Published in final edited form as:

J Neurochem. 2011 March ; 116(5): 874–880. doi:10.1111/j.1471-4159.2010.07042.x.

Histone acetylation-mediated glycosyltransferase gene regulation in mouse brain during development

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Abstract

Gangliosides are sialic acid-containing glycosphingolipids abundant in the central nervous tissues. The quantity and expression pattern of gangliosides in brain change drastically during early development and are mainly regulated through stage-specific expression of glycosyltransferase (ganglioside synthase) genes. It is still unclear, however, how the transcriptional activation of glycosyltransferase genes is regulated during development. In this study, we investigated the epigenetic regulation of two key glycosyltransferases, N-acetylgalactosaminyltransferase I (GA2/GM2/GD2/GT2-synthase) and sialyltransferase II (GD3-synthase), in embryonic, post-natal, and adult mouse brains. Combined bisulfite restriction analysis assay showed that DNA methylation in the 5' regions of these glycosyltransferase genes was not associated with their expression patterns. On the other hand, chromatin immunoprecipitation assay of both glycosyltransferase genes showed that their histone H3 acetylation was highly correlated to their mRNA expression levels during development. In fact, we confirmed that the expression patterns of gangliosides and glycosyltransferases in neuroepithelial cells were changed after treatment with a histone deacetylase inhibitor, sodium butyrate. Our studies provide the first evidence that efficient histone acetylation of the glycosyltransferase genes in mouse brain contributes to the developmental alteration of ganglioside expression.

Keywords

DNA methylation; epigenetics; gangliosides; glycosyltransferase; histone acetylation; neural stem cell

Gangliosides are sialic acid-containing glycosphingolipids that are abundant in the plasma membranes of CNS-forming cells (Yu *et al.* 2009). They have been suggested to play an essential role in various functions in the development of the nervous system (Ledeen *et al.* 1998; Yu *et al.* 2009). During development, the quantity and species of gangliosides in mouse brains change drastically; the expression patterns of gangliosides shift from the

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The authors declare no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. ChIP assay of GalNAcT, ST-II, and β -actin genes in mouse developing brains.

Figure S2. RT-PCR of GalNAcT and ST-II in NECs treated with or without NaBu for 0, 1, 2, 4, 8 and 24 h.

Figure S3. Sequences of primer sets used for COBRA assay, ChIP assay, and RT-PCR.

simplest gangliosides (GM3 and GD3) to the more complex gangliosides (GM1, GD1a, GT1a, and GT1b) (Yu *et al.* 1988; Bouvier and Seyfried 1989; Ngamukote *et al.* 2007). Therefore, gangliosides have been used as stage-specific markers of certain lineages of cells such as neural stem cells (NSCs) and their progeny (Yanagisawa and Yu 2007; Nakatani *et al.* 2010). It has been considered that the developmental change of gangliosides is mainly because of the stage-specific expression of glycosyltransferase genes (Ngamukote *et al.* 2007). So far, studies on the 5' end promoter regions of several representative glycosyltransferase genes have revealed the following characteristics: (i) 5' end promoter regions of glycosyltransferase genes are usually TATA-less, lack a CCAAT box, but contain GC-rich regions; (ii) they contain a set of *cis*-acting elements and binding sites for transcription factors, including Sp1, activator protein 2, and cAMP-response element binding protein; and (iii) some of the promoters also contain negative-regulatory regions (Furukawa *et al.* 1996; Tencomnao *et al.* 2004; Xia *et al.* 2005; Zeng and Yu 2008). However, the molecular mechanisms underlying stage-specific transcriptional activation of glycosyltransferase genes during development are still not fully elucidated.

Recently, studies using NSCs, which are capable of differentiating into brain-forming cells, have shown that stage-specific gene expression in developing mammalian brains is regulated by intracellular programs such as epigenetic modifications, including DNA methylation and histone acetylation (Hsieh and Gage 2004; Hsieh *et al.* 2004; Fukuda and Taga 2005; Kohyama *et al.* 2008; Namiyama *et al.* 2008). DNA methylation (addition of methyl groups to cytosine moieties of CpG dinucleotides) is involved in suppression of gene expression by inhibiting the binding of transcription factors or by providing binding sites for transcriptional repressor proteins (i.e., methyl-CpG-binding proteins). For instance, a signal transducer and activator 3 (STAT3)-binding site on the promoter region of the glial fibrillary acidic protein (GFAP; an astrocytic marker) gene is hypermethylated in neuroepithelial cells (NECs) at embryonic day 12 (E12), which are not responsive to the STAT3 activation signal to express GFAP, but the promoter is barely methylated in NECs at E14, which can express GFAP in response to STAT3 activation (Takizawa *et al.* 2001). On the other hand, histone acetylation (addition of acetyl groups to lysine moieties of the amino-terminal tails of core histones) is the most frequent post-translational histone modification. Acetylation of histone H3 can alter the interaction between histones and DNA and allow relaxation of chromatin. Thus, changes in histone acetylation status are associated with transcriptional activation (Hsieh and Gage 2004; Mehler 2008). In NSCs, induction of histone acetylation promotes neuronal differentiation and inhibits glial differentiation through up-regulation of neurogenic transcription factors (Hsieh *et al.* 2004). Therefore, DNA methylation and histone acetylation are important molecular mechanisms underlying gene regulation during brain development.

In the present context, it is known that treatment of rat hepatoma cells with sodium butyrate (NaBu) can alter the expression of ganglioside patterns and the activities of several glycosyltransferases including sialyltransferase I (GM3 synthase) (Taki *et al.* 1979). Alteration of endogenous glycosyltransferase activities with NaBu was also found in other cell lines such as Chinese hamster ovary cells (Datti and Dennis 1993) and Caco-2 human colonic adenocarcinoma cells (Amano and Oshima 1999). NaBu is well known as an inhibitor of histone deacetylase, which can increase histone acetylation levels *in vivo* and *in vitro* (Boffa *et al.* 1978). These reports suggest the possibility that histone acetylation could regulate transcriptional activation of glycosyltransferase genes. For this reason, we initiated the current study to examine whether ganglioside biosynthesis in developing mouse brain was also regulated by epigenetic mechanisms. We focused on DNA methylation and histone acetylation profiles of two key glycosyltransferase genes, GalNAcT (*N*-acetylgalactosaminyltransferase I or GA2/GM2/GD2/GT2-synthase) and ST-II (sialyltransferase II or GD3-synthase), that are known to govern ganglioside biosynthesis

through different pathways in mouse brain during development. The mRNA expression levels of both glycosyltransferases were highly correlated to histone H3 acetylation levels of the genes, but not to DNA methylation patterns in their 5' regions, during development. Using NECs that are rich in NSCs as a model system, we showed that the expression patterns of gangliosides and glycosyltransferases were altered after treatment with NaBu. This study is the first report providing direct evidence for the involvement of epigenetic modifications in glycosyltransferase gene expression.

Materials and methods

Animals

ICR mice (Harlan, Indianapolis, IN, USA) used in this study were treated in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Medical College of Georgia.

Real-time PCR

Real-time PCR was performed as previously described (Dasgupta *et al.* 2009). In brief, total RNA samples were isolated from mouse brains during development using a Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNAs were synthesized from the total RNAs as templates using Superscript III reverse transcriptase (Invitrogen). Quantitative PCR assays were performed using an iQcycler real-time quantitative thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) with SYBR green (Bio-Rad Laboratories) with the following settings: 94°C for 2 min; 26–30 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 40 s; and 72°C for 5 min. Primer sets used for PCR are listed in Figure S3. All primers were custom synthesized by Integrated DNA Technologies (Coralville, IA, USA). β -Actin was detected as a control housekeeping gene.

Combined bisulfite restriction analysis assay

DNA methylation levels of genes in developing mouse brains were measured by combined bisulfite restriction analysis (COBRA) assay (Xiong and Laird 1997). In brief, genomic DNAs were isolated from E12 and post-natal day 10 (P10) mouse brains using a NucleoSpin Tissue Kit (Macherey-Nagel, Bethlehem, PA, USA). DNA bisulfite conversions were performed using an EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA, USA). The sequences of the primer sets for COBRA assay are shown in Figure S3. PCR products were then digested with *Bst*UI, and the diagnostic fragments were detected on 2% agarose gels containing SYBR Safe DNA Gel stain (Invitrogen). The intact DNAs treated with or without bisulfite were used as control.

Chromatin immunoprecipitation assay

Genomic DNAs and histones (i.e., chromatin) prepared from E12, P10, P30, and adult mouse brains were cross-linked at 37°C for 10 min, lysed, and sonicated with 10–12 pulses for 20 s each at 30–50% of maximum power with a Sonic Dismembrator model 100 (Thermo Fisher Scientific, Waltham, MA, USA). The chromatin was then immunoprecipitated using 2 μ g of anti-acetylhistone H3 antibody, anti-RNA polymerase II antibody, or normal mouse IgG, and reverse cross-linked using an EZ-Magna chromatin immunoprecipitation (ChIP) A Chromatin Immunoprecipitation kit (Millipore, Billerica, MA, USA). Proteins in the immunoprecipitates were digested with proteinase K, and the recovered DNA was purified for quantitative PCR as described above. Primers used for ChIP are shown in Figure S3. DNA recovered from chromatin that was not immunoprecipitated (input) or immunoprecipitated by anti-RNA-polymerase II antibody (positive control) or normal mouse IgG (negative control) were used as controls.

NEC culture

Neuroepithelial cells, which are known to be rich in NSCs (Fukuda and Taga 2005), were isolated from telencephalons of ICR mouse embryos (E14) as previously described (Nakashima *et al.* 1999). NECs were cultured in N2-supplemented Dulbecco's modified Eagle's medium/F12 medium (Invitrogen) containing 10 ng/mL of basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA) on dishes coated with poly-L-ornithine (Sigma-Aldrich, St Louis, MO, USA) and bovine fibronectin (Sigma-Aldrich) at 37°C in a humidified 5% carbon dioxide atmosphere. NECs were treated with 1 mM NaBu (Sigma-Aldrich) at 37°C for 0–24 h for RT-PCR or for 72 h for analysis by TLC prior to harvest.

Ganglioside purification and TLC

Gangliosides were purified from NaBu-treated NECs (Ngamukote *et al.* 2007). In brief, total lipids were extracted from 2.5×10^7 NECs by repeatedly disrupting the cells in chloroform/methanol (C/M, 2 : 1, 1 : 1, and 1 : 2, v/v) and then C/M/water (C/M/W, 1 : 2 : 0.8, v/v/v) by sonication. The extracted total lipid solutions were combined and adjusted to the ratio of C/M/W (65 : 30 : 8, v/v/v). The total lipid solution was applied to anion exchange column chromatography using a diethylaminoethyl-Sephadex A-25 (acetate form; 3 mL bed volume; Sigma-Aldrich) column. Neutral and acidic lipids (gangliosides) were separately eluted from the column with 40 mL of C/M/W (30 : 60 : 8, v/v/v) and 40 mL of C/M/0.8 mol/L sodium acetate in water (30 : 60 : 8, v/v/v), respectively. Gangliosides in the acidic lipid fraction were purified by alkali treatment with sodium hydroxide at 37°C for 2 h, neutralized with acetic acid and desalted using a Sep-Pak C18 column (Waters Corporation, Milford, MA, USA) (Kubo and Hoshi 1985). Purified gangliosides were developed on a TLC plate (Silica Gel 60; Merck Chemicals, Darmstadt, Germany) using a solvent system of C/M/0.2% aq. calcium chloride in water (55 : 45 : 10, v/v/v) and visualized with an orcinol-sulfuric acid reagent.

Results and discussion

GalNAcT and ST-II expression and their DNA methylation in developing brains

The ganglioside compositional change during brain development is mainly regulated by the activities of two key glycosyltransferases, GalNAcT and ST-II, which are positioned at a branching point of the ganglioside biosynthetic pathways (Fig. 1a). To confirm the precise expression levels of GalNAcT and ST-II, we performed quantitative real-time PCR using cDNAs prepared from E12, P10, P30, and adult mouse brains as templates. As shown in Fig. 1(b), the expression level of GalNAcT was drastically increased during development. In comparison, the ST-II expression level revealed only a slight increase during development (Fig. 1c). These results are consistent with our previous report indicating that the GalNAcT, rather than ST-II, is more closely associated with the conversion of simple gangliosides (GM3 and GD3) in the late embryonic mouse brain to complex gangliosides (GM1, GD1a, GT1a, and GT1b) in adult mouse brain (Ngamukote *et al.* 2007).

Next, to elucidate the molecular mechanism underlying the stage-specific transcriptional activation of GalNAcT and ST-II, we analyzed DNA methylation in the 5' regions of the GalNAcT and ST-II genes in E12 and P10 mouse brains by COBRA, a sensitive quantitative DNA methylation assay (Xiong and Laird 1997). As shown in Fig. 1(d–i), no difference was detected in the DNA methylation levels and patterns in the 5' regions of the GalNAcT and ST-II genes of E12 and P10 mouse brains; that is, DNA methylation patterns in the 5' regions of the GalNAcT and ST-II genes were not associated with their expression patterns. These results suggest that transcriptional activation of GalNAcT and ST-II genes in developing mouse brains is not regulated by DNA methylation, at least in the 5' regions analyzed in this study.

Changes of acetylation levels of histone H3 in GalNAcT and ST-II genes in developing brains

We then analyzed histone acetylation levels in the GalNAcT and ST-II genes in E12 and P30 brains by ChIP assay using five primer sets designed for different regions of each gene (Fig. 2a and b). As shown in Fig. 2(c and d), the histone H3 acetylation levels were increased in all five regions of both the GalNAcT and ST-II genes in P30 rather than E12 mouse brains. These increased histone H3 acetylation levels were robust in the region that includes the transcription start site (+0) in the two glycosyltransferase genes. Thus, to further compare the levels of histone H3 acetylation in the GalNAcT and ST-II genes, we analyzed gene regions including the transcription start site (+0) in E12, P10, P30, and adult mouse brains. As shown in Fig. 3(a–c), the level of histone H3 acetylation was increased in GalNAcT and ST-II genes during development. A parallel increase was also found in the β -actin gene. In fact, ChIP signals of these genes in immunoprecipitates with anti-RNA-polymerase II antibody were increased in all of the genes during development (Figure S1), suggesting that histone H3 acetylation and the promoter occupancy of RNA-polymerase II in mouse brain are generally increased during development. Therefore, to more precisely compare the difference of histone H3 acetylation levels in the GalNAcT and ST-II genes during development, we normalized the data of histone H3 acetylation in GalNAcT and ST-II genes by those expressed in β -actin. As shown in Fig. 3(d), the normalized levels of histone H3 acetylation in the GalNAcT gene were significantly increased. However, the histone H3 acetylation levels in the ST-II gene were not, consistent with the mRNA expression pattern (Fig. 3e). These results suggest that changes of glycosyltransferases, especially GalNAcT, in mouse developing brains are regulated by histone acetylation.

The effect of a histone deacetylase inhibitor on ganglioside expression in NECs

Neuroepithelial cells are rich in NSCs that are capable of differentiating into brain-forming cells; for this reason, they represent an excellent model system for studying brain development. In order to confirm whether changes in histone acetylation are involved in expression of the GalNAcT and ST-II genes, we analyzed expression of gangliosides and glycosyltransferases in NECs treated with an histone deacetylase inhibitor, NaBu, which is capable of increasing levels of acetylated histone H3 in NSCs (Hsieh *et al.* 2004). As shown in Fig. 4(a), NECs treated with NaBu for 24 h resulted in neurite extension and a decrease in cell number. TLC of the gangliosides in NECs (Fig. 4b and c) revealed a significant decrease in the expression of GM3 and the upper band of GD3 in the NaBu-treated NECs. There was a concomitant increase in the expression of complex ganglioside (GD1a, GD1b, and GT1b) in the NaBu-treated NECs. Furthermore, the expression of GalNAcT mRNA was increased in NECs after treatment with NaBu in a time-dependent manner (Figure S2). The expression level of ST-II was slightly decreased (Figure S2). These results suggest that histone acetylation enhanced by NaBu modifies the ganglioside expression patterns by increasing the expression of GalNAcT, but not of ST-II, in NECs.

In this study, we examined the possibility that DNA methylation and histone acetylation regulate glycosyltransferase and ganglioside expression in mouse brain during development. Consistent changes of glycosyltransferase and ganglioside expression in NaBu-treated NECs were clearly demonstrated, indicating that transcriptional activation of glycosyltransferases occurs by histone acetylation, but not by DNA methylation. Clearly, other regulatory mechanisms, such as that effected by transcription factors, are also expected to be involved in transcriptional activation. As ganglioside expression patterns and levels are associated not only with brain development but also with pathogenic mechanisms of diseases such as cancers (Hakomori 1996) and Alzheimer's disease (Ariga *et al.* 2008; Bernardo *et al.* 2009), further epigenetic studies seem to be a fertile area for future exploration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Quansheng Du, Medical College of Georgia, for his technical help. This work was supported by USPHS grants (NS11853, NS26994) and a grant from the Children's Medical Research Foundation, Chicago, IL (RKY) and a start-up fund from Medical College of Georgia (MY).

Abbreviations

C/M/W	chloroform/methanol/water
ChIP	chromatin immunoprecipitation
COBRA	combined bisulfite restriction analysis
E	embryonic day
GalNAcT	<i>N</i> -acetylgalactosaminyltransferase I (GA2/GM2/GD2/GT2-synthase)
GFAP	glial fibrillary acidic protein
NaBu	sodium butyrate
NEC	neuroepithelial cell
NSCs	neural stem cells
P	post-natal day
ST-II	sialyltransferase II (GD3-synthase)

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Fig. 1.

(a) Structure and metabolic pathways of a- and b-series gangliosides. Cer, ceramide; LacCer, lactosylceramide. The nomenclature for gangliosides is based on that of Svennerholm (1963). Quantitative PCR analysis of GalNAcT (b) and ST-II (c) mRNAs in mouse developing brains ($n = 3$). Maps of 5' regions of GalNAcT (d) and ST-II (e) genes. Restriction sites of BstUI used for COBRA assay are indicated by arrowheads. The results of COBRA assay of GalNAcT (regions a, b, and c) and ST-II genes in E12 and P10 mouse brains are represented in (f), (g), (h), and (i), respectively. Upper and lower panels show before (positive control) and after treatment with bisulfite.

Fig. 2.

ChIP assay of GalNAcT and ST-II genes in mouse E12 and P30 brains. Regions of GalNAcT (a) and ST-II (b) genes amplified by PCR after ChIP are indicated as horizontal bars. Genomic DNAs and histones (i.e., chromatin) were sonicated and precipitated by anti-acetylated histone H3 antibody. The regions of GalNAcT (c) and ST-II (d) genes (shown in a and b, respectively) in immunoprecipitated acetylated chromatin were detected by quantitative real-time PCR. The data, normalized to those of input signals, indicate histone H3 acetylation levels of the genes.

Fig. 3.

ChIP assay of GalNAcT (a), ST-II (b), and β -actin (c) genes in mouse developing brains ($n = 3$). Regions including the transcription start sites of GalNAcT and ST-II genes (Fig. 2a and b) in acetylated chromatin immunoprecipitated with anti-acetylated histone H3 antibody were detected by quantitative real-time PCR. The data were normalized to the input signals. Representative data of ChIP assay of GalNAcT (d) and ST-II (e) genes normalized by the data of β -actin (c).

Fig. 4. Effects of an histone deacetylase inhibitor, NaBu, on ganglioside expression in NECs. (a) Phase microscopic views of NECs treated with or without 1 mM of NaBu. Scale bar represents 100 μ m. (b) Gangliosides purified from NECs (2.5×10^7 cells) treated with or without 1 mM of NaBu for 72 h were separated by TLC and visualized by orcinol-sulfuric acid reagent. The positions of standard gangliosides isolated from adult mouse brains are indicated on the left. Lane 1, adult mouse brain gangliosides (major: GM1 and GD1a, GD1b, and GT1b); 2, gangliosides purified from NECs not treated with NaBu (control); 3, gangliosides purified from NECs treated with 1 mM of NaBu. (c) The intensities of ganglioside bands in (b) were quantified by Image J software ($n = 3$).