Sialic acid metabolites preclude the development of myopathic phenotype in the DMRV/hIBM mouse model

May Christine V. Malicdan, Satoru Noguchi, Yukiko K. Hayashi, Ikuya Nonaka, Ichizo Nishino
Supplementary Fig. 1. NeuAc and ManNAc are rapidly excreted in the urine after a single dose administration by intraperitoneal and intragastric routes. (a) After a single dose (32 μmol) of NeuAc via the intraperitoneal route, a peak in the serum level is
detected within less than 30 min. (b) 90% of NeuAc is excreted into the urine within minutes after intraperitoneal route, compared to 75% excretion when the same drug is administered via intragastric route. Levels of NeuGc in serum (c) and urine (d) are shown for comparison. (e) After a single dose administration of ManNAc, peak levels are found in the serum within 5 min when intraperitoneal route is used, compared to 10 min when intragastric route is used. (f) After 120 min, almost all ManNAc is excreted in the urine when the intraperitoneal route is used. Excretion in the urine is slower when ManNAc is given by intragastric route, as 90% is excreted after 240 minutes.
Supplementary Fig. 2

(a) Number of breaks

(b) Time (minutes)

(c) Body weight ratio
**Supplementary Fig. 2.** High dose, medium dose, and low dose ManNAc administration significantly improves muscle performance in DMRV/hIBM mice, in terms of endurance (a) and hanging test (b). No dose dependence efficacy was observed. After treatment with ManNAc, there is an increase in total body weight (c) in the DMRV/hIBM mice in comparison with littermates. The key for panels are: L, littermate; P, placebo; LD, low dose; MD, medium dose; HD, high dose.
**Supplementary Fig. 3.** Tibialis anterior muscle weight (a) and CSA (b), contractile properties including specific isometric (c) and specific tetanic forces (d) are improved after high dose, medium dose, and low dose ManNAc treatment. The key for panels are:

L, littermate; P, placebo; LD, low dose; MD, medium dose; HD, high dose.
Supplementary Fig. 4. ManNAc treatment reduces immunoreactive signals of Lamp-2 and LC3, markers which are used to characterize the presence of autophagy.
**Supplementary Fig. 5.** Sialic acid metabolites increases membrane bound sialic acid in brain, kidney, and liver of DMRV/hIBM mice. (a) Membrane-bound sialic acid in liver and kidney after treatment with ManNAc in three doses. (b) Membrane-bound sialic acid in spleen, liver, kidney, heart, and brain after administration of low dose ManNAc, NeuAc, and sialyllactose. Asterisks represent $P < 0.05$ when comparing treatment group
and placebo. The key for panels are shown on right upper corner of each graph. The sialic acid determination was repeated with omission of hydrolysis to check for contamination from administered sugars. Without hydrolysis, sialic acid was undetectable in the muscle while around 5 percent was detected in liver and kidneys, supporting the notion that the levels detected in the bound fraction of tissues are not contaminated (data not shown).
Supplementary Fig. 6. Low dose sialic acid metabolites significantly improve the ability of mice to sustain a constant workload and to hang on an inverted meshwire. (a) Endurance test (b) hanging test. Asterisks represent $P < 0.05$ when comparing treatment group and placebo.
Supplementary Fig. 7. Low dose sialic acid metabolites lead to improvement in tibialis anterior muscle properties and increases gastrocnemius muscle twitch/tetanic ratio. (a) tibialis specific isometric force (b) tibialis specific tetanic force (c) twitch/tetanic ratio. Asterisks represent $P < 0.05$ when comparing treatment group and placebo.
Supplementary Fig. 8

Supplementary Fig. 8. Autophagic vacuolar marker, LC3, was detected on western blot of muscle homogenates from treated mice and placebo. The key for panels are shown on right lower corner of the figure.
Supplementary Fig. 9. The amount of amyloid in the muscle is decreased after treatment with low-dose sialic acid metabolites. The C99 fragment of β-amyloid precursor protein was detected on whole homogenates by using 6E10 antibody. After treatment with either ManNAc, NeuAc, or sialyllactose, the c99 fragment is reduced but not completely abrogated. The key for panels are shown on right lower corner of the figure.
Supplementary Table.

Effect of sialic acid metabolites on kidney and liver function tests.

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<th>Administration of ManNAc in three doses</th>
<th>AST (IU l⁻¹)</th>
<th>ALP (IU l⁻¹)</th>
<th>BUN (IU l⁻¹)</th>
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<td>Mean</td>
<td>SD</td>
<td>s.e.m</td>
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<th>ALP (IU l⁻¹)</th>
<th>BUN (IU l⁻¹)</th>
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<td>Sialyllactose</td>
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</table>
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Supplementary Method:

Sialic acid and ManNAc pharmacokinetics.

For this experiment, wild type mice were used (N=4). After collection of blood from tail vein and urine for baseline data, NeuAc (32 µmol) or ManNAc (180 µmol) was given as a single dose via intraperitoneal or intragastric route. Urine and blood were then serially collected after 5, 10, 30, 60, 120, 240, and 480 min. At the end of the experiment, the mice were sacrificed by CO₂ asphyxiation. Urine specimen were frozen and kept in -20 °C until processing. Whole blood was centrifuged to separate serum, which was also kept in -20 °C until processing. To quantify ManNAc, we added p-aminobenzoic acid ethyl ester (ABEE) to sample. Mixture was then vortexed and incubated in 80 °C for 1 h, and then cooled to room temperature. Equal volume of distilled water and chloroform...
were added and after vigorous vortexing, the mixture was centrifuged for 1 min. The ABEE-converted monosaccharides in the upper aqueous layer were then determined by reverse-phase high performance liquid chromatography according to manufacturer’s instructions (Seikagakukogyo).

**Analysis of motor performance and contractile properties of the muscle.**

The motor performance was evaluated using treadmill exercise as previously reported\(^\text{16}\). Briefly, after 7 days of acclimation on the treadmill, two exercise tests were performed on separate days. The endurance exercise consisted of a 30-min treadmill run at 30 m/min with a 7° incline, during which the number of beam breaks or rests were recorded. The performance test began with a speed of 20 m min\(^{-1}\), which was gradually increased by 10 m min\(^{-1}\) every min until the mouse was already exhausted and could no longer run. The time of exhaustion was used to calculate the distance the mice covered during the exercise. Both tests were done three times, with 3–4 days period of rest in between.

**Immunohistochemical Analysis.**

We immunostained six µm-thick cryosections from gastrocnemius muscles using the following primary antibodies: mouse antibody to β–amyloid,1–16 (6E10, Covance);
rabbit polyclonal antibody to β–amyloid 1–42 (Millipore); rabbit polyclonal antibody to β–amyloid beta 1–40 (Millipore); rabbit polyclonal antibody to LC3 (Novus Biologicals); mouse anti PHF TAU (AT8, Innogenetics); mouse monoclonal antibody to ubiquitinylated proteins (clone FK1, Biomol International). We applied secondary Alexa fluor–conjugated goat antibody to rabbit and mouse IgG1 as appropriate (Invitrogen) for 30 min at room temperature (25 °C). We used digitized images for analysis. For measuring single cross-sectional area, we stained the muscle sarcolemma with rabbit polyclonal antibody to caveolin 3 (BD Transduction Laboratories) for an hour, followed by Alexa fluor-conjugated goat IgG antibody to rabbit (Invitrogen) for 30 min. We used six randomly selected digital images to evaluate single fiber cross-sectional area of 1000–1500 as previously reported\textsuperscript{12}.

**Analysis of contractile properties of the muscle.**

We measured the contractile properties of the gastrocnemius and TA muscles were performed according to previous protocols\textsuperscript{16,30} using a muscle testing apparatus obtained from Nihon Kohden. After deeply anesthetizing the mouse with intraperitoneal sodium pentobarbital (40 mg per kg bodyweight), we isolated the entire skeletal muscles along with the proximal bone of origin intact. We subsequently mounted the muscle in a vertical chamber and connected to a force-displacement transducer
(TB-652T for gastrocnemius / TB-653T for TA) and length servosystem. Square wave pulses 0.2 ms in duration were generated by a stimulator (SEN-3301) and amplified (PP-106H), and subsequently, muscle length was adjusted to the length ($L_0$) that resulted in maximal twitch force ($P_t$) as the muscles were bathed in a physiologic solution. With the muscle held at $L_0$ and duration changed to 3 ms, the force developed during trains of stimulation pulses (10 to 100 Hz) was recorded and the maximum absolute tetanic force ($P_o$) was determined. Absolute force was normalized with the physiologic cross-sectional area (CSA; muscle weight divided by the product of $L_0$ and $1.066 \text{ g cm}^{-3}$), to obtain specific force ($P_t/\text{CSA}$ and $P_o/\text{CSA}$).

**Preparation of membrane fractions for sialic acid measurement.**

After organs were harvested, these were immediately frozen on dry ice and kept in $-80 \degree \text{C}$ until use. Organs were crushed and homogenized by using Dounce homogenizer in a buffer containing 75 mM KCl, 10 mM Tris, 2 mM MgCl$_2$, 2mM EGTA, and protease inhibitor cocktail (Complete Mini Protease Inhibitor Tablet, Roche) at pH 7.4. Equal amounts of homogenized tissues were centrifuged for an hour at 30,000 g, 4 $\degree \text{C}$. We used the pellet, which represented the protein fractions, for sialic acid measurement and protein analysis. After two washes in the same buffer, we one fraction of the pellet was resuspended in 50 mM H$_2$SO$_4$, sonicated, and subjected to hydrolysis at 80 $\degree \text{C}$. We
used the other pelleted fraction for analysis of total protein amount by extracting protein with SDS buffer (10% SDS, 10 mM EDTA, 5% β-mercaptoethanol, 0.7 M Tris-HCl, pH 6.7). To check for contamination from administered sugars we omitted hydrolysis of samples; after washing with buffer, the pellet was resuspended in water and sonicated. After centrifugation for an hour at 30,000 g, 4 °C, we used the supernatant for sialic acid measurement.

**Preparation of muscle tissues for analysis of protein expression.**

We homogenized fifty pieces of 10 µm-thick gastrocnemius cryosections in SDS buffer.

After boiling, we used the supernatant for western blotting for LC3-I and LC3-II isoforms (LC3 antibody, Novus Biologicals).