Dermatan sulfotransferase Chst14/D4st1, but not chondroitin sulfotransferase Chst11/C4st1, regulates proliferation and neurogenesis of neural progenitor cells

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Summary
Chondroitin sulfates (CSs) and dermatan sulfates (DSs) are enriched in the microenvironment of neural stem cells (NSCs) during development and in the adult neurogenic niche, and have been implicated in mechanisms governing neural precursor migration, proliferation and differentiation. In contrast to previous studies, in which a chondroitinaseABC-dependent unselective deglycosylation of both CSs and DSs was performed, we used chondroitin 4-O-sulfotransferase-1 (Chst11/C4st1)- and dermatan 4-O-sulfotransferase-1 (Chst14/D4st1)-deficient NSCs specific for CSs and DSs, respectively, to investigate the involvement of specific sulfation profiles of CS and DS chains, and thus the potentially distinct roles of CSs and DSs in NSC biology. In comparison to wild-type controls, deficiency for Chst14 resulted in decreased neurogenesis and diminished proliferation of NSCs accompanied by increased expression of GLAST and decreased expression of Mash-1, and an upregulation of the expression of the receptors for fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF). By contrast, deficiency in Chst11 did not influence NSC proliferation, migration or differentiation. These observations indicate for the first time that CSs and DSs play distinct roles in the self-renewal and differentiation of NSCs.

Key words: Chondroitin sulfate, Dermatan sulfate, Sulfotransferases, Neural stem cells, Proliferation, Neurogenesis

Introduction
Self-renewal and multipotency are two major characteristics of neural stem cells (NSCs), which are regulated by a combination of intrinsic pathways modifying the ligand dependent activity of transcriptional factors and signaling pathways in the microenvironment of NSCs (Li and Zhao, 2008; Miller and Gauthier, 2007; Shi et al., 2008). Chondroitin sulfate (CS) and dermatan sulfate (DS) polysaccharide chains are important glycosaminoglycans (GAGs) of the extracellular matrix (ECM). They are composed of the alternating disaccharides N-acetylgalactosamine (GalNAc) and glucuronic acid (GlcA) or iduronic acid (IdoA) (Bulow and Hobert, 2006), which can be sulfated at different positions and have been shown to be present in the neurogenic regions of the embryonic and adult central nervous system (Ida et al., 2006; Kabos et al., 2004; Sugahara and Mikami, 2007).

CSs and DSs have important roles in neural precursor proliferation, neuronal differentiation, migration and neuroprotection (Sato et al., 2008; Sirko et al., 2007; Sugahara and Mikami, 2007; von Holst et al., 2006). Previous studies indicated that the functions of CSs and DSs on NSCs are determined by their sulfation patterns. A sulfate-dependent arrangement of the cell surface-associated CS or DS motifs recognized by the monoclonal antibody 473HD has been observed in a subpopulation of radial glial cells and plays an important role in neurosphere formation (von Holst et al., 2006). Treatment of NSCs from the embryonic mouse telencephalon with chondroitinaseABC (ChaseABC), which unselectively degrades CSs and DSs, resulted in diminished proliferation and impaired neuronal differentiation of NSCs, with a concomitant increase in astrogensis (Sirko et al., 2007).

Because CSs and DSs are composed of various repeats of sulfated disaccharide units generating CS-A, CS-B (DS), CS-C, CS-D, CS-E and CS-H patterns, we addressed which specific CS and DS patterns generated by NSCs themselves – and not by their microenvironment in the neurogenic niche – influence the properties of these cells in an autocrine or paracrine fashion. To investigate the roles of different endogenous CS and DS motifs, we generated transgenic mice deficient in the key enzymes that distinctly affect the synthesis of CS versus DS chains.
The sulfation of CSs and DSs is mediated by several sulfotransferases. Among the 4- O-sulfotransferases involved in the sulfation of the carbon 4 position of GalNAc in CSs and DSs, chondroitin 4- O-sulfotransferase-1 (Chst11/C4st1) (Okuda et al., 2000b) uses predominantly GalNAc residues flanked by GlcA to generate CS chains. By contrast, dermatan 4- O-sulfotransferase-1 (Chst14/D4st1) (Evers et al., 2001) far more efficiently transfers sulfate to GalNAc residues flanked by IdoA to generate DSs (Mikami et al., 2003). Both Chst11 and Chst14 are expressed in the neurogenic regions of the embryonic and adult central nervous system as well as in cultures of neurospheres (Akita et al., 2008). Using Chst11- and Chst14-deficient mice, we investigated the roles of these two enzymes in NSC biology and elucidated the functions of endogenous CS and DS patterns independently in CNS development, in contrast to the previous ChaseABC-based investigations that did not discriminate between the effects of CSs and DSs. Importantly, in addition to previous investigations, our study addresses the distinct roles of CSs and DSs not only in vitro, but also in vivo. Our results show that Chst14 deficiency results in impaired neuronal differentiation and diminished NSC proliferation both in vitro and in vivo, associated with an upregulation of the expression of growth factor receptors and changes in distinct subpopulations of radial glial cells. By contrast, Chst11 deficiency does not show any influence on NSC properties. These observations indicate for the first time that DSs and CSs play distinct roles in the biology of NSCs.

**Results**

**Differential gene expression of the HNK-1 sulfotransferase family members in neurospheres derived from Chst11+/− or Chst14+/− NSCs**

To elucidate whether the ablation of Chst11 or Chst14 causes a dysregulation of the expression of other members of the HNK-1 sulfotransferase family in NSCs, differential gene expression of the HNK-1 sulfotransferase family members Chst8/GalNAc-4-st1 (Hiraoka et al., 2001; Okuda et al., 2000a; Okuda et al., 2003; Xia et al., 2000), Chst9/GalNAc-4-st2 (Hiraoka et al., 2001; Kang et al., 2001; Okuda et al., 2003), Chst10/HNK1-st (Bakker et al., 1997; Ong et al., 1998), Chst11/C4st1 (Hiraoka et al., 2000; Okuda et al., 2000b; Yamauchi et al., 2000), Chst12/C4st2 (Hiraoka et al., 2000), Chst13/C4st3 (Kang et al., 2002) and Chst14/D4st1 (Evers et al., 2001) was analyzed in NSCs derived from the ganglionic eminence of E13.5 embryonic brains of homozygous Chst11−/− and Chst14−/− mice versus Chst11+/+ and Chst14+/+ littermates by qRT-PCR.

Expression of Chst8 and Chst13 was below the detection limit in all groups. As expected, in Chst11−/− NSCs, mRNA expression of Chst11 was reduced below the detection limit (Fig. 1A). No differences in expression were detected for Chst9 and Chst10 in NSCs derived from Chst11−/− mice and Chst11+/+ mice, whereas mRNA levels of Chst12 and Chst14 were significantly reduced in Chst11−/− NSCs versus Chst11+/+ NSCs (Chst12, 0.55±0.04 versus 1±0.23; n=4; P<0.01; Chst14, 0.75±0.08 versus 1±0.14; n=4; P<0.05). Furthermore, Chst12 and Chst14 expression of the HNK-1 sulfotransferase family members and 473HD epitope in Chst11−/− and Chst14−/− NSCs.

Expression of the HNK-1 sulfotransferase family members and 473HD epitope in Chst11−/− and Chst14−/− NSCs. Expression of the HNK-1 sulfotransferase family members in NSCs isolated from Chst11+/+ (+/+ or Chst14+/+ (+/+ and Chst11−/− (−/−) (A) or Chst14−/− (−/−) (B) embryos at day E13.5 was analyzed by qRT-PCR. The average of the expression levels of these genes in +/+ animals, respectively, was set to 1.0 and relative expression in homozygous −/− NSCs was calculated (mean ± s.d.; n=4). (C) Western blot analysis of expression of the 473HD epitope in neurospheres isolated from Chst11+/+ (mean ± s.d.; n=3) or Chst14+/+ (mean ± s.d.; n=3) and Chst11−/− and Chst14−/− littermate mice at developmental stage E13.5 is shown. An actin antibody was used as a control for protein loading. (D) Quantification of western blot measuring expression of the 473HD epitope in neurospheres. Mean arbitrary units (AU) are shown. The average of expression levels of 473HD epitope in +/+ animals was set to 1.0 and relative expression was calculated accordingly. Student’s t-test was performed for statistical analysis. *P<0.05; **P<0.01; ***P<0.001. n.d., not detectable.
in Chst14−/− NSCs, the expression of Chst14 mRNA was reduced below the detection limit (Fig. 1B) whereas the mRNA levels of other HNK-1-ST family members were not altered when compared with NSCs derived from their Chst14+/− littermates. These results indicate that other HNK-1 sulfotransferase family members are not upregulated in a compensatory manner in Chst11−/− or Chst14−/− versus Chst11+/+ or Chst14+/+ NSCs.

Expression of the 473HD epitope in Chst11−/− or Chst14−/− NSCs
A unique CS–DS structure containing CS-A, CS-D and DS patterns is recognized by the monoclonal antibody 473HD (Clement et al., 1998; Ito et al., 2005) as a marker for radial glial cells (Kabos et al., 2004). To investigate the influence of Chst11 or Chst14 depletion on the expression of CS–DS structures in neurospheres, western blot analysis was performed using the 473HD antibody on protein extracts of neurospheres originating from the embryonic forebrain of Chst11−/− and Chst14−/− mice in comparison with their wild-type littermates. After ablation of Chst11, expression of the 473HD epitope was reduced to 11±6% compared with Chst11+/− neurospheres (Fig. 1C,D). In neurospheres generated from Chst14−/− mice, expression of the 473HD epitope was decreased to 58±7% of the expression level of Chst14+/+ neurospheres (Fig. 1C,D). This result indicates that deficiency in both Chst11 and Chst14 alters CS–DS structures.

Deficiency in Chst14, but not Chst11, impairs proliferation of NSCs
Because the 473HD epitope is important for neurosphere formation by NSCs (von Holst et al., 2006) and deficiency either in Chst11 or Chst14 reduced expression of the 473HD epitope, NSCs were cultured at a low density in the presence of growth factors and the number of neurospheres was counted using the 473HD antibody on protein extracts of neurospheres with regard to size to examine whether the ablation of Chst11 or Chst14 reduced expression of the 473HD epitope. NSCs were cultured in adherent cell culture under three different culture conditions: in medium containing only FGF-2, only EGF, or both FGF-2 and EGF. After culturing for 24 hours and labeling with BrdU for 8 hours, Chst11−/− NSCs showed the same proliferative activity as wild-type controls (Fig. 3A,B), whereas Chst14−/− NSCs showed decreased numbers of BrdU+ cells (Fig. 3A,C) under the three culture conditions (FGF-2+EGF, 59±2% versus 70±1%; FGF-2, 49±3% versus 54±3%; EGF, 52±2% versus 60±3% BrdU+ cells, Chst14−/− versus Chst14+/+ NSCs), confirming the results obtained in neurospheres. Thus, DS structures modified by Chst14 but not CS structures modified by Chst11 play important roles in self-renewal and proliferation of NSCs.

To analyze whether the effects of Chst14 on proliferation of NSCs are also seen in vivo, BrdU was administrated intraperitoneally to pregnant mice at E13.5 and to adult mice to label the neurogenic regions. Twenty four hours after BrdU labeling, the density of BrdU+ proliferating cells in the embryonic ganglionic eminence was slightly, but statistically not significantly, reduced in Chst14−/− embryos when compared with Chst14+/+ controls (supplementary material Fig. S1). In adult mice, 4 hours after BrdU labeling, the density of BrdU+ proliferating neural progenitors was reduced in Chst14−/− mice in comparison with control mice, both in the dentate gyrus of the hippocampus (58±4 cells/mm² in Chst14−/− versus 39±2 cells/mm² in Chst14+/+ mice; Fig. 4A,B) and in the subventricular zone (177±104 cells/mm² in Chst14−/− versus 132±280 cells/mm² in Chst14+/+ mice, Fig. 4C,D). In the olfactory bulb, 4 weeks after BrdU labeling, Chst14−/− mice had a lower density of BrdU+ cells in all regions as well as the total area of the olfactory bulb compared with Chst14+/+ mice, but only in central regions of the olfactory bulb (granule cell layer, internal plexiform layer and mitral cell layer) the difference was significant (supplementary material Fig. S2). Thus, DS patterns modified by Chst14 influence proliferation of NSCs in vitro and in vivo.

Finally, to address the question of whether the reduced cell proliferation had an effect on neuronal numbers in adult Chst14−/− mice, we analyzed neuron numbers in several brain structures. No differences were found in the volume and thickness of the motor cortex and the density of NeuN+ neurons and S100+ astrocytes between Chst14−/− and Chst14+/+ mice (supplementary material Fig. S3). The volumes of the CA1 and dentate gyrus regions of hippocampus were also analyzed, and no difference between the two groups of mice was revealed (supplementary material Fig. S4). Furthermore, the analysis of the density of the principal neurons in the hippocampal subregions CA1 (pyramidal cells) and dentate gyrus (granule cells) also showed no significant difference between the genotypes (supplementary material Fig. S4). Thus, morphometrical analysis did not reveal any differences between adult Chst14−/− and Chst14+/+ mice.

Ablation of Chst14 results in upregulation of growth factor receptors in neurospheres
Because Chst14 deficiency, but not Chst11 deficiency, negatively influences proliferation of NSCs cultured in the presence of FGF-2, EGF, or combined FGF-2 and EGF, expression of growth factor receptors in neurospheres was analyzed. Western blot analysis revealed that Chst14−/− neurospheres expressed higher levels of epidermal growth factor receptor (EGFR) (Fig. 3D) and fibroblast growth factor receptor 1 (FGFR-1) (Fig. 3E) when compared with Chst14+/+ neurospheres. By contrast, no differences in expression of EGFR and FGFR-1 were observed in Chst11−/− versus Chst11+/+ neurospheres (Fig. 3D,E).
Deficiency in Chst14, but not Chst11, influences neurogenesis

To investigate the effects of CS and DS patterns modified by Chst11 or Chst14, respectively, on neural fate decision in vitro, we analyzed the percentages of tubulin-βIII⁺ neurons, GFAP⁺ astrocytes and CNPase⁺ oligodendrocytes 7 days after induction of differentiation by growth factor withdrawal (Fig. 5A). A reduction in the percentage of neurons among total cells was observed in Chst14⁻/⁻ versus Chst14⁺/+ cells (12.2±1.6% versus 16.7±1.2% (Fig. 5C)), whereas the percentage of GFAP⁺ astrocytes was
slightly but not significantly increased in 

$$\text{Chst14}^{2/-}$$ compared with 

$$\text{Chst14}^{+/+}$$ cells (Fig. 5C). Oligodendroglial differentiation was not different between the genotypes (Fig. 5C). No differences in neural differentiation were observed between 

$$\text{Chst11}^{2/-}$$ and 

$$\text{Chst11}^{-/-}$$ cells (Fig. 5B).

We also analyzed the effects of Chst14 deficiency on neuronal differentiation in vivo. The percentage of cells positive for both tubulin-βIII and BrdU (tubulin-βIII+BrdU+) among the total number of BrdU+ cells in the ganglionic eminence of E14.5 embryonic brains 24 hours after BrdU administration was not different in 

$$\text{Chst14}^{2/-}$$ mice versus 

$$\text{Chst14}^{+/+}$$ littermates (supplementary material Fig. S1).

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$$\text{Chst14}^{2/-}$$ mice versus 

$$\text{Chst14}^{+/+}$$ littermates (supplementary material Fig. S1).

Furthermore, neurogenesis was assessed in the adult CNS in vivo. In the olfactory bulb, the density and the percentage of Neun+ neurons and GFAP+ astrocytes among the total number of BrdU+ cells (Fig. 6A–D) were not different between 

$$\text{Chst14}^{2/-}$$ and 

$$\text{Chst14}^{+/+}$$ mice 28 days after BrdU injection. By contrast, immunohistochemical analysis revealed a decreased density of Dcx+ newborn neurons in the dentate gyrus of the hippocampus (Fig. 6E) in 

$$\text{Chst14}^{2/-}$$ versus 

$$\text{Chst14}^{+/+}$$ mice (620±67 cells/mm² versus 736±55 cells/mm²; Fig. 6F), whereas 4 weeks after BrdU injection, no difference in the number of NeuN+BrdU+ cells was observed between the genotypes (Fig. 6G). Thus, Chst14 might accelerate adult hippocampal neurogenesis in vivo, but does not alter the number of newborn NeuN+BrdU+ neurons. The effect of Chst11 depletion on neurogenesis could not be investigated in adult animals, because 

$$\text{Chst11}^{-/-}$$ mice die at birth.

To investigate whether the differences in neuronal differentiation observed in vitro and in vivo were influenced by distinct mechanisms of apoptosis in 

$$\text{Chst14}^{2/-}$$ versus 

$$\text{Chst14}^{+/+}$$ mice or 

$$\text{Chst11}^{2/-}$$ versus 

$$\text{Chst11}^{-/-}$$ mice, the percentage of caspase-3+ cells was measured in vitro 7 days after induction of differentiation by withdrawal of growth factors (supplementary material Fig. S5), as well as in the olfactory bulb in vivo.
No differences in the percentage of caspase-3+ cells were observed between Chst11−/− or Chst14−/− and the respective wild-type controls in vitro or in vivo, indicating that reduced neurogenesis in Chst14−/− NSCs was not due to increased apoptosis during differentiation.

Effects of Chst14 and Chst11 on migration of NSCs
The effects of Chst11 or Chst14 deficiency on migration of NSCs were examined in vitro by measuring the distance of cells that had migrated from neurospheres 4 and 24 hours after seeding (supplementary material Fig. S7). No differences were observed in cell migration from Chst11−/− or Chst14−/− compared with Chst11+/+ and Chst14+/+ neurospheres. Furthermore, migration of NSCs in the dentate gyrus (supplementary material Fig. S8) and olfactory bulb (supplementary material Fig. S2) did not differ between adult Chst14−/− and Chst14+/+ mice.

Deficiency in Chst14 influences the composition of distinct subpopulations of radial glial cells
Previous studies showed that cleavage of CS-GAGs by ChaseABC reduced proliferation and neuronal differentiation of NSCs and increased astrocytic differentiation, associated with a change in the composition of subpopulations of radial glial cells: After removal of the CSs and DSs, neurospheres contained fewer BLBP+ and nestin+ radial glial cells and more GLAST+ and RC2+ radial glial cells (Sirko et al., 2007). In the present study, expression levels of different radial glial cell markers were determined in Chst11−/− and Chst14−/− neurospheres. Expression of the transcription factor Mash-1 was decreased to 0.66±0.28% in Chst14−/− versus control neurospheres, whereas GLAST expression was increased 1.34±0.29-fold in Chst14−/− versus Chst14+/+ neurospheres (Fig. 7A,C). The expression of nestin was not altered. Immunohistochemical analysis of neurospheres revealed increased percentages of GLAST+ cells in Chst14−/− neurospheres versus Chst14+/+ neurospheres (40.7±4.8% versus 29.8±3.8%; Fig. 7D,E), although no remarkable increase was detected in the percentage of RC2+ cells. None of the investigated radial glial cell markers was differentially expressed in Chst11−/− neurospheres compared with Chst11+/+ mice (Fig. 7A,B). Thus, deficiency in Chst14, but not Chst11, alters the composition of distinct subpopulations of radial glial cells.

Discussion
Previous studies have shown that multiple chondroitin and dermatan sulfotransferases are expressed in the neurogenic regions of the embryonic and adult CNS and that complex CS and DS chains play important roles in NSC biology (Akita et al., 2008). Two of those sulfotransferases, Chst11/C4st1 and Chst14/D4st1, belonging to the HNK1-ST family, target different GAG motifs: Chst11 preferentially sulfates GalNAc residues flanked by GlcA and is therefore involved in CS biosynthesis. Chst14 mainly targets GalNAc residues flanked by IdoA and is essential for the formation of iduronic acid blocks in DS sulfation patterns. Chst14 is indispensable in the biosynthesis of functional sulfation domains in DSs and can thus not be compensated by other 4-O-sulfotransferases (Pacheco et al., 2009). Therefore, investigations of NSCs from Chst11−/− and Chst14−/− mice were expected to provide insights into the roles of different endogenous CS and DS.
sulfation patterns in neural development. The consequences of this depletion for NSC biology were analyzed in view of possible compensatory mechanisms exerted by other HNK-1 sulfotransferase family members. Neurospheres derived from the ganglionic eminence of E13.5 $\text{Chst11}^{-/-}$ mice exhibited a reduced mRNA expression of $\text{Chst12}$ and $\text{Chst14}$, whereas no differences of other HNK-1 sulfotransferase family members were detected between $\text{Chst14}^{-/-}$ and $\text{Chst14}^{+/+}$ neurospheres. Because the genes encoding Chst11, Chst12 and Chst14 are located on different chromosomes, a positional effect at the genomic level can be excluded. Furthermore, potential feedback mechanisms among members of the HNK-1 sulfotransferase family have not yet been described. Based on the similar substrate specificity of Chst11, Chst12 and Chst14, and the importance of Chst11 in sulfation of CS chains, it is possible that chondroitin sulphate proteoglycans (CSPGs) modified by Chst11 negatively influence the expression of Chst12 and Chst14.

CS and DS chains show variation in length, arrangement of repeating disaccharide units and sulfation patterns (Sugahara and Yamada, 2000). Studies on the disaccharides of CSs and DSs in the brain indicated that blocks of IdoA, which are the principal structures of DS patterns, are dramatically increased during development of the cerebellum, accompanied by a downregulation of CS patterns (Mitsunaga et al., 2006). These observations suggest that DS chains play important roles in CNS development. Recently, the 473HD epitope, a unique CS–DS structure recognized by the antibody 473HD, was shown to be expressed in the germinative ventricular zone of E13.5 mouse brains and in neurospheres derived from embryonic forebrains.

Fig. 5. $\text{Chst14}^{-/-}$ NSCs, but not $\text{Chst11}^{-/-}$ NSCs, show impaired neuronal differentiation. (A) Distribution of neurons, astrocytes and oligodendrocytes differentiated from $\text{Chst11}^{-/-}$ or $\text{Chst14}^{-/-}$ NSCs and from $\text{Chst11}^{+/+}$ and $\text{Chst14}^{+/+}$ NSCs were analyzed with antibodies against different markers: Tuj for neurons (shown in red), GFAP for astrocytes (green) and CNPase for oligodendrocytes (red). Nuclei were counterstained with DAPI (blue). (B) Quantification of differentiation of NSCs derived from $\text{Chst11}^{-/-}$ mice and $\text{Chst11}^{+/+}$ mice. Note that NSCs derived from $\text{Chst11}^{-/-}$ mice are not different from $\text{Chst11}^{+/+}$ NSCs in neural differentiation (mean ± s.d.; n=3). (C) Quantification of differentiation of NSCs derived from $\text{Chst14}^{-/-}$ mice and $\text{Chst14}^{+/+}$ mice. Neuronal differentiation is decreased in $\text{Chst14}^{-/-}$ NSCs versus $\text{Chst14}^{+/+}$ NSCs (mean ± s.d.; n=3). Statistical significance was assessed by Student’s $t$-test. **$P<0.01$. Scale bars: 100 µm.
This epitope, consisting of CS-A, CS-D and DS patterns, has been shown to be a marker for radial glial cells and to be crucial for neurosphere formation (von Holst et al., 2006). Immunoisolated 473HD+ NSCs formed significantly more neurospheres than control NSCs that had not been enriched for 473HD expression. In the present study, expression of the 473HD epitope was decreased both in \( \text{Chst11}^{+/+} \) and \( \text{Chst14}^{+/+} \) NSCs, which is consistent with the view that Chst11 and Chst14 are involved in the biosynthesis of the 473HD epitope. Comparison between NSCs from \( \text{Chst11}^{-/-} \) or \( \text{Chst14}^{-/-} \) mice and NSCs from their wild-type littermates showed that the expression of the 473HD epitope was decreased by 90% in \( \text{Chst11}^{-/-} \) NSCs and by 42% in \( \text{Chst14}^{-/-} \) NSCs. Ito and colleagues (2005) showed that the 473HD epitope consists of A–D and/or D–A structures. The A-unit defines the monosulfated GlcUA-GalNAc(4S) and the D-unit defines the disulfated GlcUA(2S)–GalNAc(6S) structures. Additionally, these authors found that the 473HD epitope was eliminated from DSD-1-PG (also known as protein tyrosine phosphatase, receptor type Z, polypeptide 1) by digestion with chondroitinaseB, suggesting the close association of L-iduronic acid with the 473HD epitope. They also discussed the probability that the iduronic acid was not directly included in the epitope, but located on the reducing end in the parent chain. This could explain why we detect a 90% reduction of the 473HD epitope expression in \( \text{Chst11}^{-/-} \) NSCs, because Chst11 is the predominant enzyme in the production of the A-unit which is present in the 473HD epitope core, and see only a 50% reduction in \( \text{Chst14}^{-/-} \) NSCs because Chst14 is the predominant enzyme in the production of the B-unit, which is probably exposed on the reducing end in the parent chain and not on the epitope core itself.

CS and DS GAGs are important for the self-renewal and proliferation of NSCs. Removal of CSs and DSs by ChaseABC treatment, which unselectively cleaves CSs and DSs from CS-DSPGs, impaired neurosphere formation, decreased proliferation of NSCs in vitro and in the ventricular zone of E14.5 mouse forebrains (Sirko et al., 2007), and diminished in vitro proliferation of NSCs derived from the E16 rat forebrain (Gu et al., 2009). Although the influence of different CS and DS patterns on NSCs has been reported by studying isolated CSs.
and DSs, these investigations made use of either chemically synthesized CS and DS oligosaccharides or CSs and DSs extracted mostly from non-neural organs, as well as from different animal species with features possibly different from those derived from neural tissues (Gama et al., 2006; Ida et al., 2006; Sato et al., 2008). These compounds were added to cultures of NSCs and thus did not allow insights into the potentials of CSs and DSs endogenously synthesized by NSCs.

GAGs exert their biological effects by interacting with growth factors or cytokines and their receptors, thereby influencing various signaling pathways (Sasisekharan et al., 2006). CS and DS chains interact with several growth factors or cytokines that regulate maintenance and self-renewal of NSCs (Crespo et al., 2007): deglycosylation of CS-DSPGs by ChaseABC treatment impaired FGF-2-mediated neurosphere formation and proliferation. Furthermore, FGF-2-dependent MAP kinase activation in mouse embryonic cortical NSCs was diminished after ChaseABC digestion (Sirko et al., 2010). CS-DSPGs influence FGF-2- and EGF-dependent signaling pathways, thereby modulating NSC proliferation by binding to these molecules in the NSC microenvironment (Properzi et al., 2005), or by serving as co-factors for growth factor or cytokine receptors (Bandtlow and Zimmermann, 2000). DSs extracted from pig skin and highly sulfated CS-E motifs from shark cartilage promoted FGF-2-mediated proliferation of NSCs (Ida et al., 2006). CS-E and DS motifs thus serve as docking molecules for growth factors and thereby regulate their activity towards NSCs (Deepa et al., 2002; Penc et al., 1998).

In the present study, in vitro analysis revealed that deficiency in Chst14, but not Chst11, impaired neurosphere formation. The analysis of NSC proliferation in neurospheres revealed a decreased proliferation of NSCs in Chst14-deficient versus wild-type neurospheres making it very likely that the differences in the formation of neurospheres were caused by differences in...
proliferation of cells and not by differences in the adhesion of cells. Additionally, deficiency in Chst14, but not Chst11, reduced proliferation of NSCs cultured in adherent cell culture under the influence of FGF-2 or EGF alone, or both FGF-2 and EGF. In vivo, deficiency in Chst14 reduced the density of proliferating neural progenitors in the subventricular zone of the lateral ventricles and the hippocampal dentate gyrus of adult mice. In the E14.5 ganglionic eminence, a tendency towards a reduction of proliferating cells was observed in Chst14/−/− mice. The expression of growth factor receptors EGFR and FGFR-1 was upregulated in Chst14/−/− NSCs compared with Chst14+/− NSCs but was not altered in Chst11−/− versus Chst11+/− neurospheres in vitro. Combined with the results from our in vitro proliferation analysis, upregulation of EGFR and FGFR-1 expression in Chst14−/− NSCs suggests that DS motifs synthesized by Chst14 modify the interaction between the growth factors EGF and FGF-2 and their respective receptors. CS and DS variants are involved in different cellular processes, such as binding to growth factors and neurotrophic factors. In growth factor binding, variations in CS lead to varying binding capacities (Crespo et al., 2007), whereas the presence of IdoA results in increased binding levels (Hileman et al., 1998). The reason for the higher binding capacity to the growth factor is believed to be caused by the tendency of the pyranose ring of IdoA in dermatan sulfate to form various conformations. However, for more efficient binding, a domain consisting of both GlcA and IdoA appears to be essential (Nandini and Sugahara, 2006), which suggests the importance of Chst14 in the modulation of growth factor binding.

Depletion of Chst14 reduced neuronal differentiation of NSCs in vitro, associated with a tendency towards an increased differentiation into astrocytes which was, however, statistically not significant. In vivo, less Dcx-positive newborn neurons were observed in the dentate gyrus of the adult hippocampus of Chst14−/− mice, whereas the number of newborn NeuN+Brdu+ cells was not altered, suggesting that Chst14 might accelerate adult hippocampal neurogenesis in vivo. Because adult neurogenesis in the hippocampus is important for learning and memory (Jessberger et al., 2009; Shors et al., 2001), the question whether this acceleration effect driven by DS patterns formed by Chst14 plays a role in learning and memory will be addressed in a separate study. In the olfactory bulb of adult mice, a statistically insignificant reduction in the density of Brdu+ cells in whole olfactory bulb was observed in Chst14−/− mice compared with Chst14+/− littermates, whereas the percentages of newly generated neurons and newborn astrocytes in the total number of Brdu+ cells were unaltered.

The question remains how DS motifs modified by Chst14 influence the fate of NSCs. Previous studies showed that the combined removal of both CS and DSs from NSCs using ChaseABC inhibits neurogenesis and promotes gliogenesis, which is associated with a reduction of the percentage of BLBP+ and nestin+ radial glial cells and an increased percentage of GLAST+ and RC2+ radial glial cells (Sirko et al., 2007). Because BLBP is regulated by Notch signaling, which crosstalks with FGF and EGF signaling (Anthony et al., 2005), and because GLAST is expressed in adult parenchymal astrocytes (Mori et al., 2005), it has been suggested that CSs and DSs are involved in Notch signaling, and that treatment with ChaseABC affects the subpopulations of radial glial cells: nestin+ and BLBP+ precursors represent a proliferative and neurogenic precursor fraction, whereas GLAST+ radial glial cells preferentially show gliogenesis and decreased proliferation (Sirko et al., 2007). In the present study, the mechanisms by which Chst14 affects differentiation of NSCs was analyzed by measuring the expression of nestin, Mash-1 and GLAST in neurospheres derived from Chst14−/− mice and Chst14+/+ littermate controls. Mash-1 is an activator-type basic helix-loop-helix (bHLH) gene that is critical for neurogenesis, and defects in neurogenesis have consistently been observed in Mash1-deficient mice (Nieto et al., 2001; Tomita et al., 2000). Mash-1 is also involved in Notch signaling, which promotes self-renewal and inhibits neurogenesis of NSCs by regulating transcription factors such as Hes1, which inhibits neurogenesis and promotes astrogenesis by repressing expression of Mash-1 (Gaiano and Fishell, 2002). In the present study, a downregulation of Mash-1 was associated with an upregulation of GLAST in Chst14−/− mice, while Chst11−/− neurospheres expressed similar amounts of nestin, Mash-1 and GLAST as Chst11+/+ neurospheres. Immunocytochemical analysis of Chst14−/− neurospheres showed that they contained more GLAST+ cells compared with Chst14+/+ neurospheres, whereas the percentage of RC2+ cells was not significantly altered. Considering the diminished proliferation and impaired neurogenesis in Chst14−/− NSCs, we conclude that endogenously generated DS patterns modified by Chst14 might exert their effects on the fate determination of NSCs by crosstalking with the Notch signaling pathway, thereby influencing the FGF-EGF signaling pathway and thus potentially altering the occurrence of the radial glial cell subpopulations. Because the total number of neurons in the analyzed brain regions did not differ between Chst14−/− and Chst14+/+ mice, the influence of Chst14 on neurogenesis might be transient in that the effects of Chst14 deficiency are compensated by as yet unknown mechanisms.

CSs and DSs have been proposed to provide repulsive cues that inhibit cell migration (Carulli et al., 2005). ChaseABC treatment of NSCs removing CSs and DSs enhances the migration of NSCs in vitro (Gu et al., 2009; Sirko et al., 2010), a feature that is influenced by EGF signaling (Deepa et al., 2002). Also, RNA interference (RNAi) knockdown of uronyl 2-O-sulfotransferase and N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase, which are responsible for the synthesis of highly sulfated CS-D and CS-E motifs, respectively, resulted in disturbed migration of cortical neurons. However, knockdown of Chst14 did not lead to migratory defects (Ishii and Maeda, 2008). These observations are in agreement with the results of this study, showing that deficiency in Chst14 did not affect NSC migration. Analysis of Chst11−/− NSCs indicated that CS patterns modified by Chst11 also did not influence migration of NSCs. The observations that DSs formed by Chst14, as well as CSs formed by Chst11, did not affect cell migration indicate that sulfation of the carbon 4 position of GalNAc within CS or DS chains is not crucial for cell migration. Further investigations of other carbohydrate sulfotransferases targeting CSs or DSs are expected to allow insights into the sulfation patterns involved in migration of NSCs.

Materials and Methods

Animals

Chst11/C4st1-deficient (Chst11−/−) and Chst14/D4st1-deficient (Chst14−/−) mice were generated by homologous recombination using standard techniques and maintained in C57BL/6 and 129sv background (supplementary material Figs S9, S10). Briefly, using the recombination cloning (REC) system (Zhang et al., 2002) a 129/SvJ mouse 2K0-2 genomic plasmid library was screened, two clones containing either the Chst11 or Chst14 genomic regions were isolated, the third
exon of the Chst11 gene and the unique exon of the Chst14 gene were deleted and a neomycin-kanamycin expression cassette was introduced. These targeting vectors were used for electroporation of R1 ES cells (Nagy et al., 1993) and lines of Chst11−/− and Chst14−/− mice were established. The Chst11-deficient mouse line was backcrossed twice to C57BL/6 and the Chst14-deficient mouse line was backcrossed once to C57BL/6 before conducting experiments. The expected germine manipulations of the Chst11 and Chst14 loci were confirmed using multiplex PCR, Southern and northern blot analysis (supplementary material Figs S9 and S10). Southern and northern blot analyses were performed as described (Sambrook et al., 1989). The probes were amplified from genomic DNA or cDNA using the Phu DNA polymerase (Promega, Mannheim, Germany), confirmed by sequencing and labelled with 32P using the Megaprime DNA labelling kit (Amersham, Freiburg, Germany) according to the manufacturer's instructions. The plasmid pCMV-DSRed-Express (Clontech, Heidelberg, Germany) was digested with the restriction endonucleases NcoI and NolI and the 0.7 kb DsRed-express fragment was isolated after agarose gel electrophoresis and labelled as described above. Multiplex PCR was performed with Taq DNA polymerase (Invitrogen, Darmstadt, Germany) using standard cycling parameters to determine the genotype of the offspring (supplementary material Table S1). All animal experiments were performed according to the rules and regulations of the University and State of Hamburg Animal Care Committees.

**Antibodies**

The monoclonal antibodies 487 and 473HD (kindly provided by Andreas Faissner, Ruhr University Bochum, Bochum, Germany), and polyclonal antibody against the glutamate aspartate transporter (GLAST) (kindly provided by Niels Danbolt, University of Oslo, Oslo, Norway) have been described (Faissner et al., 1994; Lehre et al., 1995; Streit et al., 1996). All commercial primary antibodies are listed in supplementary material Table S2. All secondary antibodies were purchased from Jackson ImmunoResearch (Newmarket, UK) and Santa Cruz Biotechnology (Santa Cruz, CA).

**Neural stem cell culture**

NSCs were isolated from the ganglionic eminence of embryonic day 13.5 (E13.5) mice and cultured as floating neurospheres in neurosphere formation medium as described previously (Dichter et al., 1998). The formation of neurospheres was analyzed as described previously (Sirkko et al., 2007). Briefly, dissociated NSCs were seeded at a clonal density of 2 × 10^3 cells in 6 ml culture medium in the presence of FGF-2 and EGF (20 ng/ml each; PreproTech, Rocky Hill, NJ), and semi-quantitative determination of the diameter of neurospheres (<100, 100–200, >200 µm in diameter) was performed after 4 days in culture. Images were acquired with a Kontron microscope (Carl Zeiss, Jena, Germany) and data was analyzed with the AxioVision Rel. 4.6 software (Carl Zeiss). All in vitro analyses were confirmed in at least three independent experiments conducted on independent cell preparations.

**Quantitative real-time PCR**

The RNA isolation levels of all HKN-1 sulfotransferase family members were analyzed by quantitative real-time PCR (qRT-PCR). Total RNAs were isolated from neurospheres generated from Chst11+/− and Chst14+/− brains as well as Chst11+/− and Chst14+/− littermates using the RNasy Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis was performed using 0.5–5 µg total RNA, random hexamers and SuperScript II RNaseH Reverse Transcriptase (Invitrogen), and qRT-PCR was performed using 0.025 µg reverse transcribed total RNA, specific primer pairs (0.1 µM each; Metabion, Martinsried) and qPCR Core kit for SYBER Green I (Eurogentec, Köln, Germany) in an ABI 7900 HT system (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. The primer sequences are presented in supplementary material Table S3. The qRT-PCR data were analyzed using the standard curve method (Applied Biosystems). We used four housekeeping genes encoding hypoxanthine guanine phosphoribosyl transferase (Hprt), b-actin, glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and RNA polymerase II (P0r2a) as endogenous references and calculated the normalization factor with the software geNORM (Vandesompele et al., 2002).

**Proliferation, differentiation and apoptosis of NSCs in vitro**

For in vitro analysis, neurospheres were mechanically dissociated and plated at a density of 5 × 10^5 cells/well onto 15 mm glass coverslips coated with 0.01% (w/v) poly-L-lysine (PLL) (Sigma, Munich, Germany). For assessment of proliferation of NSCs derived from Chst11+/− or Chst14+/− mice and wild-type (Chst11+/+ and Chst14+/+) respectively, cells were adherently cultured in defined medium containing either FGF-2 (20 ng/ml), or EGF (20 ng/ml), or a mixture of FGF-2 and EGF (each 20 ng/ml) for 24 hours. One dose of 5-bromo-2-deoxyuridine (BrdU) (10 µM; Sigma) was added to the medium 8 hours before fixation. Proliferation was analyzed by determining the percentage of BrdU-immunoreactive cells of all 4’,6-diamidino-2-phenylindole (DAPI) (Sigma)-positive cells.

To assess differentiation and apoptosis, dissociated NSCs were cultured on coverslips in the absence of growth factors for 7 days. Antibodies recognizing different cell markers and apoptosis marker caspase-3 were applied to determine NSC differentiation and cell apoptosis.

**Migration of NSCs in vitro**

To analyze the radial migration of neuronal stem cells, neurospheres were seeded onto 0.02% (w/v) PLL-coated coverslips and cultured in the absence of growth factors. The migration distance was defined as the distance between the cell bodies of the farthest migrated cells and the edge of the neurosphere. After culture for 4 and 24 hours, the longest distances between the edge of a neurosphere and the cell bodies were measured in each quadrant of the sphere perimeter, and at least ten neurospheres were analyzed for each group and each time point. Images were acquired on a Kontron microscope and analyzed with the AxioVision Rel. 4.6 software system (Carl Zeiss).

**BrdU Incorporation**

Proliferating cells in vivo were labeled by intraperitoneal administration of BrdU, which was diluted in a sterile solution of 0.9% (w/v) NaCl and 1.75% (w/v) Na3H (Sigma). For analysis of incorporation of BrdU by embryonic cells, pregnant heterozygous Chst14+/− mice were injected intraperitoneally with BrdU (100 mg/kg body weight) at embryonic day 13.5 (E13.5) 24 hours before collection of embryos. For adult mice, 2- to 3-month-old Chst14+/− mice and Chst14−/− mutants were injected with BrdU (100 mg/kg body weight) at 2 days of age. Two different previously described protocols (Saghatelyan et al., 2004) were used: (1) to assess proliferation in the subventricular zone and dentate gyrus of the hippocampus and initial migration of adult NSCs in the dentate gyrus of the hippocampus, a single dose of BrdU was applied to the mice 4 hours before perfusion; (2) to determine the number of newborn neurons of ependyma, differentiation and cell survival of newborn cells in the olfactory bulb, which originated from the subventricular zone, four consecutive injections of BrdU were given at intervals of 2 hours, and mice were sacrificed 28 days later.

**Immunocytochemistry and immunohistochemistry**

Immunocytochemistry and immunohistochemistry were performed as previously described (Hargus et al., 2008). For immunohistology with cell type-specific markers, coverslips with adherent cells were washed with phosphate buffered saline (PBS) (pH 7.4, PAA Laboratories, Colbe, Germany), fixed with 4% (w/v) formaldehyde in 0.1 M cacodylate buffer (pH 7.3; Sigma) for 30 minutes, blocked with 5% (w/v) normal goat serum (NGS) (Jackson ImmunoResearch) in PBS containing 0.2% (v/v) Triton X-100 and 0.02% (w/v) sodium azide (both from Sigma) for 30 minutes, and incubated with primary antibodies in 0.5% (w/v) lambda casein in PBS containing 0.02% (w/v) sodium azide at 4°C overnight followed with Cy2- or Cy3-coupled secondary antibodies diluted 1:1000 in carrageenan buffer for 1 hour. Nuclei were counterstained with 50 µg/ml DAPI at room temperature for 2 minutes and coverslips were mounted with Fluoromount-G (Southern Biotech, Birmingham, AL). As primary antibodies, tubulin-BIII, GFAP, CNPase, caspase-3, BrdU, Ki67, nestin, RC2, GLAST and pan-neuro287 were applied. For the BrdU staining, cells were incubated with 2 M HCl at 37°C for 30 minutes to denature DNA before blocking with 5% (v/v) NGS in PBS with 0.2% Triton X-100 (v/v) and 0.02% sodium azide for 1 hour at room temperature. On each coverslip at least 1000 DAPI+ cells were counted using the NeuroLucida software-controlled computer system (MicroBrightField, Europe, Magdeburg, Germany). The immunolabeled slide preparation was scanned using an Olympus Fluoview™ FV1000 confocal microscope equipped with UV epifluorescence (Olympus, Hamburg, Germany).

For histological analysis of embryos, brains of embryos at E14.5 were fixed with 4% (w/v) formaldehyde for 2 hours, incubated sequentially in 15% and 30% (w/v) sucrose solution in 0.1 M cacodylate buffer (pH 7.4) at 4°C overnight, and cut in serial frontal sections (14 µm each) on a cryostat (Leica, Wetzlar, Germany) and every fifth section was used for further analysis. For histological analysis of adult mouse brains, mice were deeply anesthetized with an overdose of Narcoren (Merial, Hallbergmoos, Germany) and perfused intracardially with 4% (w/v) formaldehyde (Sigma). The brains were removed and immersed in the same fixative at 4°C overnight, incubated in 15% (w/v) sucrose in 0.1 M cacodylate buffer at 4°C overnight, cut in serial frontal sections (25 µm each) on a Leica cryostat and every tenth section was used for further analysis. For immunohistochemistry, antigen retrieval was performed by incubation in 10 mM sodium citrate (pH 9.0; Sigma) at 80°C for 30 minutes, followed by blocking with 5% (v/v) NGS or normal donkey serum (NDS) (Jackson ImmunoResearch) at room temperature for 1 hour and incubation at 4°C overnight with the primary antibodies against the following proteins: BrdU for proliferating cells; caspase-3 for cells undergoing apoptosis; Dcx for immature neurons; BrdU and NeuN double staining or BrdU and GFAP double staining for analysis of newly generated neurons and astrocytes, respectively. Subsequently, slides were washed with PBS and incubated with specific fluorochrome-coupled secondary antibodies for 2 hours at room temperature. Slides were labelled with bisbenzimide (1:10,000; Sigma) for 10 minutes to identify cell nuclei, and mounted with Fluoromount-G. For BrdU
immunofluorescence slides were incubated in 0.1 M HCl instead of antigen-retrieval buffer at 60°C for 20 minutes to denature DNA before blocking with NGS, and cell nuclei were labeled with bisbenzimide for 20 minutes. The density of single- or double-labeled cells was analyzed using the Neurolucida system (MicroBrightField Europe) and an Olympus confocal laser microscope (Olympus) was used for image scanning.

Western blot analysis
Expression of growth factor receptors EGFR and FGFR-1 and radial glial cell markers including nestin, Mash-1 and GLAST were analyzed by western blot analysis. Protein extracts were harvested by lysing neurospheres generated from Chst11+ or Chst14+ mice and Chst14+ animals with RIPA lysis buffer (150 mM NaCl, 1 mM Na3PO4, 1 mM NaF, 1 mM EDTA, 1 mM PMSE, 2 mM NaVO3, 1% NP-40, 50 mM Tris, pH 7.5) with complete EDTA-free protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany) at 4°C for 1 hour. The protein samples were boiled in SDS sample buffer for 10 minutes before loading onto 10% SDS-PAGE gel or Prolong Tris-Glycine 4–20% gradient gels (Anamed Elektrophores, Groß-Bieberau, Germany) as 20 μg for each lane and transferred onto nitrocellulose membrane (Protran, Victoria, Australia). For immunoblotting, membranes were blocked with 5% (w/v) non-fat milk powder in PBS and incubated at 4°C overnight with primary antibodies diluted in PBST [PBS with 0.05% (v/v) Tween-20] against the following proteins: 473HD, actin, EGFR, FGFR-1, GLAST, Mash-1, nestin. After washing with PBST, membranes were incubated with specific HRP-conjugated secondary antibodies for 1 hour at room temperature followed with extended washes with PBST. Immunoblot reactions were visualized using chemiluminescent substrate (Perbio Science, Bonn, Germany) on Kodak BioMax light films (Kodak, Rochester, USA). The intensities of the bands were densitometrically quantified with the image software TINA 2.10.

Statistical analysis
All experiments were repeated independently at least three times and performed in a blinded manner, and analyzed by comparison of the mean values ± s.d. of deficient and wild-type mice using Student’s t-test for independent samples.

Acknowledgements
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References


SUPPLEMENTARY FIGURES

Fig. S1

A

Chst14+/o  Chst14−/−

B

Chst14+/o  Chst14−/−

C

Chst14+/o  Chst14−/−

D

Chst14+/o  Chst14−/−
Fig. S2

A

GCL+IPL+MCL  EPL  GL

Chst14<sup>+/+</sup>  

Chst14<sup>−/−</sup>

B

![Graph A](image)

C

![Graph B](image)
<table>
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<th>Experiment</th>
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<th>Primer direction</th>
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