Learning/Memory Impairment and Reduced Expression of the HNK-1 Carbohydrate in β4-Galactosyltransferase-II-deficient Mice⁎⁻‡

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The glycosylation of glycoproteins and glycolipids is important for central nervous system development and function. Although the roles of several carbohydrate epitopes in the central nervous system, including polysialic acid, the human natural killer-1 (HNK-1) carbohydrate, α2,3-sialic acid, and oligomannosides, have been investigated, those of the glycan backbone structures, such as Galβ1–4GlcNAc and Galβ1–3GlcNAc, are not fully examined. Here we report the generation of mice deficient in β4-galactosyltransferase-II (β4GalT-II). This galactosyltransferase transfers Gal from UDP-Gal to a nonreducing terminal GlcNAc to synthesize the Gal β1–4GlcNAc structure, and it is strongly expressed in the central nervous system. In behavioral tests, the β4GalT-II−/− mice showed normal spontaneous activity in a novel environment, but impaired spatial learning/memory and motor coordination/learning. Immunohistochemistry showed that the amount of HNK-1 carbohydrate was markedly decreased in the brain of β4GalT-II−/− mice, whereas the expression of polysialic acid was not affected. Furthermore, mice deficient in glucuronontransferase (GlCat-P), which is responsible for the biosynthesis of the HNK-1 carbohydrate, also showed impaired spatial learning/memory as described in our previous report, although their motor coordination/learning was normal as shown in this study. Histological examination showed abnormal alignment and reduced number of Purkinje cells in the cerebellum of β4GalT-II−/− mice. These results suggest that the Galβ1–4GlcNAc structure in the HNK-1 carbohydrate is mainly synthesized by β4GalT-II and that the glycans synthesized by β4GalT-II have essential roles in higher brain functions, including some that are HNK-1-dependent and some that are not.

The glycosylation of glycoproteins, proteoglycans, and glycolipids is important for their biological activities, stability, transport, and clearance from circulation, and cell-surface glycans participate in cell-cell and cell-extracellular matrix interactions. In the central nervous system, several specific carbohydrate epitopes, including polysialic acid (PSA),⁶ the human natural killer-1 (HNK-1) carbohydrate, α2,3-sialic acid, and oligomannosides play indispensable roles in neuronal generation, cell migration, axonal outgrowth, and synaptic plasticity (1). Functional analyses of the glycan backbone structures, like lactosamine core (Galβ1–4GlcNAc), neolactosamine core (Galβ1–3GlcNAc), and polylactosamine (Galβ1–4GlcNAcβ1–3) have been carried out using gene-deficient mice in β4-galactosyltransferase-I (β4GalT-I) (2, 3), β4GalT-V (4), β3-N-acetylglucosaminyltransferase-II (β3GnT-II) (5), β3GnT-III (Core1-β3GnT) (6), β3GnT-V (7), and Core2GnT (8). However, the roles of these glycan backbone structures in the nervous system have not been examined except the olfactory sensory system (9). β4GalTs synthesize the Galβ1–4GlcNAc structure via the β4-galactosylation of glycoproteins and glycolipids; the β4GalTs transfer galactose (Gal) from UDP-Gal to a nonreducing terminal N-acetylglucosamine (GlcNAc) of N- and O-glycans with a β-1,4-linkage. The β4GalT family has seven members (β4GalT-I to VII), of which at least five have similar Galβ1–4GlcNAc-synthesizing activities (10, 11). Each β4GalT has a tissue-specific expression pattern and substrate specificity with overlapping, suggesting each β4GalT has its own biological role as well as redundant functions. β4GalT-I and β4GalT-II share the highest identity (52% at the amino acid level) among the β4GalTs (12), suggesting these two galactosyltransferases can compensate for each other. β4GalT-I is strongly and ubiquitously expressed in various non-neural tissues, whereas β4GalT-II is strongly expressed in neural tissues (13, 14). Indeed, the β4GalT activity in the brain of β4GalT-I-deficient (β4GalT-I−/−) mice remains as high as 65% of that of wild-type mice, and the expression levels of PSA and the HNK-1 carbohydrate in the brain of these mice are normal (15). These results

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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The abbreviations used are: PSA, polysialic acid; HNK-1, human natural killer-1; β4GalT, β4-galactosyltransferase; GlCat-P, glucuronontransferase; mAb, monoclonal antibody; DG, dentate gyrus; BrdUrd, 5-bromo-2′-deoxyuridine; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; NCAM, neural cell adhesion molecule.
suggest β4GalTs other than β4GalT-I, like β4GalT-II, are important in the nervous system.

Among the β4GalT family members, only β4GalT-I−/− mice have been examined extensively; this was done by us and another group. We reported that glycans synthesized by β4GalT-I play various roles in epithelial cell growth and differentiation, inflammatory responses, skin wound healing, and IgA nephropathy development (2, 16–18). Another group reported that glycans synthesized by β4GalT-I are involved in anterior pituitary hormone function and in fertilization (3, 19). However, no other nervous system deficits have been reported in these mice, and the role of the β4-galactosylation of glycoproteins and glycolipids in the nervous system has not been fully examined.

In this study, we generated β4GalT-II−/− mice and examined them for behavioral abnormalities and biochemical and histological changes in the central nervous system. β4GalT-II−/− mice were impaired in spatial learning/memory and motor coordination/learning. The amount of HNK-1 carbohydrate was markedly decreased in the β4GalT-II−/− brain, but PSA expression was not affected. These results suggest that the Galβ1–4GlcnAc structure in the HNK-1 carbohydrate is mainly synthesized by β4GalT-II and that glycans synthesized by β4GalT-II have essential roles in higher brain functions, including ones that are HNK-1 carbohydrate-dependent and ones that are independent of HNK-1.

**EXPERIMENTAL PROCEDURES**

**Generation of β4GalT-II−/− Mice**—The strategy for the β4GalT-II gene targeting was to replace exons 1 and 2 and part of exon 3 with the neomycin resistance gene (20). Because exon 2 contains the translation initiation codon, ATG, as well as the Golgi retention signal, the deletion of exon 2 was expected to result in null function. A targeting vector with an upstream DT-A cassette (21) was used to transfected E14-1 embryonic stem cells (22) by electroporation, and the transformed cells were selected with G418 (2). Disruption of the β4GalT-II gene was verified by Southern blot hybridization using external 5′ and 3′ probes. Chimeric mice were generated by the aggregation method (23) and mated to C57BL/6 mice to confirm germ line transmission. The absence of β4GalT-II gene expression in the homozygous mutant mice was confirmed by Northern blot hybridization using a specific probe and by reverse transcription-PCR with a primer set that detected the deleted region. The mice used for the experiments were obtained by backcrossing male homozygous and female heterozygous mutant mice. In most cases, mice backcrossed to the C57BL/6J strain for eight generations were used in the behavioral analyses, and other experiments were carried out using mice of a mixed 129/C57BL/6 strain. The exceptional cases are 129/SvJae mice and exam-

**Preparation of Brain Homogenate and Membrane Fraction**—Whole brains from postnatal day 0 (P0), 2-week-old, and 11-week-old mice were homogenized with a Polytron homogenizer in 9 volumes of 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and protease inhibitors (Nacalai Tesque, Kyoto, Japan). The homogenate was spun at 1,000 × g for 10 min at 4 °C to remove the nuclei and then spun again at 105,000 × g for 1 h at 4 °C, and the resulting pellet was used as the membrane fraction. After being resuspended in PBS, the protein concentration in the homogenate and membrane fraction was measured using the DC-Protein Assay Reagent (Bio-Rad), according to the manufacturer's protocol.

**SDS-PAGE, Western Blot, and Lectin Blot**—Western blotting and lectin blotting were carried out as described previously (24). Proteins were separated by 5–20% gradient SDS-PAGE using the Laemml buffer system and then transferred to nitrocellulose membranes. For Western blots, after being blocked with 5% skim milk (or, for the detection of PSA, 2% bovine serum albumin) in PBS containing 0.05% Tween 20, the membranes were incubated with primary antibodies followed by incubation with HRP-conjugated secondary antibodies. For lectin blots, after being blocked with 0.1% Tween 20 in TBS (T-TBS), the membranes were incubated with HRP- or biotin-conjugated lectins in T-TBS. To detect biotinylated lectins, we used the Vectastain-ABC kit (Vector Laboratories), according to the manufacturer's protocol. Protein bands were detected with SuperSignal West Pico (Pierce) using a Luminoimage Analyzer LAS-3000 (Fuji, Tokyo, Japan). In these analyses, an anti-HNK-1 monoclonal antibody (mAb) was purchased from American Type Culture Collection, Manassas, VA; the antipoly sacial acid mAb (clone 12E3) was a generous gift from Dr. T. Seki (Juntendo University). The rat anti-mouse neural cell adhesion molecule (NCAM) mAb (clone H28) was kindly provided by Dr. K. Ono (National Institute for Physiological Sciences). The fluorescein isothiocyanate-conjugated anti-mouse IgM was purchased from Cappel Laboratories, and the HRP-conjugated anti-mouse IgM, anti-rabbit IgG, and anti-rat IgG were obtained from Zymed Laboratories Inc.. Biotinylated lec-
tins (Maackia amurensis agglutinin and Sambucus sieboldiana agglutinin) and HRP-conjugated lectins (concanavalin A and RCA120) were purchased from Seikagaku Corp., Tokyo, Japan. Concanavalin A, RCA120 (Ricinus communis agglutinin), M. amurenensis agglutinin, and S. sieboldiana agglutinin recognized mammone, Galβ1–4GlcnAc, Siaα2–3Gal, and Siaα2–6Gal, respectively.

**Histological Procedures**—Two-week- and 11-week-old and 2-month- and 6-month-old mice were deeply anesthetized with diethyl ether and perfused with PBS containing 0.1% heparin and then with 4% paraformaldehyde in PBS. The brain was post-fixed overnight and then sunk in 30% sucrose solution or embedded in paraffin. Cryo- or paraffin sections were cut in the sagittal or coronal plane. For a broad morphologic investiga-
tion, Kluer-Barrera’s stain was used with 0.1% Luxol Fast Blue and 0.1% Cresyl Violet solutions.

**Immunohistochemistry**—Immunohistochemistry was used to investigate the morphologic features of neural cells, the number of each cell type, and their glycosylation status. The primary antibodies were as follows: anti-HNK-1 mAb (TIB-20; ATCC, Manassas, VA); anti-PSA mAb (12E3); anti-NeuN mAb (MAB377; Chemicon, CA); anti-Nestin mAb (MAB353; Chemicon, CA); and anti-calbindin D-28K mAb (CB-955; C9848, Sigma). Sections were incubated with primary antibodies overnight at 4 °C, rinsed twice in PBS, and then incubated with Alexa Fluor-488- or fluorescein isothiocyanate-conju-
gated secondary antibody for 2 h at room temperature. Immu-
nofluorescence was evaluated by confocal microscopy (LSM 5 Pascal, Zeiss, Oberkochen, Germany; Fluoview laser confocal microscope system, Olympus Corp., Tokyo, Japan). For the calbindin D-28K immunohistochemical staining, the number of calbindin-positive cells in the 4th and 5th cerebellar lobules was counted manually. Disturbed alignment of cerebellar Purkinje cells was evaluated by counting the number of disturbed lobules using the sections in which apparent eight lobules were observed. For this quantitative analysis, five sagittal sections (0.12–0.84 mm lateral to the midline) (25) per animal were investigated, and the total number of 40 lobules in an animal (five sections by eight lobules) was examined. For the assessment of BrdUrd incorporation, 100 mg/kg BrdUrd solution was injected once a day intraperitoneally for 5 days, and the animals were perfused intracardially 24 h after the last injection. BrdUrd immunostaining was carried out using a BrdUrd labeling and detection kit II (Roche Diagnostics), and the sections were counterstained with nuclear fast red.

Behavioral Tests, General Procedures—Three-month-old male β4GalT-II−/− mice (n = 15), β4GalT-II+/− littermates (n = 13), GlcAT-P−/− mice (n = 11), and GlcAT-P+/+ mice (n = 9), kept under specific pathogen-free conditions at the Institute for Experimental Animals (Advanced Science Research Center, Kanazawa University), were used for behavioral analyses. The subjects were housed individually with ad libitum access to food and water 1 week before the behavioral testing. All tests were conducted during the light period of the light/dark cycle (08:45–20:45 h). To evaluate the behavioral responses in every test situation, the products of O’Hara & Co., Ltd. (Tokyo, Japan) were used. In addition, for the quantitative analysis in the open field and Morris water maze test, we used ImageJ LD4 (O’Hara & Co., Ltd.), a modified software based on the public domain ImageJ program (developed at the National Institutes of Health and available on line at www.nih.gov). Each animal was subjected to a battery of the following five behavioral paradigms with an 1-week rest period between each test. The animal experiments were conducted according to Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and to the safety guidelines for gene manipulation experiments at Kanazawa University. All efforts were made to minimize both the number and the suffering of the animals used.

Open Field Test—The general activity of animals in a novel environment was measured in an open field situation. The open
field chamber (60 × 60 × 40 cm) was made of gray vinyl chloride, and the illumination of the center of the floor was kept at 50 lux by white LEDs set just above the chamber. The mice were allowed free exploration of the apparatus for 10 min per day on 3 successive days. The moving distance, speed, staying area, and number of rearing were analyzed.

**Morris Water Maze Task**—The Morris water maze test (26) was used to evaluate spatial learning. The apparatus consisted of a pool (100 cm in diameter) filled with opaque water (23 ± 2 °C) and containing a submerged hidden goal platform (10 cm in diameter) 1 cm below the surface of the water. Mice were given six training trials (inter-trial interval, 10–15 min) per day for 8 consecutive days. The trials were initiated from each of four possible start locations in a randomized manner, and the mice were required to reach the platform within 60 s. Following 2 days of extinction trials, a “landmark task,” with a visible cue on the platform was carried out to determine visual impairment. The latency to reach the platform and the length of the swimming pass were analyzed as learning performance.

**Passive Avoidance Response**—The passive avoidance apparatus consisted of a vinyl chloride chamber (30 × 10 × 9 cm) with a steel-grid floor that was divided into dark and light compartments. In the test trial, mice were placed in the light room and received scrambled electrical foot shocks (0.22 mA) for 5 s through the grid floor of the dark room when they entered it. To test memory retention, each animal was again placed into the light room 24 h after the training. The step-through latency was measured without electric foot shocks as an indicator of the memory of shock experience. An upper cutoff of 300 s was set.

**Rota-rod Test**—To investigate the motor coordination/motor learning abilities, the accelerating rota-rod paradigm (27) was used. Animals were tested in three trials per day for 3 consecutive days with a 300-s accelerating program (from 5 to 40 rpm). The latency to fall from the rod was recorded. On day 4, the number of hind limb slips at 7 rpm was measured to examine whether there was a motility defect.

**Balance Beam Test**—Motor coordination and balance were evaluated by a balance beam apparatus (28). The beam consisted of a 100-cm-long horizontal steel bar (28 or 11 mm in diameter) placed 50 cm above the floor. Following the habituation trials, the mice were placed at the starting point of the bar. The number of hind limb slips off the beam before reaching an enclosed goal box was counted. Every mouse was tested using both bar diameters.

**Statistical Analysis**—In the behavioral experiments statistical significance was determined by two-way analysis of variance followed by a post hoc protected least significant difference test or unpaired t test. Performance in the open field test and Morris water maze task was analyzed by two-way analysis of variance in which the trial/day effect was a within-subject factor and genotype was a between-subject factor. In the other behavioral tests, the effect of genotype was verified by the t test.

## RESULTS

**Generation of β4GalT-II**−/−** Mice**—The targeting strategy for the deletion of the β4GalT-II gene is shown in Fig. 1A. The targeting vector was introduced into E14-1 embryonic stem cells by electroporation, and G418-resistant colonies were picked up. Three embryonic stem clones were selected by Southern blot screening of 600 colonies and were verified to have the desired homologous recombination by Southern blot analyses, respectively. In Fig. 1B, Northern blot analyses showed that β4GalT-II mRNA in the brain were detected in β4GalT-II−/− mice generated from two independent embryonic stem clones, and behavioral experiments were conducted using mice that had been backcrossed to C57BL/6 mice for eight generations. The targeted deletion of exons 1 and 2 of the β4GalT-II gene and the loss of β4GalT-II mRNA in the brain were confirmed by Southern blot analysis (data not shown). β4GalT-II−/− mice were generated from two independent embryonic stem clones, and behavioral experiments were conducted using mice that had been backcrossed to C57BL/6 mice for eight generations. The targeted deletion of exons 1 and 2 of the β4GalT-II gene and the loss of β4GalT-II mRNA in the brain were confirmed by Southern blot analysis (data not shown), and exhibited no obvious behavioral phenotypes in an ordinary home-cage environment.

**Histological Features of the Brain**—The brain weight of β4GalT-II−/− mice on a C57BL/6 genetic background was nearly identical to that of the β4GalT-II−/− mice at least until 2 months of age. However, the brain weight was significantly reduced in the β4GalT-II−/− mice at 6–9 months of age (Fig.
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FIGURE 3. Immunohistochemical analysis of the brain. A, NeuN-positive neurons located in the cerebellar granular cell layer (gc), cerebellar nuclei (cn), and CA region/dentate gyrus (DG) of the hippocampus. B, Nestin-positive cells in the DG (left) and rostral migratory stream near the olfactory bulb (OB) (right) were the same in both genotypes. C, calbindin-positive Purkinje cells in the cerebellum. The section from a β4GalT-II−/− mouse shows ectopic Purkinje cells (arrowheads) and their disturbed alignment (circled area). D, upper, ratio of disturbed cerebellar lobules (total 40 lobules per a mouse) was counted (+/−, n = 4; −/−, n = 4). The β4GalT-II−/− mice had significantly more disturbed cerebellar lobules (*, p < 0.05). D, lower, number of Purkinje cells was significantly reduced in the mutant mice(+/−, n = 4; −/−, n = 4, *, p < 0.05). E, BrdUrd-labeled cells (arrowhead) in the subventricular zone (SVZ) and DG.
Kluver-Barrera’s staining (Fig. 2, B and C) revealed that the thickness of the cerebral cortex was slightly reduced, and the lateral ventricle was enlarged in the 8-month-old β4GalT-II−/− mice, indicating that the cerebral cortex of these mice atrophied slightly with age. In the cerebellum, no prominent morphological differences were observed between the genotypes.
Brain sections from adult mice were immunostained with several neuronal markers. NeuN-positive mature neurons were appropriately located in the cerebellar granular cell layer, cerebellar nuclei, and CA region/dentate gyrus (DG) of the hippocampus in both the /H92524GalT-II/H11001/H11002 and /H92524GalT-II/H11002/H11002 mice (Fig. 3A). Nestin-positive neural progenitor cells were present in the DG and rostral migratory stream near the olfactory bulb in both genotypes (Fig. 3B). In the cerebellum of the /H92524GalT-II/H11002/H11002 mice, however, several calbindin-positive Purkinje cells were ectopically located, and their alignment was partially disturbed (Fig. 3C). Quantitative analysis showed that the /H92524GalT-II/H11002/H11002 mice had significantly more disturbed cerebellar lobules than /H92524GalT-II/H11001/H11002 mice (Fig. 3D, upper). In addition, the number of Purkinje cells was significantly reduced in the 4th and 5th cerebellar lobules of the /H92524GalT-II/H11002 mice compared with /H92524GalT-II/H11002 mice (Fig. 3D, lower). In the subventricular zone and DG of the hippocampus of the /H92524GalT-II/H11002 mice, the pattern of BrdUrd-labeled cells was the same as in the /H92524GalT-II/H11002 mice (Fig. 3E).

Reduced Expression of the HNK-1 Carbohydrate in the /H92524GalT-II/H11002 Mouse Brain—Because the HNK-1 carbohydrate and PSA, which are well known functional carbohydrates in the nervous system, are expressed on the β4-galactose epitope, we investigated the expression of these carbohydrates in the /H92524GalT-II/H11002 mouse brain. Immunohistochemical staining with an anti-HNK-1 antibody revealed strong and widespread expression of the HNK-1 carbohydrate in the 2-week-old /H92524GalT-II/H11001/H11001 mouse brain, including the cerebral cortex and hippocampus, and comparatively less expression in the cerebellum (Fig. 4A). In contrast, the immunoreactivity of the HNK-1 carbohydrate in the 2-week-old /H92524GalT-II/H11002/H11002 mice was markedly reduced in the cerebral cortex and hippocampus. In the 11-week-old /H92524GalT-II/H11002/H11002 mouse brain (Fig. 4B), the HNK-1 reactivity was barely detectable only in the DG of the hippocampus, layers III–IV of the cerebral cortex, and the cerebellar lobule. In the cerebellar nuclei of the /H92524GalT-II/H11002 mice, no reduction of the HNK-1 carbohydrate was apparent, in contrast to the other brain regions.

FIGURE 5. PSA expression in the brain. Sections of the brain from /H92524GalT-II+/− and /H92524GalT-II−/− of 2-week-old (A) and 11-week-old mice (B) were immunostained with an anti-PSA mAb (bar, 200 μm). C, brain membrane fractions from postnatal day 0 mice were subjected to SDS-PAGE and then to Coomassie Brilliant Blue (CBB) staining (left panel) or Western blotting with the anti-PSA mAb (middle panel) or with an anti-NCAM mAb (right panel). Thirty micrograms of protein were loaded in each lane.

Brain sections from adult mice were immunostained with several neuronal markers. NeuN-positive mature neurons were appropriately located in the cerebellar granular cell layer, cerebellar nuclei, and CA region/dentate gyrus (DG) of the hippocampus in both the /H92524GalT-II+/− and /H92524GalT-II−/− mice (Fig. 3A). Nestin-positive neural progenitor cells were present in the DG and rostral migratory stream near the olfactory bulb in both genotypes (Fig. 3B). In the cerebellum of the /H92524GalT-II−/− mice, however, several calbindin-positive Purkinje cells were ectopically located, and their alignment was partially disturbed (Fig. 3C). Quantitative analysis showed that the /H92524GalT-II−/− mice had significantly more disturbed cerebellar lobules than /H92524GalT-II+/− mice (Fig. 3D, upper). In addition, the number of Purkinje cells was significantly reduced in the 4th and 5th cerebellar lobules of the /H92524GalT-II−/− mice compared with /H92524GalT-II+/− mice (Fig. 3D, lower). In the subventricular zone and DG of the hippocampus of the /H92524GalT-II−/− mice, the pattern of BrdUrd-labeled cells was the same as in the /H92524GalT-II+/− mice (Fig. 3E).
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By Western blot analysis (Fig. 4C), we further confirmed that the HNK-1 carbohydrate in the β4GalT-II−/− mouse brain largely disappeared, except for faint bands of >250 kDa at every postnatal stage. On the other hand, the expression of NCAM, which is one of major carrier proteins of the HNK-1 carbohydrate, did not differ among the wild-type, β4GalT-II+/+, and β4GalT-II−/− genotypes.

Expression of PSA Is Not Changed in the β4GalT-II−/− Mouse Brain—Immunohistochemical staining with an anti-PSA antibody showed that PSA was highly expressed in the hippocampus and corpus callosum and at lower levels in the cerebral cortex and cerebellar lobule of the 2-week-old β4GalT-II+/+ mouse brain (Fig. 5A). Weak PSA expression was detected in the DG and CA3 fields of the hippocampus of the 11-week-old β4GalT-II+/+ mouse (Fig. 5B). No obvious change in PSA expression was observed in the β4GalT-II−/− brain (Fig. 5, A and B). The comparable PSA expression in the mutant was verified by Western blot analysis (Fig. 5A) using the P0 mouse brain. The expression of NCAM also did not differ among the wild-type, β4GalT-II+/+, and β4GalT-II−/− genotypes (Fig. 5C).

To examine the carbohydrate structures in the β4GalT-II−/− brain, lectin blot analysis using concanavalin A, RCA120, S. sieboldiana agglutinin, and M. amurensis agglutinin was performed. Although a few bands disappeared in the membrane fractions of the β4GalT-II−/− brain, most of the band patterns in the β4GalT-II+/+ and β4GalT-II−/− brains of 2- and 11-week-old mice were comparable (supplemental Fig. 1).

Spontaneous Activity of β4GalT-II−/− Mice in the Open Field Test—To assess their spontaneous activity in a novel environment, the mice were subjected to an open field test for 3 days. Their spontaneous activity was typically and usually repressed at the initial phase of day 1 (Fig. 6A), probably because the animals became nervous in a novel environment. On every trial day, the greatest distance moved was recorded during the first phase of the trial (except for day 1), and this activity gradually decreased. This behavioral pattern did not significantly differ between the β4GalT-II+/+ and β4GalT-II−/− mice. The β4GalT-II−/− mice also showed similar spontaneous activity in terms of their speed (Fig. 6C) and the number of rearing (Fig. 6D) as their heterozygous littermates, although the time spent in the central area of the chamber was slightly greater for the β4GalT-II−/− mice (Fig. 6B).

Performance of β4GalT-II−/− Mice Is Impaired in the Morris Water Maze but Not in the Passive Avoidance Response Test—In the Morris water maze paradigm, the swimming latency gradually decreased with the number of training trials and days for both the β4GalT-II+/+ and β4GalT-II−/− mice (Fig. 7A). In the late phase of training, especially after day 6, the β4GalT-II−/− mice showed a significantly prolonged escape latency compared with the β4GalT-II+/+ mice. We also observed that β4GalT-II−/− mice tended to swim near the pool wall (Fig. 7C, statistically not significant) with a particular swimming path (Fig. 7B). The swimming latency of the two genotypes was not different in visible landmark trials (Fig. 7A), +/−, 6.02 ± 0.48 s; −/−, 7.16 ± 0.72 s; p = 0.38, suggesting that swimming ability was normal in β4GalT-II−/− mice. On the other hand, in the passive avoidance response, another learning/memory paradigm, the β4GalT-II−/− mice performed the same as the +/− mice in a retention trial given 24 h after the electrical shock trial (Fig. 7D).

β4GalT-II−/− Mice Show Motor Learning Retardation with Impaired Motor Coordination but GcAT-P−/− Mice Do Not—Motor function was assessed by the rota-rod and balance beam tests. Rota-rod performance, in which the latency to fall from the rod was measured, improved across trials and days for both the β4GalT-II−/− and β4GalT-II+/+ mice (Fig. 8A). However, although the β4GalT-II−/− mice showed improved motor learning, their performance was always poorer than that of the β4GalT-II+/+ mice. A relatively poor motor performance by the β4GalT-II−/− mice was also revealed by the balance beam test, in which a significantly greater number of hind foot slips-offs on the narrow (11 mm diameter) beam was observed.
In contrast, the hind paw movement of the β4GalT-II−/− mice was not impaired in a low speed rota-rod paradigm (7 rpm), as shown in Fig. 8C. To clarify whether GlcAT-P−/− mice also had impaired motor function, these mice were subjected to the same tests. Unlike in the Morris water maze, the GlcAT-P−/− mice showed the same performance in both the rota-rod and balance beam tests as the GlcAT-P+/+ mice (Fig. 8, D and E).

**DISCUSSION**

In this study, we generated the first β4GalT-II−/− mice and analyzed their behavior, the histology, and immunohistochemical characteristics of their central nervous system. The β4GalT-II−/− mice grew normally and were fertile with no overt phenotypes, probably because β4GalT-I could compensate for the β4GalT activity in most tissues. However, spatial learning/memory and motor coordination/learning as well as the expression of the HNK-1 carbohydrate in the brain, where β4GalT-I is barely expressed, were severely impaired in the β4GalT-II−/− mice. Surprisingly, the amount of HNK-1 carbohydrate was markedly decreased in the cerebral cortex and hippocampus, moderately decreased in the cerebellar lobules, and unchanged in the cerebellar nuclei of the β4GalT-II−/− mice compared with their β4GalT-II+/+ littermates. In Western blot analysis of whole-brain lysates, the HNK-1 carbohydrate was barely detectable in the β4GalT-II−/− mice, except as >250-kDa bands, although the NCAM expression was unchanged. The HNK-1 carbohydrate is mostly expressed on the Galβ1–4GlcNAc backbone, which can be synthesized by some β4GalTs. Our results indicate that β4GalT-II is indispensable for the expression of the HNK-1 carbohydrate in the brain. It is possible that β4GalT-II is solely responsible for the synthesis of Galβ1–4GlcNAc in the brain, even though β4GalT-III and -V are also expressed there (10, 13, 14). However, this is unlikely because RCA120-reactive bands, corresponding to Galβ1–4GlcNAc, were detected in the β4GalT-II−/− brain lysates at levels comparable with those in wild-type brain lysates (supplemental Fig. 1). Another possibility is that only the Galβ1–4GlcNAc that is synthesized by β4GalT-II can serve as a substrate for the HNK-1 carbohydrate, because of an unknown mechanism. In contrast, PSA expression in the brain was not affected in the β4GalT-II−/− mice, suggesting that β4GalT-II is dispensable for PSA expression. It is interesting that NCAM is glycosylated normally with PSA, but much less glycosylated with the HNK-1 carbohydrate in the β4GalT-II−/− mice, although NCAM is known to be modified with both carbohydrates. Among six putative N-glycosylation sites on NCAM,
two of them (Asn-441 and Asn-470) and four of them (Asn-297, Asn-329, Asn-441, and Asn-470) could be modified by PSA and the HNK-1 carbohydrate, respectively (29). Our results suggest that β4GalT-II and other β4GalTs are responsible for the synthesis of N-glycans with the HNK-1 carbohydrate and N-glycans with PSA, respectively. If each β4GalT discriminates N-glycosylation sites on the same protein, it will be interesting.

The spontaneous activity of β4GalT-II-/- mice in a novel environment, assessed by the open field test, was comparable with that of control mice. Our results indicated that the β4GalT-II-/- mice had normal locomotor activity and could habituate to a novel environment with improvement seen in a single day and across days. In contrast, the performance of the β4GalT-II-/- mice in the Morris water maze was inferior to that of the controls. Taking into account their normal spontaneous activities in the open field situation, normal paw movement in the low speed rota-rod paradigm, and the same escape latency in the visible landmark water maze paradigm, impaired Morris water maze performance indicates impaired spatial learning/memory but not motor deficit in β4GalT-II-/- mice. In contrast, β4GalT-II-/- mice formed an appropriate passive response, another hippocampus-dependent paradigm, as well as the control mice. Thus, their learning/memory impairment was specific for spatial information processing and was not a broad spectrum learning/memory impairment.

There are previous findings showing the importance of HNK-1 carbohydrate and HNK-1-dependent relief in the hippocampal synaptic plasticity (30–32) and hippocampus-dependent learning/memory (33, 34). We and others expanded these previous studies using the mice deficient in GlcAT-P and HNK-1 sulfotransferase, which are responsible for glucuronic acid transfer and sulfate group transfer of the HNK-1 carbohydrate, respectively. These mutant mice exhibit a spatial learning/memory defect, as evaluated by the Morris water maze task, and also show reduced long term potentiation at the Schaffer-collateral-CA1 synapses, indicating that the HNK-1 carbohydrate plays a pivotal role in the synaptic plasticity of the hippocampus (35, 36). Based on these previous reports, the impaired spatial learning/memory of the β4GalT-II-/- mice in this study can be accounted for by the reduced expression of the HNK-1 carbohydrate in the brain of the mice, especially in the hippocampus.

Neural cell adhesion molecules, including NCAM, L1, tenascin-R, and telencephalin, play important roles in neuronal generation, cell migration, axonal outgrowth, and synaptic plasticity, and their functions are regulated by glycosylation structures such as the HNK-1 carbohydrate and PSA. NCAM, one of the best characterized members of the Ig superfamily, and the functions of PSA on NCAM have been extensively examined using NCAM-, ST8SiaII-, and ST8SiaIV-deficient mice (37–39). Some neuronal functions are dependent on PSA, and some others depend on NCAM. ST8SiaII and ST8SiaIV double-deficient (PSA-deficient), but not NCAM-deficient mice, die as neonates; this lethality is because of abnormal homophilic binding of NCAM in the absence of the negatively charged PSA (40). Because the HNK-1 carbohydrate is also neg-
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Atively charged and expressed on NCAM, a similar mechanism might be at work in mice deficient in HNK-1 carbohydrate production.

Motor coordination/learning, evaluated by the rota-rod and balance beam tests, was also impaired in the β4GalT-II−/− mice. Although the β4GalT-II−/− mice showed improved performance in the rota-rod test during successive trials and days, the latency to fall was always shorter than that of the control mice. The hind paw movement of the β4GalT-II−/− mice in the 7-rpm rota-rod test was no different from that of the +/- mice, suggesting that their motor ability was normal, at least in this low speed paradigm. These results suggest that the β4GalT-II−/− mice had retarded motor learning that was based on impaired motor coordination, although they had normal paw movement. In contrast, the GlcAT-P−/− mice performed normally in the rota-rod and balance beam tests. Therefore, the impaired motor coordination/learning of the β4GalT-II−/− mice was independent of the HNK-1 carbohydrate, unlike the impairment in spatial learning/memory. Because the band patterns of lectin blot (supplemental Fig. 1) were slightly different between the β4GalT-II−/− and β4GalT-II+/− mice, carbohydrate structures of some proteins could be altered in the β4GalT-II−/− brains. Unknown functional carbohydrates other than the HNK-1 carbohydrate and PSA are likely to play an important role in motor coordination/learning.

Although the NeuN-positive mature neurons in the cerebellar granular cell layer and hippocampus and the Nestin-positive neural progenitor cells in the DG and rostral migratory stream near the olfactory bulb appeared normal, the Purkinje cells in the cerebellum of the β4GalT-II−/− mice showed abnormalities. The number of Purkinje cells in the cerebellum was significantly reduced, and their alignment was partially disturbed in these mutant mice. These abnormalities in the Purkinje cells might be responsible for the impaired motor coordination/learning of the β4GalT-II−/− mice. Unknown functional carbohydrates synthesized by β4GalT-II could be involved in the development and maintenance of Purkinje cells.

The brain weight of the β4GalT-II−/− mice became significantly reduced at 6–9 months old, and the cerebral cortex showed slight atrophy at 8 months old. Because the present behavioral analysis was performed when the mice were between 3 and 6 months old, the reduction of brain size might occur during the behavioral tests. It is, however, unclear whether reduced brain size affected the behavioral performance of the β4GalT-II−/− mice. Previous studies reported that NCAM modified by PSA and PSA itself are important in cellular migration/lamination in the cerebral cortex and in the differentiation of neural precursors (41). However, neither PSA expression nor BrdUrd incorporation was altered in the brain of the β4GalT-II−/− mice in this study. Another possibility is that other adhesion molecules, which could be glycosylated, have key roles in brain formation and behavioral output. For example, mutations in the human L1 gene cause a severe neurological disease termed CRASH (acronym for corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraplegia, and hydrocephalus) (42) and mouse mutations in the L1 gene also develop malformations of the nervous system (43, 44). Although the severity of brain malformations of L1−/− mice is different depending on genetic background, L1−/− mice with milder phenotypes display dilated lateral ventricles and reduced brain size as well as impaired Morris water maze performance (44). It might be possible that aberrant glycosylation of L1 changes L1 functions, resulting in dilated lateral ventricles and reduced brain size in β4GalT-II−/− mice. Further in vivo and in vitro analyses will be needed to address this issue.

In summary, the expression of the HNK-1 carbohydrate was markedly reduced in the brain of β4GalT-II−/− mice, suggesting that the expression of the HNK-1 carbohydrate, but not of PSA, was dependent on β4GalT-II. β4GalT-II−/− mice were impaired in spatial learning/memory and motor coordination/learning, indicating that carbohydrates synthesized by β4GalT-II play important roles in these higher brain functions. The spatial learning/memory could be dependent on the HNK-1 carbohydrate, but the motor coordination/learning was independent of it. Further investigation will be necessary to elucidate the roles of these and other carbohydrates in higher brain functions.

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