Oversulfated Chondroitin Sulfate Plays Critical Roles in the Neuronal Migration in the Cerebral Cortex

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Chondroitin sulfate (CS) proteoglycans bind with various proteins through CS chains in a CS structure-dependent manner, in which oversulfated structures, such as iB (IdoA(2-O-sulfate)α1–3GalNAc(4-O-sulfate)), D (GlcA(2-O-sulfate)β1–3GalNAc(6-O-sulfate)), and E (GlcAβ1–3GalNAc(4,6-O-disulfate)) units constitute the critical functional module. In this study, we examined the expression and function of three CS sulfotransferases in the developing neocortex: uronyl 2-O-sulfotransferase (UST), N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase (4,6-ST) and dermatan 4-O-sulfotransferase-1 (D4-ST), which are responsible for the synthesis of oversulfated structures. The CS chains of the neocortex of mouse embryos contained significant amounts of D and E units that are generated by UST and 4,6-ST, respectively. UST and 4,6-ST mRNAs were expressed in the ventricular and subventricular zones, and their expression increased during late embryonic development. In utero electroporation experiments indicated that knockdown of UST and 4,6-ST resulted in the disturbed migration of cortical neurons. The neurons electroporated with the short hairpin RNA constructs of UST and 4,6-ST accumulated in the lower intermediate zone and in the subventricular zone, showing a multipolar morphology. The cDNA constructs of UST and 4,6-ST rescued the defects caused by the RNA interference, and the neurons were able to migrate radially. On the other hand, knockdown of D4-ST, which is involved in the biosynthesis of the iB unit, caused no migratory defects. These results revealed that specific oversulfated structures in CS chains play critical roles in the migration of neuronal precursors during cortical development.

Chondroitin sulfate (CS) proteoglycans are major components of the extracellular matrix and cell surface in the central nervous system (CNS) (1). It has been revealed that CS proteoglycans play important roles in the maintenance of neural stem cells (2–4), neuronal migration (5), neurite extension (6–10), neural plasticity (11), and axonal regeneration (12, 13). The CS portions are considered to be the major determinants of function, because digestion with chondroitinase ABC (CHase ABC) diminishes many of the activities of CS proteoglycans (1). Recent biochemical studies have shown that CS proteoglycans bind with various growth factors, chemokines, and extracellular matrix molecules via CS chains in a CS structure-dependent manner (14–19). However, little is known about the significance of structural variation of CS chains in the nervous system.

CS chains are synthesized in the Golgi apparatus by sequential modifications after the polymerization of repeating disaccharide units of GlcA and GalNAc (O unit) (20). In the process of biosynthesis, some of the GlcA residues are converted to IdoA by chondroitin-glucuronate C5-epimerase, producing the iO unit (IdoAα1–3GalNAc), and then the CS precursors are heavily sulfated by various sulfotransferases (Fig. 1A). Although most of the disaccharide units are monosulfated ones that are classified as the A unit (GlcAβ1–3GalNAc(4-O-sulfate)) and C unit (GlcAβ1–3GalNAc(6-O-sulfate)), a minor but significant portion is disulfated (2, 21, 22). Recent studies revealed that the disulfated disaccharide units, which are classified into iB (IdoA(2-O-sulfate)α1–3GalNAc(4-O-sulfate)), D (GlcA(2-O-sulfate)β1–3GalNAc(6-O-sulfate)), and E (GlcAβ1–3GalNAc(4,6-O-disulfate)) units (Fig. 1A), constitute the binding sites for various heparin-binding growth factors, regulating their physiological activities (15, 17, 18).

Several in vitro studies suggested that oversulfated structures in CS chains are involved in neuritogenesis and neuronal migration (5, 8, 9, 23, 24). Notably, by using a Boyden chamber cell migration assay, we previously demonstrated that D unit- and E unit-rich CS preparations strongly inhibited the pleiotrophin/midkine-induced migration of cortical neurons (5, 25). However, no in vivo evidence has been presented for the function.
tions of oversulfated structures in the CNS. Three sulfotransferases, uronyl 2-O-sulfotransferase (UST) (26), N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase (4,6-ST) (27), and dermatan 4-O-sulfotransferase-1 (D4-ST) (28), play critical roles in the biosynthesis of oversulfated structures (Fig. 1A). D units are generated by UST from C units, and E units are synthesized through sulfation of A units by 4,6-ST. IiB units are generated by UST from A units (IdoA1–3GalNAc(4-O-sulfate)) that are generated by sulfation of 1o units by D4-ST. In this study, we analyzed the expression of these sulfotransferases and tried to reveal the in vivo functions of CS-disulfated disaccharide structures in the developing cortex using in utero RNAi. By knockdown or overexpression of UST and 4,6-ST, we could down- or up-regulate the expression of disulfated disaccharide units in the CS chains. Knockdown of UST and 4,6-ST resulted in severe defects in the radial migration of cortical neurons, and the cDNA constructs of these sulfotransferases rescued this defect. These observations suggest that the expression of CS-oversulfated structures changes the behavior of neurons, possibly by modifying their modes of interaction with extracellular environments.

**EXPERIMENTAL PROCEDURES**

**Animals**—Pregnant ICR mice were purchased from Japan SLC Inc. (Shizuoka, Japan). All of the animal experiments were performed with the approval by the Animal Use and Care Committee of the Tokyo Metropolitan Institute for Neuroscience.

**Disaccharide Composition Analysis of CS Chains**—After ether anesthesia, cerebella of embryonic day 16 (E16), E18, postnatal day 1 (P1), P7, P10, P14, and P20 mice were dissected out, embedded in OCT compound (Sakura, Tokyo, Japan), and frozen in liquid nitrogen. They were cut into 16-μm-thick sagittal sections, which were collected onto glass slides. The hippocampal and striatal regions were scraped off with a microsurgical blade under a stereomicroscope. The CS disaccharide composition was analyzed using trimmed sections 40–60 mm² in total area. The sections on the glass slides were fixed with 1 ml of methanol/aceton (1:1, v/v) at room temperature for 5 min. After the solution was discarded, the slides were further treated with 1 ml of the same solution for 5 min twice. The sections were air-dried and then washed twice with distilled water. They were again air-dried and treated with 10% bovine serum albumin (BSA) in distilled water for 15 min. After three washes with distilled water, the sections were equilibrated with 100 mM ammonium acetate for 2 min. They were then treated with 100 μl of 0.3 units/ml CHase ABC (Seikagaku, Tokyo, Japan) in 100 mM ammonium acetate for 2 h at 37 °C. After the solution was discarded, the sections were collected into microcentrifuge tubes, and the sections were extracted with 500 μl of distilled water. The combined solutions were centrifuged at 15,000 × g for 15 min, and the supernatants were dried by SpeedVac lyophilization. The dried materials were treated with 2-aminobenzamide and analyzed with anion exchange HPLC as described above.

**Quantitative Real Time RT-PCR**—After ether anesthesia, the cerebral cortices were dissected out from ICR mice, and the total RNA was purified using an RNAsol minikit (Qiagen, Hilden, Germany). For the synthesis of first strand cDNA, 2 μg of the total RNA was treated with ReverTra Ace and an oligo(dT)20 RT primer (Toyobo, Osaka, Japan). Primer sequences of CS sulfotransferases are as follows: for UST, 5’-AGACA-TGGTCACCCTTCACTTCACTT-3’ (forward) and 5’-CAAAGGCGAGCAGAATACCTATCAGATTAGA-3’ (reverse); for 4,6-ST, 5’-ATAATGGTTTCTGTGTAATCCTCCAGCAAA-3’ (forward) and 5’-AGTAGAGCACGTAGGATGGTGTCAG-3’ (reverse); for D4-ST, 5’-GCCCTGTCTAACTGGAAAACG-3’ (forward) and 5’-CCTGCCAGACACGAACCAAGTCA-3’ (reverse). Quantitative real time RT-PCR was performed using SYBR Green Master Mix (QuantiTect SYBR Green PCR Kit; Qiagen) and an ABI PRISM 7500 (Applied Biosystems). The expression levels of the genes were normalized to that of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) using the following primers: 5’-AGTCTCTGCAACTGGGTCTACACC-3’ (forward) and 5’-AGTTGTCATATTTTCTCGGTGTT-3’ (reverse).

**In Situ Hybridization**—Fragments of CS sulfotransferase genes were amplified by PCR from a mouse brain cDNA library (Clontech, Mountain View, CA) using the following primer sets: for UST (685 bp), 5’-GCTACCCCTGGTGTTCTCTCT-3’ (forward) and 5’-CAGAGGAGAATTTGTTGTT-3’ (reverse); for 4,6-ST (681 bp), 5’-GCTCGAGGATTGATGGACCATAC-3’ (forward) and 5’-GCCGAGCTGTCTCTCTCTGAGAC-3’ (reverse); for D4-ST (393 bp), 5’-TTCCTCCCAGGCTCTCAGGCT-3’ (forward) and 5’-TCCACCGGCAAGTCACCA-3’ (reverse). The amplified CS sulfotransferase gene fragments were inserted into the pBluescript II KS(+) vector (Strategene, La Jolla, CA), and the antisense or sense probes were produced using T7 or T3 RNA polymerase (Roche Applied Science) in the presence of fluorescein RNA labeling mix (Roche Applied Science).

After ether anesthesia, brains of E14, E16, and E18 embryos were dissected out and immersion-fixed in Bouin’s solution and then embedded in paraffin after dehydration through a graded alcohol series. The paraffin-embedded brains were cut into 5-μm-thick sagittal sections, which were deparaffinized and then pretreated with 0.2 N HCl and 20 μg/ml proteinase K. The COS-7 cells cultured under a serum-free condition on 6-cm diameter dishes were washed with 5 ml of phosphate-buffered saline (PBS) three times and then fixed with 1 ml of methanol/aceton (1:1, v/v) at room temperature for 5 min. The cells were scraped off using a cell scraper, and the cell suspensions were centrifuged at 15,000 × g for 15 min. The cell pellets were washed with 500 μl of methanol twice and then air-dried. The cells were treated with 100 μl of 0.3 units/ml CHase ABC in 100 mM ammonium acetate for 2 h at 37 °C. The cell suspensions were centrifuged at 15,000 × g for 15 min, and the resultant supernatants were mixed with three volumes of ethanol. After incubation at −20 °C for 30 min, the solutions were centrifuged at 15,000 × g for 15 min, and the supernatants were dried by SpeedVac lyophilization. The dried materials were treated with 2-aminobenzamide and analyzed with anion exchange HPLC as described above.

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Pack PA-03 column (YMC, Kyoto, Japan) according to the method described by Mitsunaga et al. (21).
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After prehybridizing in 50% formamide, 5 × SSC, they were hybridized with the riboprobes in the hybridization buffer (50% formamide, 5 × SSC, 1 × Denhardt’s solution, 0.1 mg/ml heparin, 10 mM dithiothreitol, 10% dextran sulfate, 0.1 mg/ml salmon sperm DNA, 0.1 mg/ml yeast tRNA) at 70 °C for 18 h. The washing step was performed sequentially as follows: 1) 4 × SSC, 2) 2 × SSC at 65 °C for 30 min (twice), 3) 0.1 × SSC at 70 °C for 1 h (twice), and 4) 100 mM Tris-HCl, pH 7.5, 150 mM NaCl for 5 min (twice). After washing, the sections were incubated in the blocking reagent and then with mouse anti-fluorescein antibody (Roche Applied Science) (1:200) at 4 °C overnight. Next, the sections were incubated with Alexa Fluor 488 anti-mouse IgG (Molecular Probes, Eugene, OR) (1:200). The samples were observed under an FV1000 confocal laser-scanning microscope (Olympus, Tokyo, Japan). Digital images were processed for publication using Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA) with minimal adjustments of brightness and contrast applied to the whole images.

Construction of Plasmids for RNAi and Evaluation of Their Knockdown Effects—The targeted regions, which only matched with UST, 4,6-ST, and D4-ST cDNA sequences, were selected and inserted into U6 promoter-based short hairpin RNA (shRNA) expression vector, pRNAT-U6.1 (GenScript, Piscataway, NJ), which expresses a GFP marker along with shRNA under the control of the CMV promoter. We used two or three regions that had sufficient knockdown effect on the targeted genes. The targeted 19-nucleotide sequences are as follows: for UST, 5′-GGACTACGGTGTCTCAG-3′ (UST-sh-1) and 5′-GAATATAGTACCGGCAGA-3′ (UST-sh-2); for 4,6-ST, 5′-AGACAGAAGTTGCATATG-3′ (4,6-ST-sh-1), 5′-GGACTATCCGAGCATTAAA-3′ (4,6-ST-sh-2), and 5′-GAGGTCTAGCAGCTACT-3′ (4,6-ST-sh-3); for D4-ST, 5′-GGACAGATCCTAAGGCTTAG-3′ (D4-ST-sh-1) and 5′-GATGGTGCCGTTAAGCAGTGT-3′ (4,6-ST-sh-2). The data obtained by using the shRNA plasmids constructed with UST-sh-1, 4,6-ST-sh-1, and D4-ST-sh-1 were presented, unless otherwise stated. As controls, scrambled sequences for UST-sh-1, 4,6-ST-sh-1, and D4-ST-sh-1 were inserted into the pRNAT vector: for UST, 5′-GGACTCTGACATAGGTTG-3′ (UST-scr); for 4,6-ST, 5′-AGACAGTTGCATATGTAAG-3′ (4,6-ST-scr); for D4-ST, 5′-GGATCCTGACAAAGCCTAG-3′ (D4-ST-scr). The underlined sequences represent the rearranged segments.

To evaluate the efficiency of shRNA constructs, full-length mouse UST, 4,6-ST, and D4-ST cDNAs were inserted into the pIRE2-DsRed-Express vector (Clontech) (UST-pIRES, 4,6-ST-pIRES, and D4-ST-pIRES, respectively). COS-7 cells in 6-cm dishes were co-transfected with each sulfotransferase cDNA construct and the corresponding shRNA construct (1:4 for 4,6-ST and D4-ST and 1:8 for UST) and cultured for 72 h under serum-free conditions. The CS disaccharide composition was determined as described above.

For the rescue experiments, we prepared the following sulfotransferase cDNA constructs, in which six mutations were inserted into the target sequences for the shRNAs with no amino acid sequence alteration. For UST, the target region of UST-sh-1 in UST-pIRES was mutated to 5′-TGATTATGGGTTTTGATG-3′. For 4,6-ST, the target region of 4,6-ST-sh-2 in 4,6-ST-pIRES was mutated to 5′-GGATATCCGAGTATGAAGG-3′. The underlined nucleotides represent the mutations introduced.

In Utero Electroporation—In utero electroporation was performed according to the methods described by Tabata and Nakajima (29). Briefly, timed pregnant ICR mice (E14) were anesthetized with sodium pentobarbital at 40 mg/kg of body weight, and the uterine horns were exposed. A plasmid DNA solution (4 μg/μl) containing 0.01% Fast Green was injected (~2 μl) into the left side of the lateral ventricle with heat-pulled glass micropipettes. The heads of embryos in the uterus were placed between the forceps-type electrode (5 mm in diameter, CUY650P5, Nepa Gene, Chiba, Japan), and four electrical pulses (35 V, 50 ms in duration at intervals of 950 ms) were delivered using an electroporator (CUY21; Nepa Gene). All of the electroporations were done targeting the dorsolateral region of the neocortex adjacent to the lateral ventricle. The uterine horns were returned into the abdominal cavity, and the embryos were allowed to develop for appropriate periods. For the rescue experiments, the embryos were co-electroporated with the mixture (~2 μl) of shRNA construct (4 μg/μl) and rescue construct (8 μg/μl). For the histological analyses, the mice were anesthetized with ether, and the embryos were dissected out. The brains were removed from the embryos and immersion-fixed in 4% paraformaldehyde/PBS at 4 °C for 6–8 h. After being washed with PBS, they were incubated in 30% sucrose/PBS for cryoprotection at 4 °C overnight. They were embedded in OCT compound and cut into 20–30-μm-thick coronal sections. The sections were counterstained with TOPRO-3 (Molecular Probes) and observed under an FV1000 confocal laser-scanning microscope. For the analyses of neuronal migration, the images of the coronal sections were divided evenly into 10 compartments from the outermost region of cortical plate to the ventricular zone, and the number of GFP-positive cells in each compartment was counted. To determine the distribution of electroporated cells, the positions of 200~500 GFP-positive cells were analyzed for the each brain. For each condition, at least four independently electroporated brains were analyzed.

Immunohistochemistry—After ether anesthesia, the heads of embryos were dissected, immersion-fixed in 4% paraformaldehyde, 0.1 M sodium phosphate buffer, pH 7.4, at 4 °C for 6–8 h and embedded in paraffin. The samples were cut into 5-μm-thick sagittal sections, which were then deparaffinized and embedded in paraffin. The sections were divided for publication using Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA) with minimal adjustments of brightness and contrast applied to the whole images.

Immunohistochemistry—After ether anesthesia, the heads of embryos were dissected, immersion-fixed in 4% paraformaldehyde, 0.1 M sodium phosphate buffer, pH 7.4, at 4 °C for 6–8 h and embedded in paraffin. The samples were cut into 5-μm-thick sagittal sections, which were then deparaffinized and treated with 2.5% hydrogen peroxide/PBS for 15 min. After blocking with 4% normal goat serum, 1% BSA/PBS for 30 min, the sections were incubated with MO-225 monoclonal antibody (Seikagaku) (1:200) in 1% BSA/PBS at room temperature for 90 min. The sections were treated with biotinylated antimouse IgM (GE Healthcare) (1:200) at room temperature for 60
min and then processed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). After being washed with PBS, they were treated with 0.02% 3,3′-diaminobenzidine, 0.02% hydrogen peroxide, 50 mM Tris-HCl, pH 7.5. The sections were observed under a Zeiss Axioskop, and the images were captured using an AxioCAM HRc CCD camera (Carl Zeiss, Oberkochen, Germany) and processed as described above.

The electroporated brain samples were fixed in 4% paraformaldehyde, 0.1 mM sodium phosphate buffer, pH 7.4, at 4 °C for 6–8 h, embedded in OCT compound, and then cut into 15-μm thick coronal sections. After blocking with 4% normal goat serum, 1% BSA/PBS, the sections were incubated with MO-225 (1:200) at 4 °C overnight. After a wash with PBS, the sections were incubated with Alexa Fluor 594 goat anti-mouse IgM (Molecular Probes) (1:200) and Hoechst 33258 (Dojindo, Kumamoto, Japan) (2 μg/ml) for 30 min at room temperature. For double fluorescent labeling, the sections were treated with 5% normal goat serum, 0.5% Triton X-100/PBS for 1 h at room temperature and then incubated with a mixture of monoclonal anti-GFAP (Clone G-A-5; Sigma) (1:400) and rabbit anti-GFP (Chemicon, Temecula, CA) (1:400), or a mixture of monoclonal anti-β-III tubulin (TUJ1) (Covance, Emeryville, CA) (1:500) and anti-GFP (1:400) in 1% BSA/PBS at 4 °C overnight. After a wash with PBS, the sections were treated with Alexa Fluor 594 goat anti-mouse IgG (1:200) and Alexa Fluor 488 goat anti-rabbit IgG (1:200) in 1% BSA/PBS for 1 h at 4 °C.

![FIGURE 1. Schematic representation of CS biosynthetic pathway and the structural change of CS in the developing cerebral cortex.](image)

**A**. CS chains are synthesized in the Golgi apparatus by sequential modifications after the polymerization of the repeating disaccharide units of GlcA and GalNAc (O unit). Many of the GalNAc residues are 4-O-sulfated (A unit) by chondroitin 4-O-sulfotransferases (C4-ST) or 6-O-sulfated (C unit) by chondroitin 6-O-sulfotransferases (C6-ST). Although A and C units are the major components of CS chains, a portion of the disaccharide units have two or three sulfate residues, which are called oversulfated structures: D unit, E unit, and T unit. D units are synthesized by UST from C units, and E units are generated by sulfation of A units by 4,6-ST. In addition, some of the GlcA residues are converted to 1,6-diazoxy-glucuronate 6-sulfate (CS-EP), which leads to the production of iA unit and iB unit structures through subsequent sulfation by D4-ST and UST. Highly iduronated CS chains are often called dermatan sulfate. B and C, the cortical sections from animals of various ages were treated with CHase ABC, and the resultant unsaturated disaccharides were labeled with 2-aminobenzamide and analyzed by HPLC. The developmental changes in the unsaturated disaccharide composition are shown in B. A and iA units and B and iB units are collectively quantified as ΔA unit and ΔB unit, respectively, because CHase ABC cannot distinguish between them producing common degradation products. The developmental changes in the amounts of oversulfated structures are shown separately in C. Data were obtained from triplicate experiments. Each bar represents the mean ± S.D.

**TABLE 1**

Disaccharide composition of CS chains in the developing cerebral cortex

| Disaccharide composition | E16 | E18 | P1  | P7  | P10 | P14 | P20 |
|-------------------------|-----|-----|-----|-----|-----|-----|-----|
| %                       | %   | %   | %   | %   | %   | %   | %   |
| ΔO unit                 | 30.3 ± 1.3 | 28.0 ± 4.1 | 15.5 ± 5.6 | 16.3 ± 1.5 | 13.4 ± 0.7 | 11.2 ± 0.9 | 7.8 ± 1.3 |
| ΔA unit                 | 53.2 ± 1.8 | 57.7 ± 3.1 | 64.2 ± 3.5 | 58.9 ± 0.2 | 67.8 ± 0.3 | 76.1 ± 1.2 | 83.4 ± 1.2 |
| ΔC unit                 | 12.2 ± 0.5 | 10.2 ± 0.7 | 16.2 ± 2.1 | 21.5 ± 1.4 | 15.8 ± 0.4 | 9.9 ± 0.6 | 5.9 ± 0.2 |
| ΔD unit                 | 0.18 ± 0.06 | 0.25 ± 0.02 | 0.23 ± 0.03 | 0.18 ± 0.01 | 0.19 ± 0.02 | 0.19 ± 0.01 | 0.23 ± 0.03 |
| ΔE unit                 | 0.52 ± 0.01 | 0.32 ± 0.02 | 0.61 ± 0.07 | 0.71 ± 0.02 | 0.69 ± 0.03 | 0.80 ± 0.06 | 0.89 ± 0.05 |
| ΔF unit                 | 3.4 ± 0.2 | 3.4 ± 0.4 | 3.0 ± 0.1 | 2.3 ± 0.1 | 2.0 ± 0.1 | 1.7 ± 0.1 | 1.6 ± 0.1 |
| ΔT unit                 | 0.11 ± 0.03 | 0.15 ± 0.02 | 0.11 ± 0.03 | 0.12 ± 0.01 | 0.13 ± 0.01 | 0.11 ± 0.02 | 0.11 ± 0.02 |
| Total amounts (pmol/mm²) | 216 ± 13 | 281 ± 27 | 181 ± 21 | 482 ± 60 | 526 ± 19 | 582 ± 156 | 488 ± 31 |
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that CS of rat telencephalon was relatively enriched with the ΔE unit at E14 and P8.

Expression of CS Sulfotransferase mRNAs in the Developing Cortex—The expression levels of three CS sulfotransferase genes (UST, 4,6-ST, and D4-ST), which are involved in the synthesis of disulfated disaccharides, were analyzed using a real time RT-PCR analysis (Fig. 2). The expression level of UST mRNA gradually increased from E14 to P10 and decreased thereafter (Fig. 2A). 4,6-ST mRNA remarkably increased from E14 to E18 and then decreased during postnatal development (Fig. 2B). There were low levels of D4-ST expression during the fetal stage, and the expression gradually decreased postnatally (Fig. 2C). The expression of 4,6-ST mRNA correlated well with that of the D unit in CS chains (Figs. 1C and 2B). The expression of UST mRNA also roughly correlated with that of the D unit, although the increase of UST mRNA from E16 to E18, and the decrease after P14 did not reflect upon the expression of D unit (Figs. 1C and 2A).

Next, we performed in situ hybridization experiments to reveal the spatiotemporal expression of these sulfotransferase mRNAs in the developing neocortex. During the middle and late embryonic development, the neocortical neural progenitors called radial glia proliferate in the ventricular zone (VZ) and generate many bipolar precursor neurons (2, 3). These bipolar cells transit into a multipolar stage in the subventricular zone (SVZ), and then change into a bipolar shape and migrate radially from the IZ to cortical plate (CP) along radial glial fibers. The radial glial fibers are radially oriented processes of radial glia and serve as a scaffold for neuronal migration (5).

At E14, the signals of the mRNAs for UST, 4,6-ST, and D4-ST were clearly detected in the VZ (Fig. 3A–C). At E16, the signals for these sulfotransferase genes were observed in the...
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SVZ and VZ (Fig. 3, D–F). At E18, the signals became weak in the SVZ, but the cells lining the ventricular surface strongly expressed these genes (Fig. 3, G–I). In the CP, weak expression of UST was observed from E14 to E16; however, the expression levels of the other genes were low. These results suggest that D and E units generated by UST and 4,6-ST, respectively, play significant roles in the SVZ and VZ during middle and late embryonic development. Akita et al. (4) also observed the expression of UST and D4-ST mRNAs in the VZ at E13.

Effects of the Knockdown of CS Sulfotransferases on the CS Structure—To investigate the functions of UST, 4,6-ST and D4-ST in the development of the cortex, we tried to decrease the expression of these enzymes in the VZ and SVZ cells by using shRNA. For each sulfotransferase, three shRNA constructs were designed, in which shRNAs were driven by the U6 promoter in the pRNAT vector. To evaluate the knockdown effects of each construct, the shRNA construct was co-transfected with the target sulfotransferase cDNA construct into COS-7 cells, and the mRNA level of the target gene was checked with the samples co-transfected with the mock or the scrambled shRNA construct (only the results obtained by using UST-sh-1 and D4-ST-sh-1 are shown in Fig. 4A). On the other hand, all three shRNA constructs of 4,6-ST showed significant knockdown effects (only the results obtained by using 4,6-ST-sh-1 are shown in Fig. 4A).

Next, we checked whether the knockdown of sulfotransferases could change the structure of CS chains (Fig. 4, C–E). CS chains of COS-7 cells were composed of ~70% ΔA, ~20% ΔC, ~8% ΔE, and trace amounts of ΔB and ΔD units (Fig. 4, C–E, mRNA was evaluated by semiquantitative RT-PCR (top). Compared with the cells co-transfected with the control scrambled shRNA construct (scr) or the empty pRNAT vector (mock), the mRNA level of UST was significantly decreased in the cells co-transfected with the shRNA construct (sh) (a). The shRNA constructs of 4,6-ST and D4-ST also decreased the mRNA levels of mouse 4,6-ST and D4-ST in the COS-7 cells, respectively (b and c). The expression of GAPDH mRNA was not altered by any of the treatments (bottom). In these experiments, more than 80% of the cells were positive for GFP expression. B, the embryonic brains were electroporated with the shRNA construct of UST (sh) at E14 and harvested after 2 days. The GFP-positive cortical regions were dissected under a fluorescent stereomicroscope. The corresponding cortical regions were dissected from the brains electroporated with the control scrambled shRNA construct of UST (scr) or empty pRNAT vector (mock). Total RNA was prepared from these tissue samples, and the expression level of UST mRNAs was evaluated by semiquantitative RT-PCR (top). a. The UST mRNA level was decreased by the shRNA compared with the scrambled and mock controls. The electroporation of shRNA constructs of 4,6-ST and D4-ST also decreased the levels of 4,6-ST and D4-ST mRNAs, respectively (b and c). The expression of GAPDH mRNA was not altered by any of the treatments (bottom). C–E, the unsaturated disaccharide composition of CS chains in COS-7 cells co-transfected with the empty pires and pRNAT vectors (open columns), with the cDNA construct of CS sulfotransferase and the empty pRNAT vector (filled columns) or with the cDNA construct of CS sulfotransferase, and its shRNA construct (gray columns) was analyzed using anion exchange HPLC. Increased expression of UST significantly increased the amounts of 2-O-sulfated ΔB and ΔD units, and the shRNA construct of UST down-regulated the expression of these units to near the control levels. D, overexpression of 4,6-ST significantly increased the amount of ΔE unit, and the shRNA construct of 4,6-ST down-regulated the expression of this unit to near the control levels. E, neither overexpression nor RNAi of D4-ST altered the unsaturated disaccharide composition of CS chains. Data were obtained from triplicate experiments. Each bar represents the mean ± S.D. (*, p < 0.05; **, p < 0.005; t test).

FIGURE 4. The knockdown effects of shRNA constructs. A, COS-7 cells were co-transfected with the cDNA construct of mouse UST and its shRNA construct. The cells were cultured for 48 h, and the expression level of mouse UST...
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empty columns). When UST was overexpressed in COS-7 cells, the amounts of 2-O-sulfated ΔB and ΔD disulfated disaccharide units significantly increased (from 0.5 ± 0.06 to 4.3 ± 0.7% for ΔB unit; from 1.8 ± 0.6 to 3.5 ± 0.8% for ΔD unit) without a change in the amount of ΔE unit (Fig. 4C, filled columns). On the other hand, when the UST cDNA and its shRNA construct were co-transfected, the amounts of ΔB and ΔD units decreased (1.8 ± 0.5% for ΔB unit; 2.4 ± 0.7% for ΔD unit) compared with the UST-overexpressing samples (Fig. 4C, gray columns). When 4,6-ST was overexpressed in the COS-7 cells, the amount of ΔE unit significantly increased compared with the control (from 10.2 ± 1.3 to 15.2 ± 1.3%) without changes in the amounts of ΔB and ΔD units (Fig. 4D, filled columns). The amount of ΔA unit was significantly decreased by the overexpression of 4,6-ST, probably because the E unit was produced using a part of the A unit as a precursor. When 4,6-ST cDNA and its shRNA constructs were co-transfected, the amounts of the ΔE unit significantly decreased (11.9 ± 2.5%) compared with the 4,6-ST-overexpressing samples (Fig. 4D, gray columns). In contrast, overexpression and knockdown of D4-ST did not change the amounts of any of the unsaturated disaccharide units (Fig. 4E). However, it should be noted that our HPLC analysis could not distinguish between A and iA units in CS chains, so they were collectively quantified as ΔA units, because CHase ABC degrades both structures, producing common degradation products. Thus, there is a possibility that overexpression and knockdown of D4-ST changed the ratio of the amounts of A to iA units. These results indicated that knockdown of UST and 4,6-ST down-regulated the expression of 2-O-sulfated disaccharide units and E unit, respectively, although the effects of D4-ST knockdown were unclear.

Effects of the Knockdown of CS Sulfotransferases on the Neuronal Migration—To analyze the functions of disulfated CS structures in cortical development, shRNA constructs of CS sulfotransferases were injected into the lateral ventricle of E14 embryos. Application of electrical pulses across the heads of embryos resulted in the electroporation of the DNAs into a subset of VZ cells lining the ventricle. The brains of embryos were harvested 4 days later (E18), and the electroporated cells expressing GFP were observed using a confocal laser-scanning microscope (Fig. 5).

As in the case of COS-7 cells, each shRNA construct decreased the mRNA expression for the target gene in the electroporated areas (Fig. 4B). We could not determine the changes in CS disaccharide composition in the electroporated areas because of a lack of sensitivity in the HPLC analysis. When the empty pRNAT vector was electroporated, the GFP-positive cells actively migrated from the VZ to the upper region of the CP (Fig. 5H). In contrast, electroporation of the UST shRNA construct disturbed migration of these cells, which were accumulated in the SVZ and in the lower IZ (Fig. 5, B and I, and 6, A and B). These accumulated cells showed multipolar morphology (Fig. 5I, arrows), which was similar to that of multipolar neurons seen in the SVZ and IZ of the control sections (Fig. 5K, arrow). There were few GFP-positive cells in the upper region of the CP, although several radially migrating neurons were observed in the IZ and in the deep CP (Fig. 5, B and I, arrowhead). Electroporation of the construct containing the scram-bled shRNA sequence of UST resulted in no defects in neuronal migration (Figs. 5F and 6I). Knockdown of 4,6-ST led to more severe defects in neuronal migration (Figs. 5C and 6, C–E). Most of the GFP-positive cells were accumulated in the SVZ and in the lower IZ, and almost no labeled cells were observed in the CP. The GFP-labeled cells showed multipolar morphology in the SVZ and lower IZ (arrowhead). K, cells electroporated with the empty vector showed multipolar (arrow) and bipolar (arrowhead) morphologies in the lower IZ. In order to reveal the morphologies of individual cells, the regions where the density of GFP-labeled cells was low were shown in I, J, and K. MZ, marginal zone. Scale bar, 100 μm (A–H) and 50 μm (I–K).

To assess the off-target effects of shRNA constructs, rescue experiments were performed (Fig. 6). A mixture of the shRNA construct and the sulfotransferase cDNA construct that had six mutations in the targeted sequence for RNAi without an amino acid change was injected into the lateral ventricles of E14 embryos. The embryos were applied with electric pulses, and the GFP-positive cells in the cortex were observed as above. The cells electroporated with the sulfotransferase cDNA construct were identified by DsRed expression, and it was revealed that more than 90% of the GFP-positive cells were co-electroporated with the sulfotransferase cDNA construct. As shown in
The cortices were electroporated with shRNA constructs at E14 and harvested 4 days later. The images of the coronal cortical sections from the electroporated brains were evenly divided into 10 compartments from the outermost region of cortical plate to ventricular zone, as shown in Fig. 5D, and the number of GFP-positive cells in each compartment was counted. The electroporated brains were evenly divided into 10 compartments from the outermost region of cortical plate to ventricular zone, as shown in Fig. 5D, and the number of GFP-positive cells in each compartment was counted. The electroporated shRNA constructs were as follows. A, UST-sh-1; B, UST-sh-2; C, 4,6-ST-sh-1; D, 4,6-ST-sh-2; E, 4,6-ST-sh-3; F, D4-ST-sh-1; G, D4-ST-sh-2; H, empty pRNAT vector; I, UST-scr; J, 4,6-ST-scr; K, D4-ST-scr. Electroporation of shRNA constructs of UST (A and B) and 4,6-ST (C–E) significantly impaired neuronal migration compared with the samples electroporated with the scrambled shRNA constructs of UST (I and J). The phenotypes of UST-sh-1 and 4,6-ST-sh-2 were rescued by the co-electroporation of the CDNA constructs of UST and 4,6-ST, respectively (L and M). The two different shRNA constructs for D4-ST showed no effects on the neuronal migration (F and G). Bars, mean ± S.D. n represents the number of independent brains analyzed for each condition.

FIGURE 6. Distribution of the electroporated cells in the cerebral cortex.

The cortices were electroporated with shRNA constructs at E14 and harvested 4 days later. The images of the coronal cortical sections from the electroporated brains were evenly divided into 10 compartments from the outermost region of cortical plate to ventricular zone, as shown in Fig. 5D, and the number of GFP-positive cells in each compartment was counted. The electroporated shRNA constructs were as follows. A, UST-sh-1; B, UST-sh-2; C, 4,6-ST-sh-1; D, 4,6-ST-sh-2; E, 4,6-ST-sh-3; F, D4-ST-sh-1; G, D4-ST-sh-2; H, empty pRNAT vector; I, UST-scr; J, 4,6-ST-scr; K, D4-ST-scr. Electroporation of shRNA constructs of UST (A and B) and 4,6-ST (C–E) significantly impaired neuronal migration compared with the samples electroporated with the scrambled shRNA constructs of UST (I and J). The phenotypes of UST-sh-1 and 4,6-ST-sh-2 were rescued by the co-electroporation of the CDNA constructs of UST and 4,6-ST, respectively (L and M). The two different shRNA constructs for D4-ST showed no effects on the neuronal migration (F and G). Bars, mean ± S.D. n represents the number of independent brains analyzed for each condition.

Fig. 6L, the effects of the shRNA construct of UST were rescued by the UST cDNA construct, and many co-transfected neurons migrated to the CP. The effects of the shRNA construct of 4,6-ST were also fully rescued by the 4,6-ST cDNA construct (Fig. 6M). These results indicated that UST and 4,6-ST play critical roles in the radial neuronal migration in the neocortex.

Expression of D Structure in the Developing Cortex—MO-225 is a monoclonal antibody that recognizes CS chains containing the A–D sequence (17, 30, 31). Fig. 7A shows the immunohistochemical staining of E18 cortex with MO-225. The pial surface was strongly stained with the antibody (Fig. 7A, arrowheads), and the IZ, SVZ, and VZ showed moderate staining. Higher magnification indicated that a subset of multipolar cells in the lower IZ showed immunoreactivity (Fig. 7B, arrowheads), and the surroundings of many of the SVZ cells were also stained with MO-225 (Fig. 7C, arrowheads). Most of the cells in the VZ were poorly stained with the antibody, however, the round cells lining the ventricular surface showed immunoreactivity (Fig. 7C, arrows).

Next, to assess the structural changes of CS in the cortex after knockdown of the CS sulfotransferases, the electroporated cortices were stained with MO-225. The SVZ region, where the cells electroporated with the shRNA construct of UST were accumulated, showed reduced immunoreactivity, as compared with the regions not electroporated (Fig. 7, D and E). On the other hand, the areas electroporated with the 4,6-ST-shRNA construct showed no change in immunoreactivity (Fig. 7, G and H). These observations indicated that knockdown of UST specifically down-regulated the expression of the D structure in the SVZ. Because of the lack of specific antibody, we could not check immunohistochemically the change of E unit expression after knockdown of 4,6-ST.

Effects of the Knockdown of Sulfotransferases on Several Aspects of the Differentiation Process—The defects in neuronal migration could be caused by impaired differentiation of neural stem cells into neurons or by the disorganization of...
radial glial fiber, a scaffold for neuronal migration. The GFP-positive cells that accumulated in the lower IZ and SVZ with shRNA expression of UST and 4,6-ST were positive for TUJ1, an early neuronal marker, as control IZ/SVZ neurons (Fig. 8, A–C). Sirko et al. (3) reported that CHase ABC treatments of the developing cortex promoted the differentiation of neural stem cells into GFAP-positive astrocytes, so we immunohistochemically stained the electroporated sections with anti-GFAP antibody. However, GFP-positive cells expressing shRNAs of UST and 4,6-ST showed no GFAP immunoreactivity (Fig. 6). Although the neurons expressing shRNAs

FIGURE 8. Effects of RNAi on neuronal differentiation and the development of radial glial fibers. The embryonic brains were electroporated with the empty pRNAT vector (C and F) or the shRNA constructs of UST (A, D, and G) or 4,6-ST (B, E, and H) at E14 and harvested 4 days later. A–C, the sections were immunohistochemically stained with anti-TUJ1 monoclonal antibody (red). The GFP-positive cells expressing UST (A) and 4,6-ST (B) shRNAs showed immunoreactivity (arrowheads) in the lower IZ and SVZ. D–F, the sections were immunohistochemically stained with anti-GFAP red; arrowheads). The GFP-positive cells electroporated with the UST (D) and the 4,6-ST (E) shRNA constructs were not stained by anti-GFAP like the cells electroporated with the empty vector (F). G and H, the radial glial fibers of the cells electroporated with the shRNA construct of UST (G) or 4,6-ST (H) were observed by tracing GFP staining in the z-stack images. The radial glial fibers (arrows) extended to the pial surface (arrowheads) in both samples. Scale bars, 50 μm (A–C), 10 μm (D–F), and 100 μm (G and H).

DISCUSSION

We demonstrated here that knockdown of UST and 4,6-ST impaired the radial neuronal migration in the neocortex by using an in utero electroporation procedure. In utero electroporation is an effective way to reveal the functional roles of proteins in neuronal migration (32–36). Knockdown experiments using this procedure provide advantages that are not achievable with conventional knock-out approaches (i.e., the acute knockdown of protein expression bypasses the compensatory mechanisms that often operate in animals with a chronic germ line deletion) (35). This is quite important for the functional analysis of glycosaminoglycan sulfotransferases, because glycosaminoglycans are sulfated by many sulfotransferases that may have overlapping functions. In fact, many of the mutant animals with deleted heparan sulfate sulfotransferase genes displayed unexpectedly mild phenotypes, which might be explained by the compensatory increase in sulfation at other positions in heparan sulfate (37–39). In this study, we found that knockdown of UST specifically down-regulated the expression of 2-O-sulfated B and D units in the COS-7 cells without a change in the amount of E unit (Fig. 4). Knockdown of 4,6-ST also specifically decreased the amount of E unit with no change in the contents of other oversulfated units. Immunohistochemical analysis of the in utero electroporated cortex with MO-225 suggested that the CS structures were changed specifically by the knockdown procedure also in vivo (Fig. 7). These observations indicated that knockdown of CS sulfotransferases by in utero electroporation to be an invaluable tool with which to reveal the functions of specific CS structures.

In the developing neocortex, precursors of pyramidal neurons with a simple bipolar shape are generated in the VZ and move to the SVZ, where they temporarily enter a multipolar stage (40). These multipolar cells show meandering behavior in the SVZ and IZ for various periods and then again change into a bipolar shape and migrate radially from the IZ to CP along radial glial fibers. The cells electroporated with the shRNA constructs of UST and 4,6-ST accumulated in the SVZ and lower IZ and showed a multipolar morphology, suggesting that knockdown of these enzymes disturbed the transition of neurons from the multipolar to bipolar stage. This transition could be induced by the change in the adhesive properties of neurons or by the cytoskeletal changes stimulated by extracellular factors, such as chemokines.

Oversulfated disaccharide structures increase the negative charge density along CS chains and can potentiate their electrostatic interactions with the positively charged CS-binding sites in various proteins. If the neurons express oversulfated structure-rich CS and the radial glial fibers express CS-binding proteins, knockdown of UST and 4,6-ST should weaken the electrostatic adhesive interaction between them and thus impair radial migration. It should be noted that the knockdown of 4,6-ST showed stronger effects on neuronal migration than that of UST (Fig. 6). Although the neurons expressing shRNAs

rated with the shRNA constructs of UST and 4,6-ST actively proliferated at the VZ (data not shown). Further systematic studies on the differentiation processes are now in progress in our laboratory.
of 4,6-ST hardly reached the CP, a significant population of UST-knocked down neurons entered this region. Because the cortex contains far more E unit than D unit (Fig. 1), the knockdown of 4,6-ST should decrease the charge density of CS chains more effectively than that of UST. This might explain the difference in the effectiveness of UST and 4,6-ST RNAs. Furthermore, since B unit (iB unit) is a quite minor component of CS chains in the cortex, it is reasonable that knockdown of D4-ST exerted no effects on the neuronal migration (Figs. 1 and 6). However, the radially migrating neurons were poorly stained by MO-225 (Fig. 7), suggesting that at least the D unit may not be involved in the adhesion between neurons and radial glial fibers.

It is known that the CS oversulfated structures constitute the binding sites for various heparin-binding growth factors, such as pleiotrophin, midkine, hepatocyte growth factor (HGF), and several fibroblast growth factors (15, 18, 41). It seems that pleiotrophin, midkine, and HGF are highly relevant to the pres- sure of developing cortex, and evidence obtained in vitro suggested that they are involved in neuronal migration in the neocortex (5, 25, 42, 43). Pleiotrophin and midkine are highly related growth factors and serve as the ligands of a receptor-type protein-tyrosine phosphatase, PTP

HGF is a pleiotrophic cytokine that promotes the motility and proliferation of various types of cells. A tyrosine kinase receptor, cMet, acts as a HGF receptor, and proteoglycans are considered to work as co-receptors that are required to form the active HGF-cMet signaling complex, in which the glycosaminoglycan portion plays critical roles (41). HGF binds with CS and heparan sulfate in a structure-dependent manner, and the cMet-HGF signaling is enhanced by specific types of glycosaminoglycans (41, 45). The preferred HGF-binding sites in CS chains are composed of disulfated disaccharide units, such as iB and E units, and the activity of HGF is markedly promoted by CS enriched with these oversulfated structures (41, 45). Sun et al. (43) demonstrated that HGF induced the migration of cortical neurons by using a Boyden chamber cell migration assay. In the cortex of rat embryos, HGF was localized to the VZ/SVZ and CP, and cMet was expressed by the postmitotic neurons and neural precursor cells (43). Thus, it is plausible that the migration of postmitotic neurons in the VZ/SVZ is induced by HGF after they start to express E unit-rich CS chains. It might be that CS-oversulfated structures act as a molecular switch that triggers the transition in neuronal behavior from the multipolar to bipolar stage.

Recently, it was shown that a gradient formation of semaphorin-3A in the cortex is required for radial neuronal migration (36). It is known that CS proteoglycans fix semaphorin 5A in the extracellular matrix via CS chains and convert this protein from an attractive to an inhibitory guidance cue (46). Thus, there is a possibility that the formation of a gradient of semaphorin-3A in the cortex is also supported by CS proteoglycans. However, since only ~20% of the VZ cells were electroporated with shRNA constructs in our in utero electroporation experiments, it is unlikely that the gradient was globally disrupted by knockdown of CS sulfotransferases.

A failure of normal neuronal migration can result in various neurological disorders, such as epilepsy and mental retardation, so there is a possibility that the CS sulfotransferases are involved in such disorders. In fact, it has been suggested that chondroitin-glucuronate C5 epimerase, which converts GlcA to IdoA in CS chains (Fig. 1A), might be involved in bipolar disorder (47). Furthermore, it has been revealed that there were large changes of CS structure in the CNS after injury, although some controversy exists. Gilbert et al. (23) reported that the A unit was the major component of CS in the uninjured cortex, whereas C, E, and 2-O-sulfated disaccharide units were overexpressed in the glial scar in the injured cortex. They also demonstrated that E unit-rich CS was a potent inhibitor of neurite extension, whereas C unit-rich CS poorly inhibited it. On the other hand, Properzi et al. (48) reported that CS 6-O-sulfotransferase 1 and its biosynthetic product, the C unit, were selectively up-regulated around sites of cortical damage, and suggested that C unit-rich CS was the inhibitor of axonal regeneration. Thus, modification of the CS structure by RNAi around the damaged areas of CNS might lead to the development of a new therapy for CNS injury. Further systematic knockdown and overexpression experiments of CS sulfotransferases should reveal a new mechanism of development and regeneration of the CNS.

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