	H-O/N
Hex- 1	4.024		4.032	
2	3.305	4.817	3.304	4.818
3	3.269	4.613	3.270	4.606
4	3.622	4.283	3.624	4.276
5	3.311		3.317	
6a	3.471	4.457	3.470	4.491
6b	3.529		3.529	
Sph- 1a	3.368		3.392	
1b	3.780		3.956	
2	4.155	7.610 (HN)	3.779	7.423 (HN)
3	5.194	—	3.888	4.798
4	5.378		5.357	
5	5.698		5.536	
6	1.967		1.935	
7	1.289		1.290	
Bulk CH_2	1.234		1.234	
Terminal CH_3	0.853		0.853	
Ac- 2	1.956		—	
Fa- 2	2.046		2.024	
3	1.454		1.445	
cis-vinyl	5.319		5.319	
cis-allyl	1.977		1.977	
Bulk CH_2	1.234		1.234	
Terminal CH_3	0.853		0.853	

^{1}H chemical shifts (ppm) of hexose (Hex), sphingosine (Sph), acetyl (Ac) and fatty acyl (Fa) protons of FMC-1 and bovine brain Gal-Cer Type II (non-hydroxy fatty acids; Sigma) in DMSO-d_6 (no D_2O) at 35°C. Chemical shift standard, TMS (0.000 ppm).

TABLE 2. ^{13}C chemical shifts of FMC-1 and bovine brain Gal-Cer Type II

	FMC-1	Bovine Brain GalCer II
Hex- 1	104.34	105.13
2	70.77	71.10
3	73.51	73.55
4	68.20	68.50
5	75.46	75.69
6	60.59	60.82
Sph- 1	68.26	69.38
2	50.45	53.44
3	73.51	71.23
4	125.44	132.28
5	135.76	132.17
6	31.89	31.90
Bulk CH_2	29.23	29.15
Terminal CH_3	14.08	14.01
Ac- 1	169.44	—
2	21.07	—
Fa- 1	172.28	172.13
2	35.70	35.85
3	25.53	25.45
cis-vinyl	129.84	130.48
cis-allyl	26.73	26.69
Bulk CH_2	29.23	29.15
Terminal CH_3	14.08	14.01

^{13}C chemical shifts (ppm) of hexose (Hex), sphingosine (Sph), acetyl (Ac) and fatty acyl (Fa) carbons of FMC-1 and bovine brain Gal-Cer Type II (non-hydroxy fatty acids; Sigma) in DMSO-d_6 (no D_2O) at 35°C. Chemical shift standard, DMSO (39.96 ppm).

TABLE 3. ^{1}H chemical shifts of protons of FMC-2 and bovine brain Gal-Cer Type I

	FMC-2		Bovine Brain GalCer I	
	H-C	H-O/N	H-C	H-O/N
Hex- 1	4.054		4.071	
2	3.280	4.777	3.296	4.745
3	3.260	4.613	3.266	4.611
4	3.619	4.292	3.622	4.293
5	3.316		3.322	
6a	3.477	4.478	3.474	4.515
6b	3.521		3.528	
Sph- 1a	3.440		3.507	
1b	3.813		3.920	
2	4.176	7.435 (HN)	3.805	7.344 (HN)
3	5.267	—	3.987	4.859
4	5.369		5.364	
5	5.696		5.568	
6	1.959		1.934	
7	1.300		1.296	
Bulk CH_2	1.232		1.234	
Terminal CH_3	0.852		0.853	
Ac- 2	1.956		—	
Fa- 2	3.831	5.460	3.805	5.444
3a	1.456		1.420	
3b	1.545		1.559	
cis-vinyl	5.318		5.319	
cis-allyl	1.976		1.977	
Bulk CH_2	1.232		1.234	
Terminal CH_3	0.853		0.852	

^{1}H chemical shifts (ppm) of hexose (Hex), sphingosine (Sph), acetyl (Ac) and fatty acyl (Fa) protons of FMC-2 and bovine brain Gal-Cer Type I (hydroxy fatty acids; Sigma) in DMSO-d_6 (no D_2O) at 35°C. Chemical shift standard, TMS (0.000 ppm).

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TABLE 4. ^{13}C chemical shifts of FMC-2 and bovine brain Gal-Cer Type I

		FMC-2[a]	Bovine Brain GalCer I
Hex-	1	104	104.46
	2	ND[b]	70.94
	3	74	73.71
	4	68	68.36
	5	76	75.64
	6	61	60.83
Sph-	1	68	69.02
	2	ND	53.22
	3	73	70.94
	4	126	131.30
	5	136	131.77
	6	32	32.09
Bulk CH_2		29	29.40
Terminal CH_3		14	14.22
Ac-	1	ND	—
	2	21	—
Fa-	1	ND	174.09
	2	73	71.46
	3	ND	ND
cis-vinyl		130	129.96
cis-allyl		ND	26.87
Bulk CH_2		29	29.40
Terminal CH_3		14	14.22

^{13}C chemical shifts (ppm) of hexose (Hex), sphingosine (Sph), acetyl (Ac) and fatty acyl (Fa) carbons of FMC-2 and bovine brain Gal-Cer Type I (hydroxy fatty acids; Sigma) in DMSO-d_6 (no D_2O) at 35°C. Chemical shift standard, DMSO (39.96 ppm).

[a] Chemical shift values estimated from 2-D ^{1}H-detected ^{13}C-^{1}H gradient HSQC analysis only.

[b] ND = not determined.

H-1b through H-6, all downfield except for H-1a and H-1b, of which the largest change is observed at H-3 ($\delta\Delta = +1.306$ ppm) and the second largest at H-2 ($\delta\Delta = +0.376$ ppm). No sugar proton shifts are affected, except for a slight downfield change for β-Gal H-1. The additional acetyl group is further manifested in the ^{13}C spectrum by additional resonances at 21.07 and 169.44 ppm (typical for $^{13}CH_3$- and $^{13}C{=}O$, respectively, of a CH_3-($-C = O$)O-group). Unambiguous confirmation was obtained by the additional acquisition of a ^{1}H-detected, ^{13}C-^{1}H gradient HMBC NMR spectrum for FMC-1 (**Fig. 4**), which clearly showed long-range through-bond correlations from the ^{1}H signal at 1.956 ppm to the $-^{13}C{=}O$ signal at 169.44 ppm (two-bond correlation), and from that carbon to the sphing-4-enine H-3 signal at 5.194 (three-bond correlation).

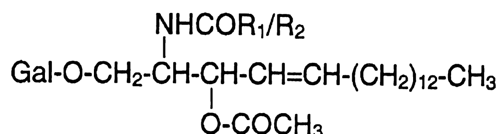
Compared with the ^{1}H NMR spectrum of GalCer Type I, that of FMC-2 (Table 3) clearly exhibits all features expected for a β-galactopyranosylceramide having 2-hydroxy-fatty-*N*-acylation (32), but with similar differences as noted for FMC-1 (deletion of the exchangeable ^{1}H resonance assigned as HO-3 of sphing-4-enine; presence of a 3-proton singlet for CH_3-($-C{=}O$)O at 1.962 ppm, etc.). In this case the presence of 2-hydroxylation on the fatty *N*-acyl components already has a significant effect on the chemical shifts of many of the protons, especially in the proximal part of the sphing-4-enine moiety (compare entries for the GalCer Type II and I standards in Tables 1 and 3, respectively), but the chemical shift changes corresponding to the *O*-acetylation are all similar in direction and magnitude to those noted above (e.g., for sphing-4-enine H-3, $\delta\Delta = +1.280$ ppm; for H-2, $\delta\Delta = +0.371$ ppm). Although a confirmatory gHMBC spectrum could not be acquired, we consider that there is sufficient NMR evidence based on analogy with FMC-1 to propose a similar structure for FMC-2, both being therefore β-galactopyranosylceramides acetylated at O-3 of the sphingosine moiety, with the only significant difference arising from the presence of 2-hydroxy-fatty-*N*-acylation in the ceramide moiety of FMC-2.

Electrospray ionization mass spectrometry of FMC-1 and FMC-2

Low resolution, positive ion mode ESI mass spectra of fractions FMC-1 and FMC-2 were acquired by direct infusion following Li^+ adduction as previously described (33). The molecular ion region of a single quadrupole mass spectrum of FMC-1 is reproduced in **Fig. 5**. The most abundant molecular adduct ions M•Li^+ are observed at *m/z* 858 and *m/z* 860 (in ~2:1 ratio), consistent with a glycosphingolipid composed of a single hexose residue and d18:1 sphingosine, *N*-acylated with 24:1 and 24:0 fatty acids, respectively, plus an increment of +42 units corresponding to additional acetylation of one hydroxy group. Less abundant M•Li^+ ions at increments of n(±14) units from these are also observed, corresponding to components differing by $(CH_2)_n$ units in either *N*-acyl or sphingosine alkyl chain. Of these, the most predominant are those having n = 2, as usually found for mammalian GSLs, in particular the M•Li^+ ion at *m/z* 776, corresponding to 18:0 fatty-*N*-acylation of d18:1 sphingosine (or 18:1 fatty-*N*-acylation of d18:0 sphinganine). Other indications of a small amount of sphinganine can be observed in some components (additional increments of +2 units, e.g., at *m/z* 890 and *m/z* 918).

In the $^+$ESI mass spectrum of FMC-2 (not shown), by far the most abundant M•Li^+ were observed at *m/z* 874 and 876 (in ~1:2 ratio); these correspond to an increment of +16 units with respect to FMC-1, consistent with an analogous mono-*O*-acetylated monohexosylceramide component, but instead *N*-acylated with 2-hydroxy fatty acids, h24:1 and h24:0, respectively. In this case the amounts of corresponding minor components are somewhat different from those found in FMC-1, with much less h18:0/d18:1 or h18:1/d18:0 (*m/z* 792), but considerably more h22:0/d18:1 or h22:1/d18:0 (*m/z* 848), fatty-*N*-acyl/sphingoid combinations.

Hence the primary structure of the two FMCs is:



R_1 and R_2 represent NFA and HFA respectively

Characterization of 3-*O*-acetyl-sphingosine in other purified FMCs by NMR-spectroscopy

Acid/alkaline hydrolysis produced a partial and/or complete conversion to GalCer of all of the purified FMCs after exposure to 0.3 N methanolic HCl and 0.1 N methanolic NaOH respectively. Determination of primary structures of the other three purified FMCs is now being carried out using NMR-spectroscopy. In addition to fatty acyl substitution(s) of galactose, a striking change from Gal-

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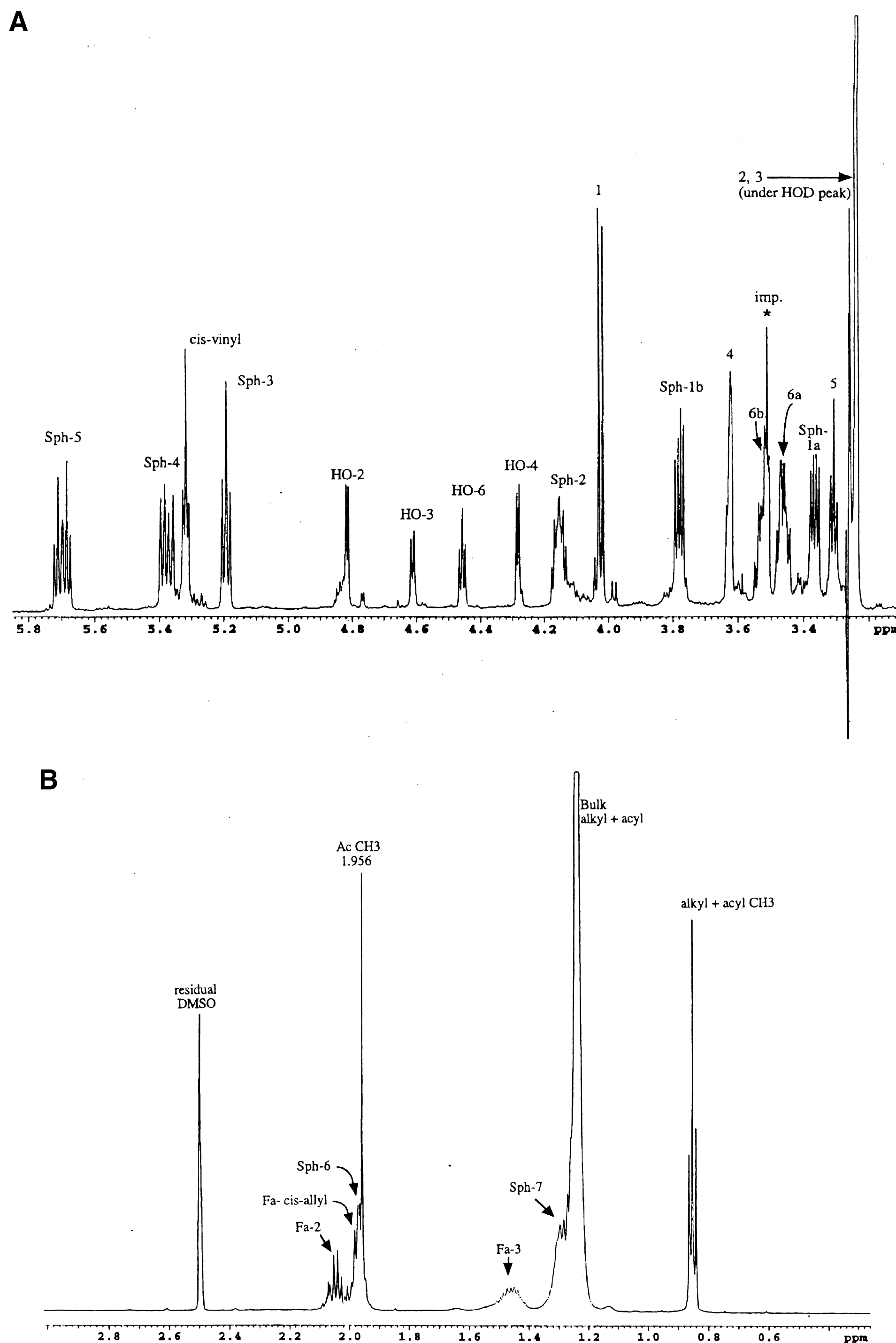

Fig. 3. One dimensional proton nuclear magnetic resonance (NMR) spectrum of FMC-1 in pure DMSO-d_6 at 308° K. A: Downfield region (3.10–5.85 ppm). B: Upfield region (0.25–3.0 ppm). "Sph" refers to protons of the sphingosine backbone; "*Cis*" refers to vinyl and allyl protons of unsaturated fatty-*N*-acyl groups; the remainder of the downfield signals are from monosaccharide hydroxyl (HO−) and ring protons.

Cer in all FMCs is the peak at δ 1.96 ppm which corresponds to the 3-*O*-acetyl substitution of sphingosine characterized in FMC-1 and FMC-2 (Table 1 and 3, δ 1.956 ppm).

MGCs in GalT1 KO mice

The MGC content of GalT1 KO mice was examined by TLC. GlcCer was the only MGC (**Fig. 6**, Lanes 2, 4, and 6) in KO brain identified by TLC-Rf. GC-MS analysis of the purified MGC from the KO mice revealed a terminal glucose (data not shown). It is noteworthy that the GlcCer purified from KO brain (characterized by GC-MS), which contains HFA showed a lower TLC-Rf than the standard NFA-GlcCer from bovine erythrocytes (Fig. 6). The rapidly migrating smear identified above GlcCer in one KO

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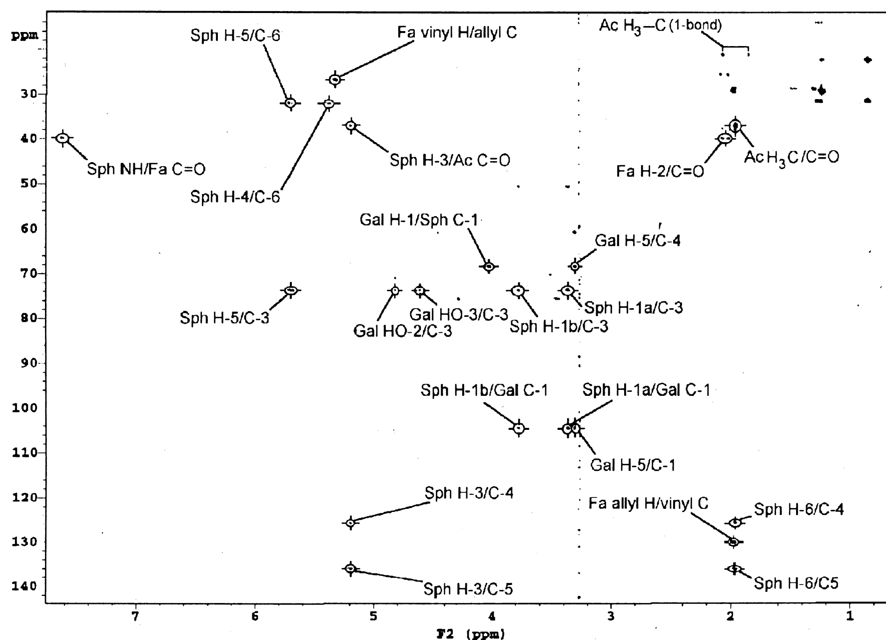

Fig. 4. Two-dimensional ^{1}H-detected, ^{13}C-^{1}H gradient heteronuclear multiple-bond correlation (HMBC) spectrum of FMC-1 in DMSO-d_6 at 308° K. "Sph" refers to nuclei of the sphingosine backbone, "Ac" to those of the *O*-acetyl group. Correlations from the fatty-*N*-acyl and *O*-acetyl carboxyl carbons (172.28 and 169.44 ppm, respectively) are folded with respect to the carbon chemical shift axis (F1). A number of internal monosaccharide and sphingosine correlations have been left unmarked.

sample (Fig. 6, lane 4) is due to a non-glycolipid contaminant. The total MGC content in KO brain is approximately 30% of total GalCer content of the control, confirming a previous report (37). No FMCs were identified in the GalT1 KO brain.

FMCs in murine and human dysmyelinating disorders

FMC and GalCer contents were decreased in both jp and qk mutants in comparison to clinically unaffected controls. The extent of loss of FMCs was greater in jp (<5% of control myelin content) than in qk (approximately 20% of control myelin content) (38) indicating that FMCs are myelin components, and that their degree of disappearance in genetic mouse disorders is proportional to the severity of myelin reduction (**Fig. 7**; jp, lane 3 and qk, lane 6). A similar finding was made in the human genetic dysmyelinating analog, Krabbé's disease, in which case there was great reduction of FMC content (90%) and loss of GalCer (75%) (**Fig. 8**, lane 3).

FMCs in CNS and PNS myelin

MGCs were purified from myelin isolated from brain and dorsal nerve roots of bovine, human, and rat. FMC bands from each lane were scanned and their concentrations were determined using a reference standard of GalCer (**Fig. 9**). It is noteworthy that FMCs are enriched in both CNS and PNS myelin of bovine, human, and rat with an average value of 20% (CNS) and 30% (PNS) of GalCer concentration. It appears that the concentration of individual FMCs varies from species to species.

DISCUSSION

Our previous study of the MGCs profile in developing rat brain was intriguing because we found that there are several GSLs, less polar than GalCer, which coappear with GalCer during the stages of active myelination (17). These compounds occur in substantial quantities in vertebrate brain and possess, as the current study shows, a unique 3-*O*-acetylation on the sphingosine long-chain base. The structure, found on numerous non-polar GSLs of this type, constitutes a series that may have considerable biological significance. Although an average concentration of FMCs in human brain has been estimated quantitatively using several (at least 8–10) samples, an occasional individual variation has been recorded, e.g., the FMC concentration of this particular normal brain (Fig. 8, lane 2) was calculated as 4.5% of the total GalCer. Similarly, there is a variation of FMCs concentration from different strains of the same species. For example, the percentage of FMCs concentration purified from control brains of KO mice [breeding of Chimeric mice with C57BL/6 mice (38)] and that from controls of jp and qk (Fig. 6, lanes 1, 3 and 5; Fig. 7, lanes 2, 4, and 5) mice varied widely.

Though these more hydrophobic MGCs have been largely overlooked in lipid analyses of the nervous system for the past 30 years, several reports have been published that hint at the existence of this molecular species. In 1963, Kochetkov et al. (15) purified several minor cerebroside components (*O*-alkenyl cerebrosides) from brain by preparative TLC followed by crystallization with methanol, and tentatively proposed a series of compounds with

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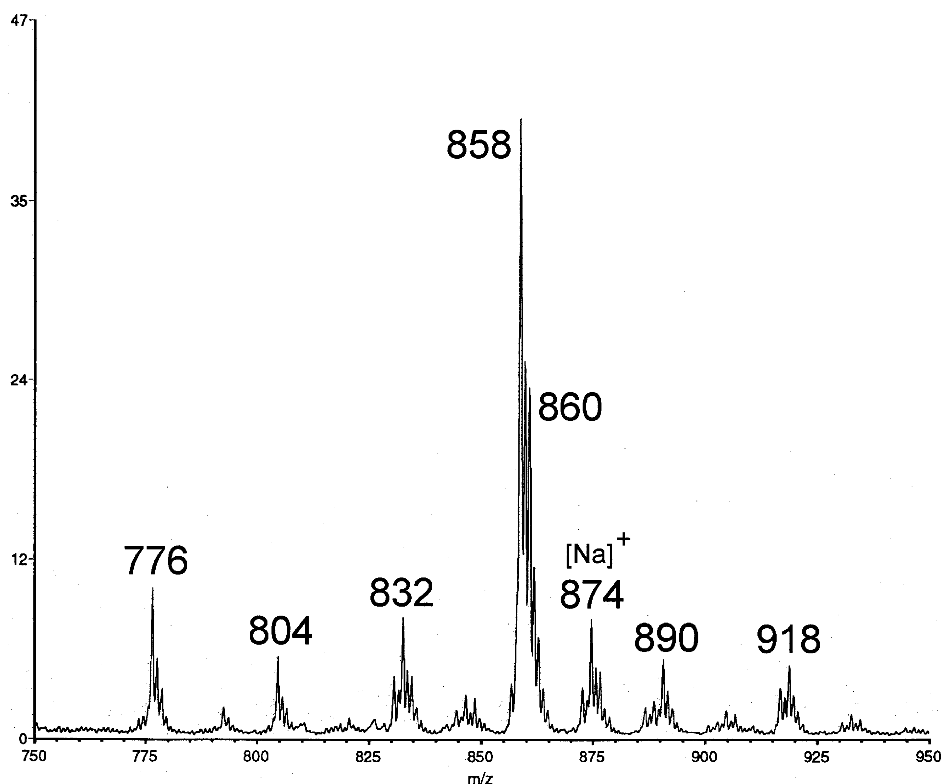

Fig. 5. Section of single quadrupole positive ion mode electrospray ionization mass spectrum of FMC-1, acquired at low orifice potential (OR = 60 V). The region shown (*m/z* 750–950) exhibits profile of lithium adducted molecular ions $[M\bullet Li]^+$. Major species are labeled with nominal, monoisotopic *m/z* (ion cluster at *m/z* 874/876 may reflect residual sodium adducts $[Na]^+$).

an alkenyl ether grouping at the C3-OH of sphingosine of GalCer and designated them as "sphingoplasmalogens." Subsequent studies did not confirm the proposed structure and these compounds, purified from brain and spinal cord, were later characterized as GalCer derivatives with fatty acyl/acetal substituents at different hydroxyls of galactose (1, 4–10). The difficulty in obtaining a precise characterization of 3-*O*-acetyl-sphingosine series FMCs appears to be a consequence of their susceptibility to mild alkali and acid, and acid/alkali methanolysis techniques that were commonly used for lipid purification and characterization. The oversight was further compounded by *1*) the methylation procedure employed, *2*) previous lack of availability of a sensitive physical/chemical technique in the 1980s for structural characterization, e.g., NMR, FAB-MS etc., together with *3*) focus upon the substitution on the Gal moiety rather than modification of the sphingoid base of ceramide. We previously purified a FMC from developing rat brain that was labile to both weak acid and alkali, and employing a modified neutral permethylation procedure we tentatively characterized the FMC as a GalCer derivative with a structural modification of the ceramide moiety (17). However, the primary structure of the compound was not completely determined. FMCs containing 3-*O*-acetyl-sphingosine have now been identified in bovine and human brain including CNS and PNS myelin. To this point, we have purified several acid-/alkali-labile FMCs from rat brain with a higher TLC-Rf than GalCer. In addition to the characterization of two rat brain FMCs described in this report, the complete structural elucidation of other FMCs including human and bovine FMCs is underway.

Both FMC-1 and FMC-2 are hydrolyzed by weak alkali and acid, and the products comigrate with standard GalCer. GC-MS of the PMAAs of the two FMCs and GalCer prepared under neutral conditions indicates that terminal galactose (2-,3-,4-,6-OMe_4Gal) is the only carbohydrate in all three GSLs. These results indicate that in addition to the GalCer backbone, FMCs contain a substitution of the ceramide sphingoid base, which is labile to both weak acid and alkali. Employing NMR-spectroscopy (1-D proton, 1-D carbon, 2-D TOCSY, 2-D H-C correlation, and 2-D H-C long-range correlation), we have unequivocally established that two FMCs contain a 3-*O*-acetyl group attached to the sphingosine of the ceramide with GalCer as the core structure (3-*O*-acetyl-sphingosine GalCer). FMC-1 differs from FMC-2 (see TLC-Rf, Fig. 1, lanes 2 and 3) by the nature of the fatty acid at the C2 *N*-acylation. FMC-1 contains NFA while HFA is the *N*-acyl derivative for the FMC-2. The α-hydroxy *N*-acylation first appears in early myelination in rat and human brain and is a myelin constituent (39–41). In addition, both FMCs contain C18 and C24 fatty *N*-acylation, which is also a distinctive feature of myelin. The evidence that FMCs are closely associated with myelin is further supported by our previous finding of their enrichment in white matter and spinal cord (16).

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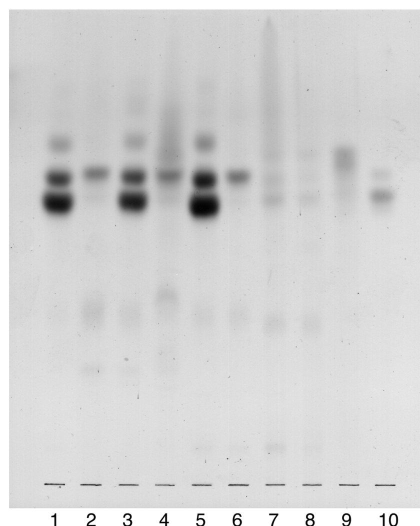

Fig. 6. Monoglycosylceramides in control and GalT1 knockout (KO) mice brain and liver. Monoglycosylceramides (MGCs), purified from normal and GalT1 KO mice brains as well as normal and KO mice livers, were examined by TLC using a 0.1 M boric acid-coated plate and resolved with chloroform-methanol-0.1 M boric acid, 65:25:3 (v/v/v). The purified fractions from brains and livers were dissolved in 4 ml/g of wet tissue and 1 ml/g of wet tissue respectively, and 10 μl were applied on a TLC plate. Bands were visualized with diphenylamine-aniline spray. Lanes 1, 3, and 5, Normal brains; Lanes 2, 4, and 6, KO brains; Lane 7, normal liver; Lane 8, KO liver; Lane 9, standard GlcCer; Lane 10, standard GalCer.

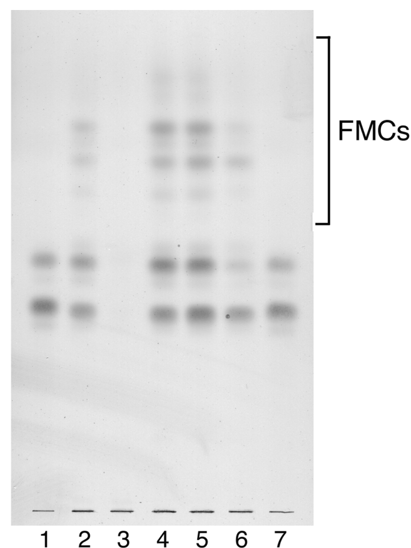

Fig. 7. Thin-layer chromatogram of monoglycosylceramides in murine demyelinating disorders [jimpy (jp) and quaking(qk)]. Monoglycosylceramides from normal, jp, and qk mice brains were purified (lane 3, jp; lane 6, qk). The purified fractions were dissolved in 4 ml/g, and 10 μl were applied on a TLC plate. The plate was developed in chloroform-methanol-water 85:15:0.5 (v/v/v) and bands were visualized with diphenylamine-aniline spray. Lanes 1 and 7: standard GalCer; Lane 2: normal brain; Lane 3: jp brain; Lane 4: normal brain; Lane 5: non-mutant (littermate) control, qk brain; Lane 6: qk brain.

Our tentative NMR-spectroscopy of the other purified FMCs indicates that each exhibits a resonance at δ 1.96 ppm, assigned to the acetyl group linked at the C3-OH of sphingosine moiety. Preliminary evidence suggests that additional fatty acyl/alkyl/acetal groups, identified in other FMCs, are linked to the different hydroxyls of the galactose. The chemical composition and primary structure of these FMCs will be reported after completion of their characterization.

The pattern of appearance of the FMCs in developing rat brain suggested that these non-polar cerebroside derivatives are regulated in concert with cerebroside (17) (for, these non-polar FMCs are myelin moieties or markers that could by virtue of their greater capacity for hydrophobic or van der Waals interactions play key roles during critical stages of myelinogenesis). They might, for example, regulate the final phases of myelin compaction to achieve the metabolically stable state that accompanies or enables its function as an insulator for the nerve fiber impulse conduction anatomico-physiological unit. In order to examine these FMCs in relation to myelin structure and function, their content was determined in the GalT1 KO mutant brain that totally lacks galactocerebroside, and in murine (jp and qk) and human (Krabbé's disease) dysmyelinating disorder. The total disappearance of FMC in GalT1 KO mice suggests that FMCs are derivatives of GalCer and that the responsible FMC synthases (transferases) are specific to GalCer. The myelin of the KO mutant deteriorates within weeks of life, and the mice manifest paranodal axon-glia disruption and an average survival of 45 days (42). This KO model established the critical importance of MGCs (GalCer and derivatives) in myelination and vertebrate brain function. However, the role of individual MGC components, e.g., GalCer, sulfatide, GM4, and FMCs, awaits further work and specific gene deletion. The genetic dysmyelinating defect leads to 95% and 80% loss in myelin in the jp and qk mutants, respectively (43, 44). FMCs, which are myelin constituents (both CNS and PNS myelin) like GalCer, a "traditional" myelin marker, decrease significantly in dysmyelination and more so in jp than in qk. A 48% decrease in FMCs

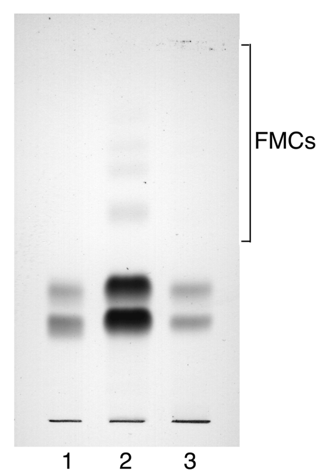

Fig. 8. Thin-layer chromatogram of monoglycosylceramides in human dysmelinating disorder (Krabbé's brain). The plate was developed in chloroform-methanol-water, 85:15:0.5 (v/v/v) and visualized by diphenylamine-aniline spray. The purified fractions were dissolved in 4 ml/g, and 10 μl were applied on a TLC plate. Lane 1: Krabbé's brain; Lane 2: normal brain; Lane 3: standard GalCer.

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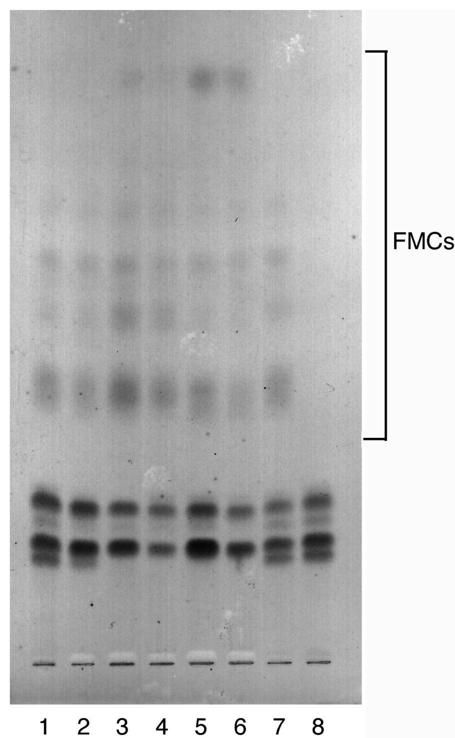

Fig. 9. Thin-layer chromatogram of monoglycosylceramides purified from myelin. The plate was developed in chloroform-methanol-water, 85:15:0.5 (v/v/v) and visualized by diphenylamine-aniline spray. The purified fractions were dissolved in 4 ml/g, and 10 μl were applied on a TLC plate. Lane 1: bovine central nervous system (CNS) myelin; Lane 2: bovine postnuclear supernatants (PNS) myelin; Lane 3: human CNS myelin; Lane 4: human PNS myelin; Lane 5: rat CNS myelin; Lane 6: rat PNS myelin; Lane 7: standard GalCer with FMCs; Lane 8: standard GalCer.

was observed in qk while the concentration of FMCs in jp is 10% compared with the normal and the littermate control mice. This is consistent with the postulation that FMCs are myelin components.

Krabbé's disease is a human dysmyelinating disorder with an average life span of 2–4 years. The disease results from a deficiency of cerebroside β1-1 glycosidase, responsible for degradation of GalCer (45, 46). Several studies have proposed that psychosine, a cytotoxic lysolipid, accumulates in early developmental stages leading to oligodendrocyte degeneration and consequent dysmyelination (47–50). A significant decrease in GalCer in Krabbé's brain has also been reported (47). In addition to a decreased GalCer concentration (75–80%), we have observed a greater decrease (95%) in FMCs in Krabbé's brain. Some FMCs contain additional fatty acid/alkyl/acetal linked to the 3-*O*-acetyl-sphingosine GalCer structure, and thus have an increased hydrophobicity that could foster interaction with other myelin lipid/protein components and which thereby could protect against myelin degradation. The extent of loss of FMCs (along with GalCer) in jp, qk, and in Krabbé's brain indicates that there is a myelin disturbance along with a loss of myelin function and premature death.

It is noteworthy that 3-*O*-acetyl GM3, i.e., the ganglioside GM3 containing a 3-*O*-acetyl group attached to the sphingosine, has been characterized in transplanted rat glioma tissue using NMR and fast atom bombardment mass spectrometry (51). 3-*O*-acetyl GM3 is immunologically less active than the parent GM3 and is resistant to endoglycoceramidase digestion (52); however, neither the neutral nor acidic GSL-series containing a 3-*O*-acetyl sphingosyl moiety has been described in brain or any other vertebrate tissue and this is the first report of the natural occurrence of a novel series of myelin GSLs in normal tissue.

The pathway of FMC synthesis has not yet been established. Based on sphingosyl acetylation, there are three possible pathways for FMC synthesis and these can be outlined as follows: *1*) GalCer- → FMC, *2*) ceramide- → 3-OAcCer- → FMC; and, *3*) sphinganine- → 3-OAc-sphinganine- → 3-OAc-dihydroCer- → 3-OAcCer- → FMC. In all of these cases, a brain 3-*O*-acetyltransferase is proposed to catalyze the acetyl group transfer to the sphingosine C3 hydroxyl of either GalCer, ceramide, or sphinganine. We anticipate that pathway 1 is more likely since neither 3-OAcCer nor 3-OAc-sphinganine has been reported in brain or elsewhere. Experiments pursuing the characterization of this novel enzyme and of 3-OAcCer/sphinganine are ongoing.

In summary, we have characterized two novel MGCs in vertebrate brain and tentatively identified a novel series of 3-*O*-acetyl sphingosyl MGCs that are myelin constituents. Their appearance in developing brain paralleling GalCer along with their disappearance (or reduction) in murine and human dysmyelinating disorder and their enrichment in purified myelin of both CNS and PNS in three species provide evidence that these 3-*O*-acetyl-sphingosine series GSLs play a role in myelin structure and undoubtedly have biological significance, though the precise role of FMCs in myelinogenesis remains to be determined. This can now be explored by the characterization of the series of FMCs, and the delineation of their precise metabolic regulation by their novel enzymes in relation to myelin structure and function, and including application of genetic approaches.

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