A Single Injection of an Optimized Adeno-Associated Viral Vector into Cerebrospinal Fluid Corrects Neurological Disease in a Murine Model of GM1 Gangliosidosis

Christian Hinderer, Brenden Nosratbakhsh, Nathan Katz, and James M. Wilson*

Gene Therapy Program, Department of Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania, USA.

GM1 gangliosidosis is a rare neurodegenerative lysosomal storage disease caused by loss-of-function mutations in the gene encoding beta-galactosidase (β-gal). There are no approved treatments for GM1 gangliosidosis. Previous studies in animal models have demonstrated that adeno-associated viral (AAV) vector-mediated gene transfer to the brain can restore β-gal expression and prevent the onset of neurological signs. We developed an optimized AAV vector expressing human β-gal and evaluated the efficacy of a single intracerebroventricular injection of this vector into the cerebrospinal fluid (CSF) of a murine disease model. The AAV vector administration into the CSF increased β-gal activity in the brain, reduced neuronal lysosomal storage lesions, prevented the onset of neurological signs and gait abnormalities, and increased survival. These findings demonstrate the potential therapeutic activity of this vector and support its subsequent development for the treatment of GM1 gangliosidosis.

Keywords: GM1 gangliosidosis, AAV, CSF, lysosomal storage disease

INTRODUCTION

GM1 gangliosidosis is a rare genetic disorder caused by mutations in the GLB1 gene, which encodes lysosomal beta-galactosidase (β-gal). In the absence of β-gal, cells are unable to catabolize polysaccharides with terminal galactose residues. Keratan sulfate, a galactose-containing glycosaminoglycan, accumulates in a variety of tissues, causing skeletal dysplasia, hepatosplenomegaly, and cardiomyopathy. In the central nervous system (CNS), the inability to catabolize GM1 ganglioside results in marked neuronal GM1 storage and subsequent neurodegeneration.

The clinical presentation of GM1 gangliosidosis varies dramatically between patients depending on the underlying GLB1 mutations. Patients expressing residual β-gal may present with neurological signs at any age and variable degrees of somatic disease. In rare cases, patients present with peripheral manifestations of β-gal deficiency without CNS involvement, a disease termed mucopolysaccharidosis type IVB.

The most common form of GM1 gangliosidosis is the infantile subtype, which occurs in patients with little or no residual β-gal expression. Infantile GM1 gangliosidosis is characterized by a uniformly rapid neurodegenerative course beginning in the first 6 months of life, with a survival of less than 5 years.1,3

Previous studies in murine and feline models of GM1 gangliosidosis demonstrated that adeno-associated viral (AAV) vectors can be used to deliver the normal GLB1 coding sequence to neurons, resulting in long-term expression of the β-gal enzyme. β-gal is secreted by transduced cells and can be taken up by surrounding cells via binding to the mannose-6-phosphate receptor, resulting in widespread distribution of the enzyme. This phenomenon allows for the correction of storage lesions throughout the CNS after a small number of vector injections into the brain parenchyma.

Studies in canine and feline models of other lysosomal storage diseases have shown that gene transfer to the brain and spinal cord can alternatively be achieved by using AAV vector delivery into the cerebrospinal fluid (CSF). This approach allows for more widespread gene transfer with a single, minimally invasive injection and appears to carry less risk of local toxicity at the injection site. The AAV delivery into the CSF achieves efficient gene

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transfer to the brain and spinal cord even in the presence of systemic neutralizing antibodies to the vector capsid, making this approach applicable to patients regardless of the presence of pre-existing AAV antibodies.\(^{9,11,12,14}\)

The aim of this study was to develop an optimized AAV vector expressing human \(\beta\)-gal and evaluate the impact of vector administration into the CSF on brain enzyme activity, lysosomal storage lesions, and neurological signs and survival by using a murine disease model.

**MATERIALS AND METHODS**

**Animal procedures**

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. \(Glb1\) knockout mice (\(Glb1^{+/+}\); RBRC00690) were obtained from RIKEN BioResource Research Center. Mice were maintained as heterozygous carriers \(Glb1^{+/-}\) in a C57BL/6J background. Mice were genotyped by PCR from tail snips at 3 weeks of age. For intracerebroventricular (ICV) injections, vectors were diluted in artificial CSF (1 mM phosphate, pH 7.2, 150 mM NaCl, 3 mM KCl, 1.4 mM CaCl\(_2\), 0.8 mM MgCl\(_2\), 0.001% Pluronic F-68.) to a volume of 5 \(\mu\)L, and injections were administered freehand on isoflurane-anesthetized mice by using a custom gastight syringe (Hamilton) and a cemented 10 mm 27 gauge needle, with plastic tubing attached to the needle base to limit penetration to a depth of 3 mm. The ICV injections were performed by a board-certified laboratory animal veterinarian. The ICV injection proficiency was verified based on 100% successful lateral ventricle delivery of a reference compound in 100 sequential injections.

Based on the historical rate of successful ICV delivery, the expected frequency of vector delivery outside of the ventricle in this study is <1%. Submandibular blood collection was performed on isoflurane-anesthetized mice. Blood was collected in serum separator tubes, allowed to clot, and separated by centrifugation before aliquoting and freezing at \(-80^\circ\)C. Enzyme activity assays

**Enzyme activity assays**

Tissues were homogenized in 0.9% NaCl, pH 4.0 by using a steel bead homogenizer (TissueLyzer; Qiagen). After three freeze-thaw cycles, samples were clarified by centrifugation and protein content was quantified by a bicinchoninic acid assay. Serum samples were used directly for enzyme assays. For the \(\beta\)-gal activity assay, a 1 \(\mu\)L sample was combined with 99 \(\mu\)L of 0.5 mM 4-methylumbelliferyl \(\beta\)-D-glucosaminide (M2133; Sigma) as a substrate and 1 \(\mu\)mol 4MU, pH 4.0 by using a steel bead homogenizer (TissueLyzer; Qiagen). After three freeze-thaw cycles, samples were clarified by centrifugation before aliquoting and freezing at \(-80^\circ\)C. Animals were euthanized at the scheduled necropsy time points or if they met one or more of the following euthanasia criteria: \(\geq 20\%\) weight loss from previous weight monitoring, inability to move right from a supine position in less than 15 s, and paralysis of two or more limbs. Determination of the need for euthanasia was made by an evaluator blinded to the treatment group at the time of assessment.

At the time of necropsy, mice were sedated with ketamine and xylazine (intraperitoneal injection, 100/10 mg/kg) and CSF was collected by suboccipital puncture by using a 32 gauge needle connected to polylethylene tubing. Euthanasia was performed by cervical dislocation. CSF, heart, lung, liver, and spleen were immediately frozen on dry ice and stored at \(-80^\circ\)C. Brains were removed, and a coronal slice of the frontal lobe was collected and frozen for biochemical studies. The remaining brain was used for histological analysis.

**Neurological examination**

Neurological assessments were adapted from a previous study of the GM1 mouse model.\(^{18}\) These assessments were selected to reflect neurological signs characteristic of this model. A blinded examiner evaluated nine different parameters: gait, forelimb position, hindlimb position, trunk position, tail position, avoidance response, rolling over, vertical righting reflex, and parachute reflex. Individual test items were assigned one of the following four scores: 0 (normal), 1 (slightly abnormal), 2 (moderately abnormal), and 3 (highly abnormal). Scores for each parameter were added to calculate a total score.

**Vector**

Vectors were constructed from cis-plasmids containing the codon-optimized human GLB1 coding sequence expressed from the chicken beta actin promoter with a cytomegalovirus enhancer (CB7), human elongation initiation factor 1 alpha promoter (EF1a), or human ubiquitin C promoter (UbC) flanked by AAV2 inverted terminal repeats. The vectors were packaged in an AAV serotype hu68 capsid by triple transfection of adherent HEK 293 cells and purified by iodixanol gradient centrifugation as previously described\(^{19}\) for the pilot expression study and by affinity chromatography followed by anion exchange chromatography for the \(Glb1\) knockout mouse study.

**Histology**

Brains were fixed overnight in 4% paraformaldehyde, equilibrated in 15% and 30% sucrose, and finally frozen in optimal cutting temperature embedding medium. Cryosections were stained with antibodies against lysosomal-associated membrane protein 1 (LAMP1) (Cat. No.
Ab24170; Abcam) overnight at 4°C. The next day, slides
were washed and incubated with an anti-rabbit IgG TritC-
conjugated secondary antibody for 1 h at room tempera-
ture. Slides were washed, and coverslips were applied.
LAMP1 staining was quantified as positive cells per field
of the whole cerebral cortex from one coronal brain sec-
tion by using VisioPharm image analysis software.

Gait analysis

Motor function assessments were performed by using
the CatWalk XT gait analysis system (Noldus Information
Technology, Wageningen, The Netherlands). The Cat-
Walk XT tracks the footprints of mice as they walk across
a glass plate. The system quantifies the dimensions of each
paw print and statistically analyzes the animal’s speed and
other features of gait.

To perform this assessment, the Catwalk XT was cali-
brated with the appropriate width of the walkway set be-
fore the start of the test. Animals were brought into the
room and allowed to acclimate in darkness for at least
30 min before running the Catwalk XT. Once acclimation
was complete, an animal was selected and placed at the
entrance of the walkway. The researcher started the ac-
quision software and allowed the animal to walk down
the walkway. The animal’s home cage was placed at the
end of the walkway for encouragement. The run was
complete when the animal had successfully walked to the
end of the catwalk within the allotted time limit; other-
wise, the run was repeated.

Animals ran three trials with a minimum duration of
0.50 s and a maximum duration of 5.00 s. Three successful
runs were needed for the trial to be considered complete.
If an animal failed to complete three runs after 10 min
of testing, only the completed runs were used for analysis.
The analyses were performed by an evaluator blinded to the
animal ID and treatment group. Runs were auto-classified
by using the Catwalk XT software, after which footprints
were checked for accuracy and proper labeling. Any non-
footprint data were manually removed. Average speed,
stride length, and hind footprint length were automatically
measured by the program. Mean values for the left and right
hind paw print lengths were calculated and analyzed for each
group. Mean values for stride length measured from
each paw were calculated and analyzed for each group.

Statistics

Analyses were performed by using Prism 7.0 (GraphPad
Software). Neurological exam scores and gait analysis
parameters (walking speed and hind print length) were
compared between groups at each time point by using a
two-way analysis of variance (ANOVA). Survival curves
were compared between groups by using a log-rank
(Mantel-Cox) test. Brain LAMP1 data were log-transformed
and compared by using a one-way ANOVA followed by
Dunnett’s test.

RESULTS

We selected the AAVhu68 capsid based on previous
studies demonstrating that clade F AAV isolates are ca-
ble of efficient gene transfer to the brain and spinal cord
after delivery into the CSF.13 We designed transgene
cestaets consisting of a codon-optimized human GLB1
cDNA driven by one of three ubiquitous promoters:
chicken beta actin with a cytomegalovirus enhancer (CB7),
elongation factor 1 alpha (EF1z) or ubiquitin C (UbC). We
packaged each cassette in an AAVhu68 capsid, and we
administered a single dose of 10^{11} genome copies (GCs) to
wild-type mice by ICV injection. Two weeks after injection,
we measured β-gal activity in brain and CSF (Supplemen-
tary Fig. S1). Only the vector carrying the UbC promoter
achieved statistically significant elevations in β-gal activity
in both the brain and CSF, with enzyme activity nearly
twofold greater than that of untreated wild-type mice in the
brain, and tenfold greater in CSF. We, therefore, selected
the AAVhu68.UbC.hGLB1 vector for further studies.

We assessed the efficacy of the optimized vector in the
Glb1^{−/−} mouse model. This mouse model of GM1 gang-
liosidosis was developed by targeted deletion of exon 15
of the Glb1 gene.20,21 Similar to infantile GM1 gangliosi-
dosis patients, these mice express no functional β-gal
and exhibit rapid accumulation of GM1 ganglioside in the
brain. Brain GM1 storage is already apparent in the first
weeks of life, and by 3 months of age, Glb1^{−/−} mice have a
similar degree of GM1 accumulation in the brain to that of
an 8-month-old infantile GM1 patient.20

The clinical phenotype of the Glb1^{−/−} mouse most
closely resembles that of infantile GM1 gangliosidosis,
with motor abnormalities appearing by 4 months of age
and severe ataxia or paralysis necessitating euthanasia
presenting by 10 months of age.20,21 The Glb1^{−/−} mouse
model does not exhibit any peripheral organ involve-
ment, unlike infantile GM1 patients who often develop
bone deformities and hepatosplenomegaly.20,21 The
Glb1^{−/−} mouse is, therefore, a representative model of the
neurological features of infantile GM1 gangliosidosis,
but not the systemic disease manifestations.

One-month-old Glb1^{−/−} mice received a single ICV
administration of AAVhu68.UbC.hGLB1 at one of four
doses: 4.4 \times 10^9 GC, 1.3 \times 10^{10} GC, 4.4 \times 10^{10} GC, or
1.3 \times 10^{11} GC. Control Glb1^{−/−} mice and normal Glb1^{−/−}
mice were administered vehicle (artificial CSF) ICV. In-
life assessments included monitoring for survival,
neurological exams, gait analysis, and evaluation of serum
transgene expression (β-gal activity). Necropsies were
performed on the day of dosing (day 1) for untreated
Glb1^{−/−} mice and normal Glb1^{−/−} mice to evaluate the
severity of baseline brain storage lesions. Vehicle- and
vector-treated mice were necropsied on day 150 and 300.

In the day 150 cohort, all mice survived to the sched-
uled necropsy except one vehicle-treated Glb1^{−/−} mouse
(Fig. 1). This animal died 2 days after vehicle adminis-
Gait analysis evaluated the stride length and hind paw print length of vehicle- and vector-treated mice at baseline (day 0) and every 60 days through day 240. Gait analysis revealed progressive abnormalities in vehicle-treated Glb1−/− mice, whereas Glb1−/− mice treated with the two highest vector doses (1.3 × 10¹¹ GC and 4.4 × 10¹⁰ GC) demonstrated consistent improvements in both gait parameters.

At baseline, the average stride length (Fig. 2A) of vehicle-treated Glb1−/− mice was significantly shorter than that of normal Glb1+/+ controls, and this abnormality persisted through day 240. The stride length abnormality was partially rescued in vector-treated Glb1−/− mice at all doses by day 120. However, by day 240, only the two highest dose groups (1.3 × 10¹¹ GC and 4.4 × 10¹⁰ GC) maintained a significantly longer average stride length compared with that of the vehicle-treated Glb1−/− mice.

On day 60, the average hind paw print length (Fig. 2B) of vehicle-treated Glb1−/− mice was significantly longer than that of normal Glb1+/+ controls, and this abnormality persisted through day 240. The hind paw print length abnormality was partially rescued by vector administration in Glb1−/− mice at the three highest doses (1.3 × 10¹¹ GC, 4.4 × 10¹⁰ GC, and 1.3 × 10¹⁰ GC), resulting in a statistically significant decrease in average hind paw print length compared with that of the vehicle-treated Glb1−/− mice by day 240.

A standardized neurological examination was performed in a blinded fashion every 60 days through day 240 (Fig. 2C). Beginning at the day 120 assessment, Glb1−/− mice administered either vehicle or the lowest dose of vector (4.4 × 10⁹ GC) exhibited progressively higher total severity scores, which was indicative of increasing severity of neurological signs. However, the total severity scores of the Glb1−/− mice administered the lowest dose were significantly lower than those of vehicle-treated Glb1−/− mice, suggesting that this dose (4.4 × 10⁹ GC) partially rescued the neurological phenotype. At the next highest dose (1.3 × 10¹⁰ GC), minimal abnormalities were detectable in 7 out of 12 (58.3%) animals at the day 240 assessment, suggesting substantial rescue of the neurological phenotype. At the two highest vector doses (1.3 × 10¹¹ GC and 4.4 × 10¹⁰ GC), neurological abnormalities were not apparent, and total severity scores for these groups were similar to those of the normal vehicle-treated Glb1+/+ controls at each time point, suggesting complete rescue of the neurological phenotype.

To evaluate the extent of lysosomal storage lesions, brain sections were stained for the lysosomal membrane protein LAMP1, and cortical cells positive for LAMP1 (i.e., cells exhibiting lysosomal distention) were quantified in scanned sections by using an automated program. Untreated Glb1−/− baseline control mice necropsied on day 1 exhibited a higher proportion of LAMP1-positive cells in the brain compared with that of normal untreated Glb1+/+ baseline controls. At both day 150 and 300, vector-treated animals exhibited a dose-dependent reduction in the proportion of LAMP1-positive cells compared with that of vehicle-treated Glb1−/− controls (Fig. 3). Of note, the two highest vector doses (1.3 × 10¹¹ GC and 4.4 × 10¹⁰ GC) reduced the proportion of LAMP1-positive cells to levels similar to those of normal vehicle-treated Glb1+/+ controls. Correction of lysosomal storage was apparent throughout
the coronal brain sections, reflecting treatment of both the superficial and deep brain structures.

β-gal activity was measured in serum on the day of dosing (day 1) and every 60 days thereafter until day 240 (Fig. 4). At necropsy, β-gal activity was measured in the brain and peripheral organs (heart, liver, spleen, lung, and kidney). In serum, average β-gal activity in Glb1−/− mice administered the highest dose of vector (1.3 × 10^{11} GC) was ∼ 10-fold greater than that of normal vehicle-treated Glb1+/− controls. At the second highest dose (4.4 × 10^{10} GC), serum β-gal activity in Glb1−/− mice was similar to that of normal vehicle-treated Glb1+/− controls. Serum β-gal activity in Glb1−/− mice for all other vector doses was similar to that of vehicle-treated Glb1+/− controls (Fig. 4).

For each tissue type examined, average β-gal activity levels within each group were similar at both time points (day 150 and 300) (Fig. 5). In the brain, β-gal activity increased in a dose-dependent manner in vector-treated Glb1−/− mice. Average β-gal activity for all dose groups was higher than that of the vehicle-treated Glb1−/−.
controls. However, only the two highest dose groups (1.3×10^{11} GC and 4.4×10^{10} GC) exhibited higher average β-gal activity than that of the normal vehicle-treated Glb1^{+/−} controls at both time points. Some peripheral organs (e.g., liver and spleen) but not all (e.g., lung and kidney) exhibited increases in β-gal activity after vector administration (Fig. 5). Of particular note, the heart displayed dose-dependent increases in β-gal activity after vector administration (Fig. 5). Of particular note, the heart displayed dose-dependent increases in β-gal activity, resulting in average levels higher than those of vehicle-treated Glb1^{+/−} mice at all doses. However, only the two highest doses (1.3×10^{11} GC and 4.4×10^{10} GC) restored β-gal activity to levels similar to or higher than those of normal vehicle-treated Glb1^{+/−} controls at both time points.

β-gal activity was measured in the CSF of all animals in the day 300 cohort that survived to the scheduled necropsy. Because none of the vehicle-treated Glb1^{−/−} animals survived to day 300 due to disease progression, brains were collected at the time of euthanasia, and data are presented as part of the day 300 cohort. Significance was evaluated by a one-way ANOVA of log-transformed data followed by Dunnett’s test. Error bars represent the SEM. LAMP1, lysosomal-associated membrane protein 1.

Figure 3. Lysosomal storage lesions in the brain of Glb1^{−/−} mice treated with vector or vehicle. On days 150±7 and 300±7, brains were collected from Glb1^{−/−} (KO) mice ICV-administered AAVhu68.UbC.hGLB1 at a dose of 1.3×10^{11} GC, 4.4×10^{10} GC, 1.3×10^{10} GC, or 4.4×10^{9} GC (N=12/group) and from Glb1^{+/−} (K0) and Glb1^{+/−} (HET) controls treated with ICV-administered vehicle (N=12/group). On day 1 (baseline cohort), brains were also collected from untreated Glb1^{−/−} (KO) and Glb1^{+/−} (HET) controls. Coronal brain sections were stained with an antibody against murine LAMP1. Automated quantification of LAMP1-positive cells in the entire cortex of one coronal brain section per animal was performed. For animals that did not survive to the scheduled day 300 necropsy due to disease progression, brains were collected at the time of euthanasia, and data are presented as part of the day 300 cohort. Significance was evaluated by a one-way ANOVA of log-transformed data followed by Dunnett’s test. Error bars represent the SEM. LAMP1, lysosomal-associated membrane protein 1.

Figure 4. Beta-galactosidase activity in serum of Glb1^{−/−} mice treated with vector or vehicle. Glb1^{−/−} (KO) mice were ICV-administered AAVhu68.UbC.hGLB1 at a dose of 1.3×10^{11} GC, 4.4×10^{10} GC, 1.3×10^{10} GC, or 4.4×10^{9} GC (N=12/group). Glb1^{+/−} (KO) and Glb1^{+/−} (HET) mice were ICV-administered vehicle as controls (N=12/group). Serum was collected on the day of dosing (day 1) and on days 60±7, 120±7, 180±7, and 240±7. Serum β-gal activity was measured by using a fluorogenic substrate. Data for the day 150 and 300 cohorts are combined by treatment and genotype. Error bars represent the SEM. β-gal, beta-galactosidase.
Figure 5. β-gal activity in tissues of Glb1<sup>−/−</sup> mice treated with vector or vehicle. Glb1<sup>−/−</sup> (KO) mice were ICV-administered AAVhu68.UbC.hGLB1 at a dose of 1.3 × 10<sup>11</sup> GC, 4.4 × 10<sup>10</sup> GC, 1.3 × 10<sup>10</sup> GC, or 4.4 × 10<sup>9</sup> GC (N=12/group). Glb1<sup>−/−</sup> (KO) and Glb1<sup>+/−</sup> (HET) mice were ICV-administered vehicle as controls (N=12/group). Brain, heart, lung, liver, spleen, and kidney were collected at necropsy on days 150±7 or 300±7. β-gal activity was measured by using a fluorogenic substrate. For animals that did not survive to the scheduled day 300 necropsy due to disease progression, tissues were collected at the time of euthanasia, and data are presented as part of the day 300 cohort. Error bars represent the SEM.
GM1 gangliosidosis patient and in murine and canine models of GM1 gangliosidosis, with no impact on the neurodegenerative course of the disease. In contrast, mice transplanted with hematopoietic stem cells engineered to overexpress β-gal exhibited improvements in neuronal storage lesions and neurologic signs, suggesting that HSCT alone is ineffective due to insufficient enzyme delivery.

Studies have explored AAV-mediated gene transfer as an alternative method to rapidly achieve robust, durable β-gal expression in the CNS. Remarkable efficacy has been demonstrated after multiple intraparenchymal AAV injections in both murine and feline disease models. However, translating this approach to patients is complicated by the invasiveness of the injection procedure as well as the challenge of scaling the number of injections to achieve similar vector distribution in the larger human brain.

This study demonstrates the potential for delivery of an optimized vector into CSF to restore β-gal activity and correct storage lesions throughout the brain. Near-complete correction of the disease phenotype was achieved at a dose of $4.4 \times 10^{10}$ GC ($1.1 \times 10^{11}$ GC/g brain mass), similar to doses employed in ongoing clinical trials for other lysosomal storage diseases (NCT03566043). A single AAV injection into the CSF of the cisterna magna results in diffuse CNS gene transfer in large animals, supporting direct translation of a similar approach to clinical studies in humans.

In addition to neurological symptoms, GM1 gangliosidosis patients exhibit variable somatic disease. AAV delivery into the CSF results in systemic vector distribution and may achieve sufficient peripheral enzyme expression to attenuate skeletal dysplasia, cardiomyopathy, and hepatosplenomegaly. Pre-existing neutralizing antibodies to the vector capsid do not impact gene transfer to the brain, but may prevent peripheral transduction, potentially leading to different degrees of peripheral β-gal expression in patients depending on neutralizing antibody status.

This study demonstrated an absence of neuronal storage lesions in Glb1−/− mice treated with an AAV vector at 4 weeks of age, when prominent brain storage lesions are already present in this model. These results suggest that gene transfer may both prevent and reverse GM1 storage in the brain. However, GM1 storage ultimately results in neuron death, meaning that it will be critical to identify and treat patients before irreversible neurodegeneration has occurred. Clinical trials of gene therapy for GM1 gangliosidosis will, therefore, face the challenge of identifying patients who are likely to develop neurological disease before symptoms manifest. Due to the high frequency of private mutations, prediction of clinical phenotype is usually not possible based on genotype alone.

Patients with infantile GM1 gangliosidosis may be an ideal population to evaluate AAV gene therapy, as they are frequently diagnosed based on subtle neurological

**DISCUSSION**

More than 40 years after researchers identified the underlying biochemical defect of GM1 gangliosidosis, there remain no disease-modifying therapies for this disease. For other neurodegenerative lysosomal storage diseases, hematopoietic stem cell transplantation (HSCT) has been successfully employed to deliver the deficient enzyme to the CNS via donor-derived cells that migrate into the brain. HSCT has been attempted in one presymptomatic CSF β-gal activity levels exceeding those of normal vehicle-treated Glb1+/− controls. β-gal activity in CSF was generally dose dependent, whereas β-gal activity in the two lowest dose groups appeared to be similar ($1.3 \times 10^{10}$ GC and $4.4 \times 10^{9}$ GC) to that of the vehicle-treated Glb1+/− controls.

The reason for similar β-gal activity levels at the two lowest doses might be related to the number of CSF samples from the animals administered the lowest vector dose ($4.4 \times 10^{9}$ GC), which was limited by this group’s high mortality; the animals that survived in this group might have had higher β-gal expression than the others that did not. In all groups, the levels of β-gal activity exceeded the range observed for CSF from historical control vehicle-treated Glb1−/− mice (gray area).

**Figure 6.** β-gal activity in CSF of vector-treated Glb1−/− mice and vehicle-treated controls. CSF was collected from Glb1−/− (KO) mice that survived to day 300 after ICV administration of AAVhu68.UbC.hGLB1 at a dose of $1.3 \times 10^{11}$ GC (N=9), $4.4 \times 10^{10}$ GC (N=9), $1.3 \times 10^{10}$ GC (N=8), or $4.4 \times 10^{9}$ GC (N=5). CSF was also collected from Glb1+/− (HET) controls after ICV administration of vehicle (N=9). β-gal activity was measured in CSF by using a fluorogenic substrate. CSF was not collected from vehicle-treated Glb1−/− (KO) mice, because none survived to day 300. The range of β-gal activity in CSF from Glb1−/− mice is based on data from 10 vehicle-treated animals from a prior study (shaded area). Error bars represent the SEM. CSF, cerebrospinal fluid.
findings that appear in the first 6 months of life before the onset of the rapid developmental regression that inevitably follows within 1–2 years.3

ACKNOWLEDGMENTS
The authors would like to acknowledge the technical assistance of Sarah Elliott, C. Angelica Medina-Jaszek, Tamara Goode, Julia Johansson, and Victoria Kehm as well as support from the Program for Comparative Medicine, Vector Core, and Morphology Core of the Gene Therapy Program, University of Pennsylvania.

AUTHOR DISCLOSURE
J.M.W. is a paid advisor to and holds equity in Scout Bio and Passage Bio; he holds equity in Surrmount Bio; and he also has sponsored research agreements with Amicus Therapeutics, Biogen, Elajab Bio, Janssen, Moderna, Passage Bio, Scout Bio, Surrmount Bio, and Ultragenyx, which are licensees of Penn technology. J.M.W. is an inventor on patents that have been licensed to various biopharmaceutical companies and for which he may receive payments. C.H. is an inventor on patents licensed to biopharmaceutical companies and holds equity in Scout Bio.

FUNDING INFORMATION
This research was supported by a sponsored research agreement with Passage Bio.

SUPPLEMENTARY MATERIAL
Supplementary Figure S1

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Received for publication October 11, 2018; accepted after revision October 6, 2020.

Published online: October 6, 2020.