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Carbohydrate–Neuroactive Hybrid Strategy for Metabolic Glycan Engineering of the Central Nervous System *in Vivo*

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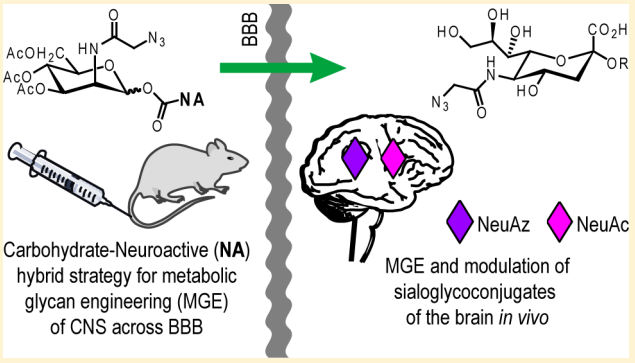
Supporting Information

ABSTRACT: Sialic acids are abundant in the central nervous system (CNS) and are essential for brain development, learning, and memory. Dysregulation in biosynthesis of sialo-glycoconjugates is known to be associated with neurological disorders, CNS injury, and brain cancer. Metabolic glycan engineering (MGE) and bioorthogonal ligation have enabled study of biological roles of glycans *in vivo*; however, direct investigations of sialoglycans in brain have been intractable. We report a simple strategy utilizing carbohydrate–neuroactive hybrid (CNH) molecules, which exploit carrier-mediated transport systems available at the blood–brain barrier, to access brain via tail vein injection in mice. Peracetylated *N*-azidoacetyl-D-mannosamine (Ac_4ManNAz) conjugated with neuroactive carriers, namely, nicotinic acid, valproic acid, theophylline-7-acetic acid, and choline, were synthesized and evaluated in SH-SY5Y (human neuroblastoma) cells for MGE. Intravenous administration of CNH molecules in mice (C57BL/6J and BALB/cByJ) resulted in robust expression of *N*-azidoacetyl-neuraminic acid (NeuAz)-carrying glycoproteins in both brain and heart, while the nonhybrid molecule Ac_4ManNAz showed NeuAz expression in heart but not in brain. Successful neuroactive carriers were then conjugated with *N*-butanoyl-D-mannosamine (ManNBut) with a goal to achieve modulation of polysialic acid (polySia) on neural cell adhesion molecules (NCAM). PolySia levels on NCAM in adult mice were reduced significantly upon administration of Ac_3ManNBut-nicotinate hybrid, but not with Ac_4ManNBut. This novel application of MGE not only offers a noninvasive tool for investigating brain glycosylation, which could be developed in to brain mapping applications, but also serves as a potential drug by which modulation of neural glycan biosynthesis and thus function can be achieved *in vivo*.



INTRODUCTION

Glycans, found in the form of glycoproteins, glycolipids, and glycosaminoglycans, play critical roles in the central nervous system (CNS) development, learning, memory, and behavior. Dysregulation of biosynthesis of these glycans is associated with neurological diseases and disorders such as Parkinson's disease, Alzheimer's disease, mucopolysaccharidosis, and autism.[1−3] Efforts to understand the role of glycans, sialic acids in particular, in brain development are at their infancy.[4] Approaches to investigate the functional roles of glycans in animals have long been limited to genetic methodologies.[5] Recently, development of metabolic glycan engineering (MGE) using non-natural monosaccharide analogues has paved the way for a complementary and versatile approach to engineer, tag, image, and intercept glycans in animals, bacteria, and plants.[6−9] MGE exploits the permissivity of the enzymes involved in biosynthetic glycosylation pathways to process monosaccharide analogues. Analogues of *N*-acetyl-D-mannosamine (ManNAc), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc), and L-fucose (Fuc) have been successfully employed for MGE.[10] The sialic acid biosynthetic pathway, wherein ManNAc is a committed precursor for the synthesis of *N*-acetyl-D-neuraminic acid (NeuAc), has been exploited for the expression of sialic acids carrying alkyl, ketone, azido, alkynyl, hydroxyl, and thiol functionalities on cell surface glycoconjugates.[11−14]

Reutter and co-workers first showed that intraperitoneal administration of *N*-propanoyl-D-mannosamine (ManNProp) to rats resulted in the expression of *N*-propanoyl-D-neuraminic acids (NeuProp) in various tissues.[15] Subsequently, Bertozzi and co-workers showed that peracetyl *N*-azidoacetyl-D-mannosamine (Ac_4ManNAz) could be metabolically converted to *N*-azidoacetyl-D-neuraminic acid (NeuAz), both *in vitro* and *in vivo*, enabling bio-orthogonal ligations of the abiotic azido-group.[16−18] MGE using Ac_4ManNAz has become an enabling technology for live imaging of glycans in *Caenorhabditis elegans*, zebra fish, and mice.[19] Ac_4ManNAz has been applied for imaging of tumor cell glycans and liposome-mediated targeting *in vivo*.[20,21] Peracetyl ManNProp (Ac_4ManNProp) has been

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shown to result in metabolic expression of NeuProp in mice.[22] Additionally, successful murine experiments have demonstrated metabolic engineering of mucin-type *O*-glycans using peracetyl *N*-azidoacetyl-D-galactosamine (Ac_4GalNAz).[23]

Three independent studies, so far, on treatment of mice *in vivo* with Ac_4ManNAz, Ac_4ManNProp, or Ac_4GalNAz have shown robust metabolic expression of engineered glycoconjugates in multiple peripheral tissues including heart, liver, and kidney. Intriguingly, very little or no expression was found in brain in all the three reports.[17,22,23] Lack of expression in the brain could arise due to two possible reasons. The rate of turnover for glycoprotein biosynthesis is not very high in brain, unlike other tissues, leading to lower incorporation.[17] Alternately, the monosaccharide analogues and their metabolites, due to their polar nature, were unable to penetrate the blood–brain barrier (BBB) and reach the brain (Figure 1). With

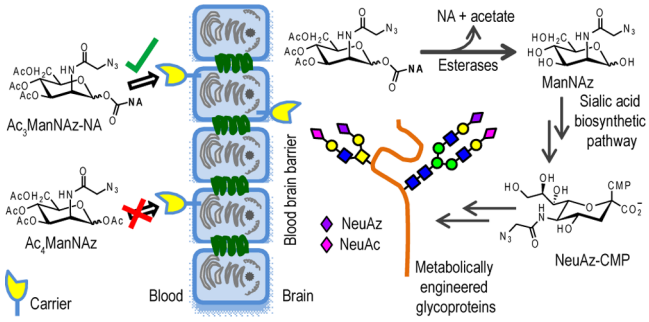

Figure 1. Piggybacking strategy using carbohydrate–neuroactive hybrid molecules. Prodrug forms of non-natural *N*-acetyl-D-mannosamine (ManNAc) analogues conjugated to neuroactive molecules could enable delivery to the central nervous system (CNS), across blood–brain barrier in mammals, exploiting the carrier-mediated transport. Once inside the CNS, metabolism of ManNAc analogues results in engineered sialic acids on glycoproteins in the brain.

the exception of D-glucose, which is transported to the brain via the glucose transporters,[24] monosaccharides and their analogues might not be able to cross the BBB by pinocytosis or passive diffusion.

The BBB consists of a compact assembly of endothelial cells, astrocytes, and pericytes that forms a formidable barrier to protect CNS. At the same time, multiple mechanisms such as receptor-mediated transport (RMT), transcytosis, pinocytosis or diffusion through the tight junctions, and carrier-mediated transport (CMT) operate across the BBB to ensure regulated supply of essential nutrients to CNS.[25,26] Particularly, cells of the BBB express a repertoire of CMT systems for sequestering of amino acids, fatty acids, vitamins, and trace elements from the blood circulation.[27] Recent efforts on drug delivery to the brain have focused mostly on vector or RMT of antibodies and liposomes.[28] On the other hand, numerous small molecule CNS drug candidates with potent activities *in vitro* suffer from poor BBB permeability.[29] Various approaches, including chemical delivery system and conjugation to glucose, have been developed for delivery of small molecules to the CNS.[27,30]

We envisaged that the poor BBB permeability of carbohydrate analogues might be overcome by piggybacking them on neuroactive carrier molecules in a prodrug form (Figure 1). Herein, we present design, synthesis, and *in vitro* and *in vivo* evaluation of carbohydrate–neuroactive hybrid (CNH) molecules of *N*-acyl-D-mannosamine derivatives carrying *N*-azidoacetyl, *N*-acetyl, and *N*-butanoyl moieties (**2**–**5**, **8**, and **9**) for MGE of CNS across the BBB in mice. Corresponding parent molecules Ac_4ManNAz (**1**), Ac_4ManNAc (**6**), and Ac_4ManNBut (**7**) were employed as nonhybrid controls (Figure 2). We

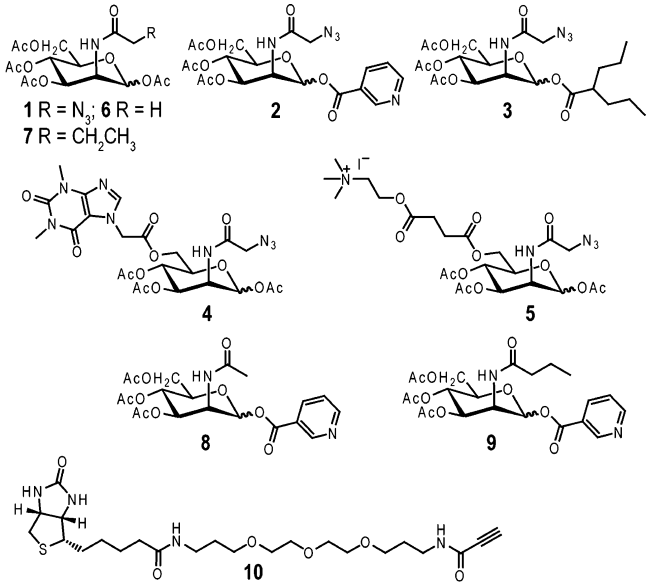

Figure 2. Design of carbohydrate–neuroactive hybrid molecules. Chemical structures of the parent peracetylated ManNAc analogues, *viz.*, *N*-azidoacetyl (**1**), *N*-acetyl (**6**), *N*-butanoyl (**7**), and their respective neuroactive hybrid molecules employed in this study. Nicotinate (**2**, **8**, and **9**) and valproate (**3**) moieties were attached at the C-1 position, while theophylline-7-acetyl (**4**) and choline (**5**) moieties were attached at the C-6 position. The biotin-linker propiolamide **10**, which exhibited improved reactivity, was used for copper(I)-mediated azide–alkyne cycloaddition (Cu-AAC) reaction.

demonstrate that these non-natural prodrug-like hybrid compounds are readily able to penetrate the BBB and incorporate in to CNS tissues as opposed to nonhybrid compounds. Although brain tissue is known to be rich in glycolipids, including gangliosides, in our investigations we focused on glycoproteins in order to make direct comparison with *in vivo* properties of **1**. A propiolamide based water-soluble biotin reagent (**10**) was synthesized which exhibited better reactivity and sensitivity in copper(I)-assisted azide–alkyne cycloaddition (Cu-AAC) reaction compared to the propargylamide derivative.

RESULTS AND DISCUSSION

Rational Design and Synthesis of CNH Molecules. First, in order to study the ability of neuroactive molecules to deliver non-natural monosaccharide analogues to the CNS, we chose Ac_4ManNAz (**1**) as the parent compound (Figure 2). Ac_4ManNAz was derivatized as a biocleavable ester with nicotinic acid (**2**) or valproic acid (**3**) at the C-1 position (see Supporting Information). Nicotinic acid (vitamin B_3, niacin), an essential building block for biosynthesis of nicotinamide adenine dinucleotide (NAD) cofactors, is known to be carried to the brain by nicotinate receptors.[31] Valproate, an established drug for treatment of epilepsy, is carried to the CNS via medium-chain fatty acid transporters.[32] Theophylline-7-acetyl moiety, a mimic of caffeine, was chosen to exploit the adenine transporters[33] and was attached at the C-6 position of ManNAz (**4**). Choline ester moiety, a known substrate for acetylcholine esterases (AChE) found abundantly at the neuromuscular

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junctions and transported by choline transporters,[34] was attached to the C-6 position of ManNAz via a succinyl linker (**5**). Notably, analogues of ManNAz hybrids with theophylline-7-acetic acid and choline attached at C-1 position were found to be unstable due to spontaneous ester cleavage. Second, peracetyl derivatives of ManNAc (Ac_4ManNAc, **6**) and *N*-butanoyl-D-mannosamine (Ac_4ManNBut, **7**) were chosen for modulation of polysialic acid.[35] Corresponding CNH molecules carrying nicotinate (**8** and **9**) at the C-1 position were designed for delivery across BBB. Third, metabolic expression of NeuAz, *in vitro* in SH-SY5Y cells and *in vivo* in C57BL/6J and BALB/cByJ mice, was characterized using a novel biotin-linker propiolamide **10** via Cu-AAC reaction.[36−40]

Metabolism of CNH Molecules *in Vitro*. SH-SY5Y (human neuroblastoma), a widely used human neuronal cell line,[41] was chosen for the *in vitro* evaluation of metabolic acceptability of **1**−**5** by flow cytometry, confocal imaging, and far-Western blotting using horseradish peroxidase-conjugated avidin (HRP-avidin), after Cu-AAC biotinylation using **10**.

Earlier studies on Cu-AAC mostly employed propargylamide derivatives for bio-orthogonal ligations.[42,43] Based on reports that propiolamides exhibit three to 50 times faster reactivity in Cu-AAC compared to propargylamides,[44] we designed the water-soluble biotin-linker-propiolamide **10**. Conditions for Cu-AAC biotinylation of total cell lysates with **10** were optimized using two cuprous ion stabilizing ligands, *viz.*, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) and tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), and various concentrations of reagents (Supporting Information Figure S1). A comparative study of the reactivity and sensitivity of biotinylation using **10** and the known biotin-linker-propargylamide (**11**)[42,43] showed that **10** was at least four times more sensitive than **11** (Supporting Information Figure S2).

Estimation of cell surface expression of NeuAz was studied by flow cytometry. SH-SY5Y cells were incubated with either dimethyl sulfoxide (DMSO (D)) or **1** (50 μM) for 48 h, harvested, fixed, biotinylated with **10** via Cu-AAC, and stained with FITC-conjugated avidin. Robust expression of NeuAz was observed on the cell surface upon treatment with **1** (Figure 3a). Cells incubated with vehicle and treated with **10** showed fluorescence levels similar to control cells indicating the high selectivity of **10** for azido- groups. Additional evidence of metabolic processing of **1** by SH-SY5Y cells was obtained by Cu-AAC biotinylation with **10** and detection using AlexaFluor-594-conjugated avidin by confocal microscopy (Figure 3b and Supporting Information Figure S3). A comparative flow cytometry study revealed that the valproate hybrid **3** incorporated at similar levels as the parent **1** while nicotinate **2** and theophylline-7-acetyl derivative **4** incorporated at a moderate level (Figure 3c). Cell surface biotinylation levels with choline derivative **5** were similar to vehicle (D) treated cells indicating poor cellular uptake of **5**, possibly due to its ionic nature.

The expression of NeuAz on sialoglycoproteins in the total cell lysates was studied by SDS-PAGE. HRP-avidin blotting of lysates of SH-SY5Y cells—incubated with D, **1**, **2**, **3**, **4**, or **5** (50 μM, 48 h), lysed, and subjected to Cu-AAC biotinylation using **10**—showed robust biotinylation (Figure 3d) with **2**− **4** indicating that CNH molecules were metabolized similar to the parent nonhybrid **1**. Consistent with the flow cytometry results, very little NeuAz levels were observed with **5** due to poor cellular uptake. No biotinylation was detected in control cells incubated with vehicle (D) indicating the high selectivity of

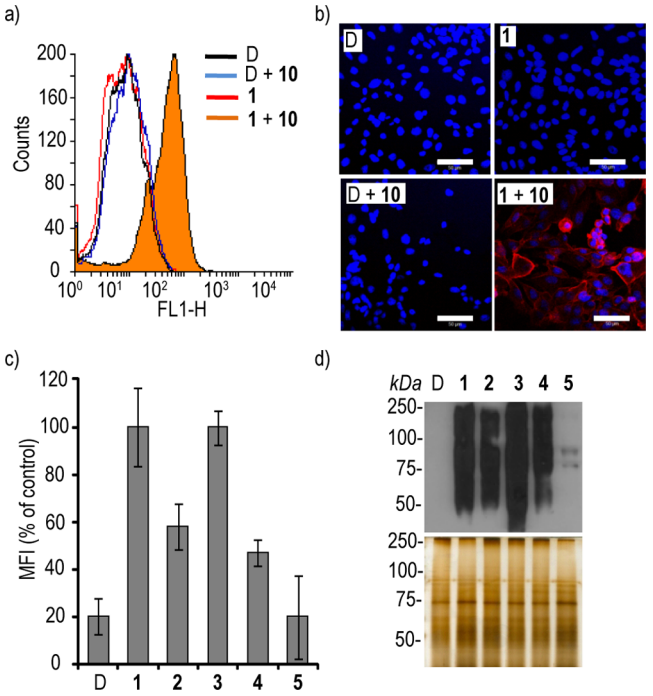

Figure 3. Metabolism of CNH molecules *in vitro*. SH-SY5Y cells were incubated with DMSO (D) or **1** (50 μM) for 48 h, fixed, subjected to Cu-AAC biotinylation with and without **10**, and stained with either (a) FITC-conjugated avidin for flow cytometry or (b) AlexaFluor-594 conjugated avidin (in red) for confocal microscopy; nuclei were stained with DAPI (in blue). Cells were incubated with D, **1**, **2**, **3**, **4**, or **5** (50 μM). After 48 h, cells were either (c) fixed, biotinylated using **10**, stained with FITC-conjugated avidin, and analyzed by flow cytometry or (d) lysed, biotinylated using **10**, and analyzed by far-Western blotting using HRP-avidin. Silver stained gel is shown as loading control. Error bars shown are standard deviation of three replicates. MFI, mean fluorescence intensity; scale bar, 50 μm.

the Cu-AAC reaction with **10** under the conditions. Silver staining of gels in parallel was used as loading control.

Evaluation of cytotoxicity and viability of SH-SY5Y cells treated with analogues **1**−**3** and **6**−**9** (0−500 μM) using MTT assay showed that 80−85% cells were viable at a concentration of 250 μM, similar to the cells treated with vehicle control. Cell viability studies, with analogues **1**−**3** (at 100 and 250 μM for 48 h), using FITC-conjugated annexin-V and propidium iodide revealed similar results (Supporting Information Figures S4 and S5).

MGE of Brain Tissue across the BBB via Tail Vein. Having confirmed efficient metabolism of CNH molecules *in vitro*, we focused on studies in mice. First, we tested metabolic expression of NeuAz-carrying glycoproteins in C57BL/6J (6 weeks old, male) mice upon administration of the parent nonhybrid **1** by various routes (Figure 4a).[17] Mice tissues, including heart and brain, were harvested after euthanasia (under ketamine/xylazine anesthesia), homogenized, and lysed. The tissue lysates were subjected to Cu-AAC biotinylation with **10**, and the proteins were precipitated. The precipitates were resolubilized and probed by HRP-avidin blotting, and silver stained gels were used as loading controls.

Intraperitoneal (i.p.) administration of **1** (300 mg/kg, once daily for 7 days), either alone or preceded by treatment with 2.0 M of D-mannitol (a hyper-osmolar agent known to disrupt the BBB), resulted in robust expression in heart, liver, and spleen, but not in the brain (Figure 4a and Supporting Information

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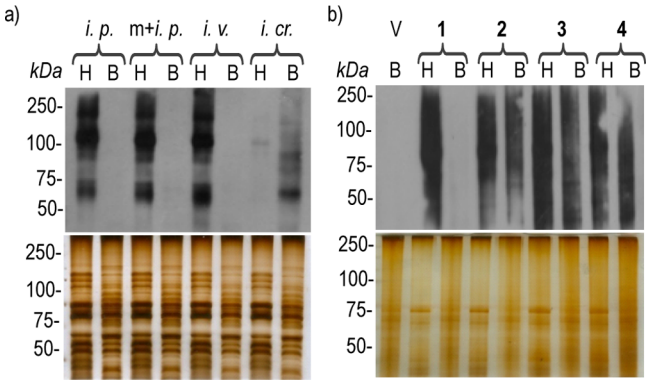

Figure 4. Metabolic glycan engineering *in vivo* of brain tissue by CNH molecules. a) HRP-avidin blots of total tissue lysates of heart (H) and brain (B) from C57BL/6J mice treated with **1** (at 300 mg/kg, once daily for 7 days) via multiple modes of administration, viz., intraperitoneal (i.p.), intraperitoneal along with D-mannitol (2.0 M) (m+i.p.), intravenous (i.v.), and intracranial (i.cr.) (2.0 mg of **1** per mouse, once daily for 3 days). Each mode of administration consisted of four animals per group except for intracranial treatment where two animals were employed. b) HRP-avidin blots of total tissue lysates of H and B from mice treated i.v. with vehicle (V), **1**, **2**, **3**, or **4** (0.26 mmol/kg, once daily for 3 days). The tissue lysates were subjected to Cu-AAC with **10**, resolved on SDS-PAGE, blotted, and detected using HRP-avidin. Blots shown are representative of at least two replicates. Silver stained gels are shown as loading controls.

Figure S6a), consistent with previous reports.[17] Mouse-to-mouse variations were tested by the analysis of heart and brain tissues from a group of four mice, treated with **1**, all of which showed absence of NeuAz expression in brain (Supporting Information Figure S6b). Bioavailability of **1** through i.p. administration might be limited due to hydrolysis of acetate groups in the intraperitoneal cavity resulting in polar partially acetylated intermediates and the free monosaccharide. Resorting to intravenous administration of **1**, in order to improve its bioavailability, again showed expression in heart but not in the brain thus confirming its inability to reach CNS. However, it is possible that metabolic pathways in CNS are more tightly regulated even if **1** were to reach CNS. This possibility was ruled out by intracranial (i.cr.) administration of **1** (2.0 mg per mouse, once daily for 3 days) which showed ready expression of NeuAz in brain sialoglycoproteins. Also, NeuAz expression was found to be minimal in the heart upon i.cr. administration indicating the inability of **1** to be reverse transported from brain to blood (Figure 4a). This showed that metabolic pathways in CNS are indeed susceptible to MGE similar to peripheral tissues and confirmed the inability of **1** to cross the BBB.

Strikingly, i.v. administration of CNH molecules **2**, **3**, or **4** (0.26 mmol/kg, corresponding to molar equivalence of 112 mg/kg of **1**, once daily for 3 days) via the tail vein in C57BL/6J mice showed robust expression of NeuAz-carrying sialoglycoproteins both in heart and brain (Figure 4b). This result showed that the simple strategy of piggybacking of carbohydrates on neuroactive carrier molecules indeed facilitated their transport across BBB and achieved MGE of the CNS. Studies in another commonly used strain of mice, namely BALB/cByJ (6 weeks old, male), showed similar results with efficient sialic acid engineering of brain tissue with **2**, **3**, and **4**, but not with **1** (Supporting Information Figure S7a).

Brain is provided with oxygen and nutrients through its extensive interpenetrating network of microcapillary blood vessels. The possibility that brain tissue might be contaminated with sialic acid engineered hematopoietic cells was eliminated by employing perfusion. Studies on brain and heart tissues from BALB/cByJ mice that were subjected to trans-cardial perfusion with PBS, prior to euthanization and tissue harvest, showed similar results thus indicating that the NeuAz observed are indeed from the brain cells (Supporting Information Figure S7b). Taken together with the absence of NeuAz in brain tissue from mice treated with the parent nonhybrid **1**, these results confirm that the observed NeuAz-labeling is not a result of contaminating hematopoietic cells. The unique and consistent ability of **2** to access and metabolically engineer brain glycoproteins, but not **1**, was confirmed with a group of BALB/cByJ mice (n = 6 for each condition) (Supporting Information Figure S8). Further support for the presence of NeuAz in brain tissues was obtained by treatment of tissue lysates with neuraminidase,[20] prior to Cu-AAC, which showed reduction in biotinylation levels as a function of neuraminidase concentration (Supporting Information Figure S9).

Mice were evaluated for *in vivo* toxicity of analogues through histology and serum parameters. An independent histopathological analysis of liver sections of mice treated with **1** or **2** (0.26 mmol/kg, i.v. once daily for 3 days and harvested on day four) did not show any visible changes compared to untreated and vehicle treated controls (Supporting Information Figure S10). Levels of creatinine and activity of liver enzymes were found to be within the standard range (Supporting Information Table S1).

Next, we turned our attention to test if the MGE of CNS induced by the metabolism of CNH molecules were dose dependent. Mice were injected i.v. with **1**, **2**, or **3** at three concentrations (molar equivalents corresponding to 75, 115, and 150 mg/kg of **1**), once daily for 3 days (Supporting Information Figure S11). HRP-avidin blots after Cu-AAC

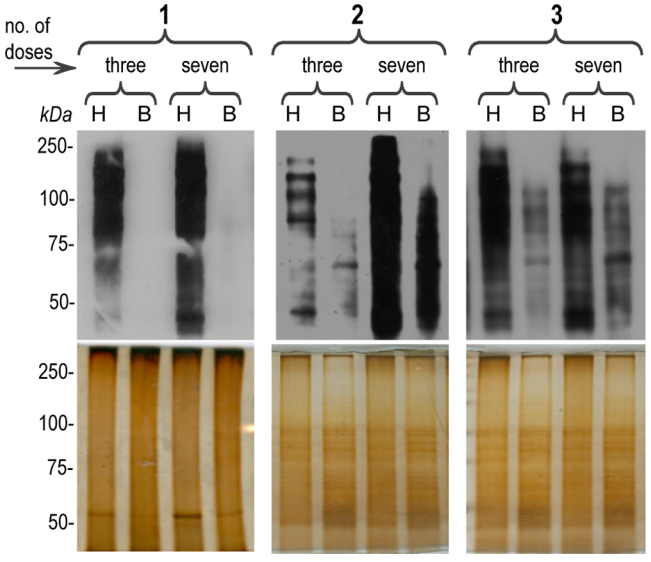

Figure 5. Effect of number of doses on MGE by CNH molecules in mice. HRP-avidin blots (top panels) and silver stained gels (bottom panels) of total lysates of heart (H) and brain (B) subjected to Cu-AAC biotinylation using **10**. BALB/cByJ mice were injected i.v. with **1**, **2**, or **3** (0.26 mmol/kg) for either 3 days or 7 days, once daily. Mice were euthanized and organs were harvested on day 4 and day 8, respectively. Four mice were studied in each group. Blots and silver stained gels (loading controls) shown are representative of at least two replicates.

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biotinylation showed intense bands in heart tissue, but not in brain, in mice treated with **1** at all concentrations. The animals treated with **2** and **3** showed robust expression in the brain but with moderate degree of dose dependency. The moderate increase in NeuAz expression as a function of concentration supported the characteristic mechanism of CMT of CNH molecules which is saturable, unlike passive diffusion.[31,32] Next, we studied the effect of number of doses at a given concentration. Mice were injected i.v. with **1**, **2**, or **3** (at 0.26 mmol/kg once daily) for three or 7 days. Again, **1** showed expression in heart but not in brain even after seven doses (Figure 5). Interestingly, significant increase in biotinylation levels were observed at seven doses compared to three doses, in brain tissues of mice treated with **2** and **3**. Thus, the saturable CMT could be overcome by increasing number of doses at a given concentration over longer periods.

***In Vitro* and *in Vivo* Modulation of PolySia on NCAM by CNH Molecules.** Apart from enabling selective tagging of glycans via MGE, non-natural monosaccharide analogues have also been successfully employed to intercept and inhibit glycan biosynthesis.[11,41,45−47] Polysialic acid (polySia) expression on the neural cell adhesion molecule (NCAM) is critical during CNS development, learning, and memory.[4,48] On the contrary, overexpression of polySia in brain tumor and metastasis presents therapeutic challenges.[41] PolySia consists of 50−200 units of NeuAc units attached in an $\alpha(2\rightarrow8)$ linkage to the IgG-V domain of NCAM, usually to the 180 kDa isoform. Earlier studies *in vitro* have shown that metabolism of *N*-butanoyl-D-mannosamine (ManNBut) results in the inhibition of biosynthesis of polySia on NCAM in NT2 neurons, SH-SY5Y cells, and chick dorsal root ganglia neurons.[35,49,50] By contrast, the natural precursor ManNAc has been shown to increase the flux of sialic acid biosynthesis.[51]

Prior to embarking on *in vivo* modulation of polySia, the analogues **6**, **7**, **8**, and **9** were evaluated *in vitro*. SH-SY5Y cells were incubated with analogues **6**−**9** (at 100 μM) on poly-L-lysine-coated glass coverslips. After 72 h, cells were fixed, permeabilized, immunostained for polySia (12F8) and NCAM (OB11), and studied by confocal microscopy (Figure 6). Results showed that the non-natural *N*-butanoyl analogues **7** and **9** clearly reduced the polySia (12F8) expression, while the natural *N*-acetyl analogues **6** and **8** showed polySia (12F8) expression similar to vehicle treated controls. NCAM (OB11) levels were unaffected in all cases. These results confirmed the ability of *N*-butanoyl analogues to selectively inhibit polySia biosynthesis. Consistent with earlier reports,[35] measurement of total sialic acid levels using periodate−resorcinol assay of cells treated with **6**−**9** did not show any reduction in total sialic acid levels (Supporting Information Figure S12).

Previous studies in mice using Ac_4ManNProp have shown very little expression of *N*-propanoyl-D-neuraminic acid in brain even after 90 doses (i.p. 200 mg/kg; twice a day for 45 days).[22] However, some conversion of polySia-NCAM to *N*-propanoyl-polySia-NCAM could be detected by Western blotting after 26 injections (i.p. 13 days, twice a day). In order to test if the piggybacking strategy could be applied for modulation of polySia-NCAM, the nicotinate hybrids **8** and **9**, respectively, carrying *N*-acetyl and *N*-butanoyl moiety were administered (i.v. at 0.36 mmol/kg on alternate days for 7 days, a total of four injections) to BALB/cByJ mice (4 weeks old, male, n = 4 for each group). Nonhybrids **6** and **7**, at equimolar doses, were employed as controls. On day eight, the brain tissues were harvested after euthanasia and probed by Western blotting.

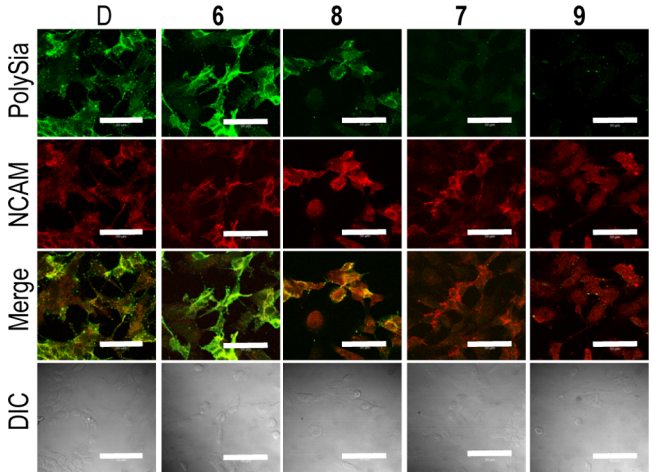

Figure 6. Effect of CNH molecules on polySia-NCAM expression. SH-SY5Y cells were treated with DMSO (D), **6**, **7**, **8**, or **9** (at 100 μM) and cultured on poly-L-lysine-coated coverslips. After 72 h, cells were fixed, permeabilized, immunostained with anti-polySia antibody (12F8) (in green) and anti-NCAM antibody (OB11) (in red) followed, respectively, by appropriate AlexaFluor-488-conjugated and Cy3-conjugated secondary antibodies and imaged by confocal microscopy. Images shown are representatives of at least two replicate samples. DIC, differential interference contrast; scale bar, 50 μm.

Anti-polySia (12F8) and anti-NCAM (OB11) antibodies were employed, respectively, to probe the levels of polySia glycans and NCAM protein levels.

The brain tissue lysates of mice treated with vehicle (V), **6**, **7**, or **8** showed diffuse bands in the 250−150 kDa range in anti-polySia (12F8) blots indicating the microheterogeneity in the chain length of polySia; anti-NCAM (OB11) showed diffuse bands from 230 to 180 kDa, sharp bands at 140 kDa, and faint bands at 120 kDa, corresponding to variable polypeptide lengths (Figure 7a). Interestingly, in the case of brain lysates of mice treated with Ac_3ManNBut-nicotinate hybrid (**9**), less diffuse

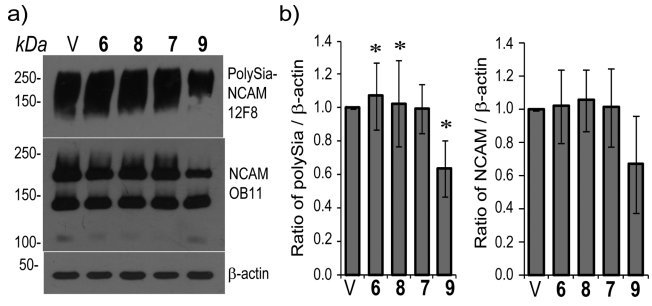

Figure 7. Modulation of polySia on NCAM *in vivo* by CNH molecules. (a) Western blots of lysates of brain tissues probed with anti-polySia (12F8) and anti-NCAM (OB11) antibodies. BALB/cByJ mice were treated i.v. with vehicle (V), **6**, **7**, **8**, or **9** (0.36 mmol/kg on alternate days, a total of four injections), euthanized on day eight, brain tissues were harvested, homogenized, and lyzed. β-actin blots are shown as loading control. Each study group consisted of four mice. Blots shown are representative of at least two replicates. (b) Densitometry analysis of the blots using ImageJ as a ratio of polySia/β-actin (left panel) and NCAM/β-actin (right panel). Error bars are standard deviation obtained from four independent blots. *One-way ANOVA with Tukey's multiple comparison post-hoc tests (n = 4; five groups) showed significantly different means between **6** vs **9** and **8** vs **9** with $p <$ 0.05. No significant differences of means were found for other groups for PolySia and for all groups for NCAM at 95% confidence interval.

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bands in the range 250−200 kDa for anti-polySia (12F8) and sharp bands at 180 kDa for anti-NCAM (OB11) were observed. The bands with retarded mobility (at higher M_r) in 12F8 blots are indicative of reduced sialylation levels and inhibition of polySia biosynthesis, which is also reflected by the absence of diffused bands in the NCAM (OB11) blots. By contrast, the nonhybrid Ac_4ManNBut (**7**) did not alter polySia levels indicating its inability to cross the BBB. Western blotting data were obtained from brain lysates of four independent groups of mice (n = 4 for each condition, five groups) (Supporting Information Figure S13).

A densitometry analysis of Western blots obtained from four independent sets of mice showed that the ratio of anti-polySia to β-actin was significantly reduced to 60% of controls upon treatment with **9** (Figure 7b). Statistical analysis revealed significant differences of the mean for **6** vs **9** and **8** vs **9** for polySia expression. No significant difference of mean was found for the rest of the groups for polySia and for all groups for NCAM expression. Considering that these studies were conducted in 4 weeks old mice, wherein polySia levels are known to be much lower than in newborn mice,[4] the reduction levels observed are quite significant. The significant reduction in polySia levels achieved using **9** in live adult mice with only four i.v. injections, clearly confirms the unique ability of CNH molecules to access and modulate the glycans of the CNS across BBB.

CONCLUSIONS

In summary, the simple strategy of piggybacking carbohydrate analogues on neuroactive molecules in the form of CNH is an efficient approach to access the CNS across BBB for MGE. Expression of abiotic NeuAz in brain was used as a read-out for identification of neuroactive carriers such as nicotinate, valproate, and theophylline-7-acetic acid. Neuroactive carrier molecules thus identified were conjugated to ManNBut and were shown to modulate expression of polySia *in vivo*, which provides an attractive tool for functional and behavioral studies (a summary of analogues used in this study and their properties is provided in Supporting Information Table S2). Recently Xie and co-workers have shown engineering of sialoglycans in brain tissue *in vivo* using liposomes loaded with 9-azidosialic acid.[52] However, it is known that the metabolic flux is more efficient with ManNAz as a precursor compared to both 9-azidosialic acid and NeuAz.[53] Notably, it is possible that liposomes given over a long period might compromise the integrity of BBB. CNH strategy using mannosamine precursors shown here provides a simple alternative approach. Very little is known about the importance of sialoglycoconjugates in CNS development, learning, memory, behavior, neurodegenerative disorders, and diseases such as autism and epilepsy. In the context of intense efforts globally for enhanced tool, imaging techniques, and understanding of brain function, the molecules and strategy presented here are an attractive platform upon which to study structure and function of CNS sialoglycoconjugates.[54,55] CNH molecules reported here have the potential to be employed as powerful tools to shed light on the functional chemical glycobiology of CNS in development and diseases.

EXPERIMENTAL PROCEDURES

Experimental Procedures for *in Vivo* Studies in Mice. *General Comments.* The animal protocols and experiments were approved by the Institutional Animal Ethics Committee (IAEC # 252/11), National Institute of Immunology (NII). Male mice of C57BL/6J and BALB/cByJ strains (4−6 weeks old) were procured from the experimental animal facility (EAF), NII and employed for the studies. Mice were provided with food and water *ad libitum* and maintained under standard conditions. Parameters of weight, health, behavior, and morbidity were monitored regularly. Studies were conducted with four mice in each group unless mentioned otherwise. No adverse effects in terms of weight or behavior were noticeable during the studies. Animals were anesthetized using intraperitoneal (i.p.) administration of ketamine and xylazine (80 mg/kg and 10 mg/kg, respectively, prepared in sterile water) solutions prior to intravenous (i.v.) and intracranial (i.cr.) injections of monosaccharide analogues, unless mentioned otherwise. Animals were euthanized by cervical dislocation under ketamine/xylazine anesthesia. For i.v. injections, stock solutions of monosaccharide analogues **1**−**9** at various concentrations were prepared in 90% DMSO (DMSO:water 9:1) and sterile filtered through 0.2 μm PTFE syringe filters. For i.p. and i.cr. studies, 70% DMSO (DMSO:water 7:3) was employed as a vehicle. Tissues were homogenized in PBS in an ice−water bath at 15000 rpm using a 7.0 mm dispersing aggregate on a homogenizer (Kinematica Cat. No. PT-DA 07).

Administration of Compounds in Mice. *Intraperitoneal (i.p.) Administration.* C57BL/6J mice (four mice per group) were injected with either vehicle (70% DMSO, DMSO:water 7:3) or **1** (at 150 and 300 mg/kg from a stock solution of 50 mg/mL), in the peritoneal cavity using a 26G needle. Treatments were administered once daily for 3 or 7 days. For studies on disruption of BBB using hyper-osmolarity, a solution of D-mannitol (100 μL of 2.0 M) in PBS was first injected peritoneally followed by i.p. injection of **1** (300 mg/kg) in 70% DMSO (DMSO:water 7:3) after 10 min.

Intravenous (i.v.) Administration. C57BL/6J or BALB/cByJ mice were first anesthetized using ketamine/xylazine i.p. injection (unless mentioned otherwise). After 10−15 min mice were injected with vehicle (90% DMSO, DMSO:water 9:1), **1**, **2**, **3**, or **4** (at 0, 75, 115, 150, 300 mg/kg equivalent of **1** corresponding to 0, 0.17, 0.26, 0.34, 0.69 mmol/kg) intravenously through the tail vein from a stock solution of 50 mg/mL in 90% aq. DMSO for 3 or 7 days (once daily).

Intracranial (i.cr.) Administration. C57BL/6J mice were anesthetized and injected with 2.0 mg of **1** per mouse from a stock solution 100 mg/mL in 70% DMSO (DMSO:water 7:3) using a 30G needle.[56] Treatments were administered once daily for 3 days carefully avoiding the previously injected sites.

Euthanization and Harvest of Organs from Mice. On the day of harvest (day 4 or day 8), mice were anesthetized using ketamine/xylazine and euthanized by cervical dislocation, and tissues were harvested. The organs were placed in ice-cold PBS and stored at −80 °C until further use. In some experiments, blood was replaced by transcardial perfusion of ice-cold PBS (20 mL over 20 min) by cardiac puncture and letting out through the jugular vein.

Processing of Tissues, Homogenization, Lysis Preparation, Biotinylation, and HRP-Avidin Blotting. Tissues were thawed to room temperature and washed thoroughly with ice-cold PBS to remove blood stains. Tissues were homogenized in PBS (5.0 mL) using a homogenizer at 15000 rpm in an ice−water bath until complete homogenization (care was exercised to prevent carry-over contamination between samples by thorough cleaning of the dispersing aggregate). Supernatants were discarded, and the homogenates were washed multiple times with PBS (5.0 mL per wash) to remove blood residuals. The pellets from the homogenates were treated with the lysis buffer (10 mM Tris, 150 mM NaCl, 1.0% NP-40, and protease inhibitor cocktail, pH 7.4) (2.0 mL). Lysates were clarified by centrifugation (16000g, 30 min) at 4 °C, and the supernatants were used for protein estimation, Cu-AAC biotinylation reaction using **10**, and probed by HRP-avidin blotting (see Supporting Information).

Neuraminidase Treatment of Tissue Lysates Prior to Cu-AAC. Tissue lysates (20 μg) prepared from the brain and heart tissues of mice treated with **3** (i.v. 135 mg/kg, once daily for 3 days) were incubated with neuraminidase from *Clostridium perfringens* (New England Biolabs Cat. No. P0720S) (0, 25, 50, and 100 units) at a reaction volume of 20 μL in G1 reaction buffer (50 mM sodium citrate, pH 6.0) at 37 °C for 12 h. Proteins were subjected to Cu-AAC biotinylation using **10**. The

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proteins were precipitated using chloroform/methanol/water, resolubilized (using 1.0% SDS in TBS), and subjected to HRP-avidin blotting (see Supporting Information).

Western Blotting for polySia and NCAM Levels in Brain Tissue of Mice. BALB/cByJ mice (4 weeks old male) were treated intravenously via tail vein with vehicle (DMSO:water 9:1), **6**, **7**, **8**, or **9** (0.36 mmol/kg) after induction of anesthesia using ketamine/xylazine. Injections were given once on alternate days for a total of four injections. Each group consisted of four mice. On day eight, mice were euthanized, brain tissues were harvested, homogenized, and lysed in RIPA buffer containing protease inhibitor cocktail, and protein concentrations were estimated. Proteins were resolved on a 7.5% SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were blocked using 5.0% nonfat milk (NFM) in PBS-T (0.1% Tween-20), incubated with primary antibodies—anti-polySia (12F8, 1:2500; Beckton-Dickinson Cat. No. 556325) or anti-NCAM (OB11, 1:5000; Sigma-Aldrich, Cat. No. C9672)—in 5.0% NFM/PBS-T overnight at 4 °C. Blots were washed with PBS-T, incubated with HRP-conjugated secondary antibodies (anti-rat IgM for 12F8, Abcam, Cat. No. ab98373; anti-mouse IgG for OB11, Jackson Immunologicals, Cat. No. 715035150), and probed using enhanced chemiluminescence (ECL) reagents. Anti-β-actin (Sigma-Aldrich, Cat. No. 1978) blots were performed in parallel as loading controls.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b08894.

Supporting results and discussion, supporting Figures S1−S13, supporting tables, experimental details for the synthesis and characterization of compounds **2**−**5** and **8**−**10**, *in vitro* studies on SH-SY5Y cells, *in vivo* studies in mice, and ^{1}H and ^{13}C NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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