Branched Sialylated N-glycans Are Accumulated in Brain Synaptosomes and Interact with Siglec-H

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ABSTRACT. Proper N-glycosylation of proteins is important for normal brain development and nervous system function. Identification of the localization, carrier proteins and interacting partners of N-glycans is essential for understanding the roles of glycoproteins. The present study examined the N-glycan A2G'2F (Galβ1-3GlcNAcβ1-2Manα1-6[Galβ1-3GlcNAcβ1-2Manα1-3]Manβ1-4GlcNAcβ1-4[Fucα1-6]GlcNAc-). A2G'2F has a branched sialic acid structural feature, and branched sialylated A2G'2F is a major N-glycan in the mouse brain. Its expression in the mouse brain increases during development, suggesting that branched sialylated N-glycans play essential roles during brain development. However, the carrier proteins, interacting partners and localization of branched sialylated N-glycans remain unknown. We previously improved our method for analyzing N-glycans from trace samples, and here we succeeded in detecting A2G'2F in small fragments excised from the two-dimensional electrophoresis gels of subcellular fractionated mouse brain proteins. A2G'2F was accumulated in mouse brain synaptosomes. We identified calreticulin as one of the candidate A2G'2F carriers and found calreticulin expression in both the endoplasmic reticulum and synaptosomal fractions. Calreticulin was observed in dendritic spines of cultured cortical neurons. Synthesized branched sialylated glycan clusters interacted with sialic acid-binding immunoglobulin-like lectin H (Siglec-H), which is known to be a microglia-specific molecule. Taken together, these results suggest that branched sialylated A2G'2F in synaptosomes plays a role in the interaction of dendritic spines with microglia.

Key words: N-glycan, subcellular fractionation, calreticulin, dendritic spine, Siglec-H

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Abbreviations: AU, arbitrary unit; CBB, Coomassie Brilliant Blue; DIV, days in vitro; DTT, dithiothreitol; ER, endoplasmic reticulum; Fuc, fucose; Gal, galactose; GFP, green fluorescent protein; GlcNAc, N-acetylglucosamine; HPLC, high performance liquid chromatography; IEF, isoelectric focusing; LAMP, limbic system-associated membrane protein; LC-MS, liquid chromatography-mass spectrometry; LeC, LewisC; Man, mannose; NeuAc, N-acetylneuraminic acid; NP, normal-phase; PA, pyridylaminated; PBS, phosphate-buffered saline; PSD, postsynaptic density; PVDF, polyvinylidene difluoride; RP, reverse-phase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Siglec, sialic acid-binding immunoglobulin-like lectin; SPR, surface plasmon resonance; TIR, transferrin receptor; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; 6SLeC, 6-sialyl-LewisC; 12w, 12-week-old.
Introduction

Glycosylation is a major posttranslational modification of proteins. In N-glycosylation the asparagine residues of proteins within an Asn-X-Ser/Thr sequence are glycosylated. N-glycans harbored on membrane proteins (glycoproteins) profoundly affect the character of these proteins by altering their structure or capacity to bind to other molecules. Abnormalities in brain development and multiple dysfunctions of the nervous system are noteworthy phenotypes of congenital disorders of glycosylation (Cylwik et al., 2013; Freeze and Aebi, 2005), which underscore the importance of N-glycans in the nervous system. Thus, the identification of N-glycans harbored on proteins is an important step in understanding protein function. Recent advances in the methodologies for determining the N-glycan structure of proteins have enabled the use of very small amounts of glycoproteins for this analysis. However, methods for identifying proteins carrying a specific N-glycan of interest have yet to be established.

We previously reported the identification of various sialylated and non-sialylated N-glycan structures in the mouse cerebral cortex (Ishii et al., 2007; Torii et al., 2014). Among the identified N-glycans, sialylated A2G'2F (Fig. 1A) is a major N-glycan which contains a type 1 antennary structure (Gal[β1-3GlcNAc-]). There are two types of α2-6-sialylated N-glycans; one attaches sialic acid to the galactose residue at the non-reducing end (Neu5Acα2-6Gal-) and the other to the GlcNAc residue in the type 1 antennary structure of N-glycans (Gal[β1-3][Neu5Acα2-6]GlcNAc-). We termed the latter branched structure (Gal[β1-3][Neu5Acα2-6]GlcNAc-) “6-sialyl-LewisC (6SLeC)”. The amount of branched sialylated A2G'2F containing 6SLeC increases during development (Ishii et al., 2007; Torii et al., 2014), suggesting that 6SLeC-containing branched sialylated A2G'2F plays a role in the development and/or maintenance of the mouse brain. However, the function of branched sialylated A2G'2F, together with its carrier protein(s), is unknown. The interacting partners of branched sialylated A2G'2F are also unknown.

To address these questions, in the present study we sought to identify branched sialylated A2G'2F carrier proteins. Carrier protein(s) for the N-glycan of interest can be identified by liquid chromatography-mass spectrometry (LC-MS) after protease treatment of all glycoproteins. However, A2G'2F (an isomer with the same molecular mass as A2G'2F and containing a type 2 antennary structure [Gal[β1-4GlcNAc-]) is abundant in the brain, so it was not appropriate to apply this standard method for identifying A2G'2F carrier proteins in the current study. We previously improved the method for analyzing N-glycans on glycoproteins from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels using high performance liquid chromatography (HPLC) after fluorescent labeling of glycoproteins (Yoshimura et al., 2012). An advantage of this method is that it is possible to separate A2G'2F from A2G'2F. In the current study we applied this method to smaller samples, such as protein spots from two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) gels of the synaptosomal fraction of mouse brain, and identified a limited number of acidic proteins with a molecular mass of 50–70 kDa that mainly carry A2G'2F. We also performed LC-MS and identified calreticulin as a candidate A2G'2F carrier protein. Furthermore, our results suggest that branched sialylated A2G'2F on calreticulin is expressed in dendritic spines and interacts with microglial sialic acid-binding immunoglobulin-like lectin H (Siglec-H). Branched sialylated A2G'2F may play a role in the interaction between synapses and microglia.

Materials and Methods

Materials

Unless otherwise noted, reagents were purchased from Wako (Osaka, Japan), Tokyo Chemical Industry (Tokyo, Japan) or Kanto Chemical (Tokyo, Japan).

Antibodies

The following primary antibodies were used in this study: rabbit anti-calreticulin IgG (ab92516, Abcam plc, Cambridge, UK), mouse anti-postsynaptic density protein (PSD)-95 IgG (7E3-1B8, Thermo Fisher Scientific Inc., Waltham, MA, USA), mouse anti-GM130 IgG (610822, BD Biosciences, Franklin Lakes, NJ, USA) and mouse anti-transferrin receptor (TfR) IgG (136800, Thermo Fisher Scientific Inc.). For immunocytocchemistry, Alexa 568-conjugated anti-rabbit IgG (Thermo Fisher Scientific Inc.) and Cy5-conjugated anti-mouse IgG (Merck Millipore, Billerica, MA, USA) were used as secondary antibodies. Anti-Siglec-H antibody (16-0333-82, eBioscience, Thermo Fisher Scientific Inc.) and iso-type control antibody (rat IgG2b, 16-4031-85, eBioscience, Thermo Fisher Scientific Inc.) were used for surface plasmon resonance (SPR) analysis.

Animals

All experimental procedures were approved by the Animal Care and Use Committee of the National Institute for Physiological Sciences, and were conducted in accordance with the Guidelines and Regulations for the Care and Use of Experimental Animals provided by the National Institute for Physiological Sciences. Timed pregnant or aged ICR mice were purchased from Japan SLIC, Inc. (Hamamatsu, Japan).

Sample preparation for SDS-PAGE and 2D-PAGE

A twelve-week-old (12w) ICR mouse was sacrificed, and the whole brain was quickly removed and washed with ice-cold
phosphate-buffered saline (PBS, pH 7.4). The tissue was homogenized in a twenty-fold volume of homogenate buffer A (20 mM Tris-HCl [pH 8.0], 2 mM EDTA and 0.32 M sucrose) using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 700 g for 10 min, and the supernatant was collected. The supernatant was then centrifuged at 20,000 g for 1 h, and the supernatant was removed by suction. The pellet was suspended in 1 ml of lysis solution B containing 9.8 M urea, 0.5 mM EDTA, 2% (w/v) NP-40 (Sigma, St. Louis, MO, USA), 100 mM dithiothreitol (DTT), 0.5% (v/v) IPG buffer pH 3-11 NL (GE Healthcare, Chicago, IL, USA) and 0.001% (w/v) bromophenol blue, and incubated for 2 h at room temperature with stirring.

Fig. 1. SDS-PAGE analysis of A2G'2F carrier proteins in the mouse brain. (A) Structure of tetra-sialylated A2G'2F. Desialylated A2G'2F (dotted box) is a major N-glycan, containing a type 1 antennary structure (Galβ1-3GlcNAc-). In the mouse brain, most A2G'2F is sialylated. Terminal Gal and GlcNAc residues of the type 1 antennary structure can be sialylated by sialyltransferases. Expression levels of A2G'2F and 6-sialyl-LewisC (6SLeC, [Galβ1-3 Neu5Acα2-6]GlcNAc-): shown boxed) increase during mouse brain development. The structure is shown as the pyridylaminated (PA-) form. Fuc, fucose; Gal, galactose; GlcNAc, N-acetylglucosamine; Man, mannose; NeuAc, N-acetylneuraminic acid. (B) Proteins in mouse brain homogenate (700 μg) were subjected to SDS-PAGE. The gel was cut into 20 pieces, and N-glycans were purified from each gel piece. (C) Purified N-glycans were subjected to NP HPLC to purify A2G'2F. A representative chromatogram of gel #7 is shown. The fraction containing A2G'2F (Fra. a) was collected. The bar under Fra. a indicates the fraction collected. (D) Fra. a was subjected to RP HPLC to isolate and quantify A2G'2F. The black line indicates the chromatogram of the A2G'2F standard. Co-elution of the A2G'2F standard with the Fra. a peak (arrow) reveals that this is the A2G'2F peak. (E–G) The peak area of N-glycans in each gel piece was calculated. N-glycans were detected in all gel pieces and the total area of N-glycans was measured using the results from the NP HPLC (E). A2G'2F was abundant in gels #7 and 8, as detected by RP HPLC (F). The ratio of A2G'2F to total N-glycans was calculated (G). The ratio of A2G'2F to total N-glycans was highest in gels #7 and 8.
**Subcellular fractionation**

This experiment was performed entirely on ice, and was performed as described previously (Carlin et al., 1980). In brief, five 12w ICR mouse brains were homogenized in a four-fold volume of homogenate buffer C (10 mM Hepes-NaOH [pH 7.4] and 0.32 M sucrose containing 0.2 mg/ml phenylmethylsulfonyl fluoride). The homogenate was centrifuged for 10 min at 1,000 g to remove the crude nuclear fraction (P1). The supernatant (S1) was centrifuged at 9,000 g for 15 min to obtain a pellet (P2) and supernatant (S2). The S2 fraction was centrifuged at 100,000 g for 1 h to obtain a pellet (P3) and supernatant (S3). The P2 fraction was resuspended in homogenate buffer C. Discontinuous sucrose gradients containing 6 ml of the resuspended P2 material and 6 ml each of 0.8, 1.0 and 1.2 M sucrose solutions in 10 mM Hepes-NaOH (pH 7.4) were centrifuged at 58,000 g for 2 h. The band between 1.0 and 1.2 M sucrose was collected as the synaptosomal fraction. This synaptosomal fraction was extracted with 0.5% (v/v) Triton X-100 in 0.16 M sucrose and 6 mM Tris-HCl (pH 8.0), then centrifuged at 33,800 g for 20 min to separate it into soluble (Triton-soluble) and insoluble fractions (PSD). Proteins of each fraction were used for 2D-PAGE, N-glycan analysis and western blot analysis.

**SDS-PAGE and western blotting**

Equal volumes of the harvested proteins were subjected to SDS-PAGE using 10% polyacrylamide gel and separated electrophoretically with a Tris-glycine buffer (25 mM Tris, 0.192 M glycine, 0.1% SDS). Gels were then subjected to Coomassie Brilliant Blue (CBB) staining or immunoblotting. For immunoblotting, separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P 0.45 μm, Merck Millipore) with a semi-dry blotting apparatus (ATTO Corporation, Tokyo, Japan) at 240 mA for 2 h at 4°C using buffer containing 192 mM glycine, 100 mM Tris and 5% methanol. In some experiments, the protein spots on the 2D-PAGE gels were also electro-blotted onto a PVDF membrane as described above. The membrane was then blocked with 0.5% (w/v) skim milk in PBS containing 0.1% (w/v) Tween-20 for 1 h at room temperature. The membrane was incubated with primary antibodies, calreticulin (1:500), PSD-95 (1:1000), GM130 (1:200) or TIR (1:1000), overnight at 4°C. The membrane was then treated with HRP-conjugated anti-mouse or rabbit IgG for 1 h at room temperature. Immunoreactive spots were detected using an enhanced chemiluminescence detection kit (ECL plus, GE Healthcare).

**Treatment of the synaptosomal fraction for 2D-PAGE**

The synaptosomal fraction was treated as described previously (Ishioka et al., 1990; Nakamura et al., 2005) for 2D-PAGE. Briefly, the synaptosomal fraction obtained from subcellular fractionation was resuspended in a nine-fold volume of 10 mM Hepes-NaOH (pH 7.4) and centrifuged at 58,000 g for 2 h. The supernatant was removed by suctioning, then the pellet was suspended in an organic solvent (methanol:chloroform, 2:1) and the suspension was centrifuged at 10,000 g for 20 min. This procedure was repeated three times. After delipidation, the pellet was washed again with 3 ml of methanol followed by washing with 3 ml of 50 mM phosphate buffer. Finally, the pellet was resuspended in 1 ml of lysis solution D containing 5 M urea, 2 M thiourea, 2% (w/v) 3-(3-cholamidopropyl) dimethylammonium)-1-propanesulfonate (Dojindo, Kamimashiki-gun, Kumamoto, Japan), 2% (w/v) sulfobetaine-10 (Amresco LLC, Solon, OH, USA), 2% (v/v) IPG buffer pH 3-11 NL, 60 mM DTT and 0.0025% (w/v) Orange G, and incubated for 1–2 h at room temperature with stirring. A total of 700 μg of protein was used for 2D-PAGE.

**2D-PAGE**

Mouse brain homogenate and synaptosomal fractions were prepared for 2D-PAGE as described previously (Ishioka et al., 1990; Nakamura et al., 2005). The isoelectric focusing (IEF, first dimension) was carried out on nonlinear immobilized pH gradients (pH 3–11). The IEF was performed at 20°C using Ettan IPGphor II (GE Healthcare) with the following two voltage programs: 500 V for 4 h, 1000 V for 1 h, 8000 V for 6 h (program E, for brain homogenate) or 500 V for 2 h, 700 V for 1 h, 1000 V for 1 h, 1500 V for 1 h, 2000 V for 1 h, 2500 V for 1 h, 3000 V for 1 h, 3500 V for 18 h (program F, for synaptosomes). After completion of electrofocusing, IPG strips were equilibrated for 30–45 min in 6 M urea, 30% glycerol, 2% (w/v) SDS, 25 mM Tris-HCl (pH 6.8) and 0.325 M DTT. IPG strips were then treated with 30% glycerol, 2% (w/v) SDS, 25 mM Tris-HCl (pH 6.8) and 4.5% (w/v) iodoacetamide. In the two-dimensional SDS-PAGE, the equilibrated IPG strip was placed on top of the 10% polyacrylamide gel and proteins on the strip were separated electrophoretically. Gels were then subjected to CBB staining and immunoblotting.

**Liquid chromatography–mass spectrometry (LC-MS)**

Protein spots stained by CBB on the 2D-PAGE gel were excised with a razor. Excess CBB dye was removed from the gel pieces by immersing in 50% (v/v) acetonitrile and 25 mM ammonium bicarbonate, and the gel pieces were further dehydrated in absolute acetonitrile, then dried. Gel pieces were then treated with reducing solution (10 mM DTT, 25 mM ammonium bicarbonate) and alkylating solution (1% [w/v] iodoacetamide, 25 mM ammonium bicarbonate), and finally dehydrated in absolute acetonitrile, followed by drying. Proteins in the gel pieces were digested with 10 μg/ml trypsin (Promega Corporation, Madison, WI, USA) and 50 mM ammonium bicarbonate overnight at 37°C. After digestion, peptide fragments were extracted from the gel pieces in 5% (v/v) trifluoroacetic acid and 50 mM ammonium bicarbonate. Peptide fragments in the supernatants were subjected to LC-MS. To analyze proteolytic peptides in gels i–iii, they were separated using a packed nano-capillary column (NTCC-360/75-3, Nikkyo Technos Co. Ltd., Tokyo, Japan) with the EASY-nLC system (pump G with 0.1% formic acid and pump H with 0.1% formic acid in ace-
tonitrile, Thermo Fisher Scientific Inc.). For analysis of peptides, separation was performed with a linear gradient from 0 to 80% (pump H) over 12 min at a flow rate of 0.3 μl/min, and mass spectra were recorded with the Orbitrap Elite system (Thermo Fisher Scientific Inc.) using a scan event (m/z 350–2000). LC-MS was performed using a capillary voltage of 1.7 kV and a capillary temperature of 250°C. LC-MS data were analyzed using the Proteome Discoverer software (Thermo Fisher Scientific Inc.).

**Sample preparation for N-glycan analysis of subcellular fractionated proteins**

N-glycans were obtained from proteins of 12w ICR mouse brains by hydrazinolysis as described previously (Torii et al., 2014; Yoshimura et al., 2012, 2017). P2, P3 and synaptosomal fractions obtained from subcellular fractionation were suspended in a three-fold volume of organic solvent (methanol:chloroform, 2:1) to remove lipids, and the suspension was centrifuged at 10,000 g for 20 min. This procedure was repeated three times. After delipidation, the pellet was washed again with 3 ml of methanol. After removing the supernatant, the pellet was dried *in vacuo* before use. Samples (2 mg) were heated with 200 μl of anhydrous hydrazine at 100°C for 10 h in a water bath.

**N-glycan analysis of glycoproteins from SDS-PAGE and 2D-PAGE gels**

In-gel hydrazinolysis was performed as described previously (Yoshimura et al., 2012). Samples were subjected to SDS-PAGE or 2D-PAGE followed by CBB staining. Target protein bands or spots were excised from gels, and the excised gels were cut into small (4-mm height × 4-mm width × 2-mm depth) pieces. Gel pieces were transferred to 5-ml glass tubes with screw caps and washed with 1 ml of ultrapure water. The water was then removed by suctioning, and the gels were treated with 400 μl of 100% methanol for 5 min. Methanol was then removed by suctioning and the gels were lyophitized. Anhydrous hydrazine (300 μl) was added to the glass tubes containing the gels, which were then heated at 100°C for 10 h in a water bath. The hydrazine solution was mixed with 1 ml of 10 mM ammonium bicarbonate buffer, and sugar chains were extracted by sonication for 10 min. This step was repeated three times for efficient sugar chain extraction. N-glycan purification and in-column N-acetylation were performed as described previously (Tanabe and Ikenaka, 2006). The reducing ends of the liberated glycans were tagged with 2-aminopyridine as described previously (Hase et al., 1978, 1981; Natsuka and Hase, 1998). Excess reagents were removed using a cellulose column according to the manufacturer’s instructions, with minor modifications as described previously (Yoshimura et al., 2012).

**Neuraminidase treatment and collection of neutral N-glycans**

Desialylation of N-glycans was performed as described previously (Torii et al., 2014; Yoshimura et al., 2012, 2017). Purified pyridyldimaminodihydrazide derived from Arthrobacter ureafaciens (Nacalai Tesque, Kyoto, Japan) at 37°C for 14 h to cleave sialic acids, followed by heating at 100°C for 5 min and filtering through a 0.20 μm spin filter (Ultrafree-MC LG, Merck Millipore). To separate neutral N-glycans from acidic ones, PA-N-glycans were passed through an anion exchange DEAE column (TSKgel DEAE-5PW, Tosoh, Tokyo, Japan) using HPLC or a Microgranular DE52-packed column (Whatman, GE Healthcare). Water adjusted to pH 9.0 with ammonia was used as a mobile solvent. Neutral N-glycans were collected in the non-adsorbed fraction.

**N-glycan analysis by HPLC**

PA-N-glycans of varying sizes were separated by HPLC using a normal-phase (NP) column (Shodex Asahipak NH2P-50 4E, 4.6 × 250 mm, Showa Denko K.K., Tokyo, Japan) at a flow rate of 0.6 ml/min at 30°C. The mobile phase consisted of solvent I (93% acetonitrile and 0.3% acetic acid titrated to pH 7.0 with 1 M aqueous ammonia) and solvent J (20% acetonitrile and 0.3% acetic acid titrated to pH 7.0 with 1 M aqueous ammonia). The column was equilibrated with mixtures of solvent I and solvent J (80:20) that were linearly increased to 49% in 240 min and then to 90% in 7 min. NP HPLC was performed using the Prominence (Shimadzu, Kyoto, Japan) or Gilson (Middleton, WI, USA) HPLC system equipped with a fluorescence detector (excitation and emission wavelengths were 310 and 380 nm, respectively). Each PA-N-glycan detected was further analyzed by reverse-phase (RP) HPLC. RP HPLC was performed on the AQUITY UPLC system (Waters, Milford, MA, USA) using the ACQUITY UPLC BEH C18 1.7 μm column (2.1 × 50 mm, Waters) at a flow rate of 0.3 ml/min at 35°C. Solvent K consisted of 5 mM ammonium acetate buffer (pH 4.0), and solvent L consisted of solvent K containing 10% acetonitrile. The column was equilibrated with a mixture of solvent K and solvent L (initially 5% L) that was increased linearly to 33% in 11 min and then to 70% in 1 min. PA-sugar chains were detected at excitation and emission wavelengths of 320 and 400 nm, respectively.

**Data quantification and analysis**

NP HPLC chromatogram data were analyzed with LC station software (Shimadzu) and Unipoint software (Gilson). RP HPLC chromatogram data were analyzed with Empower2 software (Waters).

**Immunocytochemistry of cortical neuron cultures and quantification of spine number**

Cultured cortical neurons (0.5 × 10⁶ cells/cm²) from embryonic day 16 mouse brain were seeded onto 12-mm cover slips in 4-well dishes. Neurons (12 DIV) were transfected with N2-EGFP vector using Lipofectamine 2000 (Thermo Fisher Scientific Inc.). Five days after transfection, neurons were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at room temperature for 10 min, and
treated with –20°C methanol for 10 min. The neurons were then blocked with PBS containing 10 mg/ml bovine serum albumin for 1 h at room temperature. The neurons were incubated with primary antibodies against PSD-95 (1:100) and calreticulin (1:100) overnight at 4°C. Alexa 568-conjugated anti-rabbit IgG and Cy5-conjugated anti-mouse IgG were used as secondary antibodies. Samples were visualized using a confocal laser scanning microscopy system (TCS SP5 II; Leica Microsystems GmbH, Wetzlar, Germany). We randomly chose sixteen green fluorescent protein (GFP)-expressing neurons from eight separate cultures and obtained an image of the dendrites from each neuron using LAS AF software (Leica Microsystems). To quantify the number of spines labeled with anti-PSD-95 antibody and/or anti-calreticulin antibody (the threshold for calreticulin was set at 300 fluorescence intensity) was counted. Finally, the ratio of PSD-95-positive spines to PSD-95-positive spines was calculated. Box-and-whisker plots were created to show the median and 25th and 75th percentiles.

**Purification of Siglec-H-Fc protein**

COS-7 cells were transformed with pFUSE-hIgG2-Fc1 (Invivo- gen, San Diego, CA, USA) subcloned with partial cDNA encoding the extracellular domain of mouse Siglec-H or pFUSE-hIgG2-Fc2 (Invivo- gen), and stable transformants were obtained by Zeocin resistance. After the transformants were cultured in Opti-MEM I reduced serum medium (Thermo Fisher Scientific Inc.) for 7 days, conditioned media were collected, concentrated with an ultrafiltration column (Amicon Ultra-15 10 kDa, Merck Milli- pore) and then loaded onto a HiTrap Protein A HP Column (GE Healthcare). Siglec-H-Fc or Fc protein was eluted by 0.1 M glycine-HCl (pH 2.9). The eluates were neutralized with 1.5 M Tris-HCl (pH 8.8), dialyzed against PBS overnight at 4°C, and stored at –80°C until use.

**Surface plasmon resonance (SPR) analysis**

SPR was measured by Biacore 3000 or Biacore X100 (GE Health- care) at 25°C. Fc and Siglec-H-Fc recombinant proteins were diluted to 1–2 μM with 10 mM sodium acetate (pH 4.5) and immobilized to the flow cells of a CM5 sensor chip (GE Healthcare) using an amine-coupling kit (GE Healthcare) at a flow rate of 10 μl/min for 7 min. The immobilization levels were 3666–4944 and 13370–17604 resonance units for Fc and Siglec-H-Fc, respectively. The immobilization was confirmed by injecting 0.2 μM of rat monoclonal anti-Siglec-H antibody or isotype control antibody. Various concentrations of 6SLeC cluster and control N-glycan clusters in 10 mM Hepes-NaOH (pH 7.4) containing 150 mM NaCl were injected for 1 or 2 min and then dissociation was analyzed for 1 or 2 min at a flow rate of 10 μl/min. Flow cells were regenerated with 10 mM glycine-HCl (pH 3.0) after each measurement. The sensorgrams were generated by subtraction of the Fc-immobilized flow cell from the Siglec-H-Fc-immobilized flow cell.

**Results**

**A2G’2F carriers are 50–70 kDa acidic proteins**

Almost all A2G’2F N-glycans are sialylated, and branched sialylated A2G’2F is the major type 1 N-glycan in the mouse brain (Torigi et al., 2014) (Fig. 1A). A2G’2F has 4 sialylation sites and we detected all possible sialylated forms of A2G’2F in the mouse brain (Supplementary Fig. 1). In theory, this could generate nearly 20 sialylated forms; thus it is difficult to detect sialylated A2G’2Fs on isolated proteins. Therefore, we opted to identify A2G’2F carriers after removing the sialic acids from sialylated A2G’2F and detected desialylated A2G’2F on isolated proteins. We first examined whether desialylated A2G’2F was carried on various proteins. Twelve-week-old (12w) mouse brain was homogenized, and 700 μg of protein was separated by SDS-PAGE. The gel was stained with CBB to detect proteins and cut into 20 pieces (Fig. 1B). N-glycans were purified from each gel piece by direct hydrazinolysis (Yoshimura et al., 2012), and these N-glycans were then treated with neuraminidase to remove the sialic acids. Neutral N-glycans were subjected to NP HPLC, and the A2G’2F-containing fraction (Fra. a in Fig. 1C) was collected. Fra. a was then subjected to RP HPLC, and A2G’2F was detected (Fig. 1D). All gel pieces were analyzed in the same way. N-glycans were present in all gel pieces (Fig. 1E), whereas A2G’2F was detected only in gels #1 to 9 (Fig. 1F). Gels #7 and 8 contained a high ratio of A2G’2F to total amount of N-glycans (Fig. 1G). These results indicate that the majority of A2G’2F carriers are 50–70 kDa proteins.

To further purify A2G’2F carriers, the same mouse brain homogenate sample was subjected to 2D-PAGE. Many protein spots were detected by CBB staining and the 50–70 kDa protein spots were separated into 10 areas (Fig. 2A). The N-glycans were purified and analyzed. A2G’2F was detected only in gel b, and was not detected in other gel pieces, even in the adjacent gels a and c (Fig. 2B–D). These results indicate that A2G’2F is carried on a restricted number of proteins. It is reasonable that A2G’2F carriers are acidic proteins because most A2G’2F is sialylated.

**A2G’2F and calreticulin are accumulated in mouse brain synaptosomes**

To study A2G’2F localization in the mouse brain, subcellular fractionation of 12w mouse brain was performed (Fig. 3A). N-glycans were purified from proteins in the P2, P3 and synaptosomal fractions, and the relative abundance of A2G’2F in these fractions was quantified (Fig. 3B). A2G’2F
was detected in all fractions but was more abundant in the P2 fraction than in the P3 fraction (Fig. 3B). Moreover, A2G’2F seemed to accumulate in the synaptosomal fraction. We also found that A2G’2F in the homogenate fraction was mainly contained in gel b after 2D-PAGE (Fig. 2A). To identify A2G’2F carrier proteins, the synaptosomal fraction samples were separated by 2D-PAGE (Fig. 3C). A gel piece near the location of gel b (Fig. 2A) was further separated into 3 pieces (i–iii in Fig. 3C). A2G’2F was detected in all gel pieces (Fig. 3D), but was most abundant in gel pieces ii and iii. Proteins present in each gel piece were identified by LC-MS. Calreticulin was detected among the abundant membrane proteins in these gel pieces. In its non-posttranslationally modified form, calreticulin has a molecular weight of 48 kDa and pI of 4.29. Calreticulin has amino acid consensus sequences (Asn-X-Ser/Thr) for N-glycan modification, thus it is logical that it was detected in gel b. The LC component of LC-MS detects peptides of digested proteins. The average area of the peptide peaks for calreticulin was calculated using Proteome Discoverer software (Fig. 3E). Although calreticulin was detected in all gel pieces, similar to A2G’2F, a large amount of calreticulin was detected in gel ii (Fig. 3E). Neurotrimin and limbic system-associated membrane protein (LAMP) were also detected in all three gel pieces (data not shown). These results are consistent with the results in Fig. 2, and suggest that calreticulin, neurotrimin and LAMP are candidate A2G’2F carriers.

Calreticulin is an endoplasmic reticulum (ER) protein, but it is also localized to the cell surface (Arosa et al., 1999; Raghavan et al., 2013; White et al., 1995). However, there are few reports of the localization of calreticulin in the brain, where neurotrimin and LAMP are known to be synaptic proteins contributing to synapse formation (Gil et al., 1998, 2002; Hashimoto et al., 2009; McNamee et al., 2002; Sanz et al., 2015). To examine calreticulin localization in the brain, western blot analysis of subcellular fractionated samples of 12w mouse brain was performed. A strong signal for GM130, a Golgi apparatus marker, was detected in the P3 fraction, whereas PSD-95 was detected in the PSD fraction (Fig. 3F). These results demonstrate successful subcellular fractionation. Calreticulin, a chaperone protein of the ER, was enriched in the P3 microsomal fraction as expected and also distributed to the synaptosomal-
The distribution pattern of calreticulin was similar to that of the transferrin receptor (TfR; Fig. 3F). TfR is found in the recycling endosome and is mainly expressed in the P3 fraction, although some is also present in synapses, but not in the postsynaptic density. These results indicate that calreticulin is not only expressed in the ER, but also distributed to the synaptic fraction, similar to the TfR, which is consistent with the hypothesis that calreticulin is a carrier protein of A2G'2F.
Calreticulin is observed in dendritic spines of cultured neurons

To confirm localization of calreticulin in neurons, cortical neurons from embryonic day 16 mouse brain were cultured. Neurons were transfected to express GFP to visualize dendritic shafts and spines (Fig. 4A). At 17 days in vitro (DIV), neurons were fixed and immunostained using anti-calreticulin and anti-PSD-95 antibodies (Fig. 4B–E). Calreticulin was mainly expressed in the cell bodies and dendrites (Fig. 4B), while some was also observed in the spines (Fig. 4C–E). Calreticulin signals were detected in about 10% of the PSD-95-positive spines, and these were adjacent to PSD-95 signals (Fig. 4F). These findings suggest that calreticulin is closely associated with the synaptic region but not present in the postsynaptic density.

The interaction of branched sialylated N-glycans with Siglec-H

Sialylated N-glycans are localized on the cell surface and interact with other cells through Siglec (Kleene and Schachner, 2004). However, the interacting partners of branched sialylated A2G2F remain poorly understood. Siglec-H is upregulated in activated microglia in the mouse brain (Kopatz et al., 2013), and the ligand glycan structure for Siglec-H is unknown (Zhang et al., 2006). To investigate whether 6SLeC interacts with Siglec-H, SPR analysis was performed using purified Siglec-H and synthesized 6SLeC clusters (Fig. 5A; Bao et al., 2010; Tanaka et al., 2010). Siglec-H was immobilized on the sensor chip and analyte sugar chain clusters were injected into the Biacore. A dose-dependent response, which reflects an interaction between Siglec-H and sugar chain clusters, was observed when 6SLeC clusters were injected (Fig. 5B). The signal of anti-siglec-H antibody was stronger than that of 6SLeC, possibly because of the higher molecular weight of anti-siglec-H antibody (Supplementary Fig. 2). To investigate whether Siglec-H recognizes a sialic acid on 6SLeC, 6SLeC clusters or non-sialylated LeC clusters were injected into the Biacore. An interaction between LeC clusters and Siglec-H was not observed (Fig. 5C). A response was also not observed when either sialylated or non-sialylated A2G2 clusters were injected (Fig. 5D). These results indicate that Siglec-H interacts with 6SLeC and properly distinguishes sialic acid on 6SLeC.

Fig. 4. Calreticulin was detected in some dendritic spines. (A and B) Immunocytochemistry of cultured mouse cortical neurons was performed. Neurons were manipulated to express GFP from 12 DIV, and immunostained using anti-calreticulin antibody at 17 DIV. Calreticulin (red) was strongly expressed in the cell body of GFP-expressing neurons. Calreticulin was also present in the dendrites. Scale bar: 25 μm. (C–E) Spines were visualized by GFP (green), calreticulin (red) and PSD-95 (blue), as detected immunocytochemically. Most spines had PSD-95-positive mature synapses (arrowheads in C). Fig. 4D and E are enlarged images of the white boxes 1 and 2, respectively. Some of these spines were calreticulin-positive (arrows in D and E). Calreticulin signals were adjacent to those of PSD-95. Scale bar: 10 μm. (F) Approximately 10% of the PSD-95-positive spines were also calreticulin-positive. Sixteen GFP-expressing neurons were chosen randomly from eight separate cultures for analysis.
Discussion

In this study, we sought to identify A2G'2F carrier proteins in the mouse brain. We applied our improved method (Yoshimura et al., 2012) for the detection of N-glycans from trace samples, such as subcellular fractionated proteins, protein bands from SDS-PAGE or spots from 2D-PAGE gels (Fig. 1 and Fig. 2). We showed that A2G'2F accumulated in the P2 fraction and was abundant in the synaptosomal fraction (Fig. 3B). We also successfully detected N-glycans in PAGE gels, and our results showed that A2G'2F is not ubiquitously carried by glycoproteins, but rather by a limited number of acidic proteins with a molecular mass of 50–70 kDa and mainly present in the synaptosomal fraction. We identified calreticulin, neurotrimin and LAMP as candidates for A2G'2F carrier proteins. Moreover, calreticulin was observed in the dendritic spines of cultured cortical neurons (Fig. 4) and we found that synthesized branched sialylated glycan clusters interacted with Siglec-H (Fig. 5). We therefore propose that branched sialylated A2G'2F carried by synaptic proteins plays an important role in interaction with microglial Siglec-H (Supplementary Fig. 3).

Calreticulin is known to be a chaperone protein of the ER (Nauseef et al., 1995; Peterson et al., 1995). It was reported that calreticulin is distributed in dendritic shafts of cultured rat hippocampal neurons (Sala et al., 2005). Using immunocytochemistry and western blot analysis, we revealed that calreticulin was not only localized to the ER, but was also found in the plasma membrane and in a portion of the PSD-95-positive spines (Fig. 3F and Fig. 4). This is the first report showing that calreticulin is adjacent to postsynapses. Neurotrimin and LAMP are members of the IgLON family of immunoglobulin domain-containing glycosylphosphatidylinositol-anchored cell adhesion molecules. These proteins are expressed in neurons and function in the development and stabilization of synapses (Gil et al., 1998, 2002; Hashimoto et al., 2009; McNamee et al., 2002;
Sanz et al., 2015). Our results suggest that branched sialylated A2G2F is expressed in synapses on carrier proteins, like calreticulin, neurotrimin and LAMP, and this N-glycan may contribute to synapse formation, maintenance and pruning. Branched sialylated A2G2F may be transported to the synaptic plasma membrane of neurons by proteins such as calreticulin, neurotrimin and LAMP.

The N-glycan structure of neurotrimin and LAMP in the adult rat brain has previously been reported (Itoh et al., 2008), in which the structure of the identified N-glycans was consistent with sialylated A2G'2F on neurotrimin, but not on LAMP. However, the authors did not determine the linkage between the sugar residues and thus the structural information was not complete. It is possible that the types of N-glycans that are modified on LAMP in the adult mouse brain are different from those in the adult rat brain. Although the isoelectric point of non-glycosylated neurotrimin and LAMP are neutral and their molecular weight is approximately 38 kDa, they were detected in the acidic and 50–60 kDa region on 2D-PAGE gels of the synaptosomal fraction. These results support our hypothesis that neurotrimin and LAMP are modified by sialylated N-glycans such as sialylated A2G2F.

The biosynthesis pathway of 6SLeC-containing branched sialylated A2G2F remains elusive, and it is difficult to obtain these N-glycans by using cultured cells. Therefore, we used synthesized 6SLeC to examine the interaction between 6SLeC and Siglec-H (Fig. 5). The affinity of N-glycan to protein is generally weaker than that of the protein to protein interaction. Thus, it is difficult to observe interaction between proteins and glycans by Biacore assay. To overcome this problem, we chose “sugar chain clusters” as an analyte for this assay (Bao et al., 2010; Tanaka et al., 2010), and we found that synthesized 6SLeC clusters interacted with Siglec-H (Fig. 5). In lipid rafts, the affinity of sugar chains for proteins becomes stronger, as a result of clustering of sugar chains (Taniguchi et al., 2008). Sugar chain clusters increase the local concentration of sugar chains, similar to lipid rafts, thus these are expected to show increased affinity towards proteins. Microglia play a major role in synaptic pruning (Paolicelli et al., 2011). Siglec-H is specifically expressed by microglia (Konishi et al., 2017). Microglia also express Siglec-E, and Siglec-E binds not only to α2,3- and α2,8-sialylated glycans but also to 6SLeC (Redelinglyus et al., 2011). Several studies have identified calreticulin on the cell surface of target cells where it acts as an ‘eat-me’ signal to induce phagocytosis of these cells (Arosa et al., 1999; Fricker et al., 2012; Raghavan et al., 2013; White et al., 1995). Thus, calreticulin may also play a role as an eat-me signal to induce the removal of synapses. Branched sialylated A2G2F on synaptosomal proteins may play a role in synaptic pruning in the mouse brain through the interaction with Siglec-H and Siglec-E, though we cannot neglect the possibility that branched sialylated N-glycans and Siglecs are involved in synapse formation and surveillance (Wake et al., 2013). Although a clear ortholog of Siglec-H is not identified in human, human Siglec-L2 has 42% identity with mouse Siglec-H and is a potential counterpart (Zhang et al., 2006). Further studies are necessary to reveal the signal transduction between synapses and microglia through the interaction of branched sialylated N-glycans with Siglecs. In addition, it is possible that cell surface calreticulin functions as a lectin in dendritic spines (White et al., 1995).

In conclusion, we found that branched sialylated N-glycans are accumulated in mouse brain synaptosomes. Our results support a model where branched sialylated N-glycans play an important role in the interaction between dendritic spines and microglia via microglial Siglec-H.

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Conflicts of interest

The authors declare no conflicts of interest associated with this article.

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