An iPSC-based neural model of sialidosis uncovers glycolytic impairment-causing presynaptic dysfunction and deregulation of Ca\(^{2+}\) dynamics

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1. Introduction

Sialidosis is an autosomal recessive genetic disease characterized by a deficiency in the NEU1 gene-encoding lysosomal neuraminidase and characterized by abnormal accumulation of undigested sialyl-oligoconjugates in systemic organs including brain. Although patients exhibit neurological symptoms, the underlying neuropathological mechanism remains unclear. Here, we generated induced pluripotent stem cells (iPSCs) from skin fibroblasts with sialidosis and induced the differentiation into neural progenitor cells (NPCs) and neurons. Sialidosis NPCs and neurons mimicked the disease-like phenotypes including reduced neuraminidase activity, accumulation of sialyl-oligoconjugates and lysosomal expansions. Functional analysis also revealed that sialidosis neurons displayed two distinct abnormalities, defective exocytotic glutamate release and augmented a-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPAR)-mediated Ca\(^{2+}\) influx. These abnormalities were restored by overexpression of the wild-type NEU1 gene, demonstrating causative role of neuraminidase deficiency in functional impairments of disease neurons. Comprehensive proteomics analysis revealed the significant reduction of SNARE proteins and glycolytic enzymes in synaptosomal fraction, with downregulation of ATP production. Bypassing the glycolysis by treatment of pyruvate, which is final metabolite of glycolysis pathway, improved both the synaptic ATP production and the exocytotic function. We also found that upregulation of AMPAR and L-type voltage dependent Ca\(^{2+}\) channel (VDCC) subunits in disease neurons, with the restoration of AMPAR-mediated Ca\(^{2+}\) over-load by treatment of antagonists for the AMPAR and L-type VDCC. Our present study provides new insights into both the neuronal pathophysiology and potential therapeutic strategy for sialidosis.

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**ABSTRACT**

Sialidosis is a neuropathic lysosomal storage disease caused by a deficiency in the NEU1 gene-encoding lysosomal neuraminidase and characterized by abnormal accumulation of undigested sialyl-oligoconjugates in systemic organs including brain. Although patients exhibit neurological symptoms, the underlying neuropathological mechanism remains unclear. Here, we generated induced pluripotent stem cells (iPSCs) from skin fibroblasts with sialidosis and induced the differentiation into neural progenitor cells (NPCs) and neurons. Sialidosis NPCs and neurons mimicked the disease-like phenotypes including reduced neuraminidase activity, accumulation of sialyl-oligoconjugates and lysosomal expansions. Functional analysis also revealed that sialidosis neurons displayed two distinct abnormalities, defective exocytotic glutamate release and augmented AMPAR-mediated Ca\(^{2+}\) influx. These abnormalities were restored by overexpression of the wild-type NEU1 gene, demonstrating causative role of neuraminidase deficiency in functional impairments of disease neurons. Comprehensive proteomics analysis revealed the significant reduction of SNARE proteins and glycolytic enzymes in synaptosomal fraction, with downregulation of ATP production. Bypassing the glycolysis by treatment of pyruvate, which is final metabolite of glycolysis pathway, improved both the synaptic ATP production and the exocytotic function. We also found that upregulation of AMPAR and L-type voltage dependent Ca\(^{2+}\) channel (VDCC) subunits in disease neurons, with the restoration of AMPAR-mediated Ca\(^{2+}\) over-load by treatment of antagonists for the AMPAR and L-type VDCC. Our present study provides new insights into both the neuronal pathophysiology and potential therapeutic strategy for sialidosis.
present in the lysosomes of most tissue cells where it degrades N-acetylneuraminic acid, a major form of sialic acid attached to the non-reducing end of the sugar chain (Pshezhetsky and Ashmarina, 2018). The resulting accumulation of sialyl glycoconjugates in tissues is believed to cause the characteristic dysfunctions in sialidosis (France-schetti and Canafoglia, 2016).

Sialidosis is divided into two clinical types depending on the onset and symptoms (d’Azzo et al., 2015). Sialidosis type I develops in people aged 10–30 years with progressive neurological symptoms such as myoclonic seizures, visual impairment, epilepsy, and ataxia. A characteristic cherry-red spot appears in the fundus, and urine samples show increased sialyloligosaccharides (d’Azzo et al., 2015). Sialidosis type II develops earlier and presents systemic symptoms such as coarse facial appearance, enlargement of the liver and spleen, osteogenesis imperfecta, and psychomotor development delay in addition to the same neurological symptoms as type I (d’Azzo et al., 2015). Depending on the age of onset, sialidosis type II is classified into three forms: congenital, infant, and juvenile. Patients with the congenital form die early after birth due to fetal edema. In the infant (develops within 1 year after birth) and juvenile (develops after 1 year) forms, the life prognosis is poorer in the early onset than in the late onset. There are no effective drugs for sialidosis.

Disease models are indispensable for understanding the pathophysiology and developing new drugs. Two gene-manipulated mice have been reported as animal models of sialidosis (de Geest et al., 2002; Bonten et al., 2013). Neu1 knockout mice (Neu1−/−) lack Neu1 expression throughout the body and express virtually no neuraminidase activity in the kidney, liver, lung, heart, and intestine; however, the brain retains high residual enzyme activity (de Geest et al., 2002). In mouse brain, Neu1 contributes to 30% of total neuraminidase activity and the other 70% is from the activity of Neu3 and Neu4, whereas in mouse kidney it contributes to >95% of neuraminidase activity (Pan et al., 2017, Guo et al., 2018a, Guo et al., 2018b). Consistent with the residual neuraminidase activity, the Neu1−/− mouse model exhibits only mild neurological symptoms and histological abnormalities in the brain despite the severe non-neurological symptoms and histological abnormalities in other organs. The other mouse model, Neu1−/−; NEU1V54M, was generated by crossing transgenic mice bearing the V54M mutation in their NEU1 gene (as found in sialidosis type I patients) into the Neu1−/− background (Bonten et al., 2013). This mouse retains residual neuraminidase activity that is 1–80% of that in wild-type (WT) mice, and as for the Neu1−/− mice, the residual enzyme activity is much higher in the brain (approximately 80% of that in WT mice) than in the other organs such as kidney (approximately 1% of that in WT mice). Consistently, this mouse develops the typical hydronephrosis, but no neurological symptoms. Although these models are useful for investigating the non-neurological pathophysiology in sialidosis, it is unclear whether the neuropathological phenotype accurately recapitulates the disease phenotype in human patients because of the inconsistency of severe neurological symptoms.

Sialidosis studies using patient-derived skin fibroblasts revealed the typically low neuraminidase activity and enlarged lysosomes (Kwak et al., 2015), and the brain at autopsy also revealed typical histological abnormalities (Uchihara et al., 2010). Nevertheless, these fibroblast models do not fully clarify the neuropathological picture of sialidosis, such as neural function, because of the range of cell types generally affected and the limited or no accessibility for functional analyses.

Disease-derived iPSCs can mostly generate the various tissue cells that are difficult to collect directly from patients (Taoufik et al., 2018), making them ideal tools to study disease pathogenesis at the cellular level. Indeed, there are many reports of iPSC-derived cells mimicking disease phenotypes and thereby providing novel insights into the pathogenic events and mechanisms (Taoufik et al., 2018). We therefore aimed to establish sialidosis-derived iPSCs to elucidate mechanisms underlying the range of neurological defects. Herein, we generated a new cellular model of disease and analyzed iPSC-derived neurons to reveal novel functional and molecular abnormalities in sialidosis.

2. Materials and methods

2.1. Culture of patient-derived skin fibroblasts

Fibroblasts were isolated from explants of skin biopsies collected from sialidosis patients following informed consent under protocols approved by the ethics committee assigning authors. Skin samples were minced and cultured with Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum.

2.2. Generation and maintenance of iPSCs

Disease iPSCs were generated from patient-derived skin fibroblasts as described previously (Soga et al., 2015). Briefly, 5 × 10⁵ fibroblast cells were seeded on 6-well plates and infected with Sendai virus vector carrying OCT3/4, SOX2, KLF4, and c-MYC at 10 multiplicity of infection. At 7 days after infection, cells were harvested and replated at 5 × 10⁵ cells/dish on mytomycin C (MMC)-treated mouse embryonic fibroblast feeder cells (MEF). After culturing for 11 to 18 days, colonies were picked and recultured with human iPSC medium (DMEM/F12, SIGMA, St. Louis, MO, USA) supplemented with 20% Knockout serum replacement (Invitrogen, Carlsbad, CA, USA), 5 ng/μl basic fibroblast growth factor (Wako, Osaka, Japan), 0.1 mM non-essential amino acids (SIGMA), 2 mM l-Glutamine (Life Technologies), 0.1 mM 2-mercaptoethanol (SIGMA), and 0.5% of both penicillin and streptomycin (Nacalai Tesque, Kyoto, Japan). The iPSCs were cultured at 38 °C for 3 days at passage 1 or 2 of the iPSCs to remove any Sendai virus, and then maintained on MMC-MEF with human iPSC medium. As control iPSCs, we used three healthy subject-derived iPSC lines, 201B7, 409B2, and N2–1, established as described previously (Takahashi et al., 2007, Okita et al., 2011, Fujie et al., 2014)

2.3. Neural differentiation

Differentiation of iPSCs toward neural progenitor cells (NPCs) was performed using PSC Neural Induction Medium (Life Technologies) as described previously (Matsushita et al., 2019). Briefly, detached iPSC colonies were seeded on geltrex (Life Technologies)-coated 6-well plates and cultured for 7 days with Neural Induction Medium (NIM), comprising neurobasal medium (Life Technologies) supplemented with 2% Neural Induction Supplement (Life Technologies). The NPC colonies were manually picked and recultured with NIM for 2 days. Following the dissociation of NPC colonies using Accutase (Innovative Cell Technologies, San Diego, CA, USA), NPCs were seeded on geltrex-coated 6-well plates or 60-mm dishes for culturing in Neural Expansion Medium (1,1 mixture of Neurobasal Medium and Advanced DMEM/F12, Life Technologies) supplemented with 1% Neural Induction Supplement) until use in experiments or differentiation into neurons.

NPCs were differentiated into neurons as described previously (Matsushita et al., 2019). Briefly, the NPCs were seeded onto polyethyleneimine-coated glass-bottom dishes or 12-well plates and cultured with Neural Induction Medium (Neurobasal Medium supplemented with 2% B27 supplement (Life Technologies)) including 5 μM DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester, SIGMA) for 2 weeks. Subsequently, neurons were cultured in Neural Induction Medium without DAPT for another week. After 3 weeks, the neurons were maintained in DMEM supplemented with 20% Knockout serum replacement or NeurobasalPlus medium supplemented with 2% B-27 Plus Supplement (Life Technologies) and 100 μM db-cAMP (Sigma Aldrich). Half the medium was changed once a week.
2.4. Alkaline phosphatase staining and immunostaining

Alkaline phosphatase staining was performed using the Leukocyte Alkaline Phosphatase kit (SIGMA) according to the manufacturer’s protocols.

Immunostaining was performed as described previously (Soga et al., 2015). Briefly, cells were fixed with 4% paraformaldehyde diluted with phosphate-buffered saline (PBS) for 20 min at room temperature, and then incubated with blocking buffer (PBS containing 3% bovine serum albumin and 0.2% Triton-X100) for 30 min at room temperature. After washing with PBS, the samples were incubated overnight at 4 °C with the primary antibodies diluted with blocking buffer. Following further PBS washes, secondary antibodies were applied and exposed for 1 h at room temperature, followed by washing with PBS. Immunofluorescence was observed with a fluorescence microscope or IN CELL ANALYZER 6000 (GE Healthcare, Chicago, IL, USA). The percentage of PAX6- and MAP2-positive neurites were quantified with IN Cell Developer Toolbox (GE Healthcare). All antibodies used for immunostaining are listed in Supplementary Table 1.

2.5. Immunoblotting and lectin blotting

For the immunoblotting and lectin blotting experiments, protein extraction and SDS-PAGE were performed as described previously (Soga et al., 2015). Briefly, the cells were treated in lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and protease inhibitor cocktail (Nacalai Tesque)), followed by sonication, heating (100 °C, 5 min) and centrifugation (15,000 rpm, 20 min). After collecting the supernatants as total cell lysate, the protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA) followed by Sample Buffer (65 mM Tris–HCl, 15% SDS, 12.5% mercaptoethanol, 50% glycerol, and 12.5% BPB) applied to each sample. Pellets were also diluted with 1 × Sample Buffer to yield the NP-40-insoluble fraction. The same amount of protein from each sample was separated by electrophoresis using 6–15% SDS polyacrylamide gels, and then electrophoretically transferred to a polyvinylidene fluoride membrane using a Semi-Dry Transfer Cell (BioRad). The membrane was blocked with 5% non-fat milk for immunoblotting or with a lectin-blocking solution (10 mM Tris-HCl, 0.15 M NaCl, and 0.05% Tween20) for 1 h at room temperature, before incubating the cells at 4 °C overnight with primary antibody diluted in 1% non-fat milk or with biotinylated SNA-Lectin (Vector Laboratories, Burlingame, CA, USA) diluted with lectin blocking buffer. After washing and incubation in secondary antibody diluted with 1% non-fat milk or HRP-Conjugated Streptavidin (Thermo Fisher Scientific, Waltham, MA, USA) diluted with lectin-blocking solution, protein bands on membranes were visualized by Enhanced Chemiluminescence detection (Perkin Elmer) using an Image Quant Las 4000 mini (GE Healthcare), and quantified with a CS Analyzer 3.0 (ATTO, Tokyo, Japan). All antibodies used for immunoblotting are listed in Supplementary Table 2.

2.6. Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using the Sepasol®-RNA I Super G (Nacalai Tesque) or RNAeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. Equal amounts of total RNA from each sample were then reverse transcribed into cDNA using Super Script III (Invitrogen) and random primers (Invitrogen). RT-PCR and qRT-PCR were performed with Quick Taq TM (TOYOBO, Osaka, Japan) and THUNDER BIRD TMqPCR Mix (TOYOBO) according to the manufacturer’s protocols, and then analyzed using a Step One Plus real-time PCR system (Applied Biosystems). The mRNA expressions of indicated genes were normalized to that of the β-ACTIN gene. The list of primers is supplied in Supplementary Table 3.

2.7. Karyotyping

Karyotyping of disease iPSC lines was performed by Nihon Gene Research Laboratories. Inc. (Sendai, Japan).

2.8. Teratoma formation assay

Teratoma formation was assayed as described previously (Soga et al., 2015). Briefly, detached iPSCs were injected into the testis of NOD-SCID immunodeficient mice. After 12–16 weeks, tumors were collected and fixed with 10% formalin, processed for paraffin embedding and hematoxylin-eosin staining, and then observed using a BX-9000 microscope (Keyence, Osaka, Japan). Experimental procedures conformed to the animal use guidelines of the Committee for Ethics on Animal Experiments of Kumamoto University.

2.9. Direct sequencing of the NEU1 gene

Genomic DNA was extracted from iPSCs or fibroblasts as described previously (Soga et al., 2015). PCR targeting of exons including the exon-intron junction of the NEU1 gene were performed with Quick Taq TM, and direct sequencing of PCR products was performed with BigDye® Terminator v1.1 (Applied Biosystems) according to the manufacturer’s protocols. The list of primers is supplied in Supplementary Table 4. NEU1 mutations identified in each patient were searched in the Human Gene Mutation Database Public (http://www.hgmd.cf.ac.uk/ac/index.php) and SNP database of the National Center for Biotechnology Information. The pathogenicity of novel mutations was tested bioinformatically using Polymorphism Phenotyping-2 (PolyPhen2, http://genetics.bwh.harvard.edu/pph2/).

2.10. Measurements of neuraminidase enzymatic activity

Neuraminidase activity was measured as described previously (Okumiya et al., 2006). Briefly, the cell lysates were fluorometrically measured with 4-Methylumbelliferyl (4MU)-α-D-N-acetylnearuminic acid (M8639, Sigma-Aldrich). First, 10 μl of the cell homogenate was added to 40 μl of the 4MU substrate solution in a 96-well black microtiter plate. Next, the reaction mixture was incubated at 37 °C for 1 h, and the reaction was stopped by adding 200 μl of 0.2 M glycine-NaOH buffer, pH 10.7 containing 0.1% Triton X-100. The fluorescence intensity was measured using a fluorescence plate reader (excitation 360 nm; emission 450 nm; Infinite F200 Pro, TECAN Japan, Kawasaki, Japan), and neuraminidase activity was expressed as nanomoles 4MU released per hour per milligram cellular protein (nmol/h/mg protein).

2.11. Measurements of sialic acid content

Cellular sialic acid content was determined using an EnzyChrom Sialic Acid Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer’s protocol. Briefly, 20 μl of cell homogenate was combined with 80 μl of hydrolisis buffer and incubated for 60 min at 80 °C to hydrolyze terminally bound sialic acids in the sugar chains. After adding 20 μl Neutralization Buffer, 10 μl of each sample was mixed with 93 μl Assay Buffer, 1 μl Dye Reagent, and 1 μl Enzyme in a 96-well black microtiter plate, and then incubated at room temperature for 1 h. The fluorescence intensity at λex = 560 nm and λem = 595 nm was measured with a fluorescence plate reader. Sialic acid content in each sample was normalized to the protein concentration in each sample.

2.12. LysoTracker staining

LysoTracker staining was performed as described previously.
coated 96-well culture plates. After 3 days culturing, NPCs were incubated with 50 nM LysoTracker (Life Technologies) at 37 °C for 30 min, and the staining was analyzed using the IN CELL ANALYZER 6000 and Developer Toolbox software.

2.13. Apoptosis and cell death detection assay

NPCs or neurons were detached with Accutase solution and collected in 15-ml conical tubes for centrifugation at 1200 rpm for 3 min. After washing with artificial cerebrospinal fluid (ACSF, 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 1.25 mM Na$_2$HPO$_4$, 25 mM NaHCO$_3$, and 25 mM glucose), cells were resuspended in ACSF at 5 × 10$^5$ cells/ml and cultured with either a 1/100 volume of PE-Annexin V/7-AAD or 1/100 volume of Alexa647-Annexin V/FVS450 for 30 min on ice. Annexin V stains early apoptotic cells via specific binding with extracellularly exposed phosphatidylserine, while 7-AAD and FVS450 stains the nuclei of dead cells. The fluorescence intensity of Annexin V, 7-AAD, and FVS450 staining was measured by BD FACS Aria III and the percentages of apoptotic and dead cells were calculated.

2.14. H$_2$O$_2$ oxidative stress test

H$_2$O$_2$ oxidative stress tests were performed as described previously (Matsushita et al., 2019). Briefly, NPCs were seeded on PEI-coated 96-well plates and cultured until day 66 under the neuron differentiation condition, before exposure to various concentrations of H$_2$O$_2$ (10-500 μM) for 24 h. Cell viability was then determined using the WST-8 assay with Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer’s protocol.

2.15. Exocytosis imaging with FM1–43

Exocytosis imaging with FM1–43 dye was performed as described previously (Matsushita et al., 2019). Neurons cultured over 9 weeks on glass-bottom dishes were stained with dye loading buffer containing ACSF with 5 μM FM1–43 for 30 min at 37 °C. After washing with ACSF, the fluorescence of FM 1–43 (λex = 488 nm and λem = 520 nm) was monitored using a fluorescence microscope (DMi8, Leica, Wetzlar, Germany) and captured every second. The membrane depolarization was induced by application of 50 mM KCl, and the fluorescence intensity of FM1–43 at synapse-like puncta was analyzed using the LAS X Imaging System (Leica). Exocytotic activity was evaluated by the percentage of decreased FM1–43 intensity after KCl application compared with that before application, with 1 mM pyruvate, 300 μM iodoacetate, 50 mM 2-Deoxy-d-glucose or 10 μM perampanel applied at the start of FM1–43 loading and exposed throughout the imaging assay.

2.16. Glutamate release assay

The membrane depolarization-induced glutamate release in neurons was assessed as described previously (Matsumoto et al., 2006). Briefly, neurons cultured over 9 weeks on 12-well plates were washed three times with ACSF and then incubated in ACSF for 20 min at 37 °C. The neurons were then incubated with fresh ACSF for 5 min, and supernatants were collected as “base” samples. The neurons were next incubated with ACSF supplemented with 50 mM KCl for 5 min, and the supernatants were collected as “HK” samples. Glutamate concentrations of base and HK samples were determined using a high performance liquid chromatography system consisting of two NexeraX2 LC-30 CE liquid chromatography pumps (Shimadzu, Kyoto, Japan), a DGU-20A5R degasser (Shimadzu), a SIL-30 CE autosampler (Shimadzu), a CTO-20 AC column oven (Shimadzu), and a RF-20AXS fluorescence detector (Shimadzu). Membrane depolarization-induced glutamate release was evaluated by the ratio of glutamate concentration in the HK sample to that in the base sample.

2.17. Ca$^{2+}$ imaging with Fluo-4

Ca$^{2+}$ influx was assessed by imaging of a Ca$^{2+}$-sensitive dye, Fluo-4. Neurons cultured over 9 weeks on glass-bottom dishes were stained with dye loading buffer containing ACSF with 1 mM Fluo-4 for 30 min at 37 °C. After washing with ACSF, changes in the fluorescence of Fluo-4 (λex = 488 nm and λem = 520 nm) were monitored per second using a fluorescence microscope (DMi8, Leica, Wetzlar, Germany). The Ca$^{2+}$ influx was induced by applying the indicated excitatory stimulant, and the fluorescence intensity of Fluo-4 at each time point was analyzed by the LAS X Imaging System (Leica). To test the effect of channel inhibitors, 10 μM perampanel (Eisai, Tokyo, Japan) or 30 μM nicipidine hydrochloride (Tokyo Chemical Industry, Tokyo, Japan) was exposed throughout the imaging assay. Ca$^{2+}$ influx was evaluated by the ratio of Fluo-4 intensity after stimulation to that before stimulation.

2.18. Retrovirus infection

The full-length coding region of human NEU1 (hNEU1) obtained from Kazusa DNA Res. Inst. (Chiba, Japan) was inserted into the EcoRI/Xhol sites of pMSCV-ires-deRed.T4, a retroviral vector that expresses full-length hNEU1 and DeRed.T4 using an internal ribosome entry site under the Murine Stem Cell Virus (MSCV) promoter. Production and infection of retrovirus were performed as described previously (Pear et al., 1993). Infection efficiency was then evaluated based on the percentage of DeRed.T4-positive cells using BD FACS Aria III (BD Biosciences, Franklin Lakes, NJ, USA).

2.19. Preparation of synaptosomes and proteome analysis by LC-MS

Synaptosomes of iPSC-derived neurons were prepared using Syn-PER Synaptic Protein Extraction Reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol. Briefly, the cells were first lysed with Syn-PER Synaptic Protein Extraction Reagent supplemented with protease inhibitor cocktail (Nacalai Tesque). The cell lysates were centrifuged at 1200 g for 10 min at 4 °C to remove cell debris and the supernatants were transferred to new tubes. The samples were then centrifuged again at 15000 g for 20 min at 4 °C and the resultant pellet was lysed with SDS lysis buffer (10 mM Tris-HCl, 1% SDS, 10 mM sodium pyrophosphate, 5 mM ethylenediaminetetraacetic acid, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 2 mM sodium orthovanadate) as the synaptosomal fraction. After sonication and heating at 100 °C for 5 min, the protein concentration of each sample was determined using a BCA protein assay kit (Thermo Fisher Scientific).

Equal amounts of protein were subjected to SDS-PAGE and stained with CBB for the in-gel digestion coupled with mass spectrometric analysis. Proteins regions of the gel were excised, cut into 1-mm similarly sized pieces, and then destained. Proteins in the gel pieces were reduced with DTT (Thermo Fisher Scientific), alkylated with iodoacetamide (Thermo Fisher Scientific), and digested with trypsin and lysyl endopeptidase (Promega) in 40 mM ammonium bicarbonate, pH 8.0, overnight at 37 °C. The resultant peptides were analyzed on an Advance UHPLC system (AMR/Michrom Bioscience) coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific) with the raw data processed using Xcalibur (Thermo Fisher Scientific). The raw data were analyzed against the SwissProt database restricted to Homo sapiens using Proteome Discoverer version 1.4 (Thermo Fisher Scientific) using the Mascot search engine version 2.5 (Matrix Science). A decoy database comprising either randomized or reversed sequences in the target database was used for false discovery rate (FDR) estimation, and the Percolator algorithm was used to evaluate false positives. Search results were filtered against 1% global FDR for high confidence level, and the resulting datasets were further analyzed using Scaffold 4 following cut-off values: minimum number of peptides = 2, peptide threshold = 95%, protein threshold = 1.0% FDR. Quantification was performed using the Top3 precursor intensities for each protein, and then pathway
enrichment analysis using GO terms and the KEGG pathway was performed using the DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/tools.jsp).

2.20. ATP production assay

Glucose-dependent ATP production in synaptosomes was assessed using adaptations of methods previously reported (Ikemoto et al., 2003). Following the synaptosome isolation with Syn-PER Synaptic Protein Extraction Reagent, synaptosome pellets were resuspended with ice-cold ACSF without glucose. These synaptosome solutions were divided into several tubes and then incubated with or without 10 mM glucose and indicated drugs (300 μM iodoacetate, 1 mM pyruvate, 10 μM per- ampanel) for 20 min at 37 °C. The ATP content of each sample was then measured using the “Cell” ATP ASSAY reagent (TOYO B-Net, Tokyo, Japan) according to the manufacturer’s protocol. Glucose-dependent ATP production was evaluated by the ratio of ATP content in glucose-containing samples to that in glucose-absent samples.

Cellular ATP production was assessed as described previously (Soga et al., 2015). Briefly, NPCs were seeded on geltrex-coated 24-well plates and cultured with NEM overnight at 37 °C. The medium was then changed to DMEM without glucose for a 12-h starvation, followed by recovery with NEM for 6 h at 37 °C. Finally, cells were lysed with NP-40 lysis buffer and the ATP content and protein concentration in each sample was measured. Cellular ATP content is shown as μmol/mg protein.

2.21. OCR measurements

Oxygen consumption rate (OCR) was measured with an XF24 extracellular flux analyzer (Agilent Technologies, CA, USA) according to the manufacturer’s protocol. Briefly, NPCs were seeded on geltrex-coated XF24 cell culture microplates at 1 × 10⁴ cells/well and incubated with NEM overnight at 37 °C. The NEM was then replaced with DMEM without phenol red and OCR measurements were performed using a Seahorse XF Cell Mito Stress Kit (Agilent Technologies).

2.22. Electron microscopy

Analysis by electron microscopy was performed as described previously with minor modifications (Kato et al., 2001). Cells cultured on PEI-coated cover glass were prefixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature. The specimens were examined with a transmission electron microscope (HT7700, Hitachi, Japan).

2.23. Measurement of lactate concentration

Cellular lactate content was determined using Lactate Assay Kit-WST (Dojindo, Kumamoto, Japan) according to the manufacturer’s protocol. Briefly, cells were washed with PBS and lysed with distilled water containing 0.1% Triton-X100. Cell homogenate was diluted with fresh distilled water to 10 times and 20 μl of each sample was applied in 96-well plate. Each sample was mixed with 80 μl of working solution which consist of 10% dye mixture, 90% of assay buffer and 1/50 volume of enzyme solution, and incubated for 30 min at 37 °C. Then, the optical density at 450 nm was measured with a microplate reader and the lactate concentrations were determined using a calibration curve of lactate standard. Lactate content in each sample was normalized to the protein concentration in each sample which was determined by BCA protein assay.

2.24. Statistics

Statistical analyses were performed with an unpaired, 2-tailed Student’s t-test for the comparisons the mean among two groups. Differences between means of multiple groups were analyzed by one-way ANOVA with posthoc Dunnett’s test, Tukey-Kramer test or Holm test using EZR on R Commander version 1.41 (Kanda 2013). The differences were considered to be significant at a 5% level. All graphs show mean ± SD.

3. Results

3.1. Generating iPSCs from sialidosis patients

We obtained skin fibroblasts from two sialidosis patients, namely f-Sia1 and f-Sia2. Both f-Sia1 and f-Sia2 fibroblasts exhibit significant lower neuraminidase activity in comparison with healthy control lines (Fig. S1). We generated iPSCs from f-Sia1 and f-Sia2 using Sendai virus vectors carrying the four reprogramming factors, OCT3/4, SOX2, KLF4, and c-MYC. Each iPSC line formed ES cell-like colonies and expressed pluripotent cell markers such as NANOG, OCT3/4, SSEA4, TRA1-60, and alkaline phosphatase (AP) (Fig. 1A). RT-PCR analysis also confirmed the expression of other pluripotent cell markers (Fig. 1B). Karyotypes of the iPSCs were normal (Fig. 1C), while teratoma formation analysis revealed that the iPSCs were multipotent and could give rise to three germ layers (Fig. 1D). The iPSCs, Sia1 and Sia2, carried homozygous missense mutations of c.527 A > G (p.Asp176Gly) and c.946C > T (p.Pro316Ser), respectively, in the NEU1 gene, mimicking the patient-derived fibroblasts (Fig. 1E). While the latter mutation, p. Pro316Ser, was previously reported in a juvenile-onset sialidosis type1 patient, the former mutation has not been reported in either the Human Gene Mutation Database Public or the SNP database of the National Center for Biotechnology Information (Itoh et al., 2002). We assessed the possible impact of this novel p.Asp176Gly mutation on the structure and function of neuraminidase using the bioinformatics tool, Polymorphism Phenotyping-2 (PolyPhen2), which is popularly used to speculate a molecular function to gene mutations (Adzhubei et al., 2010). PolyPhen2 predicted this mutation to be “ Probably Damaging” (Score: 0.964, Sensitivity: 0.62, Specificity: 0.92). These results show that the iPSCs established here satisfy the iPSC criteria and are derived from the sialidosis patients.

3.2. A cellular model exhibiting biochemical phenotype of sialidosis

To investigate the cellular neuropathology of sialidosis, we first differentiated the iPSCs toward NPCs. As normal controls, healthy donor-derived iPSCs established previously (201B7, 409B2 and N2–1) were used (Takahashi et al., 2007, Okita et al., 2011, Fujie et al., 2014).

Both the healthy donor- and patient-derived NPCs highly expressed the NPC markers, SOX1, PAX6, FOXG1, and NEFH (Fig. S2A and B). In both NPC populations, almost all NPCs expressed NEFH, and of these, approximately 80% of cells also expressed PAX6, a dorsal telencephalic marker. The rate of apoptosis and cell death in the sialidosis-derived NPCs were also comparable to those in healthy controls (Fig. S2C), but neither NPC lines showed significant expression of OCT3/4, pluripotency marker. These results suggested that dorsal telencephalic NPCs were dominantly and similarly present in both healthy donor- and sialidosis-derived iPSC cultures.

Sialidosis is caused by an inactivating mutation in the NEU1 gene that leads to decreased neuraminidase activity and accumulation of sialylated oligosaccharides and glycoprotein in lysosomes (Franceschetti and Canafoglia 2016). Consistently, the enzymatic activity of neuraminidase in our Sia1- and Sia2-derived NPCs was much lower than that of the healthy group-derived cells (Fig. 2A). Blotting results for...
Fig. 1. Generation of iPSCs from sialidosis patients. (A) Immunofluorescence and alkaline phosphatase (AP) staining of iPSC lines for pluripotency markers. The iPSC lines, Sia1 and Sia2, were generated from skin fibroblasts of two sialidosis patients, respectively. Scale bar: 500 μm. (B) mRNA expression of various pluripotency markers in sialidosis-iPSC lines. The expressions were determined by RT-PCR. No signal in nested RT-PCR for Sendai virus RNA confirmed elimination of the viral RNA from iPSCs (SeV-Nested). PC: positive control, DW: distilled water as negative control. (C) G-Band karyotyping of Sia1 and Sia2 iPSC lines. Both lines have normal karyotypes, 46, XX and 46, XY, respectively. (D) Histological analysis of the iPSC-derived teratomas. Hematoxylin-eosin staining showed descendants of all three germ layers in the teratomas. MP: melanin pigment, CE: columnar epithelium, M: muscle, G: glandular epithelium, C: cartilage. Scale bar: 50 μm. (E) Confirmation of NEU1 gene mutations in Sia1 and Sia2 iPSC lines. The mutations 527A > G (Asp176Gly) and 946C > T (Pro316Ser) were observed in both skin-derived fibroblasts (upper) and iPSC lines (lower) derived from patient Sia1 and Sia2, respectively.
Sambucus Nigra-lectin (SNA-lectin), which labels the derived NPCs, visualized by lectin blotting with SNA-lectins. Immunoblotting with α-TUBULIN antibody is also shown as internal control (Relative α-TUBULIN band intensity; 201B7: 1.00, 409B2: 1.05, Sia1: 1.07, Sia2: 1.27, the intensities were normalized to that of 201B7). (C) Increased intracellular sialic acid contents in the patient-derived NPCs. Bound sialic acid in sialyl-glycoconjugates in NPC lysates were hydrolyzed and the released sialic acid was measured (N = 4 wells in each line). (D) Increased lystotracker staining in the patient-derived NPCs were stained with lystotracker (representative images in left panel). Proportions of lystotracker-positive area were calculated with IN Cell Analyzer 6000. (right graph, N = 5). Scale bar: 100 μm. (E) Increased expression of lysosomal protein, LAMP1, in the patient-derived NPCs. The expressions were determined by immunoblotting (representative image in left panel). Band intensities of LAMP1 were normalized to those of α-TUBULIN (right graph, N = 3). All graphs show mean ± SD. ***P < 0.001, *P < 0.05 vs. healthy donor-derived NPCs; one-way analysis of variance with post hoc Dunnett’s test.

3.3. Characterization of sialidosis-derived mature neurons

To further explore the pathogenesis in sialidosis-derived neural cells, we generated mature neurons from the NPCs. On day 14–21, our neurons highly expressed pan-neuronal marker, MAP2, and the proportion of MAP2- and Tubulin-III (TUBB3/TUJ1)-positive cells reached 80–90% in both healthy and disease groups (Fig. S3A, B and C). To further characterize neuronal subtypes in our culture, we examined expression of subtype marker of cortical neurons because our NPCs highly express dorsal telencephalic marker, Pax6 and FoxG1 (Fig. S2A). Increased expression of CaMK2A (at day 21), a marker for cortical glutamatergic neuron, and GAD67 (at day 63), a marker for GABAergic neuron, suggesting existence of both glutamatergic and GABAergic populations (Fig. S3A). Furthermore, cortical layer markers including CTIP2 for deep layer glutamatergic and CUX1 for upper layer glutamatergic neurons also increased in the day-21 culture (Fig. S3A). The expression levels of markers in the disease group were similar to those in control group even on day 77 (Fig. S3D) Immunocytochemistry also showed that approximately 10% of MAP2-positive neurons were GAD67-positive GABAergic neurons and 20% of that were CTIP2-positive cortical neurons (Fig. S3E and F). These results suggested that our iPSC-derived neurons consisted of the mixture with various cortical neuronal subtypes and the proportion of these cell subtypes was unaffected in the sialidosis-derived neurons. There was also no appreciable difference in the proportion of apoptosis and cell death among the control and disease groups (Fig. S3G). We then assessed the outgrowth potential for neurites and detected no significant change in the sialidosis-derived neurons compared to controls (Fig. S3H). These findings suggested that the differentiation potential from NPC to neuron...
in the sialidosis group was almost equivalent to that in the healthy donor group. Similar to the NPC case, biochemical analyses revealed enhanced sialyl-glycoprotein accumulation and LAMP1 expression in the sialidosis-derived neurons relative to controls (Fig. 3A and B). Furthermore, transmission electron microscopy revealed two morphological differences in lysosomes between control and sialidosis neurons (Fig. 3C, Fig. S3I). First, abnormally enlarged lysosomes were observed in Sia1 and Sia2 neurons (Fig. 3C, Fig. S3I), and a few of these contained a large vacuole, although most were morphologically normal (Fig. 3C, Fig. S3I). Second, lysosomes filled with dotted pale contents were highly accumulated in the cytosol of Sia1 neurons, but were rarely detected in Sia2 neurons (Fig. 3C). These abnormalities were consistent with previously reported lysosomal structures in sialidosis fibroblasts (Igdaora et al., 1994). Previous studies also showed increased vulnerability to oxidative stress and impaired autophagy in some lysosomal storage diseases (Lieberman et al., 2012; Donida et al., 2017), however, we detected no vulnerability to oxidative stress in the sialidosis-derived neurons (Fig. S3J). Two markers were then analyzed for monitoring the autophagy pathway. First, we examined the expression of microtubule-associated protein 1 light chain 3B (LC3B) (Fig. S3K). A-C-terminal pro (Fig. S3J). Two markers were then analyzed for monitoring the autophagy pathway. First, we examined the expression of microtubule-associated protein 1 light chain 3B (LC3B) (Fig. S3K). C-terminal processing of LC3B produces LC3B-I, which is modified to LC3B-II with the initiation of autophagosome formation. Our sialidosis-derived neurons showed significantly decreased expression of LC3B-I and II compared with normal neurons. In addition, the ratio of LC3B-I to LC3B-II expression, indicating the initiation potential for autophagosome membrane formation, was not different between the disease and control groups. Second, we measured the insoluble form of p62/SQSTM1 (p62) to assess autophagic flux (Fig. S3L). Because p62 binds to LC3B and is degraded upon fusion with the lysosome, impaired autophagy flux results in the accumulation and aggregation of insoluble p62. Our neurons showed similar levels of insoluble p62 protein (Fig. S3L), suggesting intact autophagy flux in the sialidosis-derived neurons.

3.4. Neural dysfunction in sialidosis-derived neurons

The synapse is an essential component of neural circuits/networks. Neurotransmitter release in presynaptic site is the first step of the neurotransmission, and this presynaptic function is pathologically important because their defects cause abnormal activity in neural circuits/networks that can result in neurological and psychiatric symptoms (Taoufik et al., 2018). In our culture system, mRNA expressions of presynaptic markers such as SYNAPSIN1 (SYN1) and SYNAPTO TAGMIN1 (SYT1) significantly increased in normal neurons on day 21 and remained elevated on day 63 (Fig. S4A). Indeed, day 63 neurons contained SYN1-positive puncta, suggesting a formation of presynaptic structures (Fig. S4B), although there was no difference in the number of SYN1-positive puncta or mRNA expression level of SYN1 and SYT1 among the healthy and disease groups (Fig. S4B and C).

Exocytosis is essential step for releasing neurotransmitter at presynapse upon membrane depolarization (Li and Chinn, 2003). We therefore assessed presynaptic function in the neurons by visualizing exocytosis with the FM1–43 imaging method. As the FM1–43 dye is incorporated and released with synaptic vesicles, we can easily detect synaptic exocytosis as a sudden decrease in fluorescence intensity (Fig. 3D). The disease neurons showed significantly impeded attenuation of fluorescent intensity after membrane depolarization, indicating impaired synaptic exocytosis (Fig. 3D). The abnormal intensity of FM1–43 signal was also observed in the sialidosis neurons cultured under different condition containing NeurobasalPlus medium supplemented with 2% B-27 Plus Supplement and 100 μM db-cAMP which is optimized for the culturing of pluripotent stem cell-derived neurons, suggesting this phenotype is independent of culture conditions (Fig. S4D). To further examine the possible impairment of neurotransmitter release, we measured the extracellular content of glutamate, which is released from neurons in response to membrane depolarization. We evaluated the depolarization-evoked glutamate release as the ratio of glutamate content in the assay buffer with depolarization to that before stimulation for the depolarization. Consistent with the impaired activity of exocytosis, the ratios of glutamate content were significantly lower in both Sia1 and Sia2 neurons than in the normal neurons (Fig. 3E, Fig. S4E). Taken together, these results suggested that neurotransmitter release induced by membrane depolarization was impaired in the sialidosis-derived neurons.

We next investigated the excitatory responses to various stimulants including AMPA, glutamate, NMDA, and high KCl in our neurons using Ca²⁺ imaging with Fluo-4, a fluorescent indicator of intracellular Ca²⁺ concentration. Although all stimulants could enhance Ca²⁺ influx, the AMPA-evoked Ca²⁺ response was significantly augmented in the disease group compared to healthy controls (Fig. 3F). In contrast, there was no striking difference in the glutamate treatment among the healthy and disease-derived neurons, although the responses were significantly superior to those in healthy controls in Sia1 neurons treated with NMDA and KCl (Fig. S4F–H). Enhanced AMPA-evoked Ca²⁺ response was also observed in the sialidosis neurons cultured under different condition as described in Supplementary Fig. 4D (Fig. S4I). These results suggested that the sialidosis-derived neurons exhibited hyper-excitability on AMPA stimulation.

3.5. Restoring abnormal phenotypes by forced expression of NEU1

To validate whether NEU1 deficiency accounts for the abnormal phenotype in sialidosis-derived neural cells, we forced the over-expression of NEU1 in the sialidosis-derived NPCs using a retroviral vector carrying the DsRed.T4 gene to indicate expression. DsRed.T4-positive cells reached over 80%, which was sufficient for conducting the following experiments (Fig. S5A and B). Immunoblotting with an anti-NEU1 antibody confirmed the increased NEU1 expression following hNEU1 vector infection (Fig. S5C and D), and this forced expression of NEU1 restored the neuraminidase activity in sialidosis-derived NPCs, validating the functionality of exogenous NEU1 (Fig. 4A). Consistently, the overexpression of NEU1 also reduced the accumulation of sialyl-glycoproteins and total sialic acids in the sialidosis-derived NPCs (Fig. 4B, C and Fig. S5D, E). A significant decrease in lysotracker staining area and LAMP1 expression also suggested the restoration of lysosomal abnormalities in the sialidosis-derived NPCs overexpressing NEU1 (Fig. 4D and E). We next asked whether the forced NEU1 expression could restore the abnormal presynaptic exocytosis and AMPA-evoked Ca²⁺ response in mature neurons generated from the infected NPCs. Accordingly, NEU1 overexpression recovered the reduced exocytotic activity, decreased glutamate release and enhanced AMPA-evoked Ca²⁺ response (Fig. 4F, G, Fig. S5F). These results demonstrated that NEU1 deficiency caused the biochemical and functional abnormalities shown in our sialidosis-derived neural cells.

3.6. Proteome analysis of the synaptosomal fraction isolated from sialidosis-derived neurons

To investigate the molecular basis underlying the abnormal exocytosis in sialidosis, we analyzed possible changes in protein composition of the synaptosomal compartments in sialidosis-derived neurons, which contains full presynaptic components and fragments of post-synaptic density (Bai and Witzmann, 2007). Accordingly, quantitative mass spectrometry identified 808 proteins in the synaptosomal fractions, and while 63 proteins showed significantly upregulated expression, 108 proteins were significantly downregulated in the sialidosis groups (P < 0.05, Fig. S5A and Supplementary Data 1, 2). To identify the signaling pathways affected by the deregulated proteins, we performed KEGG pathway enrichment analysis and found that proteins involved in the “synaptic vesicle cycle” and “glycolysis/gluconeogenesis” were downregulated in sialidosis-derived neurons (Fig. 5B and Supplementary Data 3). Consistently, gene ontology (GO) enrichment analysis revealed the GO terms containing vesicle transport (including post-Golgi vesicle-
Fig. 3. Biochemical and functional abnormalities in sialidosis-derived neurons. (A) Sialyl-glycoprotein accumulation and (B) increased expression of LAMP1 in the patient-derived neurons. The NPCs were forced to differentiate into mature neurons in vitro by culturing for 77 days. Using the neurons, the same assays represented in Fig. 2B and E were conducted. (C) Electron micrographs of day-75 neurons. Arrows indicate lysosomes in the neurons. Inset of middle panel shows high power view of the rectangle in panel. Scale bar: 1 μm. (D) Impairment of depolarization-induced exocytosis in patient-derived neurons. The exocytosis was visualized in day-63 neurons with FM1–43 imaging (representative data in left panels). Each trace in the left panels represents a time-course of a change in fluorescent intensity of FM1–43 dye in single synapse-like puncta. The loss of FM1–43 intensity [(3 s before the HK application) - (7 s after the HK application)] is represented in the right graph (N = 9–10 puncta in each line). HK: high potassium solution (50 mM KCl). (E) Reduced glutamate release in patient-derived neurons at day 182. The amount of extracellular glutamate was measured by HPLC at pre- and post-depolarizations. The depolarization was evoked by application of HK solution. The ratios of glutamate after the depolarization to that pre-depolarization are shown in the graph (N = 3). (F) Enhancement of AMPA-stimulated Ca^{2+} response in patient-derived neurons. The Ca^{2+} response in day 77 neurons was determined with Ca^{2+} imaging with Fluo-4, a calcium indicator (representative image in left panels). 10 μM AMPA was applied for stimulation. Each trace in left panels corresponds with the fluorescent intensity of Fluo-4 in the cell body of a single neuron. The Fluo-4 ratio [(7 s after the stimulation)/(3 s before the stimulation)] was quantified in right graph (n = 9–10 cells in each bar). All graphs show mean ± SD. ***P < 0.001, **P < 0.01, *P < 0.05 vs. control neurons; one-way analysis of variance with posthoc Dunnett’s test (B,D,E) or Holm test (F).
Fig. 4. Forced hNEU1 expression restores abnormal phenotypes in sialidosis-derived neural cells. (A) Overexpression of hNEU1 restored neuraminidase activity in the patient-derived NPCs. N = 3 wells in each condition. (B) Sialyl-content accumulation was reduced in the hNEU1-overexpressing NPCs. Sialyl-glycoproteins (representative image in B) and total sialic acid contents (C) were significantly downregulated in the hNEU1-overexpressing NPCs. Experiments were conducted in triplicate. (D) Lysosomal abnormality was improved in the hNEU1-overexpressing NPCs. Both lysotracker-positive area (D) and LAMP1 expression (E) decreased in the hNEU1-overexpressing NPCs (N = 6 and N = 4 wells in each condition, respectively). Representative immunoblotting image (left panel) and calculated relative expression of LAMP1 (right panel) are shown in E. (F, G) Overexpression of NEU1 improved neural dysfunction in the patient-derived neurons. Exocytosis in day-95 neurons was visualized with FM1–43 imaging (F). Ca^{2+} responses were determined in day-67 neurons with Fluo-4 imaging (G). The assay and measurements were described in Fig. 3. All graphs show mean ± SD. Con: control vector, hNEU1: hNEU1 vector, ***P < 0.001, *P < 0.05; Student’s t-test (D) or one-way analysis of variance with post hoc Holm test (A, C, E, F, G).

Fig. 5. Proteome analysis in sialidosis-derived neurons. (A) Volcano plot of LC-MS-based shotgun proteomics. Proteins from synaptosomal fraction of healthy controls (201B7 and N2–1) and sialidosis (Sia1 and Sia2)-derived neurons on day 77 were analyzed. The red and blue dots indicate the significantly upregulated and downregulated proteins in synaptosomal fractions from the sialidosis-derived neurons, respectively (N = 3 wells in each sample). Gray dot indicates not significantly dysregulated proteins. Based on the P value for the fold change, gene symbols of the top 10 differentially expressed proteins between normal and disease were described. (B) KEGG enrichment analysis for dysregulated proteins in sialidosis-derived neurons. Significantly enriched KEGG pathways for downregulated and upregulated proteins in sialidosis are shown in upper and lower graphs, respectively. (C) Expression of the molecules involving in “synaptic vesicle cycle” pathway were significantly downregulated in sialidosis-derived neurons. Left panel: Protein expressions in synaptosomal fraction isolated from day-77 neurons, measured by LC-MS (N = 3 wells in each line). Right panel: mRNA expressions in day-77 neurons, measured by qRT-PCR (N = 3 wells in each line). (D) Expression of SNARE proteins such as STX1, VAMP2, and SNAP25 were significantly decreased in day-77 patient-derived neurons. Left panel: representative data determined by immunoblotting. Right graph: Relative band intensity normalized to α-TUBULIN level (N = 3 wells in each line). All graphs show mean ± SD. ***P < 0.001, **P < 0.01, *P < 0.05 vs. healthy donor controls; Student’s t-test (C) or one-way analysis of variance with post hoc Dunnett’s test (D).
mediated transport, Golgi to plasma membrane protein transport, neurotransmitter secretion, and exocytosis) and glycolysis (canonical glycolysis and glycolysis) (Fig. S6A). On the other hand, ribosomal and nucleosomal proteins were also significantly upregulated in the sialidosis groups. We first focused on “synaptic vesicle cycle”-annotated proteins because synaptic exocytosis is part of the synaptic vesicle cycle, and identified 7 proteins involved in “synaptic vesicle cycle” that showed significantly decreased expression in the sialidosis neurons (Fig. 5C), with some of the corresponding mRNA levels also downregulated (Fig. 5C). Notably, these downregulated proteins include two SNARE proteins (syntaxin 1B (STX1B) and vesicle-associated membrane protein 2 (VAMP2)) and one SNARE-binding protein (syntaxin-binding protein 1 (STXBP1)), suggesting consistent decrease of SNARE components (Fig. 5C). Synaptic SNARE proteins consisting of STX1 (STX1A/STX1B), synaptosomal nerve-associated protein 25 (SNAP25), and VAMP2 play a pivotal role in exocytosis at presynapses. Further examination by immunoblotting confirmed lower expressions of these SNARE proteins in the sialidosis-derived neurons than in normal neurons (Fig. 5D). We also examined the effect of NEU1 overexpression on expressions of SNARE proteins in sialidosis neurons (Fig. S6B). The expression levels of STX1 and VAMP2 were significantly increased by NEU1 overexpression, while that of SNAP25 showed tendency to increase (p = 0.353). Such downregulation of synaptic SNARE proteins is likely to induce exocytosis disturbance in the sialidosis-derived neurons. We finally asked whether and how the deregulated molecules identified here are relevant to sialidosis-related neurological symptoms. Searching the OMIM database revealed that 23 downregulated and 9 upregulated proteins are related to myoclonus, seizure, ataxia, or intellectual disability (Supplementary Data 4). In particular, STX1B and STXBP1 are strongly implicated in the symptoms above, supporting the notion that impaired exocytosis is critical for the typical neuropathogenesis in sialidosis (Saito et al., 2008, Schoch et al., 2001).

3.7. Impaired ATP production suppresses presynaptic exocytotic activity in sialidosis-derived neurons

The proteome analysis also revealed the downregulation of seven glycolytic enzymes in the sialidosis-derived synaptosomes (Fig. 6A), and of these, four mRNA expressions including TPI1, HK1, ALDOC, and PGK1 were significantly lower in the sialidosis-derived neurons than in normal controls (Fig. 6A). Glycolysis with the conversion from glucose to pyruvate plays a role in ATP synthesis in presynapses that is indispensable for the presynaptic function, because a large amount of ATP is required to release neurotransmitters (Ashrafi and Ryan 2017). The resultant pyruvate converts into acetyl-CoA or oxaloacetic acid, which both act as substrates in the mitochondrial citric acid cycle that is pivotal for ATP production (Ashrafi and Ryan 2017). Note that mitochondria-related term was not enriched in KEGG pathway or GO term, suggesting mitochondrial content is comparable (Fig. 5B, Fig. S6A). In accord with the low expression of glycolytic enzymes, the glucose-dependent ATP production rate was also significantly reduced in the sialidosis-derived synaptosomes compared with those in normal controls (Fig. 6B). Lactate contents were also significantly lower in sialidosis neurons than in normal neurons, suggesting a decrease of glycolytic activity (Fig. S7A). In addition, treatment with iodoacetate, an inhibitor for the GAPDH glycolytic enzyme, suppressed both glucose-dependent ATP synthesis and exocytotic activity in the healthy donor-derived neurons (Fig. 6C and D). Other type of glycolytic inhibitor, 2-Deoxy-o-glucose, which inhibits the hexokinase activity, also reduced the exocytotic activity (Fig. S7B). The results confirmed an essential role for glycolysis in synaptic exocytosis, and simultaneously suggested that low glycolytic activity caused the exocytotic dysfunction in our sialidosis-derived neurons. Thus, we next treated the sialidosis-derived neurons with exogenous pyruvate to bypass the glycolysis pathway and directly drive ATP production in mitochondria. As expected, the pyruvate treatment enhanced ATP production in the sialidosis-derived synaptosomes (Fig. 6E). Notably, the treatment also partially, but significantly, recovered the exocytotic dysfunction in the sialidosis-derived neurons (Fig. 6F). Pyruvate treatment also facilitated the ATP production and exocytotic activity even in control neurons (Fig. S7C and D). These results suggest that ATP production is critically involved in exocytotic activity and thus reduced ATP production in presynaptic compartments is responsible at least in part for the disturbance of synaptic exocytosis in sialidosis.

3.8. Blockade of AMPA receptors or L-type voltage-dependent Ca2+ channels restores increased Ca2+ influx in sialidosis neurons

Besides the exocytosis impairment in the sialidosis-derived neurons, enhanced Ca2+ influx with AMPA treatment is an intriguing phenotype because it may explain the frequent and intractable episodes of epilepsy in sialidosis patients (Rogawski 2013). AMPA treatment activates AMPA-type glutamate receptor (AMPAR), inducing an influx of intracellular cations and depolarizing plasma membranes. To elucidate the molecular basis of this increased excitability, we first examined the mRNA expressions of AMPAR subunit-encoding genes. Consistent with the functional assay, we found an over two-fold increase in AMPAR subunit-encoding genes, GRIA2 and GRIA4, in sialidosis neurons compared with control cells (Fig. 7A). GRIA2-encoding protein, GLUR2 is the most dominant subtype among AMPARs in the cerebral cortex (Kawahara et al., 2003). The protein expression of GLUR2 was also significantly upregulated in the sialidosis-derived neurons compared with the normal control (Fig. 7B). In contrast, NEU1 overexpression reduced the GLUR2 expression in sialidosis (Fig. S8A). Recently, the AMPAR antagonist, perampanel (also known as Fycompa), was approved as an anti-epileptic drug. Thus, we tested whether perampanel treatment could suppress the enhanced Ca2+ response in sialidosis-derived neurons, and found a strongly suppressed AMPA-evoked Ca2+ influx in both the sialidosis-derived and control neurons (Fig. 7C, Fig. S8B). We also examined whether perampanel treatment affects the defect in ATP production and presynaptic exocytosis in sialidosis cells, however, it did not provide any effect on ATP production and exocytotic activity (Fig. S8C and D). Although these results suggest that activation of AMPAR is essential for the AMPA-evoked Ca2+ overload in sialidosis-derived neurons, it is unlikely that the upregulation of GLUR2 directly increases the extracellular Ca2+ influx because GLUR2-containing AMPAR is Ca2+-impermeable (Isaac et al., 2007). The voltage-dependent Ca2+ channel (VDCC) and NMDA-type glutamate receptor (NMDAR), which are activated by membrane depolarization, are other possible candidates to mediate the Ca2+ influx. We therefore examined the expression of genes encoding NMDAR and VDCC subunits, and found that expression of NMDAR subunit (GRIN2B), an L-type VDCC subunit (CACNA1D), and T-type VDCC subunits (CACNA1G, CACNA1H) were significantly upregulated in both Sia1 and Sia2 neurons compared with control neurons (Fig. 7D). Among them, we focused on L-type VDCC as a candidate of Ca2+ dysregulation for three reasons. First, the CACNA1D gene was most highly upregulated, reaching approximately a two-fold increase. Second, L-type VDCC currents possess large conductance and slow inactivation kinetics, which can explain the prolonged Ca2+ influx in sialidosis neurons (Fig. 3F). Third, a previous study reported that L-type VDCC mediated AMPA-induced Ca2+ influx in primary culturing neurons (Joshi et al., 2011). To examine the involvement of L-type VDCC, we tested the effect of L-type VDCC inhibitor, nicardipine, on Ca2+ influx. Treatment with nicardipine significantly suppressed the increased AMPA-evoked Ca2+ influx in the sialidosis neurons (Fig. 7E, Fig. S8B). These results suggested that the upregulated AMPAR and L-type VDCC expressions underlie the abnormal increase in Ca2+ influx with AMPA treatment and the inhibition of AMPAR or L-type VDCC signaling improves the abnormal influx in sialidosis-derived neurons.
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Fig. 6. Impaired glycolysis affects exocytosis in sialidosis-derived neurons. (A) Expression of the molecules involved in “glycolysis/glucoseøgenesis” pathways were significantly downregulated in sialidosis-derived neurons. Left panel: Protein expressions in synaptosomal fraction isolated from day-77 neurons, measured by LC-MS (N = 3 wells in each line). Right panel: mRNA expressions in day-77 neurons, measured by qRT-PCR (N = 3 wells in each line). (B) Impaired glucose-dependent ATP production in synaptosomes of patient-derived neurons. The synaptosomes were isolated from day-77 neurons (N = 3 wells in each line). ATP production rate were calculated as 100 × (ATP content with glucose)/(ATP content without glucose). (C) Iodoacetate treatment inhibited glycolysis-dependent ATP production. Synaptosome was isolated from day-75 healthy donor-derived neurons (N2–1) treated with 300 μM iodoacetate (N = 3 wells in each condition). ATP production rates were calculated as described above. Con: no treatment, Iodo: iodoacetate treatment. (D) Iodoacetate treatment reduced exocytotic activity in normal iPSC-derived neurons. Exocytosis was visualized with FM1–43 imaging. The day-85 healthy donor-derived neurons (N2–1) were treated with 300 μM iodoacetate for 20 min before FM1–43 imaging and throughout the imaging assay. Each trace in the left panel represents a time-course of change in fluorescent intensity of FM1–43 dye in single synapse-like puncta. The loss of FM1–43 intensity (3 s before the HK application)–(7 s after the HK application) was quantified as represented in the right panel (N = 10 puncta in each condition). (E) Pyruvate treatment enhances ATP production in patient-derived neurons. Synaptosomes were isolated from day-63 patient-derived neurons. Con: Glucose alone, Pyr: Glucose plus 1 mM pyruvate. ATP production rate significantly increased in patient-derived neurons treated with the pyruvate (N = 3 wells in each condition). (F) Pyruvate treatment improved exocytosis in patient-derived neurons. The exocytosis were visualized in neurons on day 85 (upper) or day 77 (lower) with FM1–43 imaging. 1 mM pyruvate was applied throughout the imaging assay. Analysis was conducted as described in D. All graphs show mean ± SD. *** P < 0.001, ** P < 0.01, * P < 0.05 vs. healthy subjects or control group; Student’s t-test (A, C, D), one-way analysis of variance with posthoc Dunnett’s test (B) or Tukey–Kramer test (E, F).

4. Discussion

In this study, we generated disease-derived iPSC lines from the fibroblasts of sialidosis patients and developed a novel neural model mimicking the disease phenotype of sialidosis. The iPSC-derived NPCs and neurons presented the accumulation of terminally sialylated materials and lysosome enlargement previously characterized as features of sialidosis patient-derived cells (Kwak et al., 2015). SNA-lectin blotting showed that band intensity in sialidosis NPCs/neurons were stronger than that in normal ones while the contrast between sialidosis and normal was more prominent in neurons than NPCs (Fig. 3A for neurons, Figs. 2B and 4B for NPCs), suggesting higher accumulation of sialylglycoproteins in neurons. One possible explanation of the higher accumulation in neurons is the non-proliferative property of neurons. In proliferative cells including NPCs, undigested materials were divided into two daughter cells at the division, limiting the accumulation of these materials. However, it is not the case in non-proliferative neurons, implying more accumulated glycoproteins.

The sialidosis-derived neurons exhibited two distinct functional abnormalities, defective presynaptic exocytosis and excessive enhancement of AMPA-evoked Ca2+ influx. These phenotypes were recovered by the forced expression of wild-type NEU1 gene, supporting that decreased neuraminidase activity is responsible for these clinical abnormalities. The sialidosis-derived neurons also showed lower levels of glutamate release than normal neurons. At the presynaptic site, neurotransmitters are released through the depolarization-triggered exocytotic fusion of synaptic vesicles with presynaptic plasma membrane (Li and Chin 2003). The reduced glutamate thus suggested that the impaired neurotransmitter release in the sialidosis-derived neurons was due to defective exocytosis at the presynaptic site. Interestingly, similar presynaptic dysfunction was reported in disease models of other neuropathic lysosomal storage disorders, Tay-Sachs disease and mucopolysaccharidosis type IIIA (Sambri et al., 2017, Matsushita et al., 2019), possibly implying a common pathophysiology among lysosomal storage diseases. Our further analysis also revealed the downregulation of SNARE-associated proteins including STX1B, SNAP25, VAMP2, and MUNC18–1 (also known as STXBPI) in sialidosis-derived synaptosomes. SNARE proteins are membrane-associate proteins playing pivotal role in the fusion of synaptic vesicles to the presynaptic plasma membrane during exocytosis (Li and Chin 2003). Based on their localization, SNARE proteins are categorized into two types, v-SNARE (vesicle-associated) and t-SNARE (target membrane-associated). Upon stimulation, the v- and t-SNAREs form a SNARE complex to bring the vesicle and plasma membrane into juxtaposition and driving membrane fusion. In the exocytosis of synaptic vesicles, STX1 and SNAP25 serves as t-SNAREs and VAMP2 serves as a v-SNARE. MUNC18–1 acts to stabilize the SNARE complex via the binding of STX1 (Li and Chin 2003). Knockout mice of Stxb1, Snap25, Vamp2, or Munc18–1 also shows a severe defect in evoked-synaptic transmission, suggesting an essential role of these proteins in exocytosis process (Verhage et al., 2000, Schoch et al., 2001, Washbourne et al., 2002, Mishima et al., 2014). Notably, damaging mutations of STX1B, SNAP25, MUNC18–1, or VAMP2 genes cause inherited neurological diseases with symptoms that partially overlap those of sialidosis including myoclonus, ataxia, seizure, and intellectual disability (Saitu et al., 2008, Schubert et al., 2014, Shen et al., 2014, Salpietro et al., 2019). In addition, a recent study using Munc18–1 haplodeficient mice implicated a reduction in excitatory neurotransmission from the neocortex into the striatum in the occurrence of seizure (Miyamoto et al., 2019). Similar to the Munc18–1–/– mouse case, our sialidosis-derived neurons showed reduced depolarization-induced release of glutamate, a major excitatory neurotransmitter in the CNS. These data together highlight the possibility that downregulated presynaptic SNARE proteins are key players in the neurological symptoms of sialidosis via defects in neurotransmission.

In addition to the abnormal expression of SNARE proteins, we found that glucose-dependent ATP production was impaired in the synaptosomal fraction from sialidosis neurons with a consistent downregulation of seven of glycolysis enzymes. On the other hand, the mitochondria function was intact based on ATP production. Among the seven downregulated enzymes, hexokinase (HK1) and pyruvate kinase (PKM) are critical for glycolysis as rate-limiting enzymes that catalyze the irreversible step of glycolysis, and were previously shown to affect the activity of glycolysis in cancer cells (Marin-Hernandez et al., 2006, Iqbal et al., 2013). Thus, our results suggest that downregulating HK1 and PKM decreases glycolytic activity in neurons and in turn the production level of ATP at synapses to a level that is insufficient to support synaptic function during the process of neurotransmission (Rangaraju et al., 2014, Sobieski et al., 2017). Similarly, inhibition of glycolysis reduced synaptic ATP levels in the CNS, disrupting the endocytosis, recycling, and glutamate uptake into synaptic vesicles (Ikemoto et al., 2017). Damaging mutations of two others among the seven enzymes, PGK1 and ALDOA, were implicated in the onset of autosomal recessive disease associated with neurological impairments (Hurst et al., 1987, Noel et al., 2006). Consistently, we have also found that inhibiting glycolytic enzymes suppressed the evoked-exocytotic activity, supporting the requirement for glycolysis in synaptic exocytosis. Many ATPases such as the AAA ATPase family of Na+K+, H+ and Ca2+ pumps play an essential role in neurotransmission (Ly and Verstreken 2006), and in turn a lack of ATP reduces ATPase activity. Taken together, these data imply that low productivity of ATP due to downregulation of glycolytic enzymes was a causative factor in the impaired synaptic exocytosis in our iPSC models.

To bypass the impaired glycolysis, we treated the sialidosis-derived neurons with a large amount of pyruvate, which is a final metabolite of glycolysis and a major substrate of the mitochondrial TCA cycle (Asnfather and Ryan 2017). Such treatment enhanced the ATP production and partially restored the synaptic exocytosis. Indeed, sodium pyruvate is now under clinical trials for the treatment of mitochondrial disease.
Fig. 7. Antagonist against AMPA-type glutamate receptor and L-type voltage-dependent Ca\(^{2+}\) channel suppresses the abnormal Ca\(^{2+}\) response in sialidosis-derived neurons. (A) Increased mRNA expression of AMPA receptor subtypes, GRIA2 and GRIA4, in patient-derived neurons. mRNA expression of AMPA receptor subtypes including GRIA1, GRIA2, GRIA3, and GRIA4 were measured in day-63 neurons by qRT-PCR (N = 4 wells in each line). (B) Increased protein expression of GRIA2-encoding protein, GLUR2, in patient-derived neurons. GLUR2 and α-TUBULIN expressions were determined in day-63 neurons by immunoblotting (representative data in upper panel). Band intensity of GLUR2 was normalized to α-TUBULIN level (lower graph, N = 4 wells in each line). (C) Perampanel treatment suppressed abnormal enhancement of AMPA-stimulated Ca\(^{2+}\) response in patient-derived neurons. AMPA-evoked Ca\(^{2+}\) response was determined in day 72 neurons with Fluo-4 imaging. 10 \(\mu\)M AMPA was applied for stimulation. 10 \(\mu\)M perampanel, an antagonist for AMPA receptor, was applied throughout the imaging assay. Each trace in left panels is corresponding with the fluorescent intensity of Fluo-4 in the cell body of a single neuron. The Fluo-4 ratio ([7 s after the stimulation]/[3 s before the stimulation]) was quantified in right graph (n = 10 cells in each line). Con: non-treatment with perampanel as a control. (D) The mRNA expression of NMDA receptor subtypes (GRIN1, GRIN2A, and GRIN2B), and voltage-dependent Ca\(^{2+}\) channels (CACNA1C, CACNA1D, CACNA1G, CACNA1H, and CACNA1I) were measured in day-63 neurons by qRT-PCR (N = 4 wells in each line). Data was normalized to healthy donor-derived neurons (201B7). (E) Nicardipine treatment suppressed abnormal enhancement of AMPA-stimulated Ca\(^{2+}\) response in patient-derived neurons. AMPA-evoked Ca\(^{2+}\) response was determined in day-80 neurons with Fluo-4 imaging. 30 \(\mu\)M nicardipine, an antagonist for L-type VDCC, was applied throughout the imaging assay. Analysis was conducted as described in C. All graphs show mean ± SD. *** P < 0.001, ** P < 0.01, * P < 0.05 vs. control group; one-way analysis of variance with posthoc Dunnett’s test (A, B, D) or Tukey–Kramer test (C, E).
and shows sufficient tolerability thus far (Koga et al., 2019). Therefore, the enhancement of mitochondrial ATP production by sodium pyruvate is a potential therapeutic strategy for presynaptic impairment-related symptoms in sialidosis patients.

This study also showed that AMPA-evoked Ca\(^{2+}\) influx was markedly enhanced in the sialidosis-derived neurons showing upregulated expression of GLUR2. AMPAR is a glutamate-gated cation channel, formed as tetramers of GLUR1, GLUR2, GLUR3, and GLUR4, that plays a central role in receiving excitatory input in the CNS (Rogawski 2013). Generally, AMPA-induced Ca\(^{2+}\) influx is mediated by Ca\(^{2+}\)-permeable AMPAR and subsequently by activated VDCC (Joshi et al., 2011). The Ca\(^{2+}\)-permeable activity is dependent on the incorporation of GLUR2 in the AMPAR because GLUR2 provides negative regulation (Issac et al., 2007). Thus, the upregulation of GLUR2 expression is unlikely to directly enhance Ca\(^{2+}\) influx via Ca\(^{2+}\)-permeable AMPAR. Note that the GLUR2-containing AMPARs allow Na\(^+\) influx into the cell, resulting in membrane depolarization. It is possible that the upregulated GLUR2-containing AMPAR causes the excess influx of Na\(^+\) and over-depolarization, which in turn induces the secondary Ca\(^{2+}\) influx through the L-type VDCC. Increased expression of the CAGNA family gene encoding L-type VDCC could thus abnormally enhance Ca\(^{2+}\) influx in the sialidosis-derived neurons, supported by our treatment with L-type VDCC inhibitor cancelling the abnormal enhancement. We also demonstrated that the AMPA-evoked excess influx of Ca\(^{2+}\) was suppressed by perampanel, an AMPAR antagonist and approved drug for epilepsy (Rogawski 2013, Gil-Lopez et al., 2018). Indeed, AMPAR signaling has been strongly implicated in the generation of synchronized epileptic discharges (Rogawski 2013), and epilepsy manifestations such as myoclonus convulsion are a major symptom in sialidosis. To this end, our results implicate the excess influx of Ca\(^{2+}\) as a causative event for the epilepsy, and thus the potential efficacy of perampanel in treating sialidosis. In fact, a recent clinical study indicated that perampanel treatment effectively prevents the drug-resistant myoclonus convulsions in sialidosis type I patients, highlighting the possible involvement of increased AMPAR-mediated excitability in the pathogenesis of neurological symptoms (Hu et al., 2018).

Because presynaptic disturbance was still observed in the presence of perampanel (which had no effect on the ATP production), the decreased exocytotic activity was not due to the abnormal AMPA-stimulated Ca\(^{2+}\) influx in sialidosis-iPSC-derived neurons (Supplementary Fig. 8C, D). Although the relationship between AMPA-induced Ca\(^{2+}\) response and presynaptic disturbance is still unclear, a possible explanation is compensatory action during neural maturation. In general, developing neurons have a high plasticity to keep the equilibrium in neurotransmission with compensatory change including the alternation in expression levels of functional proteins (e.g. neurotransmitter receptors). For example, it has been proposed that the developmental ethanol exposure inhibits the NMDA receptor activity in developing neurons, resulting in upregulation of NMDA receptors (Valenzuela et al., 2011). Therefore, it is possible that the reduced glutamate release induces the compensatory increase of AMPA receptors in sialidosis neurons. Such a drastic alternation of neurotransmission system during developmental stage causes an unpredictable change in neural circuits.

Finally, we demonstrated that the defective NEU1 enzyme increased expression of the hyper-sialylated proteins in the sialidosis-derived neural cells. NEU1 localized in both lysosomes and plasma membrane controls the de-sialylation of membrane glycoproteins, thus critically affecting the half-life and function of glycosylated proteins (Pshezhetsky and Hinek 2011, Maurice et al., 2016). For example, sialylation regulates the signaling of many receptors including tropomyosin receptor kinase A/B (TrkA/B), insulin receptor (IR), insulin-like growth factor receptor (IGFRI/II), platelet-derived growth factor BB receptor (PDGFB), integrin j4, Fc receptors for immunoglobulin G (FcγR), Toll-like receptors (TLR2/3/4), and hyaluronic acid receptor (CD44), and its dysregulation in turn alters their signaling (Hinek et al., 2008, Amith et al., 2009, Uemura et al., 2009, Arabkhari et al., 2010, Jayanth et al., 2010, Seyranitepe et al., 2010). Alternatively, lysosomal dysfunction itself affected the same protein expressions in a mouse model of LSD, whereby in a mucopolysaccharidosis type IIIA (MPS IIIA) mouse model, the low activity of lysosomal clearance induced the aggregation of alpha-synuclein, which accelerated the SNARE protein degradation (Sambri et al., 2017). In this study, SNARE proteins, glycolysis enzymes, and AMPAR subtypes all showed dysregulated expressions in the sialidosis neurons. Future experiments will address whether hypersialylation and/or lysosomal dysfunction are responsible for such cellular dysregulation.

In summary, we demonstrated that neuraminidase deficiency underlies defective presynaptic exocytosis and AMPA-evoked excess influx of Ca\(^{2+}\), both of which can lead to the neurological symptoms of sialidosis. Sialidosis patient-derived neurons also exhibited the typical dysregulated energy metabolism, SNARE protein expression, and AMPAR expression. Based on these findings, we newly found that both NEU1 overexpression as well as pyruvate and perampanel treatments restored the impairments observed herein with sialidosis neurons. Our study provides new insight into the pathophysiology of sialidosis and suggests a novel potential therapeutic strategy for affected patients.

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Author contributions

H.O., T.N., and T.E. designed the experiments, performed the cellular and molecular experiments, and wrote and edited the manuscript. M.S. performed animal experiments and edited the manuscript. J.K. performed the imaging cytometer experiments and edited the manuscript. S.M. performed OCR measurements and edited the manuscript. R.K. and T.O. measured the neuraminidase activity and edited the manuscript. N.T. performed proteome analysis and edited the manuscript. Y.T. and T.F. performed the transmission electron microscope experiments and edited the manuscript. H.F. provided the patient samples. T.I. discussed the data and hypothesis, and edited the manuscript.

Data availability

Data supporting the figures of this manuscript are available from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors declare no competing interests.

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