Functional roles of ST8SIA3-mediated sialylation of striatal dopamine D2 and adenosine A2A receptors

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Abstract
Sialic acids are typically added to the end of glycoconjugates by sialyltransferases. Among the six ST8 α-N-acetylneuraminidase α-2,8-sialyltransferases (ST8SIA) existing in adult brains, ST8SIA2 is a schizophrenia-associated gene. However, the in vivo substrates and physiological functions of most sialyltransferases are currently unknown. The ST8SIA3 is enriched in the striatum. Here, we showed that ablation of St8sia3 in mice (St8sia3-KO) led to fewer disialylated and trisialylated terminal glycotopes in the striatum of St8sia3-KO mice. Moreover, the apparent sizes of several striatum-enriched G-protein-coupled receptors (GPCRs) (including the adenosine A2A receptor (A2AR) and dopamine D1/D2 receptors (D1R and D2R)) were smaller in St8sia3-KO mice than in WT mice. A sialidase treatment removed the differences in the sizes of these molecules between St8sia3-KO and WT mice, confirming the involvement of sialylation. Expression of ST8SIA3 in the striatum of St8sia3-KO mice using adeno-associated viruses normalized the sizes of these proteins, demonstrating a direct role of ST8SIA3. The lack of ST8SIA3-mediated sialylation altered the distribution of these proteins in lipid rafts and the interaction between D1R and A2AR. Locomotor activity assays revealed altered pharmacological responses of St8sia3-KO mice to drugs targeting these receptors and verified that a greater population of D1R formed heteromers with A2AR in the striatum of St8sia3-KO mice. Since the A2AR-D1R heteromer is an important drug target for several basal ganglia diseases (such as schizophrenia and Parkinson’s disease), the present study not only reveals a crucial role for ST8SIA3 in striatal functions but also provides a new drug target for basal ganglia-related diseases.

Introduction
Glycosylation is effected by the concerted enzymatic action of a series of glycosyltransferases, often in a protein site-specific manner with significant functional impact. Sialic acids are the abundant monosaccharide located on mammalian cell surface glycoconjugates. Sialylation is arguably the most important form of terminal glycosylation capping the glycan chains on glycoproteins and glycolipids performed by sialyltransferases (STs). While single terminal α2,3- or α2,6-sialylation is ubiquitous and has been functionally implicated in a wide range of biological processes, its further extension by the addition of sialic acids via the α2,8-linkage to make di-, tri-, oligo-, or polysialic acid chains is particularly prominent in the brain. A lack of proper sialoglycans in the brain leads to the abnormal development, maintenance, and health of the nervous system. The ST8 α-N-acetyl-neuraminidase α-2,8-sialyltransferase (ST8Sia) family catalyzes the synthesis of one or multiple α2,8-linked sialic acid chains according to their acceptor specificity. At least five ST8Sia enzymes (ST8SIA1, 2, 3, 4, and 6) have previously been identified in adult brains. These levels of these ST8Sia transferases are developmentally regulated in the brain, suggesting the importance of these enzymes in the nervous system.

ST8SIA2 and 4 are relatively well-characterized particularly for their substrate specificities towards the neural...
cell adhesion molecule (NCAM), an important regulator of neuronal plasticity in the embryonic brain tissue, and a handful of other acceptor proteins. An St8sia2 deficiency impairs hippocampal axonal targeting. Genetic studies have associated ST8SIA2 with schizophrenia. Conversely, the deletion of St8sia4 affects polysialic acids (PSA) levels and plasticity at the Schaffer collateral-CA1 synapses. In addition, ST8SIA1 and ST8SIA5 are known to be primarily responsible for mono α2,8-sialylation of GD3, GM1b, GD1a, and GT1b gangliosides. ST8SIA3 shares 26% sequence identity with ST8SIA2 and 4. All of these enzymes are considered to be oligo- and polysialyltransferases, but very little is known about the sialylation profiles mediated by ST8SIA3 in vivo.

The mouse St8sia3 gene shares 96% amino acid identity with human ST8SIA3, and is mainly expressed in the brain and testis. According to in vitro analyses, ST8SIA3 transfers PSA to NCAM with a lower efficiency than ST8SIA2 and ST8SIA1. The apo- and ligand-bound crystal structures of human ST8SIA3 were reported at 1.85-Å resolution and revealed a group of polysialyltransferase-specific structural motifs. ST8SIA3 was also suggested to be the principal sialyltransferase responsible for synthesis of the α2,8-trisialic acid (α2,8-triSia) units on gangliosides and glycoproteins in the developing mouse brain because its expression correlated with the α2,8-triSia epitope. To date, only St8sia2-knockout (KO) and St8sia4-KO, but not St8sia3-KO, mice have been generated to determine the biological roles of these enzymes in vivo. The biochemical and pathophysiological properties of ST8SIA3 remain largely unknown.

Because the striatum plays a critical role in coordinating movement and many drug targets (e.g., adenosine A3 receptors, A2A receptors, dopamine D2 receptors, D3 receptors, and dopamine D1 receptors, D1R) are enriched in the striatum, we set out to investigate whether the sialylation mediated by ST8SIA3 is essential for the functions of striatum. In the present study, we generated and characterized a mouse model that lacks ST8SIA3. Using this St8sia3-KO mouse model and adeno-associated viruses harboring St8sia3, we revealed the selective sialylation of several striatum-enriched membrane proteins (including A2AR, D2R, and D1R) by ST8SIA3, which adds α2,8-diSia and α2,8-triSia units to its substrates. The ST8SIA3-mediated sialylation of striatal proteins may affect their distributions in lipid rafts, their abilities to interact with other proteins, and ultimately the motor functions of animals in response to pharmacological modulation of these substrates.

Materials and methods

Mice

St8sia3-KO mice were generated by CRISPR/Cas9-mediated gene editing as described for details in Supplementary Materials and Methods. Male mice (10–12 weeks old) were analyzed in all experiments except stated otherwise. All animal experimental procedures were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee (IACUC) at the Institute of Biomedical Sciences, Academia Sinica.

Immunoblotting

SDS-PAGE and immunoblotting analyses were performed using previously described methods. In brief, protein samples were separated on 10% SDS-PAGE gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated overnight at 4°C with the primary antibodies listed in Supplementary Table 1, followed by a 1-h incubation with the corresponding secondary antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). After extensive washes, immunosignals were detected using the Western Lightning® Plus-ECL Enhanced Chemiluminescence Substrate (PerkinElmer, MA, USA).

Immunofluorescence staining

Brain sections (20 μm) were subjected to antigen retrieval by boiling in 10 mM citrate buffer (pH 6.0) for 20 min. The sections were subsequently incubated in 3% BSA at room temperature (RT) for 2 h. The sections were incubated with the indicated primary antibody listed in Supplementary Table 1 for 48 h at 4°C, followed by incubation with the corresponding secondary antibody conjugated with Alexa Fluor 488 or 568 (1:500; Jackson ImmunoResearch Laboratories) in 3% BSA in the dark for 2 h at RT. After extensive washing, nuclei were stained with Hoechst 33342 for 20 min. Images were captured using a Zeiss LSM 780 inverted confocal laser scanning microscope (Axio Observer Z1; Carl Zeiss, Göttingen, Germany) and analyzed using the ZEN 2012 software (Carl Zeiss).

Glycomic analysis

Glycomic sample preparation of N- and O-glycans from the striatal membrane fractions were performed exactly as described previously. The permethylated N- and O-glycans were separately subjected to nanoLC-MS2/MS3 analysis on an Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) interfaced to an Ultimate3000 RSLC nano system (Thermo Fisher Scientific) fitted with a C18 column (Acclaim PepMap® RSLC; Thermo Fisher Scientific).

Glycan microarray

To fabricating the microarray, series glycanes listed in Supplementary Table 2 were prepared and printed onto
NHS-coated glass slide (Nexterion H slide; SCHOTT North America, Elmsford, NY, USA) as described previously. S2-566 and A2B5 antibodies were incubated with microarray and the slides were finally scanned with a microarray fluorescence chip reader (GenePix 4300 A; Molecular Devices, Sunnyvale, CA, USA) and scanned images were analyzed with GenePix Pro-6.0 analysis software (Axon Instruments, Foster City, CA, USA).

Intrastriatal virus injection
The mouse St8sia3 cDNA was subcloned into an AAV expression vector (pAAV-IREs-hrGFP, Stratagene, La Jolla, CA, USA) driven by the CMV early enhancer/chicken β actin (CAG) promoter and packed into AAV serotype 8 (AAV8) particles as detailed elsewhere. Injections were performed at the following stereotaxic coordinates, measured in millimeters (mm) from the bregma: anteroposterior (AP) + 0.5, mediolateral (ML) ± 2.0, and dorsoventral (DV) −2.7 and −3.7. AAV8 particles (AAV-hrGFP or AAV-St8sia3) were injected into the mice of 5 weeks as indicated using a 30-gauge 10-μL Hamilton microsyringe in a volume of 2 μL. The injection rate was 0.5 μL/min and the needle was maintained in place for an additional 5 min after the injection before slow withdrawal of the needle. Eight microliters of viral vectors was injected into four sites in the striatum.

Preparation of lipid rafts
Lipid raft fractions were prepared from the striatum using a non-detergent method. In brief, striatal tissues were lysed in lysis buffer (1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 1% Triton X-100 and 1 mM sodium vanadate in TBS) in the presence of 1X Complete™ and EDTA-free protease inhibitor (Roche). The mixture was placed on a shaker for 2 h at 4 °C, and then centrifuged at 800 × g for 10 min. Supernatants were further centrifuged at a force of 200 K g for 18 h at 4 °C using the SW50.1 rotor (Beckman Coulter) with a discontinuous sucrose gradient (85, 35, and 5% sucrose). Fourteen fractions were separated, and raft fractions were identified with a flotillin-1 antibody by immunoblotting.

Drug-induced locomotor activity
SCH 58261 (Tocris Bioscience, Bristol, UK) was dissolved in saline containing 15% DMSO and 15% Cremophor EL (Sigma-Aldrich). L-741626 (Tocris Bioscience) was dissolved in a saline solution by adding drops of an acetic acid solution. The pH was adjusted to 7.0 with an NaOH solution. SKF 81297 (Tocris Bioscience) was dissolved in saline. All saline-based solutions also served as the vehicle control. The locomotor activity was examined in a VersaMax activity monitoring system (AccuScan Instruments, Columbus, OH, USA) and quantified for 1 h.

Statistical analysis
All experiments were reliable and independently conducted at least three times. The sample sizes were similar to other publications. The data in this study meet the assumption of normal distribution and no data was excluded. Materials were collected randomly. Behavior testing and imaging quantitation were performed blinded. Data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) software and are presented as the means ± S.E.M. Statistical analysis was performed using either Student’s t-test, one-way ANOVA or two-way ANOVA followed by post hoc Bonferroni’s multiple comparison tests as indicated. The significant difference was considered when the P-value was <0.05. Asterisks were used to indicate degree of significance (*P < 0.05; **P < 0.01; and ***P < 0.001).

Results
Expression of ST8SIA3 in the brain
St8sia3-KO mice were generated by CRISPR/Cas9-mediated gene editing and characterized as described in the Supplementary Results (Supplementary Figs. S1–S3, Supplementary Tables 3 and 4, Supplementary File 1). Immunoblotting analysis showed that ST8SIA3 was expressed in several brain regions of wild-type (WT) mice but not in St8sia3-KO mice (Fig. 1a). Moreover, the level of ST8SIA3 was higher in the striatum than in the hippocampus, cortex, and cerebellum (Fig. 1b). Double immunofluorescence staining showed that ST8SIA3 was detected in NeuN-positive neurons (Fig. 1c), but not in S100 beta-positive astrocytes or Iba1-positive glia cells (Supplementary Fig. S1E).

ST8SIA3 depletion reduced the number of disialyl and trisialyl units on striatal N- and O-glycans
To investigate how ST8SIA3 may impact the overall sialylation of glycoproteins at the glycomic level in the striatum, N- and O-glycans were sequentially released from the striatal membrane fractions of WT and St8sia3-KO mice and subjected to permethylation and nanoLC-MS²-pd-MS³ analyses. Instead of performing a full systematic analysis of individual N-glycan entities, we have resorted to semi-quantitative mapping of the complement of terminal glycotopes collectively presented by the striatal N-glycome for the current purpose of determining if any glycotopes were significantly altered in St8sia3-KO mice. As shown in our previous study, the summed signal intensity of the diagnostic MS² fragment ions representing a particular terminal glycotope from all MS² spectra was solved in saline. All saline-based solutions also served as the vehicle control. The locomotor activity was examined in a VersaMax activity monitoring system (AccuScan Instruments, Columbus, OH, USA) and quantified for 1 h.
detected at very low intensity, further MS$^3$ was critical not only to ensure true positive results but also to distinguish between the 2 isomeric disialylated glycotopes of m/z 1186 (Fig. 2b). Similar summing of the diagnostic MS$^3$ ion intensity thus provided a more reliable quantitative index since it was only derived from an MS$^2$ precursor ion that displayed a sufficient intensity to yield a productive MS$^3$ event, with the disialylated glycopeptide defined as a pair of MS$^2$→MS$^3$ transitions, namely, m/z 737→376, and m/z 1186→737, for authentic NeuAc-NeuAc- and NeuAc-NeuAc-Gal-GlcNAc-, respectively. In this context, we concluded that the reduced levels of these glycotopes in the St8sia3-KO striatum were significant, when most other glycotopes, including Lewis X (m/z 638) and the isomeric type 1 Gal-3GlcNAc chain disialylated at 2 distinct positions (m/z 1186→589), were affected to a lesser or not appreciable extent (Fig. 2b). A few MS$^2$ spectra from WT but not St8sia3-KO striatal samples contained the ion at m/z 1547 for NeuAc3Hex1HexNAc1−, albeit at very low intensity (Fig. 2c). Manual verification of one of these spectra against the accurately measured mass of the monoisotopic precursor and its assigned glycosyl composition provided evidence supporting the presence of striatal N-glycans carrying a trisialylated LacNAc, among
other antennary structures. Although ST8SIA3 was thought to contribute to the synthesis of trisialyl unit⁷, this study presents the first MS data indicating the possible occurrence of this trisialylated LacNAc antennary structure on the N-glycans (Fig. 2c). More importantly, the addition of the first NeuAc residue to a
monosialylated LacNAc was significantly affected but not completely prevented in the absence of ST8SIA3, which in turn may further impact subsequent oligo- and polysialylation mediated by other ST8SIA enzymes.

Similar nanoLC-MS2 analyses applied to the released O-glycans also revealed the presence of NeuAc-disialylated and trisialylated simple core 1 O-glycans (Supplementary Fig. S4A). Since these smaller O-glycans were chromatographically resolved, their relative amounts in WT and St8sia3-KO striatal tissues were directly quantified and compared based on their respective peak areas under the extracted ion chromatograms (Supplementary Fig. S4B), and their structures were further verified by MS2 analyses (Supplementary Fig. S4C). Overall, a small but significant reduction in the abundance of higher sialylated core 1 O-glycans was observed, suggesting that ST8SIA3 may also affect the α2-8-NeuAc-sialylation on O-glycans.

We next evaluated whether the ST8SIA3 deficiency affected the overall sialylation of the striatal glycoproteins in St8sia3-KO mice using antibodies that recognize sialic acid residues at the non-reducing terminus. The specificities of the antibodies used in the experiments described below were validated using glycan microarrays. An anti-diSia-Gal antibody, S2-566, which recognizes the Neu5Acα2-8Neu5Acα2-3Gal structures on a glycan microarray (Fig. 3a), detected fewer immunoreactive signals in the immunoblotting of striatal proteins from St8sia3-KO (−/−) mice compared with WT (+/+) control. Similarly, an anti-triSia antibody, A2B5, which recognizes the Neu5Acα2-8Neu5Acα2-8Neu5Ac structures (Fig. 3b), detected lower levels of immunoreactive bands in the striatal proteins from St8sia3-KO mice (Fig. 3d), indicating that the striatum of St8sia3-KO mice contained fewer di- and trisialylated terminal glycotopes than WT mice.
Conversely, the 12E3 antibody that recognizes (Neu5Ac)ₙ (where n is ≥ 5, Fig. 3e) and an anti-PSA antibody²⁶ (where n is ≥ 11, Fig. 3f) showed similar expression profiles for the oligo- and polysialic acid-conjugated glycoproteins, respectively, in the striatum from WT and St8sia3-KO mice. Consistent with the expression of ST8SIA3 in several brain areas, immunoblotting revealed that compared with those of WT mice, lower levels of diSia- and triSia- terminal glycotopes were detected in the hippocampus, cortex, and cerebellum of St8sia3-KO mice (Supplementary Fig. S5). Collectively, these findings suggest that ST8SIA3 mainly synthesizes di- and trisialylated terminal glycotopes in the brain.

Multiple striatum-enriched proteins are substrates of ST8SIA3

We next searched for novel protein substrate(s) of ST8SIA3. To date, there is no reported protein sequence or motif for sialylation. We selected nine membrane proteins that contain potential N-linked glycosylation sites to assess whether they are substrates of ST8SIA3. Given the enriched expression of ST8SIA3 in the striatum²⁷, we first evaluated the apparent sizes of several striatal proteins using immunoblotting analyses. We reasoned that the lack of ST8SIA3 may cause the loss of terminal di- and/or trisialylated glycotopes from its substrates and reduce their apparent molecular weights. At least four of the nine proteins tested showed changes in their apparent molecular weights but no significant change in the total levels of these proteins was observed (Fig. 4). Interestingly, these potential substrates of ST8SIA3, including the adenosine A₂ₐ receptor (A₂ₐR; Fig. 4a), type V adenyl cyclase (AC₅; Fig. 4b), dopamine D₂ receptor (D₂R; Fig. 4c) and dopamine D₁ receptor (D₁R; Fig. 4d) are all striatum-enriched proteins.²⁸–³⁰ Exogenous treatment of striatal proteins with a2-3,6,8-neuraminidase (sialidase; 37 °C for 16 h) removed terminal sialic residues and reduced the apparent molecular weights of all four proteins tested in both WT and St8sia3-KO mice (Fig. 4e–h). Most importantly, the differences in the apparent molecular weights of these proteins between WT and St8sia3-KO mice disappeared after the treatment with sialidase, suggesting that the ST8SIA3-dependent addition of sialic residues to these striatal proteins may contribute to the changes in the apparent molecular weight between WT and St8sia3-KO mice. Because treatment with sialidase further reduced the apparent molecular weights of these striatal proteins in St8sia3-KO mice, ST8SIA3 was unlikely to be the only sialyltransferase participating in the sialylation of striatal proteins. No changes in the apparent molecular weights of five glutamate receptors (including NR1, NR2A, NR2B, GluR1, and GluR2) were detected in WT and St8sia3-KO mice (Supplementary Fig. S6).

Adeno-associated virus serotype 8 (AAV8) harboring hrGFP or St8sia3 was intrastriatally injected into 5-week-old St8sia3-KO and WT mice to further confirm whether the abnormalities observed in St8sia3-KO mice were directly caused by the lack of ST8SIA3 in the striatum. Intrastriatal delivery of AAV8 particles has been shown to effectively express the transgene mainly in neurons and to a much lower level in astrocytes.²⁴ In addition, under the experimental conditions employed in the present study, intrastriatal delivery of AAV8 particles enabled the transgene to be expressed in most of the striatum, the targeted brain area (Supplementary Fig. S7). At 8 weeks post injection, striatal tissues were harvested and analyzed to determine the apparent molecular weights of A₂ₐR, AC₅, D₂R, and D₁R (Fig. 4i–l); the hrGFP group served as a control. The expression of exogenous ST8SIA3 in the striatum of St8sia3-KO mice effectively rescued the differences among the apparent molecular weights of all four proteins tested, supporting the hypothesis that these striatum-enriched G-protein-coupled receptors (GPCRs) and an effector enzyme (AC₅) are substrates of ST8SIA3. Consistent with this hypothesis, double immunofluorescence staining also indicated that these striatum-enriched GPCRs (A₂ₐR, D₂R, and D₁R) are likely to exist in ST8SIA3-expressing striatal neurons (Supplementary Fig. S8).

The lack of ST8SIA3-mediated modification altered the distributions of ST8SIA3 substrates into lipid rafts

To determine the role of ST8SIA3-mediated sialylation in the functions of its substrates, we first evaluated the amounts of these proteins in various biochemical fractions. Immunoblotting revealed that the total levels (Fig. 4a–d), the amounts in the plasma membrane fractions (Supplementary Fig. S9), and the amounts in synaptosome fractions (Supplementary Fig. S10) of A₂ₐR, AC₅, D₂R, and D₁R were similar in the striatum of WT and St8sia3-KO mice. We next assessed whether the deficient sialylation of A₂ₐR, AC₅, D₂R, and D₁R affected their abilities to move into lipid rafts, a critical signal transduction microstructure.³¹ The lipid raft fractions were isolated from the striatum of WT and St8sia3-KO mice using a nondetergent method. The amounts of the target proteins in the raft fraction were analyzed by immunoblotting. FLOT1 is a lipid raft marker.³² The amounts of ST8SIA3 substrates (A₂ₐR, AC₅, D₂R, and D₁R) in the lipid raft fractions were significantly increased (Fig. 5a–d, Supplementary Fig. S12A–C). No change in the distribution of five glutamate receptors in lipid rafts was observed (Supplementary Figs. S11 and S12D–F). Based on these findings, ST8SIA3-mediated sialylation may affect the distribution of its substrates into lipid rafts, probably changing their functions as well.
Fig. 4 Genetic ablation of St8sia3 modulated the sialylation patterns of various substrates. a–d St8sia3-KO (+/−) mice showed similar expression levels but larger shifts in the mobility of the major bands for A2AR, AC5, D1R, and D2R in immunoblots of the striatal homogenates compared with WT (+/+). e–h Differences in the mobilities of these striatum-enriched substrates were eliminated after sialidase treatment. The major bands for A2AR, AC5, D2R, and D1R in striatal samples from both genotypes migrated to lower and similar positions after the enzyme treatment (+) compared with untreated groups (−). i–l Intrastriatal injections of an AAV virus expressing mouse ST8SIA3 (AAV-St8sia3) or control (AAV-hrGFP). In the St8sia3-KO striatum, AAV-St8sia3 rescued the obviously decreased sizes of A2AR, AC5, D1R, and D2R to the original size. n = 3 for each genotype (two-tailed unpaired Student’s t-test). All values are presented as the means ± S.E.M. ACTIN was used as an internal loading control.
The lack of ST8SIA3-mediated sialylation facilitates the A2AR and D2R interaction and alters the roles of these proteins in the regulation of motor function in the striatum.

Of the four substrates of ST8SIA3, A2AR and D2R are known to form heteromers, which reciprocally regulate each other in a negative fashion and have been implicated in movement disorders (e.g., Parkinson’s disease, PD). Because the lack of ST8SIA3-mediated sialylation resulted in higher concentrations of A2AR and D2R in lipid rafts (Fig. 5a–d), we hypothesized that more A2AR-D2R complexes might exist in lipid rafts. The results of
the in situ proximity ligation assay (PLA) using anti-A2AR and anti-D2R antibodies supported the above hypothesis. More PLA signals were found in the dorsal striatum of St8sia3-KO mice than in that of WT mice (Supplementary Fig. S13). Nonetheless, no significant effect on the affinity of ligand binding to A2AR and D2R (CGS 21680 and sulpride, respectively) or forskolin-evoked AC activity was observed (Supplementary Fig. S14).

Because A2AR and D2R are known to regulate motor function, we next examined the locomotor activity of St8sia3-KO and WT mice. No changes in spontaneous locomotor activity were observed. Treatment with an A2AR-selective antagonist (SCH 58261, 1–10 mg/kg, i.p.) dose-dependently increased the locomotor activity of both WT and St8sia3-KO mice (Supplementary Fig. S15A). Interestingly, St8sia3-KO mice showed a much lower response to SCH 58261 than WT mice. The administration of a D2R-selective antagonist (L-741626, 0.25–5 mg/kg, i.p.) reduced the locomotor activity of both WT and St8sia3-KO mice in a dose-dependent manner (Supplementary Fig. S15B). In contrast to their inferior response to an A2AR antagonist, St8sia3-KO mice were more sensitive to L-741626 than WT mice. The differences in the effects of SCH 58261 and L-741626 on locomotor activity between St8sia3-KO and WT mice were rescued by restoring ST8SIA3 expression using AAV-St8sia3 (Supplementary Fig. S15D, E), confirming that the altered motor function of St8sia3-KO mice depended on ST8SIA3.

Most importantly, A2AR antagonist SCH 58261 eliminated the D2R inhibitor-induced changes in motor function between WT and St8sia3-KO mice (Fig. 5e), suggesting that this altered response to D2R blockade in St8sia3-KO mice might result from the negative impact of A2AR. This observation is consistent with the finding that more A2AR form heteromers with D2R in the striatum of St8sia3-KO mice than in WT mice. Based on these data, ST8SIA3 plays a critical role in the striatum by mediating the sialylation of specific striatal proteins, regulating their distribution in lipid rafts, affecting their interaction with other binding partners, and subsequently modulating striatal functions.

Discussion

The ST8Sia family is known to catalyze the production of α2,8-linked sialic acid chains according to the acceptor specificity of glycoproteins and gangliosides. ST8SIA3 has been proposed to deliver α2,8-triSia and polySia units onto glycoproteins in vitro. In the present study, we provided glycomic and immunoblotting evidence that ST8SIA3 mainly mediates the formation of disialylated and trisialylated terminal glycotopes on N- and O-glycans in the mouse striatum (Figs. 2 and 3a–d, Supplementary Fig. S4). Because the global levels of oligoSia and polySia units remained largely unchanged in mice lacking ST8SIA3 (Fig. 3c, f), in vivo, ST8SIA3 appears to play a role distinct from that of ST8SIA2 and ST8SIA4, enzymes that regulate the adhesive properties of NCAM by polysialylation. Notably, the level of St8sia4 transcript was slightly decreased in the striatum of St8sia3-KO mice (Supplementary Fig. S2D). Whether the level of polySia units on particular substrates of ST8SIA4 was significantly affected would require further investigation. Another interesting observation is that the global di-/trisialylation was not completely lost in St8sia3-KO mice (Fig. 3c, d), suggesting that other ST8SIA members may contribute to the di- and trisialylation of glycoproteins. Previous studies suggest that ST8SIA6 may be the other ST8SIA(s) that mediates the di- and trisialylation of glycoproteins, particularly on O-linked proteins in the striatum. Of note, no alteration in the level of St8sia6 transcripts was observed in the St8sia3-KO striatum (Supplementary Fig. S2F).

At least three GPCRs (i.e., A2AR, D2R, and D1R) and one effector (AC5) were novel sialylated substrates of ST8SIA3 in the striatum (Fig. 4). The extracellular domains of many GPCRs are glycosylated, and glycosylation-mediated regulation varies by receptor type. Specifically, A2AR contains one potential N-linked glycosylation site, while D1R and D2R contain two putative sites in their extracellular domains. The N-glycosylation sites of GPCRs may influence receptor trafficking. Nonetheless, a deficiency in ST8SIA3-mediated sialylation did not affect the total levels or trafficking of A2AR, AC5, D2R, and D1R to either the plasma membrane or synapses in the striatum (Supplementary Figs. S9 and S10). ST8SIA3-mediated sialylation appears to influence the distribution of its substrates in membrane microdomains (such as lipid rafts in Fig. 5). Lipid rafts are enriched in cholesterol and glycosphingolipids and play a critical role in controlling the signaling of GPCRs and other signaling molecules. Depending on the compositions of the available signaling molecules, lipid rafts may regulate signal transduction either positively or negatively. Previous studies suggested that D2R is largely distributed in the detergent-resistant membrane (DRM) fraction, a restricted microdomain that limits the interaction between D2R and other proteins. Biochemical characterization suggests that this DRM fraction does not have the properties of lipid rafts. Conversely, some D2Rs located in the detergent-soluble fractions are in a more fluid environment and are allowed to interact with their binding partners. Using a detergent-free method, Vanderwerf et al. demonstrated that D2R is highly enriched in the raft fraction. The distribution of A2AR in membranes has not yet been extensively characterized. Several studies have shown that A2AR colocalizes with TrkB in lipid rafts of motor neurons and regulates the function of TrkB.
In the present study, we found that removal of diSia/triSia resulted in the enrichment of A2AR, D2R, and AC5 in lipid rafts, and facilitated their complex formation in lipid rafts. ST8SIA3 may thus significantly contribute to the mechanisms regulating striatal signaling. The reason that removal of terminal diSia and triSia on glycoproteins (at least from A2AR, D2R, D1R, and AC5) would enhance their distribution in lipid rafts remains elusive. It is very likely that removal of di- and triSia would make these proteins less bulky and less negatively charged hence less repulsive. The structure and length of terminal sialic acids may play critical roles in different cellular machineries with specialized functions. Earlier studies indicated that polySia may exist as repulsive polyanionic structures and may mask recognition sites of bioactive molecules. The functions of di- and trisialyl glycotopes are largely unknown and require further investigation.

ST8SIA3-mediated sialylation also controls the interactions between its substrates, such as D2R and A2AR. This observation is of particular interest because these two receptors are known to physically interact and reciprocally regulate each other in a negative fashion in the striatum. There are two types of interactions between A2AR and D2R ligands that target heteromers. Adenosine and A2AR ligands decrease the affinity and efficacy of dopamine and D2R ligands via allosteric interaction, while dopamine or D2R agonists counteract the ability of adenosine or A2AR agonists to activate adenylyl cyclase through a canonical Gsα-Giα antagonistic interaction at the adenylyl cyclase level. In the striatum, the canonical interaction between A2AR and D2R ligands depends on the formation of complexes containing A2AR-D2R heteromers and AC5. Our findings suggest that in the striatum, the major components of this A2AR-D2R-AC5 complex are ST8SIA3-mediated sialylation in a coordinated manner. Removal of ST8SIA3-dependent sialylation might have facilitated the interaction between A2AR and D2R, as well as the negative regulation of D2R by A2AR. Since the A2A-D2R heteromer is an important drug target for several basal ganglia-related diseases (such as schizophrenia and PD) and because ST8SIA3 is capable of regulating the population of A2AR-D2R heteromers in the striatum, our findings highlight the importance of ST8SIA3 as a new drug target.

Approximately 34% of Food and Drug Administration-approved drugs target GPCRs. Three of the four ST8SIA3 substrates that we identified are GPCRs (i.e., A2AR, D2R, and D1R). Given the changes in the biochemical properties of these proteins described above, ST8SIA3-KO mice may respond abnormally to agonists or antagonists of these three receptors. In addition to A2AR and D2R as discussed above, D1R is also an important drug target for PD and psychosis. Intake of a selective D1R agonist (SKF 81297, 1–10 mg/kg, i.p., Supplementary Fig. S15C) enhanced locomotion in WT mice but not in St8sia3-KO mice in the same dose range. The restoration of ST8SIA3 expression in the striatum effectively rescued not only the sialylation of D1R (Fig. 4h) but also the abnormal responses of St8sia3-KO mice to SKF 81297, which targets D1R (Supplementary Fig. S15F). This finding suggests that locomotor-activating responses to D1R agonists are dependent on ST8SIA3-mediated sialylation. Interestingly, D1R is known to form complexes with the adenosine A1 receptor (A1R) in the striatum. Similar to the A2A-D2R heteromer, A1R-D1R heteromers are likely to form a signaling complex that contains Gsα, Gia, and AC5. Because AC5 is also a substrate of ST8SIA3, ST8SIA3-mediated sialylation may also play a critical role in the regulation of the A1R-D1R-AC5 complex as observed for the A2A-D2R-AC5 complex in the striatum. Further investigation to determine the impact of ST8SIA3-mediated sialylation on drugs targeting the A1R-D2R-AC5 complex is warranted.

In the brain, sialylation has long been recognized to play an essential role in neuronal development and regeneration. The modification of NCAM with PSA is mediated by ST8SIA2 and ST8SIA4 and is associated with development and plasticity in the brain. Although ST8SIA3 has been shown to catalyze the polysialylation of NCAM in vitro, it is not as efficient as ST8SIA2 and ST8SIA4. Such modifications with PSA may serve as a negative regulator of cell-cell interactions due to steric repulsion or a binding domain to recruit bioactive molecules such as neurotrophins and dopamine. Conversely, much less is known about the functions of di- and trisialyl glycotopes. Using an antibody (i.e., A2B5) that recognizes α2,8-triSia, triSia glycotopes were detected in both developing and adult mouse brains, as shown in Fig. 2d in the present study. Because the expression profile of α2,8-triSia is similar to that of the St8sia3 gene, ST8SIA3 has been proposed to be the primary ST8SIA family member that synthesizes α2,8-triSia on glycoproteins. Based on the results of the present study, ST8SIA3 is mainly responsible for synthesizing α2,8-triSia on glycoproteins in the striatum. To date, it has not been clearly determined whether the synthesis of PSA by any of the ST8Sia enzymes is accompanied by incompletely elongated oligosialyl chains or whether the shortest chains, namely, the di- and trisialyl units, may be specifically or preferentially added by ST8SIA3 and serve as primers for PSA.

Because ST8SIA3 is enriched in the striatum, it has been implicated in Huntington’s disease (HD), a devastating neurodegenerative disease. Compared to normal controls, the St8sia3 transcript level is significantly decreased in the caudate of patients with HD and the striatum of HD mice (R6/1). As shown in the present study, striatal signaling might be altered in patients and mice with HD due to the loss of ST8SIA3 in the striatum. Therefore, patients with...
HD and normal subjects might respond differently to therapeutic drugs targeting GPCRs that are subject to modification by ST8SIA3. This finding is potentially important because dopamine antagonists have been commonly used to treat chorea and psychosis in patients with HD<sup>24</sup>. Further investigations are required to evaluate whether the alterations in ST8SIA3 activity during HD progression may also alter the pharmacological properties of drugs targeting ST8SIA3 substrates (e.g., D<sub>2</sub>R).

In summary, ST8SIA3 is the principle ST8Sia family member that mediates the synthesis of diSia and triSia on glycoproteins and thus regulates their distribution and mode of action in the striatum. Several ST8SIA3 substrates (e.g., A<sub>2</sub>AG, D<sub>2</sub>R, and D<sub>1</sub>R) have major roles in controlling striatal functions. Our findings provide new insights into the mechanisms regulating the striatum and may pave the way for the development of novel therapeutic strategies for basal ganglia-related diseases.

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Conflict of interest
The authors declare that they have no conflict of interest.

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References
1. Spira, R. G. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. Glycobiology <b>12</b>, 43R–56R (2002).
2. Rao, F. V. et al. Structural insights into mammalian sialyltransferases. Nat. Struct. Mol. Biol. <b>16</b>, 1186–1188 (2009).
3. Sato, C. & Kitajima, K. Oligosaccharide and polysialic acids: distribution, functions and related disease. J. Biochem. <b>154</b>, 115–136 (2013).
4. Schnaar, R. L., Gerardy-Schahn, R. & Hildebrandt, H. Sialic acids in the brain: gangliosides and polysialic acid in nervous system development, stability, disease, and regeneration. <i>Physiol. Rev.</i> <b>94</b>, 461–518 (2014).
5. Paulson, J. C. & Rademacher, C. Glycan translator. Nat. Struct. Mol. Biol. <b>16</b>, 1121–1122 (2009).
6. Hardere-Lepers, A. et al. The human sialyltransferase family. Biochimie <b>83</b>, 727–737 (2001).
7. Inoko, E. et al. Developmental stage-dependent expression of analphab2-trisialic acid unit on glycoproteins in mouse brain. Glycobiology <b>20</b>, 916–928 (2010).
8. Angata, K. et al. Differential biosynthesis of polysialic acid on neural cell adhesion molecule (NCAM) and oligosaccharide acceptors by three distinct alpha2,3-sialyltransferases, ST8Sia V (PST), ST8Sia II (STX), and ST8Sia III. J. Biol. Chem. <b>275</b>, 18594–18601 (2000).
9. Angata, K. et al. Sialyltransferases ST8Sia-II assemblies a subset of polysialic acid that directs hippocampal axonal targeting and promotes fear behavior. J. Biol. Chem. <b>279</b>, 32603–32613 (2004).
10. Arii, M. et al. Association between polymorphisms in the promoter region of the sialyltransferase 8B (ST8BB8) gene and schizophrenia. Biol. Psychiatry <b>59</b>, 652–659 (2006).
11. Yang, S. Y. et al. Association between ST8SIA2 and the risk of schizophrenia and bipolar I disorder across diagnostic boundaries. <i>PLoS ONE</i> <b>10</b>, e0139413 (2015).
12. Kamien, B. et al. Characterization of a 520 kb deletion on chromosome 15q26.1 including ST8SIA2 in a patient with behavioral disturbance, autism spectrum disorder, and epilepsy. Am. J. Med. Genet. A <b>164A</b>, 782–788 (2014).
13. Eckhardt, M. et al. Mice deficient in the polysialytransferase ST8Sia/V-PST-1 allow discrimination of the roles of neural cell adhesion molecule protein and polysialic acid in neural development and synaptic plasticity. J. Neurosci. <b>29</b>, 5234–5244 (2009).
14. Kim, Y. J. et al. Molecular cloning and expression of humanalpha2,8-sialyltransferase (bST8Sia V), Biochem. Biophys. Res. Commun. <b>235</b>, 327–330 (1997).
15. Sasaki, K. et al. Expression cloning of a GM3-specific alpha-2,8-sialyltransferase (GD3 synthase). J. Biol. Chem. <b>269</b>, 15950–15956 (1994).
16. Yoshida, Y., Kojima, N., Kurosaوا, N., Hamamoto, T. & Tsuji, S. Molecular cloning of Sia alpha 2,3Gal beta 1,4GlcNAc alpha 2,8-sialyltransferase from mouse brain. J. Biol. Chem. <b>270</b>, 14628–14633 (1995).
17. Yoshida, Y. et al. Unique genomic structure and expression of the mouse alpha 2,8-sialyltransferase (ST8Sia II) gene. Glycobiology <b>6</b>, 573–580 (1996).
18. Lee, Y. C. et al. Cloning and expression of a DNA for a human Sia alpha 2,3Gal beta 1,4GlcNAc alpha 2,8-sialyltransferase (bST8Sia III). Arch. Biochem. Biophys. <b>360</b>, 41–46 (1998).
19. Volkers, G. et al. Structure of human ST8SiaII sialyltransferase provides insight into cell-surface polysialylation. Nat. Struct. Mol. Biol. <b>22</b>, 627–635 (2015).
20. Chien, T. et al. GSK3beta negatively regulates TRAX, a scaffold protein implicated in mental disorders, for NHEJ-mediated DNA repair in neurons. Mol. Psychiatry <b>23</b>, 2373–2390 (2018).
21. Hisao, C. T. et al. Advancing a high throughput glycotope-centric glycomics workflow based on nanoLC-MS/MS-2/MS3 analysis of permethylated glycans. Mol. Cell. Proteomics <b>16</b>, 2288–2298 (2017).
22. Wang, C. C. et al. Glycan microarray of Globo H and related structures for quantitative analysis of breast cancer. Proc. Natl Acad. Sci. USA <b>105</b>, 11661–11666 (2008).
23. Aschauer, D. F., Kreuz, S. & Rumpel, S. Analysis of transduction efficiency, tropism and axonal transport of AAV vectes 1, 2, 5, 6, 8 and 9 in the mouse brain. PLoS ONE <b>8</b>, e76310 (2013).
24. Pignataro, D. et al. Adeno-associated viral vectors serotype 8 for cell-specific delivery of therapeutic genes in the central nervous system. Front. Neurosci. <b>11</b>, 2 (2017).
25. Penaud-Savin, D. A., Lightcap, S. & Hanly, G. J. Isolation of rafts from mouse brain tissue by a detergent-free method. J. Lipid Res. <b>50</b>, 759–767 (2009).
26. Sato, C. et al. Characterization of the antigenic specificity of four different anti-alpha 2–>8-linked polysialic acid antibodies using lipid-conjugated oligo polysialic acids. J. Biol. Chem. <b>270</b>, 18923–18928 (1995).
27. Mazarei, G. et al. Expression analysis of novel striatal-enriched genes in Huntington disease. *Hum. Mol. Genet.* **19**, 609–622 (2010).

28. Schiffrmann, S. N., Jacobs, O. & Vanderhaegen, J. J. Striatal restricted adenosine A2 receptor (RDC8) is expressed by enkephalin but not by substance P neurons: an in situ hybridization histochemistry study. *J. Neurochem.* **57**, 1062–1067 (1991).

29. Levy, A. I. et al. Localization of D1 and D2 dopamine receptors in brain with subtype-specific antibodies. *Proc. Natl Acad. Sci. USA* **90**, 8861–8865 (1993).

30. Matsuoka, I., Suzuki, Y., Defer, N., Nakanishi, H. & Hanoune, J. Differential expression of type II IL, and V adenylyl cyclase gene in the postnatal developing rat brain. *J. Neurochem.* **68**, 498–506 (1997).

31. Simons, K. & Behehet, R. Cholesterol, lipid rafts, and disease. *J. Clin. Invest* **110**, 597–603 (2002).

32. Babuke, T. & Tikkanen, R. Dissecting the molecular function of reggie/flip domains. *Structural Biology*. **17**, e12432 (2018).

33. Harduin-Lepers, A. et al. Evolutionary history of the alpha2/3-sialyltransferase (ST8Sia) gene family: tandem duplications in early deuterostomes explain most of the diversity found in the vertebrate ST8Sia genes. *BMC Evol. Biol.* **8**, 258 (2008).

34. Takashima, S. et al. Molecular cloning and expression of a sixth type of alpha 2/3-sialyltransferase (ST8Sia VI) that sialylates O-glycans. *J. Biol. Chem.* **277**, 24030–24038 (2002).

35. Piirainen, H., Ashok, Y., Nanekar, R. T. & Jaakola, V. P. Structural features of adenosine receptors: from crystal to function. *Biochim. Biophys. Acta*. **1808**, 1233–1244 (2011).

36. Lin et al. Translational Psychiatry (2019) 9:209

37. Sebastiao, A. M., Assafaei-Lopes, N., Diogenes, M. J., Vaz, S. H. & Ribeiro, J. A. Modulation of brain-derived neurotrophic factor (BDNF) actions in the nervous system by adenosine A2A receptors and the role of lipid rafts. *Biochim. Biophys. Acta*. **1808**, 1340–1349 (2011).

38. Molinolo-Petricevic, J. et al. Protecting motor neurons from toxic insult by antagonism of adenosine A2A and Trk receptors. *J. Neurosci.* **26**, 9250–9263 (2006).

39. Varki, A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology*. **3**, 97–130 (1993).

40. Hildebrandt, H., Becker, C., Murau, M., Gerardy-Schahn, R. & Rahmann, H. Heterogeneous expression of the polysialyltransferases ST8Sia II and ST8Sia IV during postnatal rat brain development. *J. Neurochem.* **71**, 2339–2348 (1998).

41. Fevre, S., Fuxe, K., von Euler, G., Johansson, B. & Fredholm, B. B. Adenosine-dopamine interactions in the brain. *Neuroscience* **51**, 501–512 (1992).

42. Chen, J. F. et al. The role of the D2 dopamine receptor (D2R) in A2A adenosine receptor (A2AR)-mediated behavioral and cellular responses as revealed by A2A and D2 receptor knockout mice. *Proc. Natl Acad. Sci. USA* **98**, 1970–1975 (2001).

43. Fevre, S. et al. Allosteric mechanisms within the adenosine A2A-dopamine D2 receptor heterotramer. *Neuropharmacology* **104**, 154–160 (2016).

44. Navarro, G. et al. Evidence for functional pre-coupled complexes of receptor heteromers and adenylyl cyclase. *Nat. Commun.* **9**, 1242 (2018).

45. Beaulieu, J. M., Ganetdinov, R. R. & Caron, M. G. The Akt-GSK-3 signaling cascade in the actions of dopamine. *Trends Pharmacol. Sci.* **28**, 166–172 (2007).

46. Reiner, C. et al. Pharmacogenomics of GPCR drug targets. *Cell* **172**, 41–54 e19 (2018).

47. Cadet, J. L., Javanthi, S., McCoy, M. T., Beauvais, G. & Cai, N. S. Dopamine D1 receptors, regulation of gene expression in the brain, and neurodegeneration. *CNS Neurol. Disord. Drug Targets* **9**, 536–538 (2010).

48. Fevre, S., Diaz-Rios, M., Salamone, J. D. & Prediger, R. D. New developments on the adenosine mechanisms of the central effects of caffeine and their implications for neuropsychiatric disorders. *J. Caffeine Adenosine Res.* **8**, 121–131 (2018).

49. Fevre, S. An update on the mechanisms of the psychostimulant effects of caffeine. *J. Neurochem.* **105**, 1067–1079 (2008).

50. Criuella, F. et al. Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1-A2A receptor heteromers. *J. Neurosci.* **26**, 2080–2087 (2006).

51. Hildebrandt, H., Muhlenhoff, M., Weinhold, B. & Gerardy-Schahn, R. Dissecting polysialic acid and NCAM functions in brain development. *J. Neurochem.* **103**, 56–64 (2007).

52. Sato, C., Hane, M. & Kitajima, K. Relationship between ST8Sia2, polysialic acid and its binding molecules, and psychiatric disorders. *Biochim. Biophys. Acta*. **1860**, 1739–1752 (2016).

53. Kanato, Y., Kitajima, K. & Sato, C. Direct binding of polysialic acid to a brain-derived neurotrophic factor depends on the degree of polymerization. *Glycobiology*. **18**, 1044–1053 (2008).

54. Desplats, P. A. et al. Glycolipid and ganglioside metabolism imbalances in Huntington's disease. *Neurobiol. Dis.* **27**, 265–277 (2007).

55. Frank, S. Treatment of Huntington's disease. *Neurotherapeutics* **11**, 153–160 (2014).