De-*N*-acetyllactotriaosylceramide as a Novel Cationic Glycosphingolipid of Bovine Brain White Matter: Isolation and Characterization†

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ABSTRACT: A novel cationic lipid was separated from bovine brain white matter by a series of chromatographies on carboxymethyl-Sephadex and silica gel in chloroform and methanol. Its structure was identified unambiguously as de-*N*-acetyllactotriaosylceramide (deNAcLc$_3$Cer) by mass spectrometry and ^{1}H NMR. The natural occurrence of this glycolipid in white matter extract was detected by immunostaining of thin-layer chromatography with monoclonal antibody 5F5, which is directed to deNAcLc$_3$Cer and recognizes the terminal β-glucosaminyl (GlcNH$_2$) residue, having a free NH$_2$ group. A de-*N*-acetylase capable of hydrolyzing the *N*-acetyl group of Lc$_3$Cer was detected in bovine brain extract using N-[^{14}C]acetyl-labeled Lc$_3$Cer as a substrate. The biogenesis and possible functional significance of deNAcLc$_3$Cer are discussed.

The majority of glycosphingolipids (GSLs)[1] in mammalian cells and tissues are either anionic (having sialic acid or sulfate) or neutral (having no ionic group). Since sphingosine, sphingolipid breakdown products (e.g., lyso-GSLs) (*1*−*3*), and *N*-methylsphingosine (*3*) were initially implicated as signaling molecules, we have studied cationic lipids and cationic GSLs displaying positive charge through the presence of an unsubstituted amino group. We have isolated and characterized a series of novel cationic GSLs, including psychosine (*4*), plasmalopsychosine-A (PLPS-A, 3,4-cyclic plasmal of psychosine), PLPS-B (4,6-cyclic plasmal of psychosine) (*5*), and glycero-PLPS (*4*). Our studies on cationic GSLs were previously summarized in ref *6*. These cationic GSLs may serve as signaling molecules analogous to sphingosine (*1*−*3*), ceramide (for reviews, see refs *7*−*9*), and sphingosine 1-phosphate (for reviews, see refs *10*−*12*), although the functional mechanism is entirely different for each signaling molecule and process. As an example, PLPS-B strongly activates Trk A tyrosine phosphorylation, followed by prolonged Erk activation, and promotes neurite formation, in PC12 cells. Such an effect is less pronounced for PLPS-A, and psychosine has no effect on Trk A (*13*).

We hereby report results of further studies on cationic GSLs present in bovine brain white matter. A new cationic GSL, separated by a series of chromatographies with CM-Sephadex and silica gel in C/M, was identified unambiguously as de-*N*-acetyllactotriaosylceramide (deNAcLc$_3$Cer) by mass spectrometry and ^{1}H NMR. The natural occurrence of this GSL was demonstrated by immunostaining of TLC with mAb 5F5 directed to deNAcLc$_3$Cer. We also observed the presence of de-*N*-acetylase in crude bovine brain extract which specifically hydrolyzes the *N*-acetyl group of GlcNAc and converts Lc$_3$Cer to deNAcLc$_3$Cer.

MATERIALS AND METHODS

Materials. Bovine brains were purchased from Pel-Freez Biologicals (Rogers, AR). GlcCer, LacCer, and Gb$_3$Cer were from Matreya, Inc. (Pleasant Gap, PA). Gb$_4$Cer was prepared from human erythrocytes. Lc$_3$Cer and Lc$_5$Cer were prepared from nLc$_4$Cer and nLc$_6$Cer, respectively, by hydrolysis of the terminal β-Gal residue with β-galactosidase. Other reagents were from Sigma (St. Louis, MO). Iatrobeads were from Iatron Laboratories Inc. (Kanda, Tokyo, Japan). [1-^{14}C]Acetic anhydride (4.14 GBq/mmol, 39.1 mBq/mg) was from Amersham Bioscience. Lc$_3$Cer and nLc$_5$Cer were

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[1] Abbreviations: CM, carboxymethyl; C/M, chloroform/methanol mixture; ESIMS, electrospray ionization mass spectrometry; GSL, glycosphingolipid; HOHAHA, homonuclear Hartman−Hahn spectroscopy; LSIMS, liquid secondary ion mass spectrometry; PLPS, plasmalopsychosine; NLx, minor free amine (NH$_2$)-containing unknown GSL compound; ROESY, rotating frame Overhauser effect spectroscopy; TLC, thin-layer chromatography; deNAcLc$_3$Cer, de-*N*-acetyllactotriaosylceramide; GlcCer, Glcβ1,1Cer; GM3, NeuAcα2,3Galβ1,4Glcβ1,1Cer; LacCer, lactosylceramide (Galβ1,4Glcβ1,1Cer); Lc$_3$Cer, lactotriaosylceramide (see the text and Figure 8); nLc$_4$Cer, lactoneotetraosylceramide (Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Cer); nLc$_5$Cer, lactoneopentaosylceramide (GlcNAcβ1,3Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Cer). Other glycosphingolipids are abbreviated as recommended by the IUPAC−IUB Commission on Biochemical Nomenclature [(1978) *Biochem. J. 171*, 21−35, Table 1]. The suffix −Ose is omitted, but −Cer is retained. All solvent component ratios are by volume.

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prepared by treatment with sialidase and β-galactosidase of sialyl-nLc$_4$Cer and sialyl-nLc$_6$Cer, from bovine erythrocytes (*14*−*16*), respectively. Gg$_3$Cer was prepared from guinea pig erythrocytes (*17*).

Preparation of De-N-acetyl GSLs by Hydrazinolysis. Lc$_3$Cer, Gg$_3$Cer, or nLc$_5$Cer (10 μmol), prepared as described above, was dried over P_2O_5 and then dissolved in anhydrous hydrazine (1 mL) in a screw-cap culture tube. The tube was filled with N_2 gas and heated at 105 °C for 15 h (*18*). The solvent was evaporated under a N_2 stream with occasional addition of toluene (1 mL), and the residue was dried over concentrated H_2SO_4 in a vacuum desiccator overnight. The dried residue was neutralized by adding 1.0−1.5 mL of a C/M/0.1 M HCl mixture (60:30:4.5) and further dried over P_2O_5 (*23*), and the products were analyzed by TLC developed with a C/M/acetone/acetic acid/water mixture (8:2:4:2:1). The hydrazinolyzed glycolipid, i.e., Lc$_3$(NH$_2$)Cer, was purified by HPLC on an Iatrobeads 6RS-8010 column (4.6 mm × 300 mm), eluted with a linear C/M/2.5 M NH_4OH gradient (from 77:23:2.5 to 45:50:5). Elution was monitored by TLC with the C/M/acetone/acetic acid/water solvent system (8:2:4:2:1), and fractions containing de-*N*-acetyl GSLs were combined.

^{14}C N-Acetylation of DeNAcLc$_3$Cer. DeNAcLc$_3$Cer (∼50 μg) in 1 mL of anhydrous methanol was mixed with 50 μL of pyridine and 100 μCi of [^{14}C]acetic anhydride. The mixture was left at room temperature for 60 min; 1 mL of toluene was added, and the mixture was evaporated under a N_2 stream. The products were analyzed by TLC with the C/M/0.2% $CaCl_2$ solvent system (60:35:8). The reaction mixture after ^{14}C N-acetylation was separated from deNAcLc$_3$Cer by liquid chromatography on an Iatrobeads 6RS-8060 column (4 mm × 50 mm), eluted with a C/M stepwise gradient (10:1, 9:1, 8:2, 6:4, 4:6, 2:8, and 0:10). Elution was monitored by TLC with the C/M/0.2% $CaCl_2$ solvent system (60:35:8). The C/M (4:6) fraction contained ^{14}C *N*-acetyl-Lc$_3$Cer, whose specific activity was not precisely determined, but was estimated to be ∼7−8 mBq/100 μg.

Separation of the Cationic Lipid Fraction on CM-Sephadex, and Purification of NLx by Chromatography on Iatrobeads and Silica Gel. Four steps of chromatography were performed: (i) adsorption and initial separation on CM-Sephadex, and stepwise elution; (ii) adsorption on and gradient elution from CM-Sephadex; (iii) chromatography on Iatrobeads 6RS-8060; and (iv) purification on Iatrobeads 6RS-8010. For step (i), Folch's lower phase of bovine brain white matter extract was applied on a CM-Sephadex column as described previously (*5*). Cationic lipids were eluted with a methanol/water mixture (9:1) containing 0.5 M triethylamine (pH adjusted to 9.25 by gentle CO_2 gas bubbling). The eluted fractions were rotary evaporated to dryness, and the residual triethylamine was coevaporated several times with absolute ethanol. The composition of this crude fraction was checked by TLC. Cationic lipids were found to include unknown component NLx in addition to previously known PLPS-A, PLPS-B, glycero-PLPS, and psychosine. Further purification by step (ii) was performed using a second CM-Sephadex column with a linear methanol/water gradient (9:1), containing 0−1 M triethylamine. The eluate (6 mL per tube) was collected with a fraction collector over 80 tubes. The elution was monitored by TLC with the C/M/acetone/acetic acid/water solvent system (8:2:4:2:1), and fractions containing GSL were combined. For step (iii), the combined fraction containing the GSL fraction was further purified by liquid chromatography on a silica bead (Iatrobeads 6RS-8060) column (10 mm × 150 mm), eluted with a linear gradient from C/M (95:5) to C/M/water (55:45:5). For step (iv), the glycero-PLPS fraction was further purified by high-performance liquid chromatography on an Iatrobeads 6RS-8010 column (4.6 mm × 300 mm) with a linear C/M/2.5 M NH_4OH gradient (from 75:22.5:2.5 to 45:50:5). NLx was collected from a slower-eluting fraction from glycero-PLPS.

TLC of GSLs and Detection of Functional Groups. GSLs in crude extract or in eluate fraction from CM-Sephadex or Iatrobeads chromatography were separated on silica gel 60 HPTLC plates (Merck, Darmstadt, Germany) in a C/M/15 M NH_4OH (80:20:2), C/M/0.2% $CaCl_2$ (60:40:9), or C/M/acetone/acetic acid/water mixture (8:2:4:2:1), and visualized by the orcinol/sulfuric acid method for hexoses, spraying with fluorescamine in acetone and water for the amino group (*19*), or immunostaining with mAb 5F5 (see below).

Identification of Lipids Containing the Aldehyde Group, Using Schiff's Reagent. For detection of plasmal (palmital or stearal), GSLs separated on TLC were sprayed with 2.5 M HCl in 50% methanol to release the aldehyde group, and heated at 80 °C for 10 min. After cooling, the plate was briefly soaked in Schiff's reagent (reduced colorless Fuchsin in sodium bisulfide) (Sigma), and then dried to stain the reducing aldehydes with red color.

Negative-Ion LSIMS of NLx. LSIMS was performed on a Concept IH (Shimadzu/Kratos, Kyoto, Japan) mass spectrometer fitted with a cesium ion gun. Approximately 0.5 nmol of GSL in 1 μL of C/M (1:2) was mixed with 1 μL of triethanolamine as the matrix. Spectra were recorded at an accelerating voltage of 8 kV and a resolution of 1000−2000 (*20*, *21*). The scan speed was 5 s/decade.

Positive- and Negative-Ion ESIMS of NLx. Measurements were performed using an LCQ DECA ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with an ESI probe. The sample solution (∼5 nmol/mL in methanol) was directly infused into the ion source at a flow rate of 3 μL/min. Source parameters were as follows: spray voltage, 5 kV; sheath gas flow rate, 50 (arbitrary units); capillary temperature, 220 °C; and capillary voltage, 5 kV. Low-energy collision-induced dissociation was carried out on the sodiated molecules (MS^2) and their product ions sequentially (MS^n) using helium gas present in the ion trap. The relative collision energies that were used ranged from 35 to 45%.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were obtained at 400 MHz, using a GX-400 spectrometer (JEOL, Tokyo, Japan) with a probe temperature of 60 °C. The purified GSL was deuterium-exchanged with CD_3OD, dried over P_2O_5 under vacuum, and dissolved in a $(CD_3)_2SO/D_2O$ mixture (98:2). The final concentration was approximately 0.2 mM. Chemical shifts were referenced to tetramethylsilane. In the HOHAHA experiment, a data matrix of 1024 (t_2) × 256 (t_1) points was acquired with a spectral width of 2 kHz. The mixing time was 100 ms. ROESY spectra with a data matrix of 1024 (t_2) × 128 (t_1) were acquired with a 200 ms spin−lock period, with a spectral width of 2 kHz (*22*).

Establishment of mAb 5F5, and ELISA. A mouse hybridoma secreting IgM mAb 5F5 was established after

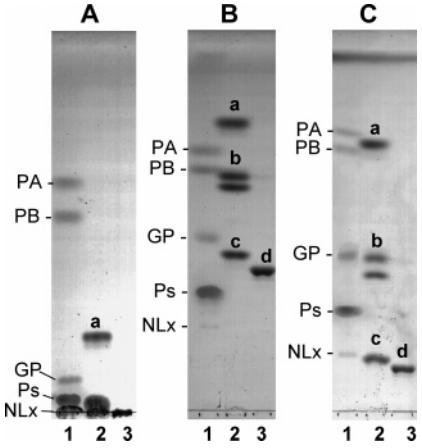

FIGURE 1: TLC of cationic GSLs from bovine brain. (A−C) TLC was developed in C/M/15 M NH_4OH (80:20:2) (solvent A, basic solvent), C/M/0.2% $CaCl_2$ (60:40:9) (solvent B, neutral solvent), and C/M/acetone/acetic acid/water (8:2:4:2:1) (solvent C, acidic solvent) mixtures, respectively, and visualized by orcinol reagent: lane 1, purified cationic GSLs as PLPS-A (PA), PLPS-B (PB), glycero-PLPS (GP), psychosine (Ps), and an unknown slow-migrating compound (NLx), from bovine brain; lane 2, GlcCer (a), LacCer (b), and Gb_4Cer (c); and lane 3, GM3 (d).

immunization of BALB/c mice with the deNAcLc$_3$Cer fraction, using TiterMax Gold (CytRx, Norcross, GA) as carrier and adjuvant according to the manufacturer's instructions. The fusion partner was NS-1. The antibody exhibited preferential reactivity with deNAcLc$_3$Cer (see Results).

Purified GSLs were dissolved in ethanol, and a 50 μL aliquot or its serially diluted solution was added to each well of 96-well flat bottom polystyrene plate, dried at 37 °C, and washed in PBS. The contents of each well were blocked with 3% BSA in PBS for 1 h, and reacted with mAb 5F5 for 1 h at room temperature. Each well was washed extensively in PBS containing 0.05% Tween 20 (T-PBS), and incubated with peroxidase-linked secondary antibody for 30 min at room temperature. After being washed in T-PBS, each well was supplemented with 100 μL of 0.05 M citric acid (pH 4.0) containing 0.5 mg/mL 2,2′-azinobis(3-ethylbenzthiazoline)sulfonic acid and 0.01% H_2O_2, and the absorbency of the solution at 630 nm was measured with a microplate reader.

TLC Immunostaining. Immunostaining was performed on HPTLC plates by a modified version of Magnani's procedure (*23*). Developed TLC plates were air-dried and reacted for 2 h with mAb at room temperature. Plates were gently washed in PBS and incubated with peroxidase-linked secondary antibody for 30 min at room temperature. Plates were gently washed in PBS, and stained with Konica Immunostain HRP-1000 (Konica, Tokyo, Japan).

De-N-acetylase Activity in Bovine Brain Extract with Lc$_3$Cer as the Substrate. The extract for determination of de-*N*-acetylase activity was prepared by homogenization of 10 g of bovine brain with 30 mL of PBS containing 10 μg/mL aprotinin. The homogenate was centrifuged at 1500*g* for 10 min, and the supernatant was further centrifuged at 100000*g* for 1 h. The sediment from the second centrifugation was dissolved in PBS containing 10 μg/mL aprotinin. [^{14}C]-*N*-Acetyl-Lc$_3$Cer (0.2 nmol) in a vial was mixed with 50 μL of the protein extract and incubated at 37 °C. The protein extract heated in boiling water bath was used as a negative control.

Table 1: Quantities of Cationic GSLs in Comparison to GalCer in Bovine Brain White Matter[a]

GSL	amount (μg/g wet weight)
psychosine	0.36
PLPS-A	0.09
PLPS-B	0.14
glycero-PLPS	0.49
deNAcLc$_3$Cer	0.01

[a] The intensity of each band, separated by TLC and reacted with orcinol/sulfuric acid reagent, was determined by Scion Image densitometry.

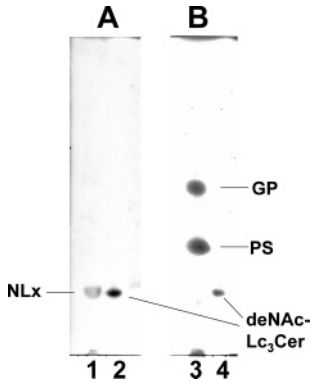

FIGURE 2: TLC immunostaining of the cationic fraction by anti-deNAcLc3Cer mAb 5F5 TLC developed in a C/M/acetone/acetic acid/water (8:2:4:2:1) mixture was visualized by immunostaining with mAb 5F5 (A) or orcinol/sulfuric acid reagent (B). Lanes 1 and 3: cationic lipid fraction of fresh bovine brain white matter containing glycero-PLPS (GP) and psychosine (PS). Lanes 2 and 4: purified deNAcLc$_3$Cer as NLx. The fraction spotted in lane 3 is ~5 times more prevalent than the fraction spotted in lane 1, but the band in lane 1 corresponding to deNAcLc$_3$Cer could be detected by immunostaining with mAb 5F5. In contrast, the band in lane 3 was very faint (not visible in the photograph), because the sensitivity to orcinol/sulfuric acid reagent was very low as compared to that of immunostaining. Only the bands for GP and PS were detected.

The following procedure was used to determine the amount of released [^{14}C]acetic acid. [^{14}C]-*N*-Acetyl-Lc$_3$Cer and protein fraction were mixed and incubated for various durations (see the abscissa of Figure 7) in an airtight vial. The vial, in the horizontal position, was punctured by an airtight syringe containing NaOH pellets, and evacuated to create negative pressure in the vial. The assembly was left for 1 h so that released acetic acid was adsorbed on NaOH in the syringe. ^{14}C activity adsorbed on NaOH was counted with a scintillation counter.

RESULTS

Pattern of Cationic GSLs in Bovine Brain White Matter. Four known cationic GSLs and one unknown, very minor cationic component were found in bovine brain white matter. Two fast-migrating components exhibited the same TLC migration rate as PLPS-A (containing 3,4-cyclic plasmal) and PLPS-B (containing 4,6-cyclic plasmal). One component showed the same TLC mobility as psychosine. The major component had the same TLC mobility as glycero-PLPS. In addition, an unknown component, termed NLx, was detected. The TLC mobility of these components is shown in Figure 1. PLPS or glycero-PLPS bands on TLC gave a red color in situ with Schiff's reagent, after HCl treatment. In contrast, psychosine or the NLx band separated on TLC did not give a red color after HCl treatment, indicating an absence of

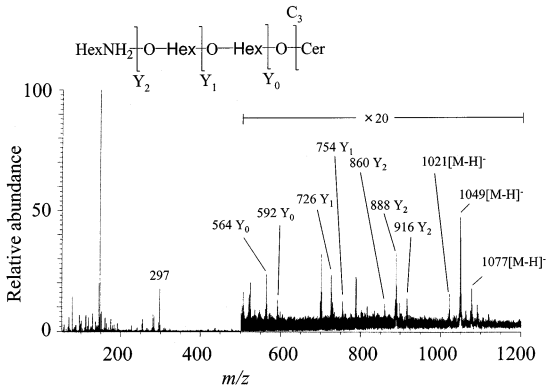

FIGURE 3: Negative-ion LSIMS spectrum of NLx. The fragmentation nomenclature is modified from that proposed for oligosaccharides by Domon and Costello (*42*).

plasmal (long chain, aliphatic aldehyde, i.e., plasmal, stearal). Relative quantities of these five cationic GSLs from bovine brain white matter were determined by Scion Image densitometry of TLC-separated bands reacted with an orcinol/sulfuric acid reagent under the same conditions. Results are shown in Table 1.

Isolation and Preliminary Characterization of NLx. After repeated cation-exchange chromatography on CM-Sephadex, retained fractions containing the components described above were combined, and further purified using Iatrobeads 6RS-8060 and 6RS-8010 with linear C/M/water and C/M/NH_4OH gradients, as described in Materials and Methods. Finally, the NLx component was purified to give a single band on TLC. Of these cationic GSLs, only the NLx component was stained by mAb 5F5 (Figure 2).

Negative-Ion LSIMS. Negative-ion LSIMS of NLx showed an intense deprotonated $[M - H]^-$ molecule at *m/z* 1049 (Figure 3). This is consistent with a structure derived from GSL containing a $HexNH_2$, two hexoses (Hex), and a Cer (18:0/d18:1). Less-abundant $[M - H]^-$ ions, corresponding to other molecular species, were also detected at *m/z* 1021 and 1077 (16:0/d18:1 and 20:0/d18:1, respectively). [Many fragments with a mass difference of 28 amu (1049 vs 1021, 916 vs 888, 754 vs 726, and 592 vs 564) may reflect the aliphatic chain difference in fatty acid.] The most abundant fragments (Y_2) at *m/z* 888, 860, and 916 resulted from elimination of $HexNH_2$. Loss of the $HexNH_2$ and Hex unit yields the fragments (Y_1) observed at *m/z* 726 and 754. The less abundant fragments were observed at *m/z* 564 and 592 (Y_0), which arise from elimination of $HexNH_2$ and two hexoses.

Positive- and Negative-Ion ESIMS. Positive-ion ESIMS produced abundant sodiated $[M + Na]^+$ molecules at *m/z* 1045 (16:0/d18:1), 1073 (18:0/d18:1), and 1101 (20:0/d18:1), supporting the conclusion that NLx includes a $HexNH_2$, two hexoses (Hex), and a Cer. MS^2 of the $[M + Na]^+$ ion at *m/z* 1073 produced abundant product ions at *m/z* 912 (Y_2 + Na) and 750 (Y_1 + Na) (Figure 4A), which resulted from elimination of $HexNH_2$ and $HexNH_2$-Hex, respectively. A less abundant product ion derived from the whole carbohydrate chain was detected at *m/z* 526 (C_3 + Na). Via a sequential product ion fragmentation experiment (MS^3) with

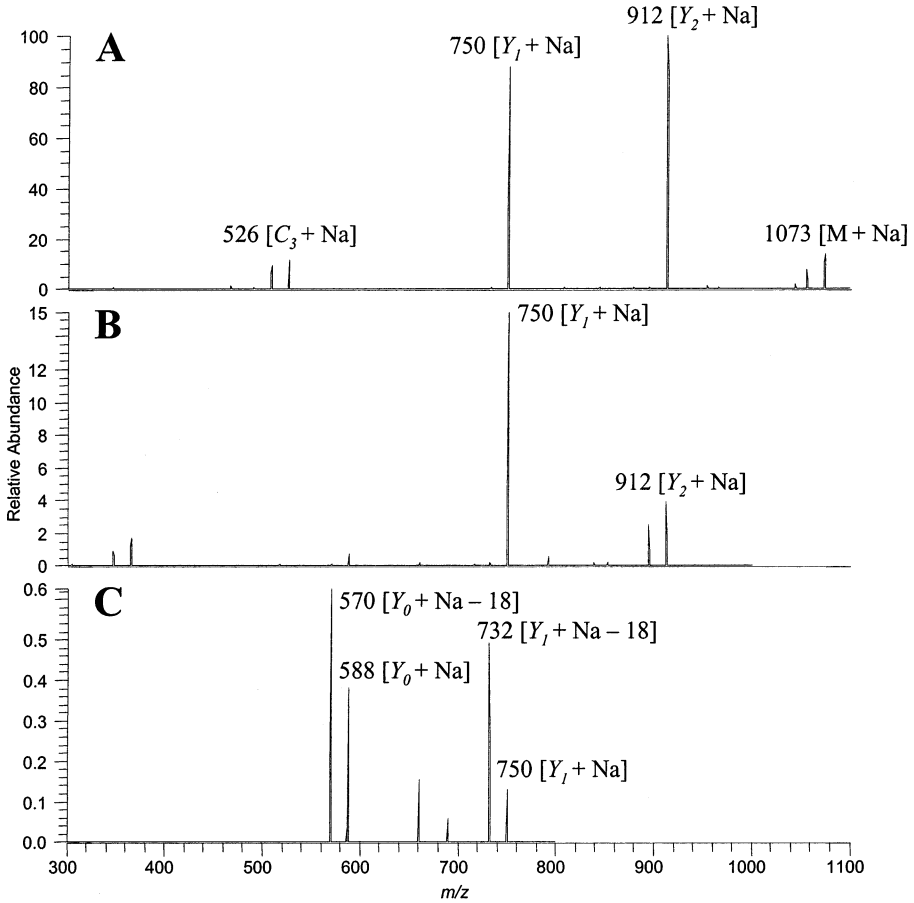

FIGURE 4: Positive-ion ESIMS spectra of NLx. (A) Product-ion spectrum (MS^2) of the $[M + Na]^+$ ion at *m/z* 1073. (B) Product-ion spectrum (MS^3) with selection of the Y_2 + Na ion at *m/z* 912, produced by MS^2 (A), as a precursor. (C) Product-ion spectrum (MS^4) with selection of the Y_1 + Na ion at *m/z* 750, produced by MS^3 (B), as a precursor. Assignments for the product ions are shown in Figure 3.

Table 2: ^{1}H Chemical Shifts (ppm) and Coupling Constants (Hz, in parentheses) of Naturally Occurring NLx and Chemically Prepared DeNAcLc$_3$Cer in a DMSO-d_6/D$_2$O (98:2) Mixture at 60 °C[a]

		chemical shift ($J_{H,H}$)	
		NLx	deNAcLc$_3$Cer
Glcβ	I-1 ($^3J_{1,2}$)	4.17 (8.1)	4.18
	I-2 ($^3J_{2,3}$)	3.06 (8.1)	3.06
	I-3 ($^3J_{3,4}$)	3.34[b]	3.3[b]
	I-4 ($^3J_{4,5}$)	3.3[b]	3.3[b]
	I-5 ($^3J_{5,6a}$)	3.29 (3.8)[b]	3.3[b]
	I-6a ($^3J_{5,6b}$)	3.61 (<1.2)	3.61
	I-6b ($^2J_{6a,6b}$)	3.76 (−12.4)	3.76
Galβ	II-1 ($^3J_{1,2}$)	4.29 (7.6)	4.29
	II-2 ($^3J_{2,3}$)	3.54 (9.7)	3.54
	II-3 ($^3J_{3,4}$)	3.48 (3.8)	3.48
	II-4 ($^3J_{4,5}$)	3.87 (<1.2)	3.87
	II-5 ($^3J_{5,6a}$)	3.56[b]	
	II-6a ($^3J_{5,6b}$)	3.5 (6.4)[b]	
	II-6b ($^2J_{6a,6b}$)	3.54 (−10.1)[b]	
GlcNβ	III-1 ($^3J_{1,2}$)	4.34 (8.1)	4.34
	III-2 ($^3J_{2,3}$)	2.44 (9.2)	2.44
	III-3 ($^3J_{3,4}$)	3.07[b]	3.1[b]
	III-4 ($^3J_{4,5}$)	3.1[b]	3.1[b]
	III-5 ($^3J_{5,6a}$)	3.12 (5.0)[b]	3.1[b]
	III-6a ($^3J_{5,6b}$)	3.46 (<1.2)	3.46
	III-6b ($^2J_{6a,6b}$)	3.66 (−12.4)	3.66
Sph	L-1a ($^2J_{1a,1b}$)	3.47 (−10.1)	
	L-1b ($^3J_{1a,2}$)	3.94 (3.2)	
	L-2 ($^3J_{1b,2}$)	3.79 (5.0)	
	L-3	3.91	
	L-4	5.37	
	L-5	5.56	

[a] Signals were assigned on the basis of the DQF-COSY and HOHAHA spectra. [b] The protons in Glcβ (I-3, I-4, and I-5), Galβ (II-5, II-6a, and II-6b), and GlcNβ (III-3, III-4, and III-5) are strongly coupled.

the Y_2 + Na ion at *m*/*z* 912 as a precursor, an intense product ion corresponding to Y_1 + Na was produced at *m*/*z* 750 (Figure 4B). By MS^4 with the Y_1 + Na ion as a precursor, intense product ions corresponding to Y_0 + Na with or without dehydration were produced at *m*/*z* 588 and 570 (Figure 4C). These MS^n experiments confirmed that NLx has a HexNH$_2$-Hex-Hex-Cer structure.

In contrast to negative-ion LSIMS, only a trace amount of $[M - H]^-$ ions are detected by negative-ion ESIMS (data not shown).

NMR Spectroscopy of NLx. ^{1}H chemical shifts and coupling constants of NLx and deNAcLc$_3$Cer are shown in Table 2. Assignments of the proton signals of NLx are given in Figure 5A. Three doublets observed in the anomeric region indicate that NLx possesses three β-hexoses ($^1J_{1,2}$ coupling constants were larger than 7 Hz), residues I, II, and III. Residue I was identified as β-Glc judging from the characteristic chemical shifts of I-3, I-4, and I-5 protons, and residue II was identified as β-Gal from the chemical shift of II-4 and the $^3J_{3,4}$ and $^3J_{4,5}$ coupling constants (*24*, *25*). As for residue III, the characteristic strongly coupled III-3, III-4, and III-5 protons show that the residue possesses the β-Glc structure, and the III-2 proton resonating in the higher field (2.44 ppm) shows that the hydroxyl group at position 2 is substituted with an amino group (*4*). The structure of acylated 4-sphingenine was determined from two multiplets at 5.37 and 5.56 ppm, and the chemical shift of the L-2 proton (3.79 ppm) (*26*).

In the ROESY spectrum, three inter-residue NOEs, I-1−L-1a, II-1−I-4, and III-1−II-3, were observed (Figure 5B). The overall results indicate a GlcNβ1−3Galβ1−4Glcβ1−1Cer structure. The chemical shifts of carbohydrate signals were in good agreement with those in deNAcLc$_3$Cer, which was produced by hydrazinolysis of Lc$_3$Cer (*27*).

Reactivity of mAb 5F5. mAb 5F5, established as described in Materials and Methods, was strongly reactive with not only deNAcLc$_3$Cer (GlcNH$_2\beta$3Galβ4GlcCer) but also deNAcLc$_5$Cer (GlcNH$_2\beta$3Galβ4GlcNAcβ3Galβ4GlcCer), both having a GlcNH$_2$ group at their termini (Figure 6). It did not react with various previously known GSLs (Lc$_3$Cer, deNAcGg$_3$Cer, Gg$_3$Cer, Gb$_4$Cer, nLc$_4$Cer, etc.) which do not have a terminal GlcNH$_2$ group.

Lc$_3$ De-N-acetylase Activity in Bovine Brain Extract. Free [^{14}C]acetic acid was observed in a mixture of protein extract and [^{14}C]acetyl-Lc$_3$Cer, but was not detected in a mixture of heated protein (as control) and [^{14}C]acetyl-Lc$_3$Cer (Figure 7).

DISCUSSION

A novel, slow-migrating, cationic GSL of bovine brain white matter, adsorbed on CM-Sephadex in C/M and eluted with trimethylamine followed by Iatrobeads chromatography, termed "NLx", was identified unambiguously as de-*N*-acetyllactotriaosylceramide (deNAcLc$_3$Cer) (structure shown in Figure 8). DeNAcLc$_3$Cer is the fifth cationic GSL found so far in extract of bovine brain white matter. The possible role of deNAcLc$_3$Cer as a signaling molecule is unknown at this time. Its quantity is only 1/10 of that of 4,6-PLPS (PLPS-B), and 1/30 of that of glycero-PLPS. Release of the signaling molecule in some cases is a rapid process, particularly when phosphorylation and dephosphorylation are involved. However, other signaling molecules behave in a different way. Ceramide is a well-known signaling molecule, derived mainly from sphingomyelin (for reviews, see refs *28* and *29*), and its metabolic turnover is relatively slow.

Release of deNAcLc$_3$Cer by de-*N*-acetylase appears to be slow, when determined in crude, total white matter extract. However, this may be an effect of "dilution" and/or the presence of unknown inhibitors; i.e., if enzyme activity could be determined in situ where it is occurring, it might appear to be much higher and more rapid. The biological significance of a signaling molecule is not related to its quantity, rapidity of formation, or rapidity of returning to precursor, but rather is related to its ability to interact with specific effector molecules in the membrane in response to a defined type of cell stimulation.

Expression of de-*N*-acetyl-GlcNAc (GlcNH$_2$ group) requires the presence of a specific de-*N*-acetylase and activation of its gene. A specific de-*N*-acetylase which converts Lc$_3$Cer to deNAcLc$_3$Cer was detected in white matter extract. It is of great interest to determine whether this enzyme is activated, and releases deNAcLc$_3$Cer, upon cell stimulation.

Three types of GlcNH$_2$ residues in glycoconjugates are known so far, each created from GlcNAc by specific de-*N*-acetylase(s).

(i) The GlcNH$_2$ residue is found in the glycosylphosphatidylinositol (GPI) anchor, having the GlcNH$_2\alpha$1−2 inositol residue of phosphatidylinositol. While this core structure is constant, the peripheral glycan of the GPI anchor shows extensive variation, through which various cell surface proteins are carried through the phosphoethanolamine linkage (*30*).

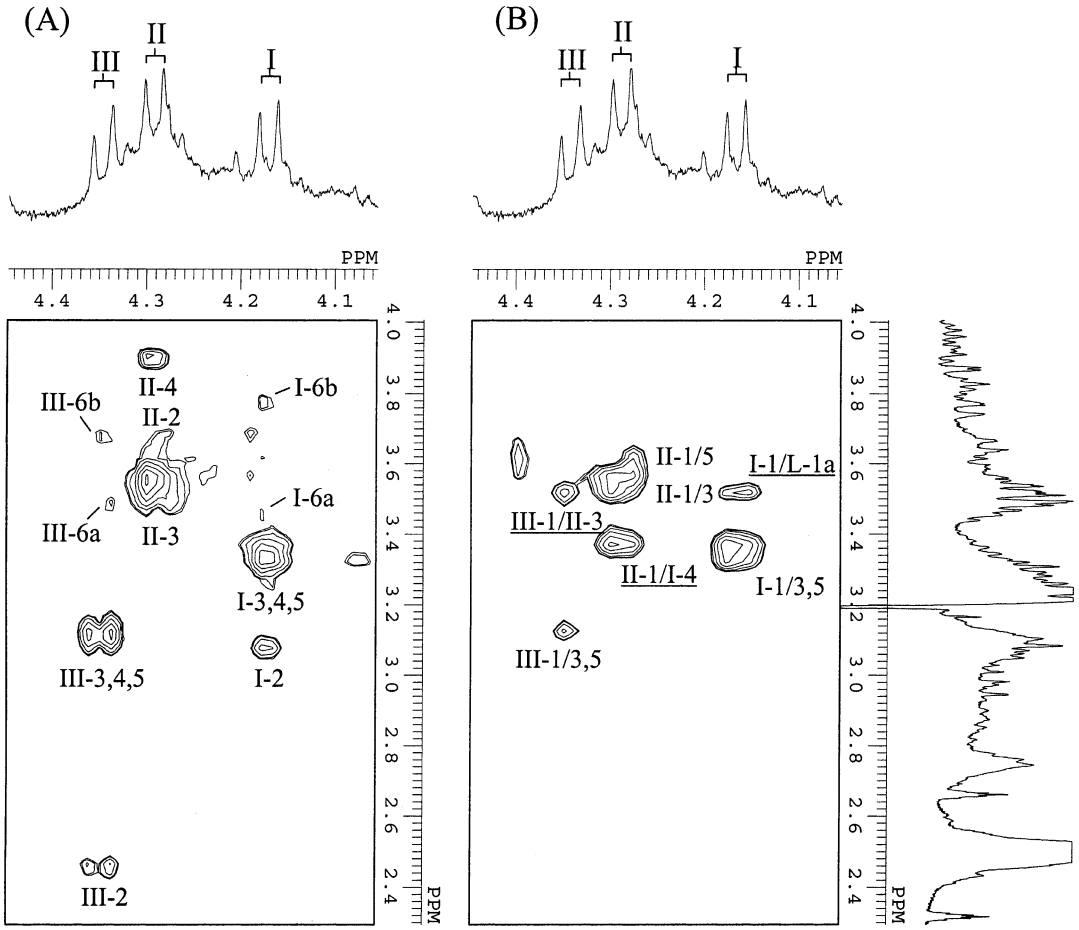

FIGURE 5: HOHAHA (A) and ROESY (B) spectra of NLx. Arabic numerals refer to the protons in three sugar residues (I, II, and III) and the long chain base (L).

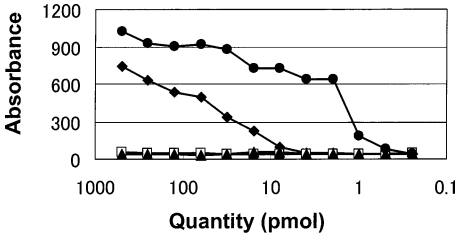

FIGURE 6: Reactivity of mAb 5F5 with various GSLs including the β-GlcNH_2 group, as determined by ELISA. Each GSL with or without a β-GlcNH_2 group (600 pmol) in an ethanol solution was placed in an ELISA plate, serially diluted in ethanol as indicated on the abscissa, air-dried, blocked with 3% BSA, and reacted with mAb 5F5. Antibody binding was assessed with a microplate reader: (◆) deNAcLc$_3$Cer, (●) deNAcLc$_5$Cer, (□) deNAcGg$_3$Cer, and (▲) Lc$_3$Cer.

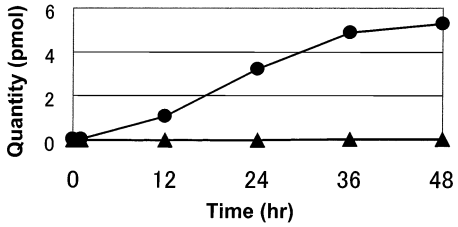

FIGURE 7: Lc3 de-*N*-acetylase activity in bovine brain extract. The release of [^{14}C]acetate from [^{14}C]-*N*-acetyl-Lc$_3$Cer by incubation with brain protein fraction, under conditions as described in Materials and Methods, is shown (●). Heat-inactivated brain protein under the same condition is also shown (▲). The abscissa is the incubation time. The ordinate is the quantity of acetate released (picomoles).

(ii) The GlcNH_2 residue is found in a novel glycosyl-inositolphosphorylceramide found recently in the pathogenic fungus *Sporothrix schenckii* (*31*) and in various filamentous fungi, particularly *Acremonium* sp. (*32*). These glycans have a common core, Glc$NH_2\alpha$1−2 myoinositol motif, regardless of their source. In addition, the GlcNH_2 residue was found in the phytosphingolipid core as GlcNH_2 → glucuronic acid → inositol → phytoceramide (*33*), and the structure was confirmed and studied in detail from various plant sources (*34*, *35*). It should be noted that phytosphingolipid glycan from higher plants has GlcNH_2 linked to glucuronic acid or glucose, rather than to myoinositol. The de-*N*-acetylase gene that releases the GlcNH_2 of GPI anchor was cloned recently (*36*).

(iii) The GlcNH_2 residue is found in heparin or heparan sulfate, most of which is converted to N-sulfated group (*37*); for a review, see ref *38*. The genes encoding the glucosaminyl *N*-deacetylase/*N*-sulfotransferase enzymes responsible for this process have been cloned (*39*).

Results of this study clearly indicate the existence of a new type of de-*N*-acetylase specific to GlcNAc, for creation of the GlcNH_2 residue in GSLs. This is in addition to the three previously known cases. Surprisingly, there is no strong homology between the genes for de-*N*-acetylases types (i) and (iii) as described above. It is possible that the de-*N*-acetylase for release of deNAcLc$_3$Cer is encoded by an independent gene.

We previously found another case of de-N-acetylation of a glycoconjugate, occurring at the sialic acid residue of GM3

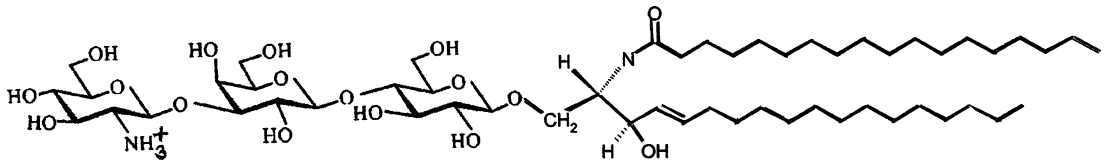

FIGURE 8: Structure of deNAcLc$_3$Cer.

ganglioside. DeNAcGM3 strongly enhanced the tyrosine kinase associated with epidermal growth factor (EGF) receptor, and promoted EGF-dependent cell growth. In striking contrast, regular GM3 inhibited EGF receptor tyrosine kinase and suppressed EGF-dependent cell growth (*40*). De-N-acetylation of sialic acid of ganglioside in human melanoma cells was also described. In this case, antibody directed to the structure inhibited cell growth, and Genistein, which promotes tyrosine phosphorylation, activated synthesis of de-*N*-acetyl ganglioside (*41*).

Two classes of cationic GSLs have been characterized so far. (I) The cationic group is the amino group of sphingosine, i.e., lyso-GSL in which the N-fatty acyl group is absent. (II) The cationic group is the amino group of GlcNAc or sialic acid. In this case, either GlcNAc or sialic acid is the major NH_2 group carrier.

The majority of class I cationic GSLs are based on β-galactosylsphingosine (psychosine), but not β-glucosylsphingosine. PLPS-A and -B (*5*), and glycero-PLPS (*4*), are the best-characterized and major constituents of this class. Interestingly, PLPS-B clearly activated Trk A tyrosine phosphorylation followed by prolonged MAPK activation, inducing neuritogenesis in PC12 cells (*13*). Class I GSLs are more prevalent in human than in bovine brain white matter (*4*, *5*). Other class I structures, such as lactosylsphingosine, can be detected as extremely minor components (*6*).

The majority of class II cationic GSLs are deNAcLc$_3$Cer, as described in this paper. It is unknown at this time whether other analogues exist, i.e., within the lacto-series structure. GlcNAc within nLc$_4$, nLc$_6$, nLc$_8$, etc., or a branched structure containing GlcNAc, could be de-N-acetylated by the same or analogous de-*N*-acetylase. mAb 5F5 was established as a probe for detecting deNAcLc$_3$Cer structure. Application of this mAb for immunohistology of brain sections was unsuccessful, presumably because the amount of antigen is too small, or the structure is closely associated with membrane protein and present in cryptic form. However, extraction of brain tissue followed by quick TLC immunostaining clearly detected the presence of deNAcLc$_3$Cer as shown in Figure 2.

Structures similar to class II GSLs are those containing deNAcGalNAc. However, these are very minor components in bovine brain white matter. DeNAcGalNAc was detected tentatively as deNAcGg$_3$Cer. Recent advances in mass spectrometry can distinguish deNAcGg$_3$Cer from deNAcLc$_3$Cer, or even Gg$_3$Cer from Lc$_3$Cer (K. Tadano-Aritomi, T. Hikita, and I. Ishizuka, unpublished data).

Typically, structural scientists are immediately asked, "What is the biological significance or function?" of a newly discovered structure. One must realize that functions of the majority of biological structures (not only glycoconjugates but also proteins) known for 50 or even 100 years are still not understood, e.g., chondroitin sulfate, hyaluronic acid, blood group ABH, Lewis antigens, Gb$_4$Cer, LacCer, serum albumin, catalase, transferrin, etc.

Our basic motivation in study of cationic GSLs is that very few examples are known; i.e., the great majority of known GSLs are acidic or neutral. There is an interesting possibility that cationic GSLs, whether class I or II, present in the membrane may affect organizational assembly of GSLs in the membrane, which are almost all either anionic or neutral. If cationic GSLs are introduced in such a membrane microdomain, perturbation may occur, which is manifested as a form of signal transduction. This possibility remains to be studied.

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