Epigenetic Regulation of a Brain-specific Glycosyltransferase N-Acetylglucosaminyltransferase-IX (GnT-IX) by Specific Chromatin Modifiers*

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Background: Epigenetic mechanisms of regulating expression of glycosyltransferase genes are largely unknown.

Results: Novel epigenetic factors were identified that specifically regulate a brain-specific glycosyltransferase gene, *GnT-IX* (*Mgat5b*).

Conclusion: *GnT-IX* is epigenetically regulated by a combination of specific chromatin modifiers (HDAC11, OGT, and TET3) and a transcriptional factor NeuroD1.

Significance: This is the first study showing that a glycosyltransferase gene is regulated by another glycosyltransferase, OGT, in combination with histone modifications.

Expression of glycosyltransferase genes is essential for glycosylation. However, the detailed mechanisms of how glycosyltransferase gene expression is regulated in a specific tissue or during disease progression are poorly understood. In particular, epigenetic studies of glycosyltransferase genes are limited, although epigenetic mechanisms, such as histone and DNA modifications, are central to establish tissue-specific gene expression. We previously found that epigenetic histone activation is essential for brain-specific expression of *N*-acetylglucosaminyltransferase-IX (GnT-IX, also designated GnT-Vb), but the mechanism of brain-specific chromatin activation around *GnT-IX* gene (*Mgat5b*) has not been clarified. To reveal the mechanisms regulating the chromatin surrounding *GnT-IX*, we have investigated the epigenetic factors that are specifically involved with the mouse *GnT-IX* locus by comparing their involvement with other glycosyltransferase loci. We first found that a histone deacetylase (HDAC) inhibitor enhanced the expression of *GnT-IX* but not of other glycosyltransferases tested. By overexpression and knockdown of a series of HDACs, we found that HDAC11 silenced *GnT-IX*. We also identified the *O*-GlcNAc transferase (OGT) and ten-eleven translocation-3 (TET3) complex as a specific chromatin activator of *GnT-IX* gene. Moreover, chromatin immunoprecipitation (ChIP) analysis in combination with OGT or TET3 knockdown showed that this OGT-TET3 complex facilitates the binding of a potent transactivator, NeuroD1, to the *GnT-IX* promoter, suggesting that epigenetic chromatin activation by the OGT-TET3 complex is a prerequisite for the efficient binding of NeuroD1.

These results reveal a new epigenetic mechanism of brain-specific *GnT-IX* expression regulated by defined chromatin modifiers, providing new insights into the tissue-specific expression of glycosyltransferases.

Glycosylation is the most abundant post-translational modification in mammals and frequently takes place in a tissue-specific manner (1). Such restricted expression of glycans is essential for maintaining various physiological functions in multicellular organisms. For instance, in the nervous system, neural specific glycans such as polysialic acid and human natural killer-1 (HNK-1) are required for higher order brain functions, including learning/memory or the formation of neural networks (2, 3). Tissue-specific glycosylation depends largely on expression of glycosyltransferases, which are responsible for glycan biosynthesis. However, it is still not clear how particular glycosyltransferase genes are expressed in specific tissues or how their expression is disturbed during disease progression.

We have been investigating the functions and gene regulation of several glycosyltransferases, especially glycan-branching enzymes acting on glycoprotein, such as *N*-acetylglucosaminyltransferases (GnT-III,[2] -V, and -IX(Vb)) or fucosyltransferase-8 (Fut8) (4). Among them, we and others recently found that GnT-IX (encoded by *Mgat5b* gene), a brain-specific glycosyltransferase, is involved in remyelination *in vivo* (5–7). GnT-IX forms a β1,6-branched structure on *O*-mannose glycans (8, 9), and the branched *O*-mannose glycans in activated astrocytes inhibit remyelination after myelin injury (5), indicating that the brain-specific expression of GnT-IX contributes to its function in brain. We also examined the regulatory mecha-

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nism of brain-specific GnT-IX expression and found that epigenetic histone activation but not DNA hypomethylation in the *GnT-IX* promoter region is pivotal for its neural specific expression (10). Two transcription factors, NeuroD1 and CTCF (CCCTC-binding factor), were also identified as transactivators for the *GnT-IX* promoter, but neural specific chromatin activation is likely to be hierarchically dominant because the chromatin activation state is critical for the binding of these transcription factors to the *GnT-IX* promoter (10). However, it remains to be clarified how chromatin at the *GnT-IX* gene is activated or repressed in a tissue- and gene-specific manner.

Epigenetic marks, such as histone modification and DNA methylation, are central to the tissue- and cell type-specific gene program (11). Covalent histone modifications (methylation, acetylation, *O*-GlcNAcylation, etc.) are dynamically regulated by an interplay of adding and removing enzyme pairs, which can influence gene transcription either through direct change of chromatin structure or through binding of effector molecules. For instance, acetylation of histone tails at specific lysine residues is catalyzed by histone acetylases (HATs), whereas acetylation is removed by histone deacetylases (HDACs). These actions are highly correlated with gene activation and silencing, respectively (12). Conversely, methylation of cytosine (5mC) in a gene promoter, which correlates strongly with transcriptional silencing, had been considered to be a static modification (13). However, the recent discovery that ten-eleven translocation (TET) family enzymes (TET1–3) can convert 5mC to 5-hydroxymethyl cytosine (5hmC) led to the hypothesis that TET-mediated hydroxylation of 5mC could be part of a DNA demethylation pathway (14–16). Although TET1 and TET2 were reported to play an important role in ES cell lineage specification (17, 18), depletion of TET3 causes impairment of neural development in *Xenopus* (19), suggesting that TET proteins, especially TET3, might be involved in tissue-specific gene transcription. Moreover, recent studies have revealed that TET proteins bind tightly with the cytosolic/nuclear glycosyl enzyme, *O*-GlcNAc transferase (OGT), at transcriptional start sites to regulate gene transcription (20–22). *O*-GlcNAcylation by OGT on histones or histone-modifying enzymes was recently added to the growing list of epigenetic modifications (23); therefore, it has been revealed that these two emerging epigenetic factors (TET and OGT) can functionally cross-talk. Few studies have linked these epigenetic modifications to glycan expression (24); however, recent works in which an epigenetic inhibitor had a great impact on the cellular *N*-glycome or composition of neural glycolipid (25–27) have suggested that many glycan-related genes can be regulated by epigenetic mechanisms.

In this study, we investigated which chromatin modifiers are involved in epigenetic regulation of the mouse *GnT-IX* gene. To this end, we focused on enzymes involved in histone acetylation and *O*-GlcNAcylation. We identified one HDAC, HDAC11, and the OGT-TET3 complex as regulators of *GnT-IX* transcription. Meanwhile, the gene expression of other glycosyltransferases (*GnT-III* and -*V* and *Fut8*) was not affected by HDAC11 or the OGT-TET3 complex. Moreover, we found that recruitment of OGT-TET3 facilitates NeuroD1-dependent up-regulation of *GnT-IX* expression. These results suggest that a set of specific epigenetic factors (TET3-OGT and HDAC11) and a

transcription factor (NeuroD1), which are highly expressed in brain, play key roles in the brain-restricted expression of *GnT-IX*.

EXPERIMENTAL PROCEDURES

Materials—The following antibodies were used: anti-H3K9Ac (07-352, Millipore), anti-acetyl H3 (06-599, Millipore), anti-actin (AC-40, Sigma), anti-FLAG (M2, Sigma), anti-OGT (sc-32921, Santa Cruz Biotechnology), anti-*O*-GlcNAc (RL2, Thermo Scientific), anti-NeuroD1 (4373, Cell Signaling Technology), anti-histone H3 (4620, Cell Signaling Technology), and normal rabbit IgG (2729, Cell Signaling Technology). siRNAs were purchased from Qiagen: control siRNA, 1027280; mouse HDAC1, SI00173523; mouse HDAC2, SI00173544; mouse HDAC3, SI00192787; mouse HDAC11, SI01063867; mouse TET1, SI00962983; mouse TET2, SI04399878; mouse TET3, SI00968555; and mouse OGT, SI00235172. Primer-probe sets for mRNA quantification were purchased from Life Technologies: control ribosomal RNA, 4308329; GnT-III, Mm00447798_s1; GnT-V, Mm00455036_m1; GnT-IX, Mm00556891_m1; Fut8, Mm00489789_m1; HDAC1, Mm02391771_g1; HDAC2, Mm00515108_m1; HDAC3, Mm00515916_m1; HDAC11, Mm01183513_m1; and TET3, Mm00805756_m1. Trichostatin A was kindly provided by Dr. Minoru Yoshida (Chemical Genetics Laboratory, RIKEN).

Cell Culture and Transfection—Neuro2A cells were cultured in DMEM supplemented with 10% fetal bovine serum. For plasmid transfection, cells plated on a 10-cm (or 6-cm) dish were transfected with 4 μg (or 1 μg) of plasmid using 10 μl (or 2.5 μl) of Lipofectamine 2000 (Life Technologies). For siRNA transfection, cells on a 10-cm (or 6-cm) dish were transfected with 200 pmol (or 80 pmol) of siRNA using 20 μl (or 8 μl) of Lipofectamine 2000. Stable HDAC transfectants were selected in the presence of antibiotics (750 μg/ml G418 for HDAC2 and 7.5 μg/m blasticidin for the other transfectants). In some experiments, cells were treated with trichostatin A (TSA) at 0.1 μM for 24 h.

Isolation of Primary Neurons and an Oligodendrocyte (OL)-rich Fraction—All the animal experiments were approved by the Animal Experiment Committee of RIKEN. Isolation and culture of primary neurons from embryonic C57BL/6 mouse brains were carried out as described previously (5) with slight modification. After plating neurons, Cytosine arabinofranoside (5 μM at final concentration, Sigma) was added to the medium at 2 days *in vitro* to remove proliferating cells. For isolation of the OL-rich fraction, brains from C57BL/6 mice (8–10 weeks old) were collected and minced. The tissue pieces were incubated in 10 ml of PBS containing 90 units of papain, 2 mg of L-cysteine, and 0.01% DNase at 37 °C for 40 min. The supernatant was passed through a 70-μm cell strainer and mixed with 2 ml of FBS. The precipitate was resuspended with 8 ml of PBS, and the resultant supernatant was passed through a 70-μm cell strainer. The passed solution was mixed with the first passage. This procedure was repeated three more times. The collected solution was centrifuged at 500 $\times$ *g* for 10 min, and the pellet was resuspended with 30 ml of PBS containing 30% (v/v) Percoll. After centrifugation at 20,000 $\times$ *g* for 30 min, the layer between the white and red layers was collected. 2 volumes of PBS were added, and the mixture was centrifuged at 700 $\times$ *g* for 12 min. The pellet was resuspended with 15 ml of PBS and

centrifuged at $500 \times g$ for 8 min. This wash step was repeated two more times, with the final pellet representing the OL-rich fraction.

RNA Extraction, Reverse Transcription, and Real-time PCR— Total RNA from cultured cells or mouse tissues was extracted using TRI Reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol. 1 μg of total RNA was reverse-transcribed using Superscript III (Life Technologies). For real-time PCR, cDNA was mixed with TaqMan Universal PCR master mix (Life Technologies) and amplified using an ABI PRISM 7900HT. The levels of mRNA were normalized to the corresponding ribosomal RNA levels.

*Chromatin Immunoprecipitation (ChIP)—*ChIP assays were carried out using a SimpleChIP enzymatic chromatin IP kit (Cell Signaling Technology) according to the manufacturer's protocol. Approximately 2×10^7 cells were used as starting material. 2% of the chromatin was reserved as input sample. After immunoprecipitation, real-time PCR was performed to quantify the amounts of the precipitated DNA. The data are shown as values relative to the corresponding control IgG sample (indicated as *Fold enrichment* in figures) or as values relative to the corresponding 2% input sample (indicated as *% of input* in figures). For ChIP assays of HDAC11-FLAG, anti-FLAG M2-agarose affinity gel (Sigma) was used. For tissue ChIP, adult male C57BL/6 mice (10–15 weeks old) were deeply anesthetized and then sacrificed. Organs were collected and immediately minced on ice. Tissue pieces (100 mg) were incubated in 1% formaldehyde, PBS for 10 min at room temperature. Glycine was then added (125 m$_M$ final concentration) followed by incubation for 5 min. After washing twice with PBS, the tissues were homogenized in 4 ml of buffer A (supplied in the above SimpleChIP kit) using a Potter homogenizer. Subsequent procedures were carried out as for cultured cells. Primers and probes for real-time PCR are listed in Table 1.

*Plasmids—*The construction of a plasmid encoding *Neurod1* was described previously (10). *Hdac* cDNAs except *Hdac2* were cloned by PCR using reverse-transcribed total RNA from mouse tissue (*Hdac1*, *-3*, *-4*, *-5*, and *-6* from testis; *Hdac7*, *-10*, and *-11* from skeletal muscle; *Hdac8* and *-9* from brain). The amplified fragments were then ligated to pCR4Blunt-TOPO (Life Technologies). All the cDNAs except *Hdac2* were subcloned into pcDNA6/Myc-His A (Life Technologies). *Hdac1* cDNA was ligated into the EcoRI site. The other *Hdac* cDNAs in pCR4Blunt-TOPO were released with NotI-SpeI and then ligated to the NotI-XbaI sites of pcDNA6/Myc-His A. *Tet3* cDNA was amplified by PCR using reverse-transcribed total RNA from mouse brain. The amplified fragment was digested and directly ligated to pcDNA6/Myc-His A using EcoRI-EcoRV. For HDAC11-FLAG and TET3-FLAG constructs, PCR-amplified cDNA fragments that lacked their respective stop codons were digested and ligated into p3×FLAG-CMV14 (Sigma) using EcoRI-BamHI and EcoRI-XbaI sites, respectively. pcDNA3/mouse HDAC2 was kindly provided by Dr. Akihiro Ito (Chemical Genetics Laboratory, RIKEN). Primers used are listed in Table 2.

*Western Blot—*Proteins were separated by 4–20% gradient SDS-PAGE using the Laemmli buffer system and then transferred to PVDF or nitrocellulose membranes. After blocking with 5% nonfat dried milk in TBS containing 0.05% Tween 20, the membranes were incubated with primary antibodies followed by HRP-conjugated secondary antibodies. Proteins were detected with SuperSignal West Dura extended duration substrate (Thermo Scientific) using an ImageQuant LAS-4000mini (GE Healthcare).

*Immunoprecipitation (IP)—*Cells were lysed and sonicated in buffer containing 20 m$_M$ Tris-HCl, pH 7.4, 0.3 $_M$ NaCl, 0.5% Nonidet P-40, and a protease inhibitor cocktail (Roche Applied Science). After ultracentrifugation at $100,000 \times g$ for 15 min, the resultant supernatant was incubated with antibody for 30 min on ice. Protein G-Sepharose 4 fast flow (GE Healthcare) was then added, and the mixture was incubated for 2 h at 4 °C with gentle rotation. For IP of FLAG-tagged TET3, anti-FLAG M2-agarose affinity gel (Sigma) was used. After washing with excess volumes of TBS containing 0.1% Nonidet P-40, the

TABLE 1
Primers and probes for ChIP analysis
FAM, 6-carboxyfluorescein; MGB, minor groove binder.

Primer (probe) name	Sequence
GnT-III primers	CAAGCACTTTGGCCAGCTCC and GCGCCTCTTTCGTGTGTATG
GnT-III probe	5'–FAM–ACACTCGCGCGTCCGATTGC–MGB–3'
GnT-V primers	GGGAATTTGCGGAACGTCCG and TCCCGGATCGACCTACCCAA
GnT-V probe	5'–FAM–TCCCTTTGCCTTGGAGAGC–MGB–3'
GnT-IX primers	TAGGAAGTTGCATGGTCCAG and CCGGGCTCCCGCGCGGCGCT
GnT-IX probe	5'–FAM–TCAGAGGGAACAATGCGGCG–MGB–3'
Fut8 primers	TCACCCGGCTGAGTCCTCA and GAGTGCGAACTGGCCAATCA
Fut8 probe	5'–FAM–TCGGCACGGGAAGCTCATCT–MGB–3'

TABLE 2
Primers for plasmid construction
Underlined sequences indicate restriction enzyme sites.

| Primer name	Sequence
HDAC1	GAGCAAGATGGCGCAGACTCAGGG and AGACCTTGCTCAGGCCAACTTGACC
HDAC3	AGCACCATGGCCAAGACCGTGGCGT and GGGGACACAGCATCCATGCTGCTCT
HDAC4	AAGGCACTGCACGCTGCTAGCAATGA and GCTTCGGGCTACAGTGGTGGTTCCT
HDAC5	GAGCCGGCATGAACTCTCCCAACGA and GGGATGGGGGCCAGGGTGTCACAG
HDAC6	TGAGGCTCTTCAGCCATGACCTCCA and GGGATTTAGTGTGAGTGGGGCATGT
HDAC7	GGCTCTCAGCCCCAACATGGAC and GAGGAGAGCCACATTTGAAGCGCGA
HDAC8	GTTGGAAGTGGGAGATGGAGATGCC and GCTGCCCAGCTCTGGGAACCTGATC
HDAC9	GGGAGCAGCTCTTGGCTCAGCAAAG and TCAGAGACATAAGCTGATAGGAAATAA
HDAC10	GTGACCACGGTGTAGCTATGGGCAC and TTATTATGGTGGAGGAGCAGCCACCT
HDAC11	TGCGGGATGCCTCACGCAAC and TAGGTAGCCGTCAAGGCACAGCACA
TET3	ATG<u>GAATTC</u>TTCCAGGTCAGATGGACTCAGGGC and GCT<u>GATATC</u>TGGCACCTAGATCCAGCGGCTGTA
HDAC11-FLAG	AGG<u>GAATTC</u>TGCGGGATGCCTCACGCAAC and TA<u>GGGATCC</u>AGGCACAGCACAGGAAAGCA
TET3-FLAG	ATG<u>GAATTC</u>TTCCAGGTCAGATGGACTCAGGGC and TGG<u>TCTAGA</u>GATCCAGCGGCTGTAGGGGC
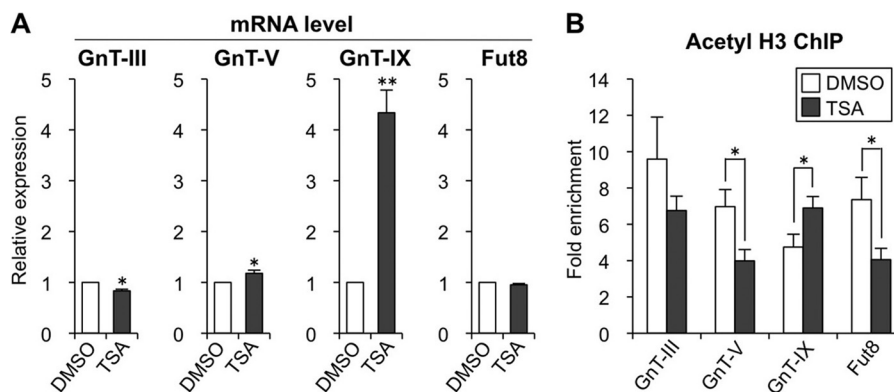
FIGURE 1. **Histone acetylation selectively up-regulates *GnT-IX*.** *A*, Neuro2A cells were treated with DMSO (vehicle), 0.1 μM TSA for 24 h, and the mRNA levels of *GnT-III*, *-V*, and *-IX* and *Fut8* were quantified and normalized to those of rRNA. The mRNA/rRNA levels of TSA-treated samples relative to those of DMSO-treated samples are shown ($n = 3$). *B*, Neuro2A cells were treated with DMSO (vehicle), 0.1 μM TSA for 24 h, and the levels of H3Ac around the transcriptional start sites of *GnT-III*, *-V*, and *-IX* and *Fut8* genes were analyzed by ChIP assay ($n = 4$). The precipitated DNA was analyzed by real-time PCR, and the amounts of DNA relative to those with control IgG are shown as -fold enrichment. All graphs show means $\pm$ S.E., *, $p < 0.05$, **, $p < 0.01$, Student's *t* test.

bound proteins were eluted by boiling with Laemmli sample buffer.

RESULTS

Histone Acetylation Selectively Up-regulates GnT-IX—We previously reported that histone acetylation is a critical factor for *GnT-IX* expression (10). To investigate whether glycosyltransferase genes are generally regulated by epigenetic histone acetylation, we first treated Neuro2A cells with a global HDAC inhibitor, TSA, that forcibly up-regulates the level of histone acetylation (28). We analyzed the expression of four glycosyltransferase genes that we divided into two groups, tissue-specific genes with the highest expression in brain (*GnT-III* and *GnT-IX*) and ubiquitously expressed genes (*GnT-V* and *Fut8*) (29, 30), respectively. As a result, only the expression of *GnT-IX* was drastically up-regulated by TSA treatment (Fig. 1*A*). To check the levels of histone acetylation around the transcriptional start sites of these genes, ChIP analysis was performed with anti-acetylated histone H3 (H3Ac) antibody. Consistent with the mRNA levels, only the *GnT-IX* gene had increased levels of H3Ac in TSA-treated cells (Fig. 1*B*). Although TSA is a pan-HDAC inhibitor, its effect on induction of histone acetylation and transcription is not global but is restricted to certain genes (31, 32), which is consistent with the varying effects of TSA on the different glycosyltransferase genes (Fig. 1*B*). The specific up-regulation of *GnT-IX* by TSA shows that *GnT-IX* can be selectively silenced by HDAC(s).

HDAC11 Silences the GnT-IX Gene—Next, we explored how *GnT-IX* gene is epigenetically silenced by HDAC(s). In mammalian cells, 11 HDACs (HDAC1–HDAC11) are expressed (33), but the precise function of each HDAC remains to be elucidated. Moreover, activity of all 11 HDACs is potentially inhibited by TSA. To examine which HDAC is involved in silencing *GnT-IX*, each HDAC enzyme was overexpressed in Neuro2A cells, and the mRNA levels of the glycosyltransferases were quantified. *GnT-IX* expression was significantly silenced by overexpression of several HDACs, such as HDAC1, -2, -3, and -11 (Fig. 2*A*), whereas other glycosyltransferases were moderately or slightly down-regulated, suggesting that *GnT-IX* gene is highly susceptible to epigenetic silencing by particular HDACs.

To further examine whether these HDACs are involved in *GnT-IX* silencing, HDAC1, -2, -3, and -11 were knocked down by siRNA (Fig. 2, *B* and *C*). Depletion of HDAC11 resulted in the highest up-regulation of *GnT-IX* gene, whereas the ubiquitously expressed *Fut8* gene was not up-regulated by knockdown of these HDACs, suggesting that HDAC11 might be a specific factor for epigenetic down-regulation of *GnT-IX* expression. ChIP analysis showed that knockdown of HDAC11 caused increased histone acetylation in the *GnT-IX* gene but not in the *Fut8* gene (Fig. 2*D*), which is consistent with the up-regulation of GnT-IX mRNA by HDAC11 knockdown, suggesting that HDAC11 deacetylates histones surrounding the *GnT-IX* gene. In addition, ChIP analysis of exogenously expressed HDAC11-FLAG demonstrated that HDAC11 is highly recruited to the *GnT-IX* gene as compared with the *Fut8* gene locus (Fig. 2*E*).

Hdac11 is known to be highly expressed in brain, kidney, heart, and muscle (34), and in brain HDAC11 was reported to regulate oligodendrocyte-specific gene expression (35). Therefore, we assumed that HDAC11 would be involved in silencing of *GnT-IX* in a specific brain cell type as well as in non-brain tissues. We, therefore, isolated primary neurons and an OL-rich fraction from mouse brain. Quantitative PCR results showed that GnT-IX mRNA is highly expressed in neurons as compared with OLs, whereas HDAC11 shows a higher level of expression in OLs as compared with neurons (Fig. 2*F*). This negative correlation suggests that HDAC11 may be implicated in cell type-specific silencing of *GnT-IX* in the brain. Collectively, these data suggest that among HDAC family members, HDAC11 may be a specific suppressor for GnT-IX but not for other glycosyltransferases.

The TET3-OGT Complex Is an Epigenetic Activator Specific for GnT-IX Gene—Next, we investigated the chromatin activation mechanism of *GnT-IX* gene. Recently, a novel chromatin activation mechanism was reported in which two epigenetic factors, OGT and TET, form a functional complex (20–22). TET molecule can recruit OGT to chromatin, and this in turn results in *O*-GlcNAcylation of surrounding chromatin molecules, including histones and host cell factor-1 (HCF-1, a com-
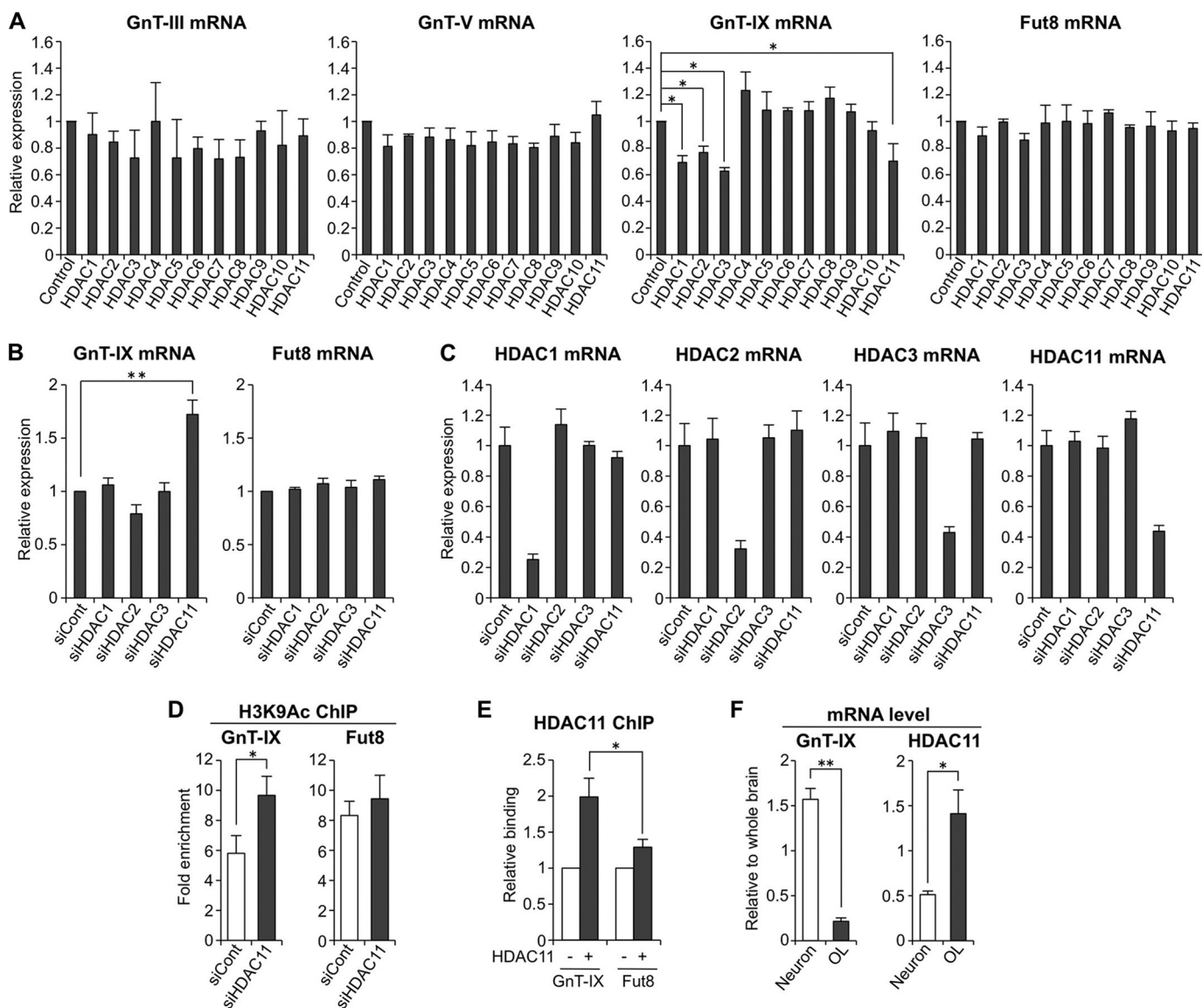
FIGURE 2. **HDAC11 silences *GnT-IX* gene.** *A*, total RNAs were extracted from Neuro2A cells (control) or transfectants stably expressing HDAC1–11. The mRNA levels of *GnT-III*, *-V*, and *-IX* and *Fut8* were quantified and normalized to those of rRNA. The mRNA/rRNA levels of HDAC-expressing samples relative to those of control samples are shown ($n = 3$, *, $p < 0.05$, Tukey-Kramer's test). *B*, Neuro2A cells were treated with control siRNA (*siCont*), HDAC1, HDAC2, HDAC3, or HDAC11 siRNA for 24 h. Total RNAs were extracted, and the mRNA levels of *GnT-IX* and *Fut8* were quantified and normalized to those of rRNA. The mRNA/rRNA levels of HDAC knockdown samples relative to those of control samples are shown ($n = 3$, **, $p < 0.01$, Tukey-Kramer's test). *C*, Neuro2A cells were treated with control siRNA, HDAC1, HDAC2, HDAC3, or HDAC11 siRNA for 24 h. Total RNAs were extracted, and the mRNA levels of *Hdac1*, *Hdac2*, *Hdac3*, and *Hdac11* were quantified and normalized to those of rRNA. The mRNA/rRNA levels of HDAC knockdown samples relative to those of control samples are shown ($n = 3$). *D*, Neuro2A cells were treated with control siRNA or HDAC11 siRNA for 24 h, and then the levels of H3K9Ac around the transcriptional start sites of *GnT-IX* and *Fut8* genes were analyzed by ChIP assays ($n = 3$, *, $p < 0.05$, Student's *t* test). *E*, HDAC11 tagged with 3×FLAG at its C terminus was overexpressed in Neuro2A cells. Recruitment of HDAC11-FLAG to the genomic region around the transcriptional start sites of *GnT-IX* and *Fut8* genes was analyzed by ChIP assays with anti-FLAG antibody (M2)-conjugated beads. The precipitated DNA was analyzed by real-time PCR, and the amount of DNA from the transfectant (+) relative to that from mock-treated cells (−) is shown ($n = 3$, *, $p < 0.05$, Student's *t* test). *F*, mRNA levels of *GnT-IX* and *Hdac11* were quantified and normalized to those of rRNA in mouse primary neurons (6 days *in vitro*) or in the oligodendrocyte-rich fraction (*OL*). The mRNA/rRNA levels are shown as relative values to those in whole brain from 20-week-old male mice ($n = 3$, *, $p < 0.05$, **, $p < 0.01$, Student's *t* test). All graphs show means ± S.E.

ponent of the H3K4 methyltransferase complex), leading to gene activation (20–22). Considering the fact that OGT is highly expressed in brain as compared with other tissues (36, 37) and that TET-generated 5hmc is most abundant in brain (38–40), we assumed that this OGT-TET machinery could be involved in chromatin activation of *GnT-IX* gene in brain. To see whether the OGT-TET complex is actually involved in activation of the glycosyltransferase genes, TET family proteins (TET1–3) were knocked down in Neuro2A cells by siRNA. As a result, we found that mRNA levels of *GnT-V* and *-IX* but not of

GnT-III and *Fut8* were down-regulated by TET3 knockdown (Fig. 3).

Next, we checked to see whether the OGT-TET3 complex activates *GnT-IX* transcription. IP experiments clearly showed that exogenously expressed TET3-FLAG can co-precipitate endogenous OGT and *vice versa* (Fig. 4*A*), which is consistent with previous studies using other cell systems (20, 21, 41). If OGT interacts with TET3 for its recruitment to chromatin and gene activation, TET3 depletion would dissociate OGT from chromatin particularly from *GnT-V* and *GnT-IX* genes. ChIP
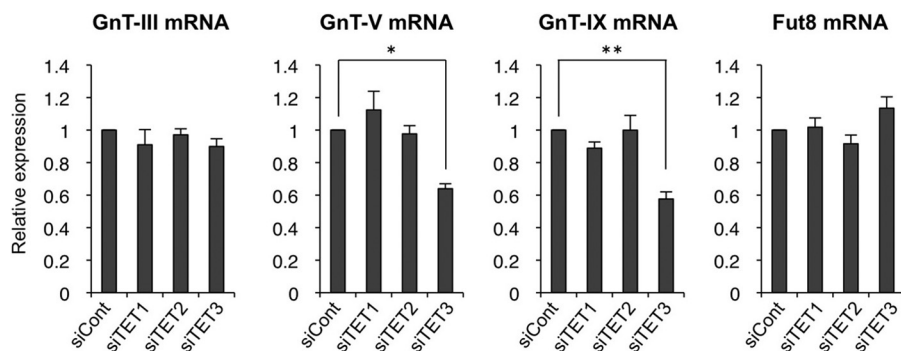
FIGURE 3. **TET3 is involved in *GnT-V* and *-IX* expression.** Neuro2A cells were treated with control siRNA (*siCont*), TET1, TET2, or TET3 siRNA for 24 h. Total RNAs were extracted, and the mRNA levels of *GnT-III*, *-V*, and *-IX* and *Fut8* were quantified and normalized to those of rRNA. The mRNA/rRNA levels of TET knockdown samples relative to those of control samples are shown. All graphs show means ± S.E. ($n = 3$, *, $p < 0.05$, **, $p < 0.01$, Tukey-Kramer's test).
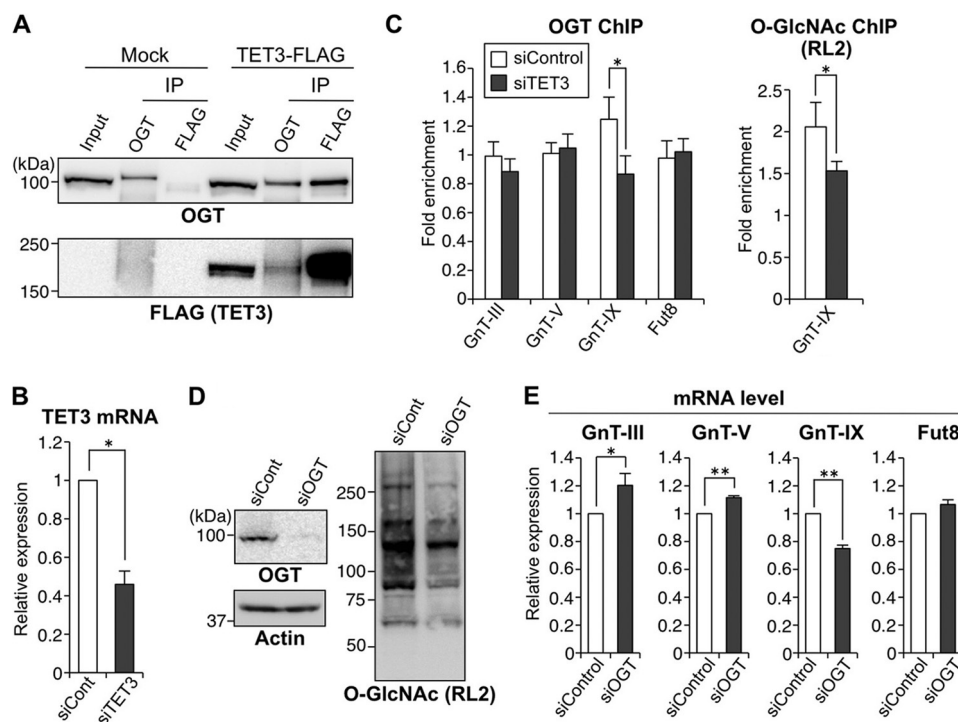

FIGURE 4. **The TET3-OGT complex is an epigenetic activator specific for *GnT-IX* gene.** *A*, Neuro2A cells were transfected with the TET3-3×FLAG expression vector or the empty vector (*Mock*). The cell lysates (*Input*) were subjected to IP with anti-OGT or anti-FLAG antibody and then Western blotted with an anti-OGT or anti-FLAG antibody. *B*, Neuro2A cells were treated with control siRNA (*siCont*) or TET3 siRNA for 24 h. Total RNAs were extracted, and the mRNA levels of TET3 were quantified and normalized to those of rRNA. The mRNA/rRNA level of TET3 knockdown sample relative to that of control sample is shown ($n = 3$, *, $p < 0.05$, Student's *t* test). *C*, Neuro2A cells were treated with control siRNA or TET3 siRNA for 24 h, and then ChIP assays were performed with anti-OGT or anti-*O*-GlcNAc (RL2) antibody. Genomic regions around the transcriptional start sites of *GnT-III*, *-V*, and *-IX* and *Fut8* genes were analyzed by real-time PCR. The amounts of DNA relative to those with control IgG are shown (*left*, $n = 5$; *right*, $n = 3$, *, $p < 0.05$, Student's *t* test). *D*, Neuro2A cells were treated with control siRNA or OGT siRNA, and the cell lysates were Western blotted with an anti-OGT, anti-actin, or anti-*O*-GlcNAc antibody. *E*, Neuro2A cells were treated with control siRNA or OGT siRNA for 24 h. Total RNAs were extracted, and the mRNA levels of *GnT-III*, *-V*, and *-IX* and *Fut8* were quantified and normalized to those of rRNA. The mRNA/rRNA levels of OGT knockdown samples relative to those of control samples are shown ($n = 4$, *, $p < 0.05$, **, $p < 0.01$, Student's *t* test). All graphs show means ± S.E.

assays with anti-OGT antibody showed that OGT was indeed dissociated from *GnT-IX* gene chromatin by TET3 depletion, together with a reduced level of *O*-GlcNAc (Fig. 4, *B* and *C*), suggesting that OGT and TET3 molecules act as a complex on the *GnT-IX* gene for epigenetic activation. Surprisingly, however, such dissociation of OGT was not observed for *GnT-V* gene, suggesting that TET3 regulates *GnT-V* expression independently of OGT or in an indirect manner. Consistent with this possibility, knockdown of OGT by siRNA revealed that only *GnT-IX* but not other glycosyltransferases was down-regulated (Fig. 4, *D* and *E*). Together, these results indicated that

the OGT-TET3 complex may be specifically required for expression of *GnT-IX* but not for other glycosyltransferases tested.

The OGT-TET3 Complex Recruits a Brain-specific Transcription Factor, NeuroD1, to the GnT-IX Promoter—We previously identified NeuroD1, a neuronal basic helix-loop-helix transcription factor (42), as a strong transactivator for the *GnT-IX* promoter (10). Overexpression of NeuroD1 markedly up-regulates *GnT-IX* transcription in Neuro2A cells, whereas NeuroD1 fails to activate *GnT-IX* in a non-neural cell line (3T3-L1), in which the *GnT-IX* chromatin is highly
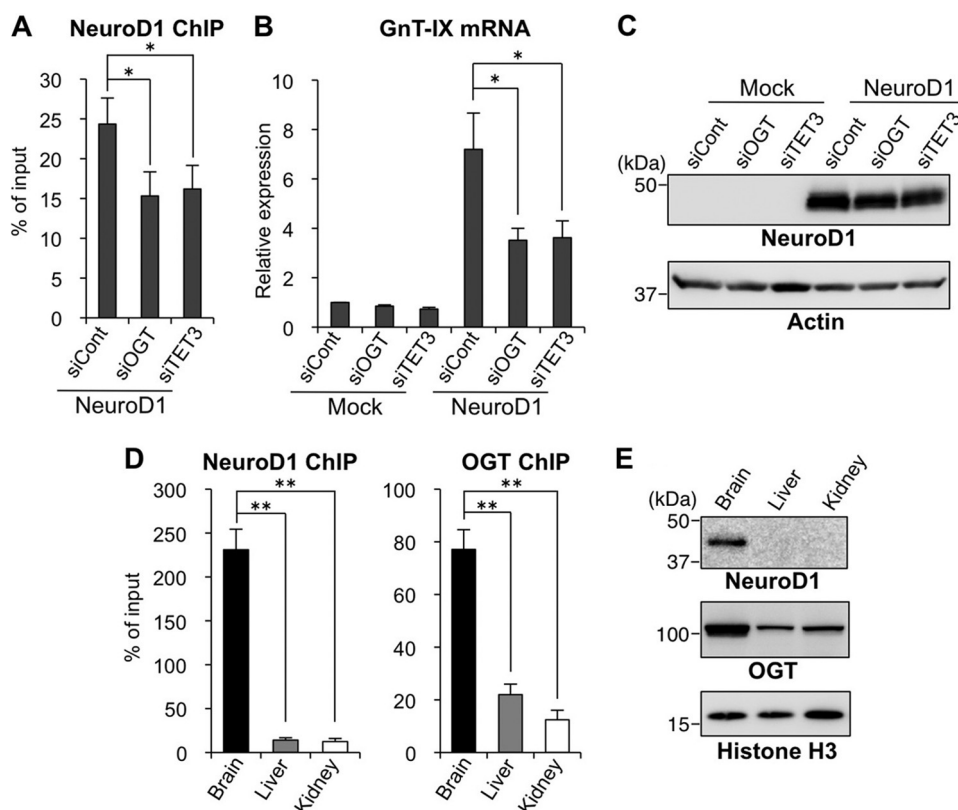
FIGURE 5. **Transactivation of *GnT-IX* by NeuroD1 is promoted by the OGT-TET3 complex.** *A*, Neuro2A cells were treated with control siRNA (*siCont*), OGT siRNA, or TET3 siRNA for 24 h and then replated on new dishes. After 24 h, the cells were transfected with the NeuroD1 expression vector followed by a further 24-h culture. Then, ChIP assays were performed with an anti-NeuroD1 antibody. A genomic region around the transcriptional start site of *GnT-IX* gene, including the NeuroD1 binding site, was analyzed by real-time PCR. The amounts of DNA relative to those of 2% input samples are shown ($n = 3$, *, $p < 0.05$, Tukey-Kramer's test). *B* and *C*, Neuro2A cells were transfected with siRNA and expression plasmid as in the case of *A*. Total RNAs were extracted, and the mRNA levels of GnT-IX were quantified and normalized to those of rRNA. The mRNA/rRNA levels relative to those of control siRNA-treated mock sample are shown ($n = 3$, *, $p < 0.05$, Tukey-Kramer's test) (*B*). Cellular proteins extracted after 24 h of NeuroD1-transfection were Western blotted with an anti-NeuroD1 or anti-actin antibody (*C*). *D*, chromatin was prepared from brain, liver, or kidney of adult mice, and ChIP assays were performed with anti-NeuroD1 or anti-OGT antibody. A genomic region around the transcriptional start site of *GnT-IX* gene, including the NeuroD1 binding site, was analyzed by real-time PCR. The amounts of DNA relative to those of 2% input samples are shown ($n = 3$, **, $p < 0.01$, Tukey-Kramer's test). *E*, homogenates of brain, liver, or kidney from adult mice were Western blotted with an anti-NeuroD1, anti-OGT, or anti-histone H3 antibody. All graphs show means ± S.E.

repressed (10). This suggests that epigenetic chromatin activation is a prerequisite for NeuroD1 binding to the *GnT-IX* promoter. Therefore, we expected that chromatin activation of *GnT-IX* by OGT-TET3 would enhance NeuroD1 recruitment. Actually, binding of NeuroD1 to the *GnT-IX* promoter and subsequent activation of *GnT-IX* transcription were both reduced by depletion of OGT or TET3 (Fig. 5, *A–C*). These results suggest that the OGT-TET3 complex is required for the efficient binding of NeuroD1 to the *GnT-IX* promoter to drive it. We also examined *in vivo* binding of NeuroD1 and OGT to the *GnT-IX* promoter. Although we failed to detect the binding of TET3 by ChIP assays with commercially available antibodies, we found that binding of endogenous NeuroD1 and OGT to the *GnT-IX* promoter was stronger in brain than in other tissues (Fig. 5*D*), which is consistent with the highest *GnT-IX* expression level being in the brain. This robust binding of NeuroD1 and OGT may be due to their high expression in brain (Fig. 5*E*). These results suggest that the OGT-TET3-dependent binding of NeuroD1 to the *GnT-IX* promoter may also be required for the brain-specific transcription of GnT-IX gene *in vivo*.

DISCUSSION

We have identified for the first time epigenetic factors involved in the regulation of *GnT-IX* gene, an epigenetic suppressor HDAC11, and an activator, the OGT-TET3 complex. The other glycosyltransferases tested were not regulated by these factors, which suggests that these chromatin modifiers selectively act on *GnT-IX* among the glycosyltransferase genes. In addition, our overexpression and knockdown data showed that the other HDACs (HDAC1–10) and TETs (TET1 and TET2) are not or are less involved in *GnT-IX* regulation, showing the specificity of HDAC11 and OGT-TET3 molecules for *GnT-IX* gene. Previous studies have revealed that TET proteins are essential for OGT recruitment to a CpG island in a gene promoter, leading to efficient *O*-GlcNAcylation of chromatin proteins (20–22, 41). Our data indicate that the OGT-TET3 complex is a prerequisite for the efficient binding of the transactivator, NeuroD1, to the *GnT-IX* promoter to drive it. Taking an overall view of our data, we present a stepwise model for the epigenetic regulation of *GnT-IX* gene (Fig. 6).

Recently, we reported that the branched *O*-mannose glycans produced by GnT-IX inhibit the development of OLs after

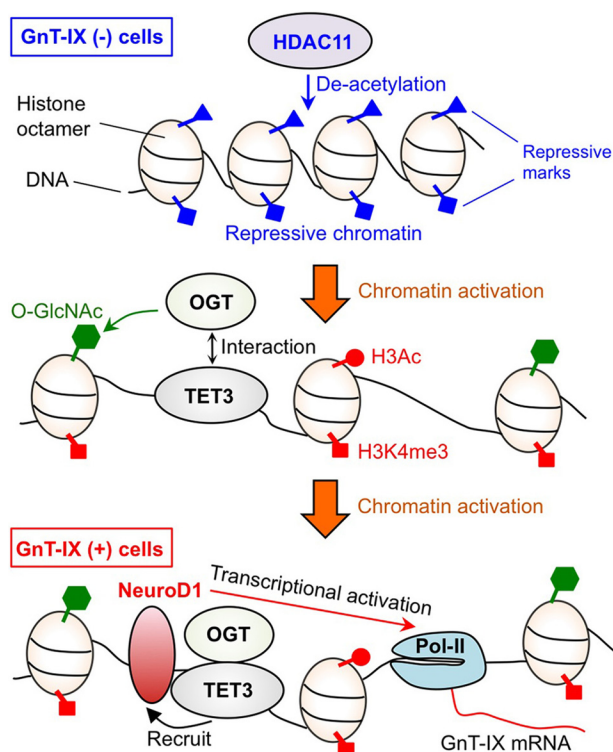## Regulation of GnT-IX Gene by Specific Chromatin Modifiers

FIGURE 6. **Schematic model for the epigenetic regulation of *GnT-IX*.** In non-GnT-IX-expressing cells, chromatin around the *GnT-IX* gene is repressed by chromatin modifiers, including HDAC11. When *GnT-IX* chromatin is activated, TET3 recruits OGT. This OGT-TET3 complex further facilitates the recruitment of NeuroD1 to GnT-IX promoter, which in turn activates *GnT-IX* transcription. *Pol-II*, RNA polymerase II.

in vivo induced myelin injury through enhanced astrocyte activation (5). Meanwhile, it was reported that HDAC11 knockdown or pharmacological suppression of HDAC activity also caused impaired development of oligodendrocytes (35, 43). This suggests that HDAC11 regulates OL development through downregulation of its target genes. The expression of *GnT-IX* in OLs *in vivo* is presumed to be silenced by HDAC11 due to the high expression of HDAC11 in these cells (Fig. 2*F*); therefore, cell lineage-specific expression of GnT-IX might be one of the downstream target genes for HDAC11 during regulation of OL development in the brain. Although the function of HDAC11 still remains largely unclear, it would be interesting to identify the direct target genes of HDAC11 in the brain. Considering that HDACs lack intrinsic DNA binding activity (33), the enrichment of HDAC11 at the *GnT-IX* gene (Fig. 2*E*) is probably maintained by the formation of a complex with another sequence-specific DNA-binding protein(s). Our next goal will be to clarify how HDAC11 acts specifically on the *GnT-IX* locus and not on other glycosyltransferases.

O-GlcNAcylation by OGT is an emerging epigenetic mark because of the recent findings that *O*-GlcNAc on histone and histone modifiers can regulate gene transcription (23, 44–46). Our present study showed that OGT is involved in epigenetic activation of *GnT-IX* gene, probably through interaction with TET3 on chromatin. Although *GnT-IX* gene is likely to be activated by OGT-TET3, probably through promoted *O*-GlcNAcylation of histone or HCF-1 on the gene promoter, as is generally seen in other genes (20–22), this complex might also facilitate

O-GlcNAcylation of NeuroD1 for *GnT-IX* activation because NeuroD1 was also reported to be modified by OGT (47). *O*-GlcNAc modification exerts a wide variety of functions depending on the OGT substrate (48). In our case, OGT, a sugar transferase, positively regulates the transcription of another sugar transferase, GnT-IX, which is connected by epigenetics. Meanwhile, OGT is considered as a master sensor for nutrients as it uses UDP-GlcNAc as the high energy donor substrate, which is the end point of the hexosamine biosynthetic pathway (48). The biosynthesis of UDP-GlcNAc integrates the metabolic pathways of glucose, an amino acid (glutamine), a fatty acid (acetyl-CoA), and a nucleotide (UTP). Therefore, nutrient flux to the cells would affect the levels of both cellular UDP-GlcNAc and cellular *O*-GlcNAc modification. Notably, other glycosyltransferases, including GnT-IX, also use UDP-GlcNAc as the donor substrate. Therefore, nutrient flux and the subsequent metabolic pathway could regulate GnT-IX activity by both donor-substrate use and OGT-mediated gene transcription. We recently proposed that cellular glycosylation forms a functional cycle called the "glycan cycle" comprising nutrient flux, biosynthesis of sugar nucleotide, sugar transfer, biological action, degradation, and recycling (49). We still do not fully understand this cycle at the molecular level, but we believe that the present study provides new insights into how epigenetic regulation of glycogenes is integrated into the glycan cycle.

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