

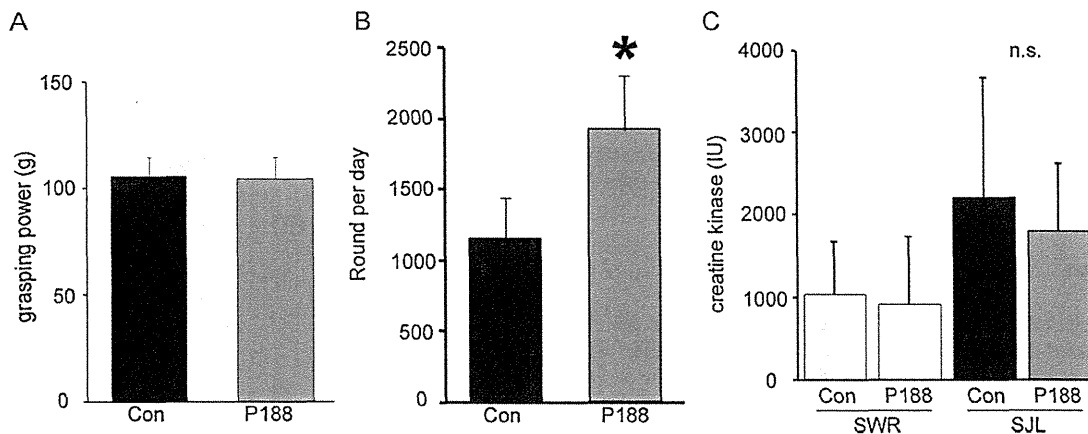
**Fig. 1.** Muscle mass loss is alleviated in P188-administered SJL mice. (A) Body mass in saline- (control: Con) and P188-administered SJL mice ( $n=10$  per group). n.s., not significant. Student's  $t$ -test,  $*p < 0.05$ . (B and C) Gastrocnemius, tibialis anterior, and soleus muscles: mass from control group and P188-administered SJL mice ( $n=10$  per group) after 2 weeks (B) and 6 weeks (C). SWR mice were also examined as a control. (D) Transitional changes in muscle mass of gastrocnemius muscle from 2 to 6 weeks after the start of administration of saline and P188 in SJL mice. n.s., not significant. Student's  $t$ -test,  $*p < 0.05$ .

**3. Results**

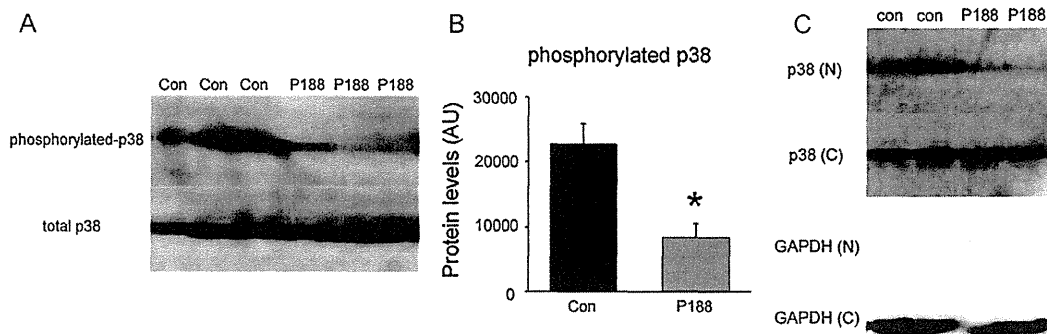
**3.1. P188 inhibits the decline of muscle mass in osmotic pump-implanted SJL mice**

The body weights of saline- (control: Con) and P188-administered SJL mice ( $n=10$  per group) did not change significantly during the 6-week experimental period (Fig. 1A). The muscle masses of saline- and P188-administered mice did not change during the first 2 weeks (Fig. 1B); however, the gastrocnemius muscles of the saline-administered mice had decreased in mass after 6 weeks. Gastrocnemius mass in the P188-administered mice, in contrast, seemed to increase significantly during those 6

weeks (Fig. 1C and D), suggesting that muscular atrophy was suppressed by P188. Compared to the SWR mice, the SJL mice muscle display several unique features including fibrosis, fiber degeneration and inflammatory cell infiltration (Suzuki et al., 2005). It has also been reported that their muscle is generally greater in mass than that of C57BL6 mice's muscle (Rayavarapu et al., 2010). In our study the SJL mice's muscle mass was greater than that of SWR mice, so we next examined the functional status of P188-administered SJL mice. We found, on average, significantly increased muscle fiber in a cross-sectional area (Supplementary Fig. 1). The forearm grip strength had not changed among saline- and P188-administered SJL mice (Fig. 2A); however, rolling cage observation revealed significantly greater running endurance in P188-administered SJL mice



**Fig. 2.** P188 improves motor function in SJL mice. (A) Grip strength is not different among saline- (control: Con) and P188-administered SJL mice ( $n=10$  per group). (B) Rolling cage observation revealed greater running function in P188-administered SJL mice ( $n=10$  per group). Student's  $t$ -test,  $*p < 0.05$ . (C) P188 did not reduce serum CK in SJL mice. SWR mice were also examined as a control.



**Fig. 3.** Phosphorylation and localization of p38 is controlled by P188 administration. (A) Representative immunoblot analysis for p38 and phosphorylated p38 in saline- (Con) and P188-administered SJL mice. (B) The amount of phosphorylated p38 in saline- (Con) and P188-administered SJL mice. (C) Representative immunoblot analysis for nuclear (N) and cytoplasmic (C) p38 and GAPDH (as a technical control).

than in saline-administered SJL mice (Fig. 2B). P188 did not significantly reduce serum CK levels in SJL mice (Fig. 2C).

### 3.2. Effect of P188 is through a p38-mediated pathway

We found reduced muscle loss in the gastrocnemius muscles of P188-administered SJL mice and increased exercise capability in the rolling cage. Next, we examined the molecular mechanism of P188's effects on muscle mass. We first examined the membrane repair mechanism using Evans blue dye (Supplementary Fig. S2) because P188 is a membrane-sealing reagent. We did not find significant differences between saline- and P188-administered SJL mice in the Evans Blue-positive muscle fibers. We also examined the rate of apoptosis in the gastrocnemius muscles (Supplementary Fig. S3) and Mac1-positive infiltrated cells (Supplementary Fig. S4), but found no significant difference between the saline- and P188-administered SJL mice.

We then examined the molecular pathway of muscular atrophy in the gastrocnemius muscle. We found reduced phosphorylated p38 in P188-administered SJL mice compared with saline-administered mice (Fig. 3A and B), as well as decreased p38 in the nuclear fraction of the gastrocnemius muscle homogenate (Fig. 3C). We also found that basal levels of p38 are low in both untreated and P188-treated wild-type mice (Supplementary Fig. S5), suggesting that the increased levels of p38 are related to the pathophysiology of SJL mice. We observed that Foxo3a, pFoxo3a, Akt, pAkt, p65, acetyl-p65, nitric oxide synthase (NOS) and the chief components of the dystrophin glycoprotein complex had not changed (Supplementary Fig. S6).

To examine the downstream events of P188 administration, we performed a microarray analysis of saline- and P188-administered SJL mice (Supplementary Table 1). We found significantly reduced expression of atrogin-1, which is an E3 ubiquitin ligase in skeletal muscle. MuRF-1 did not change significantly in the microarray analysis (data not shown). We confirmed that atrogin-1 was significantly lower in the P188-administered SJL mice by real-time PCR (Fig. 4).

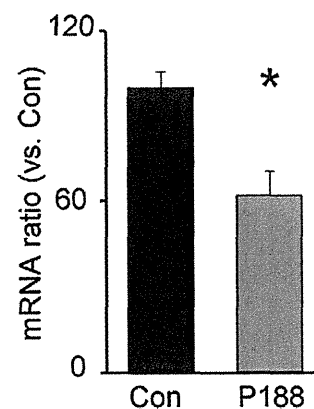
## 4. Discussion

We have demonstrated that adult dysferlin-deficient SJL mice show decreased loss of gastrocnemius muscle and higher activity levels in a rolling cage following the administration of P188 through an osmotic pump for 6 weeks. The grip strength did not vary between saline- and P188-administered mice, as instantaneous muscle strength may not be affected in a chronic model. We also reported the amount of decrease in phosphorylated and intranuclear p38 protein following the continuous administration

of P188. Furthermore, the expression of atrogin-1 was suppressed in P188-administered SJL mice.

Previous studies have established P188 as a membrane-patching polymer that interacts directly with monolayers (Wu et al., 2005) and the disrupted membrane (Maskarinec et al., 2005). P188 is effective in stabilizing membranes and enhances the recovery of a variety of cell types following an array of injury-inducing protocols (Borgens et al., 2004; Lee et al., 1992; Merchant et al., 1998; Yasuda et al., 2005); however, we found no significant changes using Evans blue dye staining. Because the sensitivity of EBD is not especially high, however, further membrane-repairing experiments (Bansal et al., 2003) are necessary to determine whether the membrane has recovered.

p38 affects the activities of transcription factors from the MyoD and MEF2 families and participates in the remodeling of chromatin at specific muscle-regulatory regions. p38 also has a crucial role in development and differentiation (Keren et al., 2006). Doxorubicin, an anti-tumor agent of the anthracycline family and a cardiotoxic drug, induces atrogin-1 through a p38-MAPK-dependent pathway in cardiac myocytes (Yamamoto et al., 2008). P188 was also reported to inhibit apoptosis and prevent necrosis of the pheochromocytoma cell line following mechanical trauma by inhibiting p38 (Serbest et al., 2006). The activation of p38 MAPK phosphorylation occurs as soon as 8 h after lipopolysaccharide injection into the skeletal muscles of mice, as well as in C2C12 myotubes with oxidant hydrogen peroxide. The inhibition of p38 by SB202190 prevented peroxide-induced atrophy, with diminished upregulation of atrogin-1 and MuRF-1 (McClung et al., 2010). These data suggest that p38 may be a mediator of the ubiquitin proteasome system,



**Fig. 4.** Semi-quantitative PCR for genes differently expressed in a microarray assay. (A) mRNA levels of atrogin-1/MAFbx ( $n = 5$ ) in gastrocnemius muscle from saline- (Con) and P188-administered SJL mice (\* $p < 0.05$ , Mann-Whitney test).

regulated by poloxamer 188 in our model. Compared with the SWR mice shown in supplementary Fig. 5, p38 is upregulated only in dysferlin-deficient SJL mice. The upregulated p38 is suppressed in SJL mice by P188, so we speculated that P188 may inhibit the dysferlin-deficiency induced p38-atrogin1-muscle atrophy pathway in SJL mice. Pathways other than p38-atrogin1 should also be examined as the downstream target of poloxamer 188.

Poloxamer 188, previously named RheothRx, significantly reduced total analgesic use, pain intensity and total days of hospitalization in human patients with moderate to severe vasoocclusive episodes (Adams-Graves et al., 1997). Poloxamer 188 was well tolerated in humans; there was no significant difference between treatment and placebo groups in terms of adverse experience. As shown in Figs. 1 and 2, P188 had no effect on muscle mass in wild-type mice. Yasuda et al. (2005) also reported that P188 has no effect on left ventricular end-diastolic volumes immediately following the infusion of P188 in control mice. Thus, we concluded that the effects of P188 on wild-type mice are minimal, if they exist at all. Poloxamer 188 administered to animals decreased myocyte injury, preserved adenosine 5'-triphosphate levels in the tissue, and improved survival following hind limb ischemia-reperfusion injury (Murphy et al., 2010). As proposed by Townsend et al. (2010), long-term administration of P188 maintains the myocardial membrane function and prevents myocardial necrosis. Our results also suggest that poloxamer 188, which has been approved by the U.S. Food and Drug Administration for other uses, could become a beneficial clinical option as a late salvage therapy.

The beneficial effects of long-term application of P188 to the skeletal muscles of SJL mice were detected in this study. The SJL mice presented a loss of muscle mass after 6 weeks of observation due to the extra weight of the infusion pump and solutions; these factors would be expected to increase the frequency of mechanically induced disruptions of membrane integrity, which are important events in the pathophysiology of dysferlin deficiency in skeletal muscle. This accelerated disease progression makes the protective effects of P188 even more notable.

Using an osmotic pump for continuous administration, we demonstrated for the first time that P188, a membrane-sealing poloxamer, is effective in reducing loss of gastrocnemius muscle mass and increasing exercise endurance. These results provide the basis for potential therapeutic strategies utilizing P188 and the p38-mediated pathway in dysferlin-deficient skeletal muscles.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neures.2011.10.005.

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# Peracetylated *N*-Acetylmannosamine, a Synthetic Sugar Molecule, Efficiently Rescues Muscle Phenotype and Biochemical Defects in Mouse Model of Sialic Acid-deficient Myopathy<sup>\*S</sup>

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**Background:** Distal myopathy with rimmed vacuoles/hereditary inclusion body myopathy (DMRV)/hIBM is a sialic acid-deficient myopathy.

**Results:** Tissue sialylation in DMRV/hIBM mice is efficiently increased by Ac<sub>4</sub>ManNAc, a synthetic compound.

**Conclusion:** Ac<sub>4</sub>ManNAc rescued muscle phenotype in DMRV/hIBM more efficiently than natural compounds.

**Significance:** Application of this compound includes its potential use in therapy and in understanding the molecular basis of sialic acid deficiency in disease.

Distal myopathy with rimmed vacuoles/hereditary inclusion body myopathy (DMRV/hIBM), characterized by progressive muscle atrophy, weakness, and degeneration, is due to mutations in *GNE*, a gene encoding a bifunctional enzyme critical in sialic acid biosynthesis. In the DMRV/hIBM mouse model, which exhibits hyposialylation in various tissues in addition to muscle atrophy, weakness, and degeneration, we recently have demonstrated that the myopathic phenotype was prevented by oral administration of *N*-acetylneuraminic acid, *N*-acetylmannosamine, and sialyllactose, underscoring the crucial role of hyposialylation in the disease pathomechanism. The choice for the preferred molecule, however, was limited probably by the complex pharmacokinetics of sialic acids and the lack of biomarkers that could clearly show dose response. To address these issues, we screened several synthetic sugar compounds that could increase sialylation more remarkably and allow demonstration of measurable effects in the DMRV/hIBM mice. In this study, we found that tetra-*O*-acetylated *N*-acetylmannosamine increased cell sialylation most efficiently, and *in vivo* evaluation in DMRV/hIBM mice revealed a more dramatic, measurable effect and improvement in muscle phenotype, enabling us to

establish analysis of protein biomarkers that can be used for assessing response to treatment. Our results provide a proof of concept in sialic acid-related molecular therapy with synthetic monosaccharides.

Distal myopathy with rimmed vacuoles (DMRV)<sup>3</sup>/hereditary inclusion body myopathy (hIBM) is a gradually progressive autosomal recessive disorder that predominantly affects distal muscles at the initial stages but also involves proximal muscles during the progression of the disease (1, 2). DMRV/hIBM has been reported as quadriceps-sparing myopathy because the quadriceps muscles are relatively spared even during the late stage of the disease (3). Skeletal muscle pathology is characterized by rimmed vacuoles in some fibers, scattered atrophic fibers, and intracellular congophilic deposits that are immunoreactive to amyloid, hyperphosphorylated tau, and various proteins (4, 5).

DMRV/hIBM is due to mutations in the UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (*GNE*) gene (6–8) that encodes the bifunctional enzyme catalyzing the two critical steps in sialic acid synthesis (9). Sialic acids are monosaccharides found at the terminal ends of and confer negative charge to glycoproteins and glycolipids and are associated with several biological functions (10–15). Because mutations in the *GNE* gene lead to significant reduction in one of the two enzymatic activities of the gene product (16, 17), it was hypoth-

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<sup>3</sup> The abbreviations used are: DMRV, distal myopathy with rimmed vacuoles; hIBM, hereditary inclusion body myopathy; *GNE*, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase; ManNAc, *N*-acetylmannosamine; Ac<sub>4</sub>ManNAc, tetra-*O*-acetylated *N*-acetylmannosamine; Ac<sub>5</sub>NeuAc, penta-*O*-acetyl-*N*-acetylneuraminic acid; Ac<sub>5</sub>NeuAc-Me, penta-*O*-acetyl-*N*-acetylneuraminic acid methyl ester; Ab, antibody; BW, body weight; CSA, cross-sectional area; NEP, neprilysin; SG, sarcoglycan; βDG, β-dystroglycan; Aβ, amyloid β; Lamp2, lysosome-associated membrane protein 2.

## Ac<sub>4</sub>ManNAc Rescues Muscle Phenotype in DMRV/hIBM Mice

esized that the sialic acid level in DMRV/hIBM is altered, and this was later demonstrated by the reduced sialic acid levels in muscle, serum, and cultured cells from patients (16). This was further supported by findings in the existing mouse model that resembles the phenotype in humans, the *Gne*<sup>-/-</sup>GNED176V-Tg mouse, hereafter referred to as “DMRV/hIBM mouse,” which showed hyposialylation of serum and other tissues from birth and exhibited late onset progressive muscle weakness and atrophy that is accompanied by mild serum creatine kinase elevation from 21 weeks of age (18). In muscle pathology, intracytoplasmic deposits comprising predominantly amyloid  $\beta$  were observed from 31 weeks of age in addition to fiber size variation. From 41 weeks onward, rimmed vacuoles were seen in scattered fibers.

We have recently reported the prophylactic effect of sialic acid-related natural molecules, *N*-acetylneuraminic acid (NeuAc) and its glycosyl conjugate sialyllactose as well as its precursor *N*-acetylmannosamine (ManNAc), on DMRV/hIBM mice (19). By oral administration of these naturally occurring molecules, the DMRV/hIBM mice showed favorable improvement in survival rate, motor performance, muscle force, muscle atrophy, and muscle degeneration, suggesting that hyposialylation is an important factor in the pathogenesis of DMRV/hIBM. More importantly, these results implied that DMRV/hIBM might be rescued by extrinsic administration of sialic acid-related molecules. However, we could not clearly define the dose effect with ManNAc, which should be expected in establishing therapeutic protocols. As related to this finding, we have shown that the sialic acid levels in plasma and the organs were not fully recovered, giving rise to some speculations that these results may reflect a limitation in the incorporation of such compounds into mouse tissues because of the rapid excretion of sialic acid metabolites or the absence of definitive markers that could show dose response. Consequently, this concept would require the use of more effective compounds for the enhancement of cellular sialylation and the search for more sensitive and specific molecular markers in establishing the proof of concept of sialic acid-related molecular therapy for DMRV/hIBM. In this study, we identified a synthetic sugar compound with a profound effect in recovering cellular sialylation in DMRV/hIBM myocytes. When applied *in vivo* to DMRV/hIBM mice, this compound can prevent the myopathic phenotype in a dose-dependent fashion, providing evidence that synthetic sugar compounds may be a good option to consider in designing therapeutic trials pending complete toxicology studies.

### EXPERIMENTAL PROCEDURES

**Mice**—The DMRV/hIBM mice were generated as reported previously (18). Mice were maintained in a barrier-free, specific pathogen-free grade facility on a 12-h light, 12-h dark cycle and had free access to normal chow and water. All animal experiments conducted in this study were approved by and carried out within the rules and regulations of the Ethical Review Committee on the Care and Use of Rodents in the National Institute of Neuroscience, National Center of Neurology and Psychiatry. These policies are based on the “Guideline for Animal Experimentation” as sanctioned by the Council of the Japanese Association of Laboratory Animal Science.

**Sialic Acid Precursors**—Sialic acid (NeuAc) and the physiological precursor ManNAc, peracetylated ManNAc (tetra-*O*-acetyl-*N*-acetylmannosamine (Ac<sub>4</sub>ManNAc)), and peracetylated NeuAc (penta-*O*-acetyl-*N*-acetylneuraminic acid (Ac<sub>5</sub>-NeuAc) and penta-*O*-acetyl-*N*-acetylneuraminic acid methyl ester (Ac<sub>5</sub>NeuAc-Me)) were purchased from Japan Food and Liquor Alliance (Kyoto, Japan), New Zealand Pharmaceuticals Ltd. (Palmerston North, New Zealand), and Nagara Science Co., Ltd. (Gifu, Japan), respectively. *N*-Acetylglucosamine (GlcNAc) (Sigma) was used as negative control.

**Cell Culture and Analysis of Cellular Sialylation and Cytotoxicity**—Cultured myoblasts from DMRV/hIBM patients, diagnosed based on clinical features, muscle pathology, and the presence of mutations (heterozygous for IVS4 + 4A→G and c.1714G→C) (16) in the *GNE* gene, were obtained with informed consent approved by the Ethical Review Board at the National Center of Neurology and Psychiatry. Primary myoblasts from DMRV/hIBM patients and DMRV/hIBM mice were prepared following standard protocols (20). Myoblasts were cultured in 10% FBS, DMEM/Ham's F-12 (Sigma) in a humidified chamber with 5% CO<sub>2</sub> at 37 °C. Myogenic differentiation was induced at confluence stage by switching the medium to 5% horse serum in DMEM/Ham's F-12. Forty-eight hours before lectin staining or sialic acid determination, the medium was replaced with serum-free DMEM/F-12 with or without GlcNAc, ManNAc, NeuAc, Ac<sub>4</sub>ManNAc, Ac<sub>5</sub>NeuAc, or Ac<sub>5</sub>NeuAc-Me and maintained in the humidified chamber for 48 h. Cells were fixed and permeabilized as described previously (16). Biotin-labeled soybean agglutinin (Seikagaku Kogyo, Tokyo, Japan), wheat germ agglutinin (Seikagaku Kogyo), and a mAb against desmin (69-181, MP Biomedicals, Solon, OH) were used for staining the cells followed either with Alexa Fluor 468-labeled (Invitrogen) Ab or TRITC-streptavidin.

For analysis of cytotoxicity, the myoblasts were cultured in 10% FBS, DMEM/Ham's F-12 with or without Ac<sub>4</sub>ManNAc or Ac<sub>5</sub>NeuAc-Me for 3 days. After removing dead cells by washing with PBS, the remaining myoblasts attached on the dish were harvested with trypsin and counted.

**Sialic Acid Measurement**—Total and bound sialic acids from the plasma, membrane-bound fractions collected from cultured cells, and pieces of different tissues were released using 25 mM sulfuric acid hydrolysis for 1 h at 80 °C. Released sialic acids were then derivatized with 1,2-diamino-4,5-methylenedioxybenzene and analyzed with reversed-phase HPLC with fluorescence detection as described previously (18, 21). Total protein was measured using the Bio-Rad Protein Assay according to the manufacturer's protocol.

**Ac<sub>4</sub>ManNAc Pharmacokinetics**—For this experiment, wild type mice were used ( $n = 2$  each group). After collection of blood from the tail vein and urine for base-line data, Ac<sub>4</sub>ManNAc (31  $\mu$ mol) was given as a single dose via an intraperitoneal, subcutaneous, intravenous, 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 CO<sub>2</sub> asphyxiation. Urine and prepared plasma were frozen and kept at -20 °C until processing. To quantify Ac<sub>4</sub>ManNAc, samples were hydrolyzed with 4 M trifluoroacetic acid for 3 h at 100 °C to release the *O*-acetyl groups.

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After cooling to room temperature, we added *p*-aminobenzoic acid ethyl ester, which reacts with ManNAc in the hydrolysate. The mixture was subsequently vortexed and then incubated in 80 °C for 1 h. After cooling the mixture to room temperature, equal volumes of distilled water and chloroform were added, and after vigorous vortexing, the mixture was centrifuged for 1 min. The *p*-aminobenzoic acid ethyl ester-converted monosaccharides in the upper aqueous layer were then analyzed by reversed-phase HPLC according to the manufacturer's instructions (Seikagaku Kogyo). To test the stability of *O*-acetylation of Ac<sub>4</sub>ManNAc *in vivo*, we directly added *p*-aminobenzoic acid ethyl ester to the sample and analyzed the amount of *O*-acetyl-free ManNAc.

**Animal Groups and Treatment Protocol**—For subcutaneous Ac<sub>4</sub>ManNAc treatment, we inserted a subcutaneous indwelling 2-French unit catheter at the back of anesthetized mice and connected this to a microinfusion pump system consisting of a Laboratory Animal Infusion kit (PUFC-C20-10, Instech Solomon, Plymouth Meeting, PA), a single axis counterbalanced swivel arm (CM375BP, Instech Laboratories), and an iPRECIO™ infusion pump (SMP101-L, Primetech, Tokyo, Japan). For this experiment, the DMRV/hIBM mice were grouped into three groups: low dose (SQAc4MN-LD) group with Ac<sub>4</sub>ManNAc infused at a rate of 3 μl/h to deliver 40 mg/kg BW/day (*n* = 5), high dose group (SQAc4MN-HD) with Ac<sub>4</sub>ManNAc infused at a rate of 10 μl/h to deliver 400 mg/kg BW/day (*n* = 4), and placebo (SQAc4MN-placebo) infused with plain normal saline (*n* = 5) at 3 μl/h. Unaffected control littermates (*n* = 14) whose genotypes are either *Gne*<sup>+/-</sup> or *Gne*<sup>+/-</sup>hGNED176V-Tg were likewise included in the cohort for analysis. We treated mice 15–23 weeks of age continuously for 23–25 weeks.

For oral Ac<sub>4</sub>ManNAc treatment, DMRV/hIBM mice, including corresponding littermates, whose ages were 11–20 weeks were included in the cohort. The DMRV/hIBM mice were divided into three groups: placebo (*n* = 6) given acidic water, low dose (Ac4MN-LD) (*n* = 6) given Ac<sub>4</sub>ManNAc at 40 mg/kg BW/day, and high dose (Ac4MN-HD) (*n* = 5) given Ac<sub>4</sub>ManNAc at 400 mg/kg BW/day. Equal numbers of control littermates (*Gne*<sup>+/-</sup> or *Gne*<sup>+/-</sup>hGNED176V-Tg) per group were also treated (placebo, *n* = 6; Ac4MN-LD, *n* = 6; and Ac4MN-HD, *n* = 5). Ac<sub>4</sub>ManNAc, computed according to the desired dose per day, was mixed with the drinking water and given continuously until the mice reached 54–60 weeks of age. In both subcutaneous and oral groups, blood was collected from the tail every month for plasma creatine kinase measurement and toxicology tests.

**Analysis of Motor Performance**—At the end of the treatment protocol, mice were exercised on a 10-lane treadmill (MK-680, Muromachi, Tokyo, Japan) with an adjustable belt speed and equipped with adjustable amperage shock bars at the rear of the belt. The mice were acclimatized to the treadmill with three 10-min running sessions at a 7° incline (5, 10, and 15 m/min) for 7 days after which two exercise tests were performed on separate days, a performance test and an endurance test. The performance test began with a speed of 15 m/min for 5 min and subsequently increased to 20 m/min, which was then gradually accelerated by 10 m/min every min until the mouse was

exhausted and could no longer run. Exhaustion was defined as the inability of the mouse to return to the treadmill belt after 10 s on the shock bars despite electrostimulation. The time of exhaustion was used to calculate the distance that the mouse ran during the exercise. The endurance exercise consisted of a 60-min treadmill run at a constant speed of 20 m/min with a 7° incline after which the number of rests or beam breaks were recorded for 3 min. A digital video camera was positioned above the treadmill to record each test, and video recordings were used for analysis. Both tests were done three times with a 3–4-day rest in between.

**Contractile Properties of Muscle**—Measurement of muscle contractile properties of gastrocnemius and tibialis anterior muscles was performed according to previous protocols (22). All materials used for *in vitro* measurement of force were acquired from Nihon Kohden (Tokyo, Japan). After weighing the mice, they were deeply anesthetized with pentobarbital sodium (40 mg/kg) intraperitoneally and given supplemental doses as necessary to maintain adequate anesthesia, which was judged by the absence of response to tactile stimuli. The entire tibialis anterior and gastrocnemius muscles were isolated, removed, and secured with a 4-0 silk suture at the distal muscle tendon and proximal bone of origin after which the mice were sacrificed by cervical dislocation. Subsequently, the muscle was mounted in a vertical chamber that was connected to a force displacement transducer (TB-653T) and positioned between a pair of platinum electrodes that delivered an electrical stimulus. Throughout the analysis, the muscle was bathed in a physiological solution consisting of 137 mM NaCl, 24 mM NaHCO<sub>3</sub>, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 11 mM glucose, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, and 0.025 mM D-tubocurarine chloride; maintained at a temperature of 20 °C; and continuously perfused with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> to maintain a pH of 7.4. Square wave pulses 0.2 ms in duration were generated by a stimulator (SEN-3301) and amplified (PP-106H). Muscle tension (length) was gradually adjusted to the length (*L*<sub>0</sub>) that resulted in maximal *P*<sub>*t*</sub>. With the muscle held at *L*<sub>0</sub> and the duration changed to 3 ms, the force developed during trains of stimulation pulses (10–200 Hz) was recorded, and the maximum absolute *P*<sub>0</sub> was determined. Absolute *P*<sub>0</sub> was normalized with the physiological cross-sectional area (CSA), which was computed as the product of the ratio of muscle weight, *L*<sub>0</sub>, and density for mammalian skeletal muscle (1.066 mg/mm<sup>3</sup>) to obtain specific forces (*P*<sub>*t*</sub>/CSA and *P*<sub>0</sub>/CSA). Data obtained were digitized and analyzed with a Leg-1000 polygraph system equipped with QP-111H software. After analysis of force generation, the muscles were removed from the chamber, trimmed off from bone and tendons, blotted dry, and weighed.

**Skeletal Muscle Histochemistry and Morphological Analysis**—Muscle tissues were processed for pathological analysis as reported previously (18, 20). Serial cryosections were stained with H&E, modified Gomori trichrome, and acid phosphatase according to standard procedures. Stained sections were visualized on a microscope (Olympus BX51, Olympus, Melville, NY), and digitized images (DP70, Olympus, Tokyo, Japan) were acquired for pathological analysis. We counted the number of rimmed vacuoles in six transverse 8-μm-thick cryosections (at

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least 100  $\mu\text{m}$  apart) stained with H&E on whole gastrocnemius sections for each group of mice.

For morphometric analyses, we stained sarcolemma of gastrocnemius muscle cryosections probed with caveolin-3 (rabbit polyclonal antibody, 610059, BD Transduction Laboratories) for 1 h followed by Alexa Fluor-conjugated goat IgG antibody to rabbit (Invitrogen) for 30 min and obtained six randomly selected digital images at  $\times 200$  magnification to evaluate single fiber CSA. From these images, individual fiber diameter was measured from 1,000–1,500 fibers with ImageJ software (National Institutes of Health), taking note of the shortest anteroposterior diameter of each myofiber.

**Skeletal Muscle Immunohistochemical Analysis**—We immunostained 6- $\mu\text{m}$ -thick cryosections from gastrocnemius muscles using the primary Abs rat mAb to lysosome-associated membrane protein 2 (Lamp2) (clone ABL-93, Developmental Studies Hybridoma Bank), rabbit polyclonal Ab to  $\text{A}\beta 1-42$  (AB5078P, Millipore, Billerica, MA), and mAb to polyubiquitin (Enzo Life Sciences, Inc. Farmingdale, NY) following published protocols (18, 20). We applied appropriate secondary Ab labeled with Alexa Fluor dyes (Invitrogen) for 30 min at room temperature. Digitized images were captured with a laser-scanning microscope (Olympus) and used for analysis.

**Preparation of Crude Membrane and Protein Fractions**—After measurement of force, the organs were harvested, immediately frozen on dry ice, and kept at  $-80^\circ\text{C}$  until use. On the day of tissue processing, organs were crushed and homogenized using a Dounce homogenizer in a buffer containing 75 mM KCl, 10 mM Tris, 2 mM  $\text{MgCl}_2$ , 2 mM EGTA, and protease inhibitor mixture (Complete Mini protease inhibitor tablet, Roche Applied Science), pH 7.4. Equal amounts of homogenized tissues were centrifuged for 1 h at  $30,000 \times g$  at  $4^\circ\text{C}$ . We used the pellet, which represented the membrane fractions, for sialic acid measurement and protein analysis. After two washes in the same buffer, one fraction of the pellet was resuspended in 50 mM  $\text{H}_2\text{SO}_4$ , sonicated, and subjected to sialic acid measurement. We used the other pelleted fraction for analysis of the total protein amount by extracting protein with SDS buffer (2% SDS, 10% glycerol, 10 mM EDTA, 5% 2-mercaptoethanol, 0.0625 M Tris-HCl, pH 6.8).

**Two-dimensional PAGE**—For the first dimension electrophoresis, membrane proteins were extracted with a solution that contained 0.5% Nonidet P-40 (Sigma), 5% 2-mercaptoethanol, ampholyte pH 3–10 (Bio-Lyte 3/10 ampholyte, Bio-Rad), and 8 M urea. Samples were applied to 4% polyacrylamide capillary gels containing 8 M urea, 0.5% Nonidet P-40, and ampholyte pH 3–10 (23). Samples were electrofocused at a constant voltage of 80 V for 30 min and then at 300 V for 120 min with 0.02 M  $\text{H}_2\text{SO}_4$  (anode) and 0.04 M NaOH (cathode) solutions. After electrofocusing, gels were equilibrated with SDS buffer for 30 min and then placed horizontally on top of a 15% SDS-polyacrylamide gel. Second dimension electrophoresis was performed at a constant current of 25 mA. The gel was later blotted into PVDF membrane.

**Immunoblotting**—Samples other than for two-dimensional PAGE were homogenized in SDS buffer. After boiling, supernatants (8  $\mu\text{g}$ ) were electrophoresed on 5–15% gradient polyacrylamide gels (Perfect NT Gel, DRC), transferred onto PVDF

membranes, blocked with 5% fat-free milk, and probed with the following Abs: anti-podocalyxin (goat polyclonal Ab, AF1556, R&D Systems), anti-actin (rabbit polyclonal Ab, 01867-96, Kantokagaku), anti-mouse neprilysin (NEP) (goat polyclonal, AF1126, R&D Systems), anti- $\alpha$ -sarcoglycan ( $\alpha\text{SG}$ ) (clone Ad1/20A6, NCL-a-SARC, Novocastra), anti- $\beta$ -dystroglycan ( $\beta\text{DG}$ ) (clone 43DAG1/8D5, NCL-b-DG, Novocastra), and anti- $\gamma$ -sarcoglycan ( $\gamma\text{SG}$ ) (clone 35DAG/21B5, NCL-g-SARC, Novocastra). Appropriate HRP-conjugated secondary Abs were used according to the manufacturer's protocol. Results were visualized with ECL (ECL Western blotting detection reagents, GE Healthcare) and digitized by ImageQuant LAS 4000mini (GE Healthcare).

**Transferrin Isoelectric Focusing**—Transferrin isoelectric focusing was performed based on standard methods (24, 25) with some modifications. Ten microliters of plasma was saturated with a mixture of 0.1 M  $\text{NaHCO}_3$  and 20 mM  $\text{FeCl}_3$  for 1 h at room temperature. Iron-saturated plasma was then diluted 10 times with 5% ampholyte (pH 3–10), 30% glycerol, and distilled water. Samples (2  $\mu\text{l}$  each) were then applied to a 6% SDS-polyacrylamide gel that contained 10% ampholyte (pH 3–10). Isoelectric focusing was done using 0.02 M NaOH as cathode buffer and 0.01 M  $\text{H}_3\text{PO}_4$  as anode buffer with the following program: 80 V for 30 min, 300 V for 60 min, and then 500 V for 30 min. After isoelectric focusing, the gel was equilibrated with 0.7% acetic acid for 15 min and then transferred to PVDF. The membrane was probed with an Ab that recognized the N terminus of murine transferrin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by appropriate secondary Ab.

**Biochemical and Toxicology Assays**—We measured plasma creatine kinase as described previously (18). We determined the activity of plasma alkaline phosphatase using the Japanese Society of Clinical Chemistry method (19) with *p*-nitrophenyl phosphate as substrate and ethylaminoethanol-HCl as buffer. We measured aspartate aminotransferase by determining the reduction of NADH (Kyowa Medics). We assayed blood urea nitrogen by an automatic clinical analyzer (Dri-Chem 3,500 V, Fuji Film). All measurements were done in triplicates.

**Amyloid Quantification**—For quantifying the amount of amyloid in myofibers, we fixed six 10- $\mu\text{m}$ -thick cryosections (each section was 100  $\mu\text{m}$  apart) from the gastrocnemius muscle in 4% paraformaldehyde for 10 min followed by postfixation in ice-cold methanol for 10 min at  $-20^\circ\text{C}$ . We immunostained sections with mouse mAb to amyloid  $\beta$  ( $\text{A}\beta$ ) (clone 6E10, SIG-39320, Covance; 1:400 dilution). We counted only the positive signals within the myofibers in the whole gastrocnemius section.

We prepared samples for ELISA according to published protocols with slight modifications (19). Homogenized proteins were extracted using equal volumes of 0.4% diethylamine and 100 mM NaCl. We centrifuged samples at  $100,000 \times g$  for 1 h at  $4^\circ\text{C}$  and neutralized the supernatant with 0.5 M Tris base, pH 6.8. We then used the supernatant for amyloid quantification with commercially available ELISA kits for  $\text{A}\beta 1-42$  (Wako) and  $\text{A}\beta 1-40$  (IBL) after brief vortexing. We normalized the amount of amyloid per mg of protein. For plasma amyloid, we



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used 25  $\mu$ l of thawed plasma. All analyses were performed in duplicates.

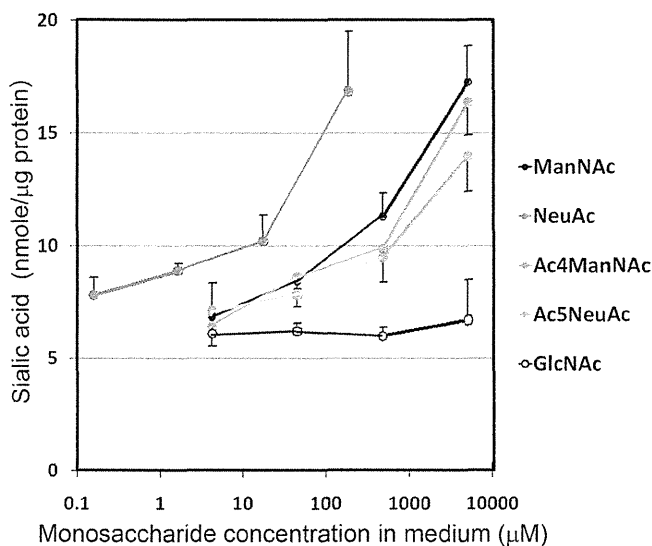
**NEP Activity**—NEP activity was measured in total homogenates from skeletal muscles according to previous reports (26–28) with slight modifications. Skeletal muscle cryosections were homogenized in 150 mM NaCl, 100 mM Tris-HCl, pH 7.8, 1% Triton X-100. Ten microliters of the supernatant ( $\sim$ 40  $\mu$ g) with or without 50  $\mu$ M Phosphoramidon was mixed with 0.17 mM NEP substrate (*N*-benzyloxycarbonyl-alanyl-alanyl-leucyl-*p*-nitroanilide) in 10 mM HEPES and 25 milliunits/ml aminopeptidase M in a total volume of 200  $\mu$ l. The mixture was incubated at 37  $^{\circ}$ C for 14 h, and the amount of chromogenic substrate released in the reaction, which reflects NEP activity, was measured by determining the absorbance at 405 nm and normalized with protein amount. Analyses were done in duplicates.

**Statistical Analyses**—All values are expressed as means  $\pm$  S.E. (or  $\pm$ S.D.) as appropriate. For survival analysis, we used the Kaplan-Meier method to draw the survival curve and used the log rank test to compare groups. For other analysis to determine significance among groups, we used one-way analysis of variance with Dunnett's post-test to compare the treatment group with the placebo group. We set the level of significance to  $p < 0.05$  for all analyses.

## RESULTS

**Screening of Peracetylated Monosaccharides to Increase Cellular Sialylation in DMRV/hIBM Myocytes**—We examined the effect of three synthetic peracetylated ManNAc and NeuAc analogs, Ac<sub>4</sub>ManNAc, Ac<sub>5</sub>NeuAc, and Ac<sub>5</sub>NeuAc-Me, on cellular sialylation in DMRV/hIBM patient myocytes. These agents were added into serum-free medium and compared with the natural compounds NeuAc and ManNAc. As treatment with Ac<sub>4</sub>ManNAc (29) and Ac<sub>5</sub>NeuAc-Me (data not shown) at concentration of 5 mM is known to cause decreased cell viability due to cell death by apoptosis, we checked the median lethal dose of both compounds on DMRV/hIBM cells and determined the LD<sub>50</sub> to be 0.2 and 0.3 mM for Ac<sub>4</sub>ManNAc and Ac<sub>5</sub>NeuAc-Me, respectively (supplemental Fig. 1, A and B). Therefore, we used the concentrations of 0.2 and 0.3 mM for Ac<sub>4</sub>ManNAc and Ac<sub>5</sub>NeuAc-Me, respectively, and 5 mM for the other compounds.

Forty-eight hours after giving the various compounds, cells were subjected to immunohistochemical analysis. Myotubes obtained from the DMRV/hIBM and that were given GlcNAc as control were strongly stained with soy bean agglutinin lectin, which recognizes peripheral  $\beta$ -*N*-acetylgalactosaminide structure, and faintly stained with wheat germ agglutinin lectin, which recognizes a cluster of sialic acids (supplemental Fig. 1C); this pattern of staining reflects hyposialylation of cells. In contrast, cells maintained in serum-free conditions and given Ac<sub>4</sub>ManNAc and Ac<sub>5</sub>NeuAc exhibited increased cellular sialylation, similar to that with ManNAc and NeuAc, by showing the opposite pattern: faint staining with soybean agglutinin and strong staining with wheat germ agglutinin. However, it is important to note that these lectin binding patterns, although not definitive, are consistent with increased sialylation for certain test compounds. The staining pattern of improved sialylation was also seen in cells incubated in 10% FBS but not in the



**FIGURE 1. Screening of compounds to increase cell sialylation.** Quantification of total sialic acid levels in DMRV/hIBM myotubes treated with increasing concentrations of monosaccharides is shown. Ac<sub>4</sub>ManNAc shows a dose-dependent increase in total sialic acid levels; a sharp increase is seen between 20 and 200  $\mu$ M, but a further increase in dose is limited by the toxicity of the compound. Total sialic acid levels are also increased by Ac<sub>5</sub>NeuAc, ManNAc, and NeuAc, but the highest level required a dose 50 times higher than Ac<sub>4</sub>ManNAc. Error bars represent S.E.

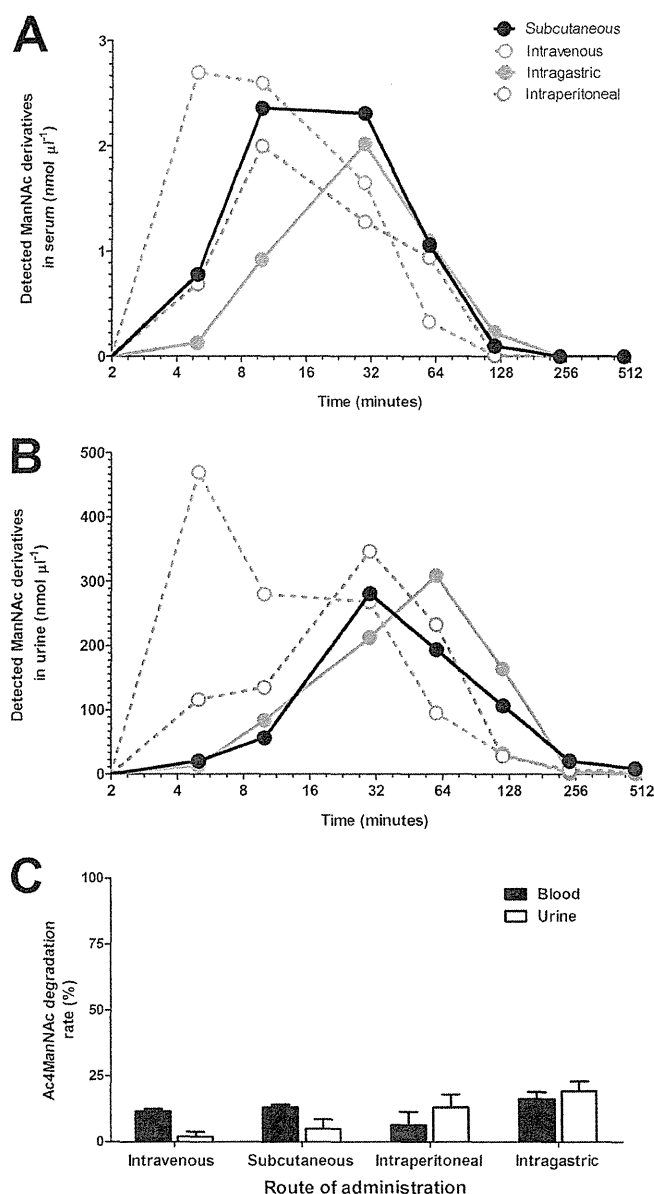
cells given Ac<sub>5</sub>NeuAc-Me. Similar results were obtained in the myocytes from DMRV/hIBM mouse (supplemental Fig. 2).

By quantifying the change in sialylation levels after treatment with various compounds, Ac<sub>4</sub>ManNAc was found to have the most robust effect in DMRV/hIBM myocytes (Fig. 1). Total sialic acid levels were also increased by Ac<sub>5</sub>NeuAc, ManNAc, and NeuAc in a dose-dependent fashion, but the highest level was obtained using a dose 50 times higher than that of Ac<sub>4</sub>ManNAc. Thus, in the succeeding *in vivo* studies, we evaluated Ac<sub>4</sub>ManNAc and its effect on increasing sialylation in various tissues.

**Ac<sub>4</sub>ManNAc Displays Rapid Rate of Excretion into Urine**—To determine the most effective route of administration, we gave a single dose (31  $\mu$ mol) of Ac<sub>4</sub>ManNAc by intravenous, intraperitoneal, subcutaneous, and intragastric routes to wild type mice and collected serum and urine samples at specified periods for analysis. The peak levels of ManNAc derivatives were found in the serum at 5 min via intravenous route, 10 min via intraperitoneal and subcutaneous routes, and 30 min via intragastric route (Fig. 2A). At 120 min, most of the ManNAc derivatives were detectable in the serum only in the intragastric group. The amount of ManNAc derivatives gradually declined over time and became virtually undetectable after 240 min. Of note, the derivatives reached a plateau in the subcutaneous route between 10 and 30 min before a gradual decrease as compared with the rapid decline in the other routes.

In terms of excretion, the peak of ManNAc derivatives was detected earliest in the urine by intravenous route at 5 min followed by the intraperitoneal and subcutaneous routes at 30 min and intragastric route at 60 min. After the peak levels, ManNAc derivatives gradually declined and were undetectable after 4 h except for the subcutaneous route where some derivatives were detectable up to 8 h (Fig. 2B). These results demon-

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**FIGURE 2. Pharmacokinetics of Ac<sub>4</sub>ManNAc.** *A*, after a single dose of 31  $\mu\text{mol}$  of Ac<sub>4</sub>ManNAc, the peak levels of ManNAc derivatives are seen in the serum at 5 min (intravenous), 10 min (intraperitoneal and subcutaneous), and 30 min (intra-gastric). After 120 min, most ManNAc derivatives are detectable only in the intra-gastric group. *B*, the levels of ManNAc derivatives in urine, reflecting Ac<sub>4</sub>ManNAc, are shown. Earliest detection is seen in the intravenous route followed by the intraperitoneal and subcutaneous routes. The peak for the intra-gastric route appeared last. A gradual decline in ManNAc derivatives is seen after the peak levels, and these become undetectable after 4 h except for the subcutaneous route where some derivatives are still seen up to 8 h. *C*, *O*-acetylation status of Ac<sub>4</sub>ManNAc calculated by measuring the ManNAc derivatives in serum or urine collected during the peak levels with or without hydrolysis reflecting the degradation rate of the compound administered. In all routes of administration used, the degradation rate was ~15–20% in both blood (closed bars) and urine (white bars), indicating that more than 80% of Ac<sub>4</sub>ManNAc was estimated to maintain its *O*-acetylation status.

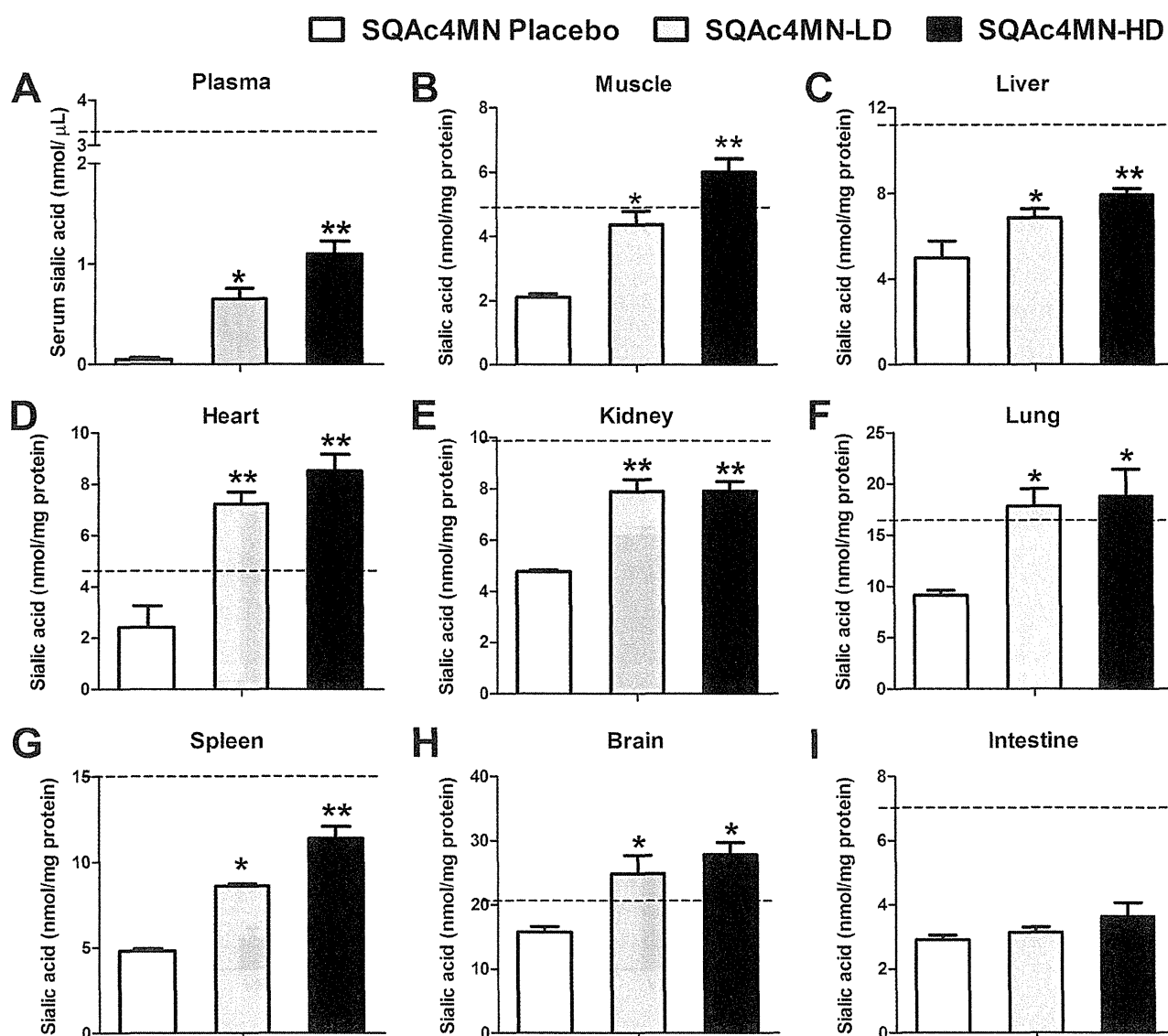
strate that administered Ac<sub>4</sub>ManNAc after being absorbed into the blood is rapidly excreted into the urine similar to ManNAc and NeuAc (19) and imply that continuous or frequent administration of Ac<sub>4</sub>ManNAc is needed to maintain effective levels in blood. Of the four routes, however, intra-gastric and subcutaneous routes appear to have a slower excretion rate.

To determine the extent to which Ac<sub>4</sub>ManNAc is subjected to esterases in the circulation, we checked the *O*-acetylation status of administered Ac<sub>4</sub>ManNAc by measuring the detection rate of ManNAc in serum or urine (at peak of detection) with or without hydrolysis. In all routes of administration used, more than 80% of Ac<sub>4</sub>ManNAc was estimated to maintain its *O*-acetylation status in both blood and urine (Fig. 2C). Interestingly, when we incubated Ac<sub>4</sub>ManNAc in the blood for 30–60 min at room temperature, the *O*-acetylation rate of Ac<sub>4</sub>ManNAc was significantly decreased (data not shown).

**Subcutaneous Infusion of Ac<sub>4</sub>ManNAc Increases Sialic Acid in Plasma and Tissues in DMRV/hIBM Mice**—To check whether Ac<sub>4</sub>ManNAc can be used to increase sialic acid in DMRV/hIBM mice *in vivo*, we performed a pilot study to evaluate whether Ac<sub>4</sub>ManNAc can be incorporated in murine tissues without causing undue toxicity. As our results on the pharmacokinetics suggested that the preferred routes of administration are either subcutaneous or intra-gastric, we continuously infused Ac<sub>4</sub>ManNAc subcutaneously using an infusion pump with the tip of the catheter surgically placed under dorsal skin in three groups of DMRV/hIBM mice that received either Ac<sub>4</sub>ManNAc at 40 mg/kg BW/day (SQAc4MN-LD), Ac<sub>4</sub>ManNAc at 400 mg/kg BW/day (SQAc4MN-HD), or plain normal saline (SQAc4MN-placebo). After 25 weeks, we analyzed sialic acid levels in tissues of treated mice. The bound forms of sialic acid were remarkably increased in almost all organs, including skeletal muscle, liver, heart, kidney, lung, spleen, brain, and plasma with dose dependence in DMRV/hIBM mice, except the intestine (Fig. 3). Notably, the increase in sialylation was more evident as compared with our previous study using natural compounds (19). Incidentally, subcutaneous treatment with Ac<sub>4</sub>ManNAc improved the survival rate (supplemental Fig. 3A) and prevented weight loss (supplemental Fig. 3B) in SQAc4MN-LD and SQAc4MN-HD as compared with SQAc4MN-placebo.

**Oral Ac<sub>4</sub>ManNAc Improves Survival and Prevents Onset of Muscle Atrophy, Weakness, and Degeneration in DMRV/hIBM Mice**—As the increase in sialic acid levels was more remarkable and dose-dependent with Ac<sub>4</sub>ManNAc as compared with natural compounds, we analyzed the effects of Ac<sub>4</sub>ManNAc using the oral route in three groups of DMRV/hIBM mice: low dose (Ac4MN-LD; 40 mg/kg BW/day), high dose (Ac4MN-HD; 400 mg/kg BW/day), and non-treated (placebo; plain acidic water). For a control, the same number of littermates was used for each group (littermate Ac4MN-LD, littermate Ac4MN-HD, and littermate placebo). Treatment with oral Ac<sub>4</sub>ManNAc improved the survival and BW in the DMRV/hIBM Ac4MN-LD group as compared with DMRV/hIBM placebo, but a more remarkable treatment effect was seen in the DMRV/hIBM Ac4MN-HD group (Fig. 4, A and B). Treated DMRV/hIBM mice showed an increase in weight (Fig. 4C) and physiologic CSA (Fig. 4D) of gastrocnemius muscles and tibialis anterior muscles (data not shown). Of note, BW and gastrocnemius mass of DMRV/hIBM mice were recovered to levels similar to littermates after treatment. DMRV/hIBM mice in both Ac<sub>4</sub>MN-LD and Ac<sub>4</sub>MN-HD groups performed better than DMRV/hIBM placebo in running on a treadmill and enduring a specific running workload (Fig. 4, E and F). Furthermore, *ex vivo* measurement of force in

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**FIGURE 3. Subcutaneous delivery of Ac<sub>4</sub>ManNAc increases sialic acid in plasma and tissues.** Total sialic acid levels in plasma (A) and membrane-bound sialic acid levels in skeletal muscle (B), liver (C), heart (D), kidney (E), lung (F), spleen (G), brain (H), and intestine (I) after 25 weeks of subcutaneous infusion are shown in three groups DMRV/hIBM mice: DMRV/hIBM placebo (PBS; open bars), SQAc4MN-LD (40 mg/kg BW/day; gray bars), and SQAc4MN-HD (400 mg/kg BW/day; black bars). Sialic acid levels are remarkably increased in a dose-dependent manner in plasma and most tissues except the intestines. Dotted horizontal lines represent mean sialic acid levels in untreated littermates. Single asterisks (\*) indicate  $p < 0.05$  (DMRV/hIBM placebo versus treated DMRV/hIBM); double asterisks (\*\*) indicate  $p < 0.001$ . Error bars represent S.E.

isolated muscles showed a marked increase not only in the peak isometric ( $P_e$ ) and peak tetanic forces ( $P_0$ ) (data not shown) but more importantly also in both the specific isometric force ( $P_e/CSA$ ) (Fig. 4G) and specific tetanic force ( $P_0/CSA$ ) (Fig. 4H) after treatment.  $P_e/CSA$  and  $P_0/CSA$  increments in DMRV/hIBM mice in Ac4MN-LD and Ac4MN-HD groups paralleled the increase in single fiber diameter as evidenced by a rightward shift of individual muscle fiber diameters, which also indicate a smaller number of atrophic fibers (Fig. 4I).

When comparing DMRV/hIBM Ac4MN-LD and Ac4MN-HD groups, dose-response correlation was noted in treadmill motor performance, endurance test, and  $P_e/CSA$  in gastrocnemius muscle. Additionally, Ac<sub>4</sub>ManNAc treatment decreased plasma creatine kinase levels from  $823.3 \pm 30.0$  IU/liter in non-treated DMRV mice to  $270.0 \pm 38.0$  and

$240.0 \pm 84.3$  IU/liter in treated mice with the low dose and high dose, respectively (supplemental Fig. 3). In all parameters tested, the littermates that were included in Ac<sub>4</sub>ManNAc-LD and Ac<sub>4</sub>ManNAc-HD groups maintained levels similar to those of non-treated littermate placebo, further indicating that treatment was not detrimental to mice at least in the doses used in this study.

In terms of toxicity, the blood urea nitrogen, plasma aspartate aminotransferase, and plasma alkaline phosphatase levels, which reflect kidney and liver functions, were within normal ranges in all mice treated (Table 1). As the use of Ac<sub>4</sub>ManNAc in cells has been associated with cell toxicity (supplemental Fig. 1), we analyzed several tissues after treatment with Ac<sub>4</sub>ManNAc with regard to reactivity to TUNEL staining but did not find any difference between treatment and placebo groups, indicating the absence of overt signs of toxicity (data not shown) and

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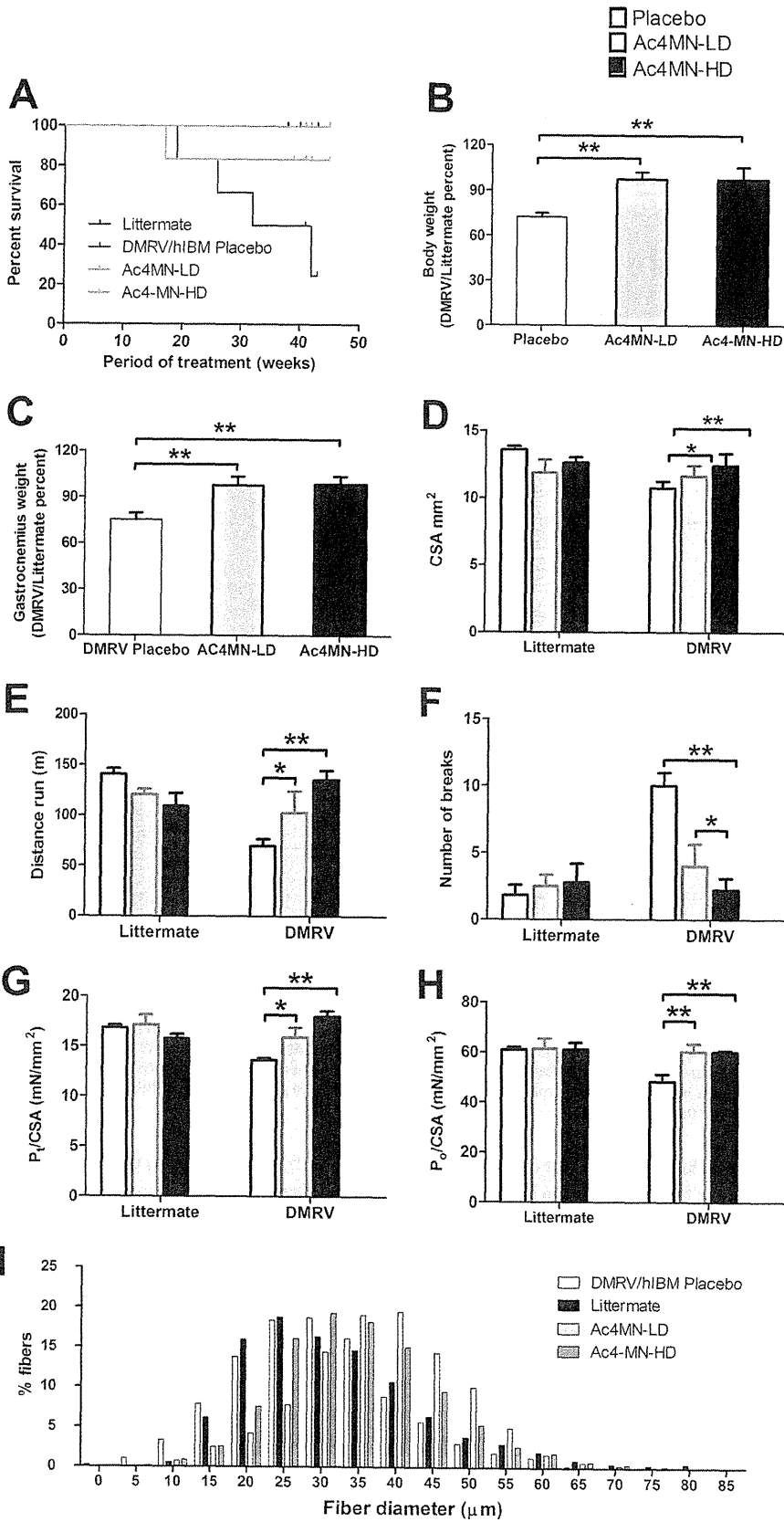


TABLE 1

Kidney and liver function tests after Ac<sub>4</sub>ManNAc

AST, plasma aspartate aminotransferase; ALP, plasma alkaline phosphatase; BUN, blood urea nitrogen.

Group	AST (mean ± S.E.)	ALP (mean ± S.E.)	BUN (mean ± S.E.)
	IU/liter	IU/liter	mg/dl
Littermate	13.20 ± 4.09	47.22 ± 4.11	92.47 ± 12.22
DMRV/hIBM placebo	12.83 ± 3.81	39.14 ± 4.72	107.60 ± 9.61
DMRV/hIBM Ac4MN-LD	14.82 ± 2.88	49.25 ± 2.88	92.44 ± 8.04
DMRV/hIBM Ac4MN-HD	12.91 ± 4.01	51.84 ± 3.65	91.01 ± 13.04

implying that Ac<sub>4</sub>ManNAc might be tolerated by mice over a prolonged period of administration.

**Administration of Ac<sub>4</sub>ManNAc Prevents Intracellular Inclusions and Rimmed Vacuole Formation in Myofibers**—Gastrocnemius muscles of non-treated DMRV/hIBM (DMRV/hIBM placebo) mice show several rimmed vacuoles (*arrow*) and intracellular deposits (*arrowhead*) in myofibers (Fig. 5A, *upper panel*). Enhanced acid phosphatase activity was also observed around rimmed vacuoles, suggesting the presence of acidic organelles that may represent autophagic vacuoles (18). These changes in muscle pathology were not observed in treated or non-treated control littermates (data not shown). Oral treatment in Ac4MN-LD and Ac4MN-HD groups led to a marked reduction in the number of rimmed vacuoles and reactivity to acid phosphatase staining in muscle cryosections (Fig. 5A, *middle and lower panels*). Quantitative analysis showed that the number of rimmed vacuoles in DMRV/hIBM Ac4MN-LD (Fig. 5B) was significantly lower than in DMRV/hIBM placebo, whereas none were seen in DMRV/hIBM Ac4MN-HD. In addition, the number of rimmed vacuoles in Ac4MN-LD DMRV/hIBM was actually lower when compared with NeuAc and ManNAc treatment (19).

Immunoreactive signals to Lamp2, a marker for lysosome; Aβ1–42, which recognizes one of the Aβ peptides; and polyubiquitin were observed in muscles of DMRV/hIBM placebo (Fig. 6A) but were not seen in control littermates (data not shown). The signals of those proteins were hardly observed in DMRV/hIBM mice after oral treatment (Fig. 6A, Ac4MN-LD and Ac4MN-HD). The immunoreaction to other proteins such as amyloid precursor protein, Aβ1–40, neurofilament proteins, phosphorylated tau, microtubule-associated protein light chain 3 (LC3; a marker for autophagosome), endoplasmic (GRP94, a marker of endoplasmic reticulum stress), and dystrophin-associated proteins, which are accumulated in myofibers of DMRV/hIBM mice (18), also disappeared after oral Ac<sub>4</sub>ManNAc treatment (data not shown). Quantification of the number of fibers with rimmed vacuoles and Aβ inclusions also showed a significant decrease in Ac4MN-LD, but these were undetectable in Ac4MN-HD (supplemental Fig. 4). The amount of LC3-II was also reduced by Ac<sub>4</sub>ManNAc treatment (data not shown).

**Sialic Acid in Plasma and Various Organs Is Increased by Oral Ac<sub>4</sub>ManNAc Treatment**—After oral treatment, sialic acid was markedly increased in the plasma and other organs of DMRV/

hIBM mice in a dose-dependent manner (Fig. 7). In the littermates included in the Ac4MN-LD and Ac4MN-HD groups, the levels of membrane-bound sialic acid were also increased when compared with the littermate placebo levels. Notably, however, levels in treated DMRV/hIBM mice were much higher than those of non-treated littermate placebo with the most prominent effects seen in lung, spleen, and brain (Fig. 7, *F–H*). In addition, higher levels of sialylation were achieved in the mice treated through the oral route as compared with the subcutaneous route (compare Fig. 7 with Fig. 3). Again, the sialic acid levels of all organs recovered after oral Ac<sub>4</sub>ManNAc treatment were higher than those after ManNAc and NeuAc treatment (19).

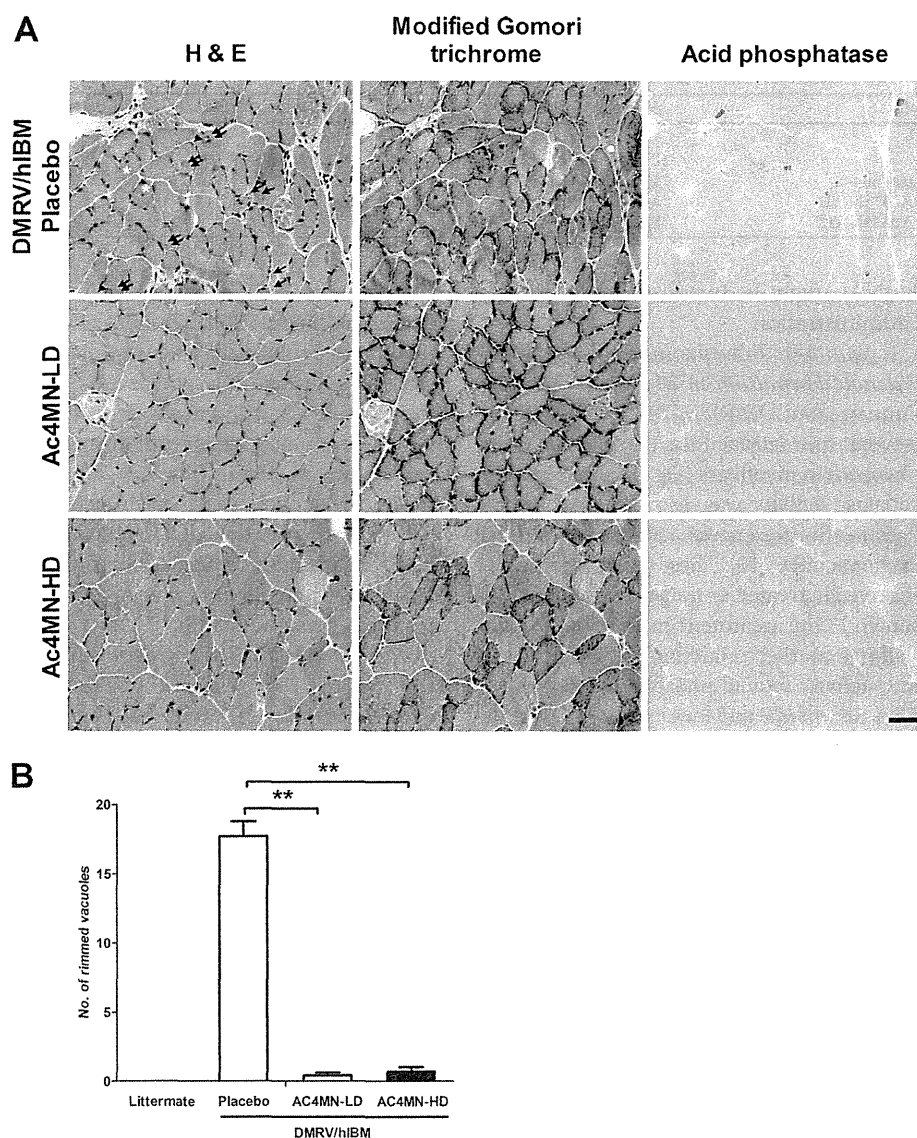
**Sialylation of Glycoproteins in DMRV/hIBM Mice Is Improved by Ac<sub>4</sub>ManNAc Treatment**—Hyposialylation of some muscle glycoproteins has been reported in DMRV/hIBM muscles (26, 30, 31). In this study, we evaluated the sialylation status of αSG, βDG, and NEP by two-dimensional PAGE analysis of skeletal muscle membrane proteins of non-treated littermates and DMRV/hIBM mice who were given either placebo, Ac4MN-LD, or Ac4MN-HD (Fig. 8A). In DMRV/hIBM placebo, when compared with non-treated littermates, the spots for αSG were shifted to the right (Fig. 8B) in reference to γSG (a non-sialylated protein), indicating αSG hyposialylation. The pattern of βDG staining was not changed. After treatment, in both Ac4MN-LD and Ac4MN-HD, αSG spots were notably shifted back to the left, indicating recovery of sialylation. In non-treated littermates, the NEP pattern comprised two spots, but in DMRV/hIBM placebo, spot 1 was not detected, whereas spot 2 was shifted to the right (basic). After treatment, however, NEP spot 1 became visible and was shifted back to the left side (acidic) together with spot 2 (Fig. 8B).

In addition to these membrane proteins, podocalyxin, the major podocyte sialoprotein, was shown to be hyposialylated in homozygous mutant *Gne* knock-in mice (*Gne*<sup>M712T/M712T</sup>) (32). In the kidney and skeletal muscle of DMRV/hIBM mice, podocalyxin from DMRV/hIBM placebo mice was indeed hyposialylated, migrating more slowly than littermate control (supplemental Fig. 5, A and B). Ac<sub>4</sub>ManNAc treatment led to more rapid dose-dependent migration as compared with DMRV/hIBM placebo, suggesting the recovery of sialylation.

In the serum, several glycoproteins are known to be sialylated, including transferrin, α<sub>1</sub>-antitrypsin, α<sub>1</sub>-acid glycopro-

FIGURE 4. Oral treatment of Ac<sub>4</sub>ManNAc improves survival and prevents onset of muscle weakness and atrophy in DMRV/hIBM mice. Three groups of mice were Ac4MN-LD (40 mg/kg BW/day) (*gray bars*), Ac4MN-HD (400 mg/kg BW/day) (*black bars*), and placebo (plain acidic water) (*white bars*). Improvement in survival (A) and BW (B) is seen in Ac4MN-HD more than Ac4MN-LD as compared with DMRV/hIBM placebo. Isolated gastrocnemius muscles had increases in weight (C) and CSA (D) in treated DMRV/hIBM mice. In terms of motor performance, DMRV/hIBM mice in Ac4MN-LD and Ac4MN-HD groups performed better than the DMRV/hIBM placebo group in running on a treadmill (E) and endurance (F). *Ex vivo* measurement of force revealed a marked increase in P<sub>0</sub>/CSA (G) and P<sub>0</sub>/CSA (H) in treated DMRV/hIBM mice. A rightward shift of individual muscle fiber diameters in DMRV/hIBM mice (I) is seen after treatment; this also indicates a smaller number of atrophic fibers. Single asterisks (\*) indicate *p* < 0.05; double asterisks (\*\*) indicate *p* < 0.001. Error bars represent S.E.

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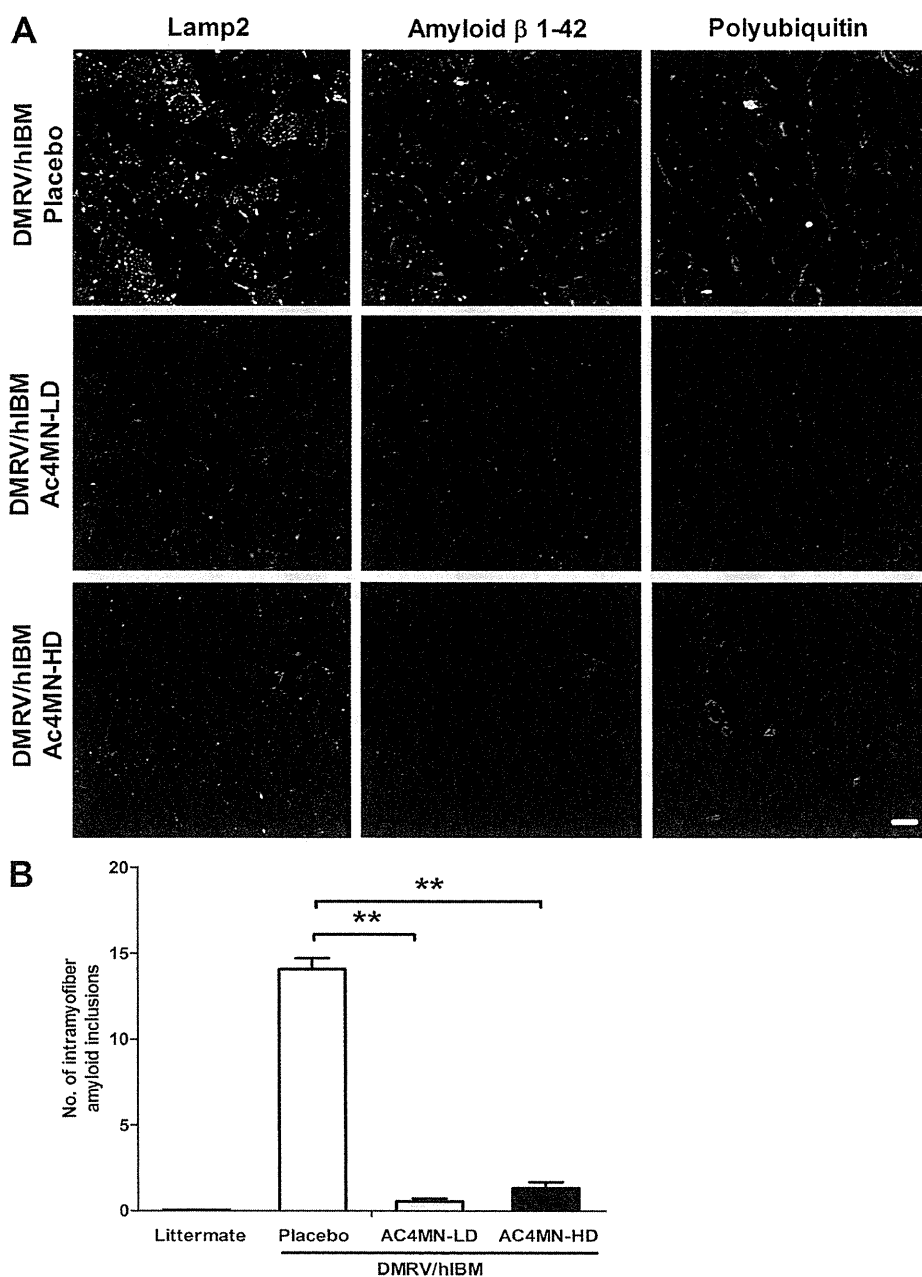


**FIGURE 5. Oral treatment with Ac<sub>4</sub>ManNAc prevents appearance of rimmed vacuoles.** *A*, histochemistry of gastrocnemius cryosections from DMRV/hIBM mice in treated and placebo groups is shown. In the placebo group, H&E staining shows variations in fiber size, scattered atrophic fibers (arrows), fibers with rimmed vacuoles (double arrows) and inclusions (arrowhead), and some fibers with internal nucleation (asterisk). Modified Gomori trichrome staining highlights rimmed vacuoles within the fibers. Acid phosphatase staining is also highlighted in fibers with rimmed vacuoles and small atrophic fibers due to the presence of acidic organelles that may represent autophagic vacuoles. These pathologies are not seen in the gastrocnemius muscles of both Ac4MN-LD and Ac4MN-HD. Bar, 50  $\mu$ m. *B*, quantitative analysis of rimmed vacuoles in gastrocnemius cryosections of non-treated control littermates (dark gray bars), non-treated DMRV/hIBM (white bars), DMRV/hIBM treated with low dose Ac<sub>4</sub>ManNAc (Ac4MN-LD; light gray bars), and DMRV/hIBM treated with high dose Ac<sub>4</sub>ManNAc (Ac4MN-HD; black bars). Double asterisks indicate  $p < 0.001$ . Error bars represent S.E.

tein, immunoglobulin G, and fibrinogen, none of which have been fully evaluated for altered sialylation level in DMRV/hIBM. In this study, we checked the sialylation pattern of transferrin in iron-saturated plasma. In littermates, the predominant tetrasialotransferrin was readily observed in addition to faint pentasialotransferrin; in contrast, DMRV/hIBM placebo mice express the asialotransferrin (non-sialylated) isoform in addition to di- and trisialotransferrin isoforms (Fig. 9A). We also confirmed the recovery of sialylation of transferrin in plasma of Ac<sub>4</sub>ManNAc-treated DMRV/hIBM mouse seen as an increase in the quantity of tri- and tetrasialotransferrin and disappearance of asialotransferrin in Ac<sub>4</sub>ManNAc-treated DMRV/hIBM (Ac4MN-LD and Ac4MN-HD) mice as compared with DMRV/hIBM placebo (Fig. 9, A and B).

*Increase in Amyloid Burden in DMRV/hIBM Mice May Be Related to Decreased NEP Activity and Is Reversed by Treatment with Ac<sub>4</sub>ManNAc*—Quantitative measurement of amyloid by amyloid ELISA showed that A $\beta$ 1–40 and A $\beta$ 1–42 levels are increased in DMRV/hIBM placebo as compared with littermates in both plasma (Fig. 10A) and muscle (Fig. 10B). After treatment, the A $\beta$ 1–40 and A $\beta$ 1–42 levels were reduced in both Ac4MN-LD and Ac4MN-HD. To understand the mechanism by which A $\beta$  peptides are generated in DMRV/hIBM, we measured  $\beta$ -secretase (generating enzyme) and NEP (catabolizing enzyme) activities, which are hypothesized to up-regulate A $\beta$  levels (26, 33).  $\beta$ -Secretase activity was not changed in DMRV/hIBM mouse muscle (data not shown), whereas the activity of NEP was decreased (Fig. 10C). By Ac<sub>4</sub>ManNAc treat-

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**FIGURE 6. Oral treatment with Ac<sub>4</sub>ManNAc prevents occurrence of intracellular inclusions in DMRV/hIBM myofibers.** *A*, in muscle cryosections from non-treated DMRV/hIBM mice (DMRV placebo), immunoreactive signals to Lamp2, A $\beta$ 1–42, and polyubiquitin were seen within myofibers. In Ac4MN-LD and Ac4MN-HD groups, immunoreactivities to Lamp2, A $\beta$ 1–42, and polyubiquitin were not seen. Bar, 50  $\mu$ m. *B*, quantitative analysis of amyloid deposits myofibers counted in several cryosections and averaged in non-treated control littermates (dark gray bars), non-treated DMRV/hIBM (white bars), DMRV/hIBM treated with low dose Ac<sub>4</sub>ManNAc (Ac4MN-LD; light gray bars), and DMRV/hIBM treated with high dose Ac<sub>4</sub>ManNAc (Ac4MN-HD; black bars) shows an increase of amyloid deposits in non-treated DMRV placebo that is markedly reduced in Ac4MN-LD and Ac4MN-HD groups after treatment. Double asterisks indicate  $p < 0.001$ . Error bars represent S.E.

ment, NEP activities in both Ac4MN-LD and Ac4MN-HD DMRV/hIBM mice were recovered to values similar to that in littermates (Fig. 10C). These results imply that neprilysin may be the major enzyme related to A $\beta$  metabolism that can be affected by cellular hyposialylation. The NEP amount as detected by Western blot analysis was not influenced by mouse genotype or treatment (data not shown).

### DISCUSSION

We have recently reported the prophylactic effect of ManNAc on myopathy of DMRV/hIBM mice but did not observe a

dose-effect correlation in using three doses of ManNAc (19). In the present study, we provide evidence that Ac<sub>4</sub>ManNAc can remarkably increase cellular sialylation *in vivo* and in a dose-dependent manner, resulting in a more robust effect on preventing the myopathic phenotype in the DMRV/hIBM mouse as compared with natural sialic acid metabolites.

It has been reported that extrinsic acylated monosaccharides can be effectively incorporated into the sialic acid biosynthetic pathway by modifying metabolic flux in the cells (34–37) to modulate the structure and function of sialic acid-bearing gly-

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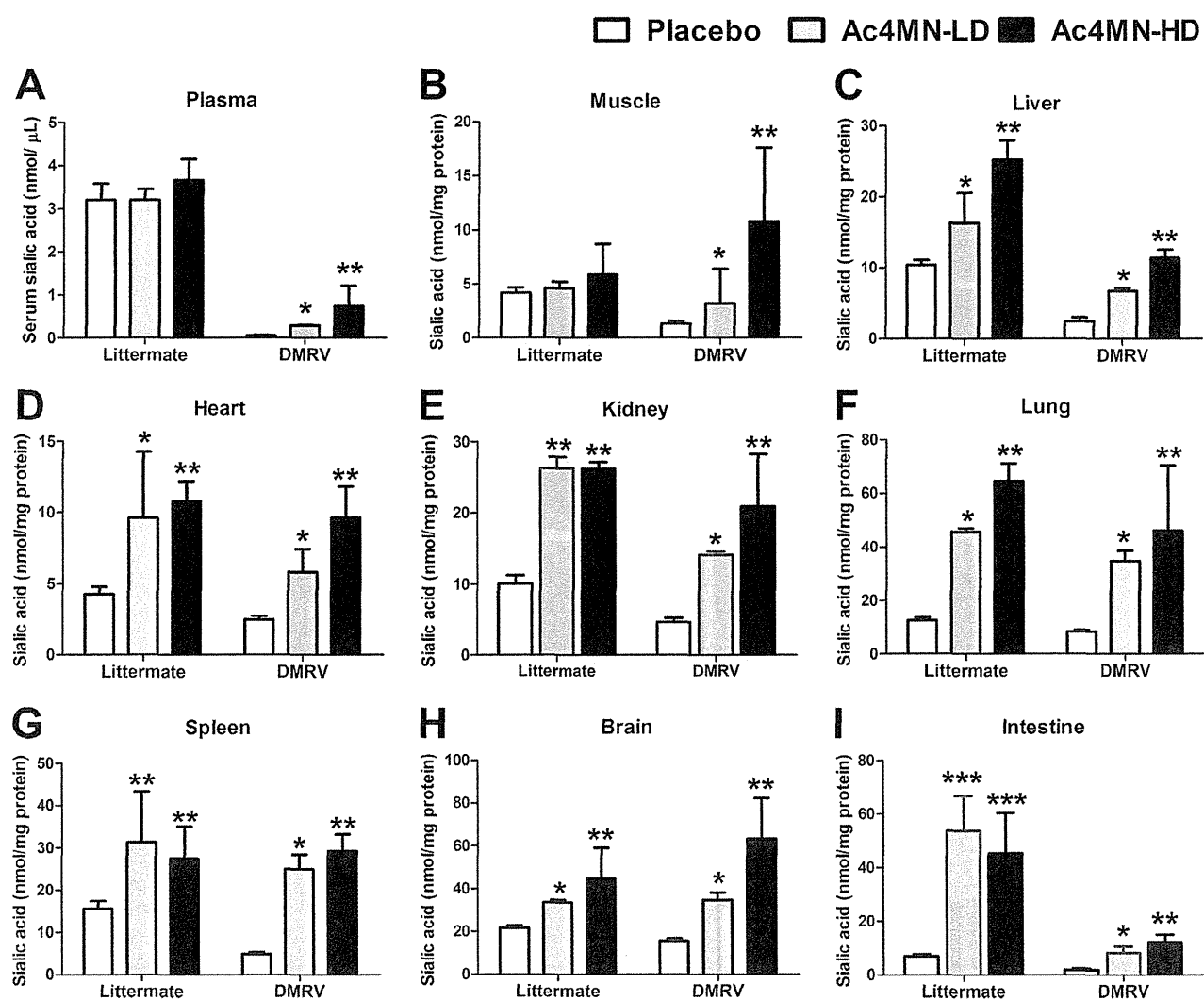


FIGURE 7. Sialylation of plasma and various tissues is increased by oral Ac<sub>4</sub>ManNAc. The total sialic acid level in plasma (A) and membrane-bound sialic acid levels in skeletal muscle (B), liver (C), heart (D), kidney (E), lung (F), spleen (G), brain (H), and intestine (I) after treatment with oral Ac<sub>4</sub>ManNAc given daily for 42–43 weeks are shown. Both DMRV and littermates were divided into three groups: placebo (acidic water), Ac4MN-LD (40 mg/kg/day; gray bars), and Ac4MN-HD (400 mg/kg/day; black bars). Note the dose-dependent increase in sialic acid levels when comparing Ac4MN-LD and Ac4MN-HD in DMRV mice. Single asterisks (\*) indicate  $p < 0.05$  (DMRV placebo versus treated DMRV and littermate versus treated littermate); double asterisks (\*\*) indicate  $p < 0.001$  (DMRV placebo versus treated DMRV and littermate versus treated littermate). Error bars represent S.E.

coproteins and lipids (38). We screened three commercially available synthetic peracetylated monosaccharides for an increase in cellular sialylation of cultured DMRV/hIBM myocytes and observed that DMRV/hIBM cells have the ability to incorporate these exogenous molecules, like ManNAc and NeuAc, and flux them into the cellular sialic metabolic pathway, coinciding with the findings in previous reports (16, 29, 36). In screening for the most effective compound, we found that Ac<sub>4</sub>ManNAc had the strongest effect on increasing sialylation even when using a comparably lower dose than the other compounds, although it induced cell death when given in higher doses, corresponding to findings reported in some cell lines (29, 35, 36).

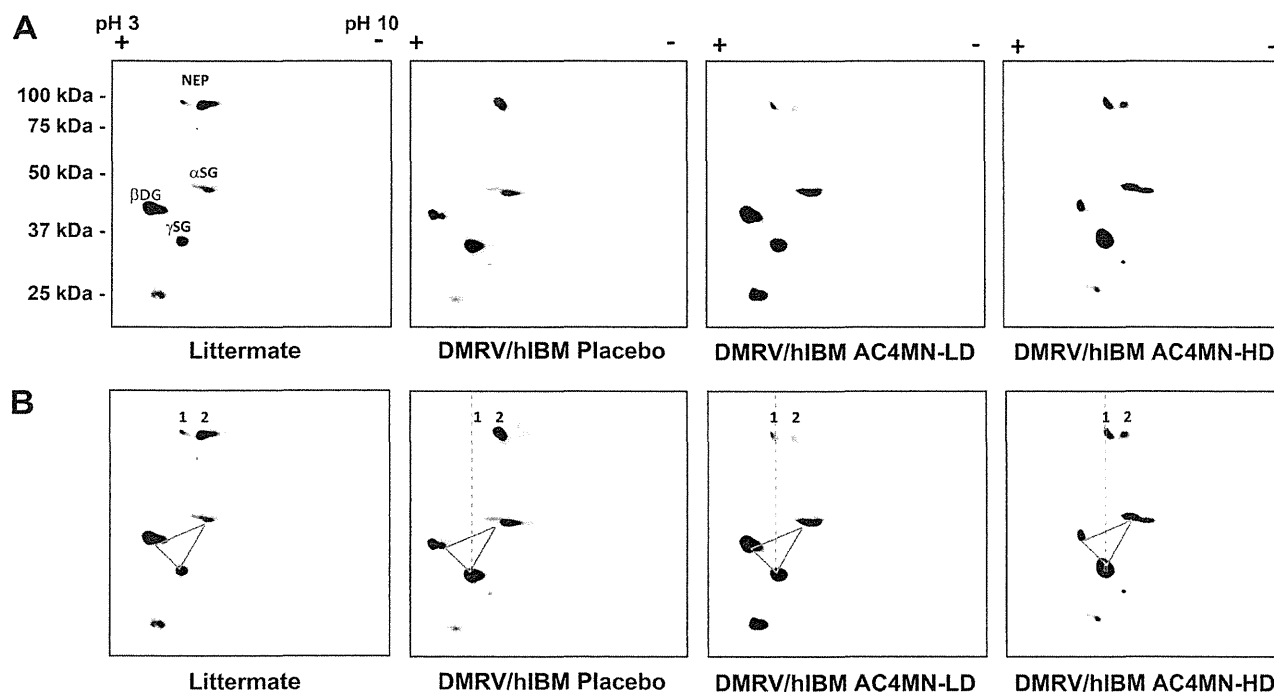
ManNAc can be modified either at its *O*-acyl site or *N*-acyl site. The use of *O*-acyl-modified (peracetylated) ManNAc analogs has been shown to increase metabolic efficiency up to 900-fold (35), and among these, Ac<sub>4</sub>ManNAc was the most efficient

in increasing total sialic acid levels (34). This distinct efficiency exhibited by Ac<sub>4</sub>ManNAc may be explained by two factors. First, *O*-acetylation of ManNAc provides hydrophobic modifications on the hydroxyl groups of the molecule because of the properties endowed by acetyl esters that facilitate passive diffusion of the compound into a cell (39). Second, *O*-acyl ManNAc analogs are believed to be sequestered in cellular membranes that serve as a “reservoir” for these compounds (35) as only a minor fraction is believed to be converted by cells to sialic acid. *N*-Acyl modifications (not used in this study) can also increase cell sialylation (40); increasing the side chains further, however, can lead to decreased sialic acid production and lower metabolic flux (35).

A caveat to the increased metabolic efficiency of hydroxyl-modified or peracetylated sugar analogs, however, is decreased cell viability upon increasing the dose or increasing the number of carbon atoms that is attributed in part to apoptosis (29). But



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**FIGURE 8. Sialylation of muscle sialoproteins is recovered by Ac<sub>4</sub>ManNAc.** *A*, membrane proteins subjected to two-dimensional electrophoresis and transferred to PVDF membrane are probed with known sialylated proteins: αSG, βDG, and NEP. γSG, a non-sialylated protein, was used as a control. The acidic end (pH 3) (+) is on the left, whereas the basic end (pH 10) (–) is on the right side of each membrane. Patterns are shown for control littermates without treatment and DMRV/hIBM mice (placebo, Ac4MN-LD, and Ac4MN-HD). *B*, duplicate image of *A* labeled to show the effect of treatment on the position of the identified proteins. Note the shift of αSG and βDG spots to the right in DMRV placebo as compared with littermate, indicating hyposialylation of proteins. After treatment, these spots are shifted to the left, indicating recovery of sialylation. For NEP, spot 1 is not detected in DMRV as compared with the littermate, and spot 2 is shifted toward the basic side. After treatment, spot 1 reappears, and spot 2 is shifted back to the left side.

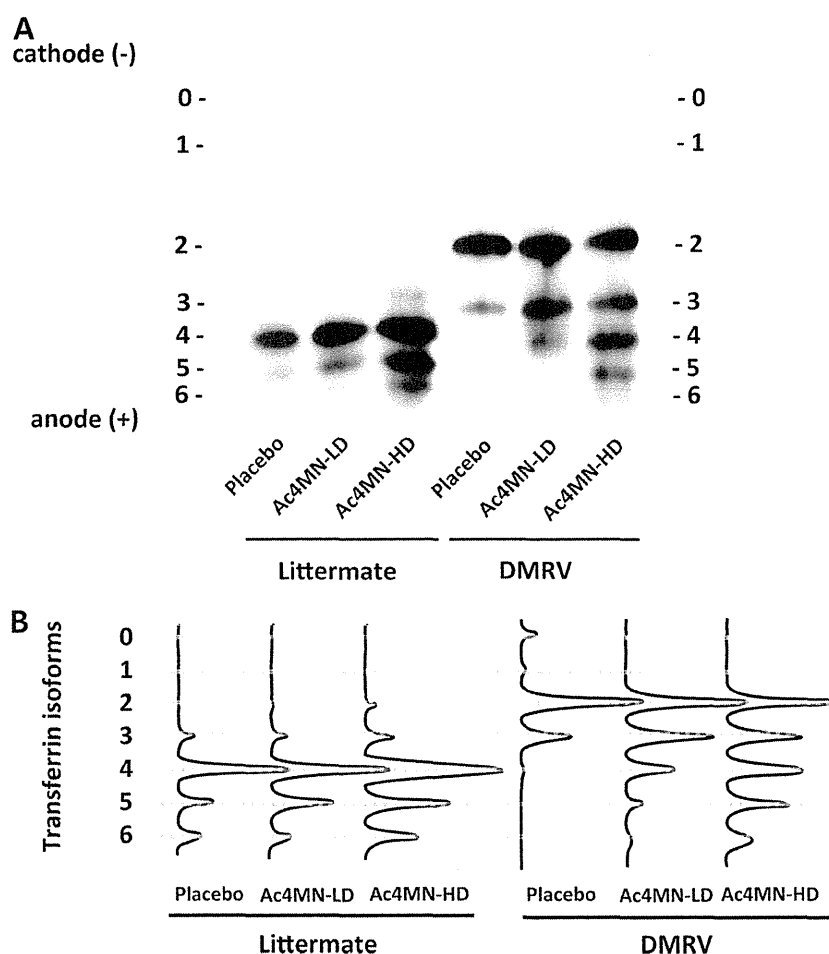
at least among all ManNAc analogs, Ac<sub>4</sub>ManNAc has been shown to be the least toxic (29). Although exogenously supplied Ac<sub>4</sub>ManNAc in Jurkat cells was shown to induce apoptosis consequently leading to the conclusion that sialic acid metabolism may be involved in apoptosis (29), we did not find any toxic effects in mice when using this compound at least in the doses that were used. This might also be partly substantiated by the absence of altered morphology in tissue of mice that were given peracetylated *N*-propanoylmannosamine (41), an agent that was shown to exert higher cellular toxicity than Ac<sub>4</sub>ManNAc (29).

Ac<sub>5</sub>NeuAc, on the other hand, had effects similar to NeuAc in terms of increasing cellular sialylation, but when Ac<sub>5</sub>NeuAc is esterified as in the case of Ac<sub>5</sub>NeuAc-Me, this effect on sialylation is lost. Interestingly, Ac<sub>5</sub>NeuAc-Me in addition to its failure to enhance cellular sialylation also induced cell death in DMRV myocytes even when smaller doses (compared with Ac<sub>4</sub>ManNAc) are given, supporting the notion that the metabolic efficiency of a substrate is highly influenced by its effect on cell viability. The differences in the sialylation effect between Ac<sub>5</sub>NeuAc and Ac<sub>4</sub>ManNAc may be related to the pathway of incorporation of each compound into cells. ManNAc is incorporated into cells by passive diffusion directly across the plasma membrane, whereas NeuAc is incorporated into cells by macropinocytosis and then transported to the cytosol via the lysosomal system (42). Ac<sub>5</sub>NeuAc may also be incorporated into the cell via same pathway as NeuAc, but it is then deacetylated within the lysosome.

Alternatively, the difference between Ac<sub>5</sub>NeuAc and Ac<sub>4</sub>ManNAc might be due to other factors. Although most enzymes in the sialic acid biosynthetic pathway are permissive to several substrates, some of the enzymes may exhibit substrate specificity. CMP synthetase, for example, was implicated to have some substrate specificity, probably depending on tissue or species specificity, as it allows the conversion of NeuAc, *N*-glycolylneuraminic acid, and 9-*O*-Ac-NeuAc but not 4-*O*-Ac-NeuAc to their respective CMP-sialic acid conjugates (43). In addition, the presence of specific sialyltransferases may influence the final incorporation of such substrates into glycoproteins (43). Another possibility is the activation of specific sialidases that can influence the final level of membrane-bound sialic acid. Further studies are needed to clarify this issue.

Pharmacokinetic profiles in mice showed the rapid excretion of administered Ac<sub>4</sub>ManNAc into urine similar to ManNAc (19). Interestingly, the majority of administered Ac<sub>4</sub>ManNAc in circulating blood and urine maintained its *O*-acetylation status, but when Ac<sub>4</sub>ManNAc was incubated in serum at room temperature, almost all acetyl groups were released (data not shown), implying that prolonged exposure of Ac<sub>4</sub>ManNAc in the serum without being mobilized for uptake by cells increases its vulnerability to the abundant esterases in the serum that can immediately degrade Ac<sub>4</sub>ManNAc. These results suggest that to keep a considerable Ac<sub>4</sub>ManNAc concentration in blood for therapeutic trials exogenous Ac<sub>4</sub>ManNAc should be supplied continuously and frequently. The rapid excretion of Ac<sub>4</sub>ManNAc might also

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**FIGURE 9. Increase in sialylated forms of plasma transferrin in DMRV/hIBM mice is seen after treatment with Ac<sub>4</sub>ManNAc.** *A*, iron-saturated plasma electrofocused in a 6% polyacrylamide gel that contained 10% ampholyte (pH 3–10), transferred to PVDF, and probed with anti-mouse transferrin demonstrates the electrophoretic profile of transferrin. Samples from control littermates and DMRV divided into three subgroups (placebo, Ac4MN-LD, and Ac4MN-HD) are shown. Six sialylated isoforms of transferrin are numbered 1–6 (1, monosialotransferrin; 2, disialotransferrin; 3, trisialotransferrin; 4, tetrasialotransferrin, main isoform; 5, pentasialotransferrin; 6, hexasialotransferrin); asialotransferrin (0) is also indicated. In control littermates, isoforms 4 and 5 are readily visible, and after treatment, isoform 6 is observed along with the increase in isoforms 4 and 5. In non-treated DMRV (*P*) mice, isoforms 2 and 3 are seen as well as the asialotransferrin. After treatment, asialotransferrin disappears with increasing detection of isoforms 4, 5, and 6. *B*, the transferrin bands are quantified to show the changes in each isoform when comparing littermates and DMRV mice with or without treatment.

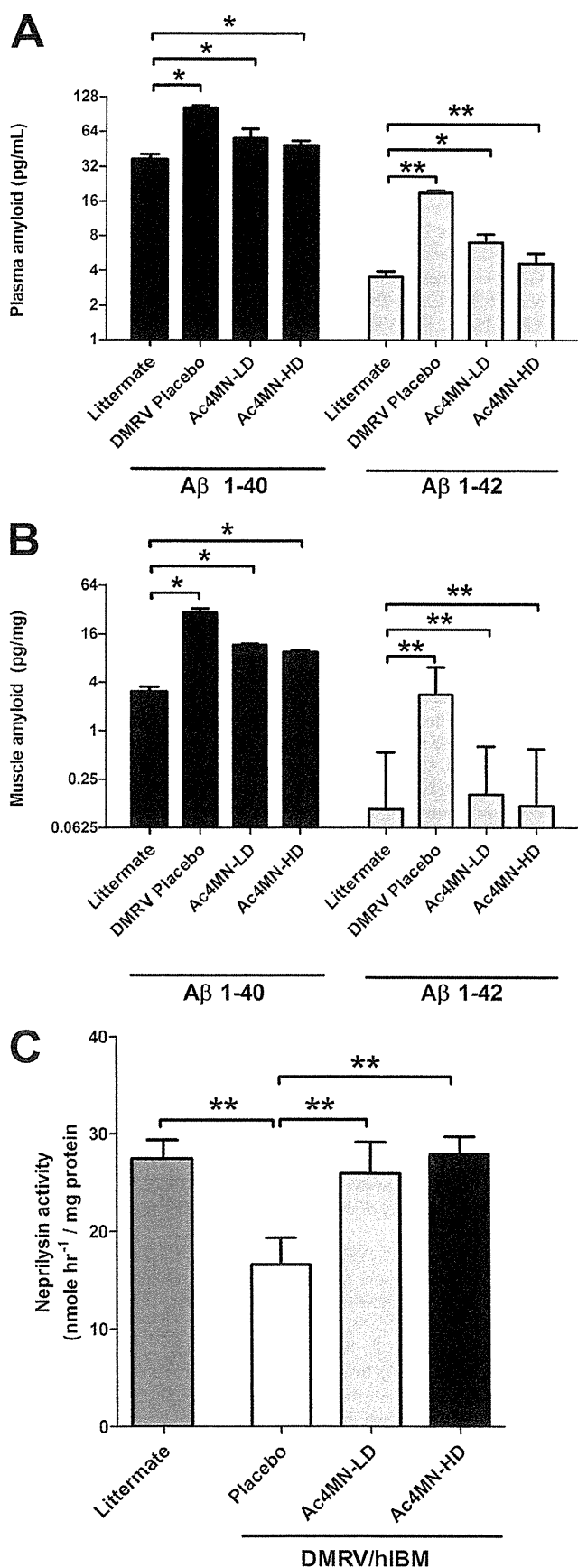
explain why it is relatively tolerated by mice *in vivo* despite its strong association with cell death by apoptosis *in vitro*. After a single administration of Ac<sub>4</sub>ManNAc, about 16% is detected in the circulation (deduced from Fig. 2*A*) because of its rapid excretion. Unlike cells that do not have any route for excreting toxic substances, mice might have some regulating mechanisms that promote excretion of substances that are deemed toxic for survival, although this notion needs further studies for discussion.

The increase in tissue sialylation in Ac4MN-LD and Ac4MN-HD both in DMRV/hIBM mice and littermates provides further evidence that ManNAc analogs can be effectively incorporated in living organisms as has been shown by a previous study of the administration of *N*-propanoylmannosamine to wild type mice (41). After subcutaneous infusion of Ac<sub>4</sub>ManNAc, however, a minimal effect was found in the intestine, indicating that the type of organ in which the sialic acid pool can be replenished may be influenced by the route of administration. As the changes in survival, motor performance,

muscle size, function, and pathology were observed with increasing sialylation of tissues, especially of skeletal muscles, our present results further underscore the importance of addressing hyposialylation in understanding the mechanism of DMRV/hIBM.

In DMRV/hIBM mice, the degree of decline in the physiological properties of the skeletal muscles seemed to be correlated with the temporal feature of atrophy and weakness. In these mice, the *P*<sub>f</sub>/CSA is reduced in middle age when intracellular inclusions that are mainly composed of amyloid were the most prominent feature, whereas there was parallel reduction of *P*<sub>f</sub>/CSA and *P*<sub>0</sub>/CSA during middle to older age (22). In this study, the gastrocnemius *P*<sub>f</sub>/CSA of DMRV/hIBM mice in Ac4MN-LD were improved to levels almost comparable with those of littermates, but a complete recovery was seen only in Ac4MN-HD. In terms of *P*<sub>0</sub>/CSA, the response in both treatment groups of DMRV/hIBM was similar to those in controls, implying that low dose Ac<sub>4</sub>ManNAc is sufficient to improve *P*<sub>0</sub>, which indicates the recovery of the contraction properties of

## Ac<sub>4</sub>ManNAc Rescues Muscle Phenotype in DMRV/hIBM Mice



myofibrils. On the other hand, Ac4MN-HD showed complete recovery of muscle contraction systems, probably including other contraction machinery besides myofibrils. This may have some implication in deciding dosages in future endeavors.

Alterations in the sialylation status of glycoproteins in skeletal muscles from DMRV/hIBM patients have been reported. In certain muscle glycoproteins, including  $\alpha$ -dystroglycan, increased reactivity to peanut agglutinin, a lectin that is reactive to desialylated forms of O-glycans, was observed (30); thus, it was proposed that the stability of such glycoproteins could be influenced by sialylation, and therefore these proteins could be involved in the pathomechanism of DMRV/hIBM. Likewise, it was also hypothesized that hyposialylation of neural cell adhesion molecule and NEP (26, 32) may contribute to the symptomatology of disease. In this study, we showed that hyposialylation of transferrin in blood,  $\alpha$ SG and NEP in skeletal muscle, and podocalyxin in both kidney and skeletal muscles of DMRV/hIBM mouse were almost completely recovered after Ac<sub>4</sub>ManNAc treatment, although until now, the relevance of changes in specific glycoproteins was still being clarified. However, hyposialylation of NEP, a catabolic enzyme for A $\beta$  peptides, has been hypothesized to cause disturbance of amyloid metabolism at least in DMRV/hIBM (26). As amyloid deposits within myofibers precede the occurrence of rimmed vacuoles and muscle degeneration in DMRV/hIBM mice (18) and amyloid has been shown to exert toxicity in muscle cells (44), the NEP hypothesis may be a reasonable platform to explain the pathomechanism of DMRV/hIBM on the basis of hyposialylation and its effect on the progression of the disease. In this study, we demonstrated that NEP is hyposialylated and has reduced enzyme activity, and this occurs together with the increase in the amyloid burden within skeletal muscles in symptomatic DMRV/hIBM mice. Of note, Ac<sub>4</sub>ManNAc treatment of DMRV/hIBM mice led to the recovery of NEP sialylation in addition to the normalization of NEP activity that may have contributed to the reduced amounts of A $\beta$  peptides, consequently leading to normal functioning of the muscle. These findings suggest that the hyposialylation of NEP might be one of the factors in the increase in A $\beta$  production in DMRV/hIBM myofibers that can possibly contribute to muscle degeneration. These results suggest the possibility that not only reduction of cellular sialylation but also the hyposialylation of certain glycoproteins, including transferrin, podocalyxin, and  $\alpha$ SG, may be related to the pathomechanism of the disease in a manner that is yet to be elucidated. Nevertheless, these proteins may be used as biomarkers for future therapeutic trials in DMRV/hIBM.

**FIGURE 10. Increase in amyloid burden may be related to decreased NEP activity in DMRV/hIBM mice and is reversed by treatment with Ac<sub>4</sub>ManNAc.** A and B, the amount of A $\beta$  peptides 1–40 and 1–42 in the plasma (A) and skeletal muscle (B) are quantified by sandwich ELISA. Samples analyzed from control littermates and DMRV/hIBM (placebo, Ac4MN-LD, and Ac4MN-HD) are shown. \*,  $p < 0.05$ ; \*\*,  $p < 0.001$ . Error bars represent standard S.E. C, analysis of NEP activity in total homogenates from skeletal muscles of littermates and DMRV/hIBM mice (placebo, Ac4MN-LD, and Ac4MN-HD). NEP activity is represented by the amount of p-nitroaniline released (nmol) in 1 h and normalized with the amount of protein homogenate (nmol/h/mg of protein). Error bars represent standard S.E.

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In this study, we provide additional information on the proof of concept for sialic acid-related molecular therapy for DMRV/hIBM. The ideal monosaccharide should be one that can control metabolic flux in the sialic acid biosynthetic pathway in a time- and dose-dependent manner. In cells, Ac<sub>4</sub>ManNAc has a very narrow concentration range (35), and its effect on cell sialylation is dependent on cell viability. But despite these findings, rapid excretion of Ac<sub>4</sub>ManNAc in mice may allow incorporation of tolerable amounts into tissues, allowing cell viability and flux into the sialic acid pathway; this is eventually translated into increased sialylation in DMRV/hIBM tissues and modulation of the onset of symptoms. Application of this molecular therapy in trials involving DMRV/hIBM patients indeed will differ from other established pharmaceutical design mainly because of the extremely rapid excretion of sialic acid compounds and the need for long term administration. Nonetheless, the penultimate choice of monosaccharide would depend on careful analysis and consideration of the structure of the substrate, route and timing of administration, dose, and safety.

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