Developmental regulation of β-1,3-galactosyltransferase-1 gene expression in mouse brain

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Abstract β-1,3-galactosyltransferase-1 (β3GalT-1) is the key enzyme to form the type 1 chain structure. Northern blot analysis indicated that β3GalT-1 was expressed predominantly in the brain. In the present study, it was revealed that the gene expression of β3GalT-1 in mouse brain was developmentally decreased. High expression levels of β3GalT-1 were found in cerebral cortex and hippocampus in both newborn and adult mice, while in cerebellum, the expression levels decreased markedly during development. In situ hybridization revealed that the absence of expression in cerebellar granual cell layers contributed to the main loss of β3GalT-1 expression in adult mouse cerebellum. Moreover, the decreased levels of β3GalT-1could affect the synthesis of type 1 chain oligosaccharides, as revealed by immunohistochemistry analysis.

Key words: Galactosyltransferase; Gene expression; Mouse brain

1. Introduction

Two types of carbohydrate chains are known to exist in the lacto-series of oligosaccharides: type 1 chains that contain the Galβ1–3GlcNAc linkage and type 2 chains containing the Galβ1–4GlcNAc structure [1,2]. The two core structures are differentially expressed in cells and organs [3] and are synthesized by at least two independent galactosyltransferase activities [4,5]. Members of the β-1,4-galactosyltransferase family enable the formation of type 2 chains while the β-1,3-galactosyltransferase family is involved in the type 1 chain synthesis [6]. The first β-1,3-galactosyltransferase gene cloned, β3GalT-1, was identified by expression cloning using mRNA from a melanoma cell line WM266-4 to direct Le^a and sialyl-Le^a expression in Burkitt lymphoma Namalwa KJM-1 cells [7]. Expression constructs of the full coding region and secreted forms of β3GalT-1 were shown to yield β3GalT activity with simple saccharides, and activity with βGlcNAc terminating lacto-series glycosphingolipids [8]. However, the kinetic properties of β3GalT-1 were much poorer than those reported for β3GalT activity found in epithelial tissues and cell lines [4,9,10], suggesting that additional β3GalT existed.

The application of the expressed sequence tag cloning strategy to search for members of this gene family has now led to the identification and cloning of six distinct β3GalTs [8,11–16]. Northern blot analysis revealed that the six β3GalTs were differentially expressed, but surprisingly β3GalT-1 was exclusively expressed in brain. High levels of β3GalT-2 were found in heart, with moderate expression levels in brain [8]. In contrast, β3GalT-3, -4, -5 and -6 showed a broad mRNA expression pattern [8,11–16]. The distinct expression pattern of β3GalT-1 raises interesting speculation as to its particular function in the brain, and to understand the roles of β3GalT-1 in the brain, it is first necessary to establish its pattern of expression in detail. This is accomplished by a combination of Northern blot and in situ hybridization analyses. In the present study, it was revealed that the gene expression of β3GalT-1 was developmentally decreased in mouse brain, and that β3GalT-1 was differentially expressed in different brain regions in newborn and adult mice. Finally, the distinct gene expression pattern of β3GalT-1 could affect the synthesis of type 1 chain oligosaccharides during brain development.

2. Materials and methods

2.1. Experimental animals

All experiments were conducted on BALB/c mice (Department of Animal Science, Shanghai Medical College of Fudan University). Animals were kept under standardized laboratory conditions in an air-conditioned room with free access to food and water. For Northern blot analysis, mice were killed under pentobarbital anesthesia. Subsequently, the skull was opened and the whole brain was removed. For regional-distribution Northern blot analysis, fresh brains were dissected in five regions: cerebral cortex, hippocampus, diencephalon, brainstem and cerebellum. The tissues of corresponding regions from three to four animals were pooled. All samples were kept on ice until total RNA isolation. For in situ hybridization and immunohistochemistry studies, mice were anesthetized and perfused intracardially with 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by fixative (4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4). The brains were immediately removed from the cranium, immersion-fixed overnight at 4°C in the same fixative and subsequently processed for sectioning.

2.2. Preparation of RNA probes

To prepare RNA probes for Northern blot hybridization and in situ hybridization analysis, reverse-transcription polymerase chain reaction (PCR) was performed on the total RNA used. Total cellular RNA was extracted from the brain using Trizol reagent (Gibco-BRL, MD, USA). The first-strand cDNA was prepared using a reverse-transcription kit (Gibco-BRL) from the 5 μg total brain RNA. A cDNA fragment was amplified by PCR using the primers 5′-TTGCCAGGAAAAACTTCACC-3′ (sense primer) and 5′-ACAGAACGGTGGGTAGTTGC-3′ (antisense primer) specific for mouse β3GalT-1,

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Abbreviations: β3GalT-1, β-1,3-galactosyltransferase-1; DIG, digoxigenin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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primers 5′-GACGACACTGCTGCTTTG-3′, 5′-TGGCATTTTTCA-GGCTCA-3′ for mouse β3GalT-2 and primers 5′-GCAAGACGT-GGGGTAGAGAG-3′, 5′-AGAGCCCAACAAACACATCC-3′ for mouse β3GalT-5. PCR amplification was carried out with an initial denaturing step at 95°C for 3 min, then 30 cycles at 95°C for 30 s, at 60°C for 45 s, and at 72°C for 45 s and a further extension at 72°C for 10 min. The PCR products were sequenced by a laser fluorescent DNA sequencer (Amersham, UK). For Northern blot analysis, the PCR-amplified cDNA fragments of mouse β3GalTs were radiolabeled with [α-^{32}P]dATP (Amersham, UK), using a Prime-A-Gene random primer labeling kit (Promega, WI, USA) according to the manufacturer's protocol. For in situ hybridization analysis, the PCR-amplified cDNA fragment of mouse β3GalT-1 was subcloned into pGEM-T Easy plasmid (Promega) and linearized with *Apa*I and *Sal*I restriction enzymes. Digoxigenin (DIG)-labeled riboprobes were obtained by in vitro transcription in the presence of SP6 or T7 RNA polymerase at 37°C for 2 h using a DIG labeling kit (Boehringer Mannheim, Mannheim, Germany).

2.3. Northern blot hybridization

For the developmental Northern blot analysis, BALB/c mice at ages 18-day-old embryo, 3 days, 1, 2, 4, 8, 12 weeks were used. Each point represented RNA prepared from brains of four animals (equal number of males and females). For regional-distribution Northern blot analysis, the brain regions were removed from newborn mice (1 week old) and adult mice (12 weeks old). Total RNA was isolated using Trizol reagent according to the manufacturer's instructions. RNA concentrations were calculated by measuring UV light absorbance at 260 nm. Each RNA sample (40 μg) was denatured and subjected to electrophoresis in 1% agarose gel containing 2.2 M formaldehyde. RNA was transferred to Hybond-N+ nylon membrane (Amersham, UK) and cross-linked by UV irradiation using a GS Gene Linker UV chamber (Bio-Rad, CA, USA). Northern blot analysis was performed as described in [17], using the [α-^{32}P]dATP-labeled β3GalT fragments as probes. The widely used cDNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1.759 bp) was adopted as an intrinsic standard during Northern blot analysis.

2.4. In situ hybridization

For analysis, 5 μm sections of 1- and 12-week-old mouse brains were used. In situ hybridization was performed as previously described [18]. In brief, deparaffinized sections were incubated in 10 μg/ml proteinase K for 15 min at 37°C and treated with 0.2 M HCl for 10 min, and 0.25% acetic anhydride/0.1 M triethanolamine (pH 8.0) for 15 min. The sections were then prehybridized in the following solution: 50% formamide, 10% dextran sulfate, 5× saline sodium citrate (SSC), 0.5% sodium dodecyl sulfate, 5× Denhardt's reagent, and 200 μg/ml of yeast tRNA in DEPC-treated water at 42°C for 60 min and hybridized at 42°C for 16 h by adding DIG-labeled antisense or sense β3GalT-1 probe in a humidified chamber. After hybridization, the slides were washed in 50% formamide/2× SSC at 42°C for 20 min three times and treated with 10 μg/ml of RNase A in buffer (10 mM Tris–HCl, pH 7.6, 500 mM NaCl, and 1 mM EDTA) at 37°C for 30 min, washed in 2× SSC at 42°C for 20 min and in 0.2× SSC at 42°C for 20 min twice; the sections were then immersed in 7.5 U/ml of an anti-DIG-alkaline phosphatase-conjugated antibody and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

2.5. Immunohistochemistry

Immunohistochemistry was performed on 5 μm sections of 1- and 12-week-old mouse brains. The standard avidin–biotin–peroxidase complex (ABC) staining method was adopted. Briefly, following deparaffination and rehydration, the sections were immersed in 0.3% (v/v) H_2O_2/methanol for 30 min at 37°C to inhibit endogenous peroxidase. Sections were then incubated with *Clostridium perfringens* neuraminidase (1 U/ml; Sigma) in 0.05 M PBS, pH 7.4, for 2 h at 37°C. The sections were washed five times with PBS and then blocked in 5% (w/v) bovine serum albumin (BSA) containing 0.1% Triton X-100, prior to incubation with monoclonal antibody specific for type 1 chain terminal structures (TE-3) (kind gift from Dr. Eric H. Holmes; characterization of TE-3 antibody was described previously [19]) in a moist chamber at 4°C overnight. Incubation of primary antibody was omitted in several sections for control. The sections were washed in PBS five times and treated for 60 min with biotinylated goat anti-mouse IgM (μ-chain specific, 10 μg/ml; Kirkegaard and Perry Labs, MD, USA). After washing three times, the sections were immersed for 60 min in ABC (Vector Labs, CA, USA; 1:200). The staining of the sections was obtained by treating the sections for 2–10 min with 50 ml Tris–HCl buffered saline containing 0.01% H_2O_2 and 25 mg diaminobenzidine.

3. Results

3.1. Gene expression of β3GalT-1 is developmentally decreased in mouse brain

Previous results by Northern blot analysis revealed that ubiquitous low levels of β3GalT-1 mRNA were expressed in all mouse organs in addition to the predominant levels in the brain. To determine whether the gene expression in mouse brain was developmentally regulated, Northern blot analysis was performed to examine the expression of β3GalT-1 during mouse brain development. The hybridization revealed a single specific 7-kb band and a significant level of β3GalT-1 transcript was present in the brains of 18-day-old mouse embryo,

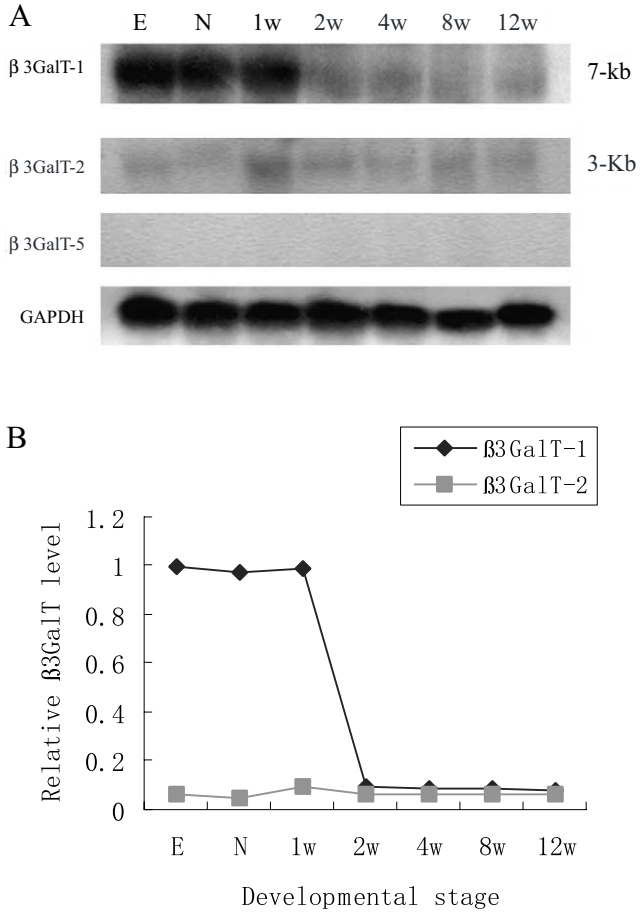

Fig. 1. Developmental expression of β3GalTs by Northern blot analysis. A: The blot containing 40 μg RNA samples from total brain tissues of 18-day-old mouse embryos (lane 1), newborn (lane 2), 1-week-old (lane 3), 2-week-old (lane 4), 4-week-old (lane 5), 8-week-old (lane 6) and (lane 7) 12-week-old mice was probed with ^{32}P-labeled β3GalTs and then GAPDH cDNA fragment after removing the previous probe thoroughly. E, 18-day-old mouse embryos; N, newborn mice (3-day-old); W, week. B: Each lane in A was scanned by laser densitometry and the absorbance of the appropriate bands was calculated. The concentration of β3GalT mRNA relative to GAPDH in each lane was determined from the ratio of their absorbance and was expressed as arbitrary units.

newborn and 1-week-old mice. Hereafter, its level decreased markedly (Fig. 1A). Densitometry analysis indicated that the β3GalT-1 band was 10-fold more intensive in the 1-week-old than in the 12-week-old mouse brain, indicating that β3GalT-1 mRNA was expressed at a much higher level in the newborn brain than in the adult (Fig. 1B). Since the β-1,3-galactosyltransferase family contains multiple isoforms involved in synthesis of type 1 chain lactosamine structure, we also examined the gene expression of two other important family members, β3GalT-2 and β3GalT-5. The expression of β3GalT-2 remained relatively low at each stage of development, with the highest level in 1-week-old mouse brain, while β3GalT-5 mRNA was not detected in mouse brain throughout development (Fig. 1A). The same results were obtained in two independent experiments. These results indicate that β3GalT-1 gene expression is developmentally regulated and that β3GalT-1 may be the key enzyme responsible for type 1 chain oligosaccharide synthesis in mouse brain.

3.2. *Differential regional distribution of β3GalT-1 mRNA expression in newborn and adult mouse brain*

Since the distinct expression of β3GalT-1 in mouse brain and the gene expression was developmentally decreased, to reveal the regional distribution of β3GalT-1 transcripts in mouse brain, and to find out whether there is a difference in the regional distribution between the newborn and adult mice, we performed Northern blot hybridization. The samples used were freshly isolated total RNA from cerebral cortex, hippocampus, diencephalon, brainstem and cerebellum of 1- and 12-week-old mouse brains, respectively. In cerebral cortex,

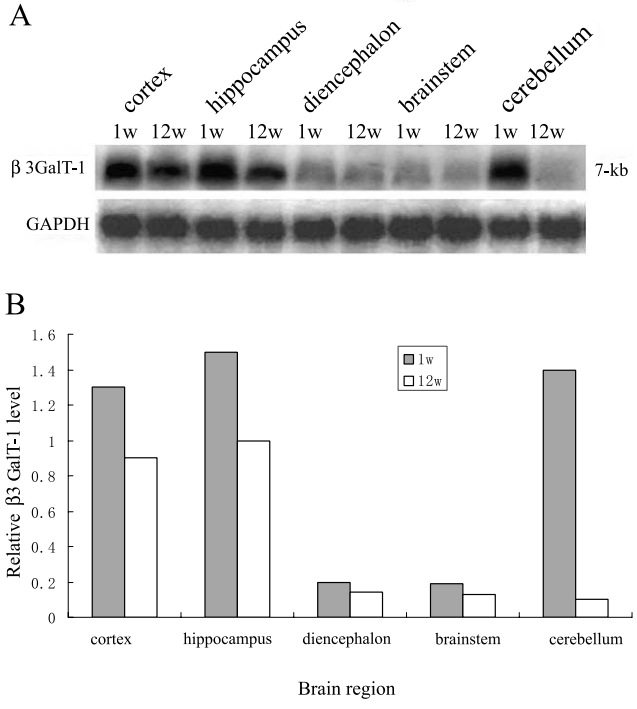

Fig. 2. Regional distribution of β3GalT-1 mRNA expression by Northern blot analysis. A: 40 μg total RNA samples from tissues of cerebral cortex, hippocampus, diencephalon, brainstem and cerebellum of 1- and 12-week-old mice were used. W, week. B: Each lane in A was scanned by laser densitometry and the relative β3GalT-1 mRNA levels were determined from the ratio of their absorbance relative to GAPDH in each lane and expressed as arbitrary units.

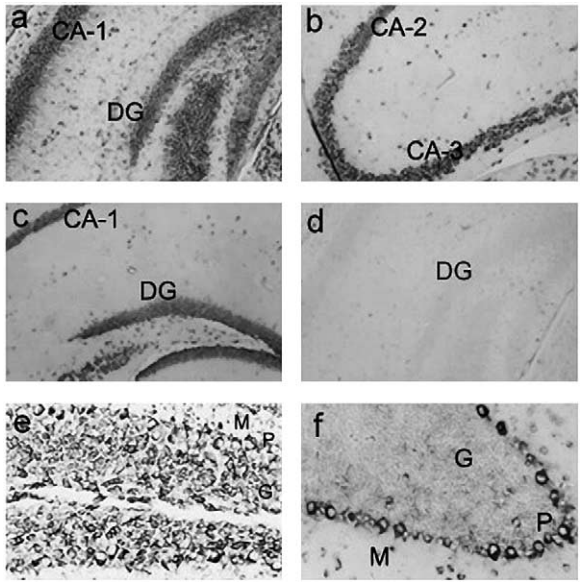

Fig. 3. Localization of β3GalT-1 mRNA in mouse brain: in situ hybridization analyses with DIG-labeled β3GalT-1 antisense RNA. a: 1-Week-old mouse hippocampus, expression in the dentate gyrus region, CA-1 region of the hippocampal formation. b: Expression in the pyramidal cell layers of the hippocampal CA2–CA3 regions. c: 12-Week-old mouse hippocampus, no apparent staining changes are visible. d: Hippocampus, sense probe, no labeling is detected. e: 1-Week-old mouse cerebellum, β3GalT-1 mRNA is strongly expressed in granule cell layer and in Purkinje cell layer. f: 12-Week-old mouse cerebellum, β3GalT-1 mRNA is expressed only in Purkinje cell layer. DG, dentate gyrus; M, molecular cell later; P, Purkinje cell layer; G, granule cell layer. Magnification 40×, except for panels e and f, 100×.

β3GalT-1 was expressed abundantly in newborn mice, with a slight decrease in adult mice, and a similar expression pattern could be detected in hippocampus (Fig. 2A). In contrast, the expression levels were low in diencephalon and brainstem in both newborn and adult mice. The cerebellum was the only area that differed significantly from the rest of the samples, with a higher expression in newborn mice, and a much lower expression in adult mice (Fig. 2A). Densitometry analysis indicated that the β3GalT-1 band was about 14-fold more intensive in the 1-week-old than in the 12-week-old mouse cerebellum (Fig. 2B).

3.3. *In situ hybridization of β3GalT-1 mRNA expression in mouse brain*

The more accurate localization of β3GalT-1 mRNA expression in mouse brain as studied by in situ hybridization was in good agreement with the general expression pattern seen with Northern blot hybridization. The overall expression of β3GalT-1 mRNA was widespread, but most areas showed an expression level that was low and often close to the detection level. The most prominent expression was seen in hippocampus and cerebellum. Strong hybridization signals were detected in the dentate gyrus region and in the pyramidal cell layers of the hippocampal CA1–CA3 regions of the hippocampal formation (Fig. 3a,b) in 1-week-old mice. No apparent difference of expression in hippocampus was detected between the 1- and 12-week-old mice (Fig. 3c), and no signals were detected in hippocampus using sense probe for β3GalT-1 transcript (Fig. 3d). In contrast, the expression pattern changed in cerebellum during development. In newborn

mice, the dense granular layer showed a strong signal (Fig. 3e), and the Purkinje cell, which is the only output element of the cerebellar cortex and forms the sole link between the cerebellar cortex and the cerebellar nuclei, also stained positively (Fig. 3e). However, in adult mice, strong signals were only detected in Purkinje cell layers and the levels in granular layers were barely detectable (Fig. 3f). This correlates well with the conclusion from the Northern blot analysis that the gene expression of β3GalT-1 decreased markedly in cerebellum during development.

3.4. Immunohistochemical localization of type 1 chain oligosaccharides in mouse brain

Since the abundance of β3GalT-1 was expressed in hippocampus and cerebellum as revealed by in situ hybridization, immunohistochemistry was performed to find out whether there is a difference between the expression of oligosaccharides with the type 1 chains and β3GalT-1 enzyme. The TE-3 antibody, which specifically binds to the type 1 chains and has no cross-reactivity with type 2 chains [19], was used. In newborn mice, the Purkinje cell layers showed strong immunopositive staining for type 1 chain oligosaccharides together with the granular cell layers (Fig. 4a,c). In contrast, in adult mice, strong immunoreactivity for type 1 chain oligosaccharides was only detected in Purkinje cell layers and in granular layers the levels were barely detectable (Fig. 4b,d). These results correlated well with the in situ analysis, showing that the expression of β3GalT-1 mRNA decreased markedly in granular layers during development. However, despite the strong expression of β3GalT-1 in the hippocampal formation, no immunoreactivity for type 1 chain oligosaccharides was detected in hippocampus in both newborn and adult mice (Fig. 4e). Furthermore, the immunoreactivity was barely detectable in mouse cerebellum without neuraminidase pretreatment (Fig. 4f), suggesting the possibility that the binding of TE-3 antibody to type 1 chain structure was disturbed or inhibited by sialic acid and that the type 1 chain oligosaccharides in mouse brain was likely to be sialylated. These results were confirmed by five more independent experiments.

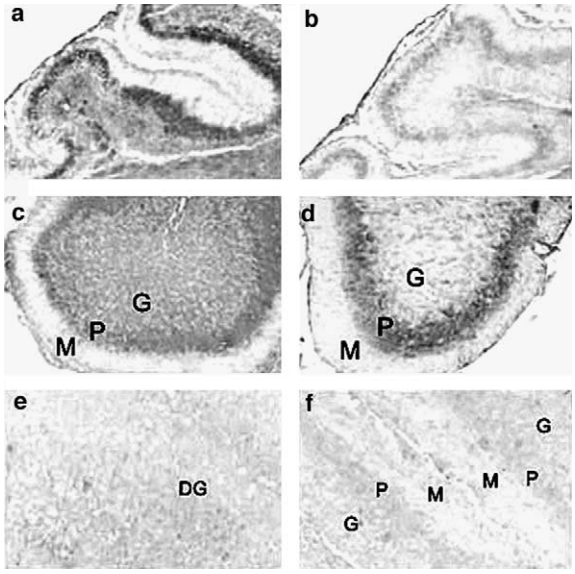

Fig. 4. Immunohistochemical localization of type 1 chain oligosaccharides in mouse brain. a,c: 1-Week-old mouse cerebellum; the expression was detectable in granule cell layer and in Purkinje cell layer. b,d: 12-Week-old mouse cerebellum; the immunopositive staining was detected only in Purkinje cell layer. e: 1-Week-old mouse hippocampus; no expression was detected. f: Sections without neuraminidase treatment. DG, dentate gyrus; M, molecular cell later; P, Purkinje cell layer; G, granule cell layer. Magnification: panels a and b, 40×; c, 200×; d–f, 100×.

4. Discussion

In the present study, we have first delineated the gene expression of β1,3-galactosyltransferases in mouse brain. We revealed that β3GalT-1, which was highly expressed in the brain, showed an abundance in the early stages and a dramatic decrease in the late stages of postnatal development. Similarly, our previous study indicated that the gene expression of β1,4-galactosyltransferase-1 was also developmentally decreased [17]. However, the β3GalT proteins represent a family that is distinct from the β4GalT proteins, as no sequence similarity can be detected between β3GalT enzymes and β4GalTs. Also, the entire protein-coding region of three murine β3GalT genes was contained in a single exon [11], while β4GalT contained multiple exons coding for protein sequence [20,21]. Although the distinctions exist between the β3GalTs and β4GalTs gene family, both can galactosylate *N*-linked oligosaccharides. Since the differential galactosylation of *N*-linked oligosaccharides will determine the subsequent glycosylation, an overall increase in the galactosylation of brain glycoproteins within the 2 weeks following birth could be important for the neural network formation, which is completed during this period. However, β4GalT-1 was expressed only at the mid-embryonic stage and the level decreased thereafter, so the β1,4-galactosylation must have been performed by other β4GalTs in later developmental stages [17,22]. In the case of β3GalT-1, which was barely detectable in the 2-week-old mouse brain, its biological function may also have been compensated by other β3GalTs. However, the low expression levels of β3GalT-2 together with the absence of β3GalT-5 expression suggested the possibility that β3GalT-1 was the major enzyme responsible for synthesis of type 1 chain oligosaccharides in mouse brain.

Since glycosyltransferases are not the final products that exert biological effects, the localization of oligosaccharides with type 1 chain was examined by immunohistochemistry. It is a noteworthy finding in the present study that no presence of type 1 chain oligosaccharides was detected using TE-3 antibody in hippocampus despite the strong expression of β3GalT-1 as revealed by in situ hybridization. In contrast, in mouse cerebellum, the presence and changes of oligosaccharides with type 1 chains were consistent with the gene expression of β3GalT-1. Since TE-3 antibody specifically binds to type 1 chain terminal structures, the lack of positive staining in hippocampus is likely due to the absence of type 1 structure. Moreover, the difference of expression seen between immunohistochemistry and in situ hybridization analysis may also be due to the abundance of β1,4-galactosyltransferases in the hippocampus, which compete with β3GalT-1 for substrate and synthesize the type 2 structure [22]. However, one possibility that must be considered in the above analyses is that while the mRNA for the β3GalT-1 gene in mouse hippocampus is clearly present, there may also be regulation of translation level. However, because of the lack of specific antibodies against β3GalT-1, it is not possible at present to determine

the distribution of β3GalT-1 proteins in mouse brain. Whether the expression pattern of β3GalT-1 proteins correlates well with that of the transcripts still needs further confirmation. Since a specific localization could give a hint to the function of the gene in vivo, the abundance of β3GalT-1 in hippocampus and cerebellum is of great interest. Neuropsychological studies and neurophysiological experiments have revealed that the hippocampus plays a key role in certain aspects of learning and memory; the strong expression of β3GalT-1 in both hippocampus and cerebellum suggests that the role of this enzyme may be essential to the normal function of these two structures and deficiency of this gene might have serious clinical consequences. In β4GalT-1 knockout mice in which the galactosylation of glycoprotein is impaired, no apparent defects in the organogenesis are observed except for the growth retardation and neonatal lethality of the mice [23,24]. However, to date, successful β3GalT-1 gene knockout mice have not been reported. Since gene disruption experiments in intact animals are likely to give the most useful biological conclusions regarding glycosyltransferase function in vivo, little is known as to the specific roles of β3GalT-1 so far.

It is also intriguing that the expression pattern of β3GalT-1 in mouse cerebellum is changed in addition to the abundance, and the marked decrease of levels in cerebellum may contribute to the main loss of β3GalT-1 expression during development. The mechanism of the regulation of β3GalT-1 expression in the cerebellum during development is to be further elucidated. In general, the gene expression of glycosyltransferase is regulated in multiple ways. Apart from the Golgi factors that can regulate glycosyltransferase function, their actual expression can be controlled at the level of RNA synthesis or turnover. Although some glycosyltransferases have a widespread, so-called 'housekeeping' type of distribution, β3GalT-1 mRNAs seem to be highly regulated in a tissue-specific and developmentally regulated manner. For the most part, it appears that differential regulation is due to the action of specific promoter regions in the 5′ region of the corresponding gene. In the case of β3GalT-1, further study is needed to elucidate the specific regulation of its gene expression.

In summary, our results provide the first evidence that the expression levels of β3GalT-1 and type 1 chain oligosaccharides in mouse brain are regulated both temporally and spatially. Although the data presented in this paper allow early insights into the possible novel physiological functions of β3GalT-1, the biological significance of the high expression level of β3GalT-1 in hippocampus remains to be elucidated. Whether β3GalT-1 has distinct roles in brain besides a galactosylation modification function is not known yet. Since in humans the expression of type 1 chains is mostly restricted to epithelia, the biological significance of the expression of type 1 chain oligosaccharides in mouse brain remains to be explored as well. Although the type 1 structure functions as a precursor to subsequent outer-chain glycosylations, specific roles for this disaccharide have not been clearly defined. Future study will focus on the distribution of β3GalT-1 proteins in mouse brain; production and analysis of individual β3GalT-1 knockout mice will help to elucidate as yet unknown functions of β3GalT-1 and type 1 chains.

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