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Developmental changes in the biochemical and immunological characters of the carbohydrate moiety of neuroglycan C, a brain-specific chondroitin sulfate proteoglycan

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Neuroglycan C (NGC), a brain-specific transmembrane proteoglycan, is thought to bear not only chondroitin sulfate but also N- and O-linked oligosaccharides on its core protein. In this study, we isolated and purified NGC from rat brains at various developmental stages by immunoaffinity column chromatography or by immunoprecipitation, and examined the structural characters of its carbohydrate moiety. The chondroitin sulfate disaccharide composition of NGC at postnatal day 10 was significantly different from those of two secreted chondroitin sulfate proteoglycans, neurocan and phosphacan, purified from the brain at the same developmental stage; higher levels of 4-sulfate unit and E unit, a disulfated disaccharide unit, and a lower level of 6-sulfate unit. The levels of both 6-sulfate and E units decreased with a compensatory increase of 4-sulfate unit with postnatal development of the brain. Lectin-blot analysis of the NGC core glycoprotein prepared by chondroitinase digestion confirmed that NGC actually bore both N- and O-linked carbohydrates, and also revealed that lectin-species reactive with NGC did not always recognize other brain-specific proteoglycans, neurocan and phosphacan, and vice versa, even though they were isolated from the brain at the same stage. The reactivity of NGC with lectins and with the HNK-1 antibody markedly changed as the brain matured. These findings indicate that the structure of the carbohydrate moiety of NGC is developmentally regulated, and differs from those of neurocan and phosphacan. The developmentally-regulated structural change of the carbohydrates on NGC may be partly implicated in the modulation of neuronal cell recognition during brain development.
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Keywords: **brain development, chondroitin sulfate proteoglycan, neuroglycan C, neurocan, phosphacan**

Abbreviations: **RPTPζ/β: receptor-like protein tyrosine phosphatase ζ/β; NGC: neuroglycan C; E17: embryonic day 17; P10: postnatal day 10; MAb: monoclonal antibody; Le^x: Lewis X; S-Le^x: sialyl Lewis X; CHase ABC: protease-free chondroitinase ABC; NANase: neuraminidase; O-Gase: O-glycosidase; N-Gase: N-glycosidase F; ΔDi-0S: 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi-4S: 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S: 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-diS$_D$: 2-acetamide-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-diS$_E$: 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4,6-bis-*O*-sulfo-D-galactose; Gal: galactose; GalNAc: *N*-acetylgalactosamine; GlcNAc: *N*-acetylglucosamine; Man: mannose; DSL: *Datura stramonium* lectin; PHA-L: *Phaseolus vulgaris* leucoagglutinin; PHA-E: *Phaseolus vulgaris* erythroagglutinin; Con A: concanavalin A; LCA: *Lens culinaris* agglutinin; AAL: *Aleuria aurantia* lectin; sWGA: succinylated wheat germ agglutinin; WGA: wheat germ agglutinin; PNA: peanut agglutinin; CBB: Coomassie Brilliant Blue; PBS: phosphate buffered saline; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF: polyvinylidene difluoride; SE: standard error; p: probability.**

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Introduction

Post-translational modifications of proteins result in a structural and functional change. Glycosylation is a typical post-translational modification of proteins [1], and a carbohydrate moiety that has itself an enormous structural diversity has been shown to be implicated in the metabolic stabilization of its core protein and modification of binding specificity and affinity of the core protein with its ligands [2–6]. In addition to these cooperative functions of carbohydrate chains with their core proteins, glycan-moieties of glycoproteins seem to play a role in various cellular events through binding with bio-active molecules such as cytokines, cell adhesion molecules, and extracellular constituents [2–6].

Proteoglycan is a glycoprotein with covalently bound-sulfated glycosaminoglycans as carbohydrate chains, and most proteoglycan core proteins also bear N- and/or O-linked oligosaccharides. In the brain, there exist many proteoglycan species both at the cell surface as transmembrane or GPI-anchored molecules and in the extracellular matrix as secreted molecules to form the micromilieu of neural cells [7]. Enormous evidence has been accumulated to demonstrate that nervous tissue proteoglycans are involved in not only developmental processes including cell migration, neurite elongation, neuronal plasticity, and synapsis formation, but also repair processes of nervous tissues through molecular interactions with their ligands [7–15]. Glycan-moieties, namely glycosaminoglycans and/or oligosaccharides, appear to be essential for many of the molecular interactions of proteoglycans with their ligand. For example, phosphacan/receptor-like protein tyrosine phosphatase ζ/β (RPTPζ/β), a brain-specific chondroitin sulfate proteoglycan, can interact with TAG-1 and growth factors such as pleiotrophin and amphoterin with a high affinity [16–18]. However, the removal of chondroitin sulfates from phosphacan results in decreased affinity, indicating that the chondroitin sulfate chains participate in these interactions [16–18]. Similarly, N-linked oligosaccharides on the phosphacan core protein have been shown to participate in the binding of phosphacan to cell adhesion molecules such as L1 and N-CAM [19].

Neuroglycan C (NGC) is a transmembrane-type of chondroitin sulfate proteoglycan, exclusively expressed in the central nervous system [20]. It has been suggested that NGC is involved in differential synaptogenesis in the cerebellum, neurite elongation, regeneration of optic nerves, and neuronal plasticity [21–23]. Since both N- and O-glycosidase digestions of NGC increase its electrophoretic mobility on SDS-PAGE, NGC should have both N- and O-linked oligosaccharides in addition to the chondroitin sulfate chain [20]. In the cerebellum, NGC was immunolocalized to the Purkinje cells at all developmental stages [21]. Interestingly, although NGC occurs in a proteoglycan form with chondroitin sulfate in the developing cerebellum, most NGC molecules do not bear the chondroitin sulfate chain in the mature cerebellum [21]. This structural change of NGC from a proteoglycan form to a non-proteoglycan form also occurs during development of the retina [24]. These findings suggest that the chondroitin sulfate moiety, and probably the oligosaccharide moieties, of NGC are involved in a particular developmental event at least during cerebellar and retinal development. However, not only the function but also the structure of NGC carbohydrates is understood very poorly.

In this study, to obtain clues about the functional roles of the carbohydrate moieties of NGC in the central nervous system development, we first analyzed the structures of both chondroitin sulfate and oligosaccharide moieties of NGC.

Materials and methods

Materials

The following antibodies were prepared in our laboratory: a monoclonal anti-NGC antibody (MAb-C5) [20], a polyclonal anti-NGC antibody [25], a monoclonal anti-phosphacan antibody (MAb-6B4) [26], and a monoclonal anti-neurocan antibody (MAb-1G2) [27]. A monoclonal anti-CD57 antibody against the HNK-1 epitope (mouse IgM, clone TB01) was purchased from Serotec (Oxford, England). The following carbohydrate antibodies and enzyme were purchased from Seikagaku Co. (Tokyo, Japan): a monoclonal anti-Lewis X antibody (mouse IgM, clone 73-30), a monoclonal anti-Sialyl Lewis X antibody (mouse IgM, clone KM-93), a monoclonal anti-proteoglycan ΔDi-0S antibody (mouse IgG_1, clone 1-B-5), a monoclonal anti-proteoglycan ΔDi-4S antibody (mouse IgG_1, clone 2-B-6), a monoclonal anti-proteoglycan ΔDi-6S antibody (mouse IgM, clone 3-B-3), and protease-free chondroitinase ABC (EC 4.2.2.4). The following biotinylated lectins and antibodies from Vector Labs. (Burlingame, CA, USA) were used: *Aleuria aurantia* lectin (AAL), concanavalin A (Con A), *Datura stramonium* lectin (DSL), Jacalin isolated from *Artcarpus integrifolia* seeds, *Lens culinaris* agglutinin (LCA), peanut agglutinin (PNA), *Phaseolus vulgaris* erythroagglutinin (PHA-E), *Phaseolus vulgaris* leucoagglutinin (PHA-L), wheat germ agglutinin (WGA), succinylated wheat germ agglutinin (sWGA), an anti-rabbit IgG antibody, an anti-mouse IgG antibody, an anti-mouse IgM antibody. The reported specificities of these lectins to carbohydrate structures are summarized in Table 1. N-glycosidase F (EC 3.5.1.52) and O-glycosidase (EC 3.2.1.97) were purchased from Roche Diagnostics (Mannheim, Germany). Neuraminidase (EC 3.2.1.18) was purchased from Nacalai Tesque (Kyoto, Japan). DEAE-Sephacel, Sepharose CL-4B, and octyl-Sepharose were purchased from Amersham Biosciences (Uppsala, Sweden).

Purification of proteoglycans

Animals were treated according to the ethical rules of our institution. All efforts were made to minimize the number of animals used and their suffering. A mixture of membrane-bound proteoglycans was prepared from a phosphate buffered saline (PBS)-insoluble fraction of 10-day-old (P10) rat brains as described previously [20]. In brief, 100 brains were homogenized

Table 1. Lectins used in this study and their major carbohydrate-binding specificities

Lectin[a]	*Glycan structure required for binding to lectin*	*Reference*
Jacalin	Galβ1-3GalNAc-*O*-Ser/Thr	[71]
DSL	*N*-acetyllactosamine repeats GlcNAcβ1-6Manα1 branching multiantennary complex type	[72]
PHA-L	GlcNAcβ1-6Manα1 branching multiantennary complex type	[73,74]
PHA-E	Complex type oligosaccharides containing a bisecting GlcNAc	[74,75]
Con A	Mannose residues in high mannose type, hybrid type, and biantennary complex type oligosaccharides	[76]
LCA	α-Fucosyl residue attached to the *N*-acetylchitobiose portion of the core oligosaccharides	[77,78]
AAL	α-Fucosyl residue on oligosaccharides	[79]
sWGA	Oligosaccharides terminating with GlcNAc	[80]
WGA	Oligosaccharides terminating with sialic acid and GlcNAc	[81]
PNA	Unsialylated Galβ1-3GalNAc-*O*-Ser/Thr	[82]

[a]Jacalin isolated from *Artcarpus integrifolia* seeds; DSL: *Datura stramonium* lectin; PHA-L: *Phaseolus vulgaris* leucoagglutinin; PHA-E: *Phaseolus vulgaris* erythroagglutinin; Con A: concanavalin A; LCA: *Lens culinaris* agglutinin; AAL: *Aleuria aurantia* lectin; sWGA; succinylated wheat germ agglutinin; WGA: wheat germ agglutinin; PNA: peanut agglutinin.

in 200 ml of ice-cold PBS that contained 20 mM EDTA, 10 mM *N*-ethylmaleimide, and 2 mM phenylmethylsulfonyl fluoride as protease inhibitors. The homogenate was centrifuged at 27,000 $\times$ *g* for 40 min at 4°C. The PBS-insoluble materials were solubilized with 200 ml of PBS containing 1% Nonidet P-40 and the protease inhibitors. The solubilized materials were applied to a sequential column chromatography on DEAE-Sephacel, Sepharose CL-4B, and octyl-Sepharose, and ultracentrifugation on a CsCl density gradient as described previously [20]. The proteoglycan mixture thus obtained was dissolved in 4 ml of PBS containing 0.5 M NaCl, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonic acid (Katayama Chemical Industries Co., Ltd., Osaka, Japan) (solution-A). The proteoglycan solution was added to 2 ml of Affi-gel 10 (Bio-Rad, Richmond, CA, USA) coupled with 10 mg of MAb-C5. The gel suspension was mixed gently overnight at 4°C and then poured into a column. After washing the column with solution-A, NGC was eluted from the column with 20 ml of 3 M potassium thiocyanate containing 0.1% 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonic acid. NGC was also purified from the adult rat brain by essentially the same method. About 5 μg (150 nmol uronic acid) of NGC was obtained from 100 P10 rat brains and from 10 adult rat brains by this procedure.

Phosphacan, a soluble spliced variant of RPTPζ/β [28], and neurocan were purified from P10 rat brains by the biochemical method described by Oohira *et al*. [29] and by the immunoaffinity column chromatography described by Matsui *et al*. [30], respectively.

Immunoprecipitation of NGC from lysates of brain membrane fraction

Brains of embryonic day 17 (E17), P10, or adult rats were homogenized in 5 volumes of 0.32 M sucrose containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM pepstatin A (solution-B) using a motor-driven Teflon glass homogenizer. The homogenate was centrifuged at 1,000 $\times$ *g* for 5 min at 4°C. The pellet was subjected to re-homogenization in 2.5 volumes of solution-B. After centrifugation, the supernatants were combined and centrifuged at 105,000 $\times$ *g* for 75 min at 4°C. The resultant pellet was dissolved in 5 volumes of PBS containing 0.2% Nonidet P-40, 0.2% sodium deoxycholate, 5 mM EDTA, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM pepstatin A and stirred overnight on a magnetic stirrer. The mixture was centrifuged at 20,000 $\times$ *g* for 15 min at 4°C. An aliquot (1 ml) of the supernatant was incubated with 5 μg of the polyclonal anti-NGC antibody for 2 h at 4°C and then with 3 μl of protein A-Sepharose (Amersham Biosciences) overnight. The immunocomplexes thus formed were precipitated by centrifugation at 20,000 $\times$ *g* for 5 min at 4°C. The immunoprecipitate was washed three times with 200 μl of PBS containing 0.2% Nonidet P-40, then once with 200 μl of a chondroitinase ABC reaction buffer (100 mM Tris-HCl, pH 7.5, containing 30 mM sodium acetate), and re-suspended in 50 μl of the chondroitinase ABC reaction buffer containing 10 mIU of protease-free chondroitinase ABC. The sample was incubated for 60 min at 37°C. The reaction was stopped by the addition of 150 μl of 95% ethanol containing 1.3% potassium acetate at 0°C. After 1 h, the core glycoprotein was collected by centrifugation at 20,000 $\times$ *g* for 15 min at 4°C. In some experiments, the core glycoprotein moiety was then treated sequentially with 1 mIU of neuraminidase at 37°C for 1 h in 50 μl of 50 mM sodium acetate buffer, pH 5.0, in the presence of 2 mM EDTA, 1 mM *N*-ethylmaleimide, 0.2 mM phenylmethylsulfonyl fluoride, and 70 μM pepstatin A. The enzymatic reaction was stopped by the addition of 150 μl of 95% ethanol containing

1.3% potassium acetate at 0°C. After 1 h, core glycoprotein was further deglycosylated by digestion with 1 IU of N-glycosidase F, and/or with 1 mIU of O-glycosidase at 37°C for 16 h after the denaturation of core protein according to the manufacturer's instructions. The core protein was dissolved in 60 μl of Laemmli sample buffer [31] containing 50 mM dithiothreitol. The mixture was boiled for 2 min. The sample was centrifuged at 20,000 × *g* for 5 min at room temperature and the supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 3% stacking gel with a 7.5% separating gel followed by immunoblotting or lectin-blot analysis.

Lectin-blot analysis

Proteins separated by SDS-PAGE were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) at 60 V overnight at 4°C in 25 mM Tris-glycine buffer, pH 8.3, that contained 20% (v/v) methanol. To block the nonspecific binding of lectins, the membrane was incubated in 3% bovine serum albumin in Tris-buffered saline. The membrane was then treated for 2 h at room temperature with a lectin solution (2.5 μg/ml biotinylated lectin in Tris-buffered saline), and the lectin-reactive bands were stained with a Vectastain ABC elite kit (Vector Labs.) and Konica Immunostaining HRP-1000 (Seikagaku Co.). The intensity of lectin-staining was quantified on a Macintosh computer via an image scanner using the public domain National Institutes of Health Image program. Statistical analysis was performed using Student's *t* test.

Prediction of O-glycosylation sites of NGC core protein

Potential sites for O-glycosylation on NGC core protein were predicted by amino acid sequence comparison with known O-glycosylated proteins listed in the O-GLYCOBASE using the NetOglyc software [32,33].

Other methods

For Western blot analysis, proteins were resolved by SDS-PAGE and electrotransferred on a PVDF membrane as described above. Immunoreactive materials were detected with various antibodies using a Vectastain ABC elite kit and Konica Immunostaining HRP-1000.

To determine the chondroitin sulfate disaccharide composition, chondroitin sulfate preparations (20 nmol hexuronate) were digested with chondroitinase ABC into unsaturated disaccharides. The separation and determination of these unsaturated disaccharides were performed by HPLC on an amine-bound silica column with a linear gradient system of sodium dihydrogen phosphate [34].

Results

Comparison of chondroitin sulfate structure of NGC with those of neurocan and phosphacan

To determine repeating disaccharide compositions of chondroitin sulfate of three brain-specific chondroitin sulfate proteoglycans, NGC, neurocan, and phosphacan, they were purified from P10 rat brains by the methods described under *Materials and methods*. In brief, NGC was purified by immunoaffinity column chromatography from a proteoglycan mixture prepared from detergent extracts of a brain-PBS insoluble fraction, and neurocan and phosphacan were purified by a sequential biochemical step from a brain-PBS soluble fraction. The purity of each proteoglycan was examined by SDS-PAGE (Figure 1). The purified NGC fraction digested with chondroitinase ABC contained faint Coomassie Brilliant Blue (CBB)-positive bands at about 150–200 kDa in addition to the main NGC band at 120 kDa (Figure 1A). The molecular sizes of these faint bands did not change before and after treatment with chondroitinase ABC. Additionally, these bands treated with chondroitinase ABC were not recognized by a mixture of three antibodies against chondroitin sulfate stubs formed on core proteins by digestion of proteoglycans with chondroitinase ABC [35]. Therefore, the purified NGC fraction contains only NGC as a chondroitin sulfate proteoglycan. NGC was also purified to the same purity from adult rat brains (data not shown). Full length-neurocan and phosphacan, a soluble form of RPTPζ/β, were both purified from P10 rat brains to a single band upon SDS-PAGE (Figure 1B and C).

Table 2 summarizes the chondroitin sulfate disaccharide compositions of these purified chondroitin sulfate proteoglycans. The composition of NGC was markedly different from those of neurocan and phosphacan even though all three

Table 2. Compositions of chondroitin sulfate disaccharides of three brain-specific proteoglycans

	Unsaturated disaccharide[a] *(%)*				
Proteoglycan	*ΔDi-0S*	*ΔDi-4S*	*ΔDi-6S*	*ΔDi-diS$_D$*	*ΔDi-diS$_E$*
NGC					
P10	8.4	76.9	10.0	0.7	4.0
Adult	6.2	89.0	2.1	0.2	2.5
Neurocan					
P10	10.2	63.0	24.5	0.8	1.5
Phosphacan					
P10	11.7	64.5	21.9	0.8	1.1

[a] ΔDi-0S: 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi-4S: 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S: 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-diS$_D$: 2-acetamide-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-diS$_E$: 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4,6-bis-*O*-sulfo-D-galactose.

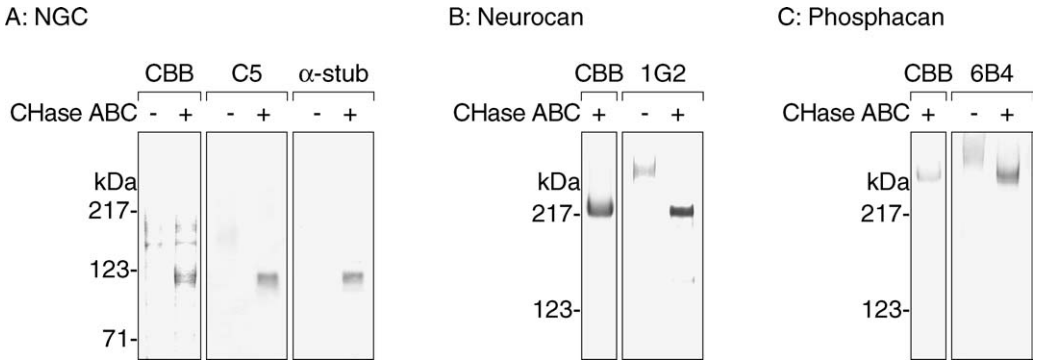

Figure 1. Purities of three brain-specific chondroitin sulfate proteoglycans prepared from P10 rat brains. NGC (A), neurocan (B), and phosphacan (C) were purified from P10 rat brains as described under *Materials and methods*. Proteoglycans were subjected to SDS-PAGE using a 7.5% separating gel for NGC, and 6% gel for the others before (−) and after (+) treatment with protease-free chondroitinase ABC (CHase ABC). Proteins were stained with Coomassie Brilliant Blue (CBB) or immunoblotted using anti-proteoglycan antibodies indicated as follows; C5, a monoclonal anti-NGC antibody; α-stub, a mixture of anti-chondroitin sulfate stub antibodies, 1-B-5, 2-B-6, and 3-B-3; 1G2, a monoclonal anti-neurocan antibody; 6B4, a monoclonal anti-phosphacan antibody. The positions of molecular mass markers are indicated at the left of the panels.

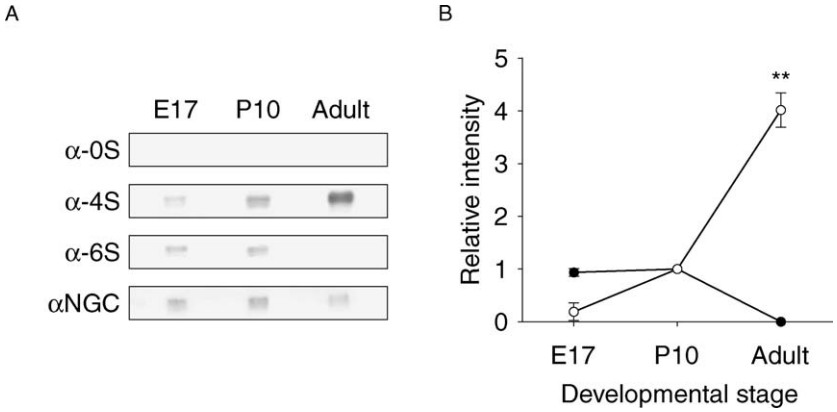

Figure 2. Structural change of chondroitin sulfate stubs on NGC treated with protease-free chondroitinase ABC during brain development. (A) Western blot analyses were performed on immunoaffinity-purified NGC from E17, P10, and adult rat brains, after digestion with protease-free chondroitinase ABC using anti-chondroitin sulfate stub antibodies and anti-NGC antibody (αNGC). α-0S, antibody to unsulfated stub; α-4S, antibody to 4-sulfated stub; α-6S, antibody to 6-sulfated stub. (B) Developmental changes in the amounts of 4-sulfated stub (open circle) and 6-sulfated stub (close circle) of NGC produced by digestion with protease-free chondroitinase ABC. Intensities of immunostains for chondroitin sulfate stubs shown in A were determined by densitometric quantification, and values were normalized to the P10 sample analyzed at the same time. Three independent determinations were carried out. Bars represent SE. $^{**}p < 0.01$ compared with the value of the E17 sample.

proteoglycans were isolated from the brain at the same developmental stage, P10. Two soluble proteoglycans, neurocan and phosphacan, showed an essentially identical composition. However, NGC had a higher proportion of 4-sulfate unit (ΔDi-4S) and a lower proportion of 6-sulfate unit (ΔDi-6S) than these soluble proteoglycans. It should be noted that NGC had a higher proportion of E-unit (ΔDi-diS_E), a highly sulfated disaccharide unit, compared with neurocan and phosphacan. The proportion of both 6-sulfate unit and E-unit decreased with postnatal development of the brain, whereas that of 4-sulfated unit increased.

Developmentally-regulated change was also observed in the structure of chondroitin sulfate linkage region on NGC (Figure 2). Extensive digestion with chondroitinase ABC produces chondroitin sulfate stubs with an unsaturated disaccharide such as ΔDi-0S, ΔDi-4S, or ΔDi-6S at the non-reducing end on the core protein. Using three monoclonal antibodies specific to each of three stub structures, we examined the immunoreactivities of the stubs remaining on the chondroitinase-digested NGC core glycoprotein at E17, P10, and adulthood (Figure 2A). The unsulfated stub was not detectable on the core glycoprotein at any developmental stages examined. The 6-sulfated stub was detectable at E17 and P10, but not at adulthood. The immunoreactivity for the 4-sulfated stub gradually increased with brain development (Figure 2B). These findings

indicate that the structures of both the linkage region and the disaccharide repeating region of chondroitin sulfate of NGC changes in a development-related manner.

Lectin-reactivity of NGC and its comparison with those of neurocan and phosphacan

Results from our previous glycosidase-digestion experiments suggest that NGC bears both O- and N-linked oligosaccharides [20]. In fact, peptide motif analysis using a computer algorithm [32,33] revealed eleven potential O-glycosylation sites on the extracellular domain on the NGC core protein of rats. Eight of eleven potential O-glycosylation sites exist on a short peptide region from Thr128 to Thr162 in the NGC ectodomain. The corresponding region of both mouse NGC and human NGC also contains a cluster of potential O-glycosylation sites, suggesting that the region has a certain functional role as a glycoprotein. Three potential N-glycosylation sites, the consensus triplet sequence Asn-Xaa-Ser/Thr, where Xaa can be any residue except proline [36], were found on the extracellular domain on NGC core protein of the rat [20]. All three potential N-glycosylation sites in the NGC ectodomain are also conserved among rats, mice, and humans.

In order to estimate the structures of O- and N-linked oligosaccharides of NGC, lectin-blot analysis of the NGC core glycoprotein produced by chondroitinase ABC-treatment was performed. Complex-carbohydrate-binding specificities of lectins used in this study are summarized in Table 1. NGC reactivity with those lectins are shown in Table 3 and partially in Figure 3. Jacalin lectin recognized NGC, indicating the occurrence of O-glycosylation on NGC as expected (Figure 3). NGC was also recognized by many lectins that can bind to carbohydrates with structures usually found in *N*-linked oligosaccharides.

It is noteworthy that NGC had a lectin-spectrum different from those of neurocan and phosphacan, even though these three central nervous system-specific proteoglycans were isolated from the brain at the same developmental stage, P10 (Table 3). NGC appeared not to bear mannosylated N-glycans, because Con A did not recognize NGC, whereas it recognized both neurocan and phosphacan. The structure of N-glycans on NGC seemed to differ from those of neurocan and phosphacan, because the NGC reactivity with DSL and PHA-L differed from those of other proteoglycans.

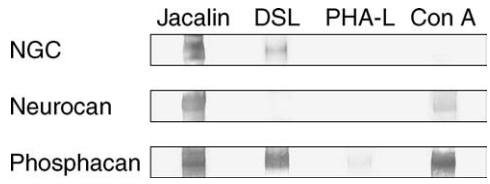

Figure 3. Lectin reactivity of core glycoproteins of three brain-specific proteoglycans. NGC, neurocan, and phosphacan were purified from P10 rat brains as described in Figure 1. Their core glycoproteins, prepared by the digestion of three proteoglycans with protease-free chondroitinase ABC, were resolved by SDS-PAGE, and then blotted to a PVDF membrane. Biotinylated lectins bound to core glycoproteins on the PVDF membrane were visualized using a combination of an ABC elite kit with a Konica kit as described under *Materials and methods*.

Developmental changes in lectin reactivity of NGC

The structure of the chondroitin sulfate moiety of NGC is developmentally regulated as described above, so it is likely that the structures of NGC oligosaccharides change in a development-regulated manner. To examine this, NGC was immunoprecipitated using an anti-NGC antibody from lysates of membrane fractions of E17, P10, and adult rat brains, and was subjected to lectin-blot analysis after digestion with chondroitinase ABC (Figure 4).

The reactivity of NGC core glycoprotein with Jacalin lectin tended to increase slightly, but was not statistically significant, suggesting that the amount and structure of NGC O-glycans do not change markedly during brain development (Figure 4A and B). In contrast, the structure of N-linked oligosaccharides of NGC appeared to change with brain development. N-glycan recognized by DSL seemed to disappear or modify as the brain matured (Figure 4A and C). On the other hand, the fucosylation of NGC *N*-glycans is promoted as the brain matures, because the LCA-reactivity of NGC increased, particularly during postnatal development (Figure 4A and D).

Developmental change in immunoreactivity of NGC for HNK-1 carbohydrate

Many proteins and gangliosides in the central nervous system bear carbohydrate chains that contain 3-*O*-sulfoglucuronic acid residues at their non-reducing ends [37–39]. These carbohydrates are known as HNK-1 carbohydrates. Both neurocan

Table 3. Summary of lectin-reactivities of three brain-specific chondroitin sulfate proteoglycans

	Jacalin	*DSL*	*PHA-L*	*PHA-E*	*Con A*	*LCA*	*AAL*	*sWGA*	*WGA*	*PNA*
NGC	+	+	−	+	−	+	+	−	+	−
Neurocan	+	−	−	+	+	+	+	−	+	+
Phosphacan	+	+	+	+	+	+	+	−	+	+

Reactive (+) and non-reactive (−) lectins with each proteoglycan core protein.

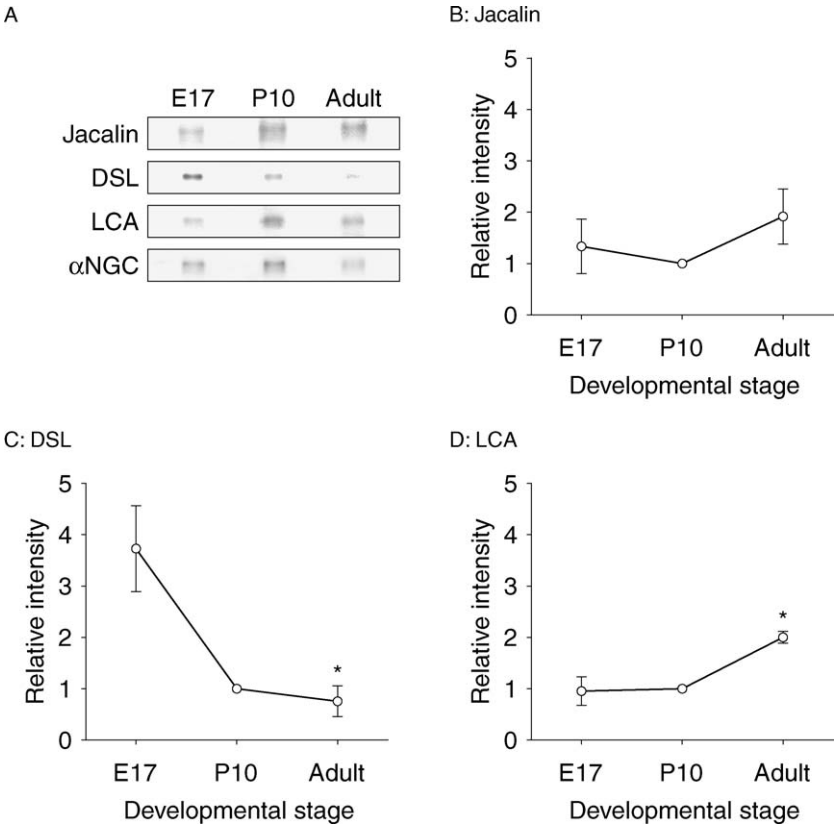

Figure 4. Developmental changes in lectin reactivity of the NGC core protein. (A) NGC was immunoprecipitated using an anti-NGC antibody from lysates of membrane fractions prepared from E17, P10, and adult rat brains. The immunoprecipitates were resolved by SDS-PAGE after digestion with protease-free chondroitinase ABC. Proteins were transferred electrophoretically to a PVDF membrane, and NGC was stained with individual lectins and with an anti-NGC antibody (αNGC). The intensities of lectin reactivity were determined by densitometric quantification. Three independent determinations were carried out, and values were normalized to the immunostain with αNGC of the same sample. Relative intensities of the lectin reactivity to that of the P10 sample were plotted at E17 and adulthood for staining with Jacalin (B), DSL (C), and LCA (D). Bars represent SE. $^{*}p < 0.05$ compared with the value of the E17 sample.

and phosphacan were immunoreactive with an antibody to the HNK-1 carbohydrate as previously reported [40]. NGC was also immunoreactive with the HNK-1 antibody, indicating that NGC bears HNK-1 carbohydrate chains (Figure 5A). The immunoreactivity decreased as the brain matured, and was very low on the NGC core glycoprotein at adulthood (Figure 5B and C). Immunoreactivity for the Lewis X (Le^X) carbohydrate, known to be attached to phosphacan [41], was not observed on the NGC core glycoprotein (Figure 5A).

To clarify whether the HNK-1 carbohydrates are O-linked or N-linked to the NGC core protein, the HNK-1 immunoreactivity of NGC isolated from P10 rat brains was examined before and after treatment with various glycosidases. As reported previously [20], the molecular size of NGC was sequentially reduced upon SDS-PAGE by sequential treatment with neuraminidase, O-glycosidase, and N-glycosidase F (Figure 6A). Although the HNK-1 carbohydrates remained on the NGC core glycoprotein after treatment with neuraminidase and O-glycosidase, they were completely removed from NGC by digestion with N-glycosidase F (Figure 6B). Since there are three potential N-glycosylation sites on the NGC core protein [20], these findings indicate that the HNK-1 epitopes exist at the non-reducing end of N-linked oligosaccharides of NGC, and that the synthesis of the HNK-1 carbohydrates of NGC is strictly regulated in a development-related manner.

Discussion

In this study, we demonstrated the developmental changes in the structures of both chondroitin sulfate and oligosaccharide moieties of NGC, a central nervous system-specific transmembrane proteoglycan. The carbohydrate structure of NGC was significantly different from those of neurocan and phosphacan, other central nervous system-specific chondroitin sulfate proteoglycans, even though they were isolated from brains at the same developmental stage.

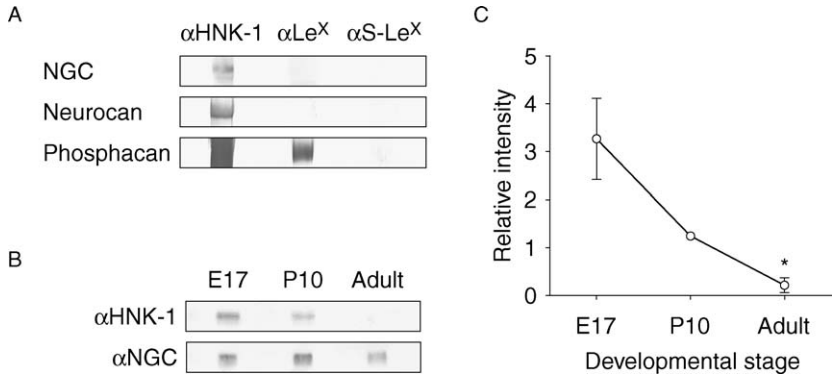

Figure 5. Existence of the HNK-1 epitope and developmental change in its expression on the NGC core glycoprotein. (A) Western blot analyses were performed on NGC, neurocan, and phosphacan purified from P10 rat brains after digestion with protease-free chondroitinase ABC using anti-HNK-1 (αHNK-1), anti-Lewis X (αLe^X), and anti-sialyl Lewis X (αS-Le^X) carbohydrate antibodies. (B) Immunoaffinity-purified NGC from E17, P10, and adult rat brains was immunoblotted using anti-HNK-1 carbohydrate antibody (αHNK-1) and anti-NGC antibody (αNGC). (C) Intensities of immunostains for the HNK-1 epitope on NGC shown in B were determined by densitometric quantification. Three independent determinations were carried out, and values were normalized to the P10 sample analyzed at the same time. Bars represent SE. $^*p < 0.05$ compared with the value of the E17 sample.

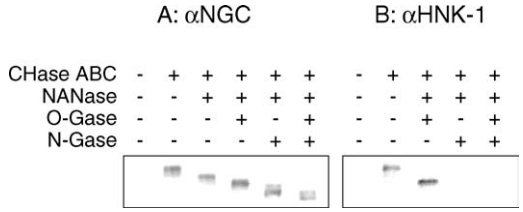

Figure 6. Effect of digestion with various glycosidases on immunoreactivity of the NGC core glycoprotein with anti-HNK-1 carbohydrate antibody. Immunoaffinity-purified NGC from P10 rat brains was digested sequentially with protease-free chondroitinase ABC (CHase ABC), neuraminidase (NANase), *O*-glycosidase (*O*-Gase), and *N*-glycosidase F (*N*-Gase). Deglycosylated materials were resolved by SDS-PAGE using a 7.5% separating gel, and proteins were immunostained with anti-NGC antibody (αNGC; A) and anti-HNK-1 carbohydrate antibody (αHNK-1; B) after blotting to a PVDF membrane.

Chondroitin sulfate of NGC

There have been few reports comparing in detail the carbohydrate structure of a proteoglycan with those of other proteoglycan species isolated from the same tissue at the same developmental stage [40]. Our results indicated that NGC had a disaccharide composition of chondroitin sulfate significantly different from those of neurocan and phosphacan, a soluble splicing variant of RPTPζ/β, although neurocan and phosphacan had an almost identical disaccharide composition. The major disaccharide unit of brain chondroitin sulfate of various animals is 4-sulfate unit, and E-unit, a highly sulfated disaccharide, is also contained in brain chondroitin sulfate preparations as a minor disaccharide unit [42–44]. This is consistent with our results that three major brain proteoglycans have chondroitin sulfate chains abundant in 4-sufate disaccharides (Table 2). It should be noted that the proportion of E-unit in chondroitin sulfate disaccharides of NGC was higher than those of neurocan and phosphacan. This is the first report that proteoglycans purified from the brain actually bear chondroitin sulfate chains containing E-units, although an artificially-expressed proteoglycan in rat C6 glioma cells has been reported to bear E-unit-rich chondroitin sulfate chains [45].

Mouse phosphacan bears chondroitin sulfate chains rich in D-unit, another oversulfated disaccharide unit, and the chondroitin sulfate promotes neurite extension in cultures of fetal rat hippocampal neurons [46]. E-unit-rich chondroitin sulfate also promotes the neurite elongation of primary cultured hippocampal neurons [47]. A number of growth factors and cell adhesion molecules have been shown to interact with high affinity with chondroitin sulfate mainly through its E-unit-rich regions [48,49]. The molecular interactions of chondroitin sulfate with its ligands are implicated in not only neurite elongation but also other cellular events such as migration and adhesion [16,44,50,51]. The search for NGC ligands, especially of chondroitin sulfate is currently in progress.

The disaccharide composition of chondroitin sulfate preparations changes with the development and aging of tissues from which chondroitin sulfates are prepared [43,52]. However, few reports have demonstrated the developmental change in disaccharide composition of a particular proteoglycan molecule purified from any tissues [40]. In this paper, we showed that the proportion of 4-sulfate disaccharides in the chondroitin sulfate moiety of NGC increased with compensatory decreases in the proportions of both 6-sulfate and E-type disaccharides. This developmental change may modify the affinity of the chondroitin sulfate chain with its ligands.

N- and O-linked oligosaccharides of NGC

Lectins, a group of proteins that recognize a particular structure of carbohydrates, are used to estimate carbohydrate structures

based on their structure-specific bindings to carbohydrates. From the lectin-spectrum reactive and non-reactive with the NGC core protein (Table 3), we can estimate some structural characters of N- and O-linked oligosaccharides on the NGC core protein. For example, in the case of N-glycosylation, NGC could bear neither the high-mannose type nor hybrid-type of oligosaccharide because Con A did not bind to NGC. The DSL-positive and PHA-L-negative binding properties of NGC suggested the occurrence of *N*-acetyllactosamine repeats in NGC carbohydrates and this structure may be required for the expression of the HNK-1 epitope. LCA and AAL, two fucosylglycan-binding lectins with different specificities, and PHA-E, a bisecting GlcNAc-binding lectin, bound to NGC. This observation indicated the presence of a complex N-glycan with α1-6-core fucosylation and a bisecting GlcNAc residue on the NGC core protein, and glycoproteins with carbohydrates of this structure have been found in rat brains [53]. The N-linked oligosaccharides on NGC would be highly sialylated at their non-reducing ends because the NGC core protein was WGA-positive but sWGA-negative. Since NGC was highly reactive with Jacalin, it bore O-linked oligosaccharides. Treatment of the NGC core protein with sialidase converted the lectin-binding character from PNA-negative to positive (data not shown), indicating that O-linked oligosaccharides are also sialylated. A computer search for potential O-glycosylation sites on the NGC core protein revealed that a large population of sites was concentrated in a peptide region from Thr128 to Thr162 of NGC. The peptide region may be implicated in molecular interactions of NGC through its O-glycan moiety. The detailed structures of both N- and O-glycans of NGC remain to be determined.

There have been few studies demonstrating the developmental changes in the carbohydrate structures on a single glycoprotein from the fetal period to adulthood [54], although some papers revealed that the carbohydrate structure of a particular glycoprotein prepared from young animals was different from those of adults [55,56]. Our data demonstrated that lectin, as well as the HNK-1 antibody-reactivity of NGC, changed in a lectin-specific manner during brain development from the fetal period to adulthood. Several lines of evidence indicate that many cytokines have lectin-like activities for binding to a particular carbohydrate structure, and that, for at least some cytokines, the binding to a particular carbohydrate at the cell surface is essential for transducing their signals into cells [57,58]. The search for a molecule that binds to the carbohydrate moiety of NGC is currently in progress.

HNK-1 epitope on NGC

HNK-1 carbohydrates, a group of carbohydrate chains with 3-O-sulfoglucuronic acid at their non-reducing end, are present on many neural recognition molecules including cell adhesion molecules, extracellular matrix molecules, proteoglycans, and glycolipids [37–39]. Although the HNK-1 carbohydrate epitope is generally expressed on both *N*-linked and O-linked oligosaccharides on glycoproteins [59–61], in the case of NGC, it was expressed only on N-linked carbohydrate chains (Figure 6). Molecular binding assays have demonstrated that HNK-1 carbohydrates can bind to various proteins such as laminin, amphoterin, and interleukin-6 [62–64]. Through these molecular interactions, HNK-1 carbohydrates are believed to be involved in various cellular events including cell migration, adhesion, and neurite elongation [65–68].

The expression of the HNK-1 carbohydrate epitope is regulated developmentally and spatially in the central nervous system [69,70]. This study demonstrated that the expression level of the HNK-1 carbohydrate epitope on NGC gradually decreased with brain development (Figure 5B and C). This finding suggests that the HNK-1 carbohydrate epitope plays a role in the development-regulated modulation of molecular NGC interactions at the neuronal cell surface.

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