View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Stem Cell Research (2015) 14, 68–78

Available online at www.sciencedirect.com
ScienceDirect
www.elsevier.com/locate/scr

Predominant expression of N-acetylglucosaminyltransferase V (GnT-V) in neural stem/progenitor cells

Makoto Hamanoue [a,b,*], Yoshitaka Ikeda [c], Toru Ogata [d], Ken Takamatsu [a,b]

[a] *Department of Physiology, Toho University School of Medicine, Tokyo 143-8540, Japan*
[b] *Advanced Medical Research Center, Toho University Graduate School of Medicine, Tokyo 143-8540, Japan*
[c] *Division of Molecular Cell Biology, Department of Biomolecular Sciences, Faculty of Medicine, Saga University, Saga 849-8501, Japan*
[d] *Department of Rehabilitation for the Movement Functions, Research Institute, National Rehabilitation Center for Persons with Disabilities, Saitama 359-8555, Japan*

Received 2 April 2014; received in revised form 27 October 2014; accepted 24 November 2014
Available online 5 December 2014

Abstract Neural stem/progenitor cells (NPCs) express a variety of asparagine-linked oligosaccharide chains, called N-glycans, on the cell surface, and mainly produce hybrid-type and complex-type N-glycans. However, the expression profiles and roles of N-acetylglucosaminyltransferase-V (GnT-V), an enzyme that forms β1,6-branched N-glycans, in NPCs remain unknown. In this study, cultured NPCs were prepared from adult or embryo cortex, and were maintained as either proliferating NPCs or differentiated cells in vitro. Analysis using reverse-transcriptase polymerase chain reaction, Western blot and lectin blot revealed that GnT-V and its reaction products were distinctly expressed in proliferating NPCs; moreover expression of GnT-V and its reaction products were markedly diminished in differentiated cells. In brain slices, many GnT-V-positive neurogenic cells were detected throughout the cerebral cortex on embryonic day 13, while only a few doublecortin (Dcx)- and GnT-V-double positive NPCs were detected around the subventricular zone of the lateral ventricle in the adult brain. However, in the mice in which motor function was spontaneously recovered after cryoinjury to the motor cortex, many Dcx- and GnT-V-double positive NPCs were found to have accumulated around the brain lesion of the adult cerebral cortex compared with the mice in which the function did not recover. These results indicate that GnT-V expression is under rigorous control during NPC differentiation. Furthermore, expression of GnT-V and its reaction products in NPCs may be necessary for the functional recovery after brain injury, and could be used as a marker for visualization of NPCs.
© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

Abbreviations: Dcx, doublecortin; DMEM/F-12 medium, Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 Ham; EGF, epidermal growth factor; E-PHA, *Phaseolus vulgaris* erythroagglutinating lectin; GFAP, glial fibrillary acidic protein; GlcNAc, N-acetylglucosamine; GnT, N-acetylglucosaminyltransferase; L-PHA, *Phaseolus vulgaris* leucoagglutinating lectin; Man, mannose; N-glycan, asparagine-linked oligosaccharides; NPCs, neural stem/progenitor cells; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; SVZ, subventricular zone.

* Corresponding author at: Department of Physiology, Toho University School of Medicine, 5-21-16 Omori-nishi, Ota-ku, Tokyo 143-8540, Japan. Fax: +81 3 3762 8225.

http://dx.doi.org/10.1016/j.scr.2014.11.004
1873-5061/© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

Introduction

Asparagine-linked oligosaccharides (N-glycan) can typically be classified into a high mannose, complex, or hybrid type, depending on the extent of processing and/or on the type of branching (Kleene and Schachner, 2004). The composition of N-glycan in adult mice differs among organs: the major part of N-glycan in the adult peripheral organs is complex- and hybrid-type N-glycan, while the majority of N-glycan in the adult brain is high mannose-type N-glycan (Chai et al., 1999; Chen et al., 1998; Zamze et al., 1998). In the adult brain, neural stem/progenitor cells (NPCs) exist in the subventricular zone (SVZ) and dentate gyrus, and have a typical stem cell phenotype, with self-renewal and multipotent capacities (for reviews see: Anderson, 2001; Gage et al., 1995; McKay, 1997; Okano, 2002). Interestingly, adult NPCs express mainly hybrid-type and complex-type N-glycans, rather than high mannose-type N-glycan. In particular, bisected complex-type N-glycans are expressed on the NPC surface, and are a useful cell surface marker that can help enrich NPCs via the lectin panning method (Hamanoue et al., 2008, 2009; Hamanoue and Okano, 2011).

Biosynthesis of both bisected and β1,6-branched complex-type N-glycans is initiated by the addition of a single N-acetylglucosamine (GlcNAc) residue to a common acceptor, typically biantennary oligosaccharide; however, different glycosyltransferases are responsible for linking GlcNAc to mannose residues in the trimannosyl core. N-acetylglucosaminyltransferase (GnT)-III, which is encoded by the Mgat3 gene, transfers a GlcNAc to β-mannose via β1,4-linkage, thus leading to the formation of bisecting GlcNAc. A β1,6-branch is formed by GnT-V, which is encoded by the Mgat5 gene, thus resulting in conversion to triantennary oligosaccharide (Saito et al., 1994; Shoreibah et al., 1993). Another glucosaminyltransferase that synthesizes β1,6-branched complex-type N-glycans in vitro is GnT-Vb (IX), a paralog of GnT-V with 53% amino acid similarity (Inamori et al., 2003; Kaneko et al., 2003). In vivo, GnT-Vb predominantly transfers GlcNAc via β1,6-linkage to O-mannose type (O-glycans) but not to N-glycan (Lee et al., 2012). GnT-III, GnT-V, and GnT-Vb are expressed throughout the brain (Abbott et al., 2008; Bhattacharyya et al., 2002; Bhaumik et al., 1995; Inamori et al., 2003; Lee et al., 2012; Partridge et al., 2004); however, the expression of these GnTs in NPCs remains unknown. In this study, we examined the expression of GnTs by PCR analysis, Western blot analysis, and immunohistochemistry, and revealed the predominant expression of GnT-V and its reaction products in proliferating NPCs. Thus, GnT-V and its reaction products could be novel markers for NPCs in living brain tissue. Furthermore, experiments using a cryoinjury model suggested that GnT-V and its reaction products may be necessary for the functional recovery of damaged brain.

Materials and methods

Materials

Pregnant and adult mice (C57BL/6J) were purchased from Sankyo Laboratory Service (Tokyo, Japan). All experiments were performed according to the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan. The Ethics Review Committee for Animal Experimentation at Toho University also approved all experimental protocols used in this study (No. 13-53-153). All chemical reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Wako Pure Chemicals Industries (Osaka, Japan) unless otherwise noted. All culture materials were purchased from Greiner Bio-One (Tokyo, Japan) unless otherwise noted.

Cell preparation

NPCs were prepared from embryonic day 13 (E13) or adult [10-week-old (P10w)] mouse brains as previously described (Hamanoue et al., 2009; Sato et al., 2008) with slight modifications. In brief, embryonic brains (cerebral hemisphere and thalamus) or adult brains (cerebral hemispheres, hippocampus, and corpus callosum) were mechanically dissociated by trituration into single-cell suspensions in Hanks' balanced salt solutions (HBSS) without Ca^{2+} or Mg^{2+}. After washing with Dulbecco's Modified Eagle's Medium (Nutrient Mixture F-12 Ham (DMEM/F-12 medium)) several times, the cells were filtered through 70-μm and 40-μm cup filters (BD Biosciences, Franklin Lakes, NJ, USA). Dissociated brain cells were cultured in growth-promoting medium containing DMEM/F-12 medium, B27 supplement (Invitrogen Corp., Carlsbad, CA, USA), epidermal growth factor (EGF; 20 ng/mL; PeproTech EC, London, UK), basic fibroblast growth factor (FGF-basic; 20 ng/mL, PeproTech EC) and penicillin and streptomycin (100 U/mL and 100 μg/mL, respectively) for seven days. The primary neurosphere was dispersed by trituration and then replated onto Petri dishes in the growth-promoting medium for an additional seven days to obtain secondary neurospheres. NPCs were then obtained by dissociation of the secondary neurospheres (Hamanoue et al., 2009; Sato et al., 2008).

The purity of NPCs was estimated by their nestin expression, and capacity for self-renewal and multipotency (Hamanoue et al., 2009). Briefly, the differentiation of NPCs was initiated by culturing them in DMEM/F12 medium with B27 supplement on poly-L-lysine and laminin-coated dishes for two or five days. The multipotency for their development into differentiated cells, including neurons and astrocytes, from NPCs was ascertained by a Western blot analysis.

PCR analysis

mRNA was purified from cultured cells via a Qiagen RNeasy kit according to the manufacturer's instructions (QIAGEN, Valencia, CA, USA). The cDNA synthesis was performed using reverse transcriptase (Takara Bio, Inc., Otsu, Japan). For PCR analysis, we used EX Taq polymerase (Takara Bio, Inc.) with the following specific primers: GnT-V, 5′-GACCTGCAGTTCCTTCTTCG-3′ and 5′-CCATGGCAGAAGTCCTGTTT-3′; GnT-Vb, 5′-CATGGCACCGTGTACTAC-3′ and 5′-TCTGGAGCTCTGCAGAAG-3′ (Inamori et al., 2006); GnT-III, 5′-GGGAAATGTAGTTTTGAGCAGG-3′ and 5′-ACAGACATCTCAGGAGAGAGCC-3′; β-actin, 5′-GACCCAGATCATGTTTGAGACC-3′ and 5′-GAGAGCATAGCCCTCGTAGA-3′. PCR was performed on a thermal cycler (Biometra GmbH, Göttingen, Germany) using the following parameters: 40 cycles of PCR (98 °C for 10 s, 60 °C for 30 s, and 72 °C for 60 s). The

PCR products separated by 12% polyacrylamide gel electrophoresis were visualized under a UV-transilluminator (ATTO Corporation, Tokyo, Japan).

Western blot analysis and lectin blot analysis

Cells were washed with PBS, and the proteins were obtained by collecting cells with Laemmli's sample buffer. After boiling, the protein samples were separated on 2–18% sodium dodecyl sulfate (SDS)-polyacrylamide gels or 5–15% Hi-QRAS Neutral gel N (Kanto Chemical Co. Inc., Tokyo, Japan). The proteins were transferred to polyvinylidene difluoride (PVDF) membranes, which were then incubated in Tris-buffered saline containing 5% skim milk and 0.1% Tween 20 or Blocking One-P (Nacalai Tesque Inc., Kyoto, Japan). After being washed, the membranes were incubated with the following antibodies: anti-GnT-V (H00004249-A01, mouse polyclonal, Abnova Co., Taipei, Taiwan), anti-GnT-III (sc-27276, goat polyclonal IgG, Santa Cruz Biotechnology, Dallas, USA), anti-GnT-Vb (sc-164500, goat polyclonal IgG, Santa Cruz Biotechnology), anti-β-actin (sc-47778, mouse monoclonal IgG1, Santa Cruz Biotechnology), anti-c-Myb (sc-517, rabbit polyclonal IgG, Santa Cruz Biotechnology), anti-Ets-1/Ets-2 (sc-112, rabbit polyclonal IgG, Santa Cruz Biotechnology), anti-nestin (MAB353, mouse monoclonal IgG1, Millipore Corp., Billerica, USA), anti-GFAP (glial fibrillary acidic protein, G9269, rabbit polyclonal IgG, Sigma-Aldrich Co.), and anti-βIII-tubulin (G7121, mouse monoclonal IgG1, Promega Corp., Madison, USA) for 16 h at 4 °C, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, anti-mouse IgG, or anti-goat IgG (1:1000, Santa Cruz Biotechnology). Chemiluminescence signals were developed by the Immunostar LD kit, and were visualized by the ImageCapture G3 system (Liponics Inc., Tokyo, Japan).

For lectin blots, samples were prepared as described above, and the transfer membrane was incubated in a buffer containing biotin-conjugated L-PHA (*Phaseolus vulgaris* leucoagglutinating lectin, 1 μg/ml, EY Laboratories, Inc., San Mateo, USA) and HRP-streptavidin (GE Healthcare Biosciences AB, Upsala, Sweden) alter blocking with Blocking One-P. As an internal standard, the filter was blotted with anti-β-actin antibody. To ascertain whether L-PHA binds to samples through oligosaccharide, we treated the protein samples with PNGase (P0704, New England BioLabs Japan Inc., Tokyo, Japan), which cleaves between the GlcNAc and asparagine residues of N-glycans, before subjecting them to SDS-PAGE. The 14-μg protein samples were treated with 1000 units of PNGase at 37 °C for 1 h, followed by heat inactivation.

We obtained the relative intensities by densitometry using a computerized analysis system (NIH Image J and the Odyssey Infrared imaging system, LI-COR Corp., Lincoln, USA). Statistical evaluations were performed using Student's t-test, and $P < 0.05$ was considered to be statistically significant.

Cryoinjury model

The cryoinjury was induced using the method reported by Steinbach et al. (1999) with slight modifications. In brief, mice (8–10 weeks old) were anesthetized with 2,2,2-tribromoethanol (avertin, 0.25 mg/g (Papaioannou and Fox, 1993)). Incisions were made along the midline to expose the skull. An aluminum probe (3 mm in diameter) was fitted to a 50-ml syringe, which was filled with liquid nitrogen. The chilled probe was placed on the parietal skull 1.5 mm lateral to the midline at the bregma for 120 s. The skin was then sutured, and mice were returned to their cages. To evaluate the motor function and coordination of the mice, we administered a rotarod test (Shichinohe et al., 2007), with some modification. In brief, mice were trained for five weeks before cryoinjury with program B (80 rpm for a maximum of 180 s, MK-670, Rota-Rod Treadmill for Rats & Mice, Muromachi Kikai Co., Ltd., Tokyo, Japan). The trial ended when the mice fell from the rotor or misstepped on the rotor, and the latency was recorded. Each mouse ran on the rotarod at least five times, and the three longest times were used for the analysis.

Histochemical analysis

Immunohistochemistry was performed as described previously (Hamanoue et al., 2009) with slight modifications. The brains were removed from paraformaldehyde (PFA)-perfused mice and further fixed in 4% PFA for 1 h, 15% sucrose for 16 h, and 30% sucrose for 8 h. Cryostat sections (14 μm) of cerebral cortex were fixed with methanol for 10 min at −20 °C. After blocking the endogenous peroxidase activity, the sections were then blocked with Blocking One Histo (Nacalai Tesque Inc.) for 10 min at room temperature. The slices were incubated with the following antibodies in 1:20-diluted Blocking One Histo:anti-doublecortin (mouse IgG1, 611706, BD Transduction Lab) for progenitor cells (Tamura et al., 2007), anti-nestin (Millipore) for neurogenic cells and anti-GnT-V (sc-19088, goat polyclonal IgG, Santa Cruz Biotechnology). After being washed, the slices were stained with secondary antibodies conjugated with Alexa Fluor 555-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR, USA) or HRP-conjugated donkey anti-goat IgG and fluorescein-conjugated TSA (NEN Life Science Products, Inc. Boston, USA). The nuclei were visualized with 13 ng/mL of 4′,6-diamidino-2-phenylindole (DAPI). We observed and counted the fluorescent signals under a confocal microscope (Nikon Corp., Tokyo, Japan).

Hematoxylin and eosin staining was performed according to the manufacturer's instructions (Merk KGaA, Darmstadt, Germany). The brain slices were incubated with Mayer's hemalum solution (109249, Merk KGaA) and Eosin Y solution (102439, Merk KGaA). We quantified the wound area using the Adobe Photoshop software package.

Results

Antigenic characterization of NPCs

NPCs have both self-renewal and multipotent capacities. To ascertain whether our cultured cells had the capacity for self-renewal, primary or secondary neurospheres prepared from embryonic and adult mouse brain were dispersed and reseeded onto Petri dishes. After seven days in vitro, multiple spheres were detected (data not shown), indicating

that our spheres had the capacity for self-renewal. The multipotent capacity of our cells was verified by a Western blot analysis. We prepared the protein samples from cells under growth-promoting conditions, and conducted a Western blot analysis using antibodies specific for each neuronal cell type. The growth-promoting cells expressed the nestin antigen, which is specific for NPCs (Supplemental Fig. 1A, day 0), but did not express βIII-tubulin or glial fibrillary acidic protein (GFAP) antigen, which is specific for immature neurons and astrocytes, respectively (Supplemental Figs. 1B, C, day 0). To induce differentiation, the growth-promoting cells were cultured on a poly-L-lysine and laminin-coated dish in growth factor-deprived medium for two or five days. A Western blot analysis of these differentiation-promoting cells showed a decrease in nestin expression and an increase in the βIII-tubulin- and GFAP-expression (Supplemental Figs. 1A, B, C, days 2 and 5). These results revealed that cultured cells obtained from mouse brains have an NPC phenotype, and are able to differentiate into neurons and glia in vitro.

Changes in GnTs mRNA expression during differentiation

To estimate the expression level of the enzyme regulating the N-glycans, mRNA was isolated from adult NPCs and embryonic NPCs, and reverse transcriptase-polymerase chain reaction (PCR) amplification was performed using specific primers for GnTs. All GnT mRNAs, including GnT-III, GnT-V, and GnT-Vb, were detected in both adult and embryonic NPCs (Fig. 1, day 0). In the adult NPCs, however, all GnT mRNA levels were reduced in differentiated cells; in particular, the levels of GnT-V and GnT-Vb mRNA were drastically reduced (Fig. 1, adult). In contrast, GnT-III and GnT-Vb mRNA levels remained unchanged during the differentiation from embryonic NPCs to differentiated cells (Fig. 1, embryo), although GnT-V mRNA expression also dramatically decreased in the differentiating cells derived from embryonic NPCs (Fig. 1, embryo). These results suggest that GnT-V mRNA, but not GnT-III or GnT-Vb mRNA, is highly regulated in both adult and embryonic NPCs during differentiation, and indicate that β1,6-branched complex-type N-glycans, rather than bisecting N-glycans, may play a role in both adult and embryonic NPCs.

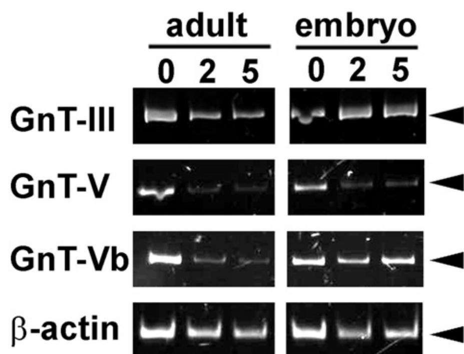


Figure 1 GnT-V mRNA is predominantly expressed in proliferating NPCs rather than in differentiated cells. mRNA was collected from adult or embryonic NPCs under growth-promoting (0 days) or differentiated (2 or 5 days) conditions. Reverse transcriptase-PCR amplification was performed using GnT-specific primers. GnT-V mRNA expression was predominantly expressed in proliferating NPCs.

Changes in the GnT protein expression during differentiation

To confirm that the GnT mRNAs are translated into proteins, protein samples were collected from adult NPCs and embryonic NPCs under growth-promoting (0 days) or differentiated (2 or 5 days) conditions, and a Western blot analysis was performed with anti-GnT antibodies. The proteins of GnT-III and GnT-V were detected in proliferating NPCs (Figs. 2A, B, day 0). During the differentiation, the expression of the GnT-V protein significantly decreased (Fig. 2B, day 2 and day 5) compared with that of proliferating NPCs (day 0). In contrast, the expression levels of the GnT-III protein (Fig. 2A) and β-actin (Fig. 2C) were unchanged during differentiation. The GnT-Vb protein (110 kDa (Inamori et al., 2003)), on the other hand, was not detected in our cultured cells (data not shown). The expression pattern of the GnT-V protein was in agreement with the expression pattern of the GnT-V mRNA.

GnT-V transfers GlcNAc to α1,6-linked mannose in the core via β1,6-linkage, and thereby forms β1,6-branched complex-type N-glycans. To ascertain whether the GnT-V protein synthesizes the β1,6-branched type of N-glycans in NPCs, protein samples from the cells were blotted with L-PHA which recognizes the pentasaccharide sequence Galβ1-4GlcNAcβ1-2(Galβ1-4GlcNAcβ1-6)Man, including β1,6-branched complex-type N-glycans (Cummings and Etzler, 2009). The binding of biotinylated L-PHA to protein samples was detected using HRP-streptavidin (Fig. 3A). To calculate the relative intensity of the L-PHA-binding proteins, the same protein samples were blotted with anti-β-actin antibody as a loading control (Fig. 3B). We found that L-PHA bound to many proteins both in NPCs and in the differentiating cells (Fig. 3A, −PNGase). To ascertain whether L-PHA binding depended on N-glycans, the protein samples were treated with PNGase, which cleaves between the innermost GlcNAc of N-glycans and asparagine residues of glycoproteins, before subjecting the samples to SDS-PAGE (Fig. 3A, +PNGase). Some of the binding of L-PHA to NPCs was diminished by PNGase treatment (Fig. 3A, +PNGase, arrows, arrowheads), but the band intensity of β-actin was not influenced by PNGase treatment (Fig. 3B); this indicated that some of the L-PHA binding proteins (p220, p135, p80, p70) were glycoproteins with β1,6-branched N-glycans. Among these glycoproteins, p220 was expressed predominantly in both adult and embryonic NPCs rather than in the differentiating cells (Fig. 3A, arrow; Fig. 3C). In addition to p220, the glycoproteins p135, p80, and p70 were also predominantly expressed in adult NPCs; however, their expression levels were higher in differentiated cells than in embryonic NPCs (Fig. 3A, arrowheads; Figs. 3D–F). These results suggest that GnT-V and the β1,6-branched complex-type N-glycans like p220 are predominantly expressed in NPCs, and play a specific role in both adult and embryonic NPCs.

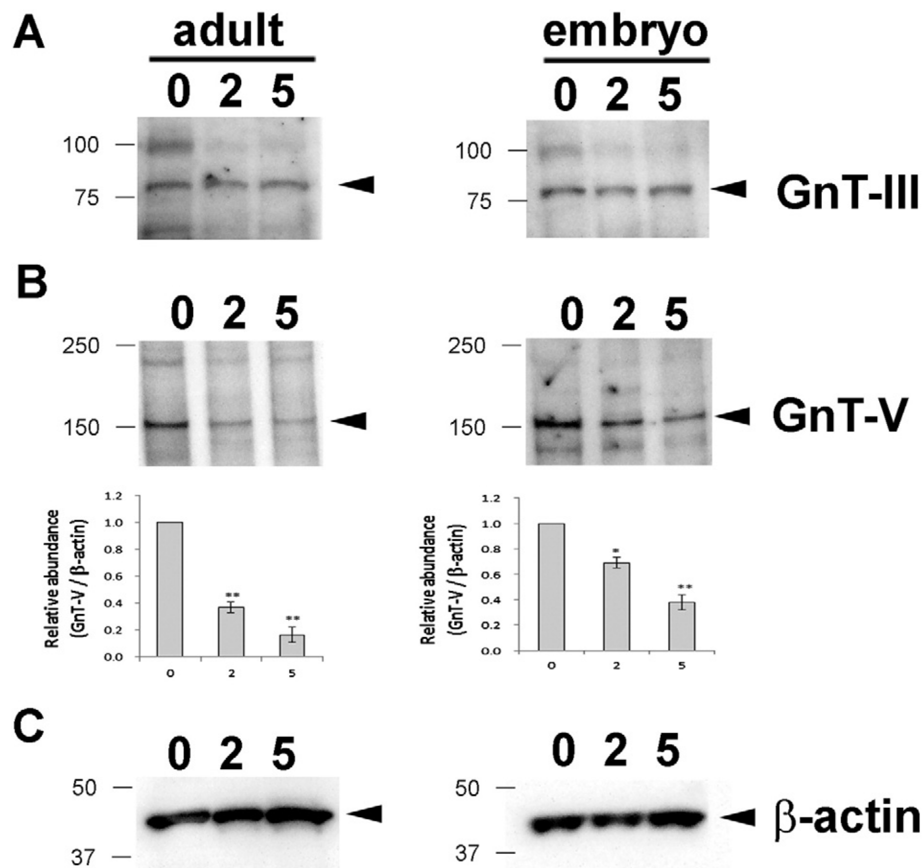


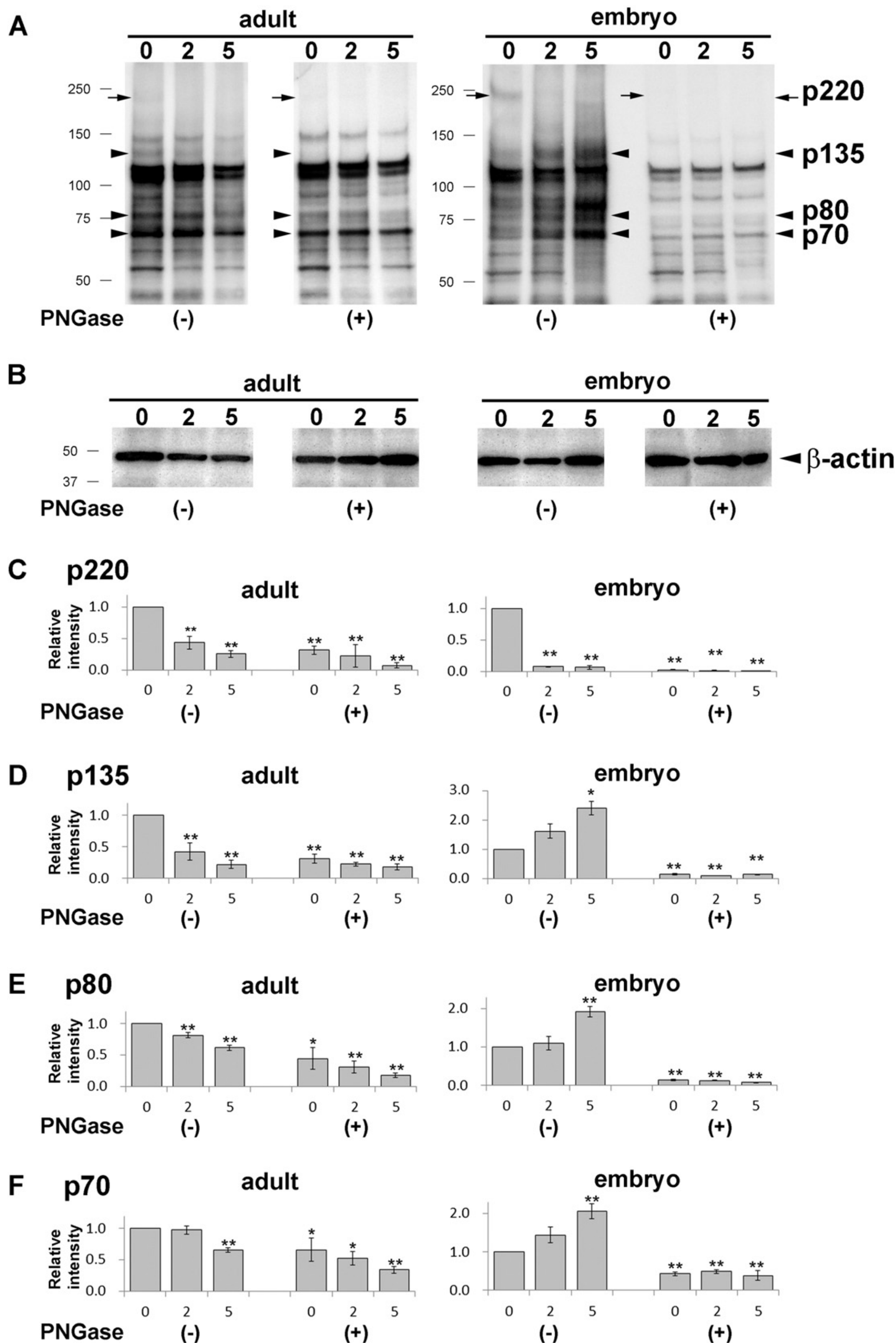
Figure 2 The GnT-V protein is predominantly expressed in the proliferating NPCs obtained from the embryonic or adult brain. Proteins were collected from embryonic or adult NPCs under growth-promoting (0 days) or differentiated (2 or 5 days) conditions. The Western blot analysis of proteins (20 μg) from NPCs was performed with an anti-GnT-III (A), anti-GnT-V (B), or anti-β-actin antibody (C). The relative intensities were obtained by densitometry with a computerized analysis system (B). Data represent the means ± SEM of three independent experiments. $*P < 0.05$, $**P < 0.01$, in comparison to the growth-promoting cells. The GnT-V protein expression (B, arrowhead, 150 kDa) was predominantly expressed in proliferating NPCs obtained from the embryonic or adult brain.

GnT-V expression in the brain and in the NPCs that accumulate around the brain lesions

To ascertain whether NPCs express GnT-V in the brain, we conducted an immunohistochemical analysis with an anti-GnT-V antibody. The embryonic brain slices were prepared from E13 mouse hemispheres during the pre-gliogenic period (Delaunay et al., 2008). Most neurogenic cells express GnT-V and nestin, and GnT-V-positive cells were detected throughout the brain (Figs. 4A, B, E13). In contrast, GnT-V expression was restricted to a few cells in the SVZ of the lateral ventricle in the adult brain (Fig. 4C, adult, SVZ), and no GnT-V-positive cells were observed in the motor cortex or cerebral cortex (Fig. 4D, adult, CTX). These GnT-V-positive cells expressed doublecortin (Fig. 4C, Dcx); thus, some adult NPCs in the brain express GnT-V, similar to the cultured NPCs.

Figure 3 β1,6-Branched N-glycan is expressed in proliferating NPCs obtained from the embryonic or adult brain. Proteins were collected from embryonic or adult NPCs under growth-promoting (0 days) or differentiated (2 or 5 days) conditions. The proteins (14 μg or 5 μg) that were pretreated with/without PNGase were blotted with biotinylated L-PHA lectin and HRP-streptavidin (A) or blotted with an anti-β-actin antibody (B), respectively. The relative intensity of the L-PHA-binding proteins (p220 (C), p135 (D), p80 (E), p70 (F)) were obtained by densitometry with a computerized analysis system and compared to the intensities obtained by Western blot analysis of β-actin. Data represent the means ± SEM of three independent experiments. $*P < 0.05$, $**P < 0.01$, in comparison to the growth-promoting cells. Several β1,6-branched N-glycans that were recognized by L-PHA were predominantly expressed in NPCs, including p220 (shown as arrow in A, left), p135, p80 and p70 in the adult (arrowheads in A, left), and p220 in the embryo (arrow in A, right). In contrast, several products were predominantly expressed in differentiated cells rather than in NPCs, including p135, p80 and p70 in the embryo (arrowheads in A, right).

To assess the function of GnT-V in NPCs, we prepared injury-model mice, because the NPCs in the SVZ of injured mice have been demonstrated to migrate toward the brain lesion (Kojima et al., 2010). Beginning one week after cryoinjury of the motor cortex, almost all mice showed impaired rotarod performance (Supplemental Fig. 2, 65–90% compared with the mice before cryoinjury). A few mice showed enduring loss of motor function (dotted lines), and parenchymal deficits in the brain lesion were observed eight weeks after injury (Figs. 5A, B, asterisk, thin dotted



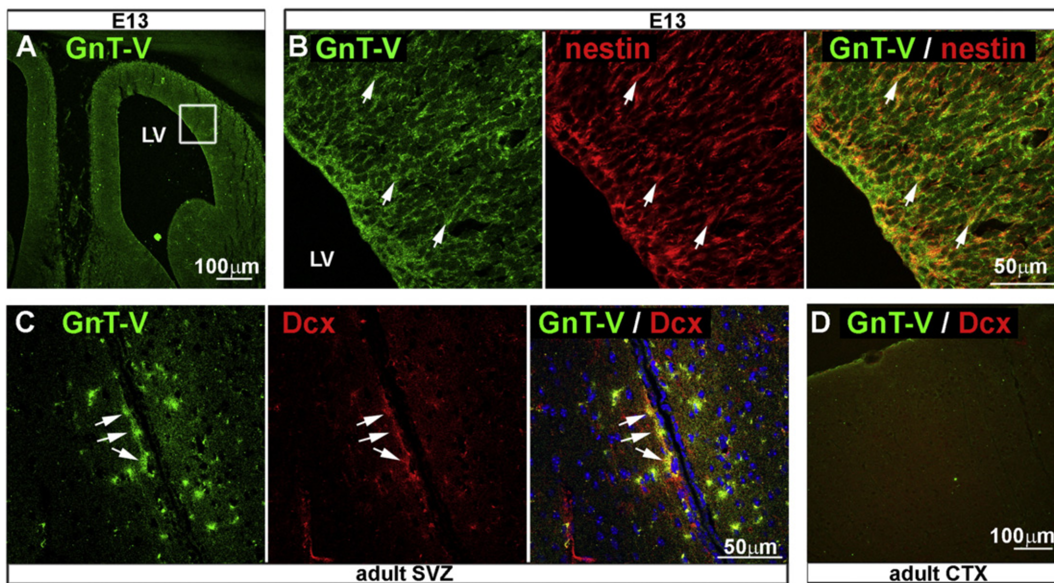


Figure 4 GnT-V is expressed in the NPCs of the embryonic or adult brain. Brain slices were prepared from E13 (A, B) or adult (C, D) mice, and an immunohistochemical analysis was performed by treating the samples with an anti-GnT-V, anti-nestin, or anti-Dcx antibody. The boxed area in (A) is shown at a higher magnification in (B). The GnT-V protein was expressed throughout the brain (A, B, E13, arrows) and, in particular, GnT-V-positive NPCs in the subventricular zone (C, adult, SVZ, arrows), but it was not expressed in the cerebral cortex (D, adult, CTX) of the adult brain.

line). Within these parenchymal deficits, we found a small cell cluster consisting of hematoxylin-positive cells (Figs. 5A, B, dotted line) in addition to the accumulation of GnT-V-positive cells (Figs. 5C, D) when compared with the control mouse brain, which had no injury (Fig. 4D, adult, CTX). However, the deficit was not filled completely by the cell cluster because the cluster was too small (Fig. 5A). Thus, poor Dcx-positive NPC colony formation could account for the decrease in functional recovery.

On the other hand, a few mice did show spontaneous functional recovery at 7 weeks after injury (Supplemental Fig. 2, solid lines, 80–106% from the impairment of motor function detected at one week after injury). In the brains of these functionally recovered mice, the large size of the bright cell cluster, which could not be detected in the functionally impaired mice, was found on the small cell cluster (Figs. 6A, B, bright cell cluster). The areas of both the bright cell cluster and small cell cluster in the functionally recovered mice were significantly larger compared with the cluster area in the functionally impaired mice (2.5 ± 0.1 fold, n = 3). Since the bright cell cluster was predominantly stained with anti-Dcx antibody (Figs. 6C, D), it was considered to be regenerated tissue, which could compensate for the observed motor dysfunction. The small cell cluster surrounding the bright cell cluster was larger compared with that observed in the functionally impaired mice (Figs. 5A, B); furthermore, many Dcx- and GnT-V-double-positive cells were also found in this surrounding cell cluster, but not in the brain parenchyma (Figs. 6C, D). These results suggest that the accumulation of GnT-V-positive NPCs in the small cell cluster surrounding the bright cell cluster could contribute to the reconstitution of regenerated tissue for the functional recovery of the damaged brain.

Discussion

Predominant expression of GnT-V in NPCs

The cells used in this study fulfilled the criteria for NPCs, including the ability for self-renewal and the capacity for multipotency. We demonstrated that GnT-III and GnT-V, which are both involved in core structure formation of complex-type N-glycan, are expressed in NPCs prepared from both adult and embryonic brain samples. These results are in agreement with previous studies, which have demonstrated the ubiquitous expression of the Mgat5 gene throughout the whole body including the adult brain (Inamori et al., 2003), in addition to the expression of bisected complex-type N-glycan in NPCs (Hamanoue et al., 2009). This study also showed that Mgat5 mRNA, GnT-V protein and its reaction products (e.g., p220) were predominantly expressed in NPCs, and were markedly reduced during the transition from NPCs to differentiated cells (e.g., neurons and glial cells). Taken together with our findings showing the accumulation of GnT-V-positive NPCs around regenerated tissue, these results not only suggest that predominant expression of Mgat5, GnT-V and β1,6-branched N-glycans, such as p220, could be necessary for the specific function of NPCs, rather than for differentiated cells, but that GnT-V and p220 could serve as valuable markers for visualization of NPCs in living brain tissue. On the other hand, some of the GnT-V reaction products besides p220 (i.e., p135, p80, p70) were discrepant in their expression patterns during differentiation between adult NPCs and embryonic NPCs. Indeed, the expression patterns of GnT-V reaction products could account for the observed adult

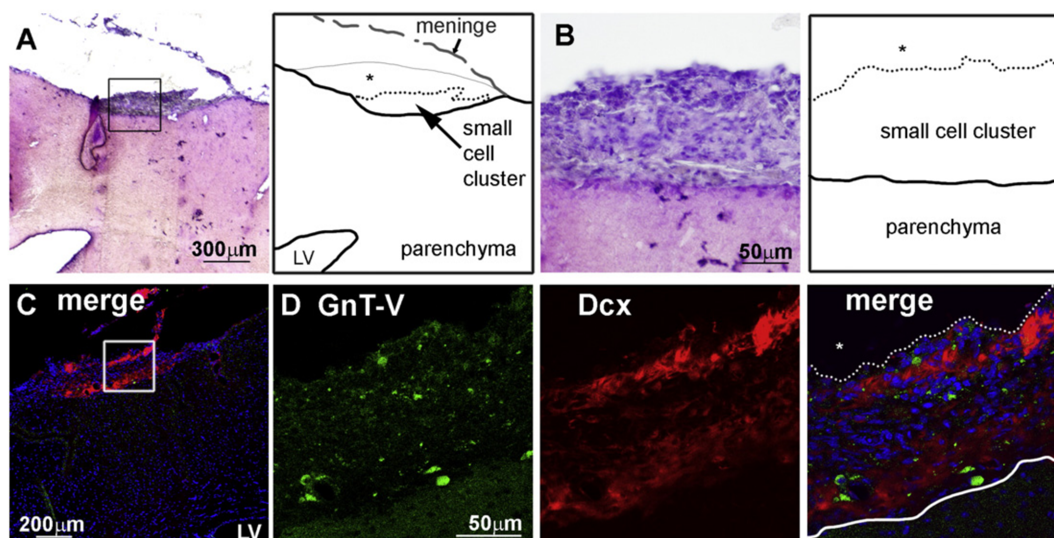


Figure 5 Several GnT-V-positive NPCs slightly accumulated in the small cell cluster of the mice in which motor function had been impaired by cryoinjury. Mice in which the motor function had been impaired were chosen based on the results of a rotarod test seven weeks after injury. Brain slices were prepared, and hematoxylin–eosin staining was performed (A, B). The boxed area in (A) is shown at a higher magnification in (B). Meninges were peeled from the brain surface through the staining procedure. The expected brain deficit area is shown (thin dotted line and asterisk). A small cell cluster was found on the cerebral parenchyma (A, B). Immunohistochemical analysis with an anti-GnT-V antibody or anti-Dcx antibody was conducted in brain sections from the injured mice (C, D). The boxed area in (C) is shown at a higher magnification in (D). The dotted and solid lines show the border between the small cell cluster and the deficit, and between the small cell cluster and the cerebral parenchyma, respectively (A, B, D). Note that limited numbers of Dcx-positive cells and GnT-V-positive cells were only detected in this cell cluster (D).

brain-specific features, such as low proliferative and regenerative abilities.

There have been questions regarding which transcriptional machinery is involved in the specific expression of GnT-V. Consensus sequences such as LF-A1, HNF1-HP1, GATA, and Pit-1 (Saito et al., 1995) and the Ets transcription factor have been reported as transcriptional regulators of GnT-V (Buckhaults et al., 1997; Kang et al., 1996; Taniguchi et al., 1999; Yamamoto et al., 2000). However, a Western blot analysis, which demonstrates Ets-1/Ets-2 expression both in NPCs and differentiated cells (Supplemental Fig. 3A), could not support the involvement of Ets transcription in NPCs. NPC-restricted GnT-V gene expression could be also regulated by histone modifications through CTCF and NeuroD1, which have been observed in GnT-Vb gene regulation (Kizuka et al., 2011). The expression pattern of GnT-Vb mRNA was inconsistent with that of GnT-V mRNA in embryonic NPCs (Fig. 1), however, suggesting that other factors may contribute to GnT-V regulation. To identify other candidate factor(s), we focused on the transcription factor binding sites in the promoter region of GnT-V. The promoter region contains several c-myb binding sites, and c-myb expression in the adult brain has been previously reported (Shin et al., 2001). In addition, mice lacking c-myb have shown a decrease in neurogenesis (Malaterre et al., 2008), prompting us to investigate whether c-myb could act as a transcription factor specific for GnT-V. In fact, a Western blot analysis of our cultured NPCs showed that c-myb appeared to be predominantly expressed in adult NPCs compared to the differentiated cells (Supplemental Fig. 3B). These findings suggest that c-myb could be a major contributor to GnT-V regulation. Thus further investigation of NPC-restricted gene regulation will be necessary for future application in stem cell biology, including the development of a NPC-specific gene expression system.

Function of GnT-V in NPCs

This study demonstrated that both mRNA and protein for GnT-V are expressed in NPCs (Figs. 1, 2). GnT-V synthesizes β1,6-branched glycan by acting as a glycosyltransferase in the Golgi apparatus in the cells (Pierce et al., 1987); furthermore, the secreted soluble form of GnT-V with a molecular weight less than 100 kDa serves to promote angiogenesis by releasing fibroblast growth factor-2 (Saito et al., 2002). Since our Western blot analysis showed a molecular weight of about 150 kDa for GnT-V expressed in the cultured NPCs (Fig. 2), it is more likely that GnT-V functions as a glycosyltransferase inside cells. In addition, our previous study demonstrated that NPCs can be selectively enriched by lectin-coated plates (Hamanoue et al., 2008) or by flow cytometry using E-PHA, which recognizes bisected N-glycan (Yamashita et al., 1983), but not using L-PHA, which recognizes a β1,6-branch structure (Hamanoue et al., 2009). These studies also support the suggestion that GnT-V and its reaction products, β1,6-branched N-glycans, in NPCs could contribute to the regulation of intrinsic biological events such as cell migration and proliferation, rather than extrinsic events such as cell–cell attachment and recognition by other cells.

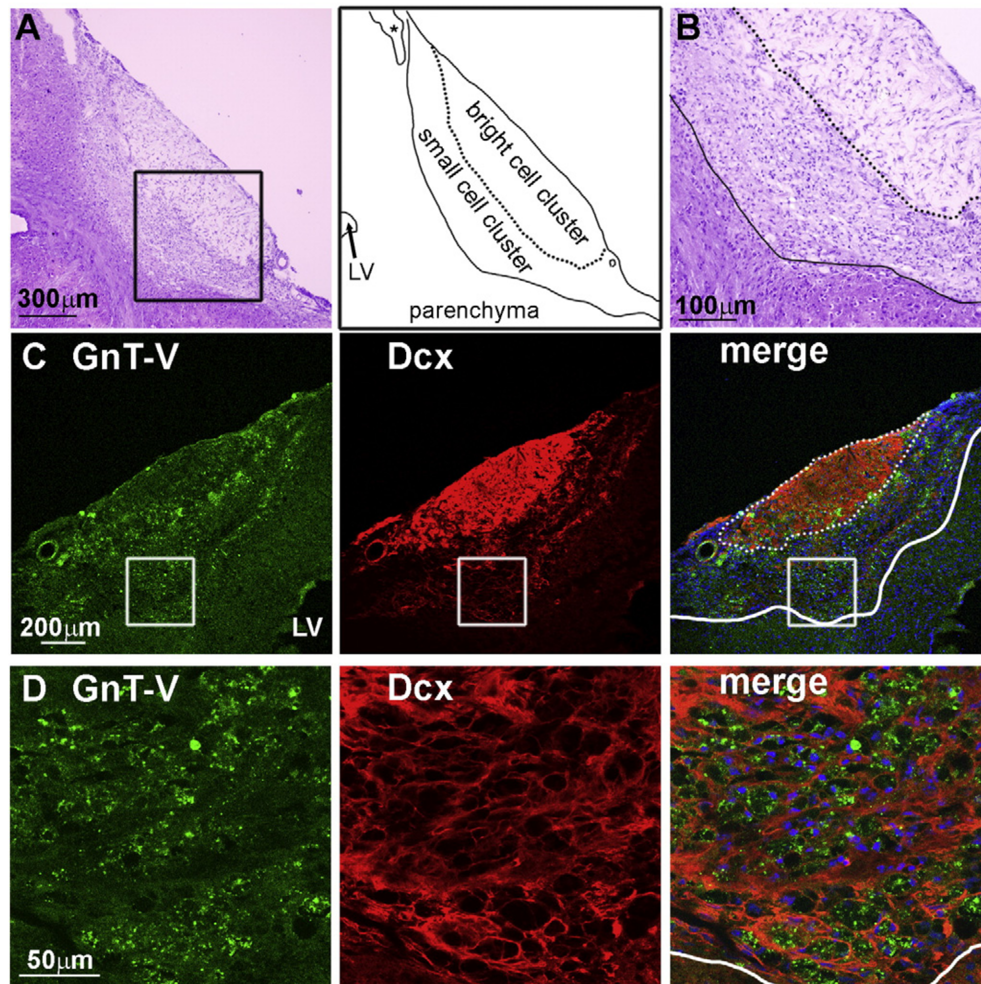


Figure 6 Many GnT-V and Dcx-double positive NPCs accumulated in the surrounding area of the regenerated tissue in the mice in which motor function had been spontaneously restored. Seven weeks after cryoinjury, mice in which the motor function had been restored were chosen based on the results of a rotarod test. Brain slices were prepared, and hematoxylin–eosin staining of the cortex was performed (A, B). The boxed area in (A) is shown at a higher magnification in (B). Dotted and solid lines show the border between the bright cell cluster and the small cell cluster, and between the small cell cluster and the cerebral parenchyma, respectively. There was a large bright cell cluster and small cell cluster on the brain parenchyma (A, B). An immunohistochemical analysis with an anti-GnT-V antibody or anti-Dcx antibody reveals the presence of Dcx-positive cells predominantly in the bright cell layer (C, D). Many GnT-V- and Dcx-double positive cells were detected in the surrounding cell cluster below the bright cell layer (C, D).

In cancer cells, GnT-V has been demonstrated to be involved in cell growth and migration by regulating integrins (Gu et al., 2009; Miyoshi et al., 2012), in epithelial–mesenchymal transition and tumor metastasis as a rate limiting step for cytokine receptor signaling (Partridge et al., 2004), and in determining the invasiveness of glioma cells (Yamamoto et al., 2000). Since NPC migration is likely necessary for normal brain development (Cayre et al., 2009; Huang, 2009), and GnT-V was expressed in the NPCs of the embryonic brain when NPC migration frequently occurred (Fig. 4), we hypothesized that GnT-V in NPCs could participate in cell migration during both normal development and pathological conditions, such as brain injury. From a study using mice lacking both the Mgat5 and Mgat5B genes, GnT-V protein expression appears to be non-essential for normal brain development, whereas both N-linked and O-Man-linked β1,6-branched glycans is totally lost (Lee et al., 2012). However, during pathological critical conditions, such as the cryoinjury model used in this study, we detected the increased accumulation of NPCs in the surrounding cell cluster of the regenerated-bright cell cluster in the functionally recovered adult brain. These results may indicate an association between the functional recovery from brain injury and GnT-V-positive NPC accumulation, probably due to the enhancement of cell migration from the brain parenchyma to the brain lesion. Similar expression patterns of GnT-V and c-myb, which maintain neurogenesis and cell cycle progression in NPCs (Malaterre et al., 2008), may also support a role of GnT-V in NPCs. Further verification of the direct relationship between NPC

accumulation (i.e., migration and proliferation) with GnT-V expression and with the reaction products of GnT-V will be necessary to evaluate this hypothesis.

Conclusions

GnT-V and its reaction products, such as p220, are predominantly expressed in NPCs rather than in differentiated cells, including neurons or glia. These results indicate that the expression of GnT-V is under rigorous control of the transcription mechanism, possibly by c-Myb, and suggest that GnT-V and its reaction products might play a specific role in NPCs. Furthermore, the lectin and cryoinjury experiments suggest that further verification of the direct relationship of NPC accumulation (migration and proliferation) with GnT-V and the reaction products of GnT-V will be necessary for the development of improved therapy employing the activation of endogenous NPCs. Finally, our results indicate that GnT-V and its reaction products could serve as marker for visualization of NPCs in the living brain tissue, a novel finding and asset to the basic research field.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2014.11.004.

Acknowledgments

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology [22500340 to M.H., 24300197 to M.H., T.O.] and a grant from the Strategic Research Foundation Grant-aided Project for Private Schools at Heisei 23rd from the Ministry of Education, Culture, Sports, Science and Technology of Japan, 2011–2015 [to M.H.].

References

Abbott, K.L., Matthews, R.T., Pierce, M., 2008. Receptor tyrosine phosphatase beta (RPTPbeta) activity and signaling are attenuated by glycosylation and subsequent cell surface galectin-1 binding. J. Biol. Chem. 283, 33026–33035. http://dx.doi.org/10.1074/jbc.M803646200.

Anderson, D.J., 2001. Stem cells and pattern formation in the nervous system: the possible versus the actual. Neuron 30, 19–35 (S0896-6273(01)00260-4 [pii]).

Bhattacharyya, R., Bhaumik, M., Raju, T.S., Stanley, P., 2002. Truncated, inactive N-acetylglucosaminyltransferase III (GlcNAc-TIII) induces neurological and other traits absent in mice that lack GlcNAc-TIII. J. Biol. Chem. 277, 26300–26309. http://dx.doi.org/10.1074/jbc.M202276200.

Bhaumik, M., Seldin, M.F., Stanley, P., 1995. Cloning and chromosomal mapping of the mouse Mgat3 gene encoding N-acetylglucosaminyltransferase III. Gene 164, 295–300.

Buckhaults, P., Chen, L., Fregien, N., Pierce, M., 1997. Transcriptional regulation of N-acetylglucosaminyltransferase V by the src oncogene. J. Biol. Chem. 272, 19575–19581.

Cayre, M., Canoll, P., Goldman, J.E., 2009. Cell migration in the normal and pathological postnatal mammalian brain. Prog. Neurobiol. 88, 41–63. http://dx.doi.org/10.1016/j.pneurobio.2009.02.001.

Chai, W., Yuen, C.T., Kogelberg, H., Carruthers, R.A., Margolis, R.U., Feizi, T., Lawson, A.M., 1999. High prevalence of 2-mono- and 2,6-di-substituted manol-terminating sequences among O-glycans released from brain glycopeptides by reductive alkaline hydrolysis. Eur. J. Biochem. 263, 879–888.

Chen, Y.J., Wing, D.R., Guile, G.R., Dwek, R.A., Harvey, D.J., Zamze, S., 1998. Neutral N-glycans in adult rat brain tissue—complete characterisation reveals fucosylated hybrid and complex structures. Eur. J. Biochem. 251, 691–703.

Cummings, R.D., Etzler, M.E., 2009. Antibodies and Lectins in Glycan Analysis. Essentials of Glycobiology. Cold Spring Harbor, NY.

Delaunay, D., Heydon, K., Cumano, A., Schwab, M.H., Thomas, J.L., Suter, U., Nave, K.A., Zalc, B., Spassky, N., 2008. Early neuronal and glial fate restriction of embryonic neural stem cells. J. Neurosci.: Off. J. Soc. Neurosci. 28, 2551–2562. http://dx.doi.org/10.1523/JNEUROSCI.5497-07.2008.

Gage, F.H., Ray, J., Fisher, L.J., 1995. Isolation, characterization, and use of stem cells from the CNS. Annu. Rev. Neurosci. 18, 159–192.

Gu, J., Isaji, T., Sato, Y., Kariya, Y., Fukuda, T., 2009. Importance of N-glycosylation on alpha5beta1 integrin for its biological functions. Biol. Pharm. Bull. 32, 780–785.

Hamanoue, M., Okano, H., 2011. Cell surface N-glycans-mediated isolation of mouse neural stem cells. J. Cell. Physiol. 226, 1433–1438. http://dx.doi.org/10.1002/jcp.22436.

Hamanoue, M., Sato, K., Takamatsu, K., 2008. Lectin panning method: the prospective isolation of mouse neural progenitor cells by the attachment of cell surface N-glycans to *Phaseolus vulgaris* erythroagglutinating lectin-coated dishes. Neuroscience 157, 762–771. http://dx.doi.org/10.1016/j.neuroscience.2008.09.054.

Hamanoue, M., Matsuzaki, Y., Sato, K., Okano, H.J., Shibata, S., Sato, I., Suzuki, S., Ogawara, M., Takamatsu, K., Okano, H., 2009. Cell surface N-glycans mediated isolation of mouse neural stem cells. J. Neurochem. 110, 1575–1584. http://dx.doi.org/10.1111/j.1471-4159.2009.06256.x.

Huang, Z., 2009. Molecular regulation of neuronal migration during neocortical development. Mol. Cell. Neurosci. 42, 11–22. http://dx.doi.org/10.1016/j.mcn.2009.06.003.

Inamori, K., Endo, T., Ide, Y., Fujii, S., Gu, J., Honke, K., Taniguchi, N., 2003. Molecular cloning and characterization of human GnT-IX, a novel beta1,6-N-acetylglucosaminyltransferase that is specifically expressed in the brain. J. Biol. Chem. 278, 43102–43109. http://dx.doi.org/10.1074/jbc.M308255200.

Inamori, K., Gu, J., Ohira, M., Kawasaki, A., Nakamura, Y., Nakagawa, T., Kondo, A., Miyoshi, E., Nakagawara, A., Taniguchi, N., 2006. High expression of N-acetylglucosaminyltransferase V in favorable neuroblastomas: involvement of its effect on apoptosis. FEBS Lett. 580, 627–632. http://dx.doi.org/10.1016/j.febslet.2005.12.089.

Kaneko, M., Alvarez-Manilla, G., Kamar, M., Lee, I., Lee, J.K., Troupe, K., Zhang, W., Osawa, M., Pierce, M., 2003. A novel beta(1,6)-N-acetylglucosaminyltransferase V (GnT-VB)(1). FEBS Lett. 554, 515–519.

Kang, R., Saito, H., Ihara, Y., Miyoshi, E., Koyama, N., Sheng, Y., Taniguchi, N., 1996. Transcriptional regulation of the N-acetylglucosaminyltransferase V gene in human bile duct carcinoma cells (HuCC-T1) is mediated by Ets-1. J. Biol. Chem. 271, 26706–26712.

Kizuka, Y., Kitazume, S., Yoshida, M., Taniguchi, N., 2011. Brain-specific expression of N-acetylglucosaminyltransferase IX (GnT-IX) is regulated by epigenetic histone modifications. J. Biol. Chem. 286, 31875–31884. http://dx.doi.org/10.1074/jbc.M111.251173.

Kleene, R., Schachner, M., 2004. Glycans and neural cell interactions. Nat. Rev. Neurosci. 5, 195–208. http://dx.doi.org/10.1038/nrn1349.

Kojima, T., Hirota, Y., Ema, M., Takahashi, S., Miyoshi, I., Okano, H., Sawamoto, K., 2010. Subventricular zone-derived neural progenitor cells migrate along a blood vessel scaffold toward the post-stroke striatum. Stem Cells 28, 545–554. http://dx.doi.org/10.1002/stem.306.

Lee, J.K., Matthews, R.T., Lim, J.M., Swanier, K., Wells, L., Pierce, J.M., 2012. Developmental expression of the neuron-specific N-acetylglucosaminyltransferase Vb (GnT-Vb/IX) and identification of

its in vivo glycan products in comparison with those of its paralog, GnT-V. J. Biol. Chem. 287, 28526–28536. http://dx.doi.org/10.1074/jbc.M112.367565.

Malaterre, J., Mantamadiotis, T., Dworkin, S., Lightowler, S., Yang, Q., Ransome, M.I., Turnley, A.M., Nichols, N.R., Emambokus, N.R., Frampton, J., Ramsay, R.G., 2008. c-Myb is required for neural progenitor cell proliferation and maintenance of the neural stem cell niche in adult brain. Stem Cells 26, 173–181. http://dx.doi.org/10.1634/stemcells.2007-0293.

McKay, R., 1997. Stem cells in the central nervous system. Science 276, 66–71.

Miyoshi, E., Terao, M., Kamada, Y., 2012. Physiological roles of N-acetylglucosaminyltransferase V(GnT-V) in mice. BMB Rep. 45, 554–559.

Okano, H., 2002. Stem cell biology of the central nervous system. J. Neurosci. Res. 69, 698–707.

Papaioannou, V.E., Fox, J.G., 1993. Efficacy of tribromoethanol anesthesia in mice. Lab. Anim. Sci. 43, 189–192.

Partridge, E.A., Le Roy, C., Di Guglielmo, G.M., Pawling, J., Cheung, P., Granovsky, M., Nabi, I.R., Wrana, J.L., Dennis, J. W., 2004. Regulation of cytokine receptors by Golgi N-glycan processing and endocytosis. Science 306, 120–124. http://dx.doi.org/10.1126/science.1102109.

Pierce, M., Arango, J., Tahir, S.H., Hindsgaul, O., 1987. Activity of UDP-GlcNAc:alpha-mannoside beta(1,6)N-acetylglucosaminyl transferase (GnT V) in cultured cells using a synthetic trisaccharide acceptor. Biochem. Biophys. Res. Commun. 146, 679–684.

Saito, H., Nishikawa, A., Gu, J., Ihara, Y., Soejima, H., Wada, Y., Sekiya, C., Niikawa, N., Taniguchi, N., 1994. cDNA cloning and chromosomal mapping of human N-acetylglucosaminyltransferase V+. Biochem. Biophys. Res. Commun. 198, 318–327.

Saito, H., Gu, J., Nishikawa, A., Ihara, Y., Fujii, J., Kohgo, Y., Taniguchi, N., 1995. Organization of the human N-acetylglucosaminyltransferase V gene. Eur. J. Biochem. 233, 18–26.

Saito, T., Miyoshi, E., Sasai, K., Nakano, N., Eguchi, H., Honke, K., Taniguchi, N., 2002. A secreted type of beta 1,6-N-acetylglucosaminyltransferase V (GnT-V) induces tumor angiogenesis without mediation of glycosylation: a novel function of GnT-V distinct from the original glycosyltransferase activity. J. Biol. Chem. 277, 17002–17008. http://dx.doi.org/10.1074/jbc.M200521200.

Sato, K., Hamanoue, M., Takamatsu, K., 2008. Inhibitors of p38 mitogen-activated protein kinase enhance proliferation of mouse neural stem cells. J. Neurosci. Res. 86, 2179–2189. http://dx.doi.org/10.1002/jnr.21668.

Shichinohe, H., Kuroda, S., Yano, S., Hida, K., Iwasaki, Y., 2007. Role of SDF-1/CXCR4 system in survival and migration of bone marrow stromal cells after transplantation into mice cerebral infarct. Brain Res. 1183, 138–147. http://dx.doi.org/10.1016/j.brainres.2007.08.091.

Shin, D.H., Lee, H.W., Jeon, G.S., Lee, H.Y., Lee, K.H., Cho, S.S., 2001. Constitutive expression of c-myb mRNA in the adult rat brain. Brain Res. 892, 203–207.

Shoreibah, M., Perng, G.S., Adler, B., Weinstein, J., Basu, R., Cupples, R., Wen, D., Browne, J.K., Buckhaults, P., Fregien, N., Pierce, M., 1993. Isolation, characterization, and expression of a cDNA encoding N-acetylglucosaminyltransferase V. J. Biol. Chem. 268, 15381–15385.

Steinbach, J.P., Weissenberger, J., Aguzzi, A., 1999. Distinct phases of cryogenic tissue damage in the cerebral cortex of wild-type and c-fos deficient mice. Neuropathol. Appl. Neurobiol. 25, 468–480.

Tamura, Y., Kataoka, Y., Cui, Y., Takamori, Y., Watanabe, Y., Yamada, H., 2007. Multi-directional differentiation of doublecortin- and NG2-immunopositive progenitor cells in the adult rat neocortex in vivo. Eur. J. Neurosci. 25, 3489–3498. http://dx.doi.org/10.1111/j.1460-9568.2007.05617.x.

Taniguchi, N., Miyoshi, E., Ko, J.H., Ikeda, Y., Ihara, Y., 1999. Implication of N-acetylglucosaminyltransferases III and V in cancer: gene regulation and signaling mechanism. Biochim. Biophys. Acta 1455, 287–300.

Yamamoto, H., Swoger, J., Greene, S., Saito, T., Hurh, J., Sweeley, C., Leestma, J., Mkrdichian, E., Cerullo, L., Nishikawa, A., Ihara, Y., Taniguchi, N., Moskal, J.R., 2000. Beta1,6-N-acetylglucosamine-bearing N-glycans in human gliomas: implications for a role in regulating invasivity. Cancer Res. 60, 134–142.

Yamashita, K., Hitoi, A., Kobata, A., 1983. Structural determinants of *Phaseolus vulgaris* erythroagglutinating lectin for oligosaccharides. J. Biol. Chem. 258, 14753–14755.

Zamze, S., Harvey, D.J., Chen, Y.J., Guile, G.R., Dwek, R.A., Wing, D.R., 1998. Sialylated N-glycans in adult rat brain tissue—a widespread distribution of disialylated antennae in complex and hybrid structures. Eur. J. Biochem. 258, 243–270.