Developmental and regional expression of heparan sulfate sulfotransferase genes in the mouse brain

Tomio Yabe, Toshihiro Hata, Jue He², and Nobuaki Maeda¹

Department of Developmental Neuroscience, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo 183-8526, Japan

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Heparan sulfate (HS) binds with various proteins including growth factors, morphogens, and extracellular matrix molecules to regulate their biological functions. These regulatory interactions are considered to be dependent on the structure of HS, which is determined by HS sulfotransferases. To gain insights into the functions of HS sulfotransferases in the development of the nervous system, we examined the expression of these enzymes (3-O-sulfotransferase-1 [3-OST-1], -2, -4; 6-OST-1, -2, -3; and N-deacetylase/N-sulfotransferase-1 [NDST-1], -2, -3) by in situ hybridization and real-time reverse transcription–polymerase chain reaction (RT–PCR). The expression of these genes was spatiotemporally regulated. In the E16 cerebrum, the expression of these genes showed two patterns: (1) selective expression at cortical plate (CP) and ventricular zone (VZ) and (2) wider expression by the cells in the marginal zone (MZ), CP, subplate (SP), and VZ. At P1, most genes showed similar expression patterns, but after P7, these genes were expressed differentially in a layer-specific manner. In the P1 cerebellum, the external granule cell layer (EGL) expressed most genes, the expressions of which were down-regulated at P7. In contrast, Purkinje cells began to express many of these genes after P7. These complex expression patterns suggest that the structure of HS is altered spatiotemporally for regulating various biological activities in the developing brain including the proliferation of neuronal progenitors, extension of axons, and formation of dendrites. We discuss possible functional roles of these sulfotransferases in the signaling of several HS-binding proteins such as fibroblast growth factors, slit, netrin, and sonic hedgehog.

Key words: brain development/heparan sulfate sulfotransferase/in situ hybridization/real-time RT–PCR

Introduction

Heparan sulfate (HS) is a highly sulfated polysaccharide ubiquitously distributed on the cell surface and in the extracellular matrix. HS has been implicated in many biological phenomena such as blood coagulation, viral infection, tumor metastasis, and various developmental processes (Bernfield et al., 1999). Biosynthetic processing leads to the enormous structural diversity of this type of glycosaminoglycan, and it has been believed that structurally different HSs display different affinities for a variety of proteins such as growth factors, enzymes, and extracellular matrix components, regulating the above biological processes.

HS is biosynthesized in the Golgi by a sequential modification involving over 20 different enzymes acting in a concerted fashion after the polymerization of repeating disaccharide units consisting of an N-acetylgalactosamine (GlcNAc) and a glucuronic acid (GlcA) by HS copolymerase, EXTs. The nascent polysaccharide chains are partly N-deacetylated and N-sulfated by glucosaminyl N-deacetylase/N-sulfotransferase (NDST). Then, some GlcA units are C5 epimerized to iduronic acid (IdoA) by glucuronyl C5-epimerase, after which O-sulfation occurs at various positions. The O-sulfation includes 2-O-sulfation of GlcA/IdoA by HS 2-O-sulfotransferase (2-OST) and 6- and 3-O-sulfation of glucosamine (GlcN) units by 6-OSTs and 3-OSTs, respectively (Figure 1).

Recently, Inatani et al. (2003) reported that disrupting the EXT1 gene in the embryonic mouse brain resulted in severe guidance errors in the major commissural tracts. Irie et al. (2002) found that specific HS structures containing a combination of 2-O- and 6-O-sulfate groups are important for regulating retinotectal axon targeting in Xenopus brain. Bulow and Hobert (2004) demonstrated that 2-O-sulfation, 6-O-sulfation, and C5 epimerization are differentially required for the correct axon guidance and neuronal migration of many types of nematode neurons. These observations suggest that specific modifications of HS chains play pivotal roles in the development of the nervous system.

Most of the HS sulfotransferases are composed of several isoforms, which gives HS further functional diversity. For example, 3-OSTs are currently known to be composed of six different isoforms in mammals and are responsible for the last step in the biosynthesis of HS (Shworrak et al., 1999; Xia et al., 2002). Although 3-O-sulfation is a rare modification in HS chains, it plays key roles in anticoagulation and viral infection. While 3-OST-1 preferentially modifies a specific precursor to create the antithrombin-binding HS sequence, 3-OST-3 produces the binding sites on the cell surface for the gD envelope protein of herpes simplex virus 1, which enables viral entry. In addition to 3-OSTs, there are three mouse isoforms of 6-OST, each of which shows a different specificity toward the isomeric hexuronic acid adjacent to the targeted N-sulfoglucosamine (Habuchi et al., 2000). Furthermore, four NDST isoforms have been identified in vertebrates, and it was found that NDST-3 and...
NDST-4 differ dramatically from NDST-1 and NDST-2 in activity and expression pattern (Aikawa et al., 2001). These findings suggest that each isoform of each sulfotransferase is involved in the production of specific HS-structural domains with distinct biological activities.

In this study, we hypothesized that each HS sulfotransferase isoform is differentially involved in the various developmental processes in the brain. So, we investigated the expression of four genes of 3-OST isoforms and all of the 6-OST and NDST isoforms to gain insight into the functional roles of specific HS structures. We observed dynamically regulated expression of these HS sulfotransferase genes during the brain development. Unexpectedly, we also found that multiple isoforms of each HS sulfotransferase were often expressed by the same cells and at the same time. Based on these observations, we discussed possible functional roles of these HS sulfotransferases in the brain development.

**Results**

**Distribution of HS sulfotransferase mRNAs detected by in situ hybridization in cerebrum**

To determine the temporal and spatial expression patterns of HS sulfotransferase mRNAs in cerebral cortex, we performed in situ hybridization on embryo (E16; n = 3), juvenile (P1, P7, P14; n = 3–5), and adult (5–6 weeks; n = 3) mouse brain sections. cRNA probes were designed so as to distinguish each isoform of the HS sulfotransferases. The signals of 3-OST-3B, 6-OST-1, and NDST-4 mRNAs were not significant at any developmental stage in the cerebral cortex (data not shown), consistent with the fact that the expressions of these genes are very weak in the cerebrum (Figure 6).

At E16, significant signals of the mRNAs for 3-OST-1, 3-OST-2, 3-OST-4, and NDST-1 (Figure 2) and weak signals of the mRNAs for NDST-2 and NDST-3 (data not shown) were observed in the cerebral cortex. Major sites of positive cells for these genes were the ventricular zone (VZ) and the cortical plate (CP). The intermediate zone (IZ) showed weak signals for these mRNAs. High magnification of the 3-OST-4 transcript was not observed in these cells. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone. Scale bars show 100 µm (A–C, I, and J), 50 µm (H and K), and 25 µm (D–G).

![Fig. 1. Schematic structure of the heparan sulfate (HS) disaccharide unit.](image)

HS is synthesized in the Golgi body through a sequential modification by many enzymes after the polymerization of repeating disaccharide units consisting of N-acetylgalactosamine (GlcNAc) (right) and glucuronic acid (GlcA) (left). The nascent polysaccharide chains are partly sulfated by N-deacetylasparagine/N-sulfotransferase (NDST). Then, some GlcA units are C5 epimerized to iduronic acid (IdoA) by glucuronyl C5 epimerase, and this is followed by O-sulfation at various positions. The O-sulfation includes 2-O-sulfation of GlcA/IdoA by 2-OST and 6-O-sulfation of glucosamine (GlcN) units by 6-OSTs and 3-OSTs, respectively.

![Fig. 2. In situ hybridization analysis of the expression of 3-OST-1 (A, D, and E), 3-OST-2 (B, F, and G), 3-OST-4 (C and H), and 6-OST-3 (I) and NDST-1 (J and K) mRNAs in the E16 cerebral cortex.](image)
gradually weakened in the CP toward the IZ (Figure 3A). In addition, the signals for 3-OST-1 were detected in the radially migrating cells in the IZ (Figure 3A). At P1 and P14, the positive signals for 3-OST-1 were observed rather uniformly in layers II–VI (Figure 3E and I).

At P1, expression pattern of 3-OST-2 was very similar to that of 3-OST-1, and several cells in the MZ also showed positive signals (Figure 3B). At P7, the expression of 3-OST-2 mRNA was intense in most neurons in layers II/III (Figures 3F and 4A). In layer IV, a subset of neurons showed strong signals for 3-OST-2 mRNA (Figure 3H). At P14, significant signals for 3-OST-2 mRNA were observed in all the layers with strong signals in a few neurons in layers II/III and V (Figure 3J). Expression pattern of the 3-OST-4 mRNA at P1–P14 was similar to that of 3-OST-2.

The expression pattern of 6-OST-3 mRNA was very similar to that of 3-OST-1 at P1 (Figure 3C), and then relatively strong signals for 6-OST-3 mRNA were detected in the cells in layers II/III and V at P7 (Figures 3G and 4C). The cells in layers IV and VI at P7 showed weaker signals than those in the other layers (Figures 3G and 4C). At P14, the positive signals for 6-OST-3 mRNA were detected in layers I and II/III, whereas in the other layers, little signal was detected compared with the negative control (Figure 3K). The expression pattern of 6-OST-2 mRNA was similar to that of 6-OST-3 at P1 and P7 (data not shown), but the signals were not observed at P14.

The expression pattern for NDST-1 at P1 was similar to that of 3-OST-1 (Figure 3D). Although the signals for NDST-1 mRNA were detected in the MZ at E16, the positive signal was not detected in this layer at P1 (Figure 3D). At P7, the signals for NDST-1 were detected in the cells in layers II/III and V as 6-OST-3 signals (Figure 3H). At P14, the positive signals for NDST-1 were observed in all the layers as 3-OST-2 signals (Figure 3L). The expression pattern of NDST-3 mRNA was similar to that of NDST-1 mRNA (data not shown).

In the adult, strong signals for 3-OST-2 and 6-OST-3 mRNAs were detected in the pyramidal neurons in layer V (Figure 5A, B, and E). While strong signals for 3-OST-2 were also observed in layers II/III, the signals for 6-OST-3 were weak in this layer. Although the signals of the expression for 3-OST-1 and 3-OST-4 were weak (Figure 5C and D), higher magnification clearly showed that they were expressed in the pyramidal neurons in layer V (Figure 5F). The signals for 6-OST-2, NDST-1, NDST-2, and NDST-3 mRNAs were not detected in the adult cerebral cortex (data not shown).
The expression levels of 11 HS sulfotransferase genes in the cerebrum were analyzed by quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR). The cerebrum were analyzed by quantitative real-time reverse transcription signals of HS sulfotransferase mRNAs in cerebrum. Quantitative real-time RT–PCR for HS sulfotransferase genes. The signals for 3-OST-1, 3-OST-2, 3-OST-4, 6-OST-1, and 6-OST-3 in the cerebellum at P1 (Figure 7A, data not shown). They all showed similar expression patterns; positive signals were detected in the external granular layer (EGL) and Purkinje cell layer (PCL). To characterize the cells in the PCL, we performed immunohistochemical staining using anti-inositol 1,4,5-trisphosphate receptor (IP3R) and anti-glutamate/aspartate transporter (GLAST) antibodies, markers of Purkinje cells and Bergmann glia, respectively (data not shown). The results suggested that both Purkinje cells and Bergmann glia express these sulfotransferase genes. The signals for 6-OST-1, NDST-1, and NDST-3 were not detected at P1 in the cerebellum (data not shown).

At P7, significant signals for 3-OST-2 mRNA were observed in the EGL and internal granular layer (IGL) (Figure 7C). In addition, the transcript of 3-OST-2 was detected in some of the cells in the molecular layer (ML) and a few Purkinje cells (Figure 7C). On the other hand, only low signals for 3-OST-1, 3-OST-4, 6-OST-2, and NDST-3 were observed in the EGL (data not shown). The signals for 6-OST-1, 6-OST-3, and NDST-1 were not detected at P7 in the cerebellar cortex (data not shown). In addition, the signals for 6-OST-2 mRNA were only detected at P1 and P7, and in the later stages of the cerebellar cortex, the signals were not detected (data not shown). The signals for NDST-3 mRNA were only detected at P7 among the examined stages of the mouse cerebellar cortex (data not shown).

At P14, the signals for 3-OST-1, 3-OST-2, and 3-OST-4 were highly detected in the IGL (Figure 8A, data not shown), and the signals for NDST-1 were weak in the IGL (data not shown). Purkinje cells began to show positive signals for 3-OST-1, 3-OST-2, 3-OST-4, and NDST-1, and a few positive interneurons were also observed in the ML (Figure 8A). Furthermore, the expression of these 3-OSTs was still detected in the cells of the EGL at P14 (Figure 8A). The signals for 6-OST-1, 6-OST-2, 6-OST-3, and NDST-3 were not detected at P14 in the cerebellar cortex (data not shown).

In the adult, signals for 3-OST-1, 3-OST-2, 3-OST-4, 6-OST-1, and NDST-1 were detected in many neurons in the granular layer (GL) and PCL (Figure 8C–F, data not shown). In the case of 3-OST-2, cells in the ML were identified as positive neurons (Figure 8C and E). The signals for 3-OST-1, 3-OST-4, 6-OST-1, and NDST-1 were weak in the ML (Figure 8D and F, data not shown). Interestingly, the signals for 6-OST-1 were not detected in the cerebellum at the earlier stages including at P14 (Figure 8B) but became prominent in the GL and PCL in the adult (Figure 8D and F). In addition, although the signals for NDST-1...
were not detected at P1 and P7 in the cerebellar cortex, the signals became prominent in the PCL and IGL at P14 and in the adult. The signals for 6-OST-2, 6-OST-3, and NDST-3 were not detected in the adult cerebellar cortex (data not shown).

Quantitative real-time RT–PCR for HS sulfotransferase mRNAs in cerebellum

The expression levels of 11 genes in the cerebellum were analyzed by quantitative real-time RT–PCR. The quantification of the transcripts revealed that 3-OST-1, 3-OST-2, 6-OST-3, and NDST-2 were highly expressed in the cerebellum throughout development (Figure 9).

Interestingly, the expression of 3-OST-1 mRNA increased significantly from P7 to P14, followed by a slight reduction at P21 (Figure 9). The expression level of 3-OST-1 at P14 was 72.0 ± 2.7 copies/pg of total RNA. In contrast, the other HS sulfotransferase mRNAs showed similar expression patterns, in which a maximal level was observed at P1 followed by a gradual decrease up to P21, except for NDST-2 and NDST-3 (Figure 9).

The expression level of NDST-2 gene was relatively high through postnatal periods in the mouse cerebellum, although the signals for NDST-2 mRNA by in situ hybridization were not detected at any developmental stages (Figure 10).
Discussion

In this study, we have demonstrated that the expression of HS sulfotransferase mRNAs was regionally and temporally regulated in the developing brain (Figure 10). The observed complex patterns of cellular and regional expression of HS sulfotransferase genes are consistent with the recent paradigm that HS with different sulfation patterns has distinct functions. This study suggests that the structure of HS changes dynamically during the development of the brain through transcriptional control of the HS sulfotransferase genes regulating the activities of many HS-binding proteins.

Comparison between the results of in situ hybridization and real-time RT–PCR experiments

In this study, there were some discrepancies between the results obtained by in situ hybridization and real-time RT–PCR experiments. For example, real-time RT–PCR indicated that NDST-2 mRNAs were highly expressed in both cerebrum and cerebellum throughout development (Figures 6 and 9). However, almost no in situ hybridization signal was observed in both tissues except for early cerebral cortex (Figure 10). Although we tried two different probes for NDST-2, strong signals were not observed. In addition, we failed to detect in situ hybridization signals of NDST-1 mRNA in the adult cerebral cortex, which expressed a substantial level of this transcript as revealed by real-time RT–PCR (Figure 6). Because of such low sensitivities, we observed no in situ hybridization signals of any of NDST isoforms in the P1 cerebellum (Figure 10). It has been considered that N-sulfation process is a prerequisite for the O-sulfation in the biosynthetic pathway of HS (Bernfield et al., 1999). If so, any cells that synthesize O-sulfated HS should express some of the NDST isoforms. Thus, the schematic data presented in Figure 10 should be carefully interpreted because “no in situ hybridization signal” does not always mean “no expression of that gene.” At present, we do not know the reason why the in situ hybridization signals of NDST isoforms were low or not detected. However, mRNAs of some HS sulfotransferase isoforms might be highly susceptible to the endogenous RNase activities and be easily degraded during the operation of animals. Further examination is necessary to localize the expression of NDST isoforms.

Expression of HS sulfotransferase isoforms in the developing brain

Recently, Nogami et al. (2004) described the expression patterns of 2-OST, 6-OST-1, and 6-OST-2 in the chick limb buds, the development and patterning of which are known to be under the spatiotemporal control of various heparin-binding proteins. They found that these HS sulfotransferases were differentially distributed in the developing limb buds. 6-OST-1 and 6-OST-2 mRNAs were preferentially localized to the anterior proximal region and to the posterior proximal region of the limb buds, respectively. 2-OST mRNAs were rather uniformly distributed throughout limb buds. They suggested that such differential distribution of 6-OST isoforms leads to the distinctive spatial distribution of iduronosyl 6-O-sulfated and glucuronosyl 6-O-sulfated residues in the limb buds regulating the morphogenetic signal transduction pathways.

On the other hand, our in situ hybridization experiments indicated that multiple isoforms of each HS sulfotransferase are often expressed in the same regions and at the same time. For example, 3-OST-1, 3-OST-2, and 3-OST-4 mRNAs were expressed in the CP and VZ of E16 cerebral cortex (Figure 2). 3-OST-1, 3-OST-2, 3-OST-4, 6-OST-2, and 6-OST-3 transcripts were expressed in the EGL and PCL of P1 cerebellum. This might suggest that brain cells synthesize complex HS chains with many different functional domains generated by multiple HS sulfotransferase isoforms. However, it should be noted that neurons show highly polarized structure with functionally distinct regions such as dendrites, axons, cell bodies, spines, and axon terminals. The development of these distinct regions are considered to be regulated by different signaling pathways, which might require structurally different HS chains. Each of the multiple HS sulfotransferase isoforms expressed in one neuron might differentially contribute to the biosynthesis of HS chains required for the development of specific region of that cell. Future fine structural analyses are necessary to evaluate
whether distinct regions in one neurons express structurally different HS chains.

**HS sulfotransferases and development of cerebrum**

Recently, Inatani et al. (2003) conditionally disrupted the HS-polymerizing enzyme \( \text{EXT1} \) in the mouse brain, in which HS was almost completely eliminated after E12.5. All the conditional mutants (Nes-\( \text{EXT1} \)-null mice) died within the first day of life and displayed several disorders in the morphogenesis of the brain including a small cerebral cortex, malformation in the caudal midbrain–cerebellum region, and the absence of major commissural tracts. The effects of HS elimination were unexpectedly selective for specific developmental events, and the major affected signaling molecules appear to be FGF-2, FGF-8, slit, and netrin. So, we will mainly focus on these molecules below.

In the Nes-\( \text{EXT1} \)-null mice, the structure of the cortex was largely normal, although the overall size and thickness were reduced (Inatani et al., 2003). In this mutant, cell proliferation in the VZ was reduced by about 30%, and the cortical progenitors exhibited reduced BrdU incorporation in response to FGF-2 and FGF-8. Allen and Rapraeger (2003) revealed that there are global changes in the structure of HS in the developing mouse embryo, and each FGF–FGF receptor pair requires an unique HS-structure for complex formation and signaling. Maccarana et al. (1993) indicated that 2-\( O \)-sulfated groups are essential for HS to bind with FGF-2 and 6-\( O \)-sulfated groups are not required. Further study by Ashikari-Hada et al. (2004) suggested that HS chains with 2-\( O \)-sulfated and \( N \)-sulfated groups show enough affinity for FGF-2. At E16, significant expression of \( \text{NDST-1} \), \( \text{NDST-2} \), and \( \text{NDST-3} \) was observed in the VZ of the cortex, but the expression of 6-\( \text{OSTs} \) was weak or undetectable (Figure 10). This pattern of expression might make this zone suitable for FGF-2 signaling. In addition, \( \text{3-OST-1} \), \( \text{3-OST-2} \), and \( \text{3-OST-4} \) were also expressed in the VZ (Figure 10). Ashikari-Hada et al. (2004) reported that FGF-8 hardly bound to any octasaccharides of HS with 2-\( O \)- and 6-\( O \)-sulfated groups. Furthermore, Loo and Salmivirta (2002) reported that complex \( \geq 14 \)-mer–heparin domains were needed to enhance cell proliferation and Erk phosphorylation by FGF-8. 3-OST isoforms expressed in the VZ might contribute to the generation of such complex structural domains involved in FGF-8 signaling.

Strong signals of \( \text{3-OST-1} \), \( \text{3-OST-2} \), and \( \text{3-OST-4} \) expression were also observed in the CP neurons at E16 (Figure 10), when these neurons extend axons making major tracts and later differentiate into deep layer neurons. Nes-\( \text{EXT1} \)-null mice lacked a corpus callosum, hippocampal commissure, and anterior commissure (Inatani et al.,

![Fig. 9. Quantitative analysis of heparan sulfate (HS) sulfotransferase transcripts in the cerebellum by real-time reverse transcription–polymerase chain reaction (RT–PCR). The expression level of each HS sulfotransferase gene was normalized to that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript. Data were obtained from triplicate experiments and are given as the mean ± standard deviation (SD).](image-url)
HS sulfotransferase expression in the mouse brain

2003), and these defects resemble those of netrin-1-deficient mice (Serafini et al., 1996), suggesting that HS is required for netrin-1 signaling. Netrins are a family of heparin-binding secreted proteins and thus appear to be HS-binding proteins. Nes-EXT1-null mice also display defects in the guidance of retinal axons at the optic chiasm, a phenotype similar to that of Slit1/Slit2 double-null mice, and genetic interaction between EXT1 and Slit2 was demonstrated (Plump et al., 2002; Inatani et al., 2003). Slit2 was shown to bind with HS, which is necessary for the repulsive activities of this protein (Hu, 2001; Ronca et al., 2001). Slit proteins were also involved in the development of several major

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**Fig. 10.** Developmental expression patterns of heparan sulfate (HS) sulfotransferase genes. The expression of the nine different HS sulfotransferase mRNAs is represented: 1, 3-OST-1; 2, 3-OST-2; 3, 3-OST-4; 4, 6-OST-1; 5, 6-OST-2; 6, 6-OST-3; 7, NDST-1; 8, NDST-2; and 9, NDST-3. The intensity of the hybridization signal is indicated by the meshed boxes as listed. The level of 3-OST-3B and NDST-4 mRNA expressions was not significant at any developmental stage. Though NDST-2 mRNA was highly expressed in both cerebrum and cerebellum throughout development as revealed by real-time reverse transcription–polymerase chain reaction (RT–PCR), the in situ hybridization signals were very low. CP, cortical plate; E, embryonic day; EGL, external granular layer; IGL, internal granular layer; IZ, intermediate zone; ML, molecular layer; MZ, marginal zone; P, postnatal day; PCL, Purkinje cell layer; VZ, ventricular zone; and WM, white matter.
forebrain tracts such as corticofugal, callosal, and thalamocortical tracts (Bagri et al., 2002). 3-OST-1, 3-OST-2, and 3-OST-4 expressed in the early CP neurons might be involved in axonal guidance in collaboration with netrin and slit proteins. Further study is necessary to investigate whether 3-O-sulfated HSs are present on the axons and to analyze the structural requirement of HS for the signaling of netrin and slit proteins.

In the postnatal period, high levels of 3-OST-1, 3-OST-2, 6-OST-3, and NDST-1 mRNAs were observed in the cerebrum (Figure 10). Notably, the expression level for 3-OST-2 peaked at P8, suggesting the importance of this enzyme in the late development of cortical neurons (Figure 6). At P7, relatively strong signal for this gene was observed on the neurons in the superficial layers, but at P14, the signal became rather uniform among almost all neurons. This postnatal period is a stage of extensive dendritic growth and branching, terminal branching of afferent axons and synaptogenesis. Recently, Whitford et al. (2002) indicated that slit proteins are involved in the dendritic patterning of cortical neurons. In addition to axonal guidance, these HS sulfotransferases might be involved in dendritic growth by controlling the slit activity. Furthermore, Ethell et al. (2001) demonstrated that a HS proteoglycan, syndecan 2, plays important roles in the maturation of dendritic spines. These sulfotransferases might modify HS chains of syndecan 2 and play a role in the dendritic spines formation.

At P1, the expression patterns of 3-OST-1, 3-OST-2, 3-OST-4, 6-OST-2, 6-OST-3, and NDST-1 were very similar (Figure 10). However, after P7, these genes displayed differential layer-specific expression patterns. At P7, 3-OST-4 had a similar distribution to 3-OST-2 with relatively strong expression at layers II/III (Figure 10). The expression of 3-OST-1 was uniformly observed in the layers II–VI. Relatively strong signals of the expression for 6-OST-3 and NDST-1 were observed in layers II/III and V. At P14, 3-OST-1, 3-OST-2, 3-OST-4, and NDST-1 displayed similar patterns of distribution, and positive signals were found uniformly in layers II–VI. In contrast, 6-OST-3 was selectively detected in layers I–II/III. Although the functional significance of such a differential distribution is not clear, it suggests that the HS structures are regulated in a layer-specific manner contributing to the developmental events in each layer. For example, terminal branching of thalamocortical axons vigorously proceeds around P7 in layer IV. The weak expression of 6-OST-3 and NDST-1 in layer IV at this stage might be related to this event. Furthermore, cortical neurons show highly diversified morphologies with variable extension patterns of dendrites and axons. As we discussed above, such morphogenesis is under the control of several heparin-binding proteins such as slit and netrin. Variable HS structures generated by different combinations of HS sulfotransferases might diversify the behavior of neurites in response to slit and netrin proteins leading to the diversified patterns of neurite extension.

**HS sulfotransferases and development of cerebellum**

Development of the mouse cerebellar cortex proceeds postnatally. The proliferation of granule cell progenitors in the EGL begins after birth, and the postmitotic granule cells migrate along Bergmann glial fibers to the IGL and at the same time extend parallel fibers into the ML. Purkinje cells begin to extend dendrites several days after birth, and then the extensive branching of dendrites and formation of synapses with parallel fibers proceed until P20. Inatani et al. (2003) reported that Nes-EXT1-null mice died at P0, and thus from this mutant we cannot know anything about the functions of HS in the postnatal cerebellar development. However, several HS-binding proteins were reported to play roles in the development of the cerebellar cortex.

Rubin et al. (2002) reported that Shh binds with HS proteoglycan, and this interaction regulates the proliferation of granule cells in the EGL. Shh is a potent mitogen for postnatal granule cells in the EGL. Although Shh is produced by Purkinje cells from as early as E17.5, it induces the proliferation of granule cells only during the early postnatal period (Lewis et al., 2004). Rubin and colleagues observed that the binding capacity of EGL to Shh increased remarkably from P3 to P6, in which most of the binding was mediated by HS proteoglycans (Rubin et al., 2002). They also demonstrated that granule cells from P3 and P6 mice exhibited quite different responses to Shh. The proliferation of P6 granule cells was more effectively promoted by Shh than that of P3 granule cells, and only the former was HS dependent. This suggests that HS proteoglycans on the surface of P3 and P6 granule cells in the EGL are functionally different. Our study indicated a strong expression of 3-OST-1, 3-OST-2, 3-OST-4, 6-OST-2, and 6-OST-3 in the EGL at P1, whereas at P7, only 3-OST-2 displayed strong signals in the EGL, and the signals of the others were weak or not detected. On the contrary, NDST-3 signals first appeared at P7 in EGL (Figure 10). So, in addition to the postnatal increase in the expression of EXT1/EXT2 genes (Rubin et al., 2002), it is highly possible that the changes in the expression of HS sulfotransferases lead to the structural changes of HS in granule cells resulting in the change of the responsiveness of this type of cells to Shh.

The Shh-induced proliferation of granule cells is synergistically enhanced by SDF-1, a heparin-binding chemokine secreted by meninges (Klein et al., 2001). CXCR-4, a receptor for SDF-1, is expressed by granule cells in both EGL and IGL (Klein et al., 2001). It has been considered that HS concentrates SDF-1 on the cell surface and also protects this chemokine against proteolysis induced by CD26/DPP IV, a serine protease that codistributes with CXCR-4 (Sadir et al., 2004). Thus, HS sulfotransferases expressed in the EGL might regulate the spatial distribution and signaling of SDF-1. SDF-1 also acts as a chemottractant for embryonic granule cells in the EGL, but not for postnatal EGL cells (Zhu et al., 2002). Ma et al. (1998) indicated that SDF-1- and CXCR-4-deficient mice showed an abnormal irregular EGL at E18.5, from which granule cells streamed and prematurely proliferated. It has been considered that SDF-1 attracts embryonic granule cells toward meninges anchoring them in the EGL. After P1, the expression pattern of HS sulfotransferase genes in the EGL changed dramatically, and this might be related to the change in the response of granule cells to SDF-1. Alcantara et al. (2000) also reported that Netrin 1 expressed in the postnatal EGL exerted strong chemorepulsive effects on parallel fibers. Postmitotic granule cells descend deep into
the EGL and extend parallel fibers, which form a ML and do not penetrate the EGL. The chemorepulsive effects of netrin are age dependent and are more drastic at P5 than at P0 (Alcantara et al., 2000). This developmental change of sensitivity might also be related to the change in the expression of HS sulfotransferases.

At P1, Purkinje cell layer exhibited signals of the expression of many HS sulfotransferase genes, which were downregulated at P7. Then the expression of HS sulfotransferases in the Purkinje cells increased afterward (Figure 10). At P7, weak expression of only ferases in the Purkinje cells increased afterward (Figure 10). They suggested that Wnt-3 plays a role in the growth and maturation of Purkinje cell dendrite (Salinas et al., 1994).

Because HS plays crucial roles in Wnt signaling (Lin, 2004), the expression of HS sulfotransferase mRNAs has long structured 5′-untranslated regions, which play roles in translational regulation. So, immunohistochemical studies using antibodies against each HS sulfotransferase and specific HS structural domains are the next important project. And, our results would provide a valuable basis for the design of functional experiments such as the knockdown of specific enzymes.

Materials and methods

Animals

BALB/c mice used in this study were purchased from SLC (Shizuoka, Japan). The mating day was considered embryonic day 0 (E0), and the day of birth was considered postnatal day 0 (P0). All experiments were conducted in accordance with the Guideline for the Care and Use of Animals (Tokyo Metropolitan Institute for Neuroscience, 2000).

In situ hybridization

For preparing hybridization probes, we amplified HS sulfotransferase gene fragments by PCR from mouse brain 5′-stretch plus cDNA library (Clontech, BD Biosciences Clontech, Mountain View, CA) using the following primers (F, forward; R, reverse): for 3-OST-1 (205 bp), 5′-GTTCCCGGTATACGACGTC-3′ (F), 5′-GATGATGATGGCCTGCGGCAG-3′ (R); for 3-OST-3 (236 bp), 5′-AAAGTTGAAAAGTTGACAGGGC-3′ (F), 5′-GGGTGTCTTGTTCAAGCTGGGTTTAC-3′ (R); for 6-OST-1 (1413 bp), 5′-GAACTCCTTACATCATCACTCAGCAG-3′ (F), 5′-TCTTCTCCTAGAGCAGATGTC-3′ (R); for 6-OST-2 (1306 bp), 5′-ATGGAGTAAGAAAGTTGACAGGGC-3′ (F), 5′-TCACCACCTGACACTTGCGA-3′ (R); for NDST-1 (235 bp), 5′-TTATGATCTCCATCTTCACTTATG-3′ (F), 5′-AGATGTTGATGATCTTGCGGATT-3′ (R); and for NDST-4 (170 bp), 5′-GGGAAAGAGCTGACAGAAGGCG-3′ (R), 5′-TCCTGTTGATAGTAGTGCCATCAT-3′ (R). The PCR products encoding the sulfotransferase gene fragments were ligated into the pPCR-ScriptTM Amp SK(+) vector (Stratagene, La Jolla, CA). The antisense or sense probes were prepared using T7 or T3 RNA polymerase (Roche Diagnostics) with a DIG RNA Labeling Kit (Roche Diagnostics, Basel, Switzerland). Both antisense and sense probes of 3-OST-2, 6-OST-2, and 6-OST-3, but not others, were truncated to ∼75 nucleotides by limited alkaline hydrolysis (Cox et al., 1984).

E16, P1, P7, P14, and adult mice were perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, at 4°C. The brains were dissected out and embedded in paraffin after dehydration through a graded alcohol series. Paraffin-embedded brains were cut into sections of 10-µm thick in the sagittal plane. Deparaffinized sections were treated with 0.2 N HCl for 20 min and Proteinase-K (Nacalai tesque, 20 µg/mL in 100 mM phosphate-buffered saline [PBS]) for 10 min at room temperature. The hybridization buffer contained 50% formamide, 10% dextran sulfate, 0.5 mg/mL of yeast tRNA, 125 µg/mL of salmon sperm DNA, 1 × Denhardt’s solution (Nacalai tesque), and 4 × standard saline citrate (SSC; 1 × SSC = 150 mM NaCl and 15 mM sodium citrate). Both the antisense and sense probes were diluted to a final concentration of 1 µg/mL. Hybridization was performed overnight at 53°C. Thereafter, the samples were washed in 2 × SSC for 30 min at 53°C, followed by two washes for 5 min in 10 mM Tris–HCl (pH 8.0) containing 0.5 M NaCl, and then treated with 20 µg/mL RNase A in 10 mM Tris–HCl (pH 8.0) containing 0.5 M NaCl for 30 min at 37°C. After a rinse in 2 × SSC twice, the samples were incubated sequentially in the following solutions: (1) 2 × SSC for 1 h at 57°C; (2) 1 × SSC for 1 h at 57°C; and (3) 0.2 × SSC for 1 h at 57°C. The digoxigenin-labeled probes were detected by overnight reaction at 4°C with alkaline phosphatase-conjugated anti-digoxigenin (Roche Diagnostics), followed by incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) at room temperature. Tissue sections were then mounted with Crystal/Mount (Biomed, Foster City, CA) and dried at 50°C for 30 min.

HS sulfotransferase expression in the mouse brain
The images of tissue sections were captured with a CCD video camera module (Zeiss AxioCam) mounted on a Zeiss Axioskop microscope (Carl Zeiss AG, Oberkochen, Germany). Digital images were processed with Adobe Photoshop 6.0.1 (Adobe Systems Incorporated, San Jose, CA) software on a Macintosh OSX.

**Immunohistochemistry**

For immunohistochemical staining, the following antibodies were used: (i) rabbit polyclonal antisera against the glutamate/aspartate transporter (1:500, Abcam, Cambridge, UK) and (ii) rat monoclonal antibody against the inositol 1,4,5-trisphosphate receptor (1:5; Maeda et al., 1989). After deparaffinization and rehydration through xylene and a descending ethanol series, internal peroxidases were blocked by 2.5% hydrogen peroxide in PBS for 30 min. After rinsing in PBS for 10 min (twice), sections were treated with 2% bovine serum albumin (BSA) and 4% goat serum in PBS to block nonspecific binding sites and then with the primary antibodies (diluted with PBS containing 0.1% BSA) overnight at 4°C. After rinsing in PBS for 10 min (twice), sections were incubated with biotinylated secondary antibodies (diluted 1:200 with PBS containing 0.1% BSA) for 2 h at room temperature. After rinsing in PBS for 10 min (twice), the sections were probed with biotinylated secondary antibodies (diluted 1:200 with PBS containing 0.1% BSA) for 2 h at room temperature. After rinsing in PBS for 10 min (twice), immunoreactivity was visualized in PBS containing 0.05% diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide for 10 min at room temperature.

**Quantitative RT–PCR analysis**

Total RNA fractions from cerebral cortex containing hippocampus (E16, P1, P8, P14, P21, and adult) and from cerebellum (P1, P7, P14, P21, and adult) were prepared using three mice at each developmental stage with an AquaPure RNA isolation kit (Bio-Rad Laboratories, Hercules, CA). Two micrograms of total RNA was treated with RNase-free DNase I (Roche Diagnostics) to remove residual DNA and reverse-transcribed with oligo(dT)20 primer (Toyobo, Osaka, Japan) and ReverTra Ace (Toyobo). Primer sequences used are as follows: for GAPDH and Dr. J. Aikawa (RIKEN) for generously donating the NDST-1–4 plasmids. This work was supported by grants from the Mizutani Foundation for Glycoscience, Brain Science Foundation and from the Ministry of Education, Science, Sports, and Culture of Japan.

**Abbreviations**

BSA, bovine serum albumin; CP, cortical plate; E, embryonic day; EGL, external granular layer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GL, granular layer; GLAST, glutamate/aspartate transporter; GlcA, glucuronic acid; GlcN, glucosamine; GlcNAc, N-acetylgalactosamine; HS, heparan sulfate; IdoA, iduronic acid; IGL, internal granular layer; IP3R, inositol 1,4,5-trisphosphate receptor; IZ, intermediate zone; ML, molecular layer; MZ, marginal zone; NDST, N-deacetylase/N-sulfotransferase; OST, O-sulfotransferase; P, postnatal day; PBS, phosphate-buffered saline; PCL, Purkinje cell layer; RT–PCR, reverse transcription–polymerase chain reaction; SP, subplate; VZ, ventricular zone.

**References**


