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Neuro-Compatible Metabolic Glycan Labeling of Primary Hippocampal Neurons in Noncontact, Sandwich-Type Neuron–Astrocyte Coculture

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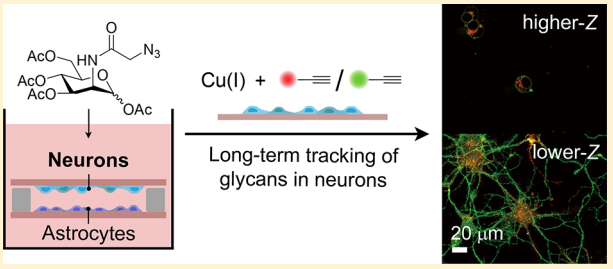
Supporting Information

ABSTRACT: Glycans are intimately involved in several facets of neuronal development and neuropathology. However, the metabolic labeling of surface glycans in primary neurons is a difficult task because of the neurotoxicity of unnatural monosaccharides that are used as a metabolic precursor, hindering the progress of metabolic engineering in neuron-related fields. Therefore, in this paper, we report a neurosupportive, neuron–astrocyte coculture system that neutralizes the neurotoxic effects of unnatural monosaccharides, allowing for the long-term observation and characterization of glycans in primary neurons in vitro. Polysialic acids in neurons are selectively imaged, via the metabolic labeling of sialoglycans with peracetylated *N*-azidoacetyl-D-mannosamine (Ac_4ManNAz), for up to 21 DIV. Two-color labeling shows that neuronal activities, such as neurite outgrowth and recycling of membrane components, are highly dynamic and change over time during development. In addition, the insertion sites of membrane components are suggested to not be random, but be predominantly localized in developing neurites. This work provides a new research platform and also suggests advanced 3D systems for metabolic-labeling studies of glycans in primary neurons.



KEYWORDS: *Metabolic labeling, bioorthogonal reaction, polysialic acid, astrocyte, primary hippocampal neuron*

Metabolic glycan labeling, developed by Reutter[1] and Bertozzi,[2] is a powerful and versatile tool for studying the spatiotemporal distribution of glycans in cells.[3] The technique has been used to label and trace glycans non-invasively in microbes,[4] mammalian cells,[5−7] plant cells,[8] and even animals, such as mice and zebrafish,[9−11] for various applications. For instance, cancer tissues, which are characterized with distinct sialic acid content, were metabolically labeled and detected in living mice, and visualized by tomographic images of fluorescence, radionuclide, and magnetic resonance.[12,13] Additionally, the metabolic labeling of *N*- and *O*-linked glycans in cancer cells, coupled with super-resolution microscopic techniques, was used to characterize the relationship between the diffusion velocity of *N*-linked sialic acids and metastatic potential.[14] However, an unfortunate limitation of metabolic labeling has been the toxicity of unnatural monosaccharides to neurons. Glycans, especially polysialic acid (PSA), are intimately involved in neuronal development and neuropathology,[15] but the neurotoxicity of metabolic glycan labeling had rendered the technique inapplicable and inaccessible to neuronal research. For example, we reported that the survival and neurite development of primary hippocampal neurons deteriorated markedly in the presence of peracetylated *N*-azidoacetyl-D-mannosamine (Ac_4ManNAz).[16] Neurotoxicity had also been observed in *N*-glycolylmannosamine pentaacetate,[17] and glycan synthesis in neurons, particularly PSA, was inhibited in neurons fed with *N*-propanoyl, *N*-butanoyl, and *N*-pentanoyl mannosamine.[18,19]

We had recently reported that while Ac_4ManNAz was harmful to primary hippocampal neurons, neurotoxicity could be neutralized if the unnatural monosaccharide was metabolized by whole hippocampal tissue prior to dissociation and cell seeding, resulting in an alternative metabolic-labeling strategy called "metabolism-by-tissues (MbT)".[16] However, although the MbT method was successful in mitigating the neurotoxicity of Ac_4ManNAz, time-lapse imaging of sialoglycans in neuron membranes was limited to the early stages of neuronal development (up to 4 DIV; DIV: days in vitro), as continual feeding could not be executed to the developing, dissociated neurons without compromising cell viability. Additionally, while the deleterious effects of unnatural monosaccharides can be decreased by lowering probe concentration or using different neuraminic acid–based sugar probes,[20] the use of Ac_4ManNAz at conventional concentrations (25−50 μM) was necessary to successfully label sialoglycans.

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Biological studies have shown that PSA, a typical sialoglycan in the nervous system and an important regulator in brain development, reversibly impedes the function of neural cell adhesion molecules and is maximally expressed from embryonic day (E) 8 to postnatal day (P) 7,[21,22] which equates to an in vitro culture time longer than possible by current metabolic-labeling systems. Therefore, a neuro-compatible platform that allows for the long-term, spatiotemporal tracking of glycans in neurons would open new opportunities for advanced neuro-glycan studies. To address this need, we, inspired by the MbT method, employed a noncontact astrocyte feeder layer, which enabled the continuous feeding of unnatural monosaccharides to dissociated neuron cells without compromising long-term cell viability. Dissociated hippocampal neurons were cocultured with a feeder layer and fed with Ac_4ManNAz for 21 days, forming stable synaptic networks, and expressing Sia5Az-presenting cell-membrane glycans, without any noticeable loss in cell viability. The recycling process of sialic acids in neurites and somata was investigated by two-color labeling. Newly synthesized sialic acids were observed predominantly in neurites rather than somata, indicating that the insertion of new membranes occurred mainly in neurites, and such insertion was found to be prevalent at later stages of neuronal development.

RESULTS AND DISCUSSION

Astrocytes, a type of glial cell, were chosen as support cells for the neuro-compatible metabolic labeling of glycans in primary hippocampal neurons because their functional relationship with neurons is well understood.[23] Dynamic astrocyte−neuron interactions are known to be a main regulator for the development, function, and repair of the nervous system by a number of signaling molecules.[24] Astrocytes also regulate fluid homeostasis from blood flow, ion concentration (including sodium and potassium), pH, transmitters, and the conversion and recycling of biomolecules.[25]

Astrocyte feeder layers were prepared for coculture, according to a conventional, noncontact coculture method with a "sandwich" configuration (Figure 1). The platform kept neurons physically separated from the astrocytes in a controlled fashion (after removing the bottom astrocyte layer).[26] Prior to neuron culture, astrocytes were obtained from the cerebral cortex of E18 rats, and a layer of astrocytes was independently prepared on poly-D-lysine (PDL)-coated glass coverslips with paraffin spacers (height <0.5 mm) and cultured for 7 days for their maturation. Primary hippocampal neurons were dissociated from the hippocampi of E18 Sprague−Dawley rat pups and seeded on separate PDL-coated coverslips. Neuron-plated coverslips were then inverted and placed facing the astrocyte-plated coverslips (separated by paraffin spacers) to form a sandwiched coculture. In this setting, fresh medium was accessible to both cell layers. As a control, hippocampal neurons were also cultured on a PDL-coated coverslip without a feeder layer. The feeding of Ac_4ManNAz was indicated with either (+) or (−) for simplicity of denotation in this paper.

The dissociated neurons were fed with Ac_4ManNAz (50 μM) for 2 days, and cell viability was monitored from 2 DIV onward. In the case of the control(+), viability gradually decreased and dropped to 19.7% at 7 DIV, in agreement with our previous report[16] (Figure 2A). In stark contrast, neurons in the sandwich coculture system survived the Ac_4ManNAz-feeding. At 7 DIV, the viability of neurons in sandwich(+) was 66.1%, which was the similar to that of the control(−) (63.1%) and sandwich(−)

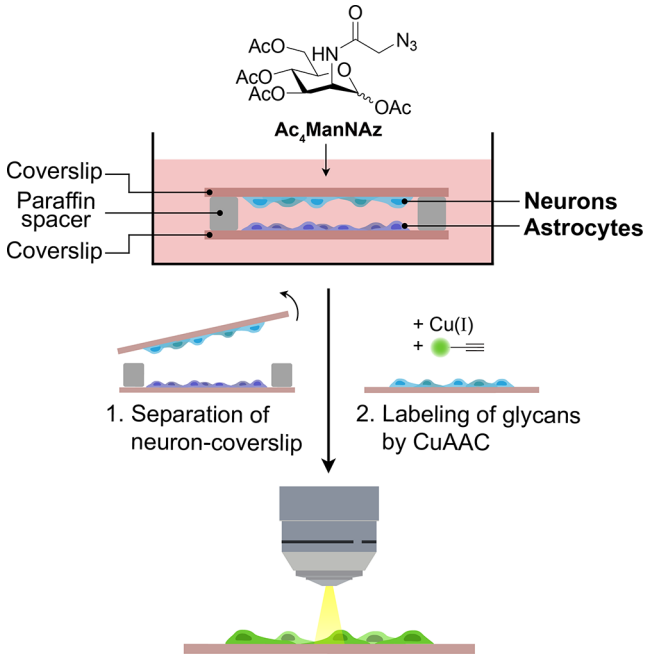

Figure 1. Scheme of the noncontact neuron-astrocyte coculture (sandwich configuration) for neuro-compatible metabolic incorporating of Ac_4ManNAz.

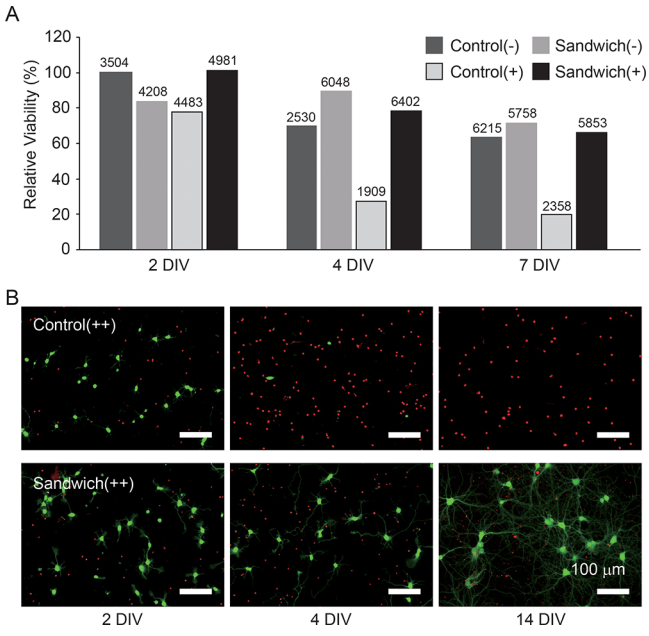

Figure 2. Diminished neurotoxicity of Ac_4ManNAz in sandwich coculture. (A) Relative viabilities of primary hippocampal neurons cultured in control(−),(+) and sandwich(−),(+) at 2, 4, and 7 DIV, compared with control(−) at 2 DIV. The number of data points is denoted. (B) CLSM images of primary neurons cultured in control(++) and sandwich(++) after live−dead assay at 2, 4, and 14 DIV.

(71.1%), confirming that the sandwich coculture system neutralized the neurotoxicity of Ac_4ManNAz. The successful in vivo metabolic sialoglycan labeling of neurons in mice,[27,28] in conjunction with the positive results of the in vitro MbT method, suggested that some aspects of the whole tissue neuro-environment were responsible for negating the deleterious effects of unnatural monosaccharides. A potential mechanism of the reduced neurotoxicity might be that astrocytes provide

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trophic support by internalizing acetate from Ac_4ManNAz and converting the molecules to acetyl-CoA, which is an energy source.[29] The sandwich coculture system clearly demonstrates that astrocytes are a key contributor in this regard. In addition, we found that other neurosupportive motifs present in tissues, such as laminin, fibronectin, and collagen type I, as well as a high density of neurons, did not alleviate the neurotoxicity of Ac_4ManNAz (see the Supporting Information, Figures S1 and S2).

After confirming the neurosupportive capabilities of the noncontact feeder layer, we stained cells using a LIVE/DEAD viability/cytotoxicity kit for mammalian cells, which stained live cells with calcein AM and dead cells with ethidium homodimer-1, and investigated the effects of long-term Ac_4ManNAz feeding on viability. Without the astrocyte layer, most neurons died after 4 days of continuous feeding (control(++) in Figure 2), whereas those in sandwich(++) remained viable and formed stable, complex neuronal circuits with strong bundles of neurites even after 14-day feeding of Ac_4ManNAz (Figure 2B, images of control(−) and sandwich(−) in Figure S3). The supporting astrocytes in the complementing slides were also alive after 14 DIV in sandwich(++) (Figure S4). To further verify healthy neuronal development and maturation at later stages of neuronal development in sandwich(++), neurons at 21 DIV (i.e., after 21-day continuous feeding of Ac_4ManNAz) were labeled with anti-β-tubulin III (red), phalloidin (green), and 4′,6-diamidino-2-phenylindole (DAPI, blue), to image microtubules, F-actin structures, and nuclei, respectively (Figure 3). Continual feeding did not affect neuronal development in sandwich(++) for up to 21 DIV, when compared with sandwich(−), and the neurons showed intact cytoskeletons (microtubules and F-actin) and healthy synaptic connections (Figure 3). While some glial cell migration to the neuron coverslips was observed, homogeneity can easily be improved with cytarabine treatment.[30]

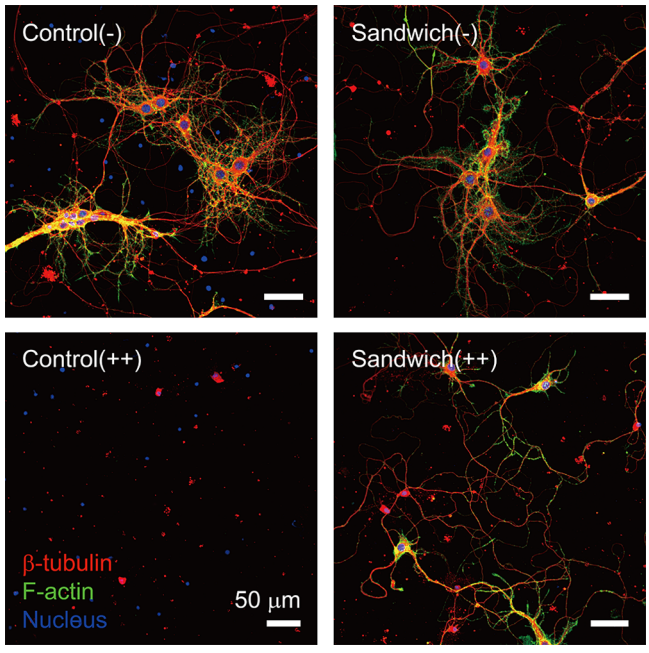

Figure 3. CLSM images of primary neurons cultured in control and sandwich with or without Ac_4ManNAz at 21 DIV, and stained with anti-β-tubulin III (red), phalloidin (green), and DAPI (blue) for detecting microtubule, F-actin, and nuclei, respectively.

Sialoglycans, including PSA, regulate several activities of neurons, including neural stem cell differentiation, migration, guidance, plasticity, cell−cell interactions, synaptogenesis, and the formation of neuronal networks.[31,32] Sialic acid is synthesized in the cytosol, activated with cytidine monophosphate (CMP) in the nucleus, and assembled and used to glycosylate proteins at the Golgi, which are transported to the cytoplasmic membrane. However, the spatiotemporal distribution of sialoglycans, as well as their insertion sites and turnover rates, remained uncertain.[33] Studies on the turnover and recycling of sialoglycans in neurons require continuous labeling and long-term tracking of sialic acids in membranes, which was not possible under conventional and MbT methods. A recent in vivo metabolic-labeling study showed that the turnover of sialic acids in the hippocampus was far slower than that of other regions in the brain (>6 h).[27] We conducted two-color labeling to investigate the spatiotemporal distribution and expression of sialic acids in developing neurons by using copper(I)-catalyzed azide−alkyne cycloaddition (CuAAC) under cytocompatible conditions[34] (for experimental details, see the Methods; Figure S5). Neurons continuously fed with Ac_4ManNAz were sequentially labeled with Alexa Fluor 594-Alkyne (red) at 3 DIV and Alexa Fluor 488-Alkyne (green) at 5 DIV, and imaged by confocal laser-scanning microscopy (CLSM) (Figure 4A). We found that the intensity ratio of green-to-red fluorescence was lower in the membranes of somata than those of neurites at 5 DIV. Red fluorescence observed in the cytoplasm (and intense red fluorescence at the center of the soma in the corresponding graph) indicated that sialic acids were internalized for turnover, which was shorter than 48 h (inset in Figure 4A). We also performed two-color labeling at 5 and 7 DIV (5,7-DIV system) and at 7 and 9 DIV (7,9-DIV system). Here, unlike in the 3,5-DIV system, the green-to-red ratio increased solely in neurites (Figure 4B and C). For example, in the case of the 5,7-DIV and 7,9-DIV systems, the green fluorescence was dominant in neurites (Figure S6). While the insertion sites of membrane vesicles in polarizing neurons were previously unknown, our observations suggested that the insertion of membrane vesicles containing newly synthesized sialoglycans (green), occurs predominantly at neurites, rather than at somata, assuming that the unnatural monosaccharides and CuAAC reaction do not disturb cellular activities. The higher green fluorescence intensity in the 7,9-DIV system, however, provided unprecedented information that the insertion site of new membrane components was neither random nor evenly distuributed, but rather spatially controlled. Additionally, we found that the rate of neurite outgrowth was not constant, concurring with previously reports,[35] indicating that neurite elongation accelerates as the neuron develops. The rapid increase in neurites would necessitate major cell membrane expansion, implying an important role of sialic acids in neurite fasciculation at later stages of development.[36]

CONCLUSION

In summary, we achieved long-term spatiotemporal tracking of surface sialoglycans in primary hippocampal neurons in vitro (21 DIV). Previously, this approach failed due to the neurotoxic effects of unnatural monosaccharides. Additionally, we found that neurite-outgrowth rate was not constant during neuronal development. Two-color labeling showed that newly synthesized sialoglycans were inserted predominantly into the membranes of growing neurites, indicating that membrane expansion during neurite outgrowth is neither exclusively

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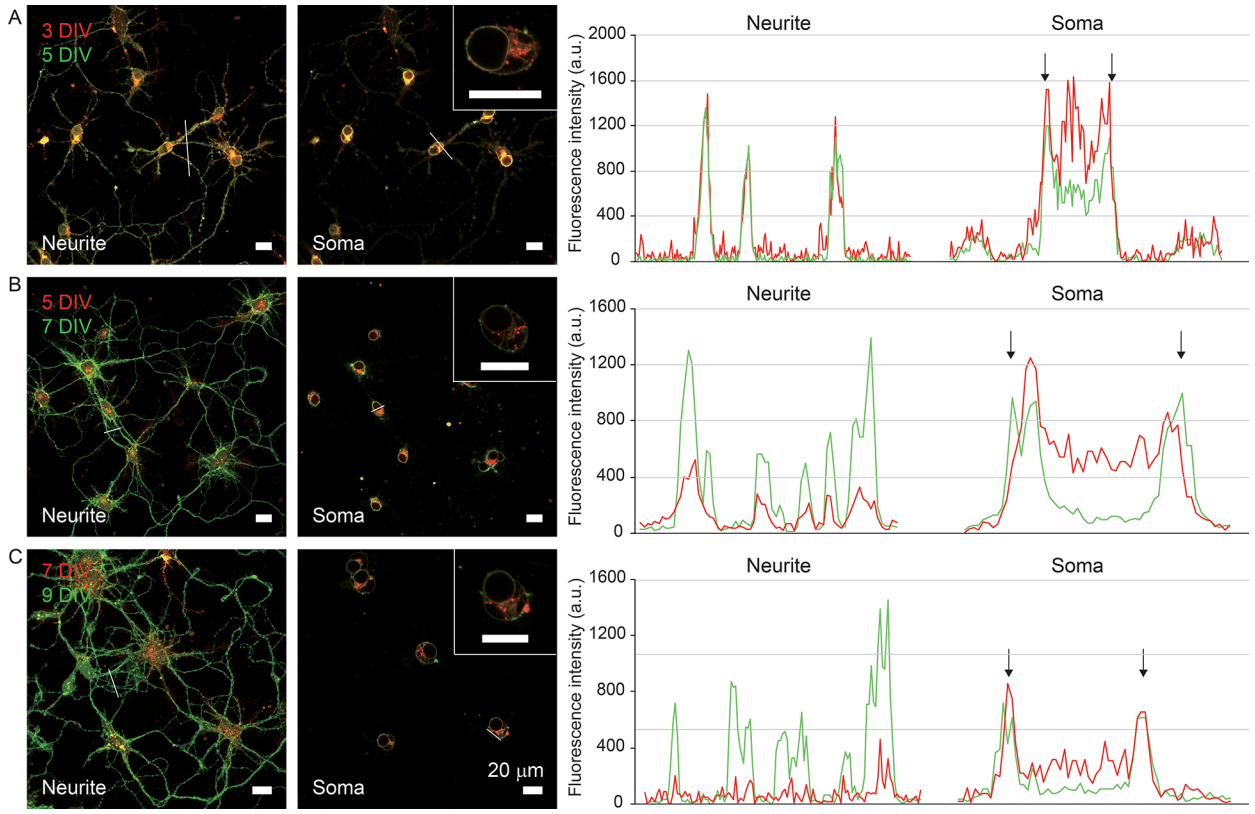

Figure 4. CLSM images and plots of fluorescence intensities of cross sections (white lines in images) of neurites and somata of primary hippocampal neurons cultured in sandwich (++) and labeled by CuAAC. (A) Neurons labeled with Alexa Fluor 594-Alkyne at 3 DIV and Alexa Fluor 488-Alkyne at 5 DIV sequentially. (B) Neurons labeled with Alexa Fluor 594-Alkyne at 5 DIV and Alexa Fluor 488-Alkyne at 7 DIV sequentially. (C) Neurons labeled with Alexa Fluor 594-Alkyne at 7 DIV and Alexa Fluor 488-Alkyne at 9 DIV sequentially. Scale bars are 20 μm. Black arrows correspond to fluorescence intensities of cell membranes.

random nor specifically localized at neurite tips.[37] The finding that astrocytes chemically support the viability of primary neurons throughout continuous feeding of unnatural monosaccharide, even without physical contact, introduces potential for other advanced research platforms, such as three-dimensional (3D) noncontact, coculture systems of electrospun fibers or microbeads, which recapitulate the in vivo neuron system more faithfully.[38]

METHODS

Cell Culture. For culturing primary astrocytes, cerebral cortices of an E18 Sprague−Dawley rat pups were dissected and dissociated to single cells in Hank's balanced salt solution (HBSS, Welgene). The cells were centrifuged for 3 min at 1000 rpm, and the cell pellet was suspended in Dulbecco's modified Eagle's medium (DMEM, Welgene) with 10% fetal bovine serum (FBS, Welgene) and 1% penicillin-streptomycin (Gibco). The cells were seeded on a cell culture flask coated with poly-D-lysine (0.1 mg/mL in deionized water, Sigma). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2, and medium was replaced every 3−4 days. After 2 weeks, the culture consisted of glial cells, astrocytes, microglia, and oligodendrocyte precursor cells. After weakly shaking the flask, the astrocytes were separated from the other cells. When the astrocytes were grown to 80−90% confluence, 0.05% trypsin-EDTA (Welgene) was added to the cell culture flask for detaching the astrocytes. The cells were then collected by centrifuging, and the cell pellet was resuspended in cell culture medium. For preparing the sandwich coculture system,[26] the astrocytes were cultured on a poly-D-lysine-coated glass coverslip with paraffin spacers at a density of 50 cells/mm^2. After 7 DIV of astrocytes culture, the cells were cocultured with neurons.

Primary hippocampal neurons from the hippocampi of E18 Sprague−Dawley rat pups were cultured in a serum-free condition. Hippocampi were dissected and dissociated to single cells in HBSS using a pipet. The cell-containing solution was centrifuged for 2 min at 1000 rpm, and the supernatant was discarded. The cell pellet was then suspended in Neurobasal medium (Gibco) with B-27 supplement (Invitrogen), 2 mM GlutaMAX (Gibco), 12.5 μM L-glutamic acid (Sigma), and 1% penicillin-streptomycin (Gibco). Dissociated primary cells were seeded on a glass coverslip coated with poly-D-lysine (0.1 mg/mL in deionized water, 1 h, Sigma) at a density of 80 cells/mm^2. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. For making the sandwich system, the neuron-attached coverslip was inverted and placed on astrocyte coverslips, separated by paraffin spacers. To feed unnatural monosaccharides to neurons, stock solution of Ac_4ManNAz in dimethyl sulfoxide was diluted in culture medium and placed in a cell culture plate, to establish a final Ac_4ManNAz concentration of 50 μM. For long-term culture of neurons in sandwich(++) system, the culture medium was replaced with fresh medium containing Ac_4ManNAz every 3−4 days. For control system, the medium was replaced every 3−4 days with L-glutamic acid-free fresh culture medium. This study was approved by IACUC (Institutional Animal Care and Use Committee) of KAIST.

Viability Assay. Viability assay was performed by using the LIVE/DEAD viability/cytotoxicity kit for mammalian cells (ThermoFisher). Neurons and astrocytes were incubated in phosphate-buffered saline (PBS) with 2 μM calcein AM and 4 μM ethidium homodimer-1 for 20 min at room temperature. After staining, the samples were washed with PBS twice and imaged with a confocal laser-scanning microscope.

Immunocytochemistry. For immunostaining, neurons were fixed in a solution of 4% (w/v) paraformaldehyde (Sigma) in PBS at room temperature and washed with PBS for 5 min. The fixed samples were treated with 0.1% Triton X-100 in PBS for 15 min to permeabilize the

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cell membrane. For blocking nonspecific binding, the samples were immersed in a solution of 6% bovine serum albumin (BSA, Chemcruz) for 30 min at room temperature. Anti-β-tubulin III (Sigma), anti-MAP2 (Sigma), and anti-GFAP (Sigma) were dissolved in 1.5% BSA solution at desired working concentrations, in which the samples were incubated for 1 h at 37 °C or overnight at 4 °C. Then, the 1.5%-BSA solutions of corresponding secondary antibodies, Alexa Fluor 594 goat anti-rabbit IgG (Life Technologies) or Alexa Fluor 488 donkey anti-mouse IgG (Life Technologies), were used to stain the targets (1 h at 37 °C). Alexa Fluor 488-phalloidin (Life Technologies) was added when staining F-actin structures. The samples were washed with PBS three times for 5 min each at every step and mounted on a slide glass with a mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI) to stain nuclei (Vector laboratories).

Metabolic Labeling of Sialoglycans. For CuAAC reaction, $CuSO_4$ (50 μM) and 2-(4-((bis((1-(*tert*-butyl)-1*H*-1,2,3-triazol-4-yl)methyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)acetic acid (BTTAA, 250 μM, synthesized according to the reported procedures) were added at a 1:5 molar ratio to PBS solution containing Alexa Fluor 594-Alkyne or Alexa Fluor 488-Alkyne (25 μM, Life Technologies) and aminoguanidine (1 mM, Sigma).[4] To the resulting solution was added a freshly prepared solution of sodium ascorbate to establish a final concentration of 2.5 mM. This reaction mixture was incubated for 10 min at 4 °C and then added to the cells, followed by incubation at 4 °C for 5 min. After the reaction, the cells were washed twice with the culture medium and observed with a confocal microscope. For two-color labeling, cells were cultured for 2 days in between the first and second fluorescence labeling.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.7b00300.

Experimental details, neurotoxicity test of Ac_4ManNAz with ECM proteins, CLSM images of neurons cultured in control(−) and sandwich(−), and astrocytes cultured in sandwich(−) and sandwich(++) (PDF)

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Author Contributions

J.Y.C., M.P., K.K., and I.S.C. conceived and designed the experiments. J.Y.C., M.P., and M.-H.K. performed the experiments and imaging analysis. H.C. contributed graphical works. J.Y.C., M.P., K.K., and I.S.C. wrote the paper.

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Notes

The authors declare no competing financial interest.

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