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Generation and Characterization of a Monoclonal Antibody Recognizing a Fetal Brain Enriched *O*-Linked Sialoglycoprotein, FOG100[1]

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With the aim of identifying molecules that are expressed specifically in the brain during neurogenesis, we tried to generate monoclonal antibodies which recognize molecules showing unique temporal expression patterns and molecular characteristics. We used a homogenate of the rat fetal forebrain (day 12 of fetal life, E12) as an immunogen, and antibodies which reacted with this preparation were screened by immunoblotting. One of the antibodies, Mab3C8, recognized a 100-kDa antigen that is enriched in fetal brain. This 100-kDa antigen was constantly expressed during fetal life (from E12 to E20) and became scarcely detectable two days after birth. The antigen was detected in the insoluble fraction of fetal brain and its isoelectric point ranged from 6 to 7, suggesting that it was a membrane-coupled glycoprotein. Analysis by glycosidase treatment and lectin blotting suggested that it was an *O*-linked glycoprotein with an α2,6 sialyl linkage. Thus, a molecule unique to the fetal brain, an *O*-linked sialoglycoprotein with a molecular mass of 100 kDa (FOG100), was found by generating an antibody.

Key words: fetal brain, FOG100, monoclonal antibody, *O*-glycosylation, sialoglycoprotein.

The neural cell population of the fetal brain proliferates and differentiates to form neurons in a precise temporal and spatial manner (*1-3*). Neural cells exist in the ventricular zone as stem, progenitor, or precursor cells, which give rise to different types of neurons, oligodendrocytes, and astrocytes; and neurogenesis is known to be completed in the mammalian cerebrum by late embryogenesis (*1, 2*). The neural network then undergoes the processes of neurite outgrowth, axon fasciculation, synaptogenesis, and myelination during the perinatal stage (*2*).

Several markers for the developing mammalian central nervous system (CNS) have been identified, and glycoconjugates seem to be important factors in the development of the mammalian CNS, being involved in the regulation of cell-to-cell interactions and the modification of synaptic activity (*4*). For example, polysialic acid is attached to the protein backbone of neural cell adhesion molecule (N-CAM) and of voltage-dependent sodium ion channels (*4, 5*), and a new dimension has thus been added to the complexity of protein structure and function. To date, however, developmentally expressed *O*-linked glycoproteins have not been reported in the mammalian CNS. We have investigated developmentally regulated genes and glycoconjugates in fetal or placental tissues (*6-10*).

In rats and mice, embryonic day 11-12 (E11-E12) seems to be a critical stage at which many multipotential stem cells and developing neuronal cells coexist (*11*). In the present study, we tried to find molecules that show unique temporal expression patterns and molecular characteristics by generating monoclonal antibodies using a homogenate of the rat E12 forebrain as an immunogen. The antigen detected by one of these antibodies possessed these attributes and was designated FOG100.

MATERIALS AND METHODS

Reagents—The reagents used were purchased as follows: pepstatin, aprotinin, leupepsin, and PNGase F (*N*-Glycosidase F; EC 3.2.2.18) from Boehringer Mannheim (Germany); urea and protein quantifying reagents (Bio-Rad Protein Assay) from Bio-Rad (Richmond, CA, USA); Nonidet P-40, acrylamide, ampholytes (pH 3-10), dithiothreitol, and polyvinylidene difluoride (PVDF) membranes from Millipore (Bedford, MA, USA); fetal calf serum from Causera (Ontario, Canada); RPMI1640 medium from GIBCO BRL Life Technology (New York, NY, USA); bovine serum albumin (BSA), streptomycin, and penicillin from Sigma Chemical (St. Louis, MO, USA); Pristane from Aldrich Chemical (Milwaukee, WI, USA); biotin-*Sam-*

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Abbreviations: Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; MAA, *Maackia amurensis* lectin; Mab, monoclonal antibody; OL, oligodendrocyte; PNA, peanut agglutinin; SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; Sia, sialic acid; SSA, *Sambucus sieboldiana* agglutinin; 2D/E, two-dimensional electrophoresis; FCS, fetal calf serum.

bucus sieboldiana agglutinin (SSA), biotin-*Maackia amurensis* lectin (MAA), and horseradish peroxidase (HRP)-peanut agglutinin (PNA) from Honen (Tokyo); HRP-streptavidin from Vector (Burlingame, CA, USA); HRP-conjugated goat anti-mouse IgG+IgM antiserum from Jackson Immunoresearch (West Grove, PA, USA); PD-10 columns and CNBr-activated Sepharose 4B from Pharmacia Biotech (Uppsala, Sweden); sialidase (source: *Arthrobacter ureafaciens*; EC 3.2.1.18) from Nacalai (Kyoto); endo-α-*N*-acetylgalactosaminidase (O-Glycanase; EC 3.2.1.97) from Genzyme (Cambridge, MA, USA); *n*-octyl-β-D-glucoside and decanoyl-*N*-methylglucamide (MEGA-10) from DOJINDO (Kumamoto); Block Ace, a blocking reagent for immunoblotting, from Snow Brand (Sapporo); ECL western blotting kit and mouse monoclonal antibody isotyping kit from Amersham (Buckinghamshire, UK); and phenylmethylsulfonyl fluoride (PMSF) and all other reagents, unless otherwise stated, from Wako Pure Chemicals (Osaka).

Animal Treatment—Adult Wistar-Imamichi rats were purchased from the Imamichi Institute for Animal Reproduction (Ibaraki). They were kept under a lighting regime of 14-h illumination and 10-h darkness (lights on between 0500 h and 1900 h) and were allowed free access to food and water. The day of insemination was designated E0. To prepare the immunogen and allow subsequent immunoblotting, the brain and other tissues of fetal (E12-20), neonatal (postnatal days P0-10), or adult rats were removed following decapitation. They were then dissected as appropriate, washed with 0.9% NaCl, frozen in liquid nitrogen, and stored at −80°C until use.

The BALB/cA mice used for monoclonal antibody production were purchased from Nihon Clea (Tokyo) and were kept as described above.

Production and Purification of the Monoclonal Antibodies—Monoclonal antibodies were generated using X63Ag8 as the parental myeloma cell line, as described by Köhler and Milstein (*12*). To prepare the immunogen, E12 rat forebrains were homogenized with 20 volumes (w/v) of homogenization buffer [10 mM phosphate-buffered saline (PBS) containing 20 IU/ml aprotinin, 1 μM leupepsin, 0.1 mg/ml pepstatin, and 0.1 mM PMSF], then diluted 10 times. Each mouse was immunized with 0.5 ml of the homogenate mixed with an equal volume of Freund's complete adjuvant. The immunization was repeated 3 times at 3-week intervals using the antigen with incomplete adjuvant. The antibodies were screened by immunoblotting on E12.5 and adult rat brain homogenates. One of these antibodies, designated Mab3C8 (IgM, κ-chain), recognized an antigen that was expressed in the E12 but not in the adult brain (Fig. 1A).

The antibody was purified from mouse ascites using a hydroxyapatite column (SH-0710M; Asahi Optical; Tokyo) connected to a Waters 600E HPLC system, in which elution was performed with a linear gradient of 100-400 mM sodium phosphate buffer (pH 6.8) containing 100 mM NaCl.

Immunoblotting—Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described previously (*13*). Briefly, tissues were homogenized with 20 volumes (w/v) of homogenization buffer, then diluted 10 times. Aliquots of 20 μl (about 20 μg protein equivalent) were subjected to SDS-PAGE (5-8% w/v gel). Mab3C8, as the supernatant of the hybridoma culture (dilution: 1:2) or purified Mab3C8 (final concentration: 1 μg/ml), was reacted for 1 h at room temperature (RT), and visualized using HRP-conjugated anti-mouse antiserum and an ECL Western blotting kit.

For subcellular fractionation, E20 cerebrum was homogenized with 5 volumes (w/v) of 3 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose (fractionation buffer). After centrifugation at 700×*g* for 10 min, the resulting precipitate was regarded as the crude nuclear fraction. The supernatant was then centrifuged at 105,000×*g* for 60 min. The resulting precipitate was regarded as the crude microsomal fraction, and the supernatant as the crude cytosolic fraction. Each fraction was adjusted to the same volume with fractionation buffer, and aliquots of the same size were subjected to immunoblotting as described above.

Two-Dimensional Electrophoresis—Two-dimensional electrophoresis (2D/E) was performed as described previously (*14*). A 50-μg equivalent of rat E20 homogenate was loaded into a capillary tube (1.2 mm diameter×150 mm length) containing 9.5 M urea, 4% (v/v) Nonidet P-40, 4.1% (w/v) acrylamide, and 5.8% (v/v) ampholytes (pH 3-10). This mixture was then loaded onto a SDS-PAGE (8% w/v) gel (150 mm×150 mm×1 mm) and run for 1.5 h at 250 V.

Purification of FOG100—Mab3C8 was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions. After centrifugation of E20 cerebral homogenate in homogenization buffer at 105,000×*g* at 4°C, the precipitate was solubilized with PBS containing 2% SDS and applied to a SDS/polyacrylamide (5% w/v) gel. The bands corresponding to 100 kDa were cut out and electroeluted at 100 V for 20 h with SDS-PAGE loading buffer. The electroeluted FOG100 was desalted in a PD-10 column, then mixed with the Mab3C8-coupled resin by end-over-end rotation in 10 mM Tris-HCl (pH 7.4) containing 140 mM NaCl and 1% (w/v) *n*-octyl-β-D-glucoside (starting buffer) at 4°C for 8 h. The gel suspension (2 ml) was then packed into a column and washed with a 10-gel volume of starting buffer. The FOG100 was eluted using 10 mM Tris-HCl (pH 7.4) containing 800 mM NaCl and 1% (w/v) *n*-octyl-β-D-glucoside.

Glycosidase Treatment—Before treating with sialidase, FOG100 was boiled for 5 min in 100 mM sodium acetate buffer (pH 5.0) containing 0.2% SDS. After adding MEGA-10 (final concentration: 2%) and PMSF (final concentration: 0.1 mM), the FOG100 was incubated with 250 mU/ml sialidase at 37°C overnight. For treatment with *N*-Glycosidase F, FOG100 was treated similarly except that 15 mM sodium phosphate buffer (pH 7.4) containing 50 mM ethylenediaminetetraacetic acid (EDTA) and 0.2% SDS was used, followed by incubation with 40 mU/ml *N*-Glycosidase F at 37°C overnight.

For *O*-Glycanase digestion, FOG100 was boiled in 15 mM sodium phosphate buffer (pH 7.4) containing 50 mM EDTA for 5 min. After adding MEGA-10 and PMSF, the sample was incubated with 250 mU/ml sialidase at 37°C for 5 h, then with 25 mU/ml *O*-Glycanase at 37°C for an additional 20 h.

Lectin Blotting—After treatment with sialidase or *O*-Glycanase, the FOG100 preparations were subjected to SDS-PAGE followed by lectin blotting using biotin-SSA, biotin-MAA, or HRP-PNA. After SDS-PAGE, the PVDF

membrane to which the blots has been transferred was treated with 0.05% (v/v) polyoxyethene sorbitan monolaurate in 10 mM Tris (pH 7.4) containing 140 mM NaCl to avoid nonspecific binding. The PVDF membrane was then incubated with each lectin at a final concentration of 1 μg/ml at RT for 1 h. Blotting with PNA and other lectins was visualized with ECL and the HRP-streptavidin-ECL system, respectively.

RESULTS

Production of Mab3C8 Recognizing FOG100—A monoclonal antibody named Mab3C8 was obtained after screening as described in "MATERIALS AND METHODS" (Fig. 1A). Mab3C8 recognized a band with a molecular weight (MW) of 100 kDa (FOG100) in the rat E12 forebrain. In contrast, FOG100 was not detected in the adult rat brain.

To investigate the ontogeny and fate of FOG100, brain homogenates obtained on various embryonic and postnatal days were subjected to immunoblotting using Mab3C8 (Fig. 1B). Interestingly, FOG100 was detected in the fetal forebrain/cerebrum on E12, 13, 14, 16, 18, and 20, and was not detected in the adult cerebrum. On the day of birth, a clear band of FOG100 was observed, and this became scarcely detectable on day 2. Thus, FOG100 was quite unique to fetal life and was only expressed during the second half of embryogenesis.

Intracellular and Tissue Distribution of FOG100—The intracellular distribution of FOG100 was investigated using the subcellular fractions prepared from E20 cerebrum (Fig. 1C). FOG100 was detected in the crude nuclear and microsomal fractions, but not in the cytosolic fraction.

The tissue distribution of FOG100 in fetal tissues (E16) is shown in Fig. 1D. FOG100 was detected in the brain, and its expression in the cerebrum was higher than that in the rest of the brain. FOG100 was scarcely detectable in the liver and the heart. Therefore, FOG100 was predominantly expressed in the fetal cerebrum.

Two-Dimensional Electrophoresis of FOG100—To determine the isoelectric point (pI) of the antigen, the E20 cerebral homogenate was subjected to 2D/E followed by immunoblot analysis (Fig. 2A). The pI of FOG100 ranged from 6 to 7 at several spots, suggesting that the core molecule of FOG100 was modified by electrically charged molecules, such as glycans.

Purification of FOG100—FOG100 was purified using preparative SDS-PAGE followed by immunoadsorption chromatography. The FOG100 fraction was separated on preparative SDS-PAGE gels and detected by immunoblotting using Mab3C8 and Coomassie Brilliant Blue staining (Fig. 2B). This partially purified FOG100 preparation was subjected to treatment with glycosidases (Fig. 2C) or to a further purification step using Mab3C8-immunoadsorption chromatography (Fig. 3A).

The FOG100 adsorbed by the column during further purification was eluted with buffer containing 800 mM NaCl, as described in "MATERIALS AND METHODS." In this fraction, a band corresponding to FOG100 was detected using silver staining (Fig. 3A). This purified preparation was used for lectin blotting (Fig. 3, B and C).

Characterization of FOG100 by Digestion with Glycosidases and Lectin Blotting—FOG100, partially purified by preparative SDS-PAGE, was treated with sialidase and subjected to immunoblotting with Mab3C8 (Fig. 2C). After sialidase treatment, the FOG100 band was not detectable on immunoblotting. Therefore, the epitope recognized by Mab3C8 contained sialic acid. Following treatment with *N*-Glycosidase F, the FOG100 band did not shift, suggesting that FOG100 does not contain *N*-linked oligosaccharides. These enzymes are specific for oligosaccharides of glycoproteins and showed no protease activity when used to analyze recombinant rat Leukemia Inhibitory Factor and Placental Lactogen, which is highly glycosylated (data not shown).

To further characterize the oligosaccharide structure of FOG100, the preparation purified on the immunoaffinity column was subjected to lectin blotting (Fig. 3). FOG100 did not react with MAA but was positively stained by SSA,

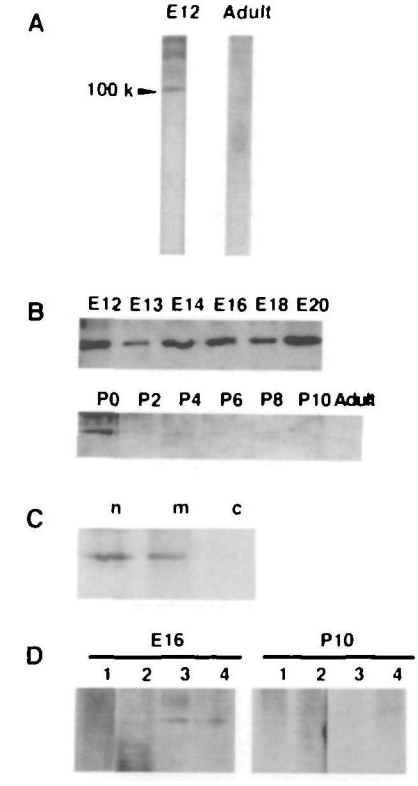

Fig. 1. **A: Mab3C8 recognized a 100-kDa antigen (FOG100).** Homogenates of brains from E12 and adult rats were subjected to SDS-PAGE under non-reducing conditions and visualized by immunoblotting, as described in "MATERIALS AND METHODS." Note that FOG100 is expressed in the rat E12 forebrain, but not in the adult cerebrum. **B: Developmental regulation of FOG100 expression.** A homogenate of the forebrain/cerebrum was prepared from E12, E13, E14, E16, E18, E20, P0, P2, P4, P6, P8, P10, or adult rats. **C: The intracellular localization of FOG100.** The E20 homogenate was fractionated into nuclear (n), microsomal (m), and cytosolic fractions (c), as described in "MATERIALS AND METHODS." The entire homogenate and the three fractions were analyzed by immunoblotting. FOG100 was expressed in the microsomal and nuclear fractions of the fetal brain, but levels had decreased dramatically by P2, and was undetectable in the adult brain. **D: Tissue distribution of FOG100.** Tissue homogenates from the heart (1), liver (2), cerebrum (3), and the rest of the brain (4) at E16, and from the heart (1), liver (2), cerebrum (3), and cerebellum (4) at P10 were subjected to SDS-PAGE followed by immunoblotting. FOG100 was expressed in the brain at E16. At P10, no expression of FOG100 was observed in any of the tissues examined.

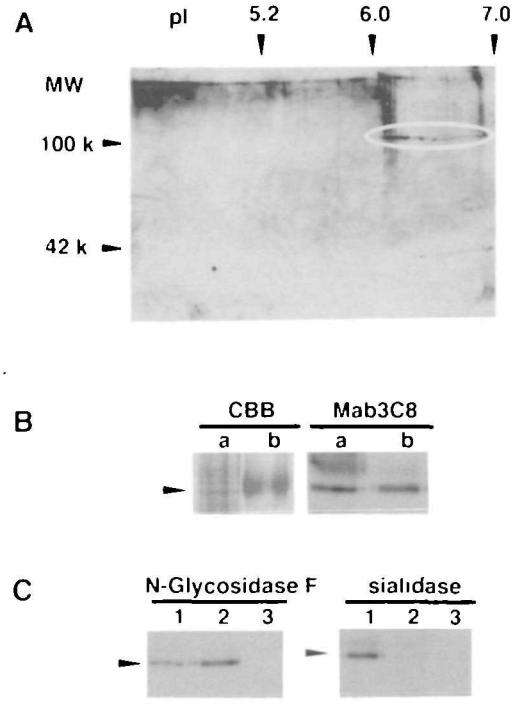

Fig. 2. **A: 2D/E analysis of FOG100.** An E20 brain homogenate was subjected to 2D/E followed by immunoblotting. FOG100 exhibited a broad isoelectric point (pI 6-7) (ellipse). **B: Partial purification of FOG100.** A fetal cerebral homogenate of (E20) (a) or FOG100 partially purified by preparative SDS-PAGE (b) was analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (CBB) or immunoblotting with Mab3C8. **C: Characterization of FOG100 by treatment with glycosidases.** The partially purified FOG100 preparation was treated with *N*-Glycosidase F or sialidase, then subjected to SDS-PAGE followed by immunoblotting. 1, FOG100; 2, FOG100 treated with the enzyme; 3, enzyme only. Arrowheads indicate FOG100.

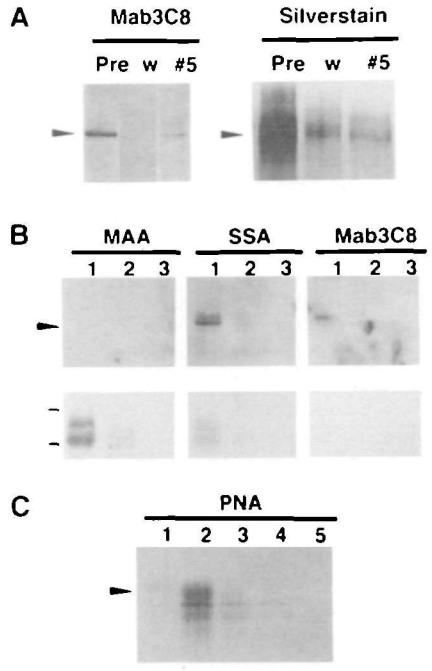

Fig. 3. **A: Purification of FOG100 on a Mab3C8-immunoaffinity column.** The FOG100 preparation (Pre: partially purified as shown in Fig. 2B) was subjected to column chromatography. The column was washed with 10 mM Tris-HCl (pH 7.4) containing 140 mM NaCl and 1% (w/v) *n*-octyl-β-D-glucoside, and FOG100 was eluted with 10 mM Tris-HCl (pH 7.4) containing 800 mM NaCl and 1% (w/v) *n*-octyl-β-D-glucoside. The washing fraction (w) and the bound fractions (500 μl each) were collected and subjected to immunoblotting with Mab3C8 and silver staining. Bound fraction #5 corresponded to purified FOG100. The arrowhead indicates FOG100. **B: (Upper Panels) Characterization of the sialyl moiety of FOG100 by lectin blotting with biotin-SSA, biotin-MAA and Mab3C8.** 1, untreated FOG100; 2, FOG100 treated with sialidase; 3, sialidase only. FOG100 was detected by SSA after treatment with sialidase. The arrowhead indicates FOG100. **(Lower Panels), FCS treated with or without sialidase was shown as controls.** 1, untreated FCS; 2, FCS treated with sialidase; 3, sialidase only. Horizontal bars indicate the molecular weights of 76 and 53 kDa. **C: Characterization of the *O*-glycosidic oligosaccharide moiety of FOG100 by lectin blotting with PNA.** 1, FOG100; 2, FOG100 treated with sialidase; 3, FOG100 sequentially treated with sialidase and *O*-Glycanase; 4, sialidase only, 5, *O*-Glycanase only. FOG100 was detected only after treatment with sialidase. The arrowhead indicates FOG100.

suggesting that it contained an α2,6 sialyl linkage. SSA also reacted with glycoproteins in the FCS, as shown in Fig. 3B, lower panels. Under the same experimental conditions, no band reacted with Mab3C8. Therefore, the epitope is not completely identical to the SSA-recognition site.

Next, the oligosaccharide core to which the sialic acids were linked was characterized using PNA. FOG100 was detected with PNA only after digestion with sialidase (Fig. 3C, lane 2), confirming that the epitope recognized by Mab3C8 contains sialic acid. This result also suggests that the core structure is Galβ1,3GalNAc-O-Ser/Thr. In accordance with this result, the band corresponding to FOG100 became scarcely detectable with PNA after O-Glycanase treatment. Therefore, FOG100 was shown to be an *O*-linked sialoglycoprotein.

DISCUSSION

The *O*-linked sialoglycoprotein with a MW of 100 kDa detected in this study was designated FOG100. FOG100 is strongly expressed in the fetal brain and differs from previously reported marker molecules for the developing CNS. The proliferation and migration of neuronal cells are completed by late embryogenesis (*1, 2*), and are followed by the processes crucial for higher brain function, including axon fasciculation, synapse formation, and myelination (*2*). Considering its expression period, FOG100 represents an interesting potential tool for the study of neurogenesis.

It is known that the *O*-linked sugars attached to the membrane proteins of most mammalian tissues have a disaccharide core, and three types of sialyl linkage, α2,3, α2,6, and α2,8, have been reported (*15*). In this study, the sialyl linkage within FOG100 was determined using SSA, which recognizes Siaα2,6GalNAc and Siaα2,6Galβ1, 4GlcNAc links in both *N*- and *O*-glycosidic oligosaccharides (*16*), and MAA, which recognizes the terminal structure Siaα2,3Gal in *N*-linked oligosaccharides (*17*). Based on the results of lectin blotting with SSA, FOG100 is thought to contain an α2,6 sialyl linkage. In addition, FOG100 became reactive with PNA only after sialidase treatment, indicating that its epitope contains an *O*-linked sialo-oligo-

saccharide. Because PNA recognizes the disaccharide core, Galβ1,3GalNAc-O-Ser/Thr (*18*), but does not react if terminal sialic acids are bound to the disaccharide, the structure of the epitope can be deduced as: Galβ1,3(Siaα2, 6)GalNAc-; Siaα2,6Galβ1,3(Siaα2,6) GalNAc-; or Siaα2, 3Galβ1,3(Siaα2,6)GalNAc-. To the best of our knowledge, however, the oligosaccharide structure Siaα2,6Galβ1, 3(Siaα2,6) GalNAc has not previously been found in native glycoproteins.

Most of the *O*-glycosylated proteins are known to contain one or more *N*-glycosidic oligosaccharides (*19*). It is, however, clear that FOG100 does not have *N*-linked oligosaccharides, and it is thus unique in terms of containing solely *O*-glycosidic oligosaccharides. N-CAM is highly polysialylated on the oligosaccharides attached to its asparagine residues during the late embryonic and early postnatal stages, but becomes less sialylated during the early embryonic and adult stages (*20*). Polysialic acid seems to be required for neurite outgrowth (*5, 21*), synaptic plasticity (*22*), and the migration of oligodendrocyte precursors (*23*), and seems to be transferred to N-CAM by an α2,8-sialyltransferase, ST8Sia II/STX (*24*). Therefore, FOG100 will be an interesting molecule with which to investigate neural development in the rat because its structure, like its expression pattern, are quite unique.

Since Mab3C8 recognizes the carbohydrate moiety containing sialic acid, the disappearance of FOG100 after birth as assessed by immunoblotting does not necessarily indicate that the whole molecule is not expressed. In other words, the structure of the carbohydrate portion may change during development. Changes in the activity or expression of sialyltransferase or sialidase in the brain might be involved in the mechanism of its apparent disappearance. Indeed, α2,6-sialyltransferase has been reported to participate in the formation of the neural network (*25*).

Several other markers expressed in fetal brain have been reported. The intermediate filaments, nestin (*26*) and vimentin (*27*), are specific to neural stem cells. Nestin is found in CNS neuronal stem cells, is detected from E11 onwards in the forebrain, and its expression continues until around P10 in the mouse. Vimentin, a class III intermediate filament found in CNS progenitor cells, is also expressed in the retinal (*28*) and olfactory epithelium (*29*) of the adult mouse. In addition, various transcription factors, such as BF-1, Emx1, Emx2, Otx1, Otx2, and Pax6, show forebrain-specific expression in the fetus (*30-34*). Considering its structure and expression pattern, FOG100 is different from these factors.

FOG100 expression decreased dramatically between P0 and P2. It is noteworthy that there are reciprocal relationships between the expression of FOG100 and oligodendrocyte (OL) markers. O-2A progenitor cells differentiate to the OL lineage at birth *via* premature OL cells (*35*). The O4 antigen, specific to the OL lineage, is first detected in the rat cerebrum at P0 (*36*). During this process, the expression of the O-2A markers, A2B5, GD3, and vimentin, decreases reciprocally (*2*). The number of O4-positive cells has been shown to increase 10-fold by P3, and galactocerebroside (GalC)-positive cells are first observed at P3 (*36*). After differentiation of the premature OLs into postmitotic OLs, a process that is defined by GalC positivity, the cells begin to synthesize glycoproteins such as myelin-associated glycoprotein, which belongs to the immunoglobulin superfamily (*37*). To date, no studies have demonstrated the existence of any neuronal cell-associated molecules that may be responsible for this process. Unlike neuronal cell populations, OLs are broadly distributed throughout the adult vertebrate nervous system, while OL precursors are initially spatially restricted (*38*). Since FOG100 is localized to the membrane fraction of the CNS throughout fetal life, a study on its participation in the mechanism of myelination may prove interesting.

In conclusion, we generated a monoclonal antibody, Mab3C8, which recognizes FOG100, an *O*-linked sialoglycoprotein which is only expressed during embryogenesis.

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