In vivo metabolic labeling of sialoglycans in the mouse brain by using a liposome-assisted bioorthogonal reporter strategy

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Mammalian brains are highly enriched with sialoglycans, which have been implicated in brain development and disease progression. However, in vivo labeling and visualization of sialoglycans in the mouse brain remain a challenge because of the blood–brain barrier. Here we introduce a liposome-assisted bioorthogonal reporter (LABOR) strategy for shuttling 9-azido sialic acid (9AzSia), a sialic acid reporter, into the brain to metabolically label sialoglycoconjugates, including sialylated glycoproteins and glycolipids. Subsequent bioorthogonal conjugation of the incorporated 9AzSia with fluorescent probes via click chemistry enabled fluorescence imaging of brain sialoglycans in living animals and in brain sections. Newly synthesized sialoglycans were found to widely distribute on neuronal cell surfaces, in particular at synaptic sites. Furthermore, large-scale proteomic profiling identified 140 brain sialylated glycoproteins, including a wealth of synapse-associated proteins. Finally, by performing a pulse–chase experiment, we showed that dynamic sialylation is spatially regulated, and that turnover of sialoglycans in the hippocampus is significantly slower than that in other brain regions. The LABOR strategy provides a means to directly visualize and monitor the sialoglycan biosynthesis in the mouse brain and will facilitate elucidating the functional role of brain sialylation.

brain | sialic acid | live imaging | glycoproteomics | histochemistry

Sialic acids are a family of negatively charged monosaccharides that are commonly expressed as outer terminal residues of cell surface glycans and widely distributed throughout mammalian tissues (1). Intriguingly, the brain is the organ with the highest level of sialylated glycans and the only organ, in mammals, with more sialic acids carried by glycolipids than glycoproteins (2). Accumulating evidence indicates that sialic acids are an essential nutrient for brain development and cognition (3). Gangliosides (i.e., glycosphingolipids containing α 2,3-linked sialic acids) undergo dramatic changes in both structural complexity and expression density as the brain develops and matures (4). Polysialic acid (PSA), a linear α 2,8-linked polymer of sialic acid, is predominantly attached to the N-glycans of neural cell adhesion molecule, which regulates neuronal differentiation and migration (5). In addition, α 2,3-linked sialic acids and, less commonly, α 2,6-linked sialic acids terminate N-glycans and O-glycans on synaptic proteins, mediating neural transmission and synaptic plasticity (6, 7). Aberrant sialylation has been implicated in cancer cell metastasis to the brain (8), lysosomal storage disorders (9), and neurodegenerative diseases (10).

Sialic acid metabolism can be probed in vivo using the recently emerged bioorthogonal chemical reporter strategy, in which analogs of sialic acid or its biosynthetic precursor *N*-acetylmannosamine (ManNAc) containing a chemical reporter (e.g., the azide) are used as metabolic tracers for labeling sialoglycans in live cells and in living animals (11). To label sialoglycans in living mice or rats, peracetylated *N*-azidoacetylmannosamine (Ac₄ManNAz) was

intraperitoneally (i.p.) injected into living animals and metabolically converted to the corresponding azido sialic acid, which was incorporated into the sialoglycans in a panel of organs, including the heart, kidney, and liver (12). Reacting the azides with an alkyne-containing fluorescent probe via bioorthogonal chemistry (e.g., copper-free click chemistry) enabled imaging of cardiac sialoglycans in intact rat hearts and revealed the up-regulation of sialylation during cardiac hypertrophy (13). However, sialoglycans in the brain cannot be labeled or visualized by using this strategy, presumably due to the inability of azidosugars to cross the blood–brain barrier (BBB).

Herein, we report the development of a liposome-assisted bioorthogonal reporter (LABOR) strategy for metabolic labeling of brain sialoglycans with 9-azido sialic acid (9AzSia), a sialic acid reporter, in living mice. In vivo copper-free click chemistry conjugated the incorporated azides with fluorescent probes and allowed for visualization of brain sialoglycans in living mice. Further, the LABOR labeling is compatible with histochemistry on brain sections, which revealed the distribution of newly synthesized sialoglycans in the brain. Click-labeling of the azide-incorporated brain with affinity tags enabled proteomic profiling of sialylated glycoproteins in the brain. Finally, we demonstrated that LABOR can be used to probe dynamic sialylation in distinct brain regions by performing pulse–chase experiments.

Significance

In mammals, the brain is the organ with the highest level of sialic acids, a family of negatively charged monosaccharides that are commonly expressed as outer terminal residues of cell-surface glycans. Brain sialoglycans play essential roles in brain development, cognition, and disease progression; however, in vivo visualization of the sialoglycan biosynthesis in the mouse brain has been impossible. Here, we introduce a liposome-assisted bioorthogonal reporter (LABOR) strategy for metabolic labeling and visualization of brain sialoglycans in living mice. Applying LABOR, we visualized the biosynthesis of brain sialoglycans by in vivo fluorescence imaging and histological analysis, and identified important sialylated glycoproteins in the brain by glycoproteomics. We discovered that the turnover of sialoglycans is spatially regulated in distinct brain regions.

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Fig. 2. Optimization of the LABOR conditions. (A) HPAEC-PAD detection of the presence of 9AzSia in the brain. After the mice were administered with 0.70 mmol/kg LP-9AzSia or 9AzSia, the brain tissues were isolated at varying time points, followed by acid hydrolysis to release the bound 9AzSia. The total 9AzSia was collected and subjected to quantitative analysis using HPAEC-PAD. The concentration of 9AzSia in the brain was calculated based on a standard curve generated by measuring 9AzSia solutions at a series of concentrations and expressed as micrograms per gram of tissue. Error bars are SD from three replicate experiments. (B) Time dependence of LABOR-mediated incorporation of 9AzSia into brain sialylated glycoproteins. The mice were injected daily with 0.70 mmol/kg LP-9AzSia for 3 d, 5 d, and 7 d. The brain tissues were collected and the tissue lysates were reacted with alkyne-biotin, followed by anti-biotin Western blot analysis. (C) Concentration dependence of metabolic incorporation of 9AzSia into brain sialylated glycoproteins. The mice were administered with LP-9AzSia or LP at varying concentrations for 7 d. The brain lysates were reacted with alkyne-Cy5, resolved by SDS/PAGE, and the gel was directly scanned in a fluorescence imager. Anti-GAPDH blot was used as the loading control in B and C. Three animals ($n = 3$) were administered in each treatment group in A–C.

after the injection and gradually increased, reaching saturation after 6 h (Fig. 2A). Because one single injection of LP-9AzSia was able to maintain the maximum concentration of 9AzSia in the brain until at least 24 h, multiple injections on a daily basis were performed for 3 d, 5 d, and 7 d. Anti-biotin Western blot analysis indicated that 9AzSia was incorporated into a variety of brain sialoglycoproteins in a time-dependent manner (Fig. 2B). Furthermore, we injected the mice with LP-9AzSia at varying concentrations. In-gel fluorescence scanning of the brain lysates reacted with alkyne-biotin showed that LP-9AzSia metabolically labeled brain sialoglycoproteins in a concentration-dependent manner (Fig. 2C). Based on these results, daily i.v. injection of LP-9AzSia at 0.70 mmol/kg for 7 d was chosen for the following experiments.

In Vivo Fluorescence Imaging of Brain Sialoglycans. Next, we sought to image brain sialoglycans in vivo by using the LABOR strategy. The mice administered with LP-9AzSia were i.v. injected with an aza-dibenzocyclooctyne-Cy5 conjugate (DBCO-Cy5; 0.14 nmol/g) at the eighth day (Fig. 3A). Copper-free click chemistry has been performed in living mice and rats and exhibited no apparent toxicity (13, 22, 23). Using mice treated with empty liposomes (LP), we determined that DBCO-Cy5 had been cleared from the bloodstream 3 h after the injection (SI Appendix, Fig. S4A). In contrast, the fluorescence signal in the brain of LP-9AzSia-treated mice remained for 12 h, indicating the covalent conjugation of DBCO-Cy5 with azides incorporated in the brain sialoglycans (SI Appendix, Fig. S4B). We therefore chose 3 h after DBCO-Cy5 administration as the time point for performing in vivo imaging on the LP-9AzSia-treated mice by whole-body fluorescence imaging (Fig. 3B and C). Robust fluorescence was observed in the brain, corresponding to the newly synthesized sialoglycans during the course of LP-9AzSia administration. In addition to the brain, strong fluorescence was also observed in the area of kidney, liver, and spleen, which is presumably due to metabolic labeling in these organs during the renal and reticuloendothelial clearance of LP-9AzSia. As two negative controls, the mice administered with 9AzSia or LP by i.v. injection exhibited no significant fluorescence in the brain (Fig. 3B and C). In addition, minimal labeling of brain

was observed in mice administered with Ac₄ManNAz by i.p. injection and DBCO-Cy5 by i.v. injection, confirming that the widely used Ac₄ManNAz is incapable of labeling brain sialoglycans in vivo.

To further rule out the possibility that the observed fluorescence in the brain was due to nonspecific binding of DBCO-Cy5, we isolated the brains from mice administered with LP-9AzSia and DBCO-Cy5. Ex vivo fluorescence imaging at the tissue level showed strong fluorescence in the brain of LP-9AzSia-treated mice, but not in the brain treated with LP (SI Appendix, Fig. S5A). Moreover, the labeled brain tissue was homogenized and subjected to in-gel fluorescence analysis (SI Appendix, Fig. S5B). A diverse repertoire of glycoproteins were resolved and fluorescently visualized on the gel. These results confirm that the 9AzSia-incorporated brain sialoglycans are covalently conjugated with DBCO-Cy5 in vivo.

Sialoglycans of Distinct Mouse Brain Regions Are Labeled. The brain is the most complex organ, with distinct regions that are functionally specialized. To evaluate LP-9AzSia labeling in distinct brain regions, we coupled LABOR with histochemistry. After fasting the LP-9AzSia-treated mice for 24 h, the brains were isolated, and tissue sections from four distinct brain regions including the pyriform cortex, septo-diencephalic region, caudal diencephalon, and rostral cerebellum were prepared (Fig. 4A). After reacting 9AzSia with alkyne-biotin and staining the nucleus with DAPI, the tissue sections were imaged using a slide fluorescence scanner. Throughout the brain, we observed robust Cy5 fluorescence in all of the four brain regions (Fig. 4B). In contrast, only minimal Cy5 labeling was observed in brain tissue sections from mice treated with LP, 9AzSia, or Ac₄ManNAz (SI Appendix, Fig. S6). These results demonstrate that LP-9AzSia metabolically labels sialoglycans throughout the entire brain.

Fig. 3. In vivo fluorescence imaging of the sialoglycans in the mouse brain. (A) Living mice that have been metabolically labeled with LP-9AzSia are i.v. injected with DBCO-Cy5, a cyclooctyne-functionalized far-red fluorophore. In vivo copper-free click chemistry allows chemoselective conjugation of Cy5 onto the brain sialoglycans that have been newly synthesized and incorporated with 9AzSia. (B) Whole-body fluorescence imaging of living mice ($n = 3$ per treatment group) administered daily with LP-9AzSia, 9AzSia, LP, Ac₄ManNAz or 70% (vol/vol) DMSO for 7 d, followed by injection with 0.14 nmol/g DBCO-Cy5 at day 8. Three hours after the injection, the mice were imaged using an in vivo imaging system. Shown are representative images in each group. The color bar indicates the fluorescence radiant efficiency, multiplied by 10⁹. (Scale bar, 1 cm.) (C) Quantitative analysis of the brain signal-to-background ratio (BBR). BBR: the contrast-to-background ratio (CBR) of the brain divided by the CBR of nearby normal tissue. Error bars are SD from three animals in each treatment group. ** $P < 0.01$; n.s., not significant (one-way ANOVA).

Fig. 4. LP-9AzSia metabolically labels distinct brain regions. (A) Representative picture of the intact mouse brain. The dashed lines indicate four distinct brain regions, from which the coronal sections were made: 1, pyriform cortex; 2, septo-diencephalic; 3, caudal diencephalon; and 4, rostral cerebellum. (Scale bar, 2 mm.) (B) Representative fluorescence images of brain tissue sections. Mice ($n = 3$) were administered with 0.70 mmol/kg LP-9AzSia daily for 7 d. The tissue sections with a thickness of 20 μm were prepared, reacted with alkyne-Cy5, and stained with 4',6-diamidino-2-phenylindole (DAPI), followed by imaging with a slide fluorescence scanner. (Scale bar, 1 mm.)

Cellular Distribution of the Newly Synthesized Sialoglycans in the Hippocampus.

The compatibility of LABOR with histological examination prompted us to analyze the cellular distribution of sialoglycans by using confocal fluorescence microscopy with a higher spatial resolution. Tissue sections from the caudal diencephalon were prepared and immunostained with the synaptic marker synaptophysin and the marker for astrocytes glial fibrillary acidic protein (GFAP), followed by reaction with DBCO-Cy5 and staining with DAPI (*SI Appendix, Fig. S7*). We focused the confocal imaging experiments on the dentate gyrus area of hippocampus within the caudal diencephalon, given that sialylation has been implicated in regulating hippocampal function (2, 24). In particular, sialoglycans have been shown to play an important role in synaptic plasticity and neurotransmission (25, 26). To better resolve the spatial distribution of 9AzSia, we zoomed in to the granule cell layer of the dentate gyrus of the hippocampus (Fig. 5). The 9AzSia labeling exhibited a fluorescence pattern that is exclusive of nucleus, indicating the labeling of cell surface sialoglycans. The punctate staining of synapses appeared to localize over the region with dense 9AzSia labeling, suggesting that many of the nascent sialoglycans are distributed in synapses (Fig. 5). In addition, 9AzSia fluorescence, although at a relatively lower level, was also observed at the locations of astrocytes, suggesting cell surface sialoglycans on astrocytes were metabolically labeled. As expected, confocal fluorescence imaging on the hippocampus of the control mice exhibited minimal Cy5 fluorescence (*SI Appendix, Fig. S8*). These results suggest that the turnover and biosynthesis of sialic acids are active in the brain, resulting in the incorporation of 9AzSia into the newly synthesized sialoglycans located on cell surfaces, including the synaptic sites.

Proteomic Analysis of Sialylated Glycoproteins in the Brain. To assess protein sialylation in the brain, we performed large-scale proteomic profiling of sialylated glycoproteins on LABOR-labeled mice. Tissue lysates of mouse brains treated with LP-9AzSia were reacted with alkyne-biotin via CuAAC for enrichment of 9AzSia-incorporated proteins with streptavidin beads, followed by gel-based proteomic identification using tandem mass spectrometry (*SI Appendix, Fig. S9*). From three independent experiments, we selectively identified 140 proteins by LP-9AzSia labeling compared with control mice treated with LP (Fig. 6A and B and *SI Appendix, Table S2*). The 140 proteins were selected using a high-confidence filter, that is, selecting proteins with $\geq$ fivefold increases of the spectral counts in the LP-9AzSia-treated samples above the LP-treated control samples.

To validate the proteins identified by mass spectrometry, we performed Western blot analysis on the LP-9AzSia-labeled and enriched brain lysates using antibodies against cell adhesion molecule 4 (CADM4) and contactin-associated protein-like 2 (CNTNAP2), confirming their robust and specific recovery dependent on 9AzSia incorporation (Fig. 6C). Among the list of identified sialylated glycoproteins, we noticed that there are a wealth of synapse-associated proteins, such as synaptotagmin 2 (Syt2), zinc transporter 3 (ZnT3), and leucine-rich, glioma-inactivated protein 1 (LGI1) (indicated by the red dots in Fig. 6A). Syt2 functions as a Ca^{2+} sensor for synchronous synaptic vesicle exocytosis (27, 28). ZnT3 is localized in clear synaptic vesicles of cortical glutamatergic terminals and involved in synaptic vesicle zinc uptake and release (29, 30). LGI1 is a secreted protein that is localized to synapses, where it modulates synaptic AMPA receptors (31, 32). We therefore performed the gene ontology analysis of the identified sialoglycoproteins, which revealed that synapse is one of the most enriched cellular localizations, indicating that many synaptic proteins are sialylated and the biosynthesis of these sialoglycoproteins is active (Fig. 6D). Notably, a list of sialylated proteins was also identified at several other cellular locations, in agreement with the imaging results.

Brain Sialylation Is Dynamically and Spatiotemporally Regulated.

Finally, we sought to investigate the spatiotemporal regulation of sialylation dynamics in the brain. By taking advantage of the metabolic labeling nature of LABOR, we performed pulse-chase experiments to image the turnover of newly synthesized sialoglycans in the brain. After administration with LP-9AzSia for 7 d, the mice were chased for 0 h or 6 h, followed by brain isolation (Fig. 7A). Brain sections were prepared and reacted with alkyne-Cy5. We examined six encephalic regions by confocal fluorescence microscopy (Fig. 7B). In most of these regions, the 9AzSia labeling had been significantly decreased within 6 h, indicating sialylation is dynamic in the brain. Remarkably, the hippocampus exhibited a significantly lower decay of the 9AzSia-incorporated sialoglycans. These results suggest that the dynamics of sialoglycan biosynthesis may be regulated in a spatiotemporally controlled manner and that the hippocampal sialoglycans possess a slow turnover rate.

Fig. 5. Cellular distribution of 9AzSia-incorporated sialoglycans in the granule cell layer of dentate gyrus in the hippocampus. The tissue sections (thickness, 10 μm) of the caudal diencephalon were made from mice ($n = 3$) treated with LP-9AzSia and immunostained with synaptophysin and GFAP, followed by reaction with DBCO-Cy5 and staining with DAPI. Images were recorded with a 63 $\times$ objective lens on a confocal fluorescence microscope. (Scale bar, 20 μm .) Images with a lower magnification showing the dentate gyrus structure are shown in *SI Appendix, Fig. S7*.

Fig. 6. Proteomic profiling of newly synthesized sialylated glycoproteins in the brain of mice treated with LP-9AzSia. (A) Tissue lysates of mouse brain treated with LP-9AzSia or LP were reacted with alkyne-biotin, enriched using streptavidin beads, and subjected to gel-based proteomic identification by tandem mass spectrometry. For each protein, the total spectral counts of LP-9AzSia samples subtracted by the total spectral counts of LP samples was plotted. Several known proteins that are associated with synapses in the brain are shown in red. (B) Pearson correlation plot for overlapping proteins from LP-9AzSia experiments (140 proteins, comparing experiment 1 with experiment 2). (C) Brain lysates were enriched using alkyne-biotin and analyzed by Western blot. All lanes are cropped from the same gel. Anti-GAPDH blot was used as the loading control. (D) Cellular localizations enriched in the newly synthesized sialoglycoproteins identified in the LP-9AzSia-treated brain. The top six enriched localizations are shown.

Discussion

Despite the functional importance of brain sialylation, *in vivo* visualization of sialoglycans in the mouse brain has been impossible. Azidosugars have been used as tracers to report the sialic acid biosynthesis in living animals (12, 13), but the BBB impedes their access to the brain. The LABOR strategy developed herein overcomes this obstacle by exploiting liposomes to shuttle 9AzSia into the brain. Previous *in vitro* studies using neuron cell culture (33) and brain tissue culture (34) have shown that neurons can uptake azidosugars and metabolically incorporate them into sialoglycans. Our results demonstrate that LABOR-mediated delivery of 9AzSia into the brain results in robust metabolic incorporation of azides into brain sialoglycans in living mice. *In vivo* copper-free click chemistry enables whole-body fluorescence imaging of brain sialylation. This strategy may be further applied to visualize sialylation in mouse models of brain tumors and neurodegenerative diseases (8, 10).

The mouse brain, which is fluorescently labeled using the LABOR strategy, is compatible with immunofluorescent histochemistry. Multicolor confocal fluorescence imaging of brain sections revealed that the newly synthesized sialoglycans are widely distributed on cell surfaces of brain cells, including neurons and glial cells. Of particular interest, dense labeling was observed on synapses, indicating active biosynthesis and turnover of sialoglycans at the synaptic sites. Sialoglycans around the synaptic cleft have been proposed to modulate neurotransmission through interaction with Ca^{2+} , which is essential in neuronal responses (26). Because neurotransmission and synaptic connectivity are dynamically regulated (28, 35), dynamic sialylation observed in this study may play an important role in these processes. Furthermore, glycoproteomic profiling can be performed on the brain tissues isolated from the LABOR-labeled mice. We have identified a list of sialylated proteins in the brain. The identified sialoglycoproteins include those involved in calcium signaling in synapses such as Syt2, supporting the hypothesis that

sialoglycans regulate neurotransmission by interacting with Ca^{2+} . Furthermore, the fact that ZnT3 is sialylated and actively synthesized in the synapse suggests that sialylation may also play a regulatory function in synaptic zinc signaling. The newly synthesized sialoglycoproteins associated with synapses contribute, at least partially, to the newly synthesized synaptic sialoglycans observed in the fluorescent histochemistry. In addition, sialoglycans can also be carried by sialylated glycolipids such as gangliosides (2). Our results indicate that those gangliosides are also metabolically labeled with 9AzSia.

The turnover of sialic acids in the brain has been a subject of extensive studies. One commonly used method is to administer the animals with ManNAc or sialic acid monosaccharide substituted with radioactive isotopes such as ^3H and ^{14}C (36–39). Isotopic labeling enables pulse–chase experiments but suffers from limited spatial resolution. Alternatively, histological analysis can be performed using lectins (40) and antibodies (41) to provide spatial information on sialoglycans at the static state. The LABOR method allows for spatiotemporal visualization of the dynamic sialylation. Our results reveal spatially distinct turnover rates among different brain regions. Of particular interest, the turnover of sialic acids in the hippocampus appears to be uniquely slow. Sialylation in the hippocampus is important for axonal growth and synaptic activity-induced neuronal–glial plasticity (42). Induction of long-term potentiation and depression at the synapses was found to be impaired if PSA is removed, thus affecting spatial learning and memory function in hippocampal regions (43). Whether the overall slow dynamics of sialylation has functional implications in these processes is an interesting topic for future studies.

Fig. 7. Pulse–chase analysis of sialoglycan turnover in distinct brain regions. (A) Schematic of the pulse–chase experimental procedures. Mice ($n = 3$ per treatment group) were injected with 0.70 mmol/kg LP-9AzSia daily for 7 d, followed by chasing for 0 h or 6 h. Brain sections with a thickness of 20 μm were prepared, reacted with alkyne-Cy5, and stained with DAPI. (B) Confocal fluorescence images of the olfactory area, corpus striatum, cerebral cortex, hippocampal formation, cerebellum, and medulla regions were recorded using a 10 $\times$ objective lens. (Scale bar, 200 μm .)

Although we focus on brain sialylation in this work, metabolic incorporation of 9AzSia in other organs, including the heart, liver, spleen, kidney, lung, and thymus, in mice treated with LP-9AzSia was also observed (*SI Appendix*, Figs. S10 and S11). In comparison with Ac₄ManNAz labeling, LP-9AzSia exhibits superior labeling efficiency in the heart, kidney, and thymus, whereas Ac₄ManNAz labels better in the intestine. These observations provide a foundation for choosing the appropriate labeling method for studying glycosylation in specific organs and suggest that LABOR will find broad applications, in addition to probing brain sialylation.

Receptor-mediated transcytosis has been explored for liposomal delivery into the brain (14). For future technical development, it will be interesting to evaluate whether ligand-targeted liposomes can be exploited for labeling brain glycosylation. For example, liposomes can also be modified with ligands such as transferrin (44), OX26 antibody (17), and short peptides (45), which target specific brain receptors. Given that the current method labels a panel of organs in addition to the brain, the ligand-targeted liposomes may improve the brain specificity.

Finally, the LABOR methodology may be further extended to probe other types of glycosylation in the mouse brain (46). Of

particular interest is the protein O-GlcNAc modification, which has been implicated in neurodegenerative diseases (47). Bio-orthogonal chemical reporters for O-GlcNAc have been developed for metabolically labeling and identifying O-GlcNAcylated proteins in live cells (48–50), which may be explored for in vivo LABOR labeling of brain O-GlcNAcylation.

Materials and Methods

The liposomes were prepared as described (15). BALB/c male mice (8 wk, 20–25 g) were administered daily for 7 d with 0.70 mmol/kg LP-9AzSia (i.v.), LP-ManNAz (i.v.), 9AzSia (i.v.), ManNAz (i.v.), Ac₄ManNAz (i.p.), or Ac₄Me9AzSia (i.p.). All animal experiments were performed in accordance with guidelines approved by the Institutional Animal Care and Use Committee of Peking University accredited by Association for Assessment and Accreditation of Laboratory Animal Care International. Further details are provided in *SI Appendix*, which also includes detailed methods for whole-body fluorescence imaging, fluorescence histochemistry, glycoproteomic analysis, Western blot analysis, and in-gel fluorescence scanning.

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- Chen X, Varki A (2010) Advances in the biology and chemistry of sialic acids. *ACS Chem Biol* 5(2):163–176.
- Schnaar RL, Gerardy-Schahn R, Hildebrandt H (2014) Sialic acids in the brain: Gangliosides and polysialic acid in nervous system development, stability, disease, and regeneration. *Physiol Rev* 94(2):461–518.
- Wang B (2009) Sialic acid is an essential nutrient for brain development and cognition. *Annu Rev Nutr* 29:177–222.
- Yu RK, Macala LJ, Taki T, Weinfield HM, Yu FS (1988) Developmental changes in ganglioside composition and synthesis in embryonic rat brain. *J Neurochem* 50(6):1825–1829.
- Rutishauser U (2008) Polysialic acid in the plasticity of the developing and adult vertebrate nervous system. *Nat Rev Neurosci* 9(1):26–35.
- Kleene R, Schnachner M (2004) Glycans and neural cell interactions. *Nat Rev Neurosci* 5(3):195–208.
- Scott H, Panin VM (2014) The role of protein N-glycosylation in neural transmission. *Glycobiology* 24(5):407–417.
- Bos PD, et al. (2009) Genes that mediate breast cancer metastasis to the brain. *Nature* 459(7249):1005–1009.
- Platt FM (2014) Sphingolipid lysosomal storage disorders. *Nature* 510(7503):68–75.
- Yamashita T, et al. (2005) Interruption of ganglioside synthesis produces central nervous system degeneration and altered axon–glial interactions. *Proc Natl Acad Sci USA* 102(8):2725–2730.
- Cheng B, Xie R, Dong L, Chen X (2016) Metabolic remodeling of cell-surface sialic acids: Principles, applications, and recent advances. *ChemBioChem* 17(1):11–27.
- Prescher JA, Dube DH, Bertozzi CR (2004) Chemical remodelling of cell surfaces in living animals. *Nature* 430(7002):873–877.
- Rong J, et al. (2014) Glycan imaging in intact rat hearts and glycoproteomic analysis reveal the upregulation of sialylation during cardiac hypertrophy. *J Am Chem Soc* 136(50):17468–17476.
- Lai F, Fadda AM, Sinico C (2013) Liposomes for brain delivery. *Expert Opin Drug Deliv* 10(7):1003–1022.
- Xie R, Hong S, Feng L, Rong J, Chen X (2012) Cell-selective metabolic glycan labeling based on ligand-targeted liposomes. *J Am Chem Soc* 134(24):9914–9917.
- Papahadjopoulos D, et al. (1991) Sterically stabilized liposomes: Improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc Natl Acad Sci USA* 88(24):11460–11464.
- Huwyler J, Wu D, Pardridge WM (1996) Brain drug delivery of small molecules using immunoliposomes. *Proc Natl Acad Sci USA* 93(24):14164–14169.
- Besanceney-Webler C, et al. (2011) Increasing the efficacy of bioorthogonal click reactions for bioconjugation: A comparative study. *Angew Chem Int Ed Engl* 50(35):8051–8056.
- Ladisch S, Gillard B (1985) A solvent partition method for microscale ganglioside purification. *Anal Biochem* 146(1):220–231.
- Garberg P, et al. (2005) In vitro models for the blood–brain barrier. *Toxicol In Vitro* 19(3):299–334.
- Kaya M, Ahishali B (2011) Assessment of permeability in barrier type of endothelium in brain using tracers: Evans blue, sodium fluorescein, and horseradish peroxidase. *Methods Mol Biol* 763:369–382.
- Chang PV, et al. (2010) Copper-free click chemistry in living animals. *Proc Natl Acad Sci USA* 107(5):1821–1826.
- Xie R, et al. (2014) Targeted imaging and proteomic analysis of tumor-associated glycans in living animals. *Angew Chem Int Ed Engl* 53(51):14082–14086.
- Eichenbaum H (2004) Hippocampus: Cognitive processes and neural representations that underlie declarative memory. *Neuron* 44(1):109–120.
- Martin PT (2002) Glycobiology of the synapse. *Glycobiology* 12(1):1R–7R.
- Wang B (2012) Molecular mechanism underlying sialic acid as an essential nutrient for brain development and cognition. *Adv Nutr* 3(3):465S–472S.
- Xu J, Mashimo T, Südhof TC (2007) Synaptotagmin-1, -2, and -9: Ca²⁺ sensors for fast release that specify distinct presynaptic properties in subsets of neurons. *Neuron* 54(4):567–581.
- Südhof TC (2013) Neurotransmitter release: The last millisecond in the life of a synaptic vesicle. *Neuron* 80(3):675–690.
- Wenzel HJ, Cole TB, Born DE, Schwartzkroin PA, Palmiter RD (1997) Ultrastructural localization of zinc transporter-3 (ZnT-3) to synaptic vesicle membranes within mossy fiber boutons in the hippocampus of mouse and monkey. *Proc Natl Acad Sci USA* 94(23):12676–12681.
- Cole TB, Wenzel HJ, Kafer KE, Schwartzkroin PA, Palmiter RD (1999) Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene. *Proc Natl Acad Sci USA* 96(4):1716–1721.
- Fukata Y, et al. (2006) Epilepsy-related ligand/receptor complex LGI1 and ADAM22 regulate synaptic transmission. *Science* 313(5794):1792–1795.
- Fukata Y, et al. (2010) Disruption of LGI1-linked synaptic complex causes abnormal synaptic transmission and epilepsy. *Proc Natl Acad Sci USA* 107(8):3799–3804.
- Graneli AEVB, et al. (2011) DmSAs is required for sialic acid biosynthesis in cultured *Drosophila* third instar larvae CNS neurons. *ACS Chem Biol* 6(11):1287–1295.
- Kang K, et al. (2015) Tissue-based metabolic labeling of polysialic acids in living primary hippocampal neurons. *Proc Natl Acad Sci USA* 112(3):E241–E248.
- Eroglu C, Barres BA (2010) Regulation of synaptic connectivity by glia. *Nature* 468(7321):223–231.
- Margolis RK, Margolis RU (1973) The turnover of hexosamine and sialic acid in glycoproteins and mucopolysaccharides of brain. *Biochim Biophys Acta* 304(2):413–420.
- Ferwerda W, Blok CM, Heijlman J (1981) Turnover of free sialic acid, CMP-sialic acid, and bound sialic acid in rat brain. *J Neurochem* 36(4):1492–1499.
- Diaz S, Varki A (1985) Metabolic labeling of sialic acids in tissue culture cell lines: Methods to identify substituted and modified radioactive neuraminic acids. *Anal Biochem* 150(1):32–46.
- Tettamanti G (2004) Ganglioside/glycosphingolipid turnover: New concepts. *Glycoconj J* 20(5):301–317.
- Sun J, et al. (2004) Myelin-associated glycoprotein (Siglec-4) expression is progressively and selectively decreased in the brains of mice lacking complex gangliosides. *Glycobiology* 14(9):851–857.
- Heffer-Laue M, Laue G, Nimrichter L, Fromholt SE, Schnaar RL (2005) Membrane redistribution of gangliosides and glycosylphosphatidylinositol-anchored proteins in brain tissue sections under conditions of lipid raft isolation. *Biochim Biophys Acta* 1686(3):200–208.
- Cremer H, et al. (2000) PSA-NCAM: An important regulator of hippocampal plasticity. *Int J Dev Neurosci* 18(2-3):213–220.
- Becker CG, et al. (1996) The polysialic acid modification of the neural cell adhesion molecule is involved in spatial learning and hippocampal long-term potentiation. *J Neurosci Res* 45(2):143–152.
- Ying X, et al. (2010) Dual-targeting daunorubicin liposomes improve the therapeutic efficacy of brain glioma in animals. *J Control Release* 141(2):183–192.
- Georgieva JV, et al. (2012) Peptide-mediated blood–brain barrier transport of polymersomes. *Angew Chem Int Ed Engl* 51(33):8339–8342.
- Laughlin ST, Bertozzi CR (2009) Imaging the glycome. *Proc Natl Acad Sci USA* 106(1):12–17.
- Yuzwa SA, Vocadlo DJ (2014) O-GlcNAc and neurodegeneration: Biochemical mechanisms and potential roles in Alzheimer's disease and beyond. *Chem Soc Rev* 43(19):6839–6858.
- Vocadlo DJ, Hang HC, Kim E-J, Hanover JA, Bertozzi CR (2003) A chemical approach for identifying O-GlcNAc-modified proteins in cells. *Proc Natl Acad Sci USA* 100(16):9116–9121.
- Boyce M, et al. (2011) Metabolic cross-talk allows labeling of O-linked beta-N-acetylglucosamine-modified proteins via the N-acetylgalactosamine salvage pathway. *Proc Natl Acad Sci USA* 108(8):3141–3146.
- Chuh KN, Zaro BW, Piller F, Piller V, Pratt MR (2014) Changes in metabolic chemical reporter structure yield a selective probe of O-GlcNAc modification. *J Am Chem Soc* 136(35):12283–12295.