

ARTICLE

Functional correction of neurological and somatic disorders at later stages of disease in MPS IIIA mice by systemic scAAV9-hSGSH gene delivery

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The reversibility of neuropathic lysosomal storage diseases, including MPS IIIA, is a major goal in therapeutic development, due to typically late diagnoses and a large population of untreated patients. We used self-complementary adeno-associated virus (scAAV) serotype 9 vector expressing human N-sulfoglucosamine sulfohydrolase (SGSH) to test the efficacy of treatment at later stages of the disease. We treated MPS IIIA mice at 1, 2, 3, 6, and 9 months of age with an intravenous injection of scAAV9-U1a-hSGSH vector, leading to restoration of SGSH activity and reduction of glycosaminoglycans (GAG) throughout the central nervous system (CNS) and somatic tissues at a dose of 5E12 vg/kg. Treatment up to 3 months age improved learning ability in the Morris water maze at 7.5 months, and lifespan was normalized. In mice treated at 6 months age, behavioral performance was impaired at 7.5 months, but did not decline further when retested at 12 months, and lifespan was increased, but not normalized. Treatment at 9 months did not increase life-span, though the GAG storage pathology in the CNS was improved. The study suggests that there is potential for gene therapy intervention in MPS IIIA at intermediate stages of the disease, and extends the clinical relevance of our systemic scAAV9-hSGSH gene delivery approach.

Molecular Therapy — Methods & Clinical Development (2016) **3**, 16036; doi:10.1038/mtm.2016.36; published online 8 June 2016

INTRODUCTION

Mucopolysaccharidosis type IIIA (MPS IIIA, Sanfilippo A) is a lysosomal storage disease caused by autosomal recessive defects in the gene encoding N-sulfoglucosamine sulfohydrolase (SGSH).^{1,2} The lack of SGSH activity results in accumulation of undegraded and partially degraded heparan sulfate (HS) or HS-derived oligosaccharides in lysosomes. Cells throughout the central nervous system (CNS) are particularly affected, resulting in severe progressive neurological manifestations.^{1,3} MPS IIIA typically can be divided into three phases.⁴ Infants appear normal at birth, but enter the first phase after 1–2 years of normal development, when a progressive developmental delay becomes apparent. The second phase, between ages 1 and 4, is marked by severe behavioral problems and cognitive deterioration, typically with hyperactivity, aggression, cognitive deterioration, rapid deterioration of social and adaptive abilities, and sleep disturbance. In the third phase, behavioral problems remit, but motor symptoms accelerate, leading to spasticity and swallowing difficulties, with death typically occurring in the second decade.^{1,3} Somatic manifestations of MPS IIIA are common, including splenohepatomegaly, coarse hair, skeletal deformation, joint stiffness, and GI issues.^{1,5} The majority of patients are

diagnosed at age 4–6 years when they have developed significant neurological disorders.

For MPS IIIA patients, palliative care has been the only option, no definite treatment is currently available. The blood–brain barrier has presented the major challenge for treating neuropathic lysosomal storage diseases. Long-term strategies for the development of MPS IIIA treatment have included recombinant enzyme replacement therapy, direct gene transfer, and gene-modified autologous stem cell transplant.^{6–10} Enzyme replacement therapy is currently being tested in patients with MPS IIIA, using intrathecal infusion of recombinant enzyme for repeated delivery of the product to the CNS (NCT02060526). Successful correction of MPS IIIA neurological disease in mouse models has also been achieved using gene-modified autologous hematopoietic stem cell transplant, *via* donor monocyte/microglial engraftment in the brain.¹⁰

Direct gene transfer of human *SGSH* (hSGSH) cDNA to the brain has shown functional correction of lysosomal storage pathology in animal models. In a recent clinical trial (NCT01474343), a rAAV serotype 10 (rAAV10) vector expressing both SGSH and SUMF1, the enzyme that activates the sulfatase catalytic center, was determined to be safe when injected into multiple sites in brain parenchyma.

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Received 20 October 2015; accepted 8 April 2016

The feasibility of all these approaches relies on the cross-correction effects of secreted SGSH, which allows significant therapeutic benefit from a small number of transduced cells within a tissue, as long as they are well distributed.

Because MPS IIIA disease manifests not only with global neuropathology in the CNS, but also in the peripheral nervous system and in virtually all somatic organs, the greatest therapeutic benefits are likely to be achieved by systemic gene delivery, providing the SGSH enzyme product directly to most tissues, including the CNS, and maintaining high levels in the circulation. Systemic administration of rAAV9 vectors has been shown to correct both CNS and peripheral pathology in mouse models of MPS IIIA and MPS IIIB, due to the ability of this serotype to cross the blood–brain barrier.^{11–14} Similar effects were reported in MPS IIIA mice treated with an intracisternal delivery of a rAAV9 vector expressing murine *SGSH*.¹⁵

The 1,509 bp h*SGSH* coding region expresses a protein of 503 amino acids, which can be accommodated in a self-complementary AAV (scAAV) vector.^{16–18} Taking advantage of both the trans-blood–brain barrier neurotropism of AAV9, and the high efficiency of the scAAV vector genome, this strategy is intended to restore SGSH activity throughout the CNS and periphery by a single systemic vector delivery, which would be expected to yield clinically beneficial outcomes in MPS IIIA patients.

Because MPS IIIA patients are usually diagnosed after neurological disorders have already occurred, it is important to determine whether therapies offer any benefit at later stages of disease progression, and to identify the general time window during which clinically meaningful benefits could be achieved. In this study, we tested scAAV9-h*SGSH* vector dosing in a MPS IIIA mouse model at early, intermediate, and late stages of the disease. Although it is difficult to draw parallels in disease progression between mice and humans, we find that we can achieve complete normalization of behavior and lifespan by treatment at early stages, partial correction at an intermediate stage, but no improvement in lifespan when treating at a very late stage. The results suggest that significant reversal of neurological disorders are possible, and may result in clinically meaningful benefits beyond stage one of the disease in humans.

RESULTS

The h*SGSH* cDNA sequence was cloned into an scAAV vector background under the control of the 250 bp murine small nuclear RNA (U1a) promoter.¹⁹ The therapeutic potential of scAAV9-U1a-h*SGSH* systemic gene delivery was tested at varying doses and ages in MPS IIIA mice. In the untreated mice, lysosomal glycosaminoglycans (GAG) storage is detectable in both the CNS and somatic tissues as early as 1 month of age. Nevertheless, there is no observable abnormality regarding the appearance, body weight, or wellbeing in MPS IIIA mice before 7 months of age, compared to wild-type (wt) littermates. Behavioral abnormalities in a Morris water are not detectable until they are 7 months old. MPS IIIA mice die between the ages of 10–17 months, compared to the 15–30-month lifespan of the wt littermates. Controls were nontreated (saline-injected) wt and MPS IIIA littermates. The animals were tested for behavior at 7–7.5 months age and observed for longevity. Tissues were analyzed for SGSH enzyme activity, GAG content, histopathology, and biodistribution. Supplementary Table S1 summarizes the overall experimental design.

Rapid expression of functional SGSH within 10 days following systemic delivery of scAAV9-h*SGSH* vector

The functionality of the scAAV-U1a-h*SGSH* vector genome (Supplementary Figure S1) was confirmed by packaging with

AAV2 capsid and infecting human MPS IIIA skin fibroblast cell cultures (GM00312) at 100 vg/cell. At 48 hours postinfection, SGSH enzyme activity was detected at approximately 2.8- and 7.3-fold above normal levels in the AAV-infected cells and media, respectively (Supplementary Table S2). This indicated that the ectopically expressed SGSH was enzymatically functional and efficiently secreted, which is critical to achieving cross-correction *in vivo*.

In a pilot *in vivo* experiment, 2-month-old MPS IIIA mice ($n = 2$) were treated with a tail vein injection of 5E12vg/kg (vector genomes per kilogram body weight) scAAV9-U1a-h*SGSH* and terminated for analyses at 10 days postinjection. The scAAV9 vector treatment resulted in the restoration of SGSH activity in the brain and multiple somatic tissues (Figure 1a,b) and the correction of lysosomal storage pathology (Figure 1c,e) and astrocytosis (Figure 1d), suggesting a rapid functional therapeutic effect from rSGSH expression following the systemic vector delivery.

Dose-dependent persistent rSGSH expression in the CNS, PNS, and somatic tissues in MPS IIIA mice treated with scAAV9-U1a-h*SGSH* vector at different ages

Young male and female MPS IIIA mice (age 1 month) were treated with an IV injection of either 5E12 or 1E12 vg/kg scAAV9-U1a-h*SGSH* vector to determine the therapeutic potential and effective vector dose for treatment at an early stage of disease progression. Animals from each dose group were terminated for analyses at age 8 months or at the humane endpoint, defined by irreversible symptoms of late-stage MPS IIIA (see Materials and Methods). At age 8 months, we detected SGSH activity at fourfold greater than wt levels in the liver in MPS IIIA mice treated at 5E12 vg/kg, and approximately 50% of wt levels in mice treated with 1E12 vg/kg vector (Figure 2a). Both of these values were statistically indistinguishable from WT after adjusting for multiple comparisons. These values decreased by approximately 10% between age 8 months and the endpoint, supporting stable vector expression. In other peripheral tissues (kidney, heart, spleen, lung, intestine, and muscle), SGSH activity was detected at 0.5- to 3-fold of wt levels at age 8 months in the 5E12 vg/kg group. Values from heart and intestine were statistically indistinguishable from WT at 8 months at the high dose. The SGSH levels decreased to less than 50% of wt levels at the endpoint. The mechanism for this decrease, particularly in postmitotic tissues like heart and skeletal muscle, is not known. In the CNS, ectopically expressed SGSH activity in the 5E12 vg/kg group was approximately 15% of the wt value at 8 months age, decreasing to approximately 5% by the endpoint. In the lower dose group, there was <5% of the wt SGSH levels at either time point in the CNS, showing a dose-dependent expression of scAAV-U1a-h*SGSH* in the brain.

To assess the vector treatment of MPS IIIA at more advanced stages of the disease, male and female mice were injected IV with 5E12 vg/kg scAAV9-U1a-h*SGSH* at ages 2, 3, 6, and 9 months (Supplementary Table S1). The vector treatments resulted in the restoration of SGSH enzyme activity to various levels in all tested tissues, at above wt levels in the liver, and at subnormal levels in the brain and other somatic tissues (Figure 2b). Age effects in rSGSH levels were observed only in mice treated at age 9 months, and only in the brain, spleen, and kidney at the endpoint.

Immunofluorescence for hSGSH was performed to assess the expression and distribution of rSGSH in the MPS IIIA mice treated at different ages. High-level rSGSH expression was detected in the liver in all age groups, with high rates of transduction remaining in the liver at 8 months age (6 months postinjection), and waning numbers of transduced cells at the humane

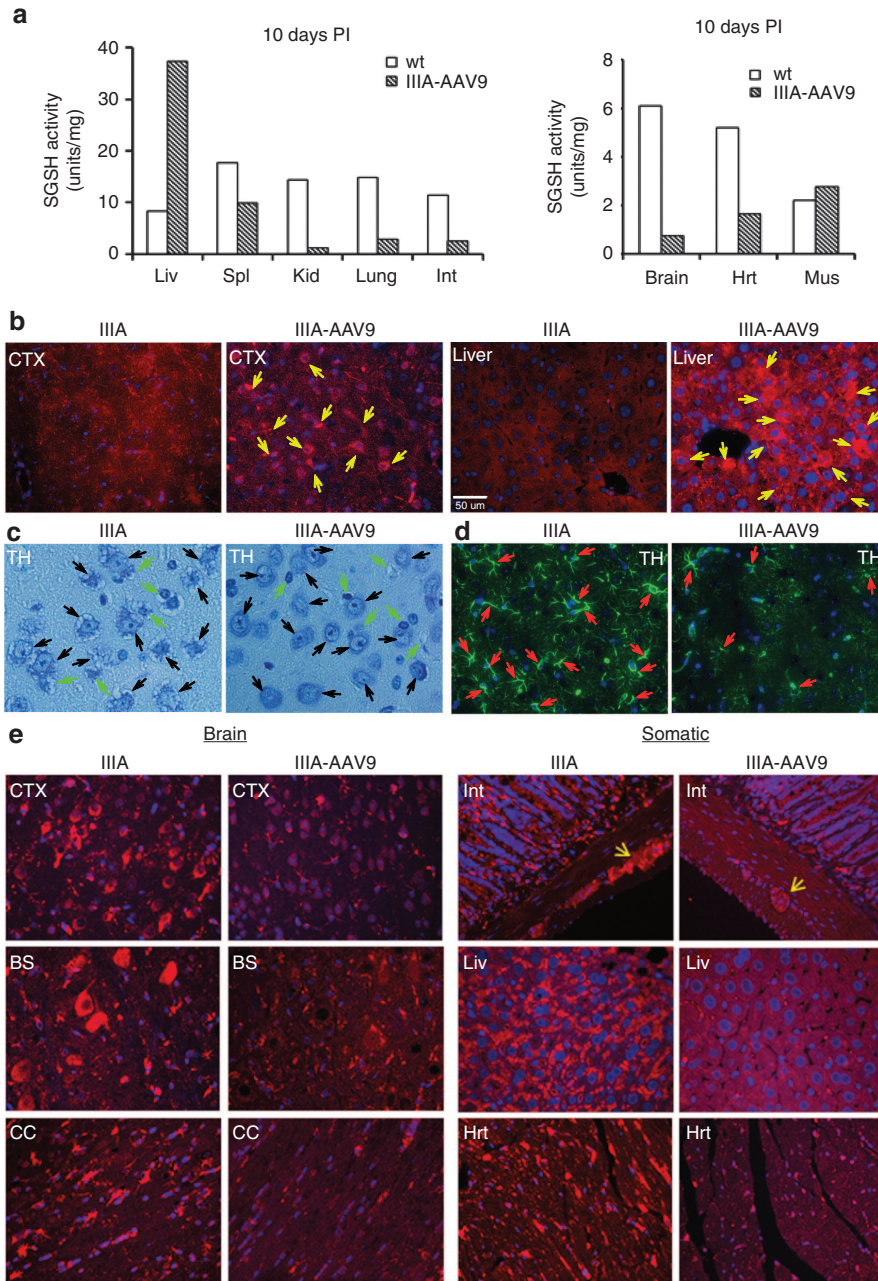


Figure 1 Expression of N-sulfoglucosamine sulfohydrolase (SGSH) enzyme and correction of CNS and somatic pathology in MPS IIIA mice at 10 days postinjection. MPS IIIA mice ($n = 2$) were injected intravenously with $5E12$ vg/kg of scAAV9-U1a-hSGSH vector at 2 months of age. Tissues were assayed at 10 days postinjection for (a) SGSH enzyme activity expressed as units/mg protein (1 unit = 1 nmol 4MU/17 hours). (b) hSGSH immunofluorescence. Red fluorescent hSGSH-positive cells indicated by yellow arrows. (c) lysosomal storage of GAGs by toluidine blue staining. Black arrows: neurons; green arrows: microglia. (d) Glial fibrillary acidic protein (GFAP). Green fluorescent GFAP-positive cells indicated by red arrows. (e) LAMP1 immunofluorescence. The indicated tissues show LAMP1 staining in red. Yellow arrows indicate myenteric neurons. BS, brain stem; cc, corpus callosum; CTX, cerebral cortex; Hrt, heart; Int, intestine; Liv, liver; TH, thalamus. Blue fluorescence: 4',6-diamidino-2-phenylindole (DAPI)-positive nuclei. Scale bar: 50 μ m.

endpoints (Figure 3a). We also detected rSGSH in other somatic tissues including: kidney, in the glomerulus and distal tubules (Supplementary Figure S2a); spleen, in abundant sinusoid endothelial cells in the red pulp and occasionally in the vasculature in white pulp (Supplementary Figure S2b); and in unidentified cells in lung (Supplementary Figure S2c). Low-level rSGSH signal was detected in various cell types throughout the brain, including neurons, glia, endothelial cells (Figure 3b), and ependymal cells of the choroid plexus (Supplementary Figure S2d), while strong

rSGSH staining was observed in neurons of the myenteric plexus and submucosal plexus in intestine (Figure 3c). The immunofluorescence results correlated with tissue SGSH activity (Figure 2).

These data indicate that the systemically delivered scAAV9 vector at $5E12$ vg/kg mediated widespread rSGSH expression in the CNS, peripheral nervous system (PNS), and somatic tissues in MPS IIIA mice, when treated at or before 6 months of age. In mice treated at 9 months, very low rSGSH expression was detected in the brain (Figure 2b).

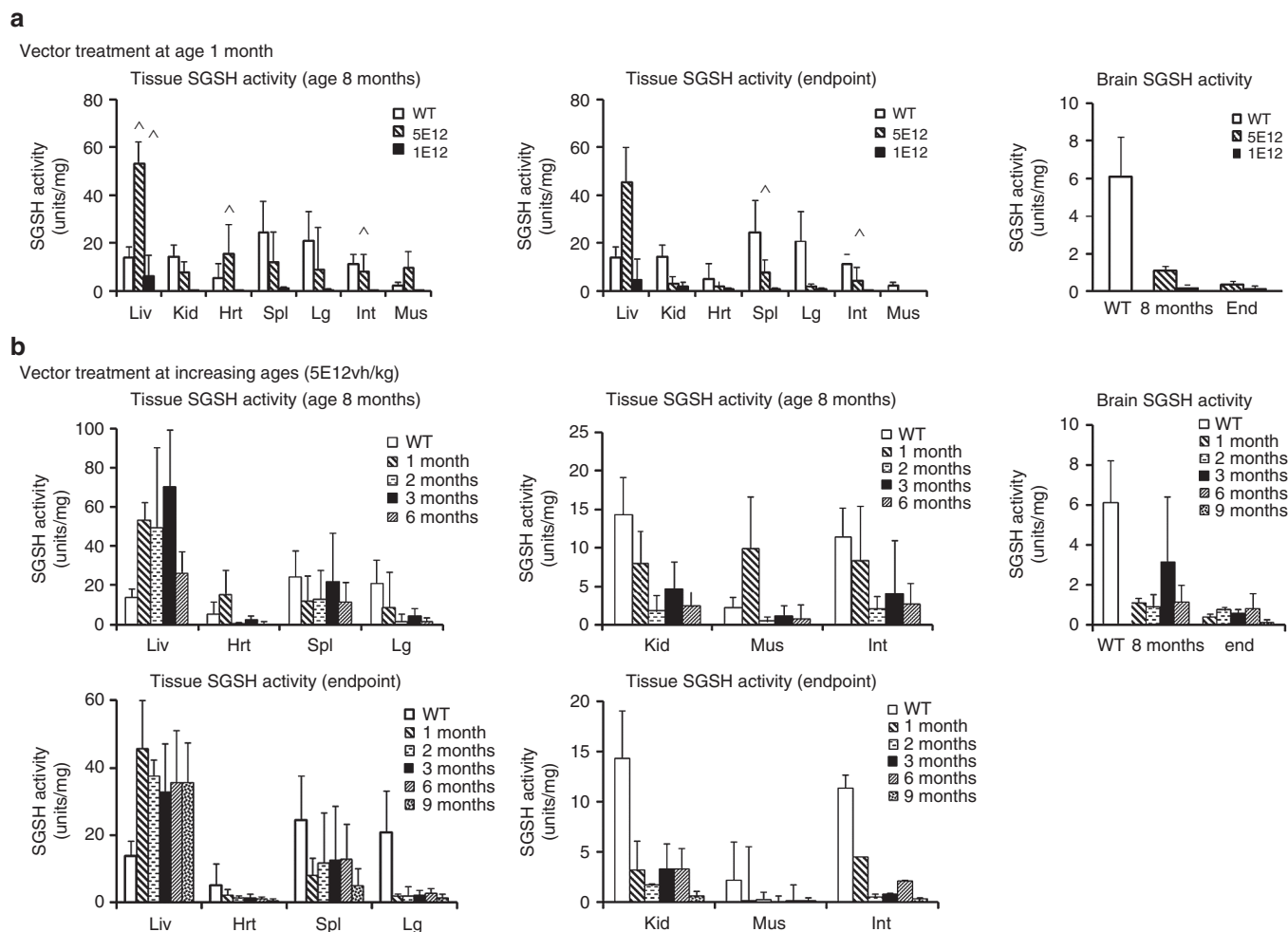


Figure 2 Tissue N-sulfoglucosamine sulfohydrolase (SGSH) enzyme activity in vector-treated MPS IIIA mice. **(a)** MPS IIIA mice were treated at 1 month age with 1E12 or 5E12 vg/kg scAAV9-U1a-hSGSH vector. Tissues were analyzed for SGSH activity at either 8 months age or at the humane endpoint for mice in the longevity study. **(b)** MPS IIIA mice were treated with 5E12 vg/kg at the indicated ages and assayed at either 8 months age or at the humane endpoint as indicated in each graph (mice treated at 9 months age were assayed only at the humane endpoint). SGSH activity is expressed as units/mg protein (1 unit = nmol 4MU released/17 hours). Data presented are means \pm SD. Background residual SGSH activity values in tissues from untreated MPS IIIA mice were <3% of wt levels, and were subtracted from the values for AAV9-treated mice to show vector-expressed enzyme activity. The indicated values in **(a)** (^) were not significantly different from wt using repeated measures analysis of variance with Bonferroni-Holm correction for group comparisons. All other values were statistically different from wt. Hrt, heart; Int, intestine; Kid, kidney; Liv, liver; Lg, lung; Mus, skeletal muscle; Spl, spleen.

rAAV9-mediated long-term clearance of lysosomal storage pathology

Tissues were assayed for GAG content to quantitate the impacts of vector treatment on lysosomal storage pathology (Figure 4a). At 5E12 vg/kg, GAG reduction was statistically different from untreated mice, after Bonferroni-Holm correction (see Materials and Methods), in liver, spleen, heart, and lung at the 8-month time point ($P < 0.05$), and statistically indistinguishable from wt mice in liver and spleen ($P > 0.05$). In mice tested at the humane endpoint, the statistically significant reduction in GAG was maintained in liver, spleen, lung, and heart. GAG levels in brain, heart, intestine, liver, and lung were statistically indistinguishable from wt mice at the endpoint. In contrast, reductions in GAG in mice treated at the low dose of 1E12 vg/kg did not reach statistical significance in any tissue compared to untreated animals.

Consistent with the high levels of rSGSH in the liver, stored GAG content in liver and heart was reduced to wt levels in MPS IIIA mice treated with 5E12 vg/kg scAAV9-hSGSH vector at all tested ages (Figure 4b). In other somatic tissues, GAG content was reduced in

all treatment groups at 8 months and at the endpoint, despite lower levels of enzyme expression. Partial GAG reduction was observed in kidney, lung, and skeletal muscle at both 8 months of age and at the endpoint in all treatment groups. Importantly, GAG levels in the brain were reduced at the 8-month time point in all age groups (mice treated at 9 months were not included in the 8-month assays). Although GAG levels increased between 8 months and the endpoint, they remained lower than untreated mice. The MPS IIIA mice treated at 9 months of age showed a similar decrease in CNS GAG contents (Figure 4b) relative to untreated mice even though only very low levels of SGSH activity were detected in the brain (Figure 2b) at the endpoint.

To visualize changes in lysosomal storage, immunofluorescence staining was performed for LAMP1 in tissues from mice at age 8 months and endpoint. Long-term reduction of LAMP1 was observed in cells throughout the brain in mice treated at or before 6 months of age, with the exception of Purkinje cells, wherein the reduction of LAMP1 was partial compared to untreated 9 month old MPS IIIA mice (Figure 5a, Supplementary Figure S3a). We

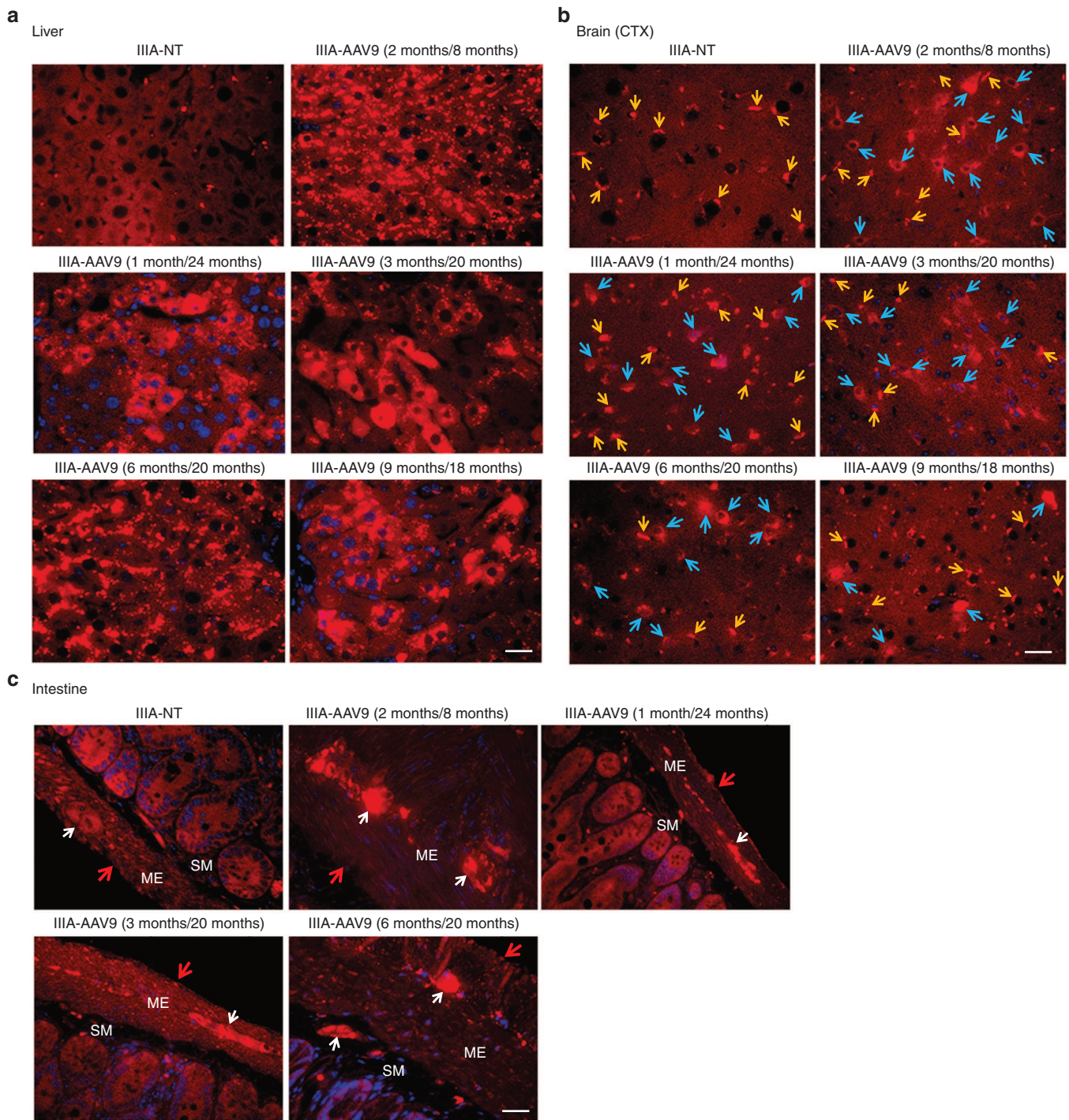


Figure 3 Persistence of tissue N-sulfoglucosamine sulfohydrolase (SGSH) expression. MPS IIIA mice were treated by an IV injection of 5E12 vg/kg scAAV9-U1a-hSGSH vector at different ages (1 to 9 months), and tissues were assayed for hSGSH by immunofluorescence at either age 8 months or the endpoint for mice in the longevity study (8 to 24 months). In each image, treatment mo/termination mo is indicated. Red fluorescence: rSGSH-positive cells and signals; blue fluorescence: DAPI-positive nuclei. (a) Liver. (b) Brain (CTX, cortex): blue arrows: SGSH-positive cells; green arrows: autofluorescent signals. (c) Intestine: SM: submucosa; ME: muscularis externa; red arrows: peritoneal surface of intestine; white arrows: SGSH positive neurons of myenteric plexus and submucosal plexus. Scale bar: 50 μ m.

also observed reduction in LAMP1 in enteric neurons in the myenteric plexus (Figure 5b). LAMP1 immunofluorescence also decreased in liver, heart, skeletal muscle, lung, fat, and capillaries of intestinal villi, with only kidney tissues unaffected (Figure 5b, Supplementary Figure S3b). Similar LAMP1 reduction was observed at the endpoint even in mice treated at 9 months

of age. The tissue LAMP1 staining results generally correlated to tissue GAG content levels (Figure 4).

Correction of astrocytosis in the CNS and PNS

Tissues were assayed by immunofluorescence staining for glial fibrillary acidic protein (GFAP) to determine the impact of scAAV9-hSGSH

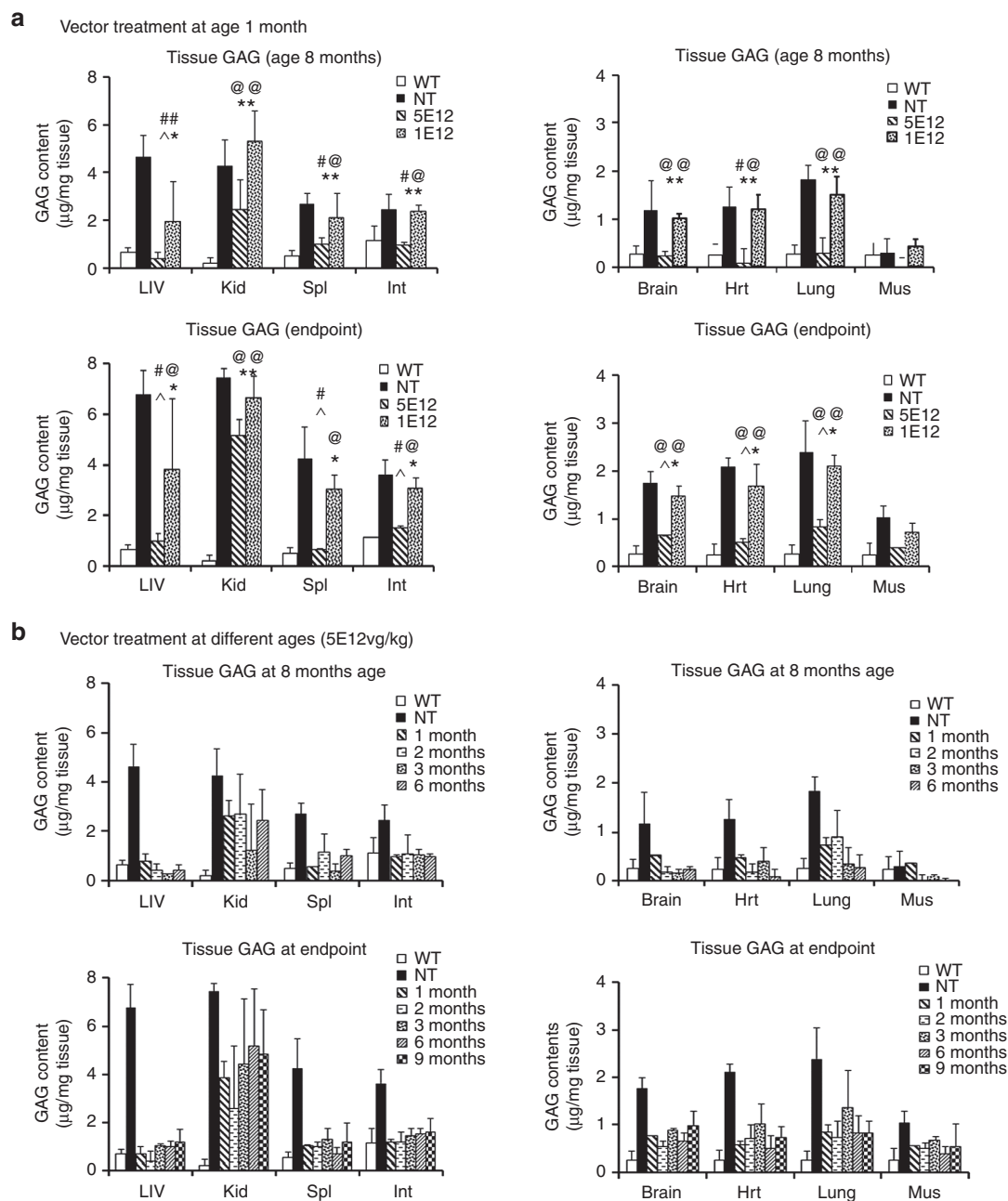


Figure 4 Reduction of stored GAGs in vector-treated MPS IIIA mice. MPS IIIA mice were treated at 1 month age with 1E12 or 5E12 vg/kg scAAV9-UTa-hSGSH vector (a), or at ages 1–9 months with 5E12 vg/kg vector (b). Tissues were analyzed for GAG content at either 8 months age or at the humane endpoint for mice in the longevity study. Data presented are means ± SD. Hrt, heart; Int, intestine; Kid, kidney; Liv, liver; Mus, skeletal muscle; NT, nontreated MPS IIIA mice; Spl, spleen; WT, wild-type mice. The following symbols indicate statistical results using repeated measures analysis of variance with Bonferroni-Holm correction for group comparisons: *difference versus wt; #difference versus IIIA; ^no statistical difference versus wt; @no statistical difference versus IIIA.

gene delivery on the hallmark astrocytosis of MPS IIIA. In the CNS, we observed a significant reduction in GFAP-positive cells and signals throughout the brain and in the gray matter of the spinal cord (Figure 6a). In the PNS, astrocytosis was reduced in the myenteric plexus and submucosal plexus in the intestine (Figure 6b), and in the retina (Figure 6c), in mice treated at or before age 6 months with 5E12 vg/kg scAAV9-hSGSH vector. The effective correction of astrocytosis, a major component of neuroinflammation, in both the CNS and PNS, supports the broad neurological benefits of the scAAV9-hSGSH gene delivery. Importantly, astrocytosis in the CNS could be corrected even at very late stages of the disease (9 months

age at treatment), and persisted to the endpoint (18 months age) (Supplementary Figure S4).

Dose-dependent behavior correction

To assess the functional benefit of the gene delivery treatment, each cohort was tested for behavioral performance in a hidden task in the Morris water maze at 7.5 months age, which is the earliest timepoint that we could readily distinguish between wt and nontreated MPS IIIA mice. Mice treated at 1 month with 5E12 vg/kg, but not the 1E12 vg/kg group, showed significantly improved latency to find a hidden

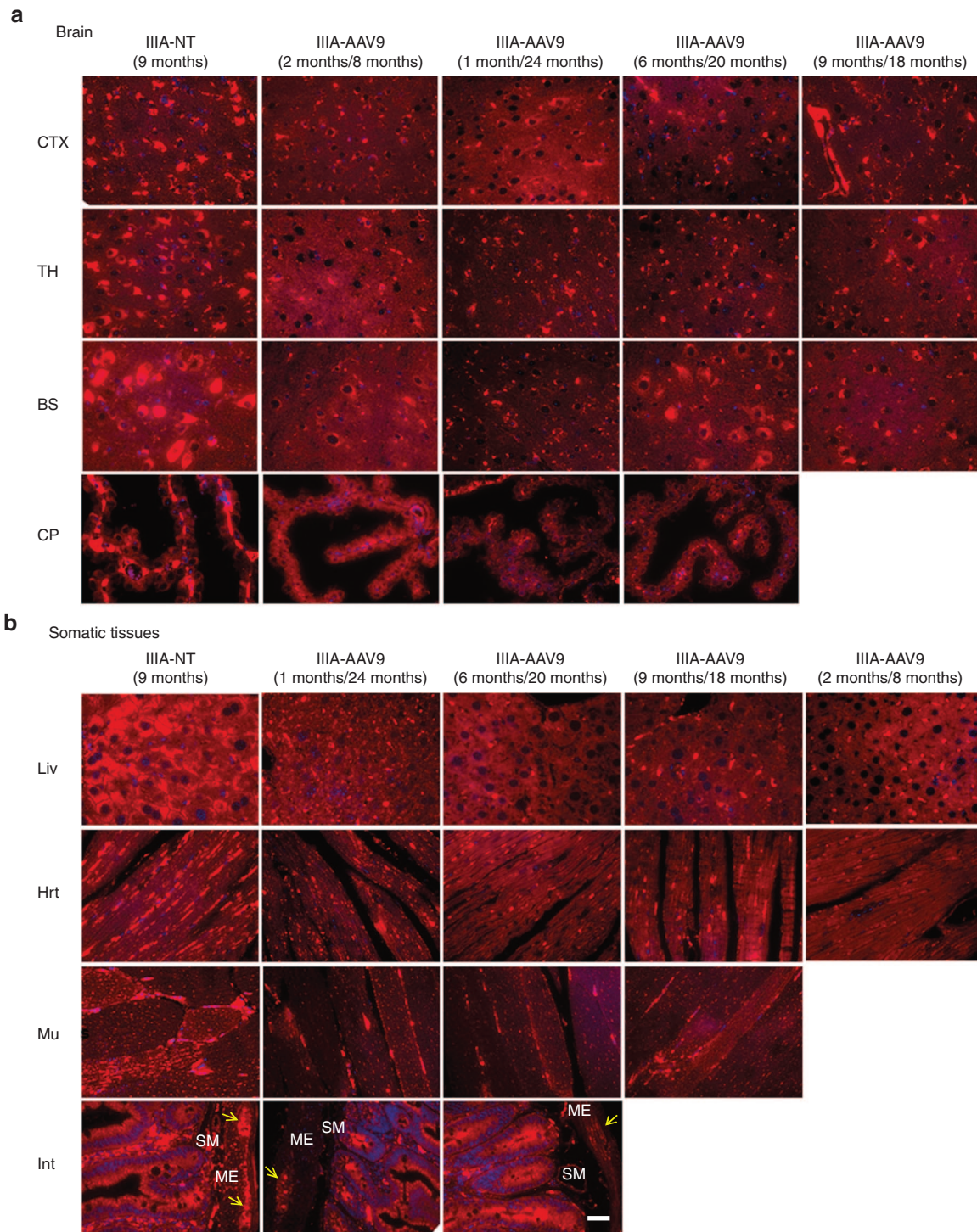


Figure 5 Reduction in lysosomal marker, LAMP1, in vector-treated MPS IIIA mice. MPS IIIA mice were treated with 5E12 vg/kg scAAV9-U1a-hSGSH vector at the indicated ages (1–9 months). Tissues were assayed for LAMP1 by immunofluorescence at either 8 months or endpoint for mice in the longevity study. In each image, treatment mo/termination mo is indicated. Red fluorescence: LAMP1-positive cell and signals; blue fluorescence: DAPI-positive nuclei. IIIA-NT: tissues from nontreated MPS IIIA mice; IIIA-AAV9; tissues from vector treated MPS IIIA mice. **(a)** Brain tissues: CTX: cortex; BS: brainstem; TH: thalamus; and CP: choroid plexus. **(b)** Somatic tissues: Liv: liver; Hrt: heart; Mu: skeletal muscle; Int: intestine; SM, submucosa; ME, muscularis externa; yellow arrows, neurons of the myenteric plexus. Scale bar: 50 μ m.

platform over 4 days of testing in pairwise comparison to nontreated MPS IIIA, though this difference did not reach statistical significance after correction for group comparisons (see Materials and Methods). (Figure 7a). Swimming speeds recorded in the Morris water maze showed statistically significant improvement over untreated mice,

and comparable to WT in the high-dose group after correction for group comparisons. Together, the data suggested a dose-dependent functional impact of systemic scAAV9-hSGSH gene delivery.

To determine the functional effects of treatment later in disease progression, the MPS IIIA mice treated at different ages with 5E12

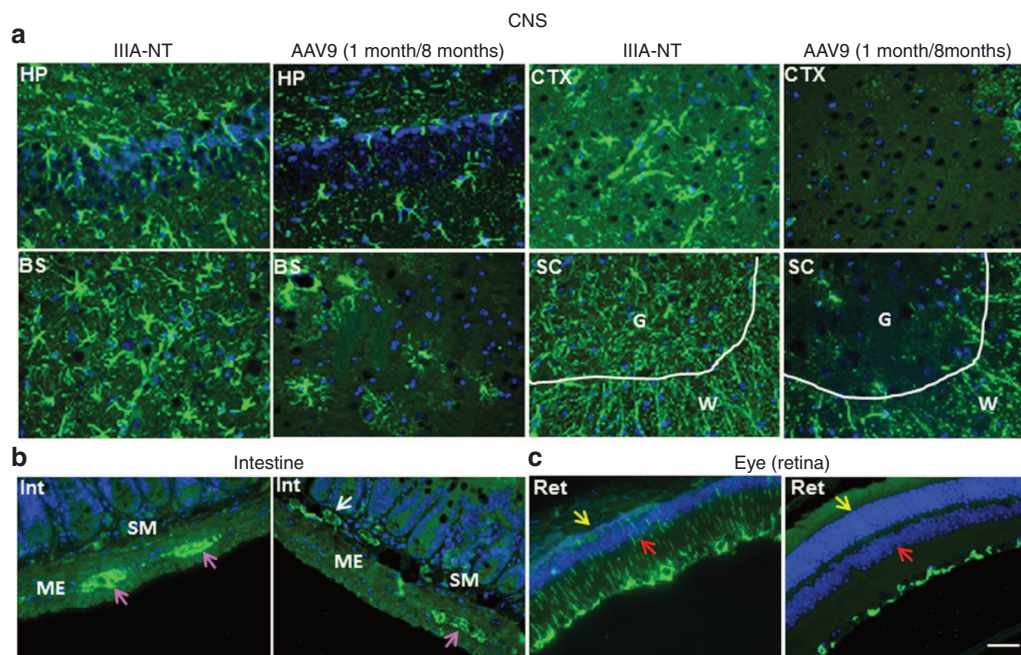


Figure 6 Correction of astrocytosis in vector-treated MPS IIIA mice. MPS IIIA mice were treated at 1 month age with an intravenous injection of 5E12 vg/kg scAAV9-U1a-hSGSH vector and tissues were assayed for glial fibrillary acidic protein (GFAP) (astrocytic marker) by immunofluorescence at age 8 months. Green fluorescence: GFAP-positive cells and signals; blue fluorescence: DAPI-positive nuclei. IIIA-NT: tissues from nontreated MPS IIIA mice; AAV9: tissues from vector-treated MPS IIIA mice. (a) CNS tissues: HP: hippocampus; BS: brain stem; CTX: cortex; and SC: spinal cord. G: Gray matter; W: white matter. (b) Intestine: SM: submucosa; ME: muscularis externa; pink arrows: neurons of myenteric plexus; white arrows: neurons in submucosal plexus. (c) Eye (retina): GFAP-positive cell processes associated with retina. Yellow arrows: outer nuclear layer; red arrows: inner nuclear layers. Scale bar: 50 μ m.

vg/kg were tested for behavioral performance at 7.5 months age (excluding those treated at 9 months). Mice treated at ages 1, 2, or 3 months all showed significant improvement in latency to find a hidden platform in the Morris water maze (Figure 7b) compared to untreated MPS IIIA mice in pairwise comparisons, though statistical significance was not reached after correction for group comparisons. Swimming speeds in the treated groups were statistically indistinguishable from WT after correction for group comparison, and different from untreated mice. In contrast, there was no suggestion of performance improvement in mice treated at 6 months age and tested at 7.5 months compared to untreated mice (Figure 7b). However, when a subset of these mice (6 of the original 10), along with small cohorts of age-matched wt ($n = 4$) and untreated MPS IIIA ($n = 3$) mice, were retested at 12 months age, latency to the hidden task was significantly improved compared to untreated mice after correction for group comparison (Figure 7c). These mice also had significantly faster swimming speed than untreated mice and were comparable to WT mice after correction for group comparison. The mice treated at age 9 months were not tested for behavior due to their poor condition. These data indicate that scAAV9-hSGSH gene delivery prevents the development of disease with treatment at or before 3 months age in MPS IIIA mice, and slows down progression of the disease in mice treated at 6 months age.

Significantly increased longevity in treated MPS IIIA mice

Groups of mice from each cohort were observed for longevity. The majority of untreated MPS IIIA mice survived 7–17 months (median = 13), while wt littermates lived to 18–31 months (median = 24). The scAAV9-hSGSH treatment resulted in a dose-dependent increase in lifespan in mice treated at 1 month age, with a minor increase in survival in the 1E12 vg/kg group

(median = 14 months), and lifespans similar to wt controls in the 5E12 vg/kg group (median = 22) (Figure 7d). The majority of mice treated at 2 and 3 months of age with 5E12 vg/kg showed significantly improved survival (median = 20), within the normal range for C57BL/6 mice. Mice treated at 5E12 vg/kg at age 6 months had improved lifespans (median = 17 months) compared to untreated MPS IIIA mice, though reduced compared to wild type. However, mice treated at 9 months age showed little improvement in lifespan compared with untreated animals. Group comparisons (see Materials and Methods) suggest that all of the increases in survival were statistically significant ($P < 0.0001$) compared to nontreated mice, including the very small increase in survival in mice treated at 9 months age. However, the lifespans of all of the treatment groups were also statistically shorter than wt ($P < 0.0001$ – 0.0008). These data further support the functional benefits of the systemic scAAV9-hSGSH gene delivery for treating MPS IIIA over a broad range of age, though not at the late stages of disease.

Broad biodistribution of scAAV9 vector genome in the CNS and somatic tissues in MPS IIIA mice following systemic scAAV9-hSGSH gene delivery

Tissues from MPS IIIA mice treated with rAAV9-U1a-hSGSH vector at different ages were analyzed for vector genome copy number by quantitative polymerase chain reaction (qPCR) detection at approximately 4 months age, 8 months, or the endpoint. Figure 8 shows vector genome copies per diploid cellular genome (VG/DG) in tissues from individual animals. High vector copy numbers were detected in the liver, correlating to high levels of SGSH expression, as was the case in most tissues. Although there was a great deal of variation between individuals and time points, we did not observe general treatment-age-related trends in vector copy number in any

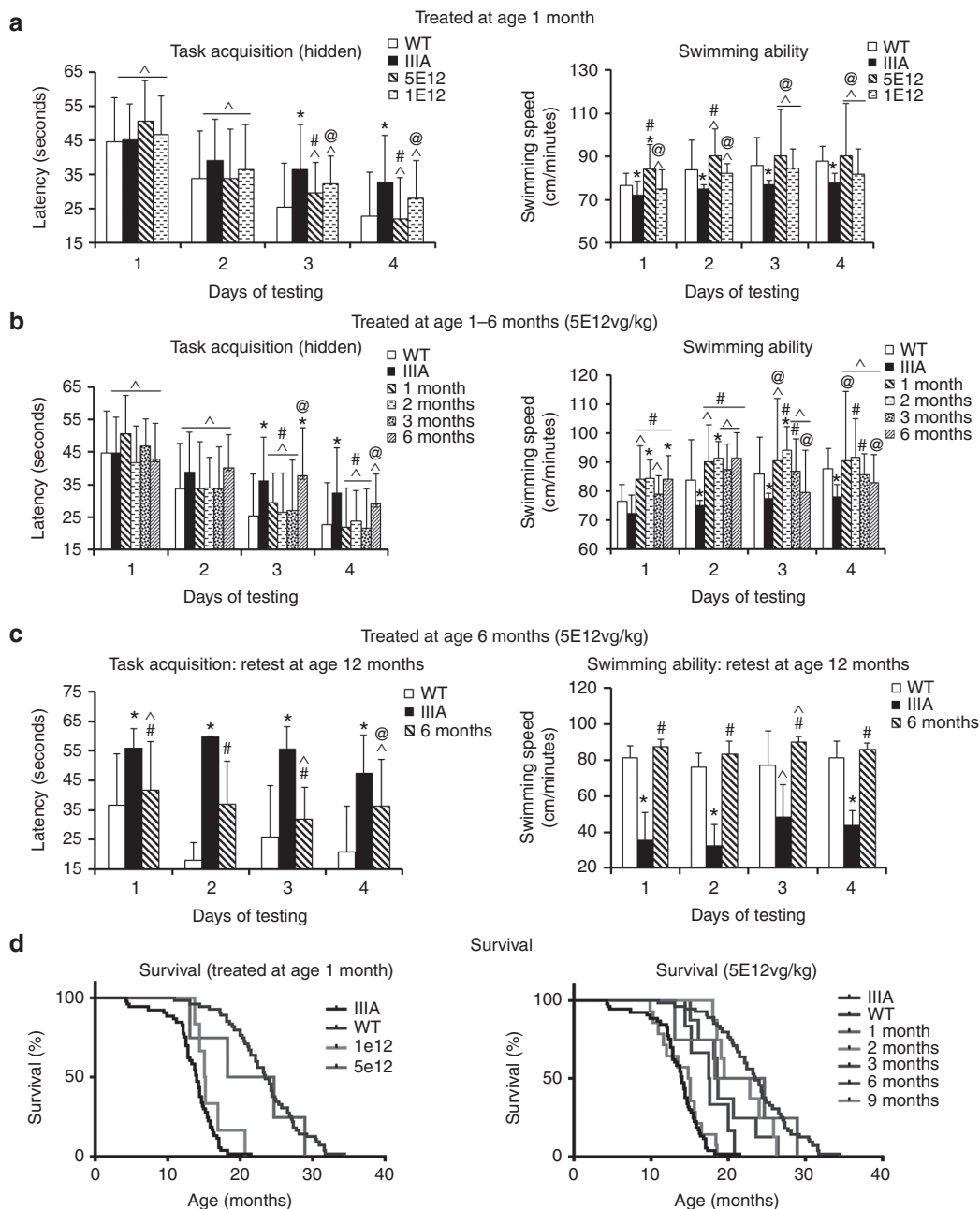


Figure 7 Improvement in behavior performance and survival in vector-treated MPS IIIA mice. MPS IIIA mice treated at 1 month age with 1E12 or 5E12 vg/kg scAAV9-U1a-hSGSH vector (**a**) or at different ages with 5E12 vg/kg vector (**b**), were evaluated for behavior performance in the Morris water maze at age 7–7.5 months. Data presented are latency to find a hidden platform (left chart) and swimming speed (right chart) over 4 days of testing. WT: wild-type mice ($n = 76$); IIIA: nontreated MPS IIIA mice ($n = 34$). (**a**) 5E12: $n = 8$; 1E12: $n = 10$. (**b**) Comparison of MPS IIIA mice treated at ages 1 ($n = 8$), 2 ($n = 12$), 3 ($n = 12$), and 6 ($n = 10$) months. (**c**) MPS IIIA mice treated at 6 months age, wild type, and untreated MPS IIIA mice, were retested at age 12 months. The following symbols indicate results of pairwise T -tests without correction for group comparisons: * $P < 0.05$ versus wt; # $P < 0.05$ versus IIIA; ^ $P > 0.05$ versus wt; @ $P > 0.05$ versus IIIA. (**d**) Kaplan-Meier graphs comparing longevity of wild-type and untreated MPS IIIA mice compared to mice treated with 1E12 or 5E12 vg/kg (left) or treated at different ages (1, 2, 3, 6, and 9 months) with 5E12 vg/kg (right).

tissue. This suggests that MPS IIIA disease progression does not specifically interfere with the ability to deliver vector to the tested tissues. There was also very little evidence for loss of vector genomes over time, with the possible exception of the spleen, wherein mice treated at the earliest age retained fewer copies than mice treated at later ages. Importantly, in mice treated at 9 months of age, vector copy numbers in the brain were similar to mice treated at earlier ages, although only very low levels of SGSH enzyme activity were detected in the brains of these animals. In combination with the

observed decrease in GAG levels noted above, this suggests that the vector may have been expressed transiently at low levels in the CNS tissue when administered at 9 months of age.

DISCUSSION

We demonstrate here the rapid therapeutic effects of a systemic scAAV9-U1a-hSGSH gene delivery. Within 10 days of treatment, an IV injection of the vector resulted in effective restoration of SGSH enzyme activity and clearance of stored GAG throughout the CNS

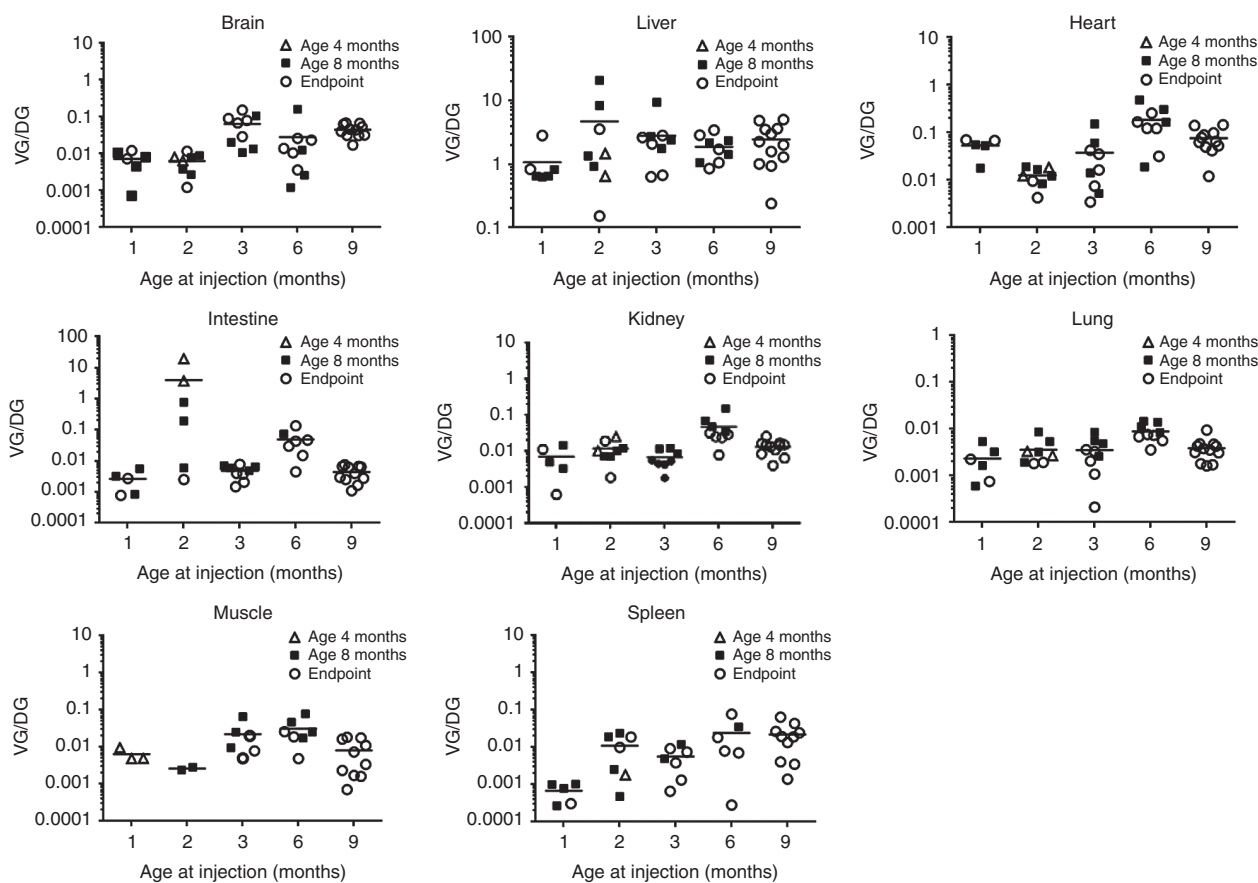


Figure 8 Persistence of sCAAV9-U1a-N-sulfoglucosamine sulfohydrolase vector genomes in MPS IIIA mice treated at different ages. At ages 4 months, 8 months, and the humane endpoint for mice in the longevity study, total DNA was extracted from the indicated tissues and assayed by quantitative polymerase chain reaction for vector and cellular genome copy numbers. Data are expressed as vector genome per diploid genome (VG/DG) from individual animals.

and in broad somatic tissues in two month old MPS IIIA mice, a point at which significant CNS histopathology has already developed. This rapid effect in already diseased tissues supports the potential of this approach for treating MPS IIIA, which may halt or slow down disease progression.

Given that the majority of patients with MPS IIIA are diagnosed when observable neurological disorders have already taken place, a critical issue in therapeutic development is the reversibility of the disease. In this study, we demonstrated that the therapeutic impacts of our approach is dose-dependent, and that a systemic sCAAV9-U1a-hSGSH delivery at an effective dose can not only slow down, but also halt the progression of MPS IIIA, depending on the age of treatment. Our minimally efficacious vector dose was 5E12 vg/kg for sCAAV9-U1a-hSGSH delivered via the systemic route. An intravenous injection of 5E12 vg/kg sCAAV9-U1a-hSGSH in 1-month-old MPS IIIA mice led to SGSH expression at approximately 15–20% of wt levels in the brain, and at supranormal or near normal levels in the liver and other somatic tissues, leading to the clearance of lysosomal GAG storage, correction of cognitive and motor functions, and extended survival, mostly within the normal range of lifespan. Furthermore, treating MPS IIIA mice at either 2 or 3 months of age with 5E12 vg/kg vector was nearly as effective as treatment at age 1 month in terms of rSGSH expression, clearance of GAGs, and functional benefits. However, treatment at 6 months at the same dose was only partially corrective, with a moderate increase in median survival from 13 to 17 months, and modest gains in cognitive function and swimming ability in a water maze,

when tested at 7–7.5 months of age (1 month postinjection), suggesting a rapid functional benefit. Importantly, when mice from the same group were retested at age 12 months, their cognitive performance had not deteriorated greatly from the 7.5-month time point, and was significantly better than a small cohort of untreated 12-month-old MPS IIIA mice that were retested and shows significant deterioration. Notably, the mice treated at age 6 months showed swimming abilities similar to that of wt mice when tested at 12 months age, suggesting that the persistent deficit in latency to the hidden platform was due to reduced cognitive function rather than motor deficiency. Whether further correction of cognitive function and longevity could be achieved with a higher dose of vector at the 6-month time point is yet to be determined. In contrast, treating MPS IIIA mice at age 9 months provided only minimal functional benefit. Notably, this is an age by which time significant neuropathology has taken place, and some of the untreated MPS IIIA mice have either died or reached the humane endpoint. Interestingly, while tissue rSGSH expression levels in mice treated at age 9 months were significantly lower than earlier treatment groups when tested at the endpoint, there was no clear correlation between tissue rSGSH levels and vector genome copies. However, tissue GAG contents were reduced to levels comparable to those in mice treated at earlier stages, suggesting effective correction and reversal of GAG storage in both the CNS and somatic tissues, despite the age at vector delivery and the variation in tissue rSGSH levels. This suggests that a systemic delivery of sCAAV9-U1a-hSGSH at 5E12 vg/kg can mediate the expression of functional rSGSH at levels

sufficient to effectively clear CNS and somatic GAG storage, regardless of the disease stage. However, the lack of functional impact in MPS IIIA mice receiving vector treatment in older animals suggest that the neuropathologies at late stages were more severe, leading to damage beyond lysosomal GAG storage, and profoundly compromised pathophysiological status.

While achieving effective global CNS and widespread somatic transduction as expected, this study demonstrates the extended neurological potential of systemic scAAV9-U1a-hSGSH gene delivery for the treatment of MPS IIIA. Transduction of the PNS was evident in MPS IIIA mice treated at any age, with rSGSH expression predominantly in enteric neurons that led to the clearance of lysosomal storage pathology and the correction of gliosis in the enteric nervous system. Importantly, we observed effective therapeutic impacts on the optic nervous system (ONS) in the vector-treated MPS IIIA mice. The IV vector treatment resulted in the resolution of Mueller glia cell activation and maintenance of the integrity of nuclear layers in the retina, indicating the correction of gliosis and neurodegeneration in the eye. This is clinically relevant because significant retinopathies, including retinal degeneration, are common in both MPS III patients and mouse models.^{20–22} These observations highlight the potential for added, therapeutically meaningful, benefits from the systemic gene delivery approach in this disease, where clinically significant somatic manifestations are normally overshadowed by the severity of the CNS neurodegeneration.

In summary, the trans-blood–brain barrier neurotropic properties of the self-complementary rAAV9 vector have made possible an effective gene therapy approach in the MPS IIIA mouse model. The results reinforce the view that effective CNS delivery can treat the global CNS neuropathologies associated with this disease. However, the widespread correction of lysosomal storage in somatic tissues and organs, and in the PNS, promise additional therapeutic benefits from the systemic delivery approach, which would be important in patient populations even when the CNS neurological manifestations have been adequately addressed.

MATERIALS AND METHODS

Animals

Spontaneous MPS IIIA mutant founder mice (B6.Cg-Sgsh^{m^{mps3a}})²³ were kindly provided by Dr. S. U. Walkley (Albert Einstein College of Medicine), and maintained on an inbred background (C57BL/6) of backcrosses from heterozygotes in the vivarium at NCH-RI. The genotypes of progeny mice were identified by PCR with restriction digest.²³ The MPS IIIA mice retain approximately 3% residual SGSH activity, and exhibit phenotypes corresponding to human disease in virtually all aspects, with characteristic lysosomal storage pathology in cells of virtually all organs, progressive neurological disorders, relatively mild somatic manifestations, and a significantly shortened lifespan.

All animal care and procedures were performed strictly following the approved protocol, in accordance with the Guide for the Care and Use of Laboratory Animals (8th Edition, 2011). MPS IIIA mice and their age-matched wildtype (wt) littermates were used in the experiments.

Recombinant AAV viral vector

An scAAV vector plasmid was constructed to produce scAAV9-U1a-hSGSH viral vector (Supplementary Figure S1). The vector genome contains minimal elements for transgene expression, including AAV2 terminal repeats, a murine small nuclear RNA promoter U1a,¹⁹ human SGSH (hSGSH) coding sequence and polyadenylation signal from SV40. The viral vectors were produced in HEK293 cells using three-plasmid cotransfection including AAV helper plasmid encoding AAV serotype 9 capsid. Vector was purified by banding on a CsCl step gradient followed by a CsCl continuous gradient, and dialysis into Tris-buffered saline (pH8.0). The titer of the vector was determined using dot-blot hybridization using the SGSH coding sequence as probe and serially diluted linearized AAV-U1a-SGSH plasmid as standard.

Systemic vector delivery

MPS IIIA mice were treated at different ages or at different doses with an intravenous injection of scAAV9-U1a-hSGSH vector (in 150–200 μ l Tris-buffered saline) via tail vein. The animals were briefly anesthetized by isoflurane inhalation for vector injection accuracy. Age and sex-matched saline-injected wt and MPS IIIA littermates were used as controls ($n = 15$ /group).

Behavioral tests: hidden task in the Morris water maze.

The scAAV9-U1a-hSGSH-treated MPS IIIA mice and controls were tested for behavioral performance at approximately 7–7.5 months of age and/or age 12 months in a hidden task in Morris water maze.²⁴ The water maze consisted of a large circular pool (diameter = 122 cm) filled with water (45 cm deep, 24–26 °C) containing 1% white TEMPERA paint, located in a room with numerous visual cues. Mice were tested for their ability to find a hidden escape platform (20 \times 20 cm) 0.5 cm under the water surface. Each animal was given four trials per day, across 4 days. For each trial, the mouse was placed in the pool at one of four randomly ordered locations, and then given 60 seconds to swim to the hidden platform. If the mouse found the platform, the trial ended, and the animal was allowed to remain 10 seconds on the platform before the next trial began. If the platform was not found, the mouse was placed on the platform for 10 seconds, and then given the next trial. Measures were taken of latency to find the platform, swimming distance (cm), and swimming speed (cm/min) through an automated tracking system (San Diego Instruments, San Diego, CA).

Longevity observation

Following the scAAV9-U1a-hSGSH vector injection, mice were continuously observed for the development of humane endpoint criteria, or until death occurred. The endpoint was when the symptoms of late stage MPS IIIA clinical manifestation (urine retention, rectal prolapse, protruding penis) became irreversible, or when mice showed significant weight loss, dehydration or morbidity.

Tissue analyses

Necropsies were carried out when mice were 8–8.5 months old and at the endpoint. Brain and multiple somatic tissues were collected, and stored either at –80 °C or in 4% paraformaldehyde (in phosphate buffered saline, pH 7.2) before being processed for analyses.

SGSH activity assay

Tissue samples were assayed for SGSH enzyme activity following previously published procedures.²⁵ The assay measures 4-methylumbelliferone (4MU), a fluorescent product formed by 2-step hydrolysis of the substrate 4-Methylumbelliferyl- α -N-sulpho-D-glucosaminide. The sample SGSH activity desulfates the substrate over a 17-hour reaction at 37 °C, followed by a secondary reaction with excess α -glucosidase, releasing the fluorescent product, 4MU over 24 hours at 37 °C. The SGSH activity is expressed as unit/mg protein. One unit is equal to 1 nmol 4MU released/17 hours.

GAG content measurement

GAGs were extracted from wet tissues following published procedures with modification.^{26,27} Dimethylmethylene blue assay was used to measure GAG content.²⁸ The GAG samples (from 0.5 to 1.0 mg tissue) were mixed with H₂O to 40 μ l before adding 35 nmol/l Dimethylmethylene blue (Polysciences, Warrington, PA) in 0.2 mmol/l sodium formate buffer (pH 3.5). The product was measured using a spectrophotometer (OD₅₃₅). The GAG content was expressed as μ g/mg tissue.

Immunofluorescence

Tissues were processed for thin paraffin sections (4 μ m) and immunofluorescence. The immunofluorescence staining was performed to identify cells expressing rSGSH, GFAP for astrocytes, or lysosomal-associated membrane protein 1 (LAMP1), using antibodies against hSGSH (Abcam, Cambridge, UK), GFAP (Millipore, Billerica, MA) or LAMP1 (Abcam), and corresponding secondary antibody conjugated with AlexaFluor⁵⁶⁸ or AlexaFluor⁴⁸⁸ (Invitrogen, Waltham, MA), following procedures recommended by the manufacturers. The sections were imaged under a fluorescence microscope.

Quantitative real-time PCR (qPCR)

Total DNA was isolated from tissue samples of scAAV9-treated and non-treated mice using Qiagen DNeasy columns. The DNA samples were analyzed by qPCR, using Absolute Blue QPCR Mix (Thermo Scientific, Waltham, MA) and Applied Biosystems 7000 Real-Time PCR System, following the procedures recommended by the manufacturer. Taqman primers specific for hSGSH were used to detect rAAV vector genomes: forward: AAGTCAGCGAGGCTACGT; reverse: GATGGTCTTCGAGCCAAAGAT; probe: (6-FAM) CCTCTAGACCTCAGCCACC (TAMRA). Genomic DNA was quantified in parallel samples using β -actin-specific primers: forward: GTCATCACTAT TGGCAACGA; reverse: CTCAGGAGTTTTGTCACCTT; probe: (6-FAM)TTCCGATG CCCTGAGGCTCT(TAMRA). Genomic DNA from tissues of nontreated mice was used as control for background levels and absence of contamination.

Histopathology

Brain and multiple somatic tissues were processed to produce thin paraffin sections (4 μ m) and then stained with hematoxylin and eosin (H&E) by the Morphology Core in the Research Institute at Nationwide Children's Hospital. The sections were examined by a board-certified veterinary pathologist from the Comparative Pathology and Mouse Phenotyping Shared Resource at The Ohio State University.

Statistics

All analyses were performed using SAS 9.2 (SAS Institute, Cary, NC), with two-sided *P* values considered statistically significant. For SGSH activity and GAG content, a natural log transformation was used on the values in order to normalize their distribution. For all analyses except survival, group comparisons were assessed using repeated measures analysis of variance. If a significant time-group or location-group interaction was found, an overall *F*-test at each location (or time) was then performed to determine at which locations significant group differences would be found. For each significant location (or time), comparisons between mice groups were then assessed, with a Bonferroni-Holm correction to account for multiple comparisons.

For survival analysis, log-rank tests were used to compare survival times between groups, after verifying the proportional hazards assumption was met.

CONFLICT OF INTEREST

Both H.F. and D.M.M. are scientific co-founders and shareholders of Abeona Therapeutics (ABEO), a publicly traded company licensed to develop gene therapy for MPS IIIA and MPS IIIB.

ACKNOWLEDGMENTS

We thank Steve U. Walkley at Albert Einstein College of Medicine for kindly providing us the MPS IIIA mouse model, and Brian Winchester and Derek Burke of Great Ormond Street Hospital for Children (UK) for sharing the protocol of SGSH activity assay. We acknowledge the contribution of the NCH Biostatistics Core for analyses of experimental data. This study was sponsored by NIH/NINDS (R21NS081173) and Sanfilippo community through A Life for Elisa - Sanfilippo Children's Research Foundation (Canada), Ben's Dream - Sanfilippo Research Foundation, Team Sanfilippo, and LivLife.

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