Molecular characterization and immunohistochemical localization of IV\(^4\)GalNAcGgOse\(_4\)Cer: a naturally occurring novel neutral glycosphingolipid in bovine brain

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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 (nLcOse\(_5\) Cer 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 \(^1\)H NMR spectroscopy and stepwise exoglycosidase digestion indicated the following pentaglycosyl structure for the oligosaccharide moiety of both Ngsls: GalNAc\(_\beta^1\)–4Gal\(_\beta^1\)–3GalNAc\(_\beta^1\)–4Gal\(_\beta^1\)–4Glc. The two Ngsls (abbreviated as IV\(^4\)GalNAcGgOse\(_4\) Cer or GalNAc-GA1), differ in their ceramide compositions, having d18:0 and d18:1 sphingosine as their long chain bases. A monoclonal 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 GgOse\(_4\) Cer) 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), GalGbOse\(_4\)Cer (Dasgupta et al., 1995b), and FucnLcOse\(_4\)Cer (Dasgupta et al., 1996). The topography of GA1 and FucnLcOse\(_4\)Cer 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 Rf than a standard pentaglycosylceramide (nLcOse\(_5\) Cer) from bovine erythrocytes (Figure 1). Ngsl A has a higher Rf 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 Rf 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,
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 was observed (Table I).

Methylation analysis of both Ngsls indicated a terminal GalNAc residue (H1 at 4.615 p.p.m., J12 8.5Hz, H4 at 3.904 p.p.m., indicative of the presence of an amino group at C2). The Gal residues were distinguished from the GalNAc (H1 at 4.615 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 β1–4-linked terminal GalNAc residue in GgOse5 (Table II) suggested as a working model for the structure of GgOse5 an extension of the β-galactosidase-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 β1–3 galactosidase, jack bean meal β-hexosaminidase and β1–4 galactosidase, yielding GlcCer as the end product (Figure 5).

TheGalNAc-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 GgOse5 and GgOse6 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 β1-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 β1–4 linked terminal GalNAc residue in GgOse5 (Table II) suggested as a working model for the structure of GgOse5 an extension of GgOse5 with a terminal β1–4 linked GalNAc residue:

GalNAcβ1–4Galβ1–3GalNAcβ1–4Galβ1–4Glc

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 GalNAc 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 J3 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 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 the...
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 (α:β ≅ 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 anomerization effect as well (δ 4.697 and 4.690, in ratio ~ 1:2). The aforementioned observations may prove useful in future conformational studies of GgOse₅.
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 (nLcOse$_5$Cer 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 immunohistochemically as

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**Peak No.** | **E.I.-M.S. fragment ions** | **Linkage** | **RT** | **Nature of carbohydrate**
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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

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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.
IV$_4$GalNAcGgOse$_4$Cer. They constitute ganglio-ganglio series Ngsls containing a repeating GalNAc$\beta$$^1–4$Gal unit. Both Ngsls have the same oligosaccharide moiety, as determined by composition and permethylation analyses. Ngsl A, the minor component of the two Ngsls, contains d18:0 sphingosine and differs from Ngsl B, the major component, only in ceramide composition. To the best of our knowledge, the occurrence of IV$_4$GalNAcGgOse$_4$Cer 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 Ngsls 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 Ngsls were purified from the total brain lipid extract by a DEAE-Sephadex column. The long-chain Ngsls fraction was then purified from other neutral lipids, phospholipids, and monoglycosylceramides by a silicic acid column. The purified Ngsl fraction was examined by ELISA using anti-GalNAc-GA1 antibody along with the purified Ngsl A/B and a Ngsl fraction prepared as described above (silicic acid chromatography followed by DEAE-Sephadex). An identical antibody titer (1/160) in both Ngsl preparations suggests that both Ngsls A and B are naturally occurring components of bovine brain.

Previously, gangliosides containing this GalNAc1-4Gal repeating unit, namely GalNAc-GM1, GalNAc-GM1b,
GalNAc-GD1α, and GalNAc-GD1β, 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 β1–4 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-GD1α 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)
Characterization and localization of IV4GalNAcGgOse4Cer (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 GalNAcβ1-4Gal 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 (V71 and 6883) specifically bind fucosylated-GM1, asialo-GM1 (GA1) and asialo-GM2 (GA2); all containing the GalNAcβ1-4Gal moiety (Krivan et al., 1988a), and with evidence that the minimal binding requirement of these bacteria is a terminal or internal GalNAcβ1-4Gal sequence unsubstituted with a sialyl residue (Krivan et al., 1988b). In this context, GalNAc-GA1 with a terminal and an internal consecutive repeating GalNAcβ1-4Gal 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, β1–3 galactosidase from bovine testis, was purchased from Boehringer-Mannheim (Indianapolis, IN) and ceramide-glycanase from V-Labs (Covington, LA). β1–4 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 × 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 × 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 × 40 cm) using chloroform:methanol:water as the eluting solvent. 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 × 40 cm) using chloroform:methanol:water 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 µg 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).

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**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 Gothenburg, 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 pH 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 pH 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 τ1 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 τ1 increment were collected. The 2D data were processed with a Lorentzian-to-Gaussian function applied in the τ2 dimension and a shifted squared sine bell function and zero-filling applied in the τ1 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-WS 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 intraorbitally 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 dianaminobenzidine 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; CNS, 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; TOCSY, total correlation spectroscopy; 2D, two-dimensional. Other designations: GaOse, GalNAcβ1–4Galβ1–4Glc; GaOse7, Galβ1–3GalNAcβ1–4Galβ1–4Glc; GaOse7, GalNAcβ1–4Galβ1–3GalNAcβ1–4Galβ1–4Glc; GA1, or GaOse7Cer, Galβ1–3GalNAcβ1–4Galβ1–4Glc;
4Galβ1→4Glcβ1→1Cer. Ganglioside and glycosphingolipid short designations (such as GM1, GD1a, NLCosE,Cer, 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