

Molecular characterization and immunohistochemical localization of IV⁴GalNAcGgOse₄Cer: a naturally occurring novel neutral glycosphingolipid in bovine brain

S.Dasgupta¹, H.van Halbeek³, S.Spicer² and E.L.Hogan

Department of Neurology and ²Department of Pathology, Medical University of South Carolina, Charleston SC, USA and ³School of Medicine, University of California San Diego, La Jolla CA, USA

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A pair of novel neutral glycosphingolipids (Ngsls) has been identified in bovine brain. Their mobilities on thin layer chromatography were slightly different from a standard pentaglycosylceramide (nLcOse₅Cer from bovine erythrocytes). The compounds were purified to homogeneity by column chromatography. Their fatty acid and base compositions, their monosaccharide compositions and sugar linkage positions were determined by gas-liquid chromatography/mass spectrometry. Carbohydrate sequence analysis by ¹H NMR spectroscopy and stepwise exoglycosidase digestion indicated the following pentaglycosyl structure for the oligosaccharide moiety of both Ngsls: GalNAcβ1–4Galβ1–3GalNAcβ1–4Galβ1–4Glc. The two Ngsls (abbreviated as IV⁴GalNAcGgOse₄Cer or GalNAc-GA1), differ in their ceramide compositions, having d18:0 and d18:1 sphingosine as their long chain bases. A mono-specific polyclonal anti-GalNAc-GA1 antibody, prepared in rabbit and purified by affinity chromatography, stained the neurons of cerebral cortex and cerebellum including Purkinje cells in adult rat brain, indicating that the novel GalNAc-GA1 is associated with cerebellar and other neurons in vertebrate central nervous system.

Key words: bovine brain/glycosphingolipid/immunohistochemistry/NMR spectroscopy/neurons

Introduction

Gangliosides, acidic glycosphingolipids (Gsls) of the ganglio-series, are abundant in nervous system while the lacto- and globo-series are the major Gsls of extraneural tissues. The major vertebrate gangliosides, e.g., GM1, GD1a, GD1b, GT1, GQ1, contain the same neutral Gsl (GA1 or GgOse₄Cer) core structure. Galactocerebroside, the predominant Ngsl of central nervous system (CNS), is a myelin marker. The molecular

species of monoglycosylceramides shift in developing brain from glucosylceramide to galactosylceramide during the transition from embryonic to postnatal life (Dasgupta *et al.*, 1997). The postpartum emergence in rat brain of several fast migrating galactocerebroside derivatives, along with other evidence, suggests that they, too, are myelin components (Dasgupta *et al.*, 1997).

At present, the role of glycoconjugates in CNS development has not been fully delineated and this is particularly true of neutral glycosphingolipids (Ngsls). In recent years, we have been examining their potential role in CNS development. Several minor long chain (i.e., containing more than three glycosyl residues) Ngsls have been identified in brain by digoxigenin immunostaining (DIG-IS) after removal of galactocerebroside (Dasgupta *et al.*, 1992). Three of them have been characterized as GA1 (Dasgupta *et al.*, 1992; Dasgupta and Hogan, 1993), GalGbOse₄Cer (Dasgupta *et al.*, 1995b), and FucnLcOse₄Cer (Dasgupta *et al.*, 1996). The topography of GA1 and FucnLcOse₄Cer has been examined immunohistochemically in rodent brain (Dasgupta *et al.*, 1996).

We now report on identification, purification and chemical characterization of another pair of minor brain Ngsls and their subsequent histochemical localization in adult rat brain. This report confirms and extends our preliminary data (Dasgupta *et al.*, 1995a).

Results

Purification and characterization of Ngsls A and B

The two Ngsls (marked A and B), purified from bovine brain by silicic acid and DEAE-Sephadex column chromatographies, appeared homogeneous when examined by TLC using two different solvent systems; namely, chloroform:methanol:water; and chloroform:methanol:2.5N ammonia and migrated at a lower R_f than a standard pentaglycosylceramide (nLcOse₅Cer) from bovine erythrocytes (Figure 1). Ngsl A has a higher R_f than Ngsl B in both solvent systems. In comparison to bovine brain ganglioside standards, Ngsl B comigrated with bovine brain ganglioside GD3 while Ngsl A migrated more closely to GM1 at neutral pH (Figure 1a). In alkaline media both Ngsls had lower R_f than GM1 and GD3 (Figure 1b). The amounts of Ngsl A and Ngsl B, purified from 30 g of acetone dried brain, were 30 µg and 100 µg, respectively.

The carbohydrate compositions of Ngsls A and B (determined by the alditol acetate method) were identical; GalNAc,

¹To whom correspondence should be addressed at: Department of Neurology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425–2232

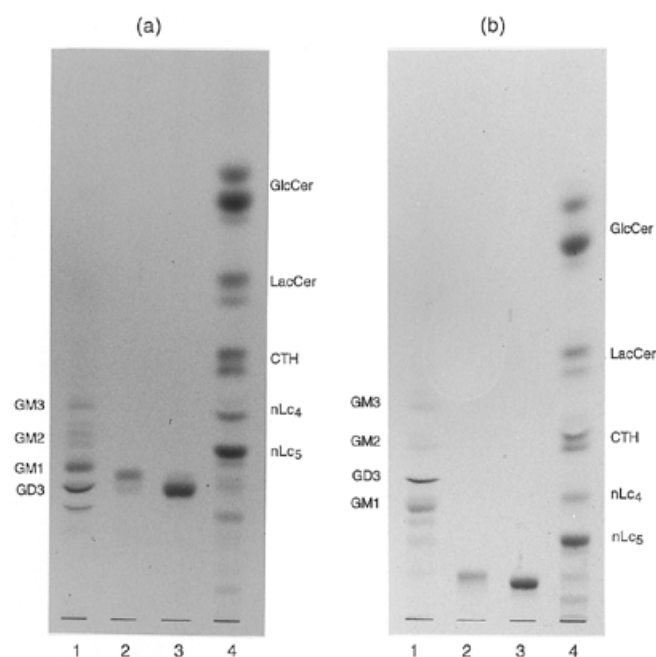


Fig. 1. Thin-layer chromatography of the purified bovine brain Ngsls A and B. Lane 1, standard bovine brain gangliosides. Lane 2, Ngsl A. Lane 3, Ngsl B. Lane 4, standard Ngsls from bovine erythrocytes. The bands were resolved using (a) chloroform:methanol:water 60:40:9 (v/v) and (b) chloroform:methanol:2.5N ammonia, 60:40:9 (v/v) and visualized by diphenylamine aniline spray.

Table I. Fatty acid and base compositions of Ngsls A and B

Fatty acid	Ngsl A (%)	Ngsl B (%)
C14:0	–	–
C16:0	2.8	–
C16:1	3.0	–
C18:0	94.2	100
Base		
C18:0	100	–
C18:1	–	100

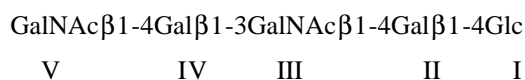
Gal, and Glc were characterized in a molar ratio of 1.6:1.8:1.0. The analysis of fatty acids (as methyl esters) and the base (as the trimethylsilyl derivative) revealed that Ngsl A contained the C18:0 base while C18:1 was the only base identified in Ngsl B (Table I). The difference in base composition between the two Ngsls correlates with their different TLC-Rf migrations (Figure 1). Some heterogeneity in the fatty acid composition of the two Ngsls was observed (Table I).

Methylation analysis of both Ngsls indicated a terminal GalNAc (3,4,6-OMe₃GalNAc), a 1–3 linked GalNAc (4,6-OMe₂GalNAc), two 1–4 linked Gal (2,3,6-OMe₃Gal), and a 1–4 linked Glc (2,3,6-OMe₃Glc) residues (Figure 2). The terminal GalNAc of both Ngsls was absent in the methylation analysis of the β -hexosaminidase hydrolyzed products and a terminal Gal appeared along with the loss of one residue of 1–4 linked Gal (Figure 3), consistent with the GA1 structure of

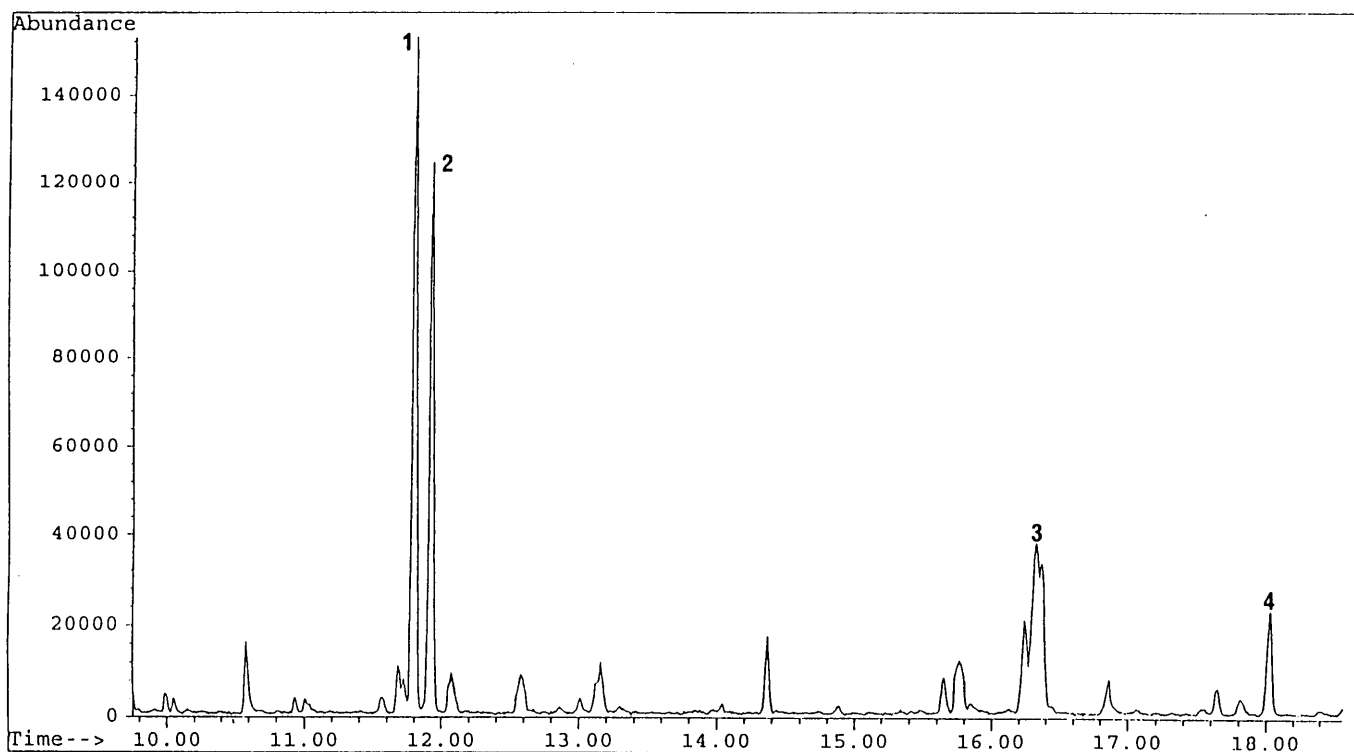
the β -hexosaminidase-digested product. The hexosaminidase-hydrolyzed products moved with a higher TLC-Rf than the original compounds and reacted with anti-GA1 antibody (Figure 4), confirming the GA1 core structure. This suggests that the original compounds (Ngsl A and B) have a β -GalNAc residue attached to GA1.

The anomeric configurations of the carbohydrate linkages were determined by sequential specific exoglycosidase digestion. Both Ngsls A and B were hydrolyzed with β -hexosaminidase from jack bean meal and subsequently by bovine testis β 1–3 galactosidase, jack bean meal β -hexosaminidase and β 1–4 galactosidase, yielding GlcCer as the end product (Figure 5).

The GalNAc-GA1 carbohydrate structure of the novel Ngsls was further confirmed by ¹H NMR spectroscopy of the oligosaccharide released from Ngsl B by ceramide glycanase treatment and purified by sizing chromatography (Biogel P2). The one-dimensional (1D) ¹H NMR spectrum of the oligosaccharide (termed GgOse₅) is shown in Figure 6. The chemical shifts of its structural reporter groups (Dasgupta *et al.*, 1994; Van Halbeek, 1994) are compiled in Table II. For comparison, the NMR characteristics of several oligosaccharides that are structurally related to GgOse₅ are included in Table II. Careful comparison of the spectra of GgOse₄ and GgOse₅ revealed the presence of all the tetrasaccharide's signals in the spectrum of GgOse₅, with only minor perturbations; in addition, the structural reporter group signals typical of a terminal β -linked GalNAc residue (H1 at 4.615 p.p.m., J₁₂ 8.5Hz, H4 at 3.904 p.p.m., and NAc at 2.056 p.p.m.) were observed. The similarity of the latter set of parameters to those for the β 1–4 linked terminal GalNAc residue in GgOse₃ (Table II) suggested as a working model for the structure of GgOse₅ an extension of GgOse₄ with a terminal β 1–4 linked GalNAc residue:



The linkage positions in GgOse₅, including the position of the terminal GalNAc-V to Gal-IV linkage, were verified by two-dimensional (2D) NMR spectroscopy. A 2D ¹H NMR TOCSY experiment was used for identification of the spin systems of the individual glycosyl residues. The TOCSY subspectra, observed in cross sections through the H1 signals, were attributed to the constituent glycosyl residues of GgOse₅ (two β -Gal residues, two β -GalNAc residues, and one Glc residue, the latter present in both α - and β -form, in a ratio of 1:2 approximately) based on their characteristic different geometries (Dasgupta *et al.*, 1994). The four well-resolved H1 doublets at δ 4.439, 4.452, 4.615 and 4.69 p.p.m. clearly belong to β -linked Gal(NAc) residues, as the TOCSY subspectra through these H1 signals showed signals for H2, H3, and H4, but not for H5, H6, and H6' due to the small (<1 Hz) Gal J₄₅ coupling (Poppe and Van Halbeek, 1991; Van Halbeek and Poppe, 1992). The Gal residues were distinguished from the GalNAc residues by virtue of the chemical shifts of their H1 and H2 signals (the Gal-II and Gal-IV H2 signals were found at δ 3.42 p.p.m.; the GalNAc H2 signals were found at 3.89 and 4.03 p.p.m., indicative of the presence of an amino group at C2). Sequential assignments of the glycosyl residues (i.e., their corresponding subsets of four-spin systems) were obtained through a ROESY experiment. The ROESY spectrum of



Peak No.	E.I.-M.S. fragment ions	Linkage	RT*	Nature of carbohydrate
1	118,162,233	4	11.803	Galactose
2	118,162,233	4	11.925	Glucose
3	117,159,161,205	terminal	16.308	NAcGalactosamine
4	117,159,161,273,318	3	18.008	NAcGalactosamine

* Retention time in minutes

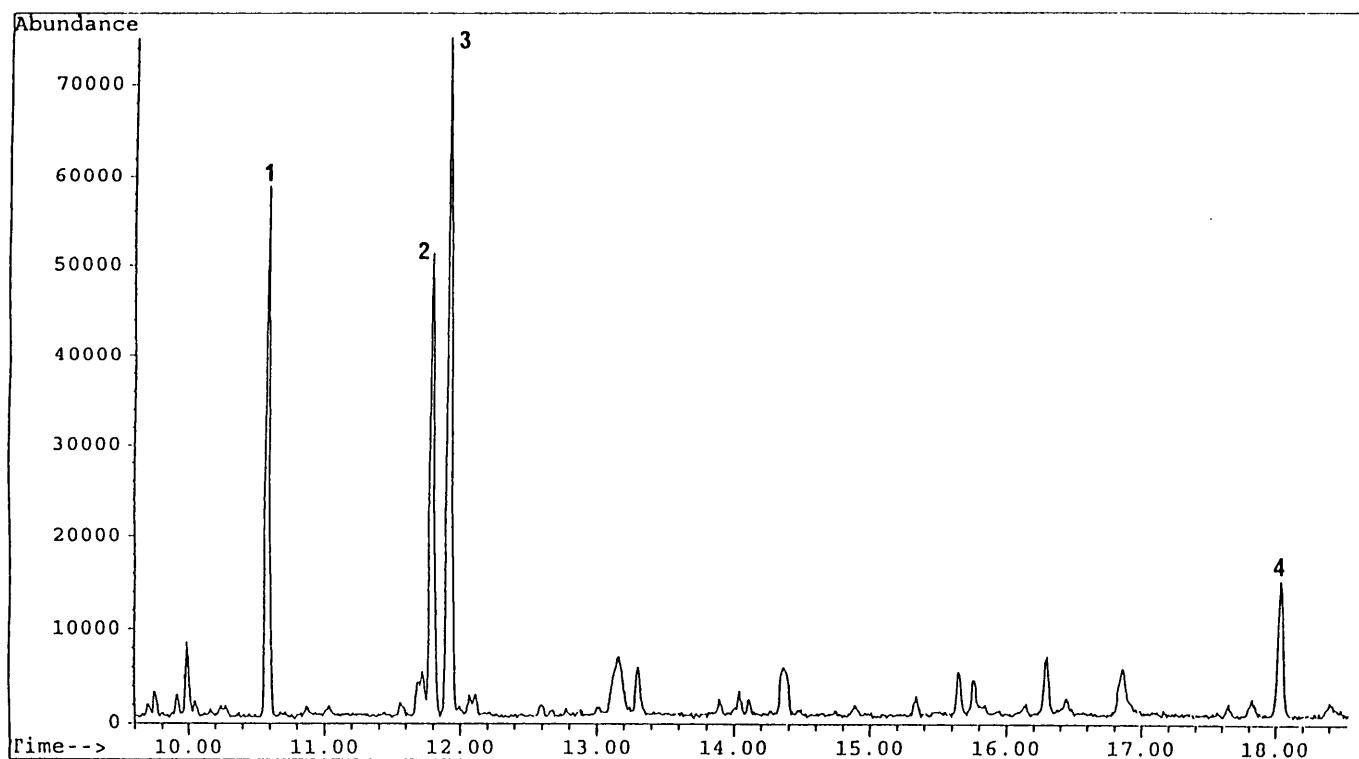
Fig. 2. GLC-MS of PMAAs Prepared from Purified Ngsls A and B. The method has been described in detail in the text. The masses of the fragment ions observed for each peak, in combination with the retention times, define the structure of the PMAAs listed.

GgOse₅ provided a number of intra-glycosyl-residue cross peaks that are compatible with, and complementary to, the TOCSY-derived assignments. For example, the triaxial H1, H3, and H5 spin systems of the β -Gal residues, and similarly those of β -GalNAc H1, H3, and H5, were assigned based on their NOE cross peaks; interestingly, for each of the residues, the NOE contact between H1 and H5 was much stronger than that between H1 and H3. More importantly, the ROESY spectrum, through its inter-residue cross peaks, confirmed the structure of the oligosaccharide as:



It is worth mentioning that the oligosaccharide GgOse₅ showed some peculiar ¹H NMR features. The presence of NAc methyl signals with total intensity equivalent to six protons (see

Figure 6) confirmed the presence of two GalNAc residues in the structure. One of the NAc signals (the singlet at 2.056 p.p.m., with intensity equivalent to three protons) was assigned to the GalNAc-V residue that is relatively remote from the anomeric center of the reducing Glc-I residue. Thus, the remaining two NAc signals (at 2.039 and 2.037 p.p.m.; combined intensity equivalent to three protons) both belong to the second GalNAc residue (GalNAc-III). This pair of singlets was observed in the anomeric intensity ratio ($\alpha:\beta \cong 1:2$), reflecting the position of the GalNAc-III residue relatively close in space to the reducing end of the oligosaccharide GgOse₅. The H1 doublet of the internal GalNAc-III residue was observed doubled by the anomeric effect as well (δ 4.697 and 4.690, in ratio $\sim 1:2$). The aforementioned observations may prove useful in future conformational studies of GgOse₅.



Peak No.	E.I.-M.S. fragment ions	Linkage	RT*	Nature of carbohydrate
1	118,161,162,205	terminal	10.586	Galactose
2	118,162,233	4	11.798	Galactose
3	118,162,233	4	11.935	Glucose
4	117,159,161,273,318	3	18.026	NACGalactosamine

* Retention time in minutes

Fig. 3. GLC-MS of PMAAs prepared from the β -hexosaminidase digests of Ngsls A and B. The method has been described in detail in the text. The masses of the fragment ions observed for each peak in combination with the retention times define the structure of the PMAAs listed.

Immunohistochemical localization of GalNAc-GA1 using anti-GalNAc-GA1 antibody

Monospecific polyclonal anti-GalNAc-GA1 antibody (anti-rabbit IgG) was used to localize the novel Ngsl in adult rat brain parasagittal sections. The antibody stained neurons in cerebral cortex and Purkinje cells in cerebellum and spared the myelin (Figure 7a,b).

Discussion

Examination of rat brain Ngsls during development and particularly during myelinogenesis using DIG-IS (Dasgupta and

Hogan, 1993, 1995b) has revealed numerous long-chain Ngsls; some are previously unidentified. The changes of these Ngsls during critical stages of brain development and their site and cell specificities (Dasgupta *et al.*, 1996) suggest that they are important biomolecules. Here we describe the characterization of two minor brain novel pentaglycosylceramides (abbreviated as Ngsl A and B) and their identification as a neuron-specific component. The purified Ngsls migrated at a lower TLC-Rf than a standard pentaglycosylceramide (nLcOse₅Cer from bovine erythrocytes). These novel compounds only differed from each other in their ceramide compositions and have been characterized chemically (by GLC-MS, NMR and stepwise exoglycosidase digestion) and immunochemically as

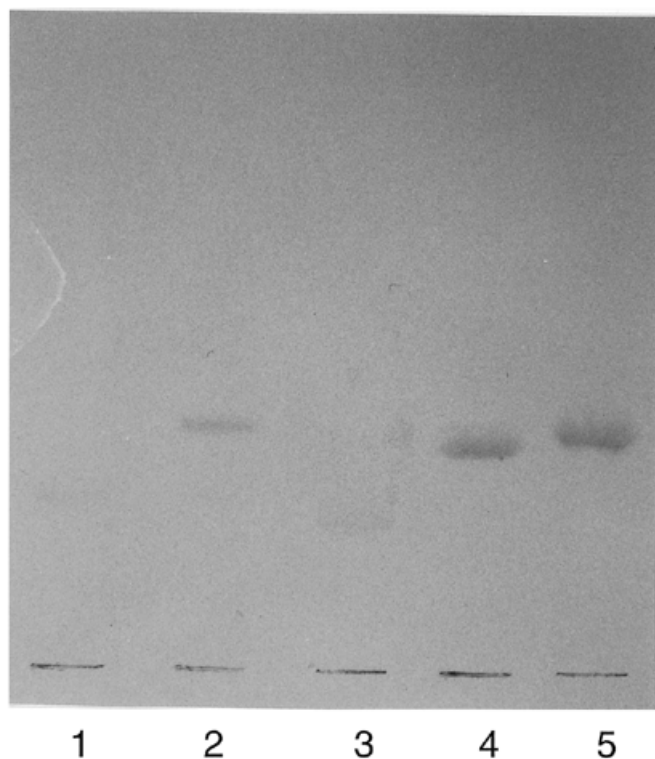


Fig. 4. TLC-IS of the parent Ngs1s A and B and of their β -hexosaminidase digests using anti-GA1 monoclonal antibody. Lane 1, Ngs1 A. Lane 2, Ngs1 A + β -hexosaminidase. Lane 3, Ngs1 B. Lane 4, Ngs1 B + β -hexosaminidase. Lane 5, standard GA1 from bovine brain. The chromatogram was developed in chloroform:methanol:water 60:40:9 (v/v) and stained with anti-GA1 antibody.

IV⁴GalNAcGgOse₄Cer. They constitute ganglio-ganglio series Ngs1s containing a repeating GalNAc β 1-4Gal unit. Both Ngs1s have the same oligosaccharide moiety, as determined by composition and permethylation analyses. Ngs1 A, the minor component of the two Ngs1s, contains d18:0 sphingosine and differs from Ngs1 B, the major component, only in ceramide composition. To the best of our knowledge, the occurrence of IV⁴GalNAcGgOse₄Cer in biological tissues has not been previously reported. Therefore, the structure is considered novel, notwithstanding the report that the core structure was previously prepared artificially by weak acid hydrolysis and/or sialidase treatment of gangliosides containing terminal GalNAc-GA1 (Krivan *et al.*, 1988a; Muthing *et al.*, 1989; Hakomori, 1993).

In order to exclude the possibility that the Ngs1s A and B were generated by the removal of sialic acid from relevant gangliosides (such as GalNAc-GM1, GalNAc-GM1b, etc.) induced by the silicic acid column during the initial purification step, the brain Ngs1s were purified from the total brain lipid extract by a DEAE-Sephadex column. The long-chain Ngs1s fraction was then purified from other neutral lipids, phospholipids, and monoglycosylceramides by a silicic acid column. The purified Ngs1 fraction was examined by ELISA using anti-GalNAc-GA1 antibody along with the purified Ngs1 A/B and a Ngs1 fraction prepared as described above (silicic acid chromatography followed by DEAE-Sephadex). An

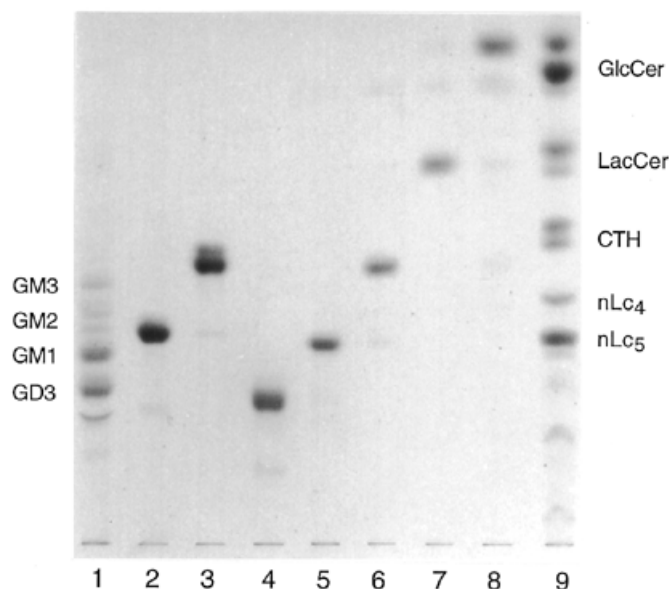


Fig. 5. Thin-layer chromatogram of stepwise specific exoglycosidase digestion of Ngs1 B. Lane 1, standard bovine brain ganglioside mixture. Lane 2, standard GA1; lane 3, standard GA2; lane 4, Ngs1 B; lane 5, lane 4 + β -hexosaminidase; lane 6, lane 5 + β 1-3 galactosidase; lane 7, lane 6 + β -hexosaminidase; lane 8, lane 7 + β 1-4 galactosidase; lane 9, standard Ngs1s mixture from bovine erythrocytes. The bands were resolved in chloroform:methanol:water 60:40:9 (v/v) and visualized by diphenylamine aniline spray.

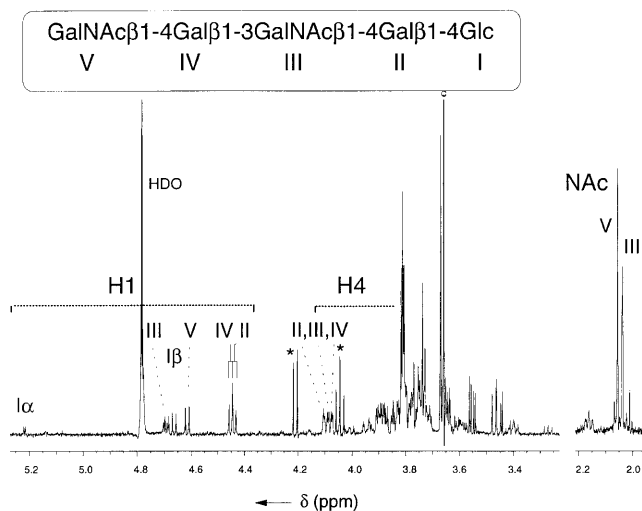


Fig. 6. One-dimensional (1D) ¹H NMR spectrum (D₂O, 600MHz, 23°C) of oligosaccharide GgOse₅ released from Ngs1 B by ceramide glycanase digestion. Roman numerals refer to the glycosyl residues in the structure of GgOse₅ shown in the inset. Signals marked by an asterisk stem from sucrose, present as contaminant in the investigated oligosaccharide sample.

identical antibody titer (1/160) in both Ngs1 preparations suggests that both Ngs1s A and B are naturally occurring components of bovine brain.

Previously, gangliosides containing this GalNAc1-4Gal repeating unit, namely GalNAc-GM1, GalNAc-GM1b,

Table II. ¹H Chemical shifts of the structural-reporter groups of oligosaccharide GgOse₃ derived from Ngsl B (this study), and its predecessors in structural complexity (GgOse₄, GgOse₃, and lactose)

Residue ^a	Reporter group	Chemical shift ^b in ^c			
		Lactose	GgOse ₃	GgOse ₄	GgOse ₅
Glc-Iβ	H1	5.222	5.219	5.220	5.219
Glc-Iβ	H1	4.659	4.665	4.665	4.664
Gal ⁴ -II	H2	3.284	3.272	3.273	3.280
GalNAc ^{4,4} -III	H1	4.447	4.441/4.439 ^d	4.443/4.441 ^d	4.439
Gal ³ -IV	H4	3.923	4.096	4.110	4.104
GalNAc ^{4,3} -V	H1	–	4.638/4.633 ^d	4.706/4.700 ^d	4.697/4.690 ^d
	H4	–	3.912	4.157	4.089
	NAc	–	2.057/2.054 ^d	2.044/2.042 ^d	2.039/2.037 ^d
	H1	–	–	4.449	4.452
	H4	–	–	3.908	4.075
	H1	–	–	–	4.615
	H4	–	–	–	3.904
	NAc	–	–	–	2.056

^aThe numbering of the glycosyl residues in the oligosaccharides is as follows:

A superscript refers to the position of the glycosidic linkage originating from the residue in question; a second superscript refers to the linkage originating from the adjacent residue. All monosaccharides are of the D-configuration.

^bChemical shifts were measured at 500 MHz and 27°C (for reference oligosaccharides) or 600MHz and 23°C (for the GgOse₃ oligosaccharide) in D₂O solutions at pD 6 – 7.

^cData for lactose, GgOse₃ and GgOse₄ are taken from Dorland *et al.*, 1986.

^dSignal is observed doubled in the anomeric intensity ratio ($\alpha:\beta \cong 1:2$).

GalNAc-GD1a, and GalNAc-GD1b, have been characterized as minor brain components. But, so far, their tissue and cell localization have not been determined because specific antibodies are unavailable. This stems from the difficulties of producing monoclonal antibodies against purified gangliosides (Ozawa *et al.*, 1992; Kotani *et al.*, 1994) and the low immunogenicity of gangliosides in rodents (Kotani *et al.*, 1994). Ozawa *et al.* developed a method to prepare anti-ganglioside monoclonal antibodies in C3/HeN mice and studied ganglioside localization in adult rat brain (Kotani *et al.*, 1994), but no ganglioside containing terminal GalNAc was examined.

We prepared a monospecific polyclonal antibody (anti-IgG) to GalNAc-GA1 in rabbit. The substrate affinity purified anti-IgG was specific for GalNAc-GA1 when determined by enzyme-linked immunosorbent assay (ELISA) and thin-layer chromatography immunostaining (TLC-IS); our antibody did not react with a series of Gsls having close structural similarity to GalNAc-GA1 used as comparison (namely GA1, GM1, GalNAc-GM1, etc.).

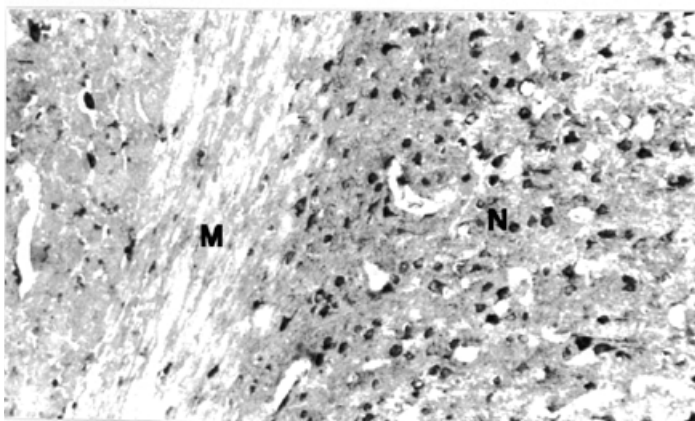
In adult rat CNS, the anti-GalNAc-GA1 antibody stained neurons in cerebral cortex and Purkinje cells, as well as other neuronal perikarya in cerebellum, suggesting that GalNAc-GA1 is neuronal cell specific. This is in marked contrast to GA1 which has been localized specifically in myelin (Dasgupta *et al.*, 1996). Our recent DIG-IS examination of Ngsls in whole brain, spinal cord, white matter and gray matter identifies GalNAc-GA1 in whole brain and gray matter but not in spinal cord or white matter (Dasgupta *et al.*, unpublished observations). Its higher concentration in gray matter than in

whole brain supports our observation and interpretation that GalNAc-GA1 is a neuron-associated Ngsl.

Several N-acetylgalactosaminyltransferases with specificity for glycoprotein and glycolipid substrates have been described previously (Basu *et al.*, 1987), but none of them catalyzes transfer of GalNAc to GA1. The characterization of GalNAc-GA1 in brain suggests that a novel galactosaminyltransferase may exist exclusively in CNS. We are actively pursuing the characterization of this enzyme and are investigating the possibility of its developmental regulation associated with neuronal cell metabolism. It is noteworthy that brains of mice, engineered to lack the β 1–4 galactosaminyl transferases responsible for synthesis of GM2/GD2, are enriched in GM3/GD3 but deficient in complex gangliosides. The brain appeared to develop normally though examined by only simple criteria of histogenesis, nerve conduction velocity, and behavioral study suggesting a compensatory role for GM3/GD3 for complex gangliosides (Takamiya *et al.*, 1996). However, since the GalNAcT specific to the synthesis of GalNAc-GA1 appears to be a different enzyme (Hashimoto *et al.*, 1993; Kanzuya *et al.*, 1994), this interesting observation would not settle the question. At this point, we have shown that GalNAc-GA1, a minor brain Ngsl with a novel carbohydrate sequence, is localized in neuronal cells and may well be a mediator of neuronal development.

An antibody to GalNAc-GD1a has been reported to occur in the serum of patients with neuropathy associated with gammopathy (Ilyas *et al.*, 1988) and in acute demyelinating inflammatory polyradiculopathy (Guillain-Barré disease)

(a) Cerebral cortex



(b) Cerebellum

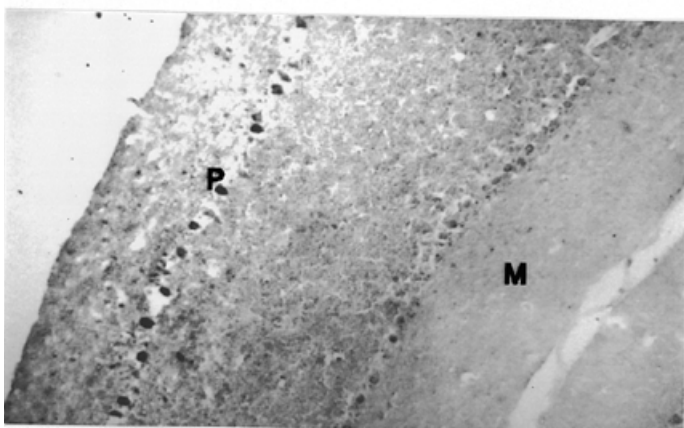


Fig. 7. Immunohistochemical localization of GalNAc-GA1 in adult rat brain: (a) cerebral cortex and (b) cerebellum. The tissues were stained with anti-GalNAc-GA1 antibody as described in the text. Control contains no primary antibody and showed no staining. M, Myelin; P, Purkinje neurons; N, Neurons and interneurons.

(Kusunoki *et al.*, 1994). Bovine brain gangliosides containing terminal GalNAc residue have been reported to be T-cell markers (Muthing *et al.*, 1989), antigens in human neuronal diseases (Kusunoki *et al.*, 1994), and putative receptors for human pathogenic bacteria (Krivan *et al.*, 1988a) which can be identified by TLC-IS using the mouse monoclonal antibody 2D4 (Muthing and Ziehr, 1990; Krivan *et al.*, 1988a). Glycosphingolipids containing GalNAc β 1-4Gal either as a terminal or internal epitope are binding receptors for *P.aeruginosa* and *P.cepacia* isolated from sputum and lung, respectively, of patients with cystic fibrosis (Krivan *et al.*, 1988a). The pulmonary pathogens *Streptococcus pneumoniae* and *Klebsiella pneumoniae*, and certain strains of *Escherichia coli* (VJI and 6883) specifically bind fucosylated-GM1, asialo-GM1 (GA1) and asialo-GM2 (GA2); all containing the GalNAc β 1-4Gal moiety (Krivan *et al.*, 1988a), and with evidence that the minimal binding requirement of these

bacteria is a terminal or internal GalNAc β 1-4Gal sequence unsubstituted with a sialyl residue (Krivan *et al.*, 1988b). In this context, GalNAc-GA1 with a terminal and an internal consecutive repeating GalNAc β 1-4Gal epitope might be a carbohydrate receptor for yet unidentified neuropathogens (microbial, viral, etc.) and is also a candidate antigen for inducing autoimmune pathogenic events and neurological disease. The specificity of the GalNAc-GA1 in neuronal development and disorder is now being examined in our laboratory.

Materials and methods

Bovine brain, collected from a local abattoir, was extracted with acetone and the acetone-dried powder was preserved at -40°C until further use. Rat brain was obtained after sacrifice. Silicic acid was purchased from Sigma Chemical Co. (St. Louis, MO); DEAE-Sephadex A50 from Pharmacia LKB Biotechnology (Uppsala, Sweden); precoated TLC plates were obtained from EM Science (Gibbstown, NJ). Enzymes, β 1-3 galactosidase from bovine testis, was purchased from Boehringer-Mannheim (Indianapolis, IN) and ceramide-glycanase from V-Labs (Covington, LA). β 1-4 Galactosidase and β -hexosaminidase were purified from jack bean meal in our laboratory (Li and Li, 1970; Li *et al.*, 1975). All reagents were of analytical grade.

Purification of neutral glycosphingolipids

Total Ngsl was purified from bovine brain as described previously (Dasgupta *et al.*, 1996). In brief, the lipid extracted from acetone-dried powder was applied to a silicic acid column (1.2 cm \times 30 cm), washed with chloroform:methanol 85:15 (v/v) to remove cerebroside and eluted with tetrahydrofuran:water 7:1 (v/v) (Dasgupta *et al.*, 1994). Ngsls and gangliosides were separated from the dialyzed eluate using a DEAE-Sephadex A50 (acetate form) column. Individual Ngsls were further fractionated on a silicic acid (1 cm \times 40 cm) column using chloroform:methanol:water as the solvent system. Twenty microliter aliquots from alternate fractions (4 ml) were examined by TLC. The fractions containing novel Ngsls were further purified through another silicic acid column (0.8 cm \times 30 cm) using chloroform:methanol: 2.5N ammonia as the eluting solvent. Fractions containing purified Ngsls were pooled, and the purified compounds were found homogeneous when reexamined by TLC using neutral and alkaline solvent systems.

Chemical composition analysis

The carbohydrate composition of Ngsl A and B was determined as alditol acetates (Bjorndal *et al.*, 1967). Carbohydrate linkage positions were determined by methylation analysis by GLC-MS of permethylated alditol acetates (PMAAs). Approximately 50 μ g of each Ngsl was permethylated (Gunnarson, 1987) and acetylated and the resulting PMAAs were separated and analyzed on a DB-1 column by a Hewlett Packard 5890 series gas chromatograph attached to a 5972 mass spectrometer (Dasgupta *et al.*, 1996). The fatty acids and bases of Ngsl A and B were analyzed by GLC as methyl esters and trimethylsilyl derivatives, respectively (Dasgupta *et al.*, 1994).

Stepwise exoglycosidase digestion

Approximately 20 µg of each Ngsl was treated with β-hexosaminidase in 50mM citrate buffer (pH 4.2) containing 0.05% sodium taurodeoxycholate (Dasgupta *et al.*, 1996). The reaction was terminated with 4 volumes of chloroform:methanol (2:1, v/v) and the lower phase was examined by TLC. The product was further treated with β1–3 galactosidase, β-hexosaminidase and β1–4 galactosidase, respectively (Dasgupta *et al.*, 1994).

Immunochemical and chemical composition analysis of the β-hexosaminidase digested Ngsls

The products obtained from Ngsl A and B after enzymatic removal of the terminal hexosamine residue and the parent compounds were resolved on a TLC plate and overlaid with anti-GA1 monoclonal antibody (a generous gift from Dr. P.Fredman, University of Goteborg, Sweden). Excess antibody was removed by thorough washing; the plate was treated with an anti-mouse IgM antibody coupled to peroxidase and the reaction was visualized with 3,3'-diaminobenzidine (Dasgupta *et al.*, 1996). Approximately 20 µg of the hexosaminidase hydrolyzed product was permethylated and the PMAAs were analyzed by GLC-MS as described above.

NMR spectroscopy of the oligosaccharide released from the Ngsl

Ngsl B (100µg) was hydrolyzed with ceramide-glycanase (Zhou *et al.*, 1989) in acetate buffer containing sodium taurodeoxycholate (0.05%). The oligosaccharide, purified by Biogel P2 chromatography (0.8 cm × 40 cm; water as eluent), was repeatedly dissolved in D₂O [Cambridge Isotope Laboratories (Andover, MA); 99.96 atom % D] at room temperature and pD 6.7, with intermediate lyophilization. Prior to NMR spectroscopic analysis the sample was redissolved in 0.5 ml of D₂O and transferred into a 5 mm NMR tube (Wilmad; 535-PP). ¹H NMR spectroscopy was performed on a Bruker AMX-600 spectrometer interfaced with an Aspect-X32 computer. The probe temperature was maintained at 23°C, with experimental details as described previously (Van Halbeek, 1994). One-dimensional (1D) data were processed on an IBM-compatible PC using Felix for Windows version 1.01 (Biosym/MSI, San Diego, CA). Chemical shifts (δ) for the oligosaccharide are expressed in "p.p.m." (parts per million) downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, measured by reference to internal acetate (δ 1.908 at pD 6.7) with an accuracy of 0.002 p.p.m..

Two-dimensional ¹H TOCSY (Braunschweiler and Ernst, 1983; Bax and Davis, 1985) and ROESY (Bothner-By *et al.*, 1984) data sets were collected in phase-sensitive mode. In each of the 2D experiments, 200 FIDs of 2048 data points were acquired. For the TOCSY experiment, 256 scans per *t*₁ increment were collected. The TOCSY pulse program contained a 200 ms MLEV-17 spin-lock pulse (Bax and Davis, 1985). The ROESY experiment used a 189 ms 2 kHz CW spin-lock pulse flanked by two 90° pulses for offset compensation (Griesinger and Ernst, 1987); 512 scans per *t*₁ increment were collected. The 2D data were processed with a Lorentzian-to-Gaussian function applied in the *t*₂ dimension and a shifted squared sine bell function and zero-filling applied in the *t*₁ dimension. Data

were processed in Felix 2.1 on a Silicon Graphics Indy workstation.

Preparation of antibody for immunohistochemical localization

Monospecific polyclonal anti-GalNAc-GA1 antibody was prepared by subcutaneous injection of the emulsified GalNAc-GA1 into New Zealand rabbits using Freund's adjuvant and keyhole limpet hemocyanin (Dasgupta *et al.*, 1996) and purified by substrate affinity chromatography (Nair *et al.*, 1993; Dasgupta *et al.*, 1995b). The purified antibody was highly specific for GalNAc-GA1 by ELISA and TLC-IS when assayed with the substrates GM1, GA1, GalNAc-GA1, and GalNAc-GM1 (the latter compound was received as a gift from Professor S. Sonnino, University of Milan, Italy).

In situ localization in adult rat brain

The immunohistochemical localization of GalNAc-GA1 in rat brain tissue was carried out according to the methods previously utilized to localize glycoconjugates in rodent brain (Nair *et al.*, 1993; Dasgupta *et al.*, 1995b). Briefly, anesthetized rats were perfused intraortically with 2000 U of heparin in 10 ml of phosphate-buffered saline (PBS) followed by 0.1 M phosphate buffer (pH 7.4) containing 2% glutaraldehyde and 2% paraformaldehyde. Parasagittal sections (10 µm) were postfixed with cold acetone (-20°C) for 10 min and stained as described (Nair *et al.*, 1993) using 0.2% Triton X-100 during staining. The fixed sections were washed with PBS, blocked with 1% normal goat serum (NGS) and incubated with the primary antibody (neat) for 24–48 h at 4°C and the antibody binding was visualized by diaminobenzidine staining.

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Abbreviations

BSA, bovine serum albumin; Cer, ceramide; CNS, central nervous system; CS, calf serum; CTH, ceramide trihexoside; DEAE, diethylaminoethyl; DIG-IS, digoxigenin immunostaining; ELISA, enzyme linked immunosorbent assay; FID, free-induction decay; GLC-MS, gas-liquid chromatography coupled with mass spectrometry; Gsl, glycosphingolipid; NGS, normal goat serum; Ngsl, neutral glycosphingolipid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; 1D, one-dimensional; PBS, phosphate-buffered saline; PMAA, partially methylated alditol acetate; p.p.m., parts per million; ROESY, rotating frame NOE spectroscopy; TLC, thin-layer chromatography; TLC-IS, thin-layer chromatography immunostaining; TOCSY, total correlation spectroscopy; 2D, two-dimensional. Other designations: GgOse₃, GalNAcβ1–4Galβ1–4Glc; GgOse₄, Galβ1–3GalNAcβ1–4Galβ1–4Glc; GgOse₅, GalNAcβ1–4Galβ1–3GalNAcβ1–4Galβ1–4Glc; GA1, or GgOse₄Cer, Galβ1–3GalNAcβ1–

4Gal β 1-4Glc β 1-1Cer. Ganglioside and glycosphingolipid short designations (such as GM1, GD1a, nLcOse₅Cer, etc.) are according to Svennerholm, L., *J. Neurochem.*, **10**, 613–623, 1963, and the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Lipid Res.*, **19**, 114–128, 1978. The glycosyl residues in a glycosphingolipid are numbered (by Roman numerals), starting at I for the Cer-linked Glc residue.

References

- Basu, M., De, T., Das, K.K., Kyle, J.W., Chon, H.C., Schaeper, R.J. and Basu, S. (1987) Biosynthesis of gangliosides and blood group glycolipids using solubilized glycosyltransferase. *Methods Enzymol.*, **138**, 575–607.
- Bax, A. and Davis, D.G. (1985) MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *J. Magn. Reson.*, **65**, 355–360.
- Bjorndal, A., Lindberg, B. and Svensson, S. (1967) Gas-liquid chromatography of partially methylated alditols as their acetates. *Acta Chem. Scand.*, **21**, 1801–1804.
- Bothner-By, A.A., Stephens, R.L., Lee, J.-M., Warren, C.D. and Jeanloz, R.W. (1984) Structure determination of a tetrasaccharide: transient nuclear Overhauser effects in the rotating frame. *J. Am. Chem. Soc.*, **106**, 811–813.
- Braunschweiler, L. and Ernst, R.R. (1983) Coherence transfer by isotropic mixing; application to proton correlation spectroscopy. *J. Magn. Reson.*, **53**, 521–528.
- Dasgupta, S. and Hogan, E.L. (1993) Molecular characterization of gangliotetraosylceramide (GA1) in normal human brain and its developmental change. *Ind. J. Biochem. Biophys.*, **30**, 341–345.
- Dasgupta, S., Van Halbeek, H. and Hogan, E.L. (1992) Ganglio-N-tetraosylceramide (GA1) of bovine and human brain: molecular characterization and presence in myelin. *FEBS Lett.*, **301**, 141–144.
- Dasgupta, S., Glushka, J., Van Halbeek, H. and Hogan, E.L. (1994) Branched monosialogangliosides of the lacto-series isolated from bovine erythrocytes: characterization of a novel ganglioside, NeuGc-iso-octaosylceramide. *Arch. Biochem. Biophys.*, **310**, 373–384.
- Dasgupta, S., Cashwell, J.M., Terry, E.C. and Hogan, E.L. (1995a) A novel brain ganglio-ganglioseries neutral glycosphingolipid. *J. Neurochem.*, **64**, S88.
- Dasgupta, S., Hogan, E.L. and Van Halbeek, H. (1995b) Chemical and immunological characterization of galactosyl- β -1-3-globoside in bovine, human and rat brain. *J. Neurochem.*, **65**, 2344–2349.
- Dasgupta, S., Spicer, S.S. and Hogan, E.L. (1996) Stage-specific expression of fuco-neolacto- (Lewis x) and ganglio-series neutral glycosphingolipids during brain development: characterization of Lewis x and related glycosphingolipids in bovine, human and rat brain. *Glycoconj. J.*, **13**, 367–375.
- Dasgupta, S., Everhart, M.B., Bhat, N.R. and Hogan, E.L. (1997) Neutral monoglycosylceramides in rat brain: occurrence, molecular expression and developmental variation. *Dev. Neurosci.*, **19**, 152–161.
- Dorland, L., Van Halbeek, H., Vliegthart, J.F.G., Schauer, R. and Wiegandt, H. (1986) A 500MHz ¹H NMR study of oligosaccharides derived from gangliosides by ozonolysis-alkaline fragmentation. *Carbohydr. Res.*, **151**, 233–245.
- Griesinger, C. and Ernst, R.R. (1987) Frequency offset effects and their elimination in NMR rotating-frame cross-relaxation. *J. Magn. Reson.*, **75**, 261–271.
- Gunnarson, A. (1987) N- and O-alkylation of glycoconjugates and polysaccharides by solid base in dimethyl sulfoxide/alkyl iodide. *Glycoconj. J.*, **4**, 239–245.
- Hakomori, S.-I. (1993) Structure and function of sphingoglycolipids in transmembrane signaling and cell-cell interactions. *Biochem. Soc. Trans.*, **21**, 583–595.
- Hashimoto, Y., Sekine, M., Iwasaki, K. and Suzuki, A. (1993) Purification and characterization of UDP-N-acetylgalactosamine GM3/GD3 N-acetylgalactosaminyltransferase from mouse liver. *J. Biol. Chem.*, **268**, 25857–25864.
- Ilyas, A.A., Li, S.-C., Chou, D.K., Li, Y.-T., Jungalwala, F.B., Dalakas, M. and Quarles, R.H. (1988) Gangliosides GM2, IV⁴GalNAcGM1b, IV⁴GalNAcGD1a as antigens for monoclonal immunoglobulin M in neuropathy associated with gammopathy. *J. Biol. Chem.*, **263**, 4369–4373.
- Kanzuya, I.-P., Hidari, J., Nagai, Y. and Sanai, Y. (1994) A unique biosynthetic pathway for gangliosides exists in *Xenopus laevis* oocytes. *FEBS Lett.*, **353**, 25–28.
- Kotani, M., Kawashima, I., Ozawa, H., Ogura, K., Ishizuka, I., Terashima, T. and Tai, T. (1994) Immunohistochemical localization of minor gangliosides in the rat central nervous system. *Glycobiology*, **4**, 855–865.
- Krivan, H., Ginsburg, V. and Roberts, D.D. (1988a) *Pseudomonas aeruginosa* and *Pseudomonas cepacia* isolated from cystic fibrosis patients bind specifically to gangliotetraosylceramide (asialo-GM1) and gangliotriaosylceramide (asialo-GM2). *Arch. Biochem. Biophys.*, **260**, 493–496.
- Krivan, H.C., Roberts, D.D. and Ginsburg, V. (1988b) Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc β 1-4Gal in some glycolipids. *Proc. Natl Acad. Sci. USA*, **85**, 6157–6161.
- Kusunoki, S., Chiba, A., Kon, K., Arisawa, K., Tate, T. and Kanazawa, I. (1994) N-acetylgalactosaminyl-GD1a is a target molecule for serum antibody in Guillain-Barré syndrome. *Ann. Neurol.*, **35**, 570–575.
- Li, S.-C. and Li, Y.-T. (1970) Studies on glycosidases of jack bean meal. III. Crystallization and properties of β -N-acetylhexosaminidase. *J. Biol. Chem.*, **245**, 8934–8942.
- Li, S.-C., Mazotta, M.Y., Chien, S.F. and Li, Y.-T. (1975) Isolation and characterization of jack bean β -galactosidase. *J. Biol. Chem.*, **250**, 6786–6791.
- Muthing, J., Schwinzler, B., Peter-Katalinic, J., Egge, H. and Muhlrath, P.F. (1989) Gangliosides of murine T lymphocyte subpopulations. *Biochemistry*, **28**, 2923–2929.
- Muthing, J. and Ziehr, H. (1990) Rapid detection of extended series gangliosides with terminal GalNAc β 1-4Gal sequence on high performance thin-layer chromatography plates. *Biomed. Chromatogr.*, **4**, 70–72.
- Nair, S.M., Prasadrao, N., Tobet, S.A. and Jungalwala, F.B. (1993) Rostracaudal expression of antibody HNK-1-reactive glycolipids in mouse cerebellum: relationship to development and compartments and loaner maturation. *J. Comp. Neurol.*, **332**, 282–292.
- Ozawa, H., Kotani, M., Kawashima, I. and Tai, T. (1992) Generation of one set of monoclonal antibodies specific for a pathway of ganglio-series gangliosides. *Biochim. Biophys. Acta*, **1123**, 184–190.
- Poppe, L. and Van Halbeek, H. (1991) The double-DANTE sequence applied to NMR of complex carbohydrates. *Magn. Reson. Chem.*, **29**, 355–361.
- Rokukawa, C., Nakamura, K. and Handa, S. (1988) Glycolipids of mouse erythroleukemia cells (Friend cells) and their alteration during differentiation. *J. Biochem. (Tokyo)*, **103**, 36–42.
- Takamiya, K., Yamamoto, A., Furukawa, K., Yamashiro, S., Shin, M., Okada, M., Fukumoto, S., Haraguchi, M., Takeda, N., Fujimura, K., Sakae, M., Kishikawa, M., Shiku, H., Furukawa, K. and Aizawa, S. (1996) Mice with disrupted GM2/GD2 synthase gene lack complex gangliosides but exhibit only subtle defects in their nervous system. *Proc. Natl Acad. Sci. USA*, **93**, 10662–10667.
- Van Halbeek, H. and Poppe, L. (1992) Conformation and dynamics of glycoprotein oligosaccharides as studied by ¹H-NMR spectroscopy. *Magn. Reson. Chem.*, **30**, S74–S86.
- Van Halbeek, H. (1994) ¹H Nuclear magnetic resonance spectroscopy of carbohydrate chains of glycoproteins. *Methods Enzymol.*, **230**, 132–168.
- Zhou, B., Li, S.-C., Laine, R.A., Huang, R.T.C. and Li, Y.-T. (1989) Isolation and characterization of ceramide glycanase from the leech *Macrobdella decora*. *J. Biol. Chem.*, **264**, 12272–12277.

