COMPOSITIONS USEFUL FOR TREATING GM1 GANGLIOSIDOSIS

BACKGROUND OF THE INVENTION

GM1 gangliosidosis, henceforth referred to as GM1, is an autosomal recessive lysosomal storage disease caused by mutations in the *GLB1* gene which encodes lysosomal acid beta galactosidase (β-gal), an enzyme that catalyzes the first step in the degradation of GM1 ganglioside and keratan sulfate (Brunetti-Pierri and Scaglia, 2008, GM1 gangliosidosis: Review of clinical, molecular, and therapeutic aspects, *Molecular Genetics and Metabolism*, 94: 391-96). The *GLB1* gene is located on chromosome 3 and leads to two alternatively spliced mRNAs, a 2.5 kb transcript encoding the β-gal lysosomal enzyme and a 2.0 kb transcript encoding the elastin binding protein (EBP) (Oshima *et al.* 1988, Cloning, sequencing, and expression of cDNA for human β-galactosidase, *Biochemical and Biophysical Research Communications*, 157: 238-44; Morreau *et al.* 1989, Alternative splicing of beta-galactosidase mRNA generates the classic lysosomal enzyme and a beta-galactosidase-related protein, *Journal of Biological Chemistry*, 264: 20655-63). β-gal is synthesized as an 85 kDa precursor that is post-translationally glycosylated to an 88 kDa form and processed into the mature 64 kDa lysosomal enzyme (D'Azzo *et al.* 1982, Molecular defect in combined beta-galactosidase and neuraminidase deficiency in man, *Proceedings of the National Academy of Sciences*, 79: 4535-39). Within lysosomes the enzyme is complexed with protective protein cathepsin A (PPCA) and neuraminidase hydrolases.

In patients carrying *GLB1* alleles that produce little or no residual β-gal, GM1 ganglioside accumulates in neurons throughout the brain, resulting in a rapidly progressive neurodegenerative disease (Brunetti-Pierri and Scaglia 2008). While the molecular mechanisms leading to disease pathogenesis are still not well understood, hypotheses include neuronal cell death and demyelination accompanied by astrogliosis and microgliosis in areas of severe neuronal vacuolation, neuronal apoptosis (Tessitore *et al.* 2004, GM1-Ganglioside-Mediated Activation of the Unfolded Protein Response Causes Neuronal Death in a Neurodegenerative Gangliosidosis, *Molecular Cell*, 15: 753-66), abnormal axoplasmic transport resulting in myelin deficiency (van der Voorn *et al.* 2004, The leukoencephalopathy of infantile GM1 gangliosidosis: oligodendrocytic loss and axonal dysfunction, *Acta Neuropathologica*, 107: 539-45), disturbed neuronal–oligodendroglial interactions (Folkerth 1999, Abnormalities of Developing White Matter in Lysosomal Storage Diseases, *Journal of Neuropathology and Experimental Neurology*, 58: 887-902;

Kaye *et al.* 1992, Dysmyelinogenesis in animal model of GM1 gangliosidosis', *Pediatric Neurology*, 8: 255-61), and inflammatory responses (Jeyakumar *et al.* 2003, Central nervous system inflammation is a hallmark of pathogenesis in mouse models of GM1 and GM2 gangliosidosis, *Brain*, 126: 974-87).

There are currently no disease-modifying therapies for GM1. Supportive care and symptomatic treatments including feeding tube placement, respiratory therapy and anti-epileptic drugs are current therapeutic approaches (Jarnes Utz *et al.* 2017, Infantile gangliosidoses: Mapping a timeline of clinical changes, *Molecular Genetics and Metabolism*, 121: 170-79). Substrate reduction therapy (SRT) with miglustat, a glucosylceramide synthase inhibitor, has been evaluated in GM1 and GM2 patients. Although miglustat is generally well tolerated, it has not resulted in marked improvement in symptom management or disease progression and some patients experience dose limiting gastro-intestinal side effects (Shapiro *et al.*, 2009, Regier *et al.*, 2016b). When used in combination with a ketogenic diet, miglustat has been shown to be well tolerated and to increase survival in some patients (Jarnes Utz *et al.*, 2017). However, it should be noted that no randomized controlled studies with miglustat have been conducted and miglustat is not approved for the treatment of GM1 gangliosidosis. There is limited experience with hematopoietic stem cell transplantation (HSCT) with bone marrow or umbilical cord blood in this disease. Bone marrow transplant performed in a patient with Type 2 GM1 resulted normalization of white cell β-galactosidase levels in a patient with presymptomatic juvenile onset GM1-gangliosidosis, did not improve long-term clinical outcome (Shield *et al.*, 2005, Bone marrow transplantation correcting β-galactosidase activity does not influence neurological outcome in juvenile GM1-gangliosidosis. *Journal of Inherited Metabolic Disease*. 28(5):797-798.). The slow time to effect of HSCT make it not suitable for rapidly progressive Type 1 GM1 disease (Peters and Steward, 2003, Hematopoietic cell transplantation for inherited metabolic diseases: an overview of outcomes and practice guidelines. *Bone Marrow Transplantation*. 31:229.). Adeno-associated virus (AAV), a member of the Parvovirus family, is a small non-enveloped, icosahedral virus with single-stranded linear DNA (ssDNA) genomes of about 4.7 kilobases (kb) long. The wild-type genome comprises inverted terminal repeats (ITRs) at both ends of the DNA strand, and two open reading frames (ORFs): *rep* and *cap*. *Rep* is composed of four overlapping genes encoding rep proteins required for the AAV life cycle, and *cap* contains overlapping nucleotide sequences of capsid proteins: VP1, VP2 and VP3, which self-assemble to form a capsid of an icosahedral symmetry.

AAV is assigned to the genus, *Dependovirus*, because the virus was discovered as a contaminant in purified adenovirus stocks. AAV's life cycle includes a latent phase at which AAV genomes, after infection, are site specifically integrated into host chromosomes and an infectious phase in which, following either adenovirus or herpes simplex virus infection, the integrated genomes are subsequently rescued, replicated, and packaged into infectious viruses. The properties of non-pathogenicity, broad host range of infectivity, including non-dividing cells, and potential site-specific chromosomal integration make AAV an attractive tool for gene transfer.

What is desirable are alternative therapeutics for treatment of conditions associated with abnormal *GLB1* gene.

SUMMARY OF THE INVENTION

A therapeutic, recombinant (r), replication-defective, adeno-associated virus (AAV) is provided which is useful for treating and/or reducing the symptoms associated with GM1 gangliosidosis in human patients in need thereof. The rAAV is desirably replication-defective and carries a vector genome comprising a *GLB1* gene encoding human(h) β-galactosidase under the control of regulatory sequences which direct its expression in targeted human cells, which may be termed as rAAV.GLB1 as used herein. In certain embodiments, the rAAV comprises an AAVhu68 capsid. This rAAV is termed herein, rAAVhu68.GLB1, but in certain instances the terms rAAVhu68.GLB1 vector, rAAVhu68.hGLB1, rAAVhu68.hGLB1 vector, AAVhu68.GLB1, or AAVhu68.GLB1 vector are used interchangeably to reference the same construct.

In one aspect, provided herein is a therapeutic regimen useful for treatment of GM1 gangliosidosis in a human patient, wherein the regimen comprises administration of a recombinant adeno-associated virus (rAAV) vector having an AAV capsid and a vector genome comprising a sequence encoding human β-galactosidase under control of regulatory sequences that direct expression thereof in target cell, the administration comprising intra-cisterna magna (ICM) injection of a single dose comprising: (i) about 1.6×10^{13} to about 1.6×10^{14} GC, wherein the patient is about 1 month to about 4 months of age; (ii) about 2.1×10^{13} to about 2.1×10^{14} GC, wherein the patient is at least 4 months to under 8 months of age; (iii) about 2.6×10^{13} to about 2.6×10^{14} GC, wherein the patient is at least 8 months up to 12 months of age; or (iv) about 3.2×10^{13} to about 3.2×10^{14} GC, wherein the patient is at least 12 months of age. In certain embodiments, the human β-galactosidase coding sequence comprises a nucleotide sequence set forth in SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6,

or SEQ ID NO: 5 or a sequence at least 95% identical to any one of SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 that encodes the mature β-galactosidase of amino acids 24 to 677 of SEQ ID NO: 4. In certain embodiments, the encoded human β-galactosidase has the sequence selected from: (a) about amino acids 1 to 677 of SEQ ID NO: 4; and (b) a synthetic human enzyme comprising a heterologous leader sequence fused to about amino acids 24 to 677 of SEQ ID NO: 4. In further embodiments, the vector genome also comprises a 5' inverted terminal repeat (ITR) sequence, a regulatory element derived from the human ubiquitin C (UbC) promoter, a chimeric intron, a polyA signal, and/or a 3' ITR sequence. In certain embodiments, the patient has been identified as having type 1 (infantile) GM1 or type 2a (late infantile) GM1. In certain embodiments, the regimen comprises administration of at least one immunosuppressive co-therapy to the patient at least one day prior to or on the day of delivery of the rAAV. The immunosuppressive co-therapy may include one or more corticosteroids, optionally oral prednisolone. In certain embodiments, the immunosuppressive co-therapy continues for at least 3 to 4 weeks following administration of the rAAV. In certain embodiments, the efficacy of treatment is assessed by one or more of a delay in the onset of seizures, a decrease in the frequency of seizures, β-galactosidase in serum and/or cerebral spinal fluid, and volumetric changes in brain tissue as measured by magnetic resonance imaging (MRI).

In one aspect, provided herein is a composition comprising a recombinant AAV(rAAV) vector comprising an AAV capsid and a vector genome comprising a human β-galactosidase coding sequence and expression control sequences that direct expression thereof in target cells, wherein the rAAV vector is formulated for intra-cisterna magna (ICM) injection to a human subject in need thereof to administer a dose of: (i) about 1.6×10^{13} to about 1.6×10^{14} GC, wherein the patient is about 1 month to about 4 months of age; (ii) about 2.1×10^{13} to about 2.1×10^{14} GC, wherein the patient is at least 4 months to under 8 months of age; (iii) about 2.6×10^{13} to about 2.6×10^{14} GC, wherein the patient is at least 8 months up to 12 months of age; or (iv) about 3.2×10^{13} to about 3.2×10^{14} GC, wherein the patient is at least 12 months of age. In certain embodiments, the human β-galactosidase coding sequence comprises a nucleotide sequence set forth in SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 or a sequence at least 95% identical to any one of SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 that encodes the mature β-galactosidase of amino acids 24 to 677 of SEQ ID NO: 4. In further embodiments, the vector genome also comprises a 5' inverted terminal repeat (ITR) sequence, a regulatory element derived from the human ubiquitin C (UbC) promoter, a chimeric intron, a polyA

signal, and/or a 3' ITR sequence. In certain embodiments, the rAAV is formulated in a suspension to deliver 3.33 x 10^{10} GC per gram of brain mass to 3.33 x 10^{11} GC per gram of brain mass, optionally wherein the volume of the administered dose is about 3.0 mL to about 5.0 mL. In certain embodiments, the rAAV is in a formulation buffer having a pH of 6 to 9, optionally wherein the pH is about 7.2. In certain embodiments, the composition is for use in a co-therapy comprising administration of at least one immunosuppressant to the patient at least one day prior to or on the day of delivery of the rAAV. The immunosuppressant may be a corticosteroid, optionally orally delivered prednisolone.

In one aspect, provided herein is a method of treating a patient with GM1 gangliosidosis, the method comprising administering a single dose of a recombinant adeno-associated virus (rAAV) to the patient by intracisternal magna (ICM) injection, wherein the rAAV comprises an AAV capsid and a vector genome comprising a sequence encoding human β-galactosidase under control of regulatory sequences that direct expression thereof in a target cell, and wherein the single dose is from $1x10^{10}$ GC to $3.4x10^{11}$ GC per gram of estimated brain mass of the patient. In certain embodiments, the patient has onset of a GM1 symptom at or before 18 months of age. In certain embodiments, the patient has onset of a GM1 symptom at 6 months of age or earlier. In certain embodiments, the patient has onset of a GM1 symptom at 6 to 18 months of age. In certain embodiments, the patient has type1 (infantile) GM1. In other embodiments, the patient has type2a (late infantile) GM1. In certain embodiments, the subject is at least 4 months of age; 4 to 36 months of age; 4 to 24 months of age; 6 to 36 months of age; 6 to 24 months of age; 12 to 36 months of age; or 12 to 24 months of age. In certain embodiments, the single dose is $3.3x10^{10}$ GC per gram of estimated brain mass of the patient. In certain embodiments, the single dose is $2.1x10^{13}$ to $2.5x10^{13}$ GC of the rAAV or $2.6x10^{13}$ to $3.1x10^{13}$ GC of the rAAV. In certain embodiments, the single dose is $3.2x10^{13}$ to $4.5x10^{13}$ GC of the rAAV. In certain embodiments, the single dose is $1.11x10^{11}$ GC per gram of estimated brain mass of the patient. In certain embodiments, the single dose is $6.8x10^{13}$ to $8.6x10^{13}$ GC of the rAAV; $8.7x10^{13}$ to $0.9x10^{14}$ GC of the rAAV; or $1.0x10^{14}$ to $1.5x10^{14}$ GC of the rAAV. In certain embodiments, the patient is 4 to 8 months of age, and the single dose is $2.1x10^{13}$ GC of the rAAV. In certain embodiments, the patient is 4 to 8 months of age, and the single dose is $6.8x10^{13}$ GC of the rAAV. In certain embodiments, the patient is 8 to 12 months of age, and the single dose is $2.6x10^{13}$ GC of the rAAV. In certain embodiments, the patient is 8 to 12 months of age, and the single dose is $8.7x10^{13}$ GC of the rAAV. In certain embodiments, the the patient is at least 12 months of age, and the single dose is $3.2x10^{13}$ GC of the rAAV. In certain

embodiments, the patient is at least 12 months of age, and the single dose is $1.0x10^{14}$ GC of the rAAV. In certain embodiments, the method further comprises the step of hematopoietic stem cell transplantation. In certain embodiments, the method further comprises the step of administering a steroid to the patient. The steroid may be a corticosteroid. In certain embodiments, the method comprises administration of a steroid daily for at least 21 days. In certain embodiments, the method comprises administration of a steroid daily for 30 days. In certain embodiments, the vector genome includes a sequence encoding human β-galactosidase comprising a nucleotide sequence set forth in SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 or a sequence at least 95% identical to any one of SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 that encodes the mature β-galactosidase of amino acids 24 to 677 of SEQ ID NO: 4. human β-galactosidase has an amino acid sequence of SEQ ID NO: 4 or a functional fragment thereof. In certain embodiments, vector genome has a sequence selected from SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15. In certain embodiments, wherein the vector genome has a sequence at least 95% identical to SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, ot SEQ ID NO: 15. In certain embodiments, the vector genome further comprises a 5' inverted terminal repeat (ITR) sequence, a regulatory element derived from the human ubiquitin C (UbC) promoter, a chimeric intron, a polyA signal, and/or a 3' ITR sequence.

In one aspect, provided herein is a pharmaceutical composition in a unit dosage form, comprising 1×10^{13} GC to 5×10^{14} of a recombinant adeno-associated virus (rAAV) vector in a buffer, wherein the rAAV comprises an AAV capsid and a vector genome comprising a sequence encoding human β-galactosidase under control of regulatory sequences that direct expression thereof in a target cell. In certain embodiments, the composition is formulated for intracisternal magna (ICM) injection. In certain embodiments, the buffer comrises sodium phosphate, sodium chloride, potassium chloride, calcium chloride, magnesium chloride, and poloxamer 188. In further embodiments, the buffer comprises 1 mM sodium phosphate, 150 mM sodium chloride, 3 mM potassium chloride, 1.4 mM calcium chloride, 0.8 mM magnesium chloride, and 0.001% poloxamer 188. In certain embodiments, the composition comprises $2.1x10^{13}$ to $2.5x10^{13}$ GC of the rAAV; $2.6x10^{13}$ to $3.1x10^{13}$ GC of the rAAV; $3.2x10^{13}$ to $4.5x10^{13}$ GC of the rAAV; $6.8x10^{13}$ to $8.6x10^{13}$ GC of the rAAV; $8.7x10^{13}$ to $0.9x10^{14}$ GC of the rAAV; or $1.0x10^{14}$ to $1.5x10^{14}$ GC of the rAAV. The pharmaceutical compositions provided include a rAAV having a vector genome with a sequence encoding human β-galactosidase that comprises a nucleotide sequence set forth in SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 or a sequence at least 95% identical to any one

of SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 that encodes the mature β-galactosidase of amino acids 24 to 677 of SEQ ID NO: 4. In certain embodiments, the human β-galactosidase has an amino acid sequence of SEQ ID NO: 4 or a functional fragment thereof. In certain embodiments, the vector genome has a sequence selected from SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15. In certain embodiments, the vector genome has a sequence at least 95% identical to SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15. In certain embodiments, the vector genome comprises a 5' inverted terminal repeat (ITR) sequence, a regulatory element derived from the human ubiquitin C (UbC) promoter, a chimeric intron, a polyA signal, and/or a 3' ITR sequence.

These and other aspects of the invention are apparent from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG 1A provides a schematic of an AAV vector genome showing 5' ITR, human ubiquitin C (UbC) promoter, chimeric intron, *GLB1* gene encoding human β-galactosidase (β-gal), SV40 late polyA signal, and 3' ITR (*i.e.*, "AAVhu68.Ubc.hGLB1co.SV40").

FIG 1B provides a schematic of a cis-plasmid containing an AAV vector genome carried by the *cis* plasmid, pAAV.UbC.hGLB1co.SV40.KanR. GLB1, β-galactosidase; ITR, inverted terminal repeats; KanR, kanamycin resistance; Ori, origin of replication; PolyA, polyadenylation; and UbC, ubiquitin C.

FIG 1C provides a schematic of a trans-plasmid comprising a coding sequence for a full-length AAV2 replicase (AAV2 Rep) encoding four proteins and the AAVhu68 VP1 capsid gene (which encodes VP1, VP2 and VP3 proteins). AAV2, adeno-associated virus serotype 2; AAVhu68, adeno-associated virus serotype hu68; Cap, capsid; KanR, kanamycin resistance; Ori, origin of replication; and Rep, replicase.

FIGs 2A and 2B illustrate β-gal activity in brain and cerebrospinal fluid (CSF), respectively, of wild-type mice treated with rAAVhu68.GLB1 expressing human β-gal using different promoters. Wild-type mice were treated with a single intracerebroventricular (ICV) injection of rAAVhu68.GLB1 expressing human GLB1 from a CB7, EF1a or UbC promoter (n = 10 per group). Untreated wild-type mice (n = 5) served as controls. Brain (frontal cortex) and CSF were collected 14 days after rAAVhu68.GLB1 administration, and β-gal activity was measured using a fluorogenic substrate. $*p < 0.05$, $**p<0.01$, $***p<0.001$, Kruskal-Wallis test followed by Dunn's test.

FIGs 3A – 3E illustrate serum and peripheral organ β-gal activity in a GLB1 knockout mouse study. Preclinical studies were conducted using a *GLB1* knockout mouse model of GM1 (mice that carry homozygous mutations in the *GLB1* gene, or GLB1-/- mice). The studies compared GLB1-/- mice treated with AAVhu68.UbC.hGLB1, GLB1-/- mice treated with vehicle (phosphate-buffered saline, or PBS) and disease-free mice that are heterozygous *GLB1* mutation carriers, or GLB1+/- mice, treated with vehicle. In this study, all mice were treated at one month of age and observed until four months of age, which is when GM1 mice typically develop marked gait abnormalities associated with brain GM1 ganglioside levels similar to those of infantile GM1 patients with advanced disease. All mice were treated with an intracerebroventricular, or ICV, injection of either test vector (denoted in the following graphics as AAV) or vehicle. Ninety days after treatment, all animals were euthanized and tissues collected, referred to as necropsy, for histological and biochemical analysis. Serum β-gal activity was measured at various time points before and following treatment (days 0, 10, 28, 60 and 90). β-gal activity in the brain, CSF and peripheral organs were evaluated at the time of necropsy. β-gal activity was measured in serum (FIG 3A) as well as lung (FIG 3B), liver (FIG 3C), heart (FIG 3D) and spleen samples (FIG 3E), respectively, using a fluorogenic substrate. PBS: phosphate buffered saline (vehicle), AAV: Adeno-associated virus (AAVhu68.UbC.hGLB1). *$p < 0.05$, **$p<0.01$ Kruskal-Wallis test followed by Dunn's test. NS: not significant. FIG 3A shows that AAVhu68.UbC.hGLB1 treated GLB1-/- mice had substantially higher serum β-gal activity following treatment than vehicle-treated GLB1-/- mice and similar β-gal activity to vehicle treated heterozygous control mice. Elevated serum β-gal activity as measured in nanomolar per milliliter per hour, or nmol/ml/h, was achieved shortly after treatment for all AAVhu68.UbC.hGLB1-treated mice and persisted throughout the study for all but two AAVhu68.UbC.hGLB1-treated mice, both of which exhibited antibodies against human β-gal. FIGs 3B – 3E show β-gal activity in the lungs, liver, heart and spleen following necropsy. In each organ, β-gal activity in the rAAV.hGLB1 GLB1-/- mice exceeded activity levels in vehicle treated GLB1-/- mice. This data supports the potential of hGLB1 to provide corrective β-gal enzyme activity to peripheral organs and suggests that treatment with the rAAV.hGLB1 vector could address both the CNS and peripheral manifestations observed in GM1 patients.

FIGs 4A – 4B illustrate β-gal activity in brain as measured in nanomolar per milligram per hour, or nmol/mg/h, and CSF following necropsy. β-gal activity in the AAVhu68.UbC.hGLB1-treated mice exceeded the vehicle-treated GLB1-/- mice in both the brain and the CSF. Brain (frontal cortex) and CSF were collected at necropsy and β-gal

activity measured using a fluorogenic substrate. PBS: phosphate buffered saline (vehicle), AAV: Adeno-associated virus (AAVhu68.UbC.hGLB1). *p < 0.05, **p<0.01 Kruskal-Wallis test followed by Dunn's test. NS: not significant. Statistical significance is important and when used herein is denoted by p-values. The p-value is the probability that the reported result was achieved purely by chance (for example, a p-value < 0.001 means that there is a less than 0.1% chance that the observed change was purely due to chance). Generally, a p-value less than 0.05 is considered to be statistically significant.

FIG 5 shows reduction of hexosaminidase (HEX) activity in brains of rAAVhu68.GLB1 -treated $GLB1^{-/-}$ mice. Brain (frontal cortex) was collected at necropsy and HEX activity measured using a fluorogenic substrate. PBS: phosphate buffered saline (vehicle), AAV: Adeno-associated virus (AAVhu68.UbC.hGLB1). *p < 0.05, **p<0.01 Kruskal-Wallis test followed by Dunn's test. NS: not significant. Correction of brain abnormalities using biochemical and histological assays was assessed following necropsy. Lysosomal enzymes are frequently upregulated in lysosomal storage diseases, an observation that has been confirmed in GM1 patients. Therefore, we measured the activity of the lysosomal enzyme HEX in brain lysates. The figure shows that the activity of HEX in rAAV.hGLB1-treated GLB1-/- mice was normalized as compared to GLB1+/- control mice, while vehicle-treated GLB1-/- exhibited elevated total HEX activity.

FIG 6 shows the correlation between β-gal activity and anti-β-gal antibodies. β-gal activity and serum anti-β-gal antibodies were measured in serum samples collected from AAV-treated mice at the time of necropsy. Each point represents an individual animal.

FIGs 7A – 7G show correction of gait abnormalities in AAV-treated $GLB1^{-/-}$ mice. FIGs 7A and 7B show that untreated $GLB1^{-/-}$ mice (n = 12) and $GLB1^{+/-}$ controls (n = 22) with an average age of 5 months were evaluated using the CatWalk system on two consecutive days. Average walking speed (FIG 7A) and length of the hind paw prints (FIG 7B) were quantified for each animal across at least 3 trials. **p < 0.01 Mann Whitney test. FIGs 7C and 7D show that four-month-old $GLB1^{+/-}$ (n = 15) or $GLB1^{-/-}$ (n = 15) mice treated with vehicle and AAV-treated $GLB1^{-/-}$ mice (n = 14) were evaluated using the CatWalk system. Average walking speed (FIG 7C) and length of the hind paw prints (FIG 7D) were quantified for each animal across at least 3 trials on the second day of testing. *p < 0.05, **p<0.01 Kruskal-Wallis test followed by Dunn's test. NS: not significant. FIGs 7E-G show representative hind paw prints for AAV-treated $GLB1^{-/-}$ mice (FIG 7G) and vehicle-treated $GLB1^{+/-}$ (FIG 7E) and $GLB1^{-/-}$ (FIG 7F) controls.

FIGs 8A and 8B show correlation between walking speed and gait parameters. $GLB1^{+/-}$ controls (n = 22) were evaluated using the CatWalk system on two consecutive days. Gait parameters measured in at least three trials on the second day of testing were recorded. Correlation analysis demonstrated a strong correlation between walking speed and gait parameters such as stride length (Spearman r = 0.7432, $p < 0.001$, FIG 8A). In contrast, hind paw print length was speed independent (Spearman r = -0.1239, p = 0.423, FIG 8B).

FIGs 9A – 9G provide β-gal activity (FIG 9A), body weight (FIG 9B), neurological examination score (neuro exam score, FIG 9C), length of hind paw print (FIG 9D), and swing time (FIG 9E) and stride length (FIG 9F) of hind limb of $GLB1^{-/-}$ mice received one of 4 doses of rAAVhu68.UbC.GLB1 (1.3×10^{11} GC, 4.4×10^{10} GC, 1.3×10^{10} GC or 4.4×10^{9} GC) or vehicle by ICV injection. $GLB1^{+/-}$ mice administered with vehicle (Het + Vehicle serves as controls. More details are provided in Example 4, Section A. FIG 9G shows average β-gal activity in serum in GLB1-/- mice administered the highest dose of rAAV.GLB1 was approximately 10-fold greater than that of normal vehicle-treated GLB1+/- controls. At the second highest dose of rAAV.h*GLB1*, 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 rAAV.*hGLB1* doses was similar to that of vehicle-treated GLB1-/- controls.

FIGs 10A - 10B provides an alignment showing the amino acid sequence of the vp1 capsid protein of AAVhu68 (SEQ ID NO: 2) (labelled hu.68.vp1 in alignment), with AAV9 (SEQ ID NO: 20), AAVhu31 (labelled hu.31 in alignment, SEQ ID NO: 21) and AAVhu32 (labelled hu.32 in alignment, SEQ ID NO: 22). Compared to AAV9, AAVhu31 and AAVhu32, two mutations (A67E and A157V) were found critical in AAVhu68 and circled in FIG 10A.

FIGs 11A - 11E provide an alignment of the nucleic acid sequence encoding the vp1 capsid protein of AAVhu68 (SEQ ID NO: 1), with AAV9 (SEQ ID NO: 23), AAVhu31 (SEQ ID NO: 24) and AAVhu32 (SEQ ID NO: 25).

FIG 12A provides an illustrative flow chart of manufacturing process for producing rAAVhu68.GLB1 drug substance. AEX, anion exchange; CRL, Charles River Laboratories; ddPCR, droplet digital polymerase chain reaction; DMEM, Dulbecco's modified Eagle medium; DNA, deoxyribonucleic acid; FFB, final formulation buffer; GC, genome copies; HEK293, human embryonic kidney 293 cells; ITFFB, intrathecal final formulation buffer; PEI, polyethyleneimine; Ph. Eur., European Pharmacopoeia; SDS-PAGE, sodium dodecyl

sulfate polyacrylamide gel electrophoresis; TFF, tangential flow filtration; USP, United States Pharmacopeia; WCB, working cell bank.

FIG 12B provides an illustrative flow chart for manufacturing process for producing rAAVhu68.GLB1 drug product. Ad5, adenovirus serotype 5; AUC, analytical ultracentrifugation; BDS, bulk drug substance; BSA, bovine serum albumin; CZ, Crystal Zenith; ddPCR, droplet digital polymerase chain reaction; E1A, early region 1A (gene); ELISA, enzyme-linked immunosorbent assay; FDP, final drug product; GC, genome copies; HEK293, human embryonic kidney 293 cells; ITFFB, intrathecal final formulation buffer; KanR, kanamycin resistance (gene); MS, mass spectrometry; NGS, next-generation sequencing; Ph. Eur., European Pharmacopoeia; qPCR, quantitative polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; $TCID_{50}$ 50% tissue culture infective dose; UPLC, ultra-performance liquid chromatography; USP, United States Pharmacopeia.

FIG 13 shows survival data of each cohort in the study through day 300 at doses of 1.3×10^{11} GC, 4.4×10^{10} GC, 1.3×10^{10} GC, and 4.4×10^{9} GC, with a vehicle control for KO and a vehicle control for a heterozygous mouse.

FIGS 14A – 14C show average total severity score for each cohort as of each neurological assessment period. FIG 14A provides stride length (cm). FIG 14B provides hind paw print length (cm). FIG 14C provides the total score of the neurological examination.

FIGS 15A – 15C provide the results of histological analysis was also performed comparing brain sections of rAAV.hGLB1-treated GLB1-/- mice, vehicle treated GLB1-/- mice and vehicle treated GLB1+/- control mice at baseline (FIG 15A, day 1, 1 month old) day 150 (FIG 15B) and day 300 (FIG 15C).

FIGs 16A provides serum β-gal activity (nmol/mL/h) and FIG 16B shows that β-gal activity was detectable in the CSF of all mice evaluated. GLB1-/- mice administered the two highest doses of the tested rAAV.hGLB1 displayed average CSF β-gal activity levels exceeding that of normal vehicle-treated GLB1+/- controls. β-gal activity in CSF was generally dose-dependent, although β-gal activity appeared to be similar in the two lowest dose groups.

FIGs 17A – FIG 17L show the results of assessing β-galactosidase activity in brain (FIG 17A, day 150 and FIG 17B, day 300), Heart (FIG 17C, day 150 and FIG 17D, day 300), Liver (FIG 17E, day 150 and FIG 17F, day 300), spleen (FIG 17G, day 150 and FIG 17H, day 300), lung (FIG 17I, day 150 and FIG 17J, day 300) or kidney (FIG 17K, day 150

and FIG 17L, day 300) of test rAAV.hGLB1 treated GLB1-/- Mice and Vehicle-Treated Controls. β-gal was detectable in the CSF of all mice evaluated. GLB1-/- mice administered the two highest doses of tested rAAV.hGLB1 displayed average CSF β-gal activity levels exceeding that of normal vehicle-treated GLB1+/- controls. β-gal activity in CSF was generally dose-dependent, although β-gal activity appeared to be similar in the two lowest dose groups.

FIGs 18A – 18B show the severity of dorsal root ganglia (DRG) and spinal cord lesions at day 120, as measured by histological analysis and scoring of severity of lesions from 0 (none) to 5 (severe). Arrows are drawn to the two animals that exhibited the most severe axon loss and fibrosis with decreased sensory nerve action potential.

FIGs 19A – 19B show median nerve axonopathy and median nerve periaxonal fibrosis at day 120, as measured by histological analysis and scoring of severity of lesions from 0 (none) to 5 (severe). Arrows are drawn to the two animals that exhibited the most severe axon loss and fibrosis with decreased sensory nerve action potential.

FIGs 20A – 20B show the change in median sensory nerve conduction as of each measuring point in the study through day 120, as measured by median sensory action potential in microvolts (MV).

FIGs 21A – 21B show results of bilateral median nerve sensory action potential amplitudes (SNAP) and conduction velocities. Juvenile NHPs received a single ICM administration of either vehicle (ITFFB; N=2/group) or rAAV.hGLB1 test vector at a dose of 3.0×10^{12} GC (low dose), 1.0×10^{13} GC (mid-dose), or 3.0×10^{13} GC (high dose) (N=3/group). Sensory nerve conduction testing was performed at BL and on Days 28±3, 60±3, 90±4, and 120±4. SNAP amplitudes and conduction velocities of the right and left median nerves are presented. Abbreviations: BL, baseline; GC, genome copies; ICM, intra-cisterna magna; ITFFB, intrathecal final formulation buffer; N, number of animals; NHP, non-human primate; SNAP, sensory nerve action potential.

FIGs 22A – 22D show the results of Human β-Galactosidase Activity in CSF and Serum of NHPs Treated with rAAV.hGLB1 test vector or Vehicle. Juvenile NHPs received a single ICM administration of either vehicle (ITFFB; N=2/group) or rAAV.GLB1 at a dose of 3.0×10^{12} GC (low dose), 1.0×10^{13} GC (mid-dose), or 3.0×10^{13} GC (high dose) (N=3/group). CSF and serum were collected at the indicated days and analyzed for human β-gal activity. Dashed line represents baseline endogenous β-gal activity levels. FIG22A shows CSF β-gal activity. FIG22B shows serum β-gal activity. FIGs 22C and 22D show an expanded view of day 14 results): Empty shapes indicate animals that were negative for

serum-circulating NAbs against the vector capsid at the time of treatment. Filled in shapes denote animals that were positive for serum-circulating NAbs against the vector capsid at the time of treatment. *Abbreviations*: β-gal, β-galactosidase; BL, baseline; GC, genome copies; ICM, intra-cisterna magna; ITFFB, intrathecal final formulation buffer; N, number of animals; NAb, neutralizing antibody; NHP, non-human primate; SEM, standard error of the mean.

FIG 23 provides Vector Biodistribution 60 Days After ICM Administration of rAAV.hGLB1 to NHPs. The indicated tissues were collected at necropsy from juvenile NHPs 60 days after a single ICM administration of rAAV.hGLB1 at a dose of 3.0×10^{12} GC (low dose), 1.0×10^{13} GC (mid-dose), or 3.0×10^{13} GC (high dose) (N=3/group). Tissues were also collected from vehicle- (ITFFB-) treated NHPs (N=2) as a control. Each bar represents mean vector genomes detected per μg of DNA. Error bars represent the SEM. The LOD was 50 GC/μg DNA. *Abbreviations*: DNA, deoxyribonucleic acid; GC, genome copies; ICM, intra-cisterna magna; ITFFB, intrathecal final formulation buffer; LOD, limit of detection; N, number of animals; NHP, non-human primate; SEM, standard error of the mean.

FIG 24 provides vector biodistribution 120 Days After ICM Administration of rAAV.hGBL1 to NHPs. The indicated tissues were collected at necropsy from juvenile NHPs 120 days after a single ICM administration of rAAV.hGLB1 at a dose of 3.0×10^{12} GC (low dose), 1.0×10^{13} GC (mid-dose), or 3.0×10^{13} GC (high dose) (N=3/group). Tissues were also collected from vehicle- (ITFFB-) treated NHPs (N=2) as a control. Each bar represents mean vector genomes detected per μg of DNA. Error bars represent the SEM. The LOD was 50 GC/μg DNA. Abbreviations: DNA, deoxyribonucleic acid; GC, genome copies; ICM, intra-cisterna magna; ITFFB, intrathecal final formulation buffer; LOD, limit of detection; N, number of animals; NHP, non-human primate; SEM, standard error of the mean.

DETAILED DESCRIPTION OF THE INVENTION

Adeno-associated virus (AAV) based compositions and methods for treating GM1 gangliosidosis (GM1) are provided herein. An effective amount of genome copies (GC) of a recombinant AAV (rAAV) having an AAVhu68 capsid and carrying a vector genome having the normal *GLB1* gene which encodes the human β-galactosidase enzyme (rAAVhu68.GLB1) is delivered to the patient. Desirably, this rAAVhu68.GLB1 is formulated with an aqueous buffer. In certain embodiments, the suspension is suitable for

intrathecal injection. In certain embodiments, rAAVhu68.GLB1 is AAVhu68.UbC.GLB1 (also termed as AAVhu68.UbC.hGLB1), in which the *GLB1* gene (*i.e.*, β-galactosidase (also termed as GLB1 enzyme, β-gal, or galactosidase as used herein) coding sequence) is under the control of regulatory sequences which include a promoter derived from human ubiquitin C (UbC). In certain embodiments, the compositions are delivered via an intra-cisterna magna injection (ICM) injection.

Nucleic acid sequences encoding the capsid of a clade F adeno-associated virus, which is termed herein AAVhu68, are utilized in the production of the AAVhu68 capsid and recombinant AAV (rAAV) carrying the vector genome. As used herein, the term "vector genome" refers to a nucleic acid molecule which is packaged in a viral capsid, for example, an AAV capsid, and is capable of being delivered to a host cell or a cell in a patient. In certain embodiments, the vector genome is an expression cassette having inverted terminal repeat (ITR) sequences necessary for packaging the vector genome into the AAV capsid at the extreme 5' and 3' end and containing therebetween a *GLB1* gene as described herein operably linked to sequences which direct expression thereof. Additional details relating to AAVhu68 are provided in WO 2018/160582, incorporated by reference in its entirety herein, and in this detailed description. The rAAVhu68.GLB1 described herein are well suited for delivery of the vector genome comprising the *GLB1* gene to cells within the central nervous system (CNS), including brain, hippocampus, motor cortex, cerebellum, and motor neurons. These rAAVhu68.GLB1 may be used for targeting other cells within the CNS and certain other tissues and cells outside the CNS. Alternatively, AAVhu68 capsid may be replaced by another capsid which is also suitable for delivering a vector genome to the CNS, for example, AAVcy02, AAV8, AAVrh43, AAV9, AAVrh08, AAVrh10, AAVbb01, AAVhu37, AAVrh20, AAVrh39, AAV1, AAVhu48, AAVcy05, AAVhu11, AAVhu32, or AAVpi02.

I. GM1 and the therapeutic GLB1 gene

GM1 gangliosidosis (*i.e.*, GM1) can be classified into three types based on the clinical phenotype: (1) type 1 or infantile form with onset from birth to 6 months, rapidly progressive with hypotonia, severe central nervous system (CNS) degeneration and death by 1-2 years of age; (2) type 2 late infantile or juvenile with onset from 7 months to 3 years, lag in motor and cognitive development, and slower progression; and (3) type 3 adult or chronic variant with late onset (3–30 years), a progressive extrapyramidal disorder due to local deposition of glycosphingolipid in the caudate nucleus (Brunetti-Pierri and Scaglia, 2008.

GM1 gangliosidosis: Review of clinical, molecular, and therapeutic aspects, *Molecular Genetics and Metabolism*, 94: 391-96). Infantile GM1 subjects with symptom onset before 6 months of age uniformly exhibit rapid and predictable progression of both motor and cognitive impairment. The majority of patients die within the first few years of life (median survival 46 months, Jarnes Utz *et al.*, 2017). Despite a shared underlying pathophysiology, the adult (Type 3) GM1 phenotype is variable and disease course is notably milder. Most patients with Type 3 GM1 first develop neurological symptoms in late childhood, with little subsequent progression in adulthood.

The severity of each type is inversely related to the residual activity of the β-gal enzyme (Brunetti-Pierri and Scaglia, 2008) which is encoded by a *GLB1* gene. Over 130 disease-causing *GLB1* mutations have been identified in human (Hofer *et al.*, 2010, Phenotype determining alleles in GM1 gangliosidosis patients bearing novel *GLB1* mutations. *Clinical Genetics*. 78(3):236-246; and Caciotti *et al.*, 2011, M1 gangliosidosis and Morquio B disease: An update on genetic alterations and clinical findings. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*. 1812(7):782-790.). While a number of *GLB1* mutations have been genetically and biochemically analyzed and correlated with clinical phenotype (Gururaj *et al.*, 2005, Magnetic Resonance Imaging Findings and Novel Mutations in GM1 Gangliosidosis. *Journal of Child Neurology*. 20(1):57-60; Caciotti *et al.*, 2011; and Sperb *et al.*, 2013, Genotypic and phenotypic characterization of Brazilian patients with GM1 gangliosidosis. *Gene*. 512(1):113-116), many *GLB1* mutations remain uncharacterized. Broadly speaking the genotype of the patient results in varying amounts of residual enzyme activity, but generally speaking, the higher the residual enzyme activity is, the less severe the phenotype is (Ou *et al.*, 2018, SAAMP 2.0: An algorithm to predict genotype-phenotype correlation of lysosomal storage diseases. *Clinical Genetics*. 93(5):1008-1014.). Diagnosis of GM1 is confirmed by either biochemical assay of β-gal and neuraminidase and/or by *GLB1* molecular analysis. However, there are limitations to the use of genotype-phenotype correlations in predicting the clinical presentation of an affected individual, as the residual enzyme activity *per se* cannot predict the disease subtypes caused by mutations in the *GLB1* gene (Hofer *et al.*, 2010, Caciotti *et al.*, 2011, Ou *et al.*, 2018). The predictive value is best for individuals bearing two severe mutations (*i.e.* mutations that show no GLB1 enzyme activity), who commonly present with a severe early onset phenotype (Caciotti *et al.*, 2011, Sperb *et al.*, 2013). Data on sibling concordance, although sparse, indicate that the clinical course in sibling with infantile GM1 is similar in terms of time to onset and prevailing disease manifestations (Gururaj *et al.*, 2005).

The gene therapy vector provided herein, *i.e.*, rAAV.GLB1 (for example, rAAVhu68.GLB1, rAAVhu68.UbC.GLB1), or the composition comprising the same is useful for treatment of conditions associated with deficiencies in normal levels of functional beta-galactosidase. As used herein, the gene therapy vector refers to a rAAV as described herein which is suitable for use in treating a patient. In certain embodiments, the gene therapy vector or the composition provided herein is useful for treating Type 1 of GM1. In certain embodiments, the gene therapy vector or the composition provided herein is useful for treating Type 2 of GM1. In certain embodiments, the gene therapy vector or the composition provided herein is useful for treating Type 3 of GM1. In certain embodiments, the gene therapy vector or the composition provided herein is useful for treating Type 1 and Type 2 of GM1. In certain embodiments, the gene therapy vector or the composition provided herein is useful for treating GM1 patients who are 18 months of age or younger. In certain embodiments, the gene therapy vector or the composition provided herein is useful for treating Type 1 and Type 2 of GM1. In certain embodiments, the gene therapy vector or the composition provided herein is useful for treating GM1 patients who are 36 months of age or younger. In certain embodiments, the gene therapy vector or the composition provided herein is for treatment of GM1 which excludes Type 3. In certain embodiments, the gene therapy vector or the composition provided herein is useful for treatment of neurological conditions associated with deficiencies in normal levels of functional β-galactosidase. In certain embodiments, the gene therapy vector or the composition provided herein is useful for amelioration of symptoms associated with GM1 gangliosidosis. In certain embodiments, the gene therapy vector or the composition provided herein is useful for amelioration of neurological symptoms associated with GM1 gangliosidosis.

In certain embodiments, the patient has infantile gangliosidosis and is 18 months of age or younger. In certain embodiments, the patients receiving the rAAV.GLB1 are 1 month to 18 months of age. In certain embodiments, the patients receiving the rAAV.GLB1 are four months to 18 months of age. In certain embodiments, the infant is under four months of age. In certain embodiments, the patients receiving the rAAV.GLB1 are about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, or about 18 months of age. In certain embodiments, the patient is a toddler, *e.g.*, 18 months to 3 years of age. In certain embodiments, the patient receiving the rAAV.GLB1 is from 3 years to 6 years of age, from 3

years to 12 years of age, from 3 years to 18 years of age, from 3 years to 30 years of age. In certain embodiments, patients are older than 18 years of age.

In certain embodiments, amelioration of symptoms associated with GM1 gangliosidosis are observed following treatment, including, *e.g.*, increased life span (survival); decreased need for feeding tube; reduction in seizure incidence, frequency, and length, delayed onset of seizures; improved quality of life, for example, as measured by PedsQL; reduction in progression towards neurocognitive decline and/or improvement in neurocognitive development, *e.g.*, improved development or improvement in adaptive behaviors, cognition, language (receptive and expressive communication), and motor function (gross motor, fine motor), as measured by the Bayley Scales of Infant and Toddler Development, Third Edition (BSID-III) and the Vineland Adaptive Behavior Scales, Second Edition (Vineland-II); earlier age-at-achievement and later age-at-loss for motor milestones; delayed increasement of brain tissue volume (cerebral cortex and other smaller structures) and ventricular volume, delayed size decrease of brain substructures including the corpus callosum, caudate and putamen as well as the cerebellar cortex, and stabilization in brain atrophy and volumetric changes; delayed progression of abnormal T1/T2 signal intensity in the thalamus and basal ganglia; increased β-gal enzyme activity in CSF and serum; reduction of CSF GM1 ganglioside concentration; reduction of serum and/or urine keratan sulfate levels, decreased hexosaminidase activity; reduce inflammatory response in the brain; delayed abnormal liver and spleen volume; delayed abnormal EEG and visual evoked potentials (VEP); and/or improvements in dysphagia, gait function, motor skills, language and/or respiratory function.

In certain embodiments, the patient receives a co-therapy following rAAV.GLB1 injection for which they would not have been eligible without the AAV therapy described herein. Such co-therapies may include enzyme replacement therapy, substrate reduction therapy (*e.g.*, with miglustat (OGT 918, N-butyl-deoxynojirimycin), tanganil (acetyl-DL-leucine) treatment, respiratory therapy, feeding tube use, anti-epileptic drugs), or hematopoietic stem cell transplantation (HSCT) with bone marrow or umbilical cord blood.

Optionally, an immunosuppressive co-therapy may be used in a subject in need. Immunosuppressants for such co-therapy include, but are not limited to, a glucocorticoid, steroids, antimetabolites, T-cell inhibitors, a macrolide (*e.g.*, a rapamycin or rapalog), and cytostatic agents including an alkylating agent, an anti-metabolite, a cytotoxic antibiotic, an antibody, or an agent active on immunophilin. The immune suppressant may include a nitrogen mustard, nitrosourea, platinum compound, methotrexate, azathioprine,

mercaptopurine, fluorouracil, dactinomycin, an anthracycline, mitomycin C, bleomycin, mithramycin, IL-2 receptor- (CD25-) or CD3-directed antibodies, anti-IL-2 antibodies, ciclosporin, tacrolimus, sirolimus, IFN-β, IFN-γ, an opioid, or TNF-α (tumor necrosis factor-alpha) binding agent. In certain embodiments, the immunosuppressive therapy may be started 0, 1, 2, 3, 4, 5, 6, 7, or more days prior to or after the rAAV.GLB1 administration. Such immunosuppressive therapy may involve administration of one, two or more drugs (*e.g.*, glucocorticoids, prednisolone, mycophenolate mofetil (MMF) and/or sirolimus (*i.e.*, rapamycin)). Such immunosuppressive drugs may be administered to a patient/subject in need once, twice or for more times at the same dose or an adjusted dose. Such therapy may involve co-administration of two or more drugs, the (*e.g.*, prednisolone, mycophenolate mofetil (MMF) and/or sirolimus (*i.e.*, rapamycin)) on the same day. One or more of these drugs may be continued after the rAAV.GLB1 administration, at the same dose or an adjusted dose. Such therapy may be for about 1 week (7 days), about 60 days, or longer, as needed. In certain embodiments, a tacrolimus-free regimen is selected.

In certain embodiments, an "effective amount" of rAAV.GLB1 (for example, rAAV.GLB1, rAAV.UbC.GLB1) as provided herein is the amount which achieves amelioration of symptoms associated with GM1 gangliosidosis. In certain embodiments, an "effective amount" of rAAV.GLB1 as provided herein is the amount which achieves one or more of the following endpoints: increased β-gal pharmacodynamics and biological activity in Cerebrospinal fluid (CSF), increased β-gal pharmacodynamics and biological activity in serum, increased average life span (survival) of the patient, delayed disease progression of GM1 gangliosidosis (assessed by one or more of age at achievement, age at loss and percentage of patients maintaining or acquiring age-appropriate developmental and motor milestones), and improvements in neurocognitive development based on one or more of change in age-equivalent cognitive, gross motor, fine motor, receptive and expressive communication scores of the Bayley Scales of Infant and Toddler Development (BSID, for example, BSID Third Edition (BSID-III)), change in standard score for each domain of the Vineland Adaptive Behavior Scales. For older children and adults, an "effective amount" of rAAV.GLB1 as provided herein may in some embodiments be an amount that improves dysphagia, gait function, motor skills, language and/or respiratory function, change in standard scores for each domain of the Vineland Adaptive Behavior Scales, Second Edition (Vineland-II), decreased seizure frequency and age of seizure onset, improved probability of feeding tube independence at 24 months of age. Examples of age-appropriate developmental and motor milestones are provided by World Health Organization (WHO). See, *e.g.*,

Wijnhoven T.M., *et al.* (2004). Assessment of gross motor development in the WHO Multicentre Growth Reference Study. *Food Nutr Bull.* 25(1 Suppl):S37-45, as well as in the table below. In certain embodiments, an "effective amount" of rAAV.GLB1 (such as, rAAVhu68, GLB1) as provided herein is the amount which achieves pharmacodynamic effects of rAAV.GLB1 on CSF and serum β-galactoside activity, CSF GM1 concentration, and serum and urine keratan sulfate; changes in brain MRI; monitoring liver and spleen volume; monitoring on EEG and visual evoked potentials (VEP).

Gross Motor Milestone	Multicenter Growth Reference Study Performance Criteria
Sitting without support	Child sits up straight with the head erect for at least 10 seconds. Child does not use arms or hands to balance body or support position.
Hands-and-knees crawling	Child alternately moves forward or backward on hands and knees. The stomach does not touch the supporting surface. There are continuous and consecutive movements, at least three in a row.
Standing with assistance	Child stands in upright position on both feet, holding onto a stable object (*e.g.*, furniture) with both hands without leaning on it. The body does not ouch the stable object, and the legs support most of the body weight. Child thus stands with assistance for at least 10 seconds.
Walking with assistance	Child is in upright position with the back straight. Child makes sideways or forward steps by holding on a stable object (*e.g.*, furniture) with one of both hands. One leg moves forward while the other supports part of the body weight. Child takes at least five steps in this manner.
Standing alone	Childs stands in upright position on both feed (not on the toes) with the back straight. The legs support 100% of the child's weigh. There is no contact with a person or objects. Child stands alone for at least 10 seconds.

Gross Motor Milestone	Multicenter Growth Reference Study Performance Criteria
Walking alone	Child takes at least five steps independently in upright position with the back straight. One leg moves forward while the other supports most of the body weight. There is no contact with a person or object.

Adapted from (Wijnhoven et al., 2004, Assessment of gross motor development in the WHO Multicentre Growth Reference Study." Food Nutr Bull. 25(1 Suppl):S37-45). *Abbreviations*: WHO, World Health Organization.

The rAAV.GLB1 described herein, and compositions comprising the same, contain a *GLB1* gene (*i.e.*, β-gal coding sequence) which encodes and expresses human β-galactosidase (which may be also termed as normal β-galactoside enzyme) or a functional fragment thereof. GLB1 enzyme catalyzes the hydrolysis of β-galactoside into monosaccharides. The amino acid sequence of human β-galactosidase (2034 bp, 677 aa, Genbank #AAA51819.1, EC3.2.1.23) is reproduced herein as SEQ ID NO: 4, which is also recognized as β-galactosidase, Isoform 1. See, for example, UniProtKB - P16278 (BGAL_HUMAN). In certain embodiments, the GLB1 enzyme may have a sequence of amino acid 24 to amino acid 677 of SEQ ID NO: 4 (i.e., mature GLB1 enzyme without signal peptide). In certain embodiments, the GLB1 enzyme may have a sequence of amino acid 31 to amino acid 677 of SED ID NO: 4 (i.e., β-galactosidase, Isoform 3). In certain embodiments, the GLB1 enzyme is Isoform 2 having an amino acid sequence of SEQ ID NO: 26. Any fragment that retains the function of the full length β-galactosidase may be encoded by the *GLB1* gene as described herein, and is referred to as a "functional fragment". For example, a functional fragment of β-galactosidase may have at least about 25%, 50%, 60%, 70%, 80%, 90%, 100% or more of the activity of the full length β-galactosidase (*i.e.*, the normal GLB1 enzyme which may be β-galactosidase having a sequence of amino acid 24 to amino acid 677 SEQ ID NO: 4, or any one of the three isoforms). Methods of evaluating the β-galactosidase activity can be found in the Examples as well as in publications. See, for example, Radoslaw Kwapiszewski, Determination of Acid β-Galactosidase Activity: Methodology and Perspectives. Indian J Clin Biochem. 2014 Jan; 29(1): 57–62. In certain embodiments, the functional fragment is a truncated β-galactosidase, which lacks about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30 or more amino acids at the N terminal and/or C terminal of the full length β-galactosidase. In certain

embodiments, the functional fragment contains about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30 or more conservative amino acid substitution(s) compared to the full length β-galactosidase. As used herein, a conservative amino acid substitution is an amino acid replacement in a protein that changes a given amino acid to a different amino acid with similar biochemical properties (*e.g.* charge, hydrophobicity and size).

In one embodiment, the *GLB1* gene has the sequence of SEQ ID NO: 5. In certain embodiments, the *GLB1* gene is engineered to have the sequence of SEQ ID NO: 6. In certain embodiments, the *GLB1* gene is engineered to have the sequence of SEQ ID NO: 7. In certain embodiments, the *GLB1* gene is engineered to have the sequence of SEQ ID NO: 8. In certain embodiments, the *GLB1* gene is engineered to have a sequence which is at least 95% identical to 99.9% identical to SEQ ID NO: 6. In certain embodiments, the *GLB1* gene is engineered to have a sequence which is at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% or at least about 99.9% identical to SEQ ID NO: 6. In certain embodiments, the *GLB1* gene is engineered to have a sequence which is at least 95% identical to 99.9% identical to SEQ ID NO: 7. In certain embodiments, the *GLB1* gene is engineered to have a sequence which is at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% or at least about 99.9% identical to SEQ ID NO: 7. In certain embodiments, the *GLB1* gene is engineered to have a sequence which is at least 95% identical to 99.9% identical to SEQ ID NO: 8. In certain embodiments, the *GLB1* gene is engineered to have a sequence which is at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% or at least about 99.9% identical to SEQ ID NO: 8. In a further embodiment, the engineered sequence encodes a full length β-galactosidase or a functional fragment thereof. In yet a further embodiment, the engineered sequence encodes amino acid 24 to amino acid 677 of SEQ ID NO: 4 or a functional fragment thereof. In another embodiment, the engineered sequence encodes an amino acid sequence of SEQ ID NO: 4 or a functional fragment thereof.

In certain embodiments, the GLB1 gene encodes a β-galactoside enzyme which comprises a signal (leader) peptide and the GLB1 mature protein, amino acids 24 to 677 of SEQ ID NO: 4. The leader sequence is preferably of human origin or a derivative of a human leader sequence, and is be about 15 to about 28 amino acids, preferably about 20 to 25 amino acids, or about 23 amino acids in length. In certain embodiments, the signal peptide is the native signal peptide (amino acids 1 to 23 of SEQ ID NO: 4). In certain embodiments, the GLB1 enzyme comprises an exogenous leader sequence in the place of the

native leader sequence (amino acids 1-23 of SEQ ID NO:4). In another embodiment, the leader may be from a human IL2 or a mutated leader. In another embodiment, a human serpinF1 secretion signal may be used as a leader peptide.

II. AAVhu68

AAVhu68 (previously termed AAV3G2) varies from another Clade F virus AAV9 by two encoded amino acids at positions 67 and 157 of vp1, based on the numbering of SEQ ID NO: 2. In contrast, the other Clade F AAV (AAV9, hu31, hu31) have an Ala at position 67 and an Ala at position 157. Provided are novel AAVhu68 capsids and/or engineered AAV capsids having valine (Val or V) at position 157 based on the numbering of SEQ ID NO: 2 and optionally, a glutamic acid (Glu or E) at position 67 based on the numbering of SEQ ID NO: 2.

As used herein, the term "clade" as it relates to groups of AAV refers to a group of AAV which are phylogenetically related to one another as determined using a Neighbor-Joining algorithm by a bootstrap value of at least 75% (of at least 1000 replicates) and a Poisson correction distance measurement of no more than 0.05, based on alignment of the AAV vp1 amino acid sequence. The Neighbor-Joining algorithm has been described in the literature. See, *e.g.*, M. Nei and S. Kumar, *Molecular Evolution and Phylogenetics* (Oxford University Press, New York (2000). Computer programs are available that can be used to implement this algorithm. For example, the MEGA v2.1 program implements the modified Nei-Gojobori method. Using these techniques and computer programs, and the sequence of an AAV vp1 capsid protein, one of skill in the art can readily determine whether a selected AAV is contained in one of the clades identified herein, in another clade, or is outside these clades. See, *e.g.*, G Gao, *et al, J Virol*, 2004 Jun; 78(10): 6381-6388, which identifies Clades A, B, C, D, E and F, and provides nucleic acid sequences of novel AAV, GenBank Accession Numbers AY530553 to AY530629. See, also, WO 2005/033321.

In certain embodiments, an AAVhu68 capsid is further characterized by one or more of the following. AAVhu68 capsid proteins comprise: AAVhu68 vp1 proteins produced by expression from a nucleic acid sequence which encodes the predicted amino acid sequence of 1 to 736 of SEQ ID NO: 2, vp1 proteins produced from SEQ ID NO: 1, or vp1 proteins produced from a nucleic acid sequence at least 70% identical to SEQ ID NO: 1 which encodes the predicted amino acid sequence of 1 to 736 of SEQ ID NO: 2; AAVhu68 vp2 proteins produced by expression from a nucleic acid sequence which encodes the predicted amino acid sequence of at least about amino acids 138 to 736 of SEQ ID NO: 2, vp2 proteins

produced from a sequence comprising at least nucleotides 412 to 2211 of SEQ ID NO: 1, or vp2 proteins produced from a nucleic acid sequence at least 70% identical to at least nucleotides 412 to 2211 of SEQ ID NO: 1 which encodes the predicted amino acid sequence of at least about amino acids 138 to 736 of SEQ ID NO: 2; and/or AAVhu68 vp3 proteins produced by expression from a nucleic acid sequence which encodes the predicted amino acid sequence of at least about amino acids 203 to 736 of SEQ ID NO: 2, vp3 proteins produced from a sequence comprising at least nucleotides 607 to 2211 of SEQ ID NO: 1, or vp3 proteins produced from a nucleic acid sequence at least 70% identical to at least nucleotides 607 to 2211 of SEQ ID NO: 1 which encodes the predicted amino acid sequence of at least about amino acids 203 to 736 of SEQ ID NO: 2.

The AAVhu68 vp1, vp2 and vp3 proteins are typically expressed as alternative splice variants encoded by the same nucleic acid sequence which encodes the full-length vp1 amino acid sequence (amino acid (aa) 1 to 736). Optionally the vp1-encoding sequence is used alone to express the vp1, vp2 and vp3 proteins. Alternatively, this sequence may be co-expressed with one or more of a nucleic acid sequence which encodes the AAVhu68 vp3 amino acid sequence (about aa 203 to 736) without the vp1-unique region (about aa 1 to about aa 137) and/or vp2-unique regions (about aa 1 to about aa 202), or a strand complementary thereto, the corresponding mRNA or tRNA (for example, the mRNA transcribed from about nucleotide (nt) 607 to about nt 2211 of SEQ ID NO: 1), or a sequence at least 70% to at least 99% (*e.g.*, at least 85%, at least 90%, at least 95%, at least 97%, at least 98% or at least 99%) identical to SEQ ID NO: 1 which encodes aa 203 to 736 of SEQ ID NO: 2. Additionally, or alternatively, the vp1-encoding and/or the vp2-encoding sequence may be co-expressed with the nucleic acid sequence which encodes the AAVhu68 vp2 amino acid sequence of SEQ ID NO: 2 (about aa 138 to 736) without the vp1-unique region (about aa 1 to about 137), or a strand complementary thereto, the corresponding mRNA or tRNA (for example, the mRNA transcribed from nt 412 to 2211 of SEQ ID NO: 1), or a sequence at least 70% to at least 99% (*e.g.*, at least 85%, at least 90%, at least 95%, at least 97%, at least 98% or at least 99%) identical to SEQ ID NO: 1 which encodes about aa 138 to 736 of SEQ ID NO: 2.

As described herein, a rAAVhu68 has a rAAVhu68 capsid produced in a production system expressing capsids from an AAVhu68 nucleic acid sequence which encodes the vp1 amino acid sequence of SEQ ID NO: 2, and optionally additional nucleic acid sequences, *e.g.*, encoding a vp 3 protein free of the vp1 and/or vp2-unique regions. The rAAVhu68 resulting from production using a single nucleic acid sequence vp1 produces the

heterogenous populations of vp1 proteins, vp2 proteins and vp3 proteins. More particularly, the AAVhu68 capsid contains subpopulations within the vp1 proteins, within the vp2 proteins and within the vp3 proteins which have modifications from the predicted amino acid residues in SEQ ID NO: 2. These subpopulations include, at a minimum, deamidated asparagine (N or Asn) residues. For example, asparagines in asparagine - glycine pairs are highly deamidated.

In one embodiment, the AAVhu68 vp1 nucleic acid sequence has the sequence of SEQ ID NO: 1, or a strand complementary thereto, *e.g.*, the corresponding mRNA or tRNA. In certain embodiments, the vp2 and/or vp3 proteins may be expressed additionally or alternatively from different nucleic acid sequences than the vp1, *e.g.*, to alter the ratio of the vp proteins in a selected expression system. In certain embodiments, also provided is a nucleic acid sequence which encodes the AAVhu68 vp3 amino acid sequence of SEQ ID NO: 2 (about aa 203 to 736) without the vp1-unique region (about aa 1 to about aa 137) and/or vp2-unique regions (about aa 1 to about aa 202), or a strand complementary thereto, the corresponding mRNA or tRNA (about nt 607 to about nt 2211 of SEQ ID NO: 1). In certain embodiments, also provided is a nucleic acid sequence which encodes the AAVhu68 vp2 amino acid sequence of SEQ ID NO: 2 (about aa 138 to 736) without the vp1-unique region (about aa 1 to about 137), or a strand complementary thereto, the corresponding mRNA or tRNA (nt 412 to 2211 of SEQ ID NO: 1).

However, other nucleic acid sequences which encode the amino acid sequence of SEQ ID NO: 2 may be selected for use in producing rAAVhu68 capsids. In certain embodiments, the nucleic acid sequence has the nucleic acid sequence of SEQ ID NO: 1 or a sequence at least 70% to 99% identical, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, identical to SEQ ID NO: 1 which encodes SEQ ID NO: 2. In certain embodiments, the nucleic acid sequence has the nucleic acid sequence of SEQ ID NO: 1 or a sequence at least 70% to 99%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, identical to about nt 412 to about nt 2211 of SEQ ID NO: 1 which encodes the vp2 capsid protein (about aa 138 to 736) of SEQ ID NO: 2. In certain embodiments, the nucleic acid sequence has the nucleic acid sequence of about nt 607 to about nt 2211 of SEQ ID NO:1 or a sequence at least 70% to 99%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, identical to nt 607 to about nt 2211 of SEQ ID NO: 1 which encodes the vp3 capsid protein (about aa 203 to 736) of SEQ ID NO: 2.

It is within the skill in the art to design nucleic acid sequences encoding this AAVhu68 capsid, including DNA (genomic or cDNA), or RNA (*e.g.*, mRNA). In certain embodiments, the nucleic acid sequence encoding the AAVhu68 vp1 capsid protein is provided in SEQ ID NO: 1. See, *also*, FIGs 11A-11E. In other embodiments, a nucleic acid sequence of 70% to 99.9% identity to SEQ ID NO: 1 may be selected to express the AAVhu68 capsid proteins. In certain other embodiments, the nucleic acid sequence is at least about 75% identical, at least 80% identical, at least 85%, at least 90%, at least 95%, at least 97% identical, or at least 99% to 99.9% identical to SEQ ID NO: 1. Such nucleic acid sequences may be codon-optimized for expression in a selected system (*i.e.*, cell type) can be designed by various methods. This optimization may be performed using methods which are available on-line (*e.g.*, GeneArt), published methods, or a company which provides codon optimizing services, *e.g.*, DNA2.0 (Menlo Park, CA). One codon optimizing method is described, *e.g.*, in US International Patent Publication No. WO 2015/012924, which is incorporated by reference herein in its entirety. See also, *e.g.*, US Patent Publication No. 2014/0032186 and US Patent Publication No. 2006/0136184. Suitably, the entire length of the open reading frame (ORF) for the product is modified. However, in some embodiments, only a fragment of the ORF may be altered. By using one of these methods, one can apply the frequencies to any given polypeptide sequence and produce a nucleic acid fragment of a codon-optimized coding region which encodes the polypeptide. A number of options are available for performing the actual changes to the codons or for synthesizing the codon-optimized coding regions designed as described herein. Such modifications or synthesis can be performed using standard and routine molecular biological manipulations well known to those of ordinary skill in the art. In one approach, a series of complementary oligonucleotide pairs of 80-90 nucleotides each in length and spanning the length of the desired sequence are synthesized by standard methods. These oligonucleotide pairs are synthesized such that upon annealing, they form double stranded fragments of 80-90 base pairs, containing cohesive ends, *e.g.*, each oligonucleotide in the pair is synthesized to extend 3, 4, 5, 6, 7, 8, 9, 10, or more bases beyond the region that is complementary to the other oligonucleotide in the pair. The single-stranded ends of each pair of oligonucleotides are designed to anneal with the single-stranded end of another pair of oligonucleotides. The oligonucleotide pairs are allowed to anneal, and approximately five to six of these double-stranded fragments are then allowed to anneal together via the cohesive single stranded ends, and then they ligated together and cloned into a standard bacterial cloning vector, for example, a TOPO® vector available from Invitrogen Corporation, Carlsbad, Calif. The construct is then sequenced by

standard methods. Several of these constructs consisting of 5 to 6 fragments of 80 to 90 base pair fragments ligated together, *i.e.*, fragments of about 500 base pairs, are prepared, such that the entire desired sequence is represented in a series of plasmid constructs. The inserts of these plasmids are then cut with appropriate restriction enzymes and ligated together to form the final construct. The final construct is then cloned into a standard bacterial cloning vector, and sequenced. Additional methods would be immediately apparent to the skilled artisan. In addition, gene synthesis is readily available commercially.

In certain embodiments, the AAVhu68 capsid is produced using a nucleic acid sequence of SEQ ID NO: 1 or a sequence at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, which encodes the vp1 amino acid sequence of SEQ ID NO: 2 with a modification (*e.g.*, deamidated amino acid) as described herein. In certain embodiments, the vp1 amino acid sequence is reproduced in SEQ ID NO: 2.

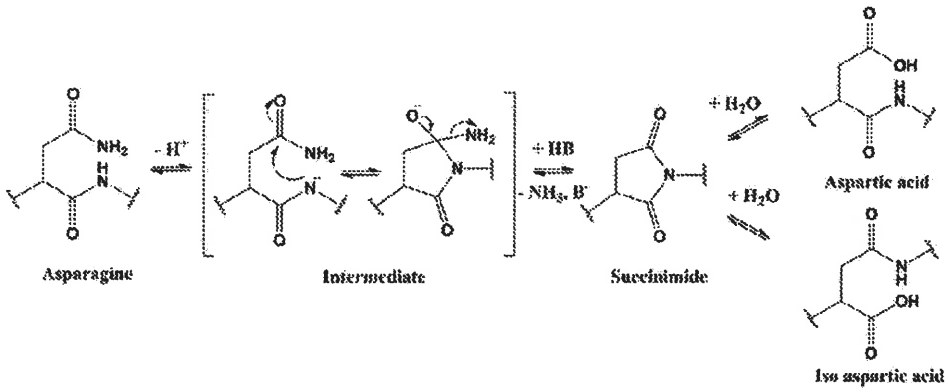
As used herein when used to refer to vp capsid proteins, the term "heterogenous" or any grammatical variation thereof, refers to a population consisting of elements that are not the same, for example, having vp1, vp2 or vp3 monomers (proteins) with different modified amino acid sequences. SEQ ID NO: 2 provides the encoded amino acid sequence of the AAVhu68 vp1 protein. The term "heterogenous" as used in connection with vp1, vp2 and vp3 proteins (alternatively termed isoforms), refers to differences in the amino acid sequence of the vp1, vp2 and vp3 proteins within a capsid. The AAV capsid contains subpopulations within the vp1 proteins, within the vp2 proteins and within the vp3 proteins which have modifications from the predicted amino acid residues. These subpopulations include, at a minimum, certain deamidated asparagine (N or Asn) residues. For example, certain subpopulations comprise at least one, two, three or four highly deamidated asparagines (N) positions in asparagine - glycine pairs and optionally further comprising other deamidated amino acids, wherein the deamidation results in an amino acid change and other optional modifications.

As used herein, a "subpopulation" of vp proteins refers to a group of vp proteins which has at least one defined characteristic in common and which consists of at least one group member to less than all members of the reference group, unless otherwise specified. For example, a "subpopulation" of vp1 proteins is at least one (1) vp1 protein and less than all vp1 proteins in an assembled AAV capsid, unless otherwise specified. A "subpopulation" of vp3 proteins may be one (1) vp3 protein to less than all vp3 proteins in an assembled AAV capsid, unless otherwise specified. For example, vp1 proteins may be a subpopulation

of vp proteins; vp2 proteins may be a separate subpopulation of vp proteins, and vp3 are yet a further subpopulation of vp proteins in an assembled AAV capsid. In another example, vp1, vp2 and vp3 proteins may contain subpopulations having different modifications, *e.g.*, at least one, two, three or four highly deamidated asparagines, *e.g.*, at asparagine - glycine pairs.

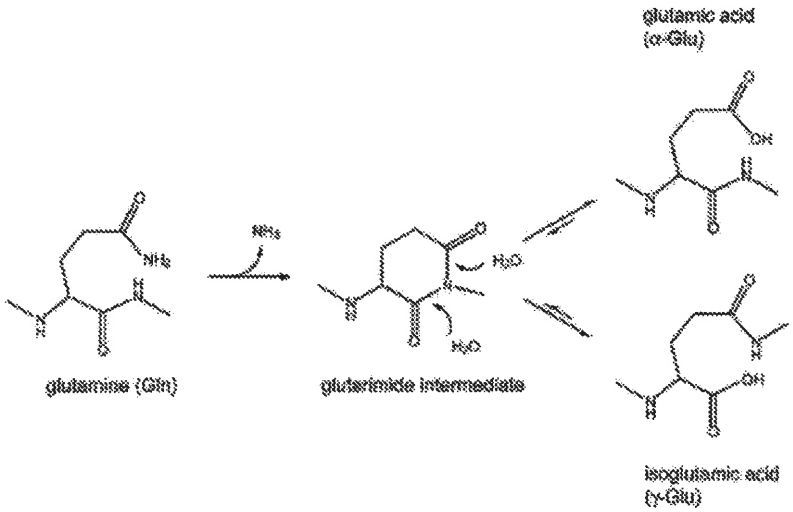
Unless otherwise specified, highly deamidated refers to at least 45% deamidated, at least 50% deamidated, at least 60% deamidated, at least 65% deamidated, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or up to about 100% deamidated at a referenced amino acid position, as compared to the predicted amino acid sequence at the reference amino acid position (*e.g.*, at least 80% of the asparagines at amino acid 57 based on the numbering of SEQ ID NO: 2 (AAVhu68) may be deamidated based on the total vp1 proteins may be deamidated based on the total vp1, vp2 and vp3 proteins). Such percentages may be determined using 2D-gel, mass spectrometry techniques, or other suitable techniques.

Without wishing to be bound by theory, the deamidation of at least highly deamidated residues in the vp proteins in the AAV capsid is believed to be primarily non-enzymatic in nature, being caused by functional groups within the capsid protein which deamidate selected asparagines, and to a lesser extent, glutamine residues. Efficient capsid assembly of the majority of deamidation vp1 proteins indicates that either these events occur following capsid assembly or that deamidation in individual monomers (vp1, vp2 or vp3) is well-tolerated structurally and largely does not affect assembly dynamics. Extensive deamidation in the VP1-unique (VP1-u) region (~aa 1-137), generally considered to be located internally prior to cellular entry, suggests that VP deamidation may occur prior to capsid assembly. The deamidation of N may occur through its C-terminus residue's backbone nitrogen atom conducts a nucleophilic attack to the Asn's side chain amide group carbon atom. An intermediate ring-closed succinimide residue is believed to form. The succinimide residue then conducts fast hydrolysis to lead to the final product aspartic acid (Asp) or iso aspartic acid (IsoAsp). Therefore, in certain embodiments, the deamidation of asparagine (N or Asn) leads to an Asp or IsoAsp, which may interconvert through the succinimide intermediate *e.g.*, as illustrated below.



As provided herein, each deamidated N in the VP1, VP2 or VP3 may independently be aspartic acid (Asp), isoaspartic acid (isoAsp), aspartate, and/or an interconverting blend of Asp and isoAsp, or combinations thereof. Any suitable ratio of α- and isoaspartic acid may be present. For example, in certain embodiments, the ratio may be from 10:1 to 1:10 aspartic to isoaspartic, about 50:50 aspartic: isoaspartic, or about 1:3 aspartic: isoaspartic, or another selected ratio.

In certain embodiments, one or more glutamine (Q) may deamidates to glutamic acid (Glu), *i.e.*, α-glutamic acid, γ-glutamic acid (Glu), or a blend of α- and γ-glutamic acid, which may interconvert through a common glutarimide intermediate. Any suitable ratio of α- and γ-glutamic acid may be present. For example, in certain embodiments, the ratio may be from 10:1 to 1:10 α to γ, about 50:50 α: γ, or about 1:3 α : γ, or another selected ratio.



Thus, an rAAV includes subpopulations within the rAAV capsid of vp1, vp2 and/or vp3 proteins with deamidated amino acids, including at a minimum, at least one subpopulation comprising at least one highly deamidated asparagine. In addition, other modifications may include isomerization, particularly at selected aspartic acid (D or Asp) residue positions. In still other embodiments, modifications may include an amidation at an Asp position.

In certain embodiments, an AAV capsid contains subpopulations of vp1, vp2 and vp3 having at least 4 to at least about 25 deamidated amino acid residue positions, of which at least 1% to 10% are deamidated as compared to the encoded amino acid sequence of the vp proteins. The majority of these may be N residues. However, Q residues may also be deamidated.

In certain embodiments, a rAAV has an AAV capsid having vp1, vp2 and vp3 proteins having subpopulations comprising combinations of two, three, four or more deamidated residues at the positions set forth in the table provided in Example 1 and incorporated herein by reference. Deamidation in the rAAV may be determined using 2D gel electrophoresis, and/or mass spectrometry (MS), and/or protein modelling techniques. Online chromatography may be performed with an Acclaim PepMap column and a Thermo UltiMate 3000 RSLC system (Thermo Fisher Scientific) coupled to a Q Exactive HF with a NanoFlex source (Thermo Fisher Scientific). MS data is acquired using a data-dependent top-20 method for the Q Exactive HF, dynamically choosing the most abundant not-yet-sequenced precursor ions from the survey scans (200–2000 m/z). Sequencing is performed

via higher energy collisional dissociation fragmentation with a target value of 1e5 ions determined with predictive automatic gain control and an isolation of precursors was performed with a window of 4 m/z. Survey scans were acquired at a resolution of 120,000 at m/z 200. Resolution for HCD spectra may be set to 30,000 at m/z200 with a maximum ion injection time of 50 ms and a normalized collision energy of 30. The S-lens RF level may be set at 50, to give optimal transmission of the m/z region occupied by the peptides from the digest. Precursor ions may be excluded with single, unassigned, or six and higher charge states from fragmentation selection. BioPharma Finder 1.0 software (Thermo Fischer Scientific) may be used for analysis of the data acquired. For peptide mapping, searches are performed using a single-entry protein FASTA database with carbamidomethylation set as a fixed modification; and oxidation, deamidation, and phosphorylation set as variable modifications, a 10-ppm mass accuracy, a high protease specificity, and a confidence level of 0.8 for MS/MS spectra. Examples of suitable proteases may include, *e.g.*, trypsin or chymotrypsin. Mass spectrometric identification of deamidated peptides is relatively straightforward, as deamidation adds to the mass of intact molecule +0.984 Da (the mass difference between –OH and $–NH_2$ groups). The percent deamidation of a particular peptide is determined by the mass area of the deamidated peptide divided by the sum of the area of the deamidated and native peptides. Considering the number of possible deamidation sites, isobaric species which are deamidated at different sites may co-migrate in a single peak. Consequently, fragment ions originating from peptides with multiple potential deamidation sites can be used to locate or differentiate multiple sites of deamidation. In these cases, the relative intensities within the observed isotope patterns can be used to specifically determine the relative abundance of the different deamidated peptide isomers. This method assumes that the fragmentation efficiency for all isomeric species is the same and independent on the site of deamidation. It is understood by one of skill in the art that a number of variations on these illustrative methods can be used. For example, suitable mass spectrometers may include, *e.g,* a quadrupole time of flight mass spectrometer (QTOF), such as a Waters Xevo or Agilent 6530 or an orbitrap instrument, such as the Orbitrap Fusion or Orbitrap Velos (Thermo Fisher). Suitably liquid chromatography systems include, *e.g.*, Acquity UPLC system from Waters or Agilent systems (1100 or 1200 series). Suitable data analysis software may include, *e.g.*, MassLynx (Waters), Pinpoint and Pepfinder (Thermo Fischer Scientific), Mascot (Matrix Science), Peaks DB (Bioinformatics Solutions). Still other techniques may be described, *e.g.*, in X. Jin *et al*, Hu Gene Therapy Methods, Vol. 28, No. 5, pp. 255-267, published online June 16, 2017.

In addition to deamidations, other modifications may occur do not result in conversion of one amino acid to a different amino acid residue. Such modifications may include acetylated residues, isomerizations, phosphorylations, or oxidations.

Modulation of Deamidation: In certain embodiments, the AAV is modified to change the glycine in an asparagine-glycine pair, to reduce deamidation. In other embodiments, the asparagine is altered to a different amino acid, *e.g.*, a glutamine which deamidates at a slower rate; or to an amino acid which lacks amide groups (*e.g.*, glutamine and asparagine contain amide groups); and/or to an amino acid which lacks amine groups (*e.g.*, lysine, arginine and histidine contain amine groups). As used herein, amino acids lacking amide or amine side groups refer to, *e.g.*, glycine, alanine, valine, leucine, isoleucine, serine, threonine, cystine, phenylalanine, tyrosine, or tryptophan, and/or proline. Modifications such as described may be in one, two, or three of the asparagine-glycine pairs found in the encoded AAV amino acid sequence. In certain embodiments, such modifications are not made in all four of the asparagine - glycine pairs. Thus, a method for reducing deamidation of AAV and/or engineered AAV variants having lower deamidation rates. Additionally, or alternative one or more other amide amino acids may be changed to a non-amide amino acid to reduce deamidation of the AAV. In certain embodiments, a mutant AAV capsid as described herein contains a mutation in an arginine - glycine pair, such that the glycine is changed to an alanine or a serine. A mutant AAV capsid may contain one, two or three mutants where the reference AAV natively contains four NG pairs. In certain embodiments, an AAV capsid may contain one, two, three or four such mutants where the reference AAV natively contains five NG pairs. In certain embodiments, a mutant AAV capsid contains only a single mutation in an NG pair. In certain embodiments, a mutant AAV capsid contains mutations in two different NG pairs. In certain embodiments, a mutant AAV capsid contains mutation is two different NG pairs which are located in structurally separate location in the AAV capsid. In certain embodiments, the mutation is not in the VP1-unique region. In certain embodiments, one of the mutations is in the VP1-unique region. Optionally, a mutant AAV capsid contains no modifications in the NG pairs, but contains mutations to minimize or eliminate deamidation in one or more asparagines, or a glutamine, located outside of an NG pair.

In certain embodiments, a method of increasing the potency of a rAAV is provided which comprises engineering an AAV capsid which eliminating one or more of the NGs in the wild-type AAV capsid. In certain embodiments, the coding sequence for the "G" of the "NG" is engineered to encode another amino acid. In certain examples below, an "S" or an

"A" is substituted. However, other suitable amino acid coding sequences may be selected. See, the table of Example 1, incorporated herein by reference.

In the AAVhu68 capsid protein, 4 residues (N57, N329, N452, N512) routinely display levels of deamidation >70% and it most cases >90% across various lots. Additional asparagine residues (N94, N253, N270, N304, N409, N477, and Q599) also display deamidation levels up to ~20% across various lots. The deamidation levels were initially identified using a trypsin digest and verified with a chymotrypsin digestion.

The AAVhu68 capsid contains subpopulations within the vp1 proteins, within the vp2 proteins and within the vp3 proteins which have modifications from the predicted amino acid residues in SEQ ID NO: 2. These subpopulations include, at a minimum, certain deamidated asparagine (N or Asn) residues. For example, certain subpopulations comprise at least one, two, three or four highly deamidated asparagines (N) positions in asparagine - glycine pairs in SEQ ID NO: 2 and optionally further comprising other deamidated amino acids, wherein the deamidation results in an amino acid change and other optional modifications. SEQ ID NO: 3 provide an amino acid sequence of a modified AAVhu68 capsid, illustrating positions which may have some percentage of deamidated or otherwise modified amino acids. The various combinations of these and other modifications are described herein.

In other embodiments, the method involves increasing yield of a rAAV and thus, increasing the amount of an rAAV which is present in supernatant prior to, or without requiring cell lysis. This method involves engineering an AAV VP1capsid gene to express a capsid protein having Glu at position 67, Val at position 157, or both based on an alignment having the amino acid numbering of the AAVhu68 vp1 capsid protein. In other embodiments, the method involves engineering the VP2 capsid gene to express a capsid protein having the Val at position 157. In still other embodiments, the rAAV has a modified capsid comprising both vp1 and vp2 capsid proteins Glu at position 67 and Val at position 157.

As used herein, an "AAV9 capsid" is a self-assembled AAV capsid composed of multiple AAV9 vp proteins. The AAV9 vp proteins are typically expressed as alternative splice variants encoded by a nucleic acid sequence of SEQ ID NO: 23 or a sequence at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% thereto, which encodes the vp1 amino acid sequence of GenBank accession: AAS99264. In certain embodiments, "AAV9 capsid" includes an AAV having an amino acid sequence which is 99% identical to AAS99264 or 99% identical to SEQ ID NO: 20. See,

also US7906111 and WO 2005/033321. As used herein "AAV9 variants" include those described in, e.g., WO2016/049230, US 8,927,514, US 2015/0344911, and US 8,734,809.

Methods of generating the capsid, coding sequences therefore, and methods for production of rAAV have been described. See, *e.g.*, Gao, et al, Proc. Natl. Acad. Sci. U.S.A. 100 (10), 6081-6086 (2003) and US 2013/0045186A1.

The term "substantial homology" or "substantial similarity," when referring to a nucleic acid, or fragment thereof, indicates that, when optimally aligned with appropriate nucleotide insertions or deletions with another nucleic acid (or its complementary strand), there is nucleotide sequence identity in at least about 95 to 99% of the aligned sequences. Preferably, the homology is over full-length sequence, or an open reading frame thereof, or another suitable fragment which is at least 15 nucleotides in length. Examples of suitable fragments are described herein.

The terms "sequence identity" "percent sequence identity" or "percent identical" in the context of nucleic acid sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence. The length of sequence identity comparison may be over the full-length of the genome, the full-length of a gene coding sequence, or a fragment of at least about 500 to 5000 nucleotides, is desired. However, identity among smaller fragments, *e.g.* of at least about nine nucleotides, usually at least about 20 to 24 nucleotides, at least about 28 to 32 nucleotides, at least about 36 or more nucleotides, may also be desired. Similarly, "percent sequence identity" may be readily determined for amino acid sequences, over the full-length of a protein, or a fragment thereof. Suitably, a fragment is at least about 8 amino acids in length and may be up to about 700 amino acids. Examples of suitable fragments are described herein.

The term "substantial homology" or "substantial similarity," when referring to amino acids or fragments thereof, indicates that, when optimally aligned with appropriate amino acid insertions or deletions with another amino acid (or its complementary strand), there is amino acid sequence identity in at least about 95 to 99% of the aligned sequences. Preferably, the homology is over full-length sequence, or a protein thereof, *e.g.*, a cap protein, a rep protein, or a fragment thereof which is at least 8 amino acids, or more desirably, at least 15 amino acids in length. Examples of suitable fragments are described herein.

By the term "highly conserved" is meant at least 80% identity, preferably at least 90% identity, and more preferably, over 97% identity. Identity is readily determined by one

of skill in the art by resort to algorithms and computer programs known by those of skill in the art.

Generally, when referring to "identity", "homology", or "similarity" between two different adeno-associated viruses, "identity", "homology" or "similarity" is determined in reference to "aligned" sequences. "Aligned" sequences or "alignments" refer to multiple nucleic acid sequences or protein (amino acids) sequences, often containing corrections for missing or additional bases or amino acids as compared to a reference sequence. In the examples, AAV alignments are performed using the published AAV9 sequences as a reference point. Alignments are performed using any of a variety of publicly or commercially available Multiple Sequence Alignment Programs. Examples of such programs include, "Clustal Omega", "Clustal W", "CAP Sequence Assembly", "MAP", and "MEME", which are accessible through Web Servers on the internet. Other sources for such programs are known to those of skill in the art. Alternatively, Vector NTI utilities are also used. There are also a number of algorithms known in the art that can be used to measure nucleotide sequence identity, including those contained in the programs described above. As another example, polynucleotide sequences can be compared using Fasta™, a program in GCG Version 6.1. Fasta™ provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences. For instance, percent sequence identity between nucleic acid sequences can be determined using Fasta™ with its default parameters (a word size of 6 and the NOPAM factor for the scoring matrix) as provided in GCG Version 6.1, herein incorporated by reference. Multiple sequence alignment programs are also available for amino acid sequences, *e.g.*, the "Clustal Omega", "Clustal X", "MAP", "PIMA", "MSA", "BLOCKMAKER", "MEME", and "Match-Box" programs. Generally, any of these programs are used at default settings, although one of skill in the art can alter these settings as needed. Alternatively, one of skill in the art can utilize another algorithm or computer program which provides at least the level of identity or alignment as that provided by the referenced algorithms and programs. See, *e.g.*, J. D. Thomson et al, Nucl. Acids. Res., "A comprehensive comparison of multiple sequence alignments", 27(13):2682-2690 (1999).

III. rAAV

Recombinant adeno-associated virus (rAAV) has been described as suitable vehicles for gene delivery. Typically, an exogenous expression cassette comprising the transgene (for example, the *GLB1* gene) for delivery by the rAAV replaces the functional *rep* genes and the

cap gene from the native AAV source, resulting in a replication-incompetent vector. These rep and cap functions are provided in *trans* during the vector production system but absent in the final rAAV.

As indicated above, a rAAV is provided which has an AAV capsid and a vector genome which comprises, at a minimum, AAV inverted terminal repeats (ITRs) required to package the vector genome into the capsid, a *GLB1* gene and regulatory sequences which direct expression therefor. In certain embodiments, the AAV capsid is from AAVhu68. The examples herein utilize a single-stranded AAV vector genome, but in certain embodiments, a rAAV may be utilized in the invention which contains self-complementary (sc) AAV vector genome.

The regulatory control elements necessary are operably linked to the gene (*e.g.*, *GLB1*) in a manner which permits its transcription, translation and/or expression in a cell which takes up the rAAV. As used herein, "operably linked" sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in *trans* or at a distance to control the gene of interest. Such regulatory sequences typically include, *e.g.*, one or more of a promoter, an enhancer, an intron, a polyA, a self-cleaving linker (*e.g.*, furin, furin-F2A, an IRES). The examples below utilize CB7 promoter (*e.g.*, SEQ ID NO:10), EF1a promoter (*e.g.*, SEQ ID NO: 11), or human ubiquitin C (UbC) promoter (*e.g.*, SEQ ID NO: 9) for expression of the *GLB1* gene. However, in certain embodiments, other promoters, or an additional promoter, may be selected.

In certain embodiments, in addition to the *GLB1* gene, a non-AAV sequence encoding another one or more of gene products may be included. Such gene products may be, *e.g.*, a peptide, polypeptide, protein, functional RNA molecule (*e.g.*, miRNA, miRNA inhibitor) or other gene product, of interest. Useful gene products may include miRNAs. miRNAs and other small interfering nucleic acids regulate gene expression via target RNA transcript cleavage/degradation or translational repression of the target messenger RNA (mRNA). miRNAs are natively expressed, typically as final 19-25 non-translated RNA products. miRNAs exhibit their activity through sequence-specific interactions with the 3′ untranslated regions (UTR) of target mRNAs. These endogenously expressed miRNAs form hairpin precursors which are subsequently processed into a miRNA duplex, and further into a "mature" single stranded miRNA molecule. This mature miRNA guides a multiprotein complex, miRISC, which identifies target site, *e.g.*, in the 3′ UTR regions, of target mRNAs based upon their complementarity to the mature miRNA.

In certain embodiments, the vector genome may be engineered to contain, in addition to the GBL1 coding sequences, one or more miR useful for detargetting dorsal root ganglion in order to improve safety and/or reduce side effects. Such drg de-targeting sequences are operably linked to the GLB1 coding sequence so as to minimize or prevent expression of the GLB1 product in dorsal root ganglion. Suitable drg-detargetting sequences are described in PCT/US19/67872, filed December 20, 2019, and entitled "Compositions for DRG-specific reduction of transgene expression".

The AAV vector genome typically comprise the *cis*-acting 5′ and 3′ inverted terminal repeat (ITR) sequences (See, *e.g.*, B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp. 155 168 (1990)). The ITR sequences are about 145 base pairs (bp) in length. Preferably, substantially the entire sequences encoding the ITRs are used in the molecule, although some degree of minor modification of these sequences is permissible. The ability to modify these ITR sequences is within the skill of the art. (See, *e.g.*, texts such as Sambrook et al, "Molecular Cloning. A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory, New York (1989); and K. Fisher et al., J. Virol., 70:520 532 (1996)). An example of such a molecule employed in the present invention is a "cis-acting" plasmid containing the transgene, in which the selected transgene sequence and associated regulatory elements are flanked by the 5′ and 3′ AAV ITR sequences. In one embodiment, the ITRs are from an AAV different than that supplying a capsid. In one embodiment, the ITR sequences are from AAV2. A shortened version of the 5' ITR, termed ΔITR, has been described in which the D-sequence and terminal resolution site (*trs*) are deleted. In certain embodiments, the vector genome includes a shortened AAV2 ITR of 130 base pairs, wherein the external A elements is deleted. The shortened ITR is reverted back to the wild type length of 145 base pairs during vector DNA amplification using the internal A element as a template. In other embodiments, the full-length AAV 5' and 3' ITRs are used. However, ITRs from other AAV sources may be selected. Where the source of the ITRs is from AAV2 and the AAV capsid is from another AAV source, the resulting rAAV may be termed pseudotyped. However, other configurations of these elements may be suitable.

In certain embodiments, an additional or alternative promoter sequence may be included as part of the expression control sequences (regulatory sequences), *e.g.*, located between the selected 5' ITR sequence and the coding sequence. Constitutive promoters, regulatable promoters (*see, e.g.*, WO 2011/126808 and WO 2013/04943), tissue specific promoters (for example, a neuron specific promoter or a glial cell specific promoter, or a CNS specific promoter), or a promoter responsive to physiologic cues may be utilized in the

rAAVs described herein. The promoter(s) can be selected from different sources, *e.g.*, human cytomegalovirus (CMV) immediate-early enhancer/promoter, the SV40 early enhancer/promoter, the JC polymovirus promoter, myelin basic protein (MBP) or glial fibrillary acidic protein (GFAP) promoters, herpes simplex virus (HSV-1) latency associated promoter (LAP), rouse sarcoma virus (RSV) long terminal repeat (LTR) promoter, neuron-specific promoter (NSE), platelet derived growth factor (PDGF) promoter, hSYN, melanin-concentrating hormone (MCH) promoter, CBA, matrix metalloprotein promoter (MPP), and the chicken beta-actin promoter. Other suitable promoter may include a CB7 promoter. In addition to a promoter, a vector genome may contain one or more other appropriate transcription initiation sequences, transcription termination sequences, enhancer sequences, efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA for example WPRE; sequences that enhance translation efficiency (*i.e.*, Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. An example of a suitable enhancer is the CMV enhancer. Other suitable enhancers include those that are appropriate for desired target tissue indications. In one embodiment, the regulatory sequences comprise one or more expression enhancers. In one embodiment, the regulatory sequences contain two or more expression enhancers. These enhancers may be the same or may differ from one another. For example, an enhancer may include a CMV immediate early enhancer. This enhancer may be present in two copies which are located adjacent to one another. Alternatively, the dual copies of the enhancer may be separated by one or more sequences. In still another embodiment, the expression cassette further contains an intron, *e.g.*, the chicken beta-actin intron. In certain embodiments, the intron is a chimeric intron (CI)– a hybrid intron consisting of human beta-globin splice donor and immunoglobulin G (IgG) splice acceptor elements. Other suitable introns include those known in the art, *e.g.*, such as are described in WO 2011/126808. Examples of suitable polyA sequences include, *e.g.*, SV40, SV50, bovine growth hormone (bGH), human growth hormone, and synthetic polyAs. Optionally, one or more sequences may be selected to stabilize mRNA. An example of such a sequence is a modified WPRE sequence, which may be engineered upstream of the polyA sequence and downstream of the coding sequence (see, *e.g.*, MA Zanta-Boussif, *et al*, *Gene Therapy* (2009) 16: 605-619). In certain embodiments, no WPRE sequence is present.

In certain embodiments, vector genomes are constructed which comprise a 5' AAV ITR - promoter – optional enhancer – optional intron – *GLB1* gene – polyA – 3' ITR. In

certain embodiments, the ITRs are from AAV2. In certain embodiments, more than one promoter is present. In certain embodiments, the enhancer is present in the vector genome. In certain embodiments, more than one enhancer is present. In certain embodiments, an intron is present in the vector genome. In certain embodiments, the enhancer and intron are present. In certain embodiments, the intron is a chimeric intron (CI)– a hybrid intron consisting of a human beta-globin splice donor and immunoglobulin G (IgG) splice acceptor elements. In certain embodiments, the polyA is an SV40 poly A (*i.e.*, a polyadenylation (PolyA) signal derived from Simian Virus 40 (SV40) late genes). In certain embodiments, the polyA is a rabbit beta-globin (RBG) poly A. In certain embodiments, the vector genome comprises a 5' AAV ITR – CB7 promoter – *GLB1* gene – RBG poly A – 3' ITR. In certain embodiments, the vector genome comprises a 5' AAV ITR – EF1a promoter – *GLB1* gene – SV40 poly A – 3' ITR. In certain embodiments, the vector genome comprises a 5' AAV ITR – UbC promoter – *GLB1* gene – SV40 poly A – 3' ITR. In certain embodiments, the *GLB1* gene has SEQ ID NO: 5. In certain embodiments, the *GLB1* gene has SEQ ID NO: 6. In certain embodiments, the *GLB1* gene has SEQ ID NO: 7. In certain embodiments, the *GLB1* gene has SEQ ID NO: 8. In certain embodiments, the vector genome has the sequence of SEQ ID NO: 12 or a sequence at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99%, to about 99.9% identical thereto. In certain embodiments, the vector genome has the sequence of SEQ ID NO: 13 or a sequence at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99%, to about 99.9% identical thereto. In certain embodiments, the vector genome has the sequence of SEQ ID NO: 14 or a sequence at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99%, to about 99.9% identical thereto. In certain embodiments, the vector genome has the sequence of SEQ ID NO: 15 or a sequence at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99%, to about 99.9% identical thereto. In certain embodiments, the vector genome has the sequence of SEQ ID NO: 16 or a sequence at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99%, to about 99.9% identical thereto.

IV. rAAV Production

For use in producing an AAV viral vector (*e.g.*, a recombinant (r) AAV), the vector genomes can be carried on any suitable vector, *e.g.*, a plasmid, which is delivered to a packaging host cell. The plasmids useful in this invention may be engineered such that they are suitable for replication and packaging *in vitro* in prokaryotic cells, insect cells, mammalian cells, among others. Suitable transfection techniques and packaging host cells

are known and/or can be readily designed by one of skill in the art. An illustrative production process is provided in FIGs 12A – 12B.

Methods for generating and isolating AAVs suitable for use as vectors are known in the art. See generally, *e.g.,* Grieger & Samulski, 2005, Adeno-associated virus as a gene therapy vector: Vector development, production and clinical applications, *Adv. Biochem. Engin/Biotechnol.* 99: 119-145; Buning *et al.,* 2008, Recent developments in adeno-associated virus vector technology, *J. Gene Med.* 10:717-733; and the references cited below, each of which is incorporated herein by reference in its entirety. For packaging a gene into virions, the ITRs are the only AAV components required in *cis* in the same construct as the nucleic acid molecule containing the gene. The *cap* and *rep* genes can be supplied in *trans.*

In one embodiment, the selected genetic element may be delivered to an AAV packaging cell by any suitable method, including transfection, electroporation, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion. Stable AAV packaging cells can also be made. The methods used to make such constructs are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. See, *e.g.,* Molecular Cloning: A Laboratory Manual, ed. Green and Sambrook, Cold Spring Harbor Press, Cold Spring Harbor, NY (2012).

The term "AAV intermediate" or "AAV vector intermediate" refers to an assembled rAAV capsid which lacks the desired genomic sequences packaged therein. These may also be termed an "empty" capsid. Such a capsid may contain no detectable genomic sequences of an expression cassette, or only partially packaged genomic sequences which are insufficient to achieve expression of the gene product (for example, β-gal). These empty capsids are non-functional to transfer the gene of interest to a host cell. In certain embodiment, the rAAV.GLB1 or the composition as described herein may be at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 99.9% free from an AAV intermediate, *i.e.,* containing less than 20%, 15%, 10%, 5%, 4%, 3%, 2%, 1%, or 0.1% AAV intermediates.

The recombinant adeno-associated virus (AAV) described herein may be generated using techniques which are known. See, *e.g.,* WO 2003/042397; WO 2005/033321, WO 2006/110689; US 7588772 B2. Such a method involves culturing a host cell which contains a nucleic acid sequence encoding an AAV capsid protein; a functional *rep* gene; an expression cassette composed of, at a minimum, AAV inverted terminal repeats (ITRs) and a transgene; and sufficient helper functions to permit packaging of the expression cassette into

the AAV capsid protein. Methods of generating the capsid, coding sequences therefor, and methods for production of rAAV viral vectors have been described. See, *e.g.,* Gao, *et al,* Proc. Natl. Acad. Sci. U.S.A. 100 (10), 6081-6086 (2003) and US 2013/0045186A1.

In one embodiment, a production cell culture useful for producing a recombinant AAV (such as rAAVhu68) is provided. Such a cell culture contains a nucleic acid which expresses the AAV capsid protein in the host cell; a nucleic acid molecule suitable for packaging into the AAV capsid, *e.g.*, a vector genome which contains AAV ITRs and a GLB1 gene operably linked to regulatory sequences which direct expression of the gene in a cell (for example, a cell in a patient in need); and sufficient AAV rep functions and adenovirus helper functions to permit packaging of the vector genome into the recombinant AAV capsid. In one embodiment, the cell culture is composed of mammalian cells (*e.g.*, human embryonic kidney 293 cells, among others) or insect cells (*e.g.*, Spodoptera frugiperda (Sf9) cells). In certain embodiments, baculovirus provides the helper functions necessary for packaging the vector genome into the recombinant AAVhu68 capsid.

Optionally the rep functions are provided by an AAV other than the capsid source AAV, AAVhu68. In certain embodiments, at least parts of the rep functions are from AAVhu68. In another embodiment, the rep protein is a heterologous rep protein other than AAVhu68 rep, for example but not limited to, AAV1 rep protein, AAV2 rep protein, AAV3 rep protein, AAV4 rep protein, AAV5 rep protein, AAV6 rep protein, AAV7 rep protein, AAV8 rep protein; or rep 78, rep 68, rep 52, rep 40, rep68/78 and rep40/52; or a fragment thereof; or another source. Any of these AAVhu68 or mutant AAV capsid sequences may be under the control of exogenous regulatory control sequences which direct expression thereof in a host cell.

In one embodiment, cells are manufactured in a suitable cell culture (*e.g.*, HEK 293 or Sf9) or suspension. Methods for manufacturing the gene therapy vectors described herein include methods well known in the art such as generation of plasmid DNA used for production of the gene therapy vectors, generation of the vectors, and purification of the vectors. In some embodiments, the gene therapy vector is a rAAV and the plasmids generated are an AAV cis-plasmid encoding the AAV vector genome comprising the gene of interest, an AAV trans-plasmid containing AAV rep and cap genes, and an adenovirus helper plasmid. The vector generation process can include method steps such as initiation of cell culture, passage of cells, seeding of cells, transfection of cells with the plasmid DNA, post-transfection medium exchange to serum free medium, and the harvest of vector-containing cells and culture media. The harvested vector-containing cells and culture media are referred

to herein as crude cell harvest. In yet another system, the gene therapy vectors are introduced into insect cells by infection with baculovirus-based vectors. For reviews on these production systems, see generally, *e.g.*, Zhang *et al.*, 2009, Adenovirus-adeno-associated virus hybrid for large-scale recombinant adeno-associated virus production, *Human Gene Therapy* 20:922-929, the contents of each of which is incorporated herein by reference in its entirety. Methods of making and using these and other AAV production systems are also described in the following U.S. patents, the contents of each of which is incorporated herein by reference in its entirety: 5,139,941; 5,741,683; 6,057,152; 6,204,059; 6,268,213; 6,491,907; 6,660,514; 6,951,753; 7,094,604; 7,172,893; 7,201,898; 7,229,823; and 7,439,065.

The crude cell harvest may thereafter be subject method steps such as concentration of the rAAV harvest, diafiltration of the rAAV harvest, microfluidization of the rAAV harvest, nuclease digestion of the rAAV harvest, filtration of microfluidized intermediate, crude purification by chromatography, crude purification by ultracentrifugation, buffer exchange by tangential flow filtration, and/or formulation and filtration to prepare bulk rAAV.

A two-step affinity chromatography purification at high salt concentration followed anion exchange resin chromatography are used to purify the rAAV drug product and to remove empty capsids. These methods are described in more detail in WO 2017/160360, International Patent Application No. PCT/US2016/065970, filed December 9, 2016 and its priority documents, US Patent Application Nos. 62/322,071, filed April 13, 2016 and 62/226,357, filed December 11, 2015 and entitled "Scalable Purification Method for AAV9", which is incorporated by reference herein.

To calculate empty and full particle content, VP3 band volumes for a selected sample (*e.g.*, in examples herein an iodixanol gradient-purified preparation where # of genome copies (GC) = # of particles) are plotted against GC particles loaded. The resulting linear equation (y = mx+c) is used to calculate the number of particles in the band volumes of the test article peaks. The number of particles (pt) per 20 µL loaded is then multiplied by 50 to give particles (pt) /mL. Pt/mL divided by GC/mL gives the ratio of particles to genome copies (pt/GC). Pt/mL–GC/mL gives empty pt/mL. Empty pt/mL divided by pt/mL and x 100 gives the percentage of empty particles.

Generally, methods for assaying for empty capsids and rAAV particles with packaged vector genomes have been known in the art. See, *e.g.*, Grimm *et al.*, *Gene Therapy* (1999) 6:1322-1330; Sommer *et al.*, Molec. Ther. (2003) 7:122-128. To test for

denatured capsid, the methods include subjecting the treated AAV stock to SDS-polyacrylamide gel electrophoresis, consisting of any gel capable of separating the three capsid proteins, for example, a gradient gel containing 3-8% Tris-acetate in the buffer, then running the gel until sample material is separated, and blotting the gel onto nylon or nitrocellulose membranes, preferably nylon. Anti-AAV capsid antibodies are then used as the primary antibodies that bind to denatured capsid proteins, preferably an anti-AAV capsid monoclonal antibody, most preferably the B1 anti-AAV-2 monoclonal antibody (Wobus et al., *J. Virol.* (2000) 74:9281-9293). A secondary antibody is then used, one that binds to the primary antibody and contains a means for detecting binding with the primary antibody, more preferably an anti-IgG antibody containing a detection molecule covalently bound to it, most preferably a sheep anti-mouse IgG antibody covalently linked to horseradish peroxidase. A method for detecting binding is used to semi-quantitatively determine binding between the primary and secondary antibodies, preferably a detection method capable of detecting radioactive isotope emissions, electromagnetic radiation, or colorimetric changes, most preferably a chemiluminescence detection kit. For example, for SDS-PAGE, samples from column fractions can be taken and heated in SDS-PAGE loading buffer containing reducing agent (*e.g.*, DTT), and capsid proteins were resolved on pre-cast gradient polyacrylamide gels (*e.g.*, Novex). Silver staining may be performed using SilverXpress (Invitrogen, CA) according to the manufacturer's instructions or other suitable staining method, i.e. SYPRO ruby or coomassie stains. In one embodiment, the concentration of AAV vector genomes (vg) in column fractions can be measured by quantitative real time PCR (Q-PCR). Samples are diluted and digested with DNase I (or another suitable nuclease) to remove exogenous DNA. After inactivation of the nuclease, the samples are further diluted and amplified using primers and a TaqMan™ fluorogenic probe specific for the DNA sequence between the primers. The number of cycles required to reach a defined level of fluorescence (threshold cycle, Ct) is measured for each sample on an Applied Biosystems Prism 7700 Sequence Detection System. Plasmid DNA containing identical sequences to that contained in the rAAV is employed to generate a standard curve in the Q-PCR reaction. The cycle threshold (Ct) values obtained from the samples are used to determine vector genome titer by normalizing it to the Ct value of the plasmid standard curve. End-point assays based on the digital PCR can also be used.

In one aspect, an optimized q-PCR method is used which utilizes a broad spectrum serine protease, e.g., proteinase K (such as is commercially available from Qiagen). More particularly, the optimized qPCR genome titer assay is similar to a standard assay, except

that after the DNase I digestion, samples are diluted with proteinase K buffer and treated with proteinase K followed by heat inactivation. Suitably samples are diluted with proteinase K buffer in an amount equal to the sample size. The proteinase K buffer may be concentrated to 2 fold or higher. Typically, proteinase K treatment is about 0.2 mg/mL, but may be varied from 0.1 mg/mL to about 1 mg/mL. The treatment step is generally conducted at about 55 °C for about 15 minutes, but may be performed at a lower temperature (*e.g.*, about 37 °C to about 50 °C) over a longer time period (*e.g.*, about 20 minutes to about 30 minutes), or a higher temperature (*e.g.*, up to about 60 °C) for a shorter time period (*e.g.*, about 5 to 10 minutes). Similarly, heat inactivation is generally at about 95 °C for about 15 minutes, but the temperature may be lowered (*e.g.*, about 70 to about 90 °C) and the time extended (*e.g.*, about 20 minutes to about 30 minutes). Samples are then diluted (*e.g.*, 1000 fold) and subjected to TaqMan analysis as described in the standard assay.

Additionally, or alternatively, droplet digital PCR (ddPCR) may be used. For example, methods for determining single-stranded and self-complementary AAV vector genome titers by ddPCR have been described. See, *e.g.*, M. Lock *et al*, Hum Gene Ther Methods. 2014 Apr;25(2):115-25. doi: 10.1089/hgtb.2013.131. Epub 2014 Feb 14.

In brief, the method for separating rAAVhu68 particles having packaged genomic sequences from genome-deficient AAVhu68 intermediates involves subjecting a suspension comprising recombinant AAVhu68 viral particles and AAVhu68 capsid intermediates to fast performance liquid chromatography, wherein the AAVhu68 viral particles and AAVhu68 intermediates are bound to a strong anion exchange resin equilibrated at a pH of about 10.2, and subjected to a salt gradient while monitoring eluate for ultraviolet absorbance at about 260 nanometers (nm) and about 280 nm. Although less optimal for rAAVhu68, the pH may be in the range of about 10.0 to 10.4. In this method, the AAVhu68 full capsids are collected from a fraction which is eluted when the ratio of A260/A280 reaches an inflection point. In one example, for the Affinity Chromatography step, the diafiltered product may be applied to a Capture Select™ Poros- AAV2/9 affinity resin (Life Technologies) that efficiently captures the AAV2/hu68 serotype. Under these ionic conditions, a significant percentage of residual cellular DNA and proteins flow through the column, while AAV particles are efficiently captured.

Also provided herein is a production vector (such as a plasmid) or a host cell for producing the vector genome and/or the rAAV.GLB1 as described herein. As used herein, a production vector carrying a vector genome to a host cell for generating and/or packaging a gene therapy vector as described herein.

The rAAV.GLB1 (for example, rAAVhu68.GLB1) is suspended in a suitable physiologically compatible composition (*e.g.*, a buffered saline). This composition may be frozen for storage, later thawed and optionally diluted with a suitable diluent. Alternatively, the rAAV.GLB1 may be prepared as a composition which is suitable for delivery to a patient without proceeding through the freezing and thawing steps.

V. Compositions and Uses

Provided herein are compositions containing at least one rAAV stock (*e.g.*, an rAAVhu68 stock or a mutant rAAVhu68 stock) and an optional carrier, excipient and/or preservative.

As used herein, a "stock" of rAAV refers to a population of rAAV. Despite heterogeneity in their capsid proteins due to deamidation, rAAV in a stock are expected to share an identical vector genome. A stock can include rAAV having capsids with, for example, heterogeneous deamidation patterns characteristic of the selected AAV capsid proteins and a selected production system. The stock may be produced from a single production system or pooled from multiple runs of the production system. A variety of production systems, including but not limited to those described herein, may be selected.

In particular, the composition is for the treatment of GM1 gangliosidosis. In one embodiment, the composition is suitable for administration to a patient having GM1 gangliosidosis or a patient having infantile gangliosidosis who is 18 months of age or younger. In one embodiment, the composition is suitable for administration to a patient having GM1 gangliosidosis or a patient having infantile gangliosidosis who is 36 months of age or younger. In one embodiment, the composition is suitable for administration to a patient in need thereof to ameliorate symptoms of GM1 gangliosidosis, or ameliorate neurological symptoms of GM1 gangliosidosis. In some embodiments, the composition is for use in the manufacture of a medication for the treatment of GM1 gangliosidosis.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Supplementary active ingredients can also be incorporated into the compositions.

In certain embodiments, provided herein is a composition comprising the rAAV.GLB1 as described herein and a pharmaceutically acceptable carrier. The phrase

"pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a host.

In certain embodiments, provided herein is a composition comprising the rAAV.GLB1 as described herein and a delivery vehicle. Delivery vehicles such as liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, may be used for the introduction of the compositions of the present invention into suitable host cells. In particular, the rAAV delivered vector genomes may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like.

In one embodiment, a composition includes a final formulation suitable for delivery to a subject/patient, *e.g.*, is an aqueous liquid suspension buffered to a physiologically compatible pH and salt concentration. Optionally, one or more surfactants are present in the formulation. In another embodiment, the composition may be transported as a concentrate which is diluted for administration to a subject. In other embodiments, the composition may be lyophilized and reconstituted at the time of administration.

A suitable surfactant, or combination of surfactants, may be selected from among non-ionic surfactants that are nontoxic. In one embodiment, a difunctional block copolymer surfactant terminating in primary hydroxyl groups is selected, *e.g.*, such as Pluronic® F68 [BASF], also known as Poloxamer 188, which has a neutral pH, has an average molecular weight of 8400. Other surfactants and other Poloxamers may be selected, *i.e.*, nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)), SOLUTOL HS 15 (Macrogol-15 Hydroxystearate), LABRASOL (Polyoxy caprylic glyceride), polyoxy 10 oleyl ether, TWEEN (polyoxyethylene sorbitan fatty acid esters), ethanol and polyethylene glycol. In one embodiment, the formulation contains a poloxamer. These copolymers are commonly named with the letter "P" (for poloxamer) followed by three digits: the first two digits x 100 give the approximate molecular mass of the polyoxypropylene core, and the last digit x 10 gives the percentage polyoxyethylene content. In one embodiment Poloxamer 188 is selected. In one embodiment, the surfactant may be present in an amount up to about 0.0005 % to about 0.001% (based on weight ratio, w/w %) of the suspension. In another embodiment, the surfactant may be present in an amount up to about 0.0005 % to about 0.001% (based on volume ratio, v/v %) of the suspension. In yet another embodiment, the surfactant may be

present in an amount up to about 0.0005 % to about 0.001% of the suspension, wherein n % indicates n gram per 100 mL of the suspension.

The rAAV.GLB1 is administered in sufficient amounts to transfect the cells and to provide sufficient levels of gene transfer and expression to provide a therapeutic benefit without undue adverse effects, or with medically acceptable physiological effects, which can be determined by those skilled in the medical arts. Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to a desired organ (*e.g.*, brain, CSF, the liver (optionally via the hepatic artery), lung, heart, eye, kidney,), oral, inhalation, intranasal, intrathecal, intratracheal, intraarterial, intraocular, intravenous, intramuscular, subcutaneous, intradermal, intraparenchymal, intracerebroventricular, intrathecal, ICM, lumbar puncture and other parenteral routes of administration. Routes of administration may be combined, if desired.

Dosages of the rAAV.GLB1 depend primarily on factors such as the condition being treated, the age, weight and health of the patient, and can thus vary among patients. For example, a therapeutically effective human dosage of the rAAV.GLB1 is generally in the range of from about 25 to about 1000 microliters to about 100 mL of solution containing from about 1×10^9 to 1×10^{16} vector genome copies per mL. In certain embodiments, a volume of about 1 mL to about 15 mL, or about 2.5 mL to about 10 mL, or about 5 mL suspension is delivered. In certain embodiments, a volume of about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, or about 15 mL suspension is delivered.

In some embodiments, the composition is for administration in a single dose. In some embodiments, the composition is for administration in multiple doses.

In certain embodiments, a dose from about 8×10^{12} genome copies (GC) of rAAV.GLB1 per patient to about 3×10^{14} GC of rAAV.GLB1 per patient is administered in the volume described herein. In certain embodiments, a dose from about 2×10^{12} GC of rAAV.GLB1 per patient to about 3×10^{14} GC of rAAV.GLB1 per patient, or from about 2×10^{13} GC of rAAV.GLB1 per patient to about 3×10^{14} GC of rAAV.GLB1 per patient, or from about 8×10^{13} GC of rAAV.GLB1 per patient to about 3×10^{14} GC of rAAV.GLB1 per patient, or about 9×10^{13} GC of rAAV.GLB1 per patient, or about 8.9×10^{12} to 2.7×10^{14} GC total is administered in the volume.

In certain embodiments, a dose from 1×10^{10} GC of rAAV.GLB1 per g brain mass (GC/g brain mass) to 3.4×10^{11} GC/g brain mass is administered in the volume as described herein. In certain embodiments, a dose from 3.4×10^{10} GC/g brain mass to 3.4×10^{11} GC/g

brain mass, or from 1.0 x 10^{11} GC/g brain mass to 3.4 x 10^{11} GC/g brain mass, or about 1.1 x 10^{11} GC/g brain mass, or from about 1.1 x10^{10} GC/g brain mass to about 3.3 x 10^{11} GC/g brain mass is administered in the volume. In certain embodiments, a dose of about 3.0 x10^{9}, about 4.0 x10^{9}, about 5.0 x10^{9}, about 6.0 x10^{9}, about 7.0 x10^{9}, about 8.0 x10^{9}, about 9.0 x10^{9}, about 1.0 x10^{10}, about 1.1 x10^{10}, about 1.5 x10^{10}, about 2.0 x10^{10}, about 2.5 x10^{10}, about 3.0 x10^{10}, about 3.3 x10^{10}, about 3.5 x10^{10}, about 4.0 x10^{10}, about 4.5 x10^{10}, about 5.0 x10^{10}, about 5.5 x10^{10}, about 6.0 x10^{10}, about 6.5 x10^{10}, about 7.0 x10^{10}, about 7.5 x10^{10}, about 8.0 x10^{10}, about 8.5 x10^{10}, about 9.0 x10^{10}, about 9.5 x10^{10}, about 1.0 x10^{11}, about 1.1 x10^{11}, about 1.5 x10^{11}, about 2.0 x10^{11}, about 2.5 x10^{11}, about 3.0 x10^{11}, about 3.3 x10^{11}, about 3.5 x10^{11}, about 4.0 x10^{11}, about 4.5 x10^{11}, about 5.0 x10^{11}, about 5.5 x10^{11}, about 6.0 x10^{11}, about 6.5 x10^{11}, about 7.0 x10^{11}, about 7.5 x10^{11}, about 8.0 x10^{11}, about 8.5 x10^{11}, about 9.0 x10^{11} GC per gram brain mass is administered in the volume. In certain embodiments, the dose reflects the minimum effective dose shown in a GM1 animal model and adjusted for use in a human patient based on genome copies per gram brain mass. In one embodiment, the dose for use in a human patient is calculated using the assumed brain masses listed in the table below.

Subject Age	Assumed brain mass (g)
≥ 4 to < 9 months	600
≥ 9 to < 18 months	1000
≥ 18 months to < 3 years	1100
≥ 3 years	1300

Age	≥1 – <4 months	≥4 – <8 months	≥8 – <12 months	≥12 months
Mean human brain weight (g)	488	610	780	960
Low dose 3.33 x 10^{10} GC/g	1.6 x 10^{13} GC	2.1 x 10^{13} GC	2.6 x 10^{13} GC	3.2 x 10^{13} GC
High dose 1.11 x 10^{11}GC/g	5.4 x 10^{13} GC	6.8 x 10^{13} GC	8.7 x 10^{13} GC	1.0 x 10^{14} GC

Maximum Feasible dose 3.33 10^{11}GC/g	1.6 x 10^{14} GC	2.1x10^{14} GC	2.6x10^{14} GC	3.2x10^{14} GC

The dosage is adjusted to balance the therapeutic benefit against any side effects and such dosages may vary depending upon the therapeutic application for which the rAAV.GLB1 is employed. The levels of expression of the transgene product (for example, β-gal) can be monitored to determine the frequency of dosage resulting in rAAV.GLB1, preferably rAAV containing the minigene (for example, the *GLB1* gene). Optionally, dosage regimens similar to those described for therapeutic purposes may be utilized for immunization using the compositions of the invention.

The replication-defective virus compositions can be formulated in dosage units to contain an amount of replication-defective virus (for example, rAAV.GLB1, rAAVhu68.GLB1, or rAAVhu68.UbC.GLB1) that is in the range of about 1.0 x 10^9 GC to about 1.0 x 10^{16} GC (to treat a subject) including all integers or fractional amounts within the range, and preferably 1.0 x 10^{12} GC to 1.0 x 10^{14} GC for a human patient. In one embodiment, the compositions are formulated to contain at least 1x10^9, 2x10^9, 3x10^9, 4x10^9, 5x10^9, 6x10^9, 7x10^9, 8x10^9, or 9x10^9 GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1x10^{10}, 2x10^{10}, 3x10^{10}, 4x10^{10}, 5x10^{10}, 6x10^{10}, 7x10^{10}, 8x10^{10}, or 9x10^{10} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1x10^{11}, 2x10^{11}, 3x10^{11}, 4x10^{11}, 5x10^{11}, 6x10^{11}, 7x10^{11}, 8x10^{11}, or 9x10^{11} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1x10^{12}, 2x10^{12}, 3x10^{12}, 4x10^{12}, 5x10^{12}, 6x10^{12}, 7x10^{12}, 8x10^{12}, or 9x10^{12} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1x10^{13}, 2x10^{13}, 3x10^{13}, 4x10^{13}, 5x10^{13}, 6x10^{13}, 7x10^{13}, 8x10^{13}, or 9x10^{13} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1x10^{14}, 2x10^{14}, 3x10^{14}, 4x10^{14}, 5x10^{14}, 6x10^{14}, 7x10^{14}, 8x10^{14}, or 9x10^{14} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1x10^{15}, 2x10^{15}, 3x10^{15}, 4x10^{15}, 5x10^{15}, 6x10^{15}, 7x10^{15}, 8x10^{15}, or 9x10^{15} GC per dose including all integers or fractional amounts

within the range. In one embodiment, for human application the dose can range from $1x10^{10}$ to about $1x10^{12}$ GC per dose including all integers or fractional amounts within the range.

These above doses may be administered in a variety of volumes of carrier, excipient or buffer formulation, ranging from about 25 to about 1000 microliters, or higher volumes, including all numbers within the range, depending on the size of the area to be treated, the viral titer used, the route of administration, and the desired effect of the method. In one embodiment, the volume of carrier, excipient or buffer is at least about 25 µL. In one embodiment, the volume is about 50 µL. In another embodiment, the volume is about 75 µL. In another embodiment, the volume is about 100 µL. In another embodiment, the volume is about 125 µL. In another embodiment, the volume is about 150 µL. In another embodiment, the volume is about 175 µL. In yet another embodiment, the volume is about 200 µL. In another embodiment, the volume is about 225 µL. In yet another embodiment, the volume is about 250 µL. In yet another embodiment, the volume is about 275 µL. In yet another embodiment, the volume is about 300 µL. In yet another embodiment, the volume is about 325 µL. In another embodiment, the volume is about 350 µL. In another embodiment, the volume is about 375 µL. In another embodiment, the volume is about 400 µL. In another embodiment, the volume is about 450 µL. In another embodiment, the volume is about 500 µL. In another embodiment, the volume is about 550 µL. In another embodiment, the volume is about 600 µL. In another embodiment, the volume is about 650 µL. In another embodiment, the volume is about 700 µL. In another embodiment, the volume is from about 700 to 1000 µL. In some embodiments, the volume is from about 1 mL to 10mL, In some embodiments, the volume is less than 15mL.

In certain embodiments, the dose may be in the range of about 1×10^9 GC/g brain mass to about 1×10^{12} GC/g brain mass. In certain embodiments, the dose may be in the range of about 3×10^{10} GC/g brain mass to about 3×10^{11} GC/g brain mass. In certain embodiments, the dose may be in the range of about 5×10^{10} GC/g brain mass to about 1.85×10^{11} GC/g brain mass.

In one embodiment, the viral constructs may be delivered in doses of from at least about least $1x10^9$ GC to about 1×10^{15}, or about 1×10^{11} to 5×10^{13} GC. Suitable volumes for delivery of these doses and concentrations may be determined by one of skill in the art. For example, volumes of about 1 µL to 150 mL may be selected, with the higher volumes being selected for adults. Typically, for newborn infants a suitable volume is about 0.5 mL to about 10 mL, for older infants, about 0.5 mL to about 15 mL may be selected. For toddlers, a volume of about 0.5 mL to about 20 mL may be selected. For children, volumes

of up to about 30 mL may be selected. For pre-teens and teens, volumes up to about 50 mL may be selected. In still other embodiments, a patient may receive an intrathecal administration in a volume of about 5 mL to about 15 mL are selected, or about 7.5 mL to about 10 mL. Other suitable volumes and dosages may be determined. The dosage may be adjusted to balance the therapeutic benefit against any side effects and such dosages may vary depending upon the therapeutic application for which the rAAV.GLB1 is employed.

The above-described rAAV.GLB1 may be delivered to host cells according to published methods. The rAAV, preferably suspended in a physiologically compatible carrier, may be administered to a human or non-human mammalian patient. In certain embodiments, for administration to a human patient, the rAAV is suitably suspended in an aqueous solution containing saline, a surfactant, and a physiologically compatible salt or mixture of salts. Suitably, the formulation is adjusted to a physiologically acceptable pH, e.g., in the range of pH 6 to 9, or pH 6.0 to 7.5, or pH 6.2 to 7.7, or pH 6.5 to 7.5, pH 7.0 to 7.7, or pH 7.2 to 7.8, or about 7.0. In certain embodiments, the formulation is adjusted to a pH of about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, about 6.9, about 7.0, about 7.1, about 7.2, about 7.3 about 7.4, about 7.5, about 7.6, about 7.7, or about 7.8. In certain embodiments, a pH of about 7.28 to about 7.32, about 6.0 to about 7.5, about 6.2 to about 7.7, about 7.5 to about 7.8, about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, about 6.9, about 7.0, about 7.1, about 7.2, about 7.3 about 7.4, about 7.5, about 7.6, about 7.7, or about 7.8 may be desired for intrathecal delivery; whereas for intravenous delivery, a pH of about 6.8 to about 7.2 may be desired. However, other pHs within the broadest ranges and these subranges may be selected for other route of delivery.

In another embodiment, the composition includes a carrier, diluent, excipient and/or adjuvant. Suitable carriers may be readily selected by one of skill in the art in view of the indication for which the transfer virus is directed. For example, one suitable carrier includes saline, which may be formulated with a variety of buffering solutions (*e.g.*, phosphate buffered saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, and water. The buffer/carrier should include a component that prevents the rAAV, from sticking to the infusion tubing but does not interfere with the rAAV binding activity *in vivo*. A suitable surfactant, or combination of surfactants, may be selected from among non-ionic surfactants that are nontoxic. In one embodiment, a difunctional block copolymer surfactant terminating in primary hydroxyl groups is selected, e.g., such as Poloxamer 188 (also known under the

commercial names Pluronic® F68 [BASF], Lutrol® F68, Synperonic® F68, Kolliphor® P188) which has a neutral pH, has an average molecular weight of 8400. Other surfactants and other Poloxamers may be selected, i.e., nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)), SOLUTOL HS 15 (Macrogol-15 Hydroxystearate), LABRASOL (Polyoxy capryllic glyceride), polyoxy -oleyl ether, TWEEN (polyoxyethylene sorbitan fatty acid esters), ethanol and polyethylene glycol. In one embodiment, the formulation contains a poloxamer. These copolymers are commonly named with the letter "P" (for poloxamer) followed by three digits: the first two digits x 100 give the approximate molecular mass of the polyoxypropylene core, and the last digit x 10 gives the percentage polyoxyethylene content. In one embodiment Poloxamer 188 is selected. The surfactant may be present in an amount up to about 0.0005 % to about 0.001% of the suspension.

In one example, the formulation may contain, *e.g.*, buffered saline solution comprising one or more of sodium chloride, sodium bicarbonate, dextrose, magnesium sulfate (*e.g.*, magnesium sulfate ·$7H_2O$), potassium chloride, calcium chloride (*e.g.*, calcium chloride ·$2H_2O$), dibasic sodium phosphate, and mixtures thereof, in water. Suitably, for intrathecal delivery, the osmolarity is within a range compatible with cerebrospinal fluid (*e.g.*, about 275 milliosmoles/liter (mOsm/L) to about 290 mOsm/L); see, *e.g.*, emedicine.medscape.com/-article/2093316-overview. Optionally, for intrathecal delivery, a commercially available diluent may be used as a suspending agent, or in combination with another suspending agent and other optional excipients. See, *e.g.*, Elliotts B® solution [Lukare Medical]. Each 10 mL of Elliotts B Solution contains: Sodium Chloride, USP - 73 mg; Sodium Bicarbonate, USP - 19 mg; Dextrose, USP - 8 mg; Magnesium Sulfate • $7H_2O$, USP - 3 mg; Potassium Chloride, USP - 3 mg; Calcium Chloride • $2H_2O$, USP - 2 mg; sodium Phosphate, dibasic • $7H_2O$, USP - 2 mg; Water for Injection, USP - qs 10 mL. Concentration of Electrolytes: Sodium (149 mEq/liter); Bicarbonate (22.6 mEq/liter); Potassium (4.0 mEq/liter); Chloride (132 mEq/liter); Calcium (2.7 mEq/liter); Sulfate (2.4 mEq/liter); Magnesium (2.4 mEq/liter); Phosphate (1.5 mEq/liter).

The formulae and molecular weights of the ingredients are:

INGREDIENT	MOLECULAR FORMULA	MOLECULAR WEIGHT
Sodium Chloride	NaCl	58.44

Sodium Bicarbonate	$NaHCO_3$	84.01
Dextrose	$C_6H_{12}O_6$	180.16
Magnesium Sulfate • $7H_2O$	$Mg_2SO_4 \cdot 7H_2O$	246.48
Potassium Chloride	KCl	74.55
Calcium Chloride • $2H_2O$	$CaCl_2 \cdot 2H_2O$	147.01
Sodium Phosphate, dibasic • $7H_2O$	$Na_2HPO_4 \cdot 7H_2O$	268.07

The pH of Elliotts B Solution is 6 to 7.5, and the osmolarity is 288 mOsmol per liter (calculated). In certain embodiments, the intrathecal final formulation buffer (ITFFB) formulation buffer comprises an artificial cerebrospinal fluid comprising buffered saline and one or more of sodium, calcium, magnesium, potassium, or mixtures thereof; and a surfactant. In certain embodiments, the surfactant comprises about 0.0005 % to about 0.001% of the suspension. In a further embodiment, the percentage (%) is calculated based on weight (w) ratio (i.e., w/w).

In certain embodiments, the composition containing the rAAVhu68.GLB1 (e.g., the ITFFB formulation) is at a pH in the range of 6.0 to 7.5, or 6.2 to 7.7, or 6.8 to 8, or 7.2 to 7.8, or 7.5 to 8. In certain embodiments, the final formulation is at a pH of about 7, or 7 to 7.4 , or 7.2. In certain embodiments, for intrathecal delivery, a pH above 7.5 may be desired, *e.g.*, 7.5 to 8, or 7.8.

In certain embodiments, a pH of about 7 is desired for intrathecal delivery as well as other delivery routes.

In certain embodiments, the formulation may contain a buffered saline aqueous solution not comprising sodium bicarbonate. Such a formulation may contain a buffered saline aqueous solution comprising one or more of sodium phosphate, sodium chloride, potassium chloride, calcium chloride, magnesium chloride and mixtures thereof, in water, such as a Harvard's buffer. The aqueous solution may further contain Kolliphor® P188, a poloxamer which is commercially available from BASF which was formerly sold under the trade name Lutrol® F68. In certain embodiment, the aqueous solution may have a pH of 7.2. In certain embodiment, the aqueous solution may have a pH of about 7.

In another embodiment, the formulation may contain a buffered saline aqueous solution comprising 1 mM Sodium Phosphate (Na_3PO_4), 150 mM sodium chloride (NaCl), 3mM potassium chloride (KCl), 1.4 mM calcium chloride ($CaCl_2$), 0.8 mM magnesium chloride ($MgCl_2$), and 0.001% poloxamer (*e.g.*, Kolliphor®) 188. In certain embodiments, the formulation has a pH of about 7.2. In certain embodiments, the formulation has a pH of

about 7. See, e.g., harvardapparatus.com/harvard-apparatus-perfusion-fluid.html. In certain embodiments, Harvard's buffer is preferred due to better pH stability observed with Harvard's buffer. The table below provides a comparison of Harvard's buffer and Elliot's B buffer.

Cerebrospinal Fluid (CSF) Compositions

Component	Units	CSF	Elliot's B	Harvard's
Na^+	mEq/L	117-137	149	150
K^+	mEq/L	2.3-4.6	4.0	3.0
Mg^+	mEq/L	2.2	2.4	0.8
Ca^{2+}	mEq/L	2.2	2.7	1.4
Cl^-	mEq/L	113-127	132	155
HCO_3^-	mEq/L	22.9	22.6	**0**
Phos	mg/dL	1.2-2.1	1.5	1.0
Glucose	mg/dL	45-80	80	-
Pluronic	%	-	0.001% (added)	0.001% (added)
Osmolarity	mOsm/L	295	288	290
pH		7.31	6.0-7.5* Drift to 9+ (8.2+ w/o titration)	7.2 (titrated to)

In certain embodiments, the formulation buffer is artificial CSF with Pluronic F68. In other embodiments, the formulation may contain one or more permeation enhancers. Examples of suitable permeation enhancers may include, *e.g.*, mannitol, sodium glycocholate, sodium taurocholate, sodium deoxycholate, sodium salicylate, sodium caprylate, sodium caprate, sodium lauryl sulfate, polyoxyethylene-9-laurel ether, or EDTA.

Optionally, the compositions of the invention may contain, in addition to the rAAV and carrier(s), other conventional pharmaceutical ingredients, such as preservatives, or chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

The compositions according to the present invention may comprise a pharmaceutically acceptable carrier, such as defined above. Suitably, the compositions described herein comprise an effective amount of one or more AAV suspended in a pharmaceutically suitable carrier and/or admixed with suitable excipients designed for delivery to the subject via injection, osmotic pump, intrathecal catheter, or for delivery by another device or route. In one example, the composition is formulated for intrathecal delivery. In one embodiment, the composition is formulated for administration via an intra-cisterna magna injection (ICM). In one embodiment, the composition is formulated for administration via a CT-guided sub-occipital injection into the cisterna magna.

As used herein, the terms "intrathecal delivery" or "intrathecal administration" refer to a route of administration for drugs via an injection into the spinal canal, more specifically into the subarachnoid space so that it reaches the cerebrospinal fluid (CSF). Intrathecal delivery may include lumbar puncture, intraventricular (including intracerebroventricular (ICV)), suboccipital/intracisternal, and/or C1-2 puncture. For example, material may be introduced for diffusion throughout the subarachnoid space by means of lumbar puncture. In another example, injection may be into the cisterna magna.

As used herein, the terms "intracisternal delivery" or "intracisternal administration" refer to a route of administration for drugs directly into the cerebrospinal fluid of the cisterna magna cerebellomedularis, more specifically via a suboccipital puncture or by direct injection into the cisterna magna or via permanently positioned tube.

In certain embodiments, an aqueous composition comprising a formulation buffer and an rAAV.GLB1 (for example, rAAVhu68.GLB1) as provided herein is delivered to a patient in need thereof. In certain embodiments, the rAAV.GLB1 has an AAV capsid (for example, an AAVhu68 capsid) and a vector genome comprising a 5' AAV ITR - promoter – optional enhancer – optional intron – GLB1 gene – polyA – 3' ITR. In certain embodiments, the ITRs are from AAV2. In certain embodiments, more than one promoter is present. In certain embodiments, the enhancer is present in the vector genome. In certain embodiments, more than one enhancer is present. In certain embodiments, an intron is present in the vector genome. In certain embodiments, the enhancer and intron are present. In certain embodiments, the polyA is an SV40 poly A. In certain embodiments, the polyA is a rabbit beta-globin (RBG) poly A. In certain embodiments, the vector genome comprises a 5' AAV ITR – CB7 promoter – GLB1 gene – RBG poly A – 3' ITR. In certain embodiments, the vector genome comprises a 5' AAV ITR – EF1a promoter – GLB1 gene – SV40 poly A – 3' ITR. In certain embodiments, the vector genome comprises a 5' AAV

ITR – UbC promoter – GLB1 gene – SV40 poly A – 3' ITR. In certain embodiments, the GLB1 gene has SEQ ID NO: 5. In certain embodiments, the GLB1 gene has SEQ ID NO: 6. In certain embodiments, the GLB1 gene has SEQ ID NO: 7. In certain embodiments, the GLB1 gene has SEQ ID NO: 8. In certain embodiments, the vector genome has the sequence of SEQ ID NO: 12. In certain embodiments, the vector genome has the sequence of SEQ ID NO: 13. In certain embodiments, the vector genome has the sequence of SEQ ID NO: 14. In certain embodiments, the vector genome has the sequence of SEQ ID NO: 15. In certain embodiments, the vector genome has the sequence of SEQ ID NO: 16.

In certain embodiments, the final formulation buffer comprises an artificial cerebrospinal fluid comprising buffered saline and one or more of sodium, calcium, magnesium, potassium, or mixtures thereof; and a surfactant. In certain embodiments, the surfactant is about 0.0005 % to about 0.001% of the suspension. In certain embodiments, the surfactant is Pluronic F68. In certain embodiments, the Pluronic F68 is present in an amount of about 0.0001% of the suspension. In certain embodiments, the composition is at a pH in the range of 7.5 to 7.8 for intrathecal delivery. In certain embodiments, the composition is at a pH in the range of 6.2 to 7.7, or 6.9 to 7.5, or about 7for intrathecal delivery. In one embodiment, the percentage (%) is calculated based on weight ratio or volume ratio. In another embodiment, the percentage represents "gram per 100ml of final volume".

In certain embodiments, treatment of the composition described herein has minimal to mild asymptomatic degeneration of DRG sensory neurons in animals and/or in human patients, well-tolerated with respect to sensory nerve toxicity and subclinical sensory neuron lesions.

In certain embodiment, the composition described herein is useful in improving functional and clinical outcomes in the subject/patient treated. Such outcomes may be measured at about 30 days, about 60 days, about 90 days, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, about 12 months, about 13 months, about 14 months, about 15 months, about 16 months, about 17 months, about 18 months, about 19 months, about 20 months, about 21 months, about 22 months, about 23 months, about 24 months, about 2.5 years, about 3 years, about 3.5 years, about 4 years, about 4.5 years and then yearly up to the about 5 years after administration of the composition. Measurement frequency may be about every 1 month, about every 2 months, about every 3 months, about every 4 months, about every 5 months, about every 6 months, about every 7 months, about every 8 months, about every 9 months, about every 10 months, about every 11 months, or about every 12 months.

In certain embodiments, the composition described herein shows pharmacodynamics and clinical efficacy measured in treated subjects compared to untreated controls.

In certain embodiments, the pharmacodynamics efficacy, clinical efficacy, functional outcomes, or clinical outcomes may be measured via one or more of the following: (1) survival, (2) feeding tube independence, (3) seizure diary, *e.g.*, incidence, onset, frequency, length, and type of seizure, (4) quality of life, for example, as measured by PedsQL, (5) neurocognitive and behavioral development, (6) β-gal enzyme expression or activity, for example in serum or CSF, and (7) other parameters as described herein. The Bayley Scales of Infant Development and Vineland Scales may be used to quantify the effects of the composition on development and/or changes in adaptive behaviors, cognition, language, motor function, and health-related quality of life.

In certain embodiments, the neurocognitive development is based on one of more of the following: change in age equivalent cognitive, gross motor, fine motor, receptive and expressive communication scores of the Bayley Scales of Infant and Toddler Development; change in standard scores for each domain of the Vineland Adaptive Behavior Scales; and pediatric quality of life by change in total score on the Pediatric Quality of Life Inventory- and the Pediatric Quality of Life Inventory Infant Scale (PedsQL and PedsQL-IS).

BSID (Bayley Scale of Infant Development): is used primarily to assess the development of infants and toddlers, ages 1–42 months (Albers and Grieve, 2007, Test Review: Bayley, N. (2006). Bayley Scales of Infant and Toddler Development– Third Edition. San Antonio, TX: Harcourt Assessment. Journal of Psychoeducational Assessment. 25(2):180-190). It consists of a standardized series of developmental play tasks and derives a developmental quotient by converting raw scores of successfully completed items to scale scores and composite scores and comparing the scores with norms taken from typically developing children of the same age. The Bayley-III has 3 main subtests; a Cognitive Scale, which includes items such as attention to familiar and unfamiliar objects, looking for a fallen object, and pretend play; a Language Scale, which assesses understanding and expression of language (*e.g.* ability to follow directions and naming objects); and a Motor Scale that measures gross and fine motor skills (*e.g.* grasping, sitting, stacking blocks, and climbing stairs). The most current version is the BSID-III.

Vineland: Assesses adaptive behavior from birth through adulthood (0–90 years) across five domains: communication, daily living skills, socialization, motor skills, and maladaptive behavior. The most current version is the Vineland III. Improvements from the

Vineland-II to the Vineland-III incorporate questions to enable better understanding of developmental disabilities.

The BSID and Vineland were chosen based on data from the only prospective study of infantile GM1 gangliosidosis patients (Brunetti-Pierri and Scaglia, 2008, GM1 gangliosidosis: Review of clinical, molecular, and therapeutic aspects. Molecular Genetics and Metabolism. 94(4):391-396.). Age-equivalent scores on the BSID-III showed a decline to the floor of the testing scale by 28 months of age for both cognitive and gross motor domains, and the scores on the Vineland-II adaptive behavior scale remained measurable, albeit far below normal, by 28 months of age. While these tools showed floor effects they were shown to be appropriate scales for measuring developmental changes in this severely impaired population, the cross-cultural validity of the scales make them appropriate for international studies.

PedsQL and PedsQL-IS: As is the case with severe pediatric diseases, the burden of the disease on the family is significant. The Pediatric Quality of Life Inventory™ is a validated a tool that assesses quality of life in children and their parents (by parent proxy reports). It has been validated in healthy children and adolescents and has been used in various pediatric diseases (Iannaccone et al., 2009, The PedsQL in pediatric patients with Spinal Muscular Atrophy: feasibility, reliability, and validity of the Pediatric Quality of Life Inventory Generic Core Scales and Neuromuscular Module. *Neuromuscular disorders : NMD.* 19(12):805-812; Absoud et al., 2011, Paediatric UK demyelinating disease longitudinal study (PUDDLS)." BMC Pediatrics. 11(1):68; and Consolaro and Ravelli, 2016, Chapter 5 - Assessment Tools in Juvenile Idiopathic Arthritis. Handbook of Systemic Autoimmune Diseases. R. Cimaz and T. Lehman, Elsevier. 11: 107-127). Therefore, the PedsQL is included to evaluate the impact of rAAV.GLB1 on the quality of life of the patient and their family. It can be applied to parents of children age 2 and above and may therefore be informative as the children age over the 5 year follow-up period. The Pediatric Quality of Life Inventory™ Infant Scale (Varni et al., 2011, "The PedsQL™ Infant Scales: feasibility, internal consistency reliability, and validity in healthy and ill infants." Quality of Life Research. 20(1):45-55.) is a validated modular instrument completed by parents and designed to measure health-related quality of life instrument specifically for healthy and ill infants ages 1– 24 months.

Given the severity of disease in the target population, subjects may have achieved motor skills by enrollment, developed and subsequently lost other motor milestones, or not yet shown signs of motor milestone development. Assessments tracks age-at-achievement

and age-at-loss for all milestones. Motor milestone achievement is defined for six gross milestones based on the WHO criteria outlined in the Table provided herein under Section I GM1 and the therapeutic *GLB1* gene. Given that subjects with infantile GM1 gangliosidosis can develop symptoms within the months of life, and acquisition of the first WHO motor milestone (sitting without support) typically does not manifest before 4 months of age (median: 5.9 months of age), this endpoint may lack sensitivity to evaluate the extent of therapeutic benefit, especially in subjects who had more overt symptoms at the time of treatment. For this reason, assessment of age-appropriate developmental milestones that can be applied to infants are also be included (Scharf et al., 2016, Developmental Milestones. Pediatr Rev. 37(1):25-37; quiz 38, 47.). One shortcoming is that the published tool is intended for use by clinicians and parents, and organizes skills around the typical age of milestone acquisition without referencing normal ranges. However, the data may be informative for summarizing retention, acquisition, or loss of developmental milestones over time relative to untreated children with infantile GM1 disease or the typical time of acquisition in neurotypical children.

As the disease progresses children can develop seizures. The onset of seizure activity enables us to determine whether treatment with rAAV.GLB1 can either prevent or delay onset of seizures or decrease the frequency of seizure events in this population. Parents are asked to keep seizure diaries, which tracks onset, frequency, length, and type of seizure.

In certain embodiments, the pharmacodynamics efficacy, clinical efficacy, functional outcomes, or clinical outcomes may also include CNS manifestations of the disease, for example, volumetric changes measured on MRI over time. The infantile phenotype of all gangliosidoses was shown to have a consistent pattern of macrocephaly and rapidly increasing intracranial MRI volume with both brain tissue volume (cerebral cortex and other smaller structures) and ventricular volume. Additionally, various smaller brain substructures including the corpus callosum, caudate and putamen as well as the cerebellar cortex generally decrease in size as the disease progresses (Regier et al., 2016s, and Nestrasil et al., 2018, as cited herein). Treatment with rAAV.GLB1 can slow or cease the progression of CNS disease manifestations with evidence of stabilization in atrophy and volumetric changes. Changes (normal/abnormal) in T1/T2 signal intensity in the thalamus and basal ganglia may also be included based on reported evidence for changes in the thalamic structure in patients with GM1 and GM2 gangliosidosis (Kobayashi and Takashima, 1994, Thalamic hyperdensity on CT in infantile GM1-gangliosidosis." Brain and Development. 16(6):472-474). In certain embodiments, the pharmacodynamics efficacy, clinical efficacy,

functional outcomes, or clinical outcomes may include changes in total brain volume, brain substructure volume, and lateral ventricle volume as measured by MRI; and/or changes in T1/T2 signal intensity in the thalamus and basal ganglia activity.

Alternatively or additionally, the pharmacodynamics efficacy, clinical efficacy, functional outcomes, or clinical outcomes may include biomarkers, for example, pharmacodynamics and biological activity of rAAV.GLB1, β-gal enzyme activity, which can be measured in CSF and serum, CSF GM1 concentration, serum and urine keratan sulfate levels, reduction of hexosaminidase activity, and brain MRI, which demonstrates consistent, rapid atrophy in infantile GM1 gangliosidosis (Regier et al., 2016b, as cited herein).

In certain embodiments, the composition described herein is useful in slowing down disease progression, for example, as assessed by age at achievement, age at loss, and percentage of children maintaining or acquiring age-appropriate developmental and motor milestones (as defined by World Health Organization [WHO] criteria).

In certain embodiments, the pharmacodynamics efficacy, clinical efficacy, functional outcomes, or clinical outcomes may include liver and spleen volume; and/or EEG and visual evoked potentials (VEP).

VI. Apparatus and Method for Delivery of a Pharmaceutical Composition into Cerebrospinal Fluid

In one aspect, the rAAV or composition provided herein may be administered intrathecally via the method and/or the device provided in this section and described in WO 2018/160582, which is incorporated by reference herein. Another suitable device is described in PCT/US20/14402, entitled Microcatheter for Therapeutic and/or Diagnostic Interventions in the Subarachnoid Space", filed 31 January 2020, which is incorporated hereby by reference. Alternatively, other devices and methods may be selected.

In certain embodiments, the method comprises the steps of CT-guided sub-occipital injection via spinal needle into the cisterna magna of a patient. As used herein, the term Computed Tomography (CT) refers to radiography in which a three-dimensional image of a body structure is constructed by computer from a series of plane cross-sectional images made along an axis.

On the day of treatment, the appropriate concentration of rAAV.GLB1 is prepared. A syringe containing the appropriate volume (e.g., 3.6 mL, 4.6 mL, or 5.6 mL) of rAAV.GLB1 at the appropriate concentration is delivered to the procedure room. The following personnel are present for study drug administration: interventionalist performing the procedure;

anesthesiologist and respiratory technician(s); nurses and physician assistants; CT (or operating room) technicians; site research coordinator. Prior to drug administration, a lumbar puncture is performed to remove a predetermined volume of CSF (e.g., about 5 mL) and then to inject iodinated contrast intrathecally (IT) to aid in visualization of relevant anatomy of the cisterna magna. Intravenous (IV) contrast may be administered prior to or during needle insertion as an adjunct to intrathecal contrast. The subject is anesthetized, intubated, and positioned on the procedure table. The injection site is prepped and draped using sterile technique. A spinal needle (e.g., a 2" or 3" 25 G spinal needle for subjects age 3 mos to 18 years) are advanced into the cisterna magna under fluoroscopic guidance. A larger introducer needle may be used to assist with needle placement. After confirmation of needle placement, the extension set are attached to the spinal needle and allowed to fill with CSF. At the discretion of the interventionalist, a syringe containing contrast material may be connected to the extension set and a small amount injected to confirm needle placement in the cisterna magna. After the needle placement is confirmed by CT guidance +/- contrast injection, the syringe containing the appropriate volume of rAAV.GLB1 is connected to the extension set. The syringe contents are slowly injected slowly (e.g., over about 1 to 2 minutes) , without excessive force applied to the syringe plunger during injection. A total of 3 mL, 4 mL, or 5 mL of rAAV.GLB1 is injected; 0.6 mL of rAAV.GLB1 remains in the apparatus. The needle, extension tubing, and syringe are slowly removed from the subject and placed onto a surgical tray for discarding into appropriate biohazard waste receptacles. The needle insertion site is examined for signs of bleeding or CSF leakage and treated as indicated by the proceduralist. The site is dressed using gauze, surgical tape and/or a transparent dressing (e.g. Tegaderm), as indicated. The subject remains in the prone position for at least 20 minutes after the bandage is placed. The subject is removed from the CT scanner and placed in the supine position onto a stretcher. Adequate staff must be present to ensure subject safety during transport and positioning. Anesthesia is discontinued, and the subject recovers as per institutional guidelines for post-anesthesia care. Neurophysiologic equipment will be removed if applicable. The head of the stretcher is lowered to approximately 20-30 degrees during the recovery period for approximately 1 hour. The subject is transported to a suitable post-anesthesia care unit as per institutional guidelines.

Additional or alternate routes of administration to the intrathecal method described herein include, for example, systemic, oral, intravenous, intraperitoneal, subcutaneous, or intramuscular administration.

In one embodiment, doses may be scaled by brain mass, which provides an approximation of the size of the CSF compartment. In a further embodiment, dose conversions are based on a brain mass of 0.4 g for an adult mouse, 90 g for a juvenile rhesus macaque, and 800 g for children 4–18 months of age. The following table provides illustrative doses for a murine MED study, NHP toxicology study, and equivalent human doses.

Dose (GC/g brain mass)	Mouse (GC)	NHP (GC)	Human (GC)
3.33×10^{11}	1.30×10^{11}	3.00×10^{13}	2.70×10^{14}
1.11×10^{11}	4.40×10^{10}	1.00×10^{13}	8.90×10^{13}
3.33×10^{10}	1.30×10^{10}	3.00×10^{12}	2.70×10^{13}
1.11×10^{10}	4.40×10^{9}	-	8.90×10^{12}

In certain embodiments, a rAAV.GLB1 is administered to a subject in a single dose. In certain embodiments, multiple doses (for example 2 doses) may be desired. For example, for infants under 6 months, multiple doses delivered days, weeks, or months, apart may be desired.

In certain embodiments, a single dose of rAAV.GLB1 is from about 1×10^9 GC/g brain mass to about 5×10^{11} GC/g brain mass. In certain embodiments, a single dose of rAAV.GLB1 is from about 1×10^9 GC/g brain mass to about 3×10^{11} GC. In certain embodiments, a single dose of rAAV.GLB1 is from about 1×10^{10} GC/g brain mass to about 3×10^{11} GC/g brain mass. In certain embodiments, the dose of rAAV.GLB1 is from 1×10^{10} GC/brain mass to 3.33×10^{11} GC/brain mass. In certain embodiments, the dose of rAAV.GLB1 is from 1×10^{11} GC/brain mass to 3.33×10^{11} GC/brain mass. In certain embodiments, a single dose of rAAV.GLB1 is from 1.11×10^{10} GC/g brain mass to 3.33×10^{11} GC/g brain mass.

In certain embodiments, a single dose of rAAV.GLB1 is from 1×10^{10} GC/g brain mass to 3.4×10^{11} GC/g brain mass. In certain embodiments, a single dose of rAAV.GLB1 is from 3.4×10^{10} GC/g brain mass to 3.4×10^{11} GC/g brain mass. In certain embodiments, a single dose of rAAV.GLB1 is from 1.0×10^{11} GC/g brain mass to 3.4×10^{11} GC/g brain mass. In certain embodiments, a single dose of rAAV.GLB1 is about 1.1×10^{11} GC/g brain mass. In certain embodiments, a single dose of rAAV.GLB1 is at least 1.11×10^{10} GC/g brain mass. In other embodiments, different doses may be selected.

In preferred embodiments, the subject is a human patient. In this case, a single dose of rAAV.GLB1 is from about 1 x 10^{12} GC to about 3 x 10^{14} GC. In certain embodiments, a single dose of rAAV.GLB1 is from 9 x 10^{12} GC to 3 x 10^{14} GC. In certain embodiments, the dose of rAAV.GLB1 is from 5 x 10^{13} GC to 3 x 10^{14} GC. In certain embodiments, a single dose of rAAV.GLB1 is from 8.90 × 10^{13} GC to 2.70 × 10^{14} GC. In certain embodiments, a single dose of rAAV.GLB1 is from 8 x 10^{12} genome copies (GC) per patient to 3 x 10^{14} GC per patient. In certain embodiments, a single dose of rAAV.GLB1 is from 2 x 10^{13} GC per patient to 3 x 10^{14} GC per patient. In certain embodiments, a single dose of rAAV.GLB1 is from 8 x 10^{13} GC per patient to 3 x 10^{14} GC per patient. In certain embodiments, a single dose of rAAV.GLB1 is about 9 x 10^{13} GC per patient. In certain embodiments, a single dose of rAAV.GLB1 is at least 8.90 × 10^{13} GC. In other embodiments, different doses may be selected.

The compositions can be formulated in dosage units to contain an amount of AAV that is in the range from about 1 x 10^{9} genome copies (GC) to about 5 x 10^{14} GC (to treat an average subject of 70 kg in body weight). In some embodiments, the composition is formulated in dosage unit to contain an amount of AAV in the range from 1 x 10^{9} genome copies (GC) to 5 x 10^{13} GC; from 1 x 10^{10} genome copies (GC) to 5 x 10^{14} GC; from 1 x 10^{11} GC to 5 x 10^{14} GC; from 1 x 10^{12} GC to 5 x 10^{14} GC; from 1 x 10^{13} GC to 5 x 10^{14} GC; from 8.9 x 10^{13} GC to 5 x 10^{14} GC; or from 8.9 x 10^{13} GC to 2.7 x 10^{14} GC. In certain embodiments, the composition is formulated in dosage unit to contain an amount of AAV at least 1 x 10^{13} GC, 2.7 x 10^{13} GC, or 8.9 x 10^{13} GC.

In one embodiment, a spinal tap is performed in which from about 15 mL (or less) to about 40 mL CSF is removed and in which rAAV.GLB1 is admixed with the CSF and/or suspended in a compatible carrier and delivered to the subject. In one example, the rAAV.GLB1 concentration is from 1 x 10^{10} genome copies (GC) to 5 x 10^{14} GC; from 1 x 10^{11} GC to 5 x 10^{14} GC; from 1 x 10^{12} GC to 5 x 10^{14} GC; from 1 x 10^{13} GC to 5 x 10^{14} GC; from 8.9 x 10^{13} GC to 5 x 10^{14} GC; or from 8.9 x 10^{13} GC to 2.7 x 10^{14} GC, but other amounts such as about 1 x 10^{9} GC, about 5 x 10^{9} GC, about 1 x 10^{10} GC, about 5 x 10^{10} GC, about 1 x 10^{11} GC, about 5 x 10^{11} GC, about 1 x 10^{12} GC, about 5 x 10^{12} GC, about 1.0 x 10^{13} GC, about 5 x 10^{13} GC, about 1.0 x 10^{14} GC, or about 5 x 10^{14} GC. In certain embodiments, the concentration in GC is illustrated as GC per spinal tap. In certain embodiments, the concentration in CG is illustrated as GC per mL.

A co-therapy may be delivered with the rAAV.GLB1 compositions provided herein. Co-therapies such as described carlier in this application are incorporated herein by reference.

One such co-therapy may be an immune modulator. Immunosuppressants for such co-therapy include, but are not limited to, a glucocorticoid, steroids, antimetabolites, T-cell inhibitors, a macrolide (e.g., a rapamycin or rapalog), and cytostatic agents including an alkylating agent, an anti-metabolite, a cytotoxic antibiotic, an antibody, or an agent active on immunophilin. The immune suppressant may include a nitrogen mustard, nitrosourea, platinum compound, methotrexate, azathioprine, mercaptopurine, fluorouracil, dactinomycin, an anthracycline, mitomycin C, bleomycin, mithramycin, IL-2 receptor- (CD25-) or CD3-directed antibodies, anti-IL-2 antibodies, cyclosporin, tacrolimus, sirolimus, IFN-β, IFN-γ, an opioid, or TNF-α (tumor necrosis factor-alpha) binding agent. In certain embodiments, the immunosuppressive therapy may be started prior to the gene therapy administration. Such therapy may involve co-administration of two or more drugs, the (e.g., prednisolone, mycophenolate mofetil (MMF) and/or sirolimus (i.e., rapamycin)) on the same day. One or more of these drugs may be continued after gene therapy administration, at the same dose or an adjusted dose. Such therapy may be for about 1 week, about 15 days, about 30 days, about 45 days, 60 days, or longer, as needed.

For example, when nutrition is a concern in GM1, placement of a gastrostomy tube is appropriate. As respiratory function deteriorates, tracheotomy or noninvasive respiratory support is offered. A power chair and other equipment may improve quality of life.

The words "comprise", "comprises", and "comprising" are to be interpreted inclusively rather than exclusively. The words "consist", "consisting", and its variants, are to be interpreted exclusively, rather than inclusively. While various embodiments in the specification are presented using "comprising" language, under other circumstances, a related embodiment is also intended to be interpreted and described using "consisting of" or "consisting essentially of" language.

The term "expression" is used herein in its broadest meaning and comprises the production of RNA or of RNA and protein. With respect to RNA, the term "expression" or "translation" relates in particular to the production of peptides or proteins. Expression may be transient or may be stable.

As used herein, the term "NAb titer" a measurement of how much neutralizing antibody (e.g., anti-AAV Nab) is produced which neutralizes the physiologic effect of its targeted epitope (e.g., an AAV). Anti-AAV NAb titers may be measured as described in,

e.g., Calcedo, R., et al., Worldwide Epidemiology of Neutralizing Antibodies to Adeno-Associated Viruses. Journal of Infectious Diseases, 2009. 199(3): p. 381-390, which is incorporated by reference herein.

In some embodiments, the administration of the AAV or composition ameliorates symptoms of GM1 gangliosidosis, or ameliorated neurological symptoms of GM1 gangliosidosis. In some embodiments, following treatment, the patient has one or more of increased average life span, decreased need for feeding tube, reduction in seizure incidence and frequency, reduction in progression towards neurocognitive decline and/or improvement in neurocognitive development.

As used herein, an "expression cassette" refers to a nucleic acid molecule which comprises a coding sequence, promoter, and may include other regulatory sequences therefor. In certain embodiments, a vector genome may contain two or more expression cassettes. In other embodiments, the term "transgene" may be used interchangeably with "expression cassette". Typically, such an expression cassette for generating a viral vector contains the coding sequence for the gene product described herein flanked by packaging signals of the viral genome and other expression control sequences such as those described herein.

The term "heterologous" when used with reference to a protein or a nucleic acid indicates that the protein or the nucleic acid comprises two or more sequences or subsequences which are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid. For example, in one embodiment, the nucleic acid has a promoter from one gene arranged to direct the expression of a coding sequence from a different gene. Thus, with reference to the coding sequence, the promoter is heterologous.

A "replication-defective virus" or "viral vector" refers to a synthetic or artificial viral particle in which a vector genome comprising an expression cassette containing a gene of interest (for example, *GLB1*) is packaged in a viral capsid (e.g., AAV or bocavirus) or envelope, where any viral genomic sequences also packaged within the viral capsid or envelope are replication-deficient; *i.e.*, they cannot generate progeny virions but retain the ability to infect target cells. In one embodiment, the genome of the viral vector does not include genes encoding the enzymes required to replicate (the genome can be engineered to be "gutless" - containing only the gene of interest flanked by the signals required for amplification and packaging of the artificial genome), but these genes may be supplied

during production. Therefore, it is deemed safe for use in gene therapy since replication and infection by progeny virions cannot occur except in the presence of the viral enzyme required for replication.

As used herein, an "effective amount" refers to the amount of the rAAV composition which delivers and expresses in the target cells an amount of the gene product from the vector genome. An effective amount may be determined based on an animal model, rather than a human patient. Examples of a suitable murine or NHP model are described herein.

It is to be noted that the term "a" or "an", refers to one or more, for example, "an enhancer", is understood to represent one or more enhancer(s). As such, the terms "a" (or "an"), "one or more," and "at least one" is used interchangeably herein.

As described above, the term "about" when used to modify a numerical value means a variation of ±10%, unless otherwise specified.

As described above, the terms "increase" "decrease" "reduce" "ameliorate" "improve" "delay" "earlier" "slow" "cease" or any grammatical variation thereof, or any similar terms indication a change, means a variation of about 5 fold, about 2 fold, about 1 fold, about 90%, about 80%, about 70%, about 60%, about 50%, about 40%, about 30%, about 20%, about 10%, about 5 % compared to the corresponding reference (*e.g.*, untreated control, corresponding level of a GM1 patient or a GM1 patient at a certain stage or a healthy subject or a healthy human without GM1)), unless otherwise specified.

"Patient" or "subject" as used herein refer to a mammalian animal, including a human, a veterinary or farm animal, a domestic animal or pet, and animals normally used for clinical research. In one embodiment, the subject of these methods and compositions is a human. In certain embodiments, the patient has GM1.

Unless defined otherwise in this specification, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art and by reference to published texts, which provide one skilled in the art with a general guide to many of the terms used in the present application.

EXAMPLES

The following examples are illustrative only and are not intended to limit the present invention.

EXAMPLE 1: AAVhu68 + Deamidation

AAVhu68 was analyzed for modifications. Briefly, AAVhu68 were produced using vector genomes which are not relevant to this study, each produced using conventional triple transfection methods in 293 cells. For a general description of these techniques, see, *e.g.*, Bell CL, *et al.*, The AAV9 receptor and its modification to improve *in vivo* lung gene transfer in mice. *J Clin Invest.* 2011;121:2427–2435. Briefly, for example, a plasmid encoding the sequence to be packaged (a transgene expressed from a chicken β-actin promoter, an intron and a poly A derived from Simian Virus 40 (SV40) late gene) flanked by AAV2 inverted terminal repeats, was packaged by triple transfection of HEK293 cells with plasmids encoding the AAV2 *rep* gene and the AAVhu68 *cap* gene and an adenovirus helper plasmid (pAdΔF6). The resulting AAV viral particles can be purified using CsCl gradient centrifugation, concentrated, and frozen for later use.

Denaturation and alkylation: To 100 µg of the thawed viral preparation (protein solution), add 2 µl of 1M Dithiothreitol (DTT) and 2µl of 8M guanidine hydrochloride (GndHCl) and incubate at 90°C for 10 minutes. Allow the solution to cool to room temperature then add 5µl of freshly prepared 1M iodoacetamide (IAM) and incubate for 30 minutes at room temperature in the dark. After 30 minutes, quench alkylation reaction by adding 1 µl of 1M DTT.

Digestion: To the denatured protein solution add 20mM Ammonium Bicarbonate, pH 7.5-8 at a volume that dilutes the final GndHCl concentration to 800mM. Add trypsin solution for a 1:20 trypsin to protein ratio and incubate at 37 °C overnight. After digestion, add TFA to a final of 0.5% to quench digestion reaction.

Mass Spectrometry: Approximately 1 microgram of the combined digestion mixture is analyzed by UHPLC-MS/MS. LC is performed on an UltiMate 3000 RSLCnano System (Thermo Scientific). Mobile phase A is MilliQ water with 0.1% formic acid. Mobile phase B is acetonitrile with 0.1% formic acid. The LC gradient is run from 4% B to 6% B over 15 min, then to 10% B for 25 min (40 minutes total), then to 30% B for 46 min (86 minutes total). Samples are loaded directly to the column. The column size is 75 cm x 15 um I.D. and is packed with 2 micron C18 media (Acclaim PepMap). The LC is interfaced to a quadrupole-Orbitrap mass spectrometer (Q-Exactive HF, Thermo Scientific) via nanoflex electrospray ionization using a source. The column is heated to 35°C and an electrospray voltage of 2.2 kV is applied. The mass spectrometer is programmed to acquire tandem mass spectra from top 20 ions. Full MS resolution to 120,000 and MS/MS resolution to 30,000.

Normalized collision energy is set to 30, automatic gain control to 1e5, max fill MS to 100 ms, max fill MS/MS to 50 ms.

Data Processing: Mass spectrometer RAW data files were analyzed by BioPharma Finder 1.0 (Thermo Scientific). Briefly, all searches required 10 ppm precursor mass tolerance, 5ppm fragment mass tolerance, tryptic cleavage, up to 1 missed cleavages, fixed modification of cysteine alkylation, variable modification of methionine/tryptophan oxidation, asparagine/glutamine deamidation, phosphorylation, methylation, and amidation.

In the following table, T refers to the trypsin and C refers to chymotrypsin.

Modification AAVhu68												
Enzyme	T	T	T	T	C	C	C	C	T	T	T	
% Coverage	93.6	92	93.1	92.5	90.2	89.7	91.1	88.9	98.9	97	94.6	92.4
+ Deamidation (Deamid)												
~N35												
N57+ Deamid	87.6	95.5	89.3	88.2	90.5	96.3	86.4	84.8	100.0	100.0	99.0	92.7
N66+ Deamid	4.7											
N94+ Deamid	11.3	10.9	11.0	5.3	11.6	10.4	10.8	5.6	5.0	11.1	5.4	16.0
N113+ Deamid			1.8									
~N253+ Deamid	17.7	22.0	21.1	15.0	17.0	22.6	20.5	15.6	4.2	5.5		
Q259+ Deamid	35.2	25.6	21.0		35.4	26.3	20.9	9.2				
~N270+ Deamid	16.4	25.1	23.2	16.6	15.9	24.9	23.5	16.1	0.2			
~N304+ Deamid	2.6	2.9	2.8	1.3	2.5	2.8	2.9	1.3	16.6	10.3		
~N314+Deamid									6.5			

N319+ Deamid	0.3	2.8	2.8	0.2		2.9	2.8	0.2				
N329+ Deamid	72.7	85.6	89.1	86.8	71.0	87.2	88.7	84.7	85.5	79.4	78.9	91.8
N336+ Deamid		30.8	9.3	100.0		31.0	9.2	95.7				
~N409+ Deamid	21.3	22.9	23.9	24.0	22.0	23.4	24.7	24.2				
N452+ Deamid	98.8	99.7	99.2	100.0	98.9	97.3	98.1	95.2	98.2	68.7	67.4	49.4
N477+ Deamid	4.4	4.3	4.3	2.6	4.5	4.4	4.3	2.6			0.8	
N512+ Deamid	97.5	97.9	95.3	95.7	92.2	91.8	99.2	96.1	99.7	98.2	87.9	75.7
~N515+ Deamid	8.2	21.0	16.0		8.3	21.0	16.5	0.0	2.5	3.0		15.1
~Q599+ Deamid	4.0	15.4	10.1	13.6	4.0	15.5	10.0	13.8	15.8			
N628+ Deamid	5.3		5.6		5.4	0.0	5.4	0.0				
N651+ Deamid	0.9	1.6	1.6						0.5			
N663+ Deamid	3.4		3.5	3.7	3.4	0.0	3.4	3.6				
N709+ Deamid	0.6	0.8	20.2	0.6	0.6	0.8	19.8	0.6	0.3	1.3	0.1	0.2
N735									25.0	42.7		21.7
+ Acetylation (Ac):												
K332 + Ac				100.0								
~K693+Ac	13.0		13.5									
~K666+Ac				93.8								
~K68+ Ac		59.2										

+ Isomerization (Iso):												
D97 + Iso	0.5	0.4	0.4	0.2	0.5		0.4	0.2				
D107 + Iso		0.3		0.3		0.3						
D384 + Iso	0.8				0.9							
+ Phosphorylation (Phos)												
S149+Phos	5.8	5.7	5.2	9.8	5.7	5.9	5.2	9.9				
~S499+ Phos				30.6								
~T569+ Phos	0.9											
~S586+ Phos		3.6										
+ Oxidation												
~W23+Oxi		4.7	5.5			4.8	5.5					
W247+Oxi	1.5	0.4	0.7		1.4							
W247+Oxi to kynurenine		0.1				0.1						
W306+Oxi	0.7	0.9	1.6	1.8	0.7	1.0	1.6	1.8				
W306+Oxidation to kynurenine			0.3				0.3					
M404+Oxi	0.1		0.2		0.1		0.2					

M436+Oxi	4.9		10.2	23.0	4.8		10.2	22.6				
~M518+ Oxi	29.9		1.5	10.6	29.9		1.5	10.5				
~M524+ Oxi	18.8	31.6	52.7		18.4	31.1	52.5	14.2				
M559+Oxi	19.0	21.6	19.6	20.9	19.6	21.3	20.1	20.9				
~M605+ Oxi	12.2	15.2			12.8	14.8						
W619+Ox i	1.0		0.6	1.5	1.0		0.6	1.5				
W619+Ox idation			20.3									
~M640+ Oxi	23.5	64.2	24.6		22.4	21.1	25.6					
W695+Ox i	0.3		0.4	0.4	0.3		0.4	0.4				
+Amidation												
~D297+A midation		72.9		73.3								

In the case of the AAVhu68 capsid protein, 4 residues (N57, N329, N452, N512) routinely display levels of deamidation >70% and it most cases >90% across various lots. Additional asparagine residues (N94, N253, N270, N304, N409, N477, and Q599) also display deamidation levels up to ~20% across various lots. The deamidation levels were initially identified using a trypsin digest and verified with a chymotrypsin digestion.

Accordingly, AAV comprising AAVhu68 capsid proteins can include a heterogeneous population of capsid proteins because the AAV can contain AAVhu68 capsid proteins displaying different levels of deamidation. The heterogenous population of AAVhu68 vp1 proteins having various levels of deamidation can be vp1 proteins produced by expression from a nucleic acid sequence which encodes the predicted amino acid sequence of 1 to 736 of SEQ ID NO:2, vp1 proteins produced from SEQ ID NO: 1, or vp1 proteins produced from a nucleic acid sequence at least 70% identical to SEQ ID NO:1

which encodes the predicted amino acid sequence of 1 to 736 of SEQ ID NO:2. The heterogenous population of AAVhu68 vp2 proteins having various levels of deamidation can be vp2 proteins produced by expression from a nucleic acid sequence which encodes the predicted amino acid sequence of at least about amino acids 138 to 736 of SEQ ID NO:2, vp2 proteins produced from a sequence comprising at least nucleotides 412 to 2211 of SEQ ID NO:1, or vp2 proteins produced from a nucleic acid sequence at least 70% identical to at least nucleotides 412 to 2211 of SEQ ID NO:1 which encodes the predicted amino acid sequence of at least about amino acids 138 to 736 of SEQ ID NO:2. The heterogenous population of AAVhu68 vp3 proteins having various levels of deamidation can be vp3 produced by expression from a nucleic acid sequence which encodes the predicted amino acid sequence of at least about amino acids 203 to 736 of SEQ ID NO:2, vp3 proteins produced from a sequence comprising at least nucleotides 607 to 2211 of SEQ ID NO:1, or vp3 proteins produced from a nucleic acid sequence at least 70% identical to at least nucleotides 607 to 2211 of SEQ ID NO:1 which encodes the predicted amino acid sequence of at least about amino acids 203 to 736 of SEQ ID NO:2.

Adult Rhesus macaques were ICM-administered AAVhu68.CB7.CI.eGFP.WPRE.rBG (3.00×10^{13} GC) and necropsied 28 days later to assess vector transduction. Transduction of AAVhu68 was observed in widespread areas of the brain (data not shown). Thus, the AAVhu68 capsid provides the possibility of cross-correction in the CNS.

EXAMPLE 2: Manufacturing - Components and Materials

Vectors are constructed from cis-plasmids containing a coding sequence for human GLB1 expressed from the chicken beta actin promoter with a cytomegalovirus enhancer (CB7) [SEQ ID NO: 10], human elongation initiation factor 1 alpha promoter (EF1a) [SEQ ID NO: 11] or human ubiquitin C promoter (UbC) [SEQ ID NO: 9] (1229bp, GenBank #D63791.1)] flanked by AAV2 inverted terminal repeats. Various coding sequences for human GLB1 [aa sequence of SEQ ID NO: 4] are constructed. The wild-type sequence is reproduced in SEQ ID NO: 5. Various engineered GLB1 coding sequences were generated and are provided in SEQ ID NO: 6, 7, or 8.

The vectors are packaged in an AAV serotype hu68 capsid by triple transfection of adherent HEK 293 cells and purified by iodixanol gradient centrifugation as previously described in Lock, M., *et al.* Rapid, Simple, and Versatile Manufacturing of Recombinant Adeno-Associated Viral Vectors at Scale. Human Gene Therapy 21, 1259-1271 (2010). The

AAV serotype Hu68 capsid was described in WO2018/160582 which is incorporated by reference in its entirety herein.

More particularly, AAVhu68.GLB1 are produced by triple plasmid transfection of human HEK293 WCB cells with: 1) the AAV *cis* vector genome plasmid, 2) the AAV *trans* plasmid termed pAAV2/hu68.KanR encoding the AAV2 replicase (rep) and AAVhu68 capsid (cap), and 3) the helper adenovirus plasmid termed pAdΔF6.KanR.

Description of Sequence Elements of the AAV *cis* Vector Genome Plasmid :

- Inverted Terminal Repeat (ITR): The ITRs are identical, reverse complementary sequences derived from AAV2 (130bp, GenBank # NC001401) that flank all components of the vector genome. The ITR sequences function as both the origin of vector DNA replication and the packaging signal of the vector genome, when AAV and adenovirus helper functions are provided in *trans*. As such, the ITR sequences represent the only *cis* sequences required for vector genome replication and packaging.
- Promoter: Regulatory element derived from human ubiquitin C (UbC) promoter: This ubiquitous promoter (1229 bp, GenBank #D63791.1) was selected to drive transgene expression in any CNS cell type.
- Coding sequence: GLB1 gene, based on maximized human codon usage, encodes beta-galactosidase. GLB1 enzyme catalyzes the hydrolysis of β-linked galactose from gangliosides (2034 bp polynucleotide for 677 aa and the stop codon, Genbank #AAA51819.1, EC3.2.1.23).
- Chimeric intron (CI) – a hybrid intron consisting of a human beta-globin splice donor and immunoglobulin G (IgG) splice acceptor elements.
- SV40 polyadenylation signal (232bp): The SV40 polyadenylation signal facilitates efficient polyadenylation of the gene mRNA in *cis*. This element functions as a signal for transcriptional termination, a specific cleavage event at the 3' end of the nascent transcript and addition of a long polyadenyl tail.

AAVhu68 *Trans* Plasmid: pAAV2/hu68.KanR

The AAV2/hu68 *trans* plasmid pAAV2/hu68.KanR was constructed in the laboratory of Dr. James M. Wilson at the University of Pennsylvania. The AAV2/hu68 *trans* plasmid encodes the four wild type (WT) AAV2 replicase (Rep) proteins required for the replication and packaging of the AAV vector genome. The AAV2/hu68 *trans* plasmid also encodes three WT AAVhu68 virion protein capsid (Cap) proteins, which assemble into a virion shell of the AAV serotype hu68 to house the AAV vector genome. The AAVhu68 sequence was obtained from human heart tissue DNA.

To create the AAV2/hu68 *trans* plasmid, the AAV9 cap gene from plasmid pAAV2/9n which encodes the wild type AAV2 rep and AAV9 cap genes on a plasmid backbone derived from the pBluescript KS vector was removed and replaced with the AAVhu68 cap gene. The ampicillin resistance (AmpR) gene was also replaced with the kanamycin resistance (KanR) gene, yielding pAAV2/hu68.KanR. The AAV p5 promoter, which normally drives rep expression, is moved from the 5' end of rep to the 3' end of cap, leaving behind a truncated p5 promoter upstream of rep. This truncated promoter serves to down-regulate expression of rep and, consequently, maximize vector production (FIG 1C). All component parts of the plasmid have been verified by direct sequencing.

pAdDeltaF6(KanR) Adenovirus Helper Plasmid:

Plasmid pAdDeltaF6(KanR) is 15,774 bp in size. The plasmid contains the regions of adenovirus genome that are important for AAV replication, namely E2A, E4, and VA RNA (the adenovirus E1 functions are provided by the HEK293 cells), but does not contain other adenovirus replication or structural genes. The plasmid does not contain the *cis* elements critical for replication such as the adenoviral inverted terminal repeats and therefore, no infectious adenovirus is expected to be generated. The plasmid was derived from an E1, E3 deleted molecular clone of Ad5 (pBHG10, a pBR322 based plasmid). Deletions were introduced in the Ad5 DNA to remove expression of unnecessary adenovirus genes and reduce the amount of adenovirus DNA from 32kb to 12kb. Finally, the ampicillin resistance gene was replaced by the kanamycin resistance gene to create pAdeltaF6(KanR). The E2, E4 and VAI adenoviral genes which remain in this plasmid, along with E1, which is present in HEK293 cells, are necessary for AAV vector production.

AAVhu68.GM1 are manufactured by transient transfection of HEK293 cells followed downstream purification. A manufacturing process flow diagram is shown in FIGs 12A – 12B. The major reagents entering into the preparation of the product are indicated on the left side of the diagram and in-process quality assessments are depicted on the right side of the diagram. A description of each production and purification step is also provided.

Cell Culture and Harvest: The cell culture and harvest manufacturing process comprise four main manufacturing steps: cell seeding and expansion, transient transfection, vector harvest and vector clarification (FIG 12A).

Cell Seeding and Expansion: A fully characterized HEK293 cell line is used for the production process.

Transient Transfection: Following approximately 4 days of growth (DMEM media + 10% FBS), cell culture media is replaced with fresh, serum-free DMEM media and the cells

are transfected with the 3 production plasmids using a polyethyleneimine (PEI)-based transfection method. Initially, a DNA/PEI mixture is prepared containing *cis* (vector genome) plasmid, *trans* (rep and cap genes) plasmid, and helper plasmid in a ratio with GMP-grade PEI (PEIPro HQ, PolyPlus Transfection SA). This plasmid ratio was determined to be optimal for AAV production in small-scale optimization studies. After mixing well, the solution is allowed to sit at room temperature for up to 25 minutes, then added to serum-free media to quench the reaction, and finally added to the iCELLis bioreactor. The reactor is temperature- and DO- controlled, and cells are incubated for 5 days.

Vector Harvesting: Transfected cells and media are harvested from the PALL iCELLis bioreactor using disposable bioprocess bags by aseptically pumping the medium out of the bioreactor. Following the harvest, detergent, endonuclease, and $MgCl_2$ (a co-factor for the endonuclease) are added to release vector and digest unpackaged DNA. The product (in a disposable bioprocess bag) is incubated at 37°C for 2 hours in a temperature-controlled single-use mixer to provide sufficient time for enzymatic digestion of residual cellular and plasmid DNA present in the harvest as a result of the transfection procedure. This step is performed to minimize the amount of residual DNA in the final vector drug product (DP). Following incubation, NaCl is added to a final concentration of 500 mM to aid in the recovery of the product during filtration and downstream tangential flow filtration (TFF).

Vector Clarification: Cells and cellular debris are removed from the product using a pre-filter and depth filter capsule (1.2/0.22 µm) connected in series as a sterile, closed tubing and bag set that is driven by a peristaltic pump. Clarification assures that downstream filters and chromatography columns are protected from fouling and bioburden reduction filtration ensures that, at the end of the filter train, any bioburden potentially introduced during the upstream production process is removed before downstream purification.

Purification Process: The purification process comprises four main manufacturing steps: concentration and buffer exchange by TFF, affinity chromatography, anion exchange chromatography, and concentration and buffer exchange by TFF. These process steps are depicted in the overview process diagram (FIG 12B). General descriptions of each of these processes are provided below.

Large-Scale Tangential Flow Filtration: Volume reduction (20-fold) of the clarified product is achieved by TFF using a custom sterile, closed bioprocessing tubing, bag and membrane set. The principle of TFF is to flow a solution under pressure parallel to a membrane of suitable porosity (100 kDa). The pressure differential drives molecules of smaller size through the membrane and effectively into the waste stream while retaining

molecules larger than the membrane pores. By recirculating the solution, the parallel flow sweeps the membrane surface, preventing membrane pore fouling and product loss through binding to the membrane. By choosing an appropriate membrane pore size and surface area, a liquid sample may be rapidly reduced in volume while retaining and concentrating the desired molecule. Diafiltration in TFF applications involves addition of a fresh buffer to the recirculating sample at the same rate that liquid is passing through the membrane and to the waste stream. With increasing volumes of diafiltration, increasing amounts of the small molecules are removed from the recirculating sample. This diafiltration results in a modest purification of the clarified product, but also achieves buffer exchange compatible with the subsequent affinity column chromatography step. Accordingly, we utilize a 100 kDa, PES membrane for concentration that is then diafiltered with a minimum of 4 diavolumes of a buffer composed of 20 mM Tris pH 7.5 and 400 mM NaCl. The diafiltered product is then further clarified with a 1.2/0.22 μm depth filter capsule to remove any precipitated material.

Affinity Chromatography: The diafiltered product is applied to a Poros™ CaptureSelect™ AAV affinity resin (Life Technologies) that efficiently captures the AAVhu68 serotype. Under these ionic conditions, a significant percentage of residual cellular DNA and proteins flow through the column, while AAV particles are efficiently captured. Following application, the column is treated with 5 volumes of a low salt endonuclease solution (250 U/mL endonuclease, 20 mM Tris pH 7.5 and 40 mM NaCl, 1.5 mM $MgCl_2$) to remove any remaining host cell and plasmid nucleic acid. The column is washed to remove additional feed impurities followed by a low pH step elution (400 mM NaCl, 20 mM Sodium Citrate, pH 2.5) that is immediately neutralized by collection into a 1/10th volume of a neutralization buffer (200 mM Bis Tris Propane, pH 10.2).

Anion Exchange Chromatography: To achieve further reduction of in-process impurities including empty AAV particles, the Poros AAV elution pool is diluted 50-fold (20 mM Bis Tris Propane, 0.001% Pluronic F68, pH 10.2) to reduce ionic strength and enable binding to a CIMultus™ QA monolith matrix (BIA Separations). Following a low-salt wash, vector product is eluted using a 60 column volume (CV) NaCl linear salt gradient (10-180 mM NaCl). This shallow salt gradient effectively separates capsid particles without a vector genome (empty particles) from particles containing vector genome (full particles) and results in a preparation enriched for full particles. The full particle peak eluate is collected, neutralized and diluted 20-fold in 20 mM Bis Tris Propane, 0.001% Pluronic F68, pH 10.2 and reapplied to the same column, which has been cleaned in place. The 10-180 mM NaCl

salt gradient is reapplied and the appropriate full particle peak is collected. The peak area is assessed and compared to previous data for determination of the approximate vector yield.

Concentration and Buffer Exchange by Hollow Fiber Tangential Flow Filtration: The pooled anion exchange intermediate is concentrated, and buffer exchanged using TFF. In this step, a 100 kDa membrane hollow fiber TFF membrane is used. During this step, the product is brought to a target concentration and then buffer exchanged into the Intrathecal Final Formulation Buffer (ITFFB, *i.e.*, artificial CSF with 0.001% Pluronic® F68). The product is sterile-filtered (0.22 μm), stored in sterile containers, and frozen at ≤ –60°C in a quarantine location until release for final fill.

Final Fill: The frozen product is thawed, pooled, and adjusted to the target concentration (dilution or concentrating step via TFF) using the final formulation buffer. The product is terminally filtered through a 0.22 μm filter and filled into sterile West Pharmaceutical's Crystal Zenith (cyclic olefin polymer) vials and stoppers with crimp seals at a fill volume to be determined. Vials are individually labeled. Labeled vials are stored at ≤ 60°C.

EXAMPLE 3

An AAV vector expressing human β-gal was developed and the impact of vector administration into the CSF was evaluated on brain enzyme activity, lysosomal storage lesions and neurological signs using a murine disease model. Neurological assessments were adapted from a previous study of the GM1 mouse model [Ichinomya, S., et al., Brain Dev 2007;29:210-216.] 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.

A. 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 were obtain from RIKEN BioResource Research Center. Mice were maintained as heterozygous carriers on a C57BL/6J background. For ICV injections, vectors were diluted in sterile phosphate buffered saline (Gibco) to a volume of 5 μL, and injections were performed freehand on isoflurane anesthetized mice 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. 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 ≤ -60° C. At the time of necropsy, mice were sedated with ketamine and xylazine and CSF was collected by suboccipital puncture using a 32-gauge needle connected to polyethylene tubing. Euthanasia was performed by cervical dislocation. CSF, heart, lung, liver and spleen were immediately frozen on dry ice and stored at ≤ -60° 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.

Vectors were generated as described in Examples 1 and 2.

Empty:Full Particle Ratio: Vector samples are loaded into cells with two-channel charcoal-epon centerpieces with 12 mm optical path length. The supplied dilution buffer is loaded into the reference channel of each cell. The loaded cells are then placed into an AN-60Ti analytical rotor and loaded into a Beckman-Coulter ProteomeLab XL-I analytical ultracentrifuge equipped with both absorbance and RI detectors. After full temperature equilibration at 20°C, the rotor is brought to the final run speed of 12,000 rpm. Absorbance at 280 nm scans are recorded approximately every 3 minutes for approximately 5.5 hours (110 total scans for each sample). The raw data is analyzed using the c(s) method and implemented in the analysis program SEDFIT. The resultant size distributions are graphed and the peaks integrated. The percentage values associated with each peak represent the peak area fraction of the total area under all peaks and are based upon the raw data generated at 280 nm; many labs use these values to calculate empty:full particle ratios. However, because empty and full particles have different extinction coefficients at this wavelength, the raw data can be adjusted accordingly. The ratio of the empty particle and full monomer peak values both before and after extinction coefficient adjustment is used to determine the empty:full particle ratio.

Replication-competent AAV Assay: A sample is analyzed for the presence of replication-competent AAV2/hu68 (rcAAV) that could potentially arise during the production process. The cell-based component consists of inoculating monolayers of HEK293 cells (P1) with dilutions of the test sample and wild type (WT) human adenovirus type 5 (Ad5). The maximal amount of the product tested is 1.0×10^{10} GC of the vector product. Due to the presence of adenovirus, rcAAV amplifies in the cell culture. After 2 days, a cell lysate is generated and Ad5 is heat-inactivated. The clarified lysate is then passed

onto a second round of cells (P2) to enhance sensitivity (again in the presence of Ad5). After 2 days, a cell lysate is generated, and Ad5 is heat-inactivated. The clarified lysate is then passed onto a third round of cells (P3) to maximize sensitivity (again in the presence of Ad5). After 2 days, cells are lysed to release DNA, which is then subjected to qPCR to detect AAVhu68 cap sequences. Amplification of AAVhu68 cap sequences in an Ad5-dependent manner indicates the presence of rcAAV. The use of a AAV2/hu68 surrogate positive control containing AAV2 rep and AAVhu68 cap genes enables the limit of detection of the assay to be determined (0.1, 1, 10, and 100 IU). Using a serial dilution of rAAV (1.0×10^{10}, 1.0×10^{9}, 1.0×10^{8}, and 1.0×10^{7} GC), the approximate quantity of rcAAV present in the test sample can be quantitated.

In Vitro Potency: To relate the ddPCR GC titer to gene expression, an *in vitro* relative potency bioassay is performed. Briefly, cells are plated in a 96-well plate and incubated at 37°C/5% CO_2 overnight. The next day, cells are infected with serially diluted AAV vector and are incubated at 37°C/5% CO_2 for up to 3 days. Cell supernatant is collected and analyzed for β-gal activity based on cleavage of a fluorogenic substrate.

Total Protein, Capsid Protein, Protein Purity and Capsid Protein Ratio: Vector samples are first quantified for total protein against a bovine serum albumin (BSA) protein standard curve using a bicinchoninic acid (BCA) assay. The determination is made by mixing equal parts of sample with a Micro-BCA reagent provided in the kit. The same procedure is applied to dilutions of a BSA standard. The mixtures are incubated at 60°C and absorbance measured at 562 nm. A standard curve is generated from the standard absorbance of the known concentrations using a 4-parameter fit. Unknown samples are quantified according to the 4-parameter regression. To provide a semi-quantitative determination of rAAV purity, the samples are normalized for genome titer, and 5.0×10^{9} GC is separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The SDS-PAGE gel is then stained with SYPRO Ruby dye. Any impurity bands are quantified by densitometry. Stained bands that appear in addition to the three AAV-specific proteins (VP1, VP2, and VP3) are considered protein impurities. The impurity mass percent as well as approximate molecular weight of contaminant bands are reported. The SDS-PAGE gel is also used to quantify the VP1, VP2, and VP3 proteins and determine their ratio.

Enzyme activity assays: Tissues were homogenized in 0.9% NaCl, pH 4.0 use a steel bead homogenizer (TissueLyzer, Qiagen). After 3 freeze-thaw cycles, samples were clarified by centrifugation and protein content was quantified by bicinchoninic acid assay

(BCA) assay. Serum samples were used directly for enzyme assays. For the β-gal activity assay, 1 µL sample was combined with 99 µL of 0.5 mM 4-Methylumbelliferyl β-D-galactopyranoside (Sigma M1633) in 0.15 M NaCl, 0.05% Triton-X100, 0.1 M sodium acetate, pH 3.58. The reaction was incubated at 37° C for 30 minutes, then stopped by addition of 150 µL of 290 mM glycine, 180 mM sodium citrate, pH 10.9. Fluorescence was compared to standard dilutions of 4MU. β-gal activity is expressed as nmol 4MU liberated per hour per mg of protein (tissues) or per ml of serum or CSF. The HEX assay was performed in the same manner as the β-gal activity assay using 1 mM 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (Sigma M2133) as substrate and sample volumes of 1 µL for tissue lysates and 2 µL for serum.

Histology: In addition to the knockout mouse model, we also performed a histological analysis comparing rAAV.hGLB1treated GLB1-/- mice to both vehicle-treated GLB1-/- mice and GLB1+/- control mice following necropsy. We evaluated lysosomal storage lesions by staining brain sections with filipin, a fluorescent molecule that binds GM1 ganglioside, as well as immunostaining for lysosomal-associated membrane protein 1. Filipin staining revealed marked GM1 ganglioside accumulation in neurons of the cortex, hippocampus and thalamus of the vehicle-treated GLB1-/- mice, which was normalized in the GLB1-/- mice treated with rAAV.hGLB1. Immunohistochemistry demonstrated increased lysosomal membrane staining in the cortex and thalamus of vehicle-treated GLB1-/- mice, which was reduced in rAAV.hGLB1-treated GLB1-/- mice similar to GLB1+/- control mice. Brains were fixed overnight in 4% paraformaldehyde, equilibrated in 15% and 30% sucrose, then frozen in OCT embedding medium. Cryosections were stained with filipin (Sigma, 10 µg/mL) or antibodies against GFAP or LAMP1.

Anti-β-gal antibody ELISA: High binding polystyrene ELISA plates were coated overnight with 100 µL per well of recombinant human β-gal (R&D Systems) at a concentration of 1 µg/mL in PBS. Plates were washed and blocked for 2 hours at room temperature with 2% bovine serum albumin in PBS. Duplicate wells were incubated with serum samples diluted 1:1,000 in PBS for one hour at room temperature. Plates were washed, incubated for one hour with a horseradish peroxidase-conjugated anti-mouse IgG polyclonal antibody diluted 1:5,000 in blocking solution, and developed using TMB substrate.

Evaluation of Treatment-Effects on Neurological Function

In order to evaluate neurological function in rAAV.hGLB1-treated GLB1-/- mice, gait analysis was performed at four months of age (three months after rAAV.hGLB.1 or

vehicle administration) over two consecutive days using the CatWalk XT gait analysis system (Noldus), a commonly used assessment of motor performance in mice, according to manufacturer's instructions. Mice were tested on two consecutive days. At least 3 complete trials were acquired for each animal on each day of testing. Trials lasting more than 5 seconds, or trials in which the animal did not traverse the entire length of the apparatus before stopping or turning around were excluded from analysis. Average walking speed and the length of the hind paw print were quantified for each animal across at least three assessments on the second day of testing. Slower speed and elongated paw prints are indicative of impaired motor performance. As shown in the figure below, walking speed and paw print length improved significantly in rAAV.hGLB1-treated GLB1-/- mice compared to vehicle- treated $GLB1^{-/-}$ mice, and were similar to the $GLB1^{+/-}$ control mice. See, e.g., FIGs 7C and 7D.

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 Day 300.

In the Day 150 cohort, all mice survived to the scheduled necropsy except one vehicle-treated $GLB1^{-/-}$ mouse (FIG 13). This animal died 2 days after vector administration due to intracranial hemorrhage likely caused by the ICV injection procedure.

In the Day 300 cohort, all 12 vehicle-treated $GLB1^{-/-}$ mice were euthanized according to the study-defined euthanasia criteria prior to the scheduled study endpoint. The mice exhibited neurological signs (i.e., ataxia, tremors, and/or limb weakness) as a result of disease progression. The median survival of vehicle-treated $GLB1^{-/-}$ mice was 268 days (ranging from 185–283 days). In the lowest dose group (4.4×10^9 GC), 5/12 (41.7%) animals were euthanized due to disease progression with survival ranging from 268–297 days. A single animal (1/12 [8.3%]) in the 1.3×10^{10} GC dose cohort was euthanized due to disease progression 290 days post treatment. All animals that received vector doses of 4.4×10^{10} GC or 1.3×10^{11} GC survived to the study endpoint.

Gait analysis evaluated the stride length and hind paw print length of vehicle- and vector-treated mice at baseline (Day –7–0) and every 60 days through Day 240. Gait analysis revealed progressive abnormalities in vehicle-treated $GLB1^{-/-}$ mice, while $GLB1^{-/-}$ mice treated with the two highest vector doses (1.3×10^{11} GC and 4.4×10^{10} GC) demonstrated consistent improvements in both gait parameters.

At baseline, the average stride length 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 rAAV.hGLB1-treated GLB1–/– mice, which displayed a statistically significant increase in average stride length compared to that of the vehicle-treated GLB1-/- mice at all doses by Day 60. However, by Day 240, only the 2 highest dose groups (1.3×10^{11} GC and 4.4×10^{10} GC) maintained a significantly longer average stride length compared to that of the vehicle treated GLB1-/- mice.

On Day 60, the average hind paw print length 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 rAAV.hGLB1 administration in GLB1-/- mice at the 3 highest doses (1.3×10^{11} GC, 4.4×10^{10} GC, 1.3×10^{10} GC), resulting in a statistically significant decrease in average hind paw print length compared to that of the vehicle treated GLB1-/- mice by Day 240.

Dose Ranging Pharmacology Study

A pharmacology study was conducted to evaluate the minimum effective dose, or MED, and β-gal expression levels in a GLB1 knockout mouse model of GM1 following ICV administration of rAAV.hGLB1. In this study, GLB1-/- mice were ICV-administered with rAAV.hGLB1at four separate dose levels. GLB1-/- mice and heterozygous GLB1 mice, or HET mice, were ICV-administered with vehicle. In this study, ICV administration of rAAV.hGLB1 resulted in stable, dose-dependent increases in transgene product expression in the brain and peripheral organs, resolution of brain lysosomal storage lesions, improvements in neurological phenotype and increased survival of GLB1-/- mice. The lowest dose evaluated is considered the MED based on statistically significant improvements in survival, neurological exam scores and brain storage lesions.

B. Results:

Transgene cassettes were designed consisting of a human GLB1 cDNA driven by chicken beta actin promoter with a cytomegalovirus enhancer (CB7), human elongation initiation factor 1 alpha promoter (EF1a) or human ubiquitin C promoter (UbC). Each cassette was packaged in an AAVhu68 capsid, and a single dose of 10^{11} genome copies (GC) was administered by intracerebroventricular (ICV) injection to wild-type mice. Two weeks after injection, β-gal activity was measured in brain and CSF (FIGs 2A - 2B). The vector carrying the UbC promoter achieved statistically significant elevations in β-gal activity in

both the brain and CSF, with enzyme activity nearly 2-fold greater than that of untreated wild-type mice in the brain, and 10-fold greater in CSF. The AAVhu68.UbC.hGLB1 vector was therefore selected for further studies.

Efficacy of the optimized vector was assessed in the $GLB1^{-/-}$ mouse model. Mouse models of GM1 gangliosidosis have been developed by targeted insertion of neomycin resistance cassettes into the 6th and/or 15th exons of the GLB1 gene. Hahn, C.N., *et al.* Generalized CNS disease and massive GM1-ganglioside accumulation in mice defective in lysosomal acid beta-galactosidase. Human molecular genetics 6, 205-211 (1997) and Matsuda, J., *et al.* Beta-galactosidase-deficient mouse as an animal model for GM1-gangliosidosis. Glycoconjugate journal 14, 729-736 (1997). Similar to infantile GM1 gangliosidosis 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 (Hahn 1997, as cited above). The clinical phenotype of the $GLB1^{-/-}$ mouse most closely models that of infantile GM1 gangliosidosis, with motor abnormalities appearing by 4 months of age and severe neurological symptoms (*e.g.*, ataxia or paralysis) necessitating euthanasia presenting by 10 months of age (Hahn 1997; Matsuda 1997, as cited above). The $GLB1^{-/-}$ mouse model does not exhibit any peripheral organ involvement, unlike infantile GM1 patients who often develop bone deformities and hepatosplenomegaly (Hahn 1997; Matsuda 1997, as cited above. The $GLB1^{-/-}$ mouse is therefore a representative model of the neurological features of infantile GM1 gangliosidosis, but not the systemic disease manifestations.

$GLB1^{-/-}$ mice were treated at one month of age, and observed until four months of age, when they would typically develop marked gait abnormalities associated with brain GM1 levels similar to those of infantile GM1 gangliosidosis patients with advanced disease (Matsuda 1997, as cited above). $GLB1^{-/-}$ mice were treated with a single ICV injection of 1.0×10^{11} genome copies (GC) of AAVhu68.UbC.hGLB1 (n = 15) or vehicle (n = 15). A group of heterozygous ($GLB1^{+/-}$) mice treated with vehicle (n = 15) served as normal controls. Serum was collected on the day of injection (Day 0) and on Days 10, 28, 60 and 90. Motor function was assessed using the CatWalk XT gait analysis system (Noldus Information Technology, Wageningen, The Netherlands) 90 days post treatment, after which animals were euthanized and tissues collected for histological and biochemical analysis. The CatWalk 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 calibrated, with the appropriate width of the walkway set, prior to the start of the test. Animals were brought into the room and allowed to acclimate in darkness for at least 30 minutes prior to running the Catwalk XT. Once acclimation was complete, an animal was selected and placed at the entrance of the walkway. The researcher started the acquisition 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, otherwise the run was repeated. Animals ran three trials with a minimum duration of 0.50 seconds and a maximum duration of 5.00 seconds. Three successful runs were needed for the trial to be considered complete. If an animal failed to complete three runs after 10 minutes of testing, only the completed runs were used for analysis. The analysis was performed by an evaluator blinded to animal ID and treatment group. Runs were auto-classified 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. Analyses were performed 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 using a two-way analysis of variance (ANOVA). Survival curves were compared between groups using a log-rank (Mantel-Cox) test. Brain LAMP1 data were log-transformed and compared using a one-way ANOVA followed by Dunnett's test.

One AAV-treated mouse died during the ICV injection procedure. All other mice survived until the 90-day study endpoint. AAV delivery into the CSF has been shown to result in vector distribution in the peripheral blood and significant hepatic transduction. (Hinderer, C., *et al.* Intrathecal gene therapy corrects CNS pathology in a feline model of mucopolysaccharidosis I. Molecular therapy : the journal of the American Society of Gene Therapy 22, 2018-2027 (2014); Gray, S.J., Nagabhushan Kalburgi, S., McCown, T.J. & Jude Samulski, R. Global CNS gene delivery and evasion of anti-AAV-neutralizing antibodies by intrathecal AAV administration in non-human primates. Gene therapy 20, 450-459 (2013); Haurigot, V., *et al.* Whole body correction of mucopolysaccharidosis IIIA by intracerebrospinal fluid gene therapy. The Journal of clinical investigation (2013); Hinderer, C., *et al.* Widespread gene transfer in the central nervous system of cynomolgus macaques

following delivery of AAV9 into the cisterna magna. Molecular therapy. Methods & clinical development 1, 14051 (2014); Hordeaux, J., *et al.* Toxicology Study of Intra-Cisterna Magna Adeno-Associated Virus 9 Expressing Human Alpha-L-Iduronidase in Rhesus Macaques. Molecular therapy. Methods & clinical development 10, 79-88 (2018)). $GLB1^{-/-}$ mice treated with AAVhu68.UbC.hGLB1 exhibited serum β-gal activity greater than that of heterozygous ($GLB1^{+/-}$) controls 10 days after vector administration (FIG 3A). Serum antibodies against human β-gal were detectable in 5/15 mice treated with AAVhu68.UbC.hGLB1 by Day 90. Elevated serum β-gal activity persisted throughout the study for all but two mice, both of which developed antibodies against human β-gal (FIG 6). Peripheral organs including the heart, lung, liver and spleen also exhibited elevated β-gal activity (FIGs 3B-3E). Some animals that developed antibodies against the human transgene product had lower β-gal activity in peripheral organs.

CSF collected at the time of necropsy demonstrated β-gal activity exceeding that of heterozygous controls in $GLB1^{-/-}$ mice treated with AAVhu68.UbC.hGLB1 (FIG 4B). β-gal activity in the brains of vector-treated mice was similar to heterozygous controls (FIG 4A). Anti-β-gal antibodies did not appear to impact brain or CSF β-gal levels.

Correction of brain abnormalities was assessed using biochemical and histological assays. Lysosomal enzymes are frequently upregulated in the setting of lysosomal storage, an observation that has been confirmed in GM1 gangliosidosis patients (Van Hoof, F. & Hers, H.G. The abnormalities of lysosomal enzymes in mucopolysaccharidoses. European journal of biochemistry 7, 34-44 (1968)). Therefore, the activity of the lysosomal enzyme hexosaminidase (HEX) was measured in brain lysates. HEX activity was elevated in brain samples from vehicle-treated $GLB1^{-/-}$ mice and was normalized in vector-treated animals (FIG 5).

To evaluate the extend of lysosomal storage lesions, brain sections were stained for the lysosomal membrane protein LAMP1 with filipin, a fluorescent molecule that binds to GM1 ganglioside, as well as immunostaining for the lysosomal-associated membrane 1 (protein LAMP1). Filipin also binds to unesterified cholesterol, though previous studies have demonstrated that filipin staining primarily reflects GM1 accumulation in $GLB1^{-/-}$ mice (Arthur, J.R., Heinecke, K.A. & Seyfried, T.N. Filipin recognizes both GM1 and cholesterol in GM1 gangliosidosis mouse brain. Journal of lipid research 52, 1345-1351 (2011)). Filipin staining revealed marked GM1 accumulation in neurons of the cortex, hippocampus and thalamus of vehicle-treated $GLB1^{-/-}$ mice which was normalized in mice treated with AAVhu68.UbC.hGLB1 (data not shown). LAMP1 immunohistochemistry demonstrated

increased lysosomal membrane staining in the cortex and thalamus of $GLB1^{-/-}$ mice, which was reduced in vector-treated mice (data not shown). Gliosis was assessed by staining for the astrocyte marker, glial fibrillary acidic protein (GFAP). Vector treated $GLB1^{-/-}$ mice exhibited markedly reduced astrogliosis in the thalamus compared to vehicle-treated controls (data not shown).

In order to evaluate neurological function in vector-treated $GLB1^{-/-}$ mice, gait analysis was performed at 4 months of age (3 months after vector or vehicle administration). Untreated $GLB1^{-/-}$ mice were previously noted to exhibit clinically apparent gait abnormalities by 3-4 months of age. Quantitative gait assessments performed using the CatWalk system on a cohort of untreated $GLB1^{-/-}$ mice and normal controls revealed a variety of abnormalities, including slower voluntary walking speed, differences in stride length, and the duration of some phases of the step cycle (FIGs 7C and 7D). Due to the significantly slower walking speed of the $GLB1^{-/-}$ mice, interpretation of many of these apparent differences was complicated by the speed dependence of most gait parameters (FIGs 8A and 8B) (Batka, R.J., *et al.* The need for speed in rodent locomotion analyses. Anatomical record (Hoboken, N.J. : 2007) 297, 1839-1864 (2014)). $GLB1^{-/-}$ mice also exhibited a consistent abnormality in the placement of the hind paws, which could be measured as an increased length of the hind paw prints (FIG 7D). This abnormality was found to be independent of walking speed, consistent with previous reports (Batka, *et al*, as cited above), making it a useful gait signature to assess speed-independent gait dysfunction in $GLB1^{-/-}$ mice (FIGs 8A and 8B). Tests conducted using the same cohort of mice on two consecutive days revealed that slower voluntary walking speed and increased hind print length are reproducible observations in untreated $GLB1^{-/-}$ mice (FIGs 7A and 7B). Vehicle treated $GLB1^{-/-}$ mice exhibited similar gait abnormalities to those previously identified in untreated animals (FIGs 7A-7G). Walking speed and print length were normalized in vector-treated $GLB1^{-/-}$ mice (FIGs 7A-7G).

Survival Data: FIG 13 shows survival data of each cohort in the study through day 300. All 12 vehicle- treated GLB1-/- mice were euthanized according to the study defined euthanasia criteria prior to the scheduled study endpoint due to disease progression with neurological signs, characterized by ataxia, tremors and limb weakness. The median survival of this group was 268 days. In the lowest dose group, 5/12 animals were euthanized due to disease progression. In the second lowest dose cohort, 1/12 was euthanized due to disease progression. All animals in the two highest dose cohorts survived to the study endpoint.

Neurological Examinations: A standardized neurological examination was performed in a blinded fashion every 60 days through day 240, and an average total severity score was obtained. FIG 14C shows average total severity score for each cohort as of each assessment period. Beginning at the Day 120 assessment, *Glb1*$^{-/-}$ mice administered either vehicle or the lowest dose of vector (4.4 x 10^9 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 that of vehicle-treated *Glb1*$^{-/-}$ mice, suggesting that this dose (4.4 x 10^9 GC) partially rescued the neurological phenotype. At the next highest dose (1.3 x 10^{10} GC), minimal abnormalities were detectable in 7/12 (58.3%) animals at the Day 240 assessment, suggesting substantial rescue of the neurological phenotype. At the two highest vector doses (1.3 x 10^{11} GC and 4.4 x 10^{10} 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.

The results of vehicle-treated *GLB1*$^{-/-}$ mice exhibited progressively higher total severity scores indicative of progressive neurological signs beginning at the day 120 assessment. At the lowest dose of rAAV.hGLB1, a progressive increase in the total severity score was also observed by the day 120 assessment, although the total severity score was significantly lower than that of the vehicle-treated GLB1$^{-/-}$ mice at the same time point. At the second lowest rAAV.hGLB1 dose, minimal abnormalities were detectable in 7/12 animals at the day 240 assessment. At the two highest doses of rAAV.hGLB1, neurological abnormalities were not apparent, and total severity scores for these groups were similar to those of the normal vehicle-treated GLB1$^{+/-}$ time point.

Histological Analysis: A histological analysis was also performed comparing brain sections of rAAV.hGLB1-treated GLB1-/- mice, vehicle treated GLB1-/- mice and vehicle treated GLB1+/- control mice at baseline, day 150 and day 300. Brain cryosections were stained with antibodies against lysosomal-associated membrane protein (LAMP1) (Abcam, Catalog #Ab4170) , overnight at 4°C. The next day, slides were washed and incubated with an anti-rabbit IgG TritC-conjugated secondary antibody for 1 hour at room temperature. Slides were washed and coverslips applied. LAMP1 staining was quantified as positive cells per field of the whole cerebral cortex from one coronal brain section using VisioPharm image analysis software. Cortical cells positive for LAMP1 (i.e., cells exhibiting lysosomal distention) were quantified in scanned sections using an automated program. 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. Untreated GLB1-/- baseline mice necropsied on day 1 exhibited a higher proportion of LAMP1-positive cells in the brain compared to that of normal untreated GLB1+/- baseline controls. At both day 150 and day 300, rAAV.hGLB1-treated mice exhibited a dose-dependent reduction in the proportion of LAMP1-positive cells compared to that of vehicle-treated GLB1-/- controls. At the two highest doses of rAAV.hGLB1 the proportion of LAMP1-positive cells were reduced to levels similar to those of normal vehicle-treated GLB1+/- controls.

β-gal Activity: β-gal activity was measured in serum on the day of dosing and every 60 days thereafter until day 240. At necropsy, β-gal activity was measured in the brain and peripheral organs (heart, liver, spleen, lung and kidney). As shown in FIG 9C, average β-gal activity in serum in $GLB1^{-/-}$ mice administered the highest dose of test hAAV.hGLB1 (1.3×10^{11} GC) was approximately 10-fold greater than that of normal vehicle-treated GLB1+/- controls. At the second highest dose of test hAAV.hGLB1(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 rAAV.hGLB1 doses was similar to that of vehicle-treated $GLB1^{-/-}$ controls.

For each tissue type examined, average β-gal activity levels within each group were similar at both time points (Day 150 and Day 300) (FIGS 17A-L). 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 (FIGS 17A-L). Of particular note, the heart displayed dose-dependent increases in β-gal activity, resulting in average levels higher than that 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 that 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, β-gal activity levels of vector-treated mice were compared to that of normal vehicle-treated $Glb1^{+/-}$ controls (Fig. 16C). 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, β-gal activity levels of vector-treated mice were compared to that of normal vehicle-treated $Glb1^{+/-}$ controls (FIG 16C). As shown in FIG 16C, β-gal activity was detectable in the CSF of all mice evaluated. GLB1-/- mice administered the two highest doses of test rAAV.hGLB1 (1.3×10^{11} GC and 4.4×10^{10} GC) displayed average CSF β-gal activity levels exceeding that of normal vehicle-treated GLB1+/- controls. β-gal activity in CSF was generally dose-dependent, although β-gal activity in the two lowest dose groups (1.3×10^{10} GC and 4.4×10^{9} GC) appeared similar to that of the vehicle-treated $Glb1^{+/-}$. 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 (4.4×10^{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.

FIGS 17A - L show β-gal activity in the brain, heart and liver following necropsy. In the brain, β-gal activity increased in a dose-dependent manner in test rAAV.hGLB1-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 exhibited higher average β-gal activity than that of the normal vehicle-treated GLB1+/- controls at both time points. Some peripheral organs also exhibited dose-dependent increases in β-gal activity after test rAAV.hGLB1 administration. The heart displayed dose-dependent increases in β-gal activity, resulting in average levels higher than that of vehicle-treated GLB1-/- mice at all doses. However, only the two highest doses restored β-gal activity to levels similar to or higher than that of normal vehicle-treated GLB1+/- controls at both time points. The liver displayed dose-dependent increases in β-gal activity after test rAAV.hGLB1 administration. At all doses except the lowest dose, average β-gal activity levels at both time points were higher than that of vehicle-treated GLB1-/- mice and similar to or higher than that of normal vehicle-treated GLB1+/- controls.

C. Discussion:

These results indicate that rAAVhu68.hGLB1 administration into the CSF increases brain β-gal activity, reduces neuronal lysosomal storage lesions, and prevents neurological decline, and gene transfer may both prevent and reverse GM1 storage in the brain.

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. Patients with infantile GM1 gangliosidosis are a suitable population for AAV gene therapy, as they are frequently diagnosed based on subtle neurological findings that appear in the first six months of life before the onset of the rapid developmental regression that inevitably follows within 1 to 2 years.

EXAMPLE 4: Animal Models

A. Identification of the minimum effective dose (MED) of AAVhu68.UbC.GLB1 in the GLB1$^{-/-}$ mouse model

The impact of different doses of rAAVhu68.UbC.GLB1 was evaluated on CNS lesions and neurological signs in the GLB1$^{-/-}$ mouse model. Efficacy was assessed by serum enzyme activity, reduction of brain lesions, neurological signs measured by automated gait analysis (for example via CatWalk system) and a standardized neurological exam (for example, 9 point assessment of posture, motor function, sensation and reflexes) performed by a blinded reviewer, and survival. Safety analyses (including blood collection and analysis) were also performed. Four-week old GLB1$^{-/-}$ mice received one of 4 doses of rAAVhu68.UbC.GLB1 (1.3×10^{11} GC, 4.4×10^{10} GC, 1.3×10^{10} GC or 4.4×10^{9} GC) or vehicle by ICV injection (n = 24 per group). Heterozygous littermates treated with vehicle (n = 24) served as normal controls.

Serum β-gal enzyme activiy, gait analysis and neurological exam were perfomred on half of the animals for each group every 60 days while the body weights were measured at least every 30 days in an observation period of 120 days. Results are ploted as FIGs 9A-9F and briefly described below.

All treated mice appeared healthy, exhibiting normal weight gain. During the observation period, no significant differences in body weights among groups were detected (FIG 9B).

Serum enzyme expression was consistent with the study discussed in Example 3. As shown in FIG 9A, β-gal enzyme activity of the vehicle treated GLB1$^{-/-}$ mice (which served as a negative control) remained around 10 nmol/mL/hour while the positive control group (which are vehicle treated GLB1$^{+/-}$ mice) demonstrated an about 100 nmol/mL/h enzyme activity. Upon treatment with rAAVhu68.UbC.GLB1 at a dose of 4.4×10^{10} GC per mouse, the β-gal enzyme activiy increased signficantly compared to the negative control on both

Day 60 and Day 120. A higher dose of rAAVhu68.UbC.GLB1 at 1.3 x 10^{11} GC per mouse resulted in a β-gal enzyme activiy higher than the positive control on Day 60 with a further elevation on Day 120.

Gait phenotype of GM1 mouse was also consistent with the previous results shown in Example 3. Neurological exam score, hind paw print length, hind limb swing time, and hind limb stride length were acquired and the results are plotted in FIGs. 9C-9F. For all four plotted parameters, there is a significant statistical difference between the negative control and the positive control, indicating those parameters may serve as good indicaters for evaluating efficacy. Compared to vehicle treated GLB1$^{-/-}$ mice, mice treated with 4.4 x 10^{10} GC of rAAVhu68.UbC.GLB1 showed significant improvements in hind paw print length, hind limb swing time and hind limb stride length. A higher dose at 1.3 x 10^{11} GC provided an increased swing time and longer stride length in hind limb, indicating successful corrections. Neurological exam is more sensitive compared to gait analysis. An dosage dependent amelioration shown by decreased neurological score with increased dose was observed as shown in FIG 9C, while treatment with 1.3 x 10^{10} GC of rAAVhu68.UbC.GLB1 displayed a statistical significance in the total score compared to that of the negative control. Evidence of phenotype correction was observed at doses as low as 1.3 x 10^{10} GC per mouse.

The same set of parameters continues being collected in this animal cohort for at least another 150 days, when all untreated animals are expected to remain alive. Survival changes relative to untreated GLB1$^{-/-}$ mice are evaluated.

The first half of animals discussed in the above paragraph are sacrificed 270 days after treatment. The remaining half animals are sacrificed 150 days after treatement. Another 24 mice are served as a baseline necropsy control. Histological and biochemical comparisons are perfomred between treated and untreated animals for all sacrificed animals. After necropsy, brains are sectioned and stained for LAMP1 to evaluate lysosomal storage lesions, which are quantified using an automated imaging system. β-gal activity is measured in the brain, serum and peripheral organs. For safety analysis, blood is collected at necropsy for complete blood counts and serum chemistry panels, and the brain, spinal cord, heart, lungs, liver, spleen, kidneys and gonads are collected for evaluation of histopathology by a board certified veterinary pathologist. The lowest dose of rAAVhu68.UbC.GLB1 that achieves a significant reduction of brain storage lesions relative to vehicle-treated GLB1$^{-/-}$ mice are selected as the minimum effective dose (MED).

Results: A single intracerebroventricular (ICV) administration of rAAVhu68.UbC.GLB1 at dose levels ranging from 4.40 x 10^{9} genome copies (GC) to 1.30 x

10^{11} GC in GLB1-/- mice at 4 weeks of age resulted in the resolution of brain storage lesions, corroborative with increased β-galactosidase activity measured in the brain. Survival, resolution of brain storage lesions and neurological function, as measured by automated gait analysis and a standardized neurological examination, improved in a dose-dependent manner. Hepatic transduction and serum β-galactosidase activity in rAAVhu68.UbC.GLB1 treated mice significantly exceeded that of heterozygous controls (Glb+/- mice). Biochemical correction in peripheral organs was observed with rAAVhu68.UbC.GLB1 treatment, indicating the possibility of treating both central and peripheral disease with a single ICV administration. The lowest dose evaluated (4.4×10^9 GC) was considered the MED based on statistically significant improvements in survival, neurological exam scores, and brain storage lesions.

B. Toxicology study in nonhuman primates (NHPs)

Rhesus monkeys were selected for toxicology studies because they best replicate the size and CNS anatomy of the patient population (infants 4–18 months of age) and can be treated using the clinical route of administration (ROA). Juvenile animals were selected to be representative of the pediatric trial population. In one embodiment, the juvenile rhesus monkeys are 15 to 20 months of age. The similarity in size, anatomy, and ROA resulting in representative vector distribution and transduction profiles, allow for accurate assessment of toxicity. In addition, more rigorous neurological assessments are performed in NHPs than in rodent models, allowing for more sensitive detection of CNS toxicity.

A 120 day GLP-compliant safety study was conducted in juvenile rhesus macaques to investigate the toxicology of AAVhu68.UbC.GLB1 following ICM administration. The 120-day evaluation period was selected as this allows sufficient time for a secreted transgene product to reach stable plateau levels following ICM AAV administration. The study design is outlined in Table below. Rhesus macaques receive one of three dose levels: 3.0×10^{12} GC total, 1.0×10^{13} GC total, or 3.0×10^{13} GC total (n=6/dose) or vehicle (n=4). Dose levels were selected to be equivalent to those that are evaluated in the MED study when scaled by brain mass (assuming 0.4 g for mouse and 90 g for rhesus monkey). Baseline neurologic examinations, clinical pathology (cell counts with differentials, clinical chemistries, and coagulation panel), CSF chemistry and CSF cytology were performed. After AAVhu68.UbC.GLB1 or vehicle administration, the animals were monitored daily for signs of distress and abnormal behavior.

Blood and CSF clinical pathology assessments and neurologic examinations were performed on a weekly basis for 30 days following rAAVhu68.UbC.GLB1 or vehicle administration, and every 30 days thereafter. At baseline and at each 30-day timepoint thereafter, neutralizing antibodies to AAVhu68 and cytotoxic T lymphocyte (CTL) responses to AAVhu68 and the AAVhu68.UbC.GLB1 transgene product were assessed by an interferon gamma (IFN-γ) enzyme-linked immunospot (ELISpot) assay.

Rhesus macaque Good Laboratory Practice (GLP) Toxicology Study

Group Designation	1	2	3	4
Number of macaques	4	6	6	6
Sex/age	M+F/juvenile	M+F/juvenile	M+F/juvenile	M+F/juvenile
Test article	Vehicle	AAVhu68.UbC.GLB1	AAVhu68.UbC.GLB1	AAVhu68.UbC.GLB1
Route of administration	ICM	ICM	ICM	ICM
Vector Dose (total dose)	N/A	3.0×10^{12} GC	1.0×10^{13} GC	3.0×10^{13} GC
Necropsy Day	60 (3) 120 (3)	60 (3) 120 (3)	60 (3) 120 (3)	60 (3) 120 (3)

After administration of either rAAVhu68.UbC.GLB1 or vehicle, half of the animals were euthanized on Day 60 and half are euthanized on Day120. Tissues were harvested for comprehensive microscopic histopathological examination. The histopathological examination focused on central nervous system tissues (brain, spinal cord, and dorsal root ganglia) and the liver because these are the most heavily transduced tissues following ICM administration of AAVhu68 vectors. In addition, lymphocytes were harvested from the spleen and bone marrow to evaluate the presence of T cells reactive to both the capsid and transgene product in these organs at the time of necropsy.

Vector biodistribution was evaluated by quantitative PCR in tissue samples. Vector genomes were quantified in serum and CSF samples.

Results:

The 120-day good laboratory practice, or GLP, -compliant toxicology study was conducted in NHPs to assess the safety, tolerability and biodistribution and excretion (shedding) profile of vector following ICM administration. Juvenile male and female rhesus macaques received a single ICM administration of vehicle or one of three dose levels of

rAAV.hGLB1. Animals from each cohort were euthanized either 60 or 120 days following administration.

In-life evaluations included clinical observations performed daily, multiple scheduled physical exams, standardized neurological monitoring, sensory nerve conduction studies, or NCS, body weights, clinical pathology of the blood and CSF, evaluation of serum-circulating neutralizing antibodies and assessment of vector pharmacokinetics and vector excretion.

Animals were necropsied, and tissues were harvested for a comprehensive histopathological examination, measurement of T-cell responses and biodistribution analysis.

C. Sensory neuron toxicity in nonclinical AAV studies

Nonclinical studies evaluating systemic and intrathecal (IT) administration of AAV have consistently demonstrated efficient transduction of sensory neurons within dorsal root ganglia (DRG), and in some cases, evidence of toxicity involving these cells. Intrathecal administration could allow for sensory neuron transduction because their central axons are exposed to CSF, or the rAAV may directly reach the cell body since the DRG is exposed to the spinal CSF. The results of the nonclinical studies suggest that ICM administration of rAAV.hGLB in subjects aged 1 to 24 months with GM1 gangliosidosis will increase central beta-galactosidase levels and prevent disease progression. The nonclinical toxicology data indicate that clinical safety monitoring should consist of those assessments typically utilized for other AAV gene therapies, with the addition of peripheral nerve safety monitoring.

Sensory nerve conduction studies were performed for all animals prior to rAAV.hGLB administration and monthly thereafter to measure bilateral median nerve sensory action potential amplitudes and conduction velocities. Animals were sedated with a combination of ketamine/dexmedetomidine. Sedated animals were placed in lateral or dorsal recumbency on a procedure table with heat packs to maintain body temperature. Electronic warming devices were not used due to the potential for interference with electrical signal acquisition.

Sensory nerve conduction studies (NCS) were performed using the Nicolet EDX® system (Natus Neurology) and Viking® analysis software. Briefly, the stimulator probe was positioned over the median nerve with the cathode closest to the recording site. Two needle electrodes were inserted subcutaneously on digit II at the level of the distal phalanx (reference electrode) and proximal phalanx (recording electrode), while the ground electrode was placed proximal to the stimulating probe (cathode). A WR50 Comfort Plus Probe pediatric stimulator (Natus Neurology) was used. The elicited responses were

differentially amplified and displayed on the monitor. The initial acquisition stimulus strength was set to 0.0 mA in order to confirm a lack of background electrical signal. In order to find the optimal stimulus location, the stimulus strength was increased up to 10.0 mA, and a train of stimuli were generated while the probe was moved along the median nerve until the optimal location was found as determined by a maximal definitive waveform. Keeping the probe at the optimal location, the stimulus strength was progressively increased up to 10.0 mA in a step-wise fashion until the peak amplitude response no longer increased. Each stimulus response was recorded and saved in the software. Up to 10 maximal stimuli responses were averaged and reported for the median nerve. The distance (cm) from the recording site to the stimulation cathode was measured and entered into the software. The conduction velocity was calculated using the onset latency of the response and the distance (cm). Both the conduction velocity and the average of the sensory nerve action potential (SNAP) amplitude were reported. The median nerve was tested bilaterally. All raw data generated by the instrument were retained as part of the study file.

For SNAP amplitudes, inter- and intra-animal variability was apparent, though values typically remained within the range of baseline measurements (FIGS 21A-21B). One animal administered the mid-dose (Animal 17-226 [1.0 x 10^{13} GC, Group 7]) and one animal administered the high dose (Animal 17-205 [3.0 x 10^{13} GC, Group 8]) exhibited a marked reduction in bilateral median nerve sensory amplitudes 28 days after rAAV.GLB administration, which persisted until the time of necropsy (FIGS 21A-21B). There were no abnormal clinical findings in these animals, but the findings did correlate with histopathology findings in the peripheral nerves. In animals presenting a marked reduction in SNAP (Animal 17-226 [1.0 x 1013 GC, Group 7] and Animal 17-205 [3.0 x 1013 GC, Group 8]), the onset latency could not be determined, therefore precluding the measurement of conduction velocity. For all other animals, no significant changes in median nerve conduction velocities were observed throughout the study (FIGS 21A-21B).

Histopathologic Findings

A. Histopathology was assessed by Hematoxylin and Eosin Staining or Trichrome staining.

Hematoxylin and Eosin Staining: All tissues and any gross lesions were collected and labeled according to SOP 4019. The samples in pre-labeled cassettes were fixed in 10% Neutral Buffer Formalin, modified Davidson's solution (eyes), or Davidson's solution (testis) according to SOP 4003. All wet tissues were sent to Histo-Scientific Research Laboratories for tissue processing, embedding, sectioning, and

hematoxylin and eosin (H&E) staining. For histopathological evaluation, histopathology slides were initially evaluated by the Primary Study Pathologist, who prepared a preliminary pathology report based on the histologic assessment, gross necropsy findings, pertinent clinical pathology results, and any supporting data that aided in the interpretation of histopathologic findings. Once the primary review was complete, the draft pathology report, slides, and any supporting materials used to generate the draft report were submitted to the Peer-Review Pathologist for peer-review. A pathology peer-review memo was generated, signed, and dated by the Peer-Review Pathologist. The memo included documentation of the materials, methods, and conduct of the peer-review process, and the Peer-Review Pathologist's general agreement with the Primary Study pathologist's pathology report. Differences, if any, between the Primary Study and Peer-Review Pathologist were reconciled, and upon completion of the peer-review, the final report was prepared. The final study report incorporated input from the peer-review report and was reviewed for quality assurance by the Quality Assurance Unit (QAU) of the GTP.

For Trichrome Staining: At the discretion of the Study Pathologist, Masson's trichrome histochemical stain was employed to further evaluate findings of interest identified through H&E staining (i.e., periaxonal fibrosis). Slides of the left and right proximal median nerves were stained using Masson's Trichrome Stain Kit (Polysciences, Inc.; Catalog number: 25088-1). For histopathological evaluation, slides were examined by light microscopy and scored by the Primary Study Pathologist in blinded fashion using the same semi-quantitative scoring system used for H&E-stained slides. Slides were also digitally scanned using an Aperio VERSA Scanning System (Leica Biosystems) and quantified using VIS image analysis software (Visiopharm; Hoersholm, Denmark; Version 2019.07.0.6328).

B. Histopathology Findings

Test article-related findings were observed primarily within the DRG, trigeminal ganglia (TRG), dorsal white matter tracts of the spinal cord, and peripheral nerves. These findings consisted of neuronal degeneration within the DRG/TRG and axonal degeneration (i.e., axonopathy) within the dorsal white matter tracts of the spinal cord and peripheral nerves. Overall, these findings were observed across all GTP-203-treated groups; however, the incidence and severity tended to be higher in individual animals from the mid-dose (1.0×10^{13} GC) and high dose (3.0×10^{13} GC) groups at both time points. Other test article-related findings generally included small foci of gliosis in various nuclei and white

matter tracts of the brain, in addition to mononuclear cell infiltrates in the skeletal muscle and adipose tissue at the injection site.

Test article-related histopathologic findings observed across all dose groups at the Day 60 and Day 120 time points consisted of neuronal cell body degeneration with mononuclear cell infiltration in the DRG, which project axons centrally into the dorsal white matter tracts of the spinal cord and peripherally to peripheral nerves. Similar findings were observed in the TRG as well. At the Day 60 time point, the incidence and severity of the DRG/TRG degeneration was slightly lower in the low dose group (none to minimal [3.0×10^{12} GC, Group 2, 1/3 animals]) compared to that of both the mid-dose (1.0×10^{13} GC, Group 3, 3/3 animals) and high dose (3.0×10^{13} GC, Group 4, 2/3 animals) groups (none to moderate), suggesting a dose-dependent response. At the Day 120 time point, the severity of the DRG/TRG degeneration was lowest in the low dose group (none to minimal [3.0×10^{12} GC, Group 6, 2/3 animals]) and increased from the mid-dose (none to mild [1.0×10^{13} GC, Group 7, 3/3 animals]) to the high dose group (none to moderate [3.0×10^{13} GC, Group 8, 3/3 animals]), also indicating a dose-dependent response. Comparing across time points, the incidence and severity of the DRG/TRG neuronal degeneration was relatively similar between rAAV.GLB1-treated groups, suggesting the absence of a time-dependent response. The lack of time-dependent response suggests that further progression of the DRG/TRG neuronal degeneration does not occur from the Day 60 to Day 120 time points.

The DRG degeneration resulted in an axonopathy of the dorsal white matter tracts of the spinal cord and peripheral nerves, which were microscopically consistent with axonal degeneration. At the Day 60 time point, a dose-dependent response was not observed for the dorsal white matter tract axonopathy as the overall incidence and severity (none to mild) was similar across all rAAV.hGLB1-treated groups. At the Day 120 time point, a dose-dependent response was observed as the incidence and severity of the dorsal white matter tract axonopathy was lowest in the low dose group (minimal [3.0×10^{12} GC, Group 6, 2/3 animals]) compared to that of both the mid-dose (1.0×10^{13} GC, Group 7, 3/3 animals) and high dose (3.0×10^{13} GC, Group 8, 3/3 animals) groups (minimal to moderate). Comparing across time points, the severity and, to a lesser extent, incidence of the dorsal white matter tract axonopathy increased in both the mid-dose (1.0×10^{13} GC) and high dose (3.0×10^{13} GC) groups from the Day 60 to Day 120 time points, indicating a time-dependent response and progression of findings. However, an important caveat to this conclusion is that at the Day 120 time point, 1/3 animals in the mid-dose group (Animal 17-226

[1.0 x 10^{13} GC, Group 7]) and 1/3 animals in the high dose group (Animal 17-205 [3.0 x 10^{13} GC, Group 8]) had significantly higher severities than the other animals from both groups, which affects the interpretation of these results. In contrast, the incidence and severity was decreased in the low dose group (3.0 x 10^{12} GC) from Day 60 to Day 120, indicating that the dorsal white matter tract axonopathy did not progress at this dose.

In regards to the peripheral nerve axonopathy at the Day 60 time point, a dose-dependent response was observed as the severity was lowest in the low dose (3.0 x 10^{12} GC, Group 2; 20/24 nerves; 3/3 animals) and mid-dose (1.0 x 10^{13} GC, Group 3; 22/24 nerves; 3/3 animals) groups (minimal to mild) compared to the severity observed in the high dose group (minimal to moderate [3.0 x 10^{13} GC, Group 4; 20/24 nerves; 3/3 animals]). At the Day 120 time point, the severity of the peripheral nerve axonopathy was higher in the mid-dose (1.0 x 10^{13} GC, Group 7; 30/30 nerves; 3/3 animals) and high dose (3.0 x 10^{13} GC, Group 8; 30/30 nerves; 3/3 animals) groups (minimal to marked) compared to the severity observed in the low dose (3.0 x 10^{12} GC, Group 6; 29/30 nerves; 3/3 animals) and vehicle-treated (ITFFB, Group 5, 30/30 nerves; 2/2 animals) groups (minimal), indicating a dose-dependent response. The vehicle-treated animals (ITFFB, Group 5; 30/30 nerves; 3/3 animals) at the Day 120 time point exhibited minimal axonopathy, which was observed in peripheral nerves as well as DRG axons. The extent of axonopathy observed in these vehicle-treated animals was comparable to that of most peripheral nerves and DRG axons in 3/3 animals in the low dose group (3.0 x 10^{12} GC, Group 6) and 1/3 animals in the mid-dose group (1.0 x 10^{13} GC, Group 7) at the Day 120 time point. Comparing the peripheral nerve axonopathy across time points, the incidence and severity increased from Day 60 to Day 120 across all groups, indicating a time-dependent response; however, the difference was most dramatic at the mid dose (1.0 x 10^{13} GC).

A predominant difference in the peripheral nerve findings at the Day 120 time point compared to the Day 60 time point was the presence of periaxonal fibrosis (minimal to marked), which was only observed in the mid-dose group (1.0 x 10^{13} GC, Group 7; 2/3 animals) and high dose group (3.0 x 10^{13} GC, Group 8; 3/3 animals). While a dose-dependent response in the periaxonal fibrosis was observed, the highest severity was seen in the mid-dose group (1.0 x 10^{13} GC, Group 7). Given the absence of periaxonal fibrosis at the Day 60 time point, a time-dependent response was observed.

To further evaluate the peripheral nerve periaxonal fibrosis observed by H&E staining, Masson's trichrome staining was performed. This stain highlights fibrous connective tissue from surrounding muscles and other tissues. The proximal portion of the

left and right median nerve was selected for trichrome staining due to its large circumference, which allowed for additional recuts. Trichrome staining was performed on all animals at Day 120, as periaxonal fibrosis was absent from all animals at Day 60. Semi-quantitative scoring of trichrome staining by a blinded evaluator confirmed the presence of dose-dependent periaxonal fibrosis in the mid-dose group (1.0×10^{13} GC, Group 7; 2/3 animals) and high dose group (3.0×10^{13} GC, Group 8; 3/3 animals). The severity ranged from none to marked in the mid-dose group (1.0×10^{13} GC, Group 7; 3/3 animals) and minimal to marked in the high dose group (3.0×10^{13} GC; Group 8; 3/3 animals). Consistent with the finding based on H&E, the highest severity periaxonal fibrosis (moderate to marked) occurred in 1/3 animals at the mid-dose (Animal 17-226; 1.0×10^{13} GC, Group 7) and 1/3 animals at the high dose (Animal 17-205; 3.0×10^{13} GC, Group 8), which correlated with a marked reduction in SNAP amplitudes in these animals observed on Day 28 through Day 120. Furthermore, quantification of trichrome staining using VIS image analysis software revealed a dose-dependent decrease in nerve tissue volume and dose-dependent increase in white space within the tissue sections for the mid-dose (1.0×10^{13} GC, Group 7) and high dose groups (3.0×10^{13} GC, Group 8) when compared to that of the low dose (3.0×10^{12} GC, Group 6) and vehicle-treated groups (ITFFB, Group 5). These findings were indicative of axonal loss in the mid-dose (1.0×10^{13} GC, Group 7) and high dose groups (3.0×10^{13} GC, Group 8).

Other test article-related findings in the CNS included mild gliosis and satellitosis in the ventral horns of the lumbar spinal cord at Day 60 for a single animal administered the high dose (Animal 17-216 [3.0×10^{13} GC, Group 4]). Minimal gliosis with or without satellitosis was sporadically observed in the brain of animals across all rAAV.hGLB1-treated groups at both time points. At the Day 60 time point, the incidence of gliosis with and without satellitiosis slightly higher in the high dose group (3.0×10^{13} GC, Group 4; 2/3 animals), especially in Animal 17-213, compared to the low dose (3.0×10^{12} GC, Group 2; 1/3 animals) and mid-dose (1.0×10^{13} GC, Group 3; 1/3 animals) groups. At the Day 120 time point, minimal perivascular infiltrates and small foci of gliosis were observed sporadically across all rAAV.hGLB1-treated groups; however, the incidence of these findings was decreased at the Day 120 time point compared to the Day 60 time point, suggesting resolution.

Localized injection site findings within the skeletal muscle and adipose tissue over the ICM/CSF collection site were observed across all groups, including vehicle-treated animals (ITFFB, Group 1) at the Day 60 time point. However, at the Day 60 time point, the

composition of the infiltrates varied, and the severity was increased in rAAV.hGLB1-treated animals. Infiltrates in the Day 60 vehicle-treated group (ITFFB, Group 1; 1/2 animals) were mostly composed of histiocytes (minimal), while GTP-203-treated animals had mainly lymphocytes and plasma cells (minimal to moderate) with or without minimal to mild myofiber changes. Myofiber changes that included degeneration and atrophy were only seen at Day 60 in the high dose group (3.0×10^{13} GC, Group 4; 1/3 animals). At the Day 120 time point, all rAAV.hGLB1-treated animals exhibited mononuclear cell infiltrates within the skeletal muscle and/or adipose tissue, which ranged from minimal at the low dose (3.0×10^{12} GC, Group 6; 3/3 animals) to minimal to mild at the mid-dose (1.0×10^{13} GC, Group 7; 3/3 animals) and high dose (3.0×10^{13} GC, Group 8; 3/3 animals), possibly suggestive of a dose-dependent response. The severity of the injection site findings at Day 120 (minimal to mild) was decreased compared to the severity observed at Day 60 (minimal to moderate with myofiber changes), which is indicative of resolution and suggestive of a time-dependent response. While these findings likely resulted from the initial injection with possible contribution from repetitive CSF collection, there was possibly a component stemming from a local response to the test article.

Vector Pharmacokinetics and Excretion

Following ICM administration, rAAV.hGLB1 vector DNA was detectable in CSF and peripheral blood, with peak concentrations in CSF correlating with dose. The concentration of rAAV.hGLB1 in CSF rapidly declined following the first time point evaluated (Day 7) and was undetectable by Day 60 in most animals with the exception of one animal in the high dose group (Animal 17-212 [3.0×10^{13} GC, Group 8]) for whom the rAAV.hGLB1 vector DNA concentration in CSF was downward trending at the time of necropsy on Day 60. rAAV.hGLB1vector DNA concentrations in blood declined more slowly, which may be attributed to transduction of peripheral blood cells.

At Day 0, rAAV.hGLB1 vector DNA was detected in the CSF, but not the blood, of two animals in the high dose group (Animals 17-197 and 17-205 [3.0×10^{13} GC, Group 8]). The CSF samples positive for rAAV.hGLB1on Day 0 were retested to confirm the results. The detection of rAAV.hGLB1vector DNA in the CSF on Day 0 was likely due to CSF sample contamination during the ICM administration procedure.

rAAV.GLB1 vector DNA was detectable in urine and feces on Day 5 after vector administration. Peak levels were generally proportional to the dose administered. rAAV.hGLB1vector DNA was undetectable in urine and feces of all animals by Day 60 after vector administration.

Evaluation of Transgene Expression

Human β-gal activity was measured in CSF and serum. Briefly, 1–10 µL of either CSF or serum is mixed with 99 µL of the reaction mix (0.5 mM 4-Methylumbelliferyl β-D-galactopyranoside [Sigma M1633], 0.15 M NaCl, 0.05% Triton-X100, and 0.1 M sodium acetate, pH 3.58) in a 96-well black plastic assay plate. The plate is sealed and incubated at 37°C for 30 minutes, and the reaction is stopped by the addition of 150 µL of the stop solution (290 mM glycine and 180 mM sodium citrate, pH 10.9). Fluorescence from the reaction product is measured at an emission wavelength of 450 nm upon excitation at 365 nm.

Transgene product expression (β-gal activity) in major organs could not be evaluated due to high levels of endogenous rhesus β-gal enzyme activity in normal NHPs. Lower levels of endogenous rhesus β-gal enzyme are present in CSF and serum, allowing transgene expression analysis of CSF on Days 0, 7, 14, 28, 60, 90, and 120 and serum at baseline and Days 14, 28, 60, 90, and 120. However, it should be noted that analysis of transgene product activity in CSF and serum of NHPs was limited by the nature of the assay, which could not distinguish human β-gal enzyme versus endogenous rhesus β-gal enzyme. This limited sensitivity and required the use of each animal's baseline endogenous β-gal activity levels for analysis (denoted by the dashed lines in FIG 22A – FIG 22D). The analysis was also complicated by the rapid loss of transgene product activity after Day 14, which was likely due to an antibody response to the human transgene product (FIG 22A).

Despite these caveats, β-gal activity in the CSF and serum was detected above baseline levels in animals from all dose groups 14 days after rAAV.hGLB1administration (FIG 22B). In the CSF, animals receiving the two higher doses (1.0×10^{13} GC [1.1×10^{11} GC/g brain] or 3.0×10^{13} GC [3.3×10^{11} GC/g brain]) displayed β-gal activity levels that were approximately 2-fold and 4-fold higher than the levels of vehicle-treated controls, respectively. Furthermore, expression in the CSF was not affected by the presence of pre-existing NAbs to the vector capsid, supporting the potential to achieve therapeutic activity in the target organ system (CNS) in infantile/late infantile GM1 patients regardless of NAb status.

In serum, animals lacking pre-existing NAbs to the vector capsid (denoted by empty shapes in FIG 22B) trended towards higher β-gal enzyme activity compared to that of either the vehicle-treated controls or animals positive for pre-existing NAbs to the vector capsid (denoted by filled-in shapes in FIG 22B). This result suggests the potential for therapeutic activity in peripheral organs for NAb-negative infantile/late infantile GM1 patients.

Biodistribution: At the time of necropsy, tissues were collected for biodistribution, placed on dry ice in a labeled vial, and stored at ≤–60°C prior to analysis. DNA was extracted from tissues by trained operators, and TaqMan qPCR reactions were performed by following SOP 3001. Briefly, tissues were mechanically homogenized and digested with Proteinase K. Samples were treated with RNAse A, and cells were lysed by incubation for 1 h at 70°C in Buffer AL (Cat. #19075, QIAGEN). DNA was extracted and purified on QIAGEN spin columns. Following dilution to a concentration of ≥90 and ≤110 ng/µl, qPCR reactions were performed in duplicate using vector- and/or transgene-specific primers. Signal was compared to a standard curve of linearized plasmid DNA in a background of a known concentration of DNA from a naïve or negative control animal from the same study. Genome copies per microgram of DNA were calculated. Additional controls were utilized to rule out cross-contamination and sample interference in the PCR reaction. Raw data were analyzed based upon pre-defined acceptance criteria for Ct values, and a limit of quantification was determined for each run. All data were included in and/or attached to a batch record form.

Vector genomes were detected at high levels in the brain, spinal cord, DRG, liver, and spleen at Day 60 (FIG 23) and Day 120 (FIG 24), which is consistent with previous studies of ICM AAV administration. The quantity of vector genomes detected in CNS tissues was generally observed to be dose-dependent. Vector genomes in CNS tissues appeared stable between 60 and 120 days after administration. At Day 120, all three animals enrolled in the mid-dose group (1.0×10^{13} GC, Group 7) had baseline NAbs to AAVhu68. which correlated with very low vector distribution to the liver. Vector genomes were detected in a few samples from two vehicle-treated control animals (Animals 17-199 [Group 1] and 17-204 [Group 5]). These samples were tested twice to confirm the presence of vector genomes.

Conclusions:

ICM administration of rAAV.hGLB was well-tolerated at all doses evaluated. rAAV.hGLB produced no adverse effects on clinical and behavioral signs, body weight, or neurologic and physical examinations. There were no abnormalities of blood and CSF clinical pathology related to rAAV.hGLB administration except for a mild transient increase in CSF leukocytes in some animals.

rAAV.hGLB administration resulted in asymptomatic degeneration of TRG and DRG sensory neurons and their associated central and peripheral axons. The severity of these lesions was typically minimal to mild. These findings were dose-dependent with a trend of

more severe lesions in the mid-dose (1.0×10^{13} GC) and high dose (3.0×10^{13} GC) groups.

Degeneration of sensory neuron cell bodies was less severe at Day 120 than Day 60. This result indicated these lesions are not progressive, although the subsequent axon degeneration and fibrosis may continue to evolve over several months. Consistent with these findings, two animals that exhibited the most severe axon loss and fibrosis of median nerves upon necropsy at Day 120 (Animals 17-226 and 17-205) exhibited a reduction in median nerve sensory action potential amplitudes by Day 28 with no subsequent progression. Due to the presence of asymptomatic sensory neuron lesions in all dose groups, a NOAEL was not defined. The highest dose evaluated (3.0×10^{13} GC) was considered the MTD. The two animals that exhibited the most severe axon loss and fibrosis with decreased sensory nerve action potential are shown with arrows. (FIGS 18A- 18B, 19A-19B. FIGS 20A – 20B show the change in median sensory nerve conduction as of each measuring point in the study, as measured by median sensory action potential in microvolts.

Transgene expression (i.e., β-gal enzyme activity) in CSF and serum was detectable above baseline levels in animals from all dose groups 14 days after ICM administration of rAAV.hGLB. In CSF, animals receiving the two higher doses (1.0 x 1013 GC or 3.0 x 1013 GC) displayed β-gal activity levels approximately 2-fold and 4-fold higher than the levels of vehicle-treated controls, respectively. Expression in the CSF was not affected by the presence of pre-existing NAbs to the vector capsid, supporting the potential to achieve therapeutic activity in the target organ system (CNS) in infantile/late infantile GM1 patients regardless of NAb status.

ICM administration of rAAV.hGLB resulted in vector distribution in the CSF and high levels of gene transfer to the brain, spinal cord, and DRG. rAAV.hGLB also reached significant concentrations in peripheral blood and liver.

Evaluation of rAAV.hGLB DNA excretion demonstrated detectable vector DNA in urine and feces 5 days after administration, which reached undetectable levels within 60 days.

T cell responses to the vector capsid and/or human transgene product were detectable in the PBMCs and/or tissue lymphocytes (liver, spleen, bone marrow) in the majority of rAAV.hGLB -treated animals. T cell responses were not generally associated with any abnormal clinical or histological findings.

Pre-existing NAbs to the vector capsid were detectable in some animals and did not appear to influence gene transfer to the brain and spinal cord, although the presence of pre-existing NAbs correlated with markedly reduced hepatic gene transfer.

EXAMPLE 5: A Phase 1/2 Open-Label, Multi-Center Dose Escalation Study To Assess The Safety And Tolerability Of Single Doses Of rAAVhu68.GLB1 Delivered Into The Cisterna Magna (ICM) Of Pediatric Subjects With Infantile GM1 Gangliosidosis

GM1 subjects up to 24 months of age with symptom onset in the first 18 months are enrolled. This will include subjects with Type 1 (Infantile) and Type 2a (Late infantile) GM1. Type 1 (Infantile) subjects may show symptoms at birth. Therefore, treatment should start as early as possible to maximize potential benefit, and this study includes subjects who are at least one-month old. Another consideration in selecting the lower age limit is to ensure that the ICM procedure can be safely performed. The proposed ICM procedure includes pre-procedure MRI and MR angiogram of the brain and CT/CTA-guided ICM injection. There are no age-specific safety concerns with performing ICM administration in infants > 1 month of age.

ICM vector administration results in immediate vector distribution within the CNS compartment. Thus, clinical doses were determined by scaling according to brain mass, which provides an approximation of the size of the CNS compartment. Both efficacy and toxicity are expected to be related to CNS vector exposure. Dose conversions will be based on a brain mass of 0.4 g for a juvenile-adult mouse, 90 g for juvenile and adult rhesus macaques (Herndon 1998) and a range of 370 g to 1080 g for human infants aged 0 to 30 months (Dekaban, 1978). Non-clinical and equivalent human doses are shown in the following table.

Comparison of Doses for Non-Clinical Studies

Dose (GC/g brain mass)	Juvenile Mouse MED Study (GC)	Juvenile Rhesus Macaque Toxicology Study (GC)
3.33×10^{11}	1.30×10^{11}	3.00×10^{13}
1.11×10^{11}	4.40×10^{10}	1.00×10^{13}
3.33×10^{10}	1.30×10^{10}	3.00×10^{12}
1.11×10^{10}	4.40×10^{9}	-

Abbreviations: GC, genome copies; MED, minimum effective dose; NHP, nonhuman primate.

Given that brain weights differ (e.g., there is an approximately 3-fold difference between a newborn and a 2-year old subject), a sliding scale will be used to determine the amount of drug product (in gene copies [GC]) to be administered to individual subjects in the FIH study based on published mean brain weights for infants and children up to 24 months of age. In this manner, subjects will be administered a volume of drug product that best approximates the intended dose in gene copies / estimated grams of brain weight.

Age	1 – 4 months	>4 – <8 months	≥8 – 12 months	≥12 months
Mean human brain weight (g)	488	610	780	960
Low dose 3.33 x 10^{10} GC/g	1.6 x 10^{13} GC	2.1 x 10^{13} GC	2.6 x 10^{13} GC	3.2 x 10^{13} GC
High dose 1.11 x 10^{11}GC/g	5.4 x 10^{13} GC	6.8 x 10^{13} GC	8.7 x 10^{13} GC	1.0 x 10^{14} GC
Maximum Feasible dose 3.33 x 10^{11}GC/g	1.6 x 10^{14} GC	2.1x10^{14} GC	2.6x10^{14} GC	3.2x10^{14} GC
Delivered Dosing Volume	3.0 mL	4.0 mL	5.0 mL	5.0 mL

1. Dose basis: 3.33e10 GC/gram of brain
2. Dose basis: 1.11e11 GC/gram of brain

GC: genome copies

Pediatric Administration Kit

Part	Description
BD™ Spinal Needles	25 G × 2 in. BD™ Spinal Needle with Quincke Bevel,
	25 G × 3 in. BD™ Spinal Needle with Quincke Bevel,

Baxter T-Connector Extension Set	T-Connector Extension Set with a Retractable T-Connector, an INTERLINK Injection Site, Female Luer Lock Adapter, non-DEHP, Approximate Volume 0.60 mL, Approximate Length 6.7" (17 cm)

Note: Multiple spinal needles are listed as options for pediatric administration to account for anatomical differences

Adult Administration Kit

Part	Description
BD™ Spinal Needles	22 G × 5 in. BD™ Spinal Needle with Quincke Bevel,
	18 G × 3 in. BD™ Spinal Needle with Quincke Bevel Introducer for the dosing 22G spinal needle,
Baxter T-Connector Extension Set	T-Connector Extension Set with a Retractable T-Connector, an INTERLINK Injection Site, Female Luer Lock Adapter, non-DEHP, Approximate Volume 0.60 mL, Approximate Length 6.7" (17 cm)

The study is a Phase 1/2, open-label, dose escalation study of AAVhu68.GLB1 to evaluate the safety, tolerability, and exploratory efficacy endpoints following a single dose of AAVhu68.GLB1 delivered into the cisterna magna (ICM) of pediatric subjection with the infantile form of GM1 (Type 1) or late infantile (Type 2a). This study enrolls up to 24 pediatric subjects and subjects receive a single dose of ICM-administered AAVhu68.GLB1.

Type 1 (Infantile) GM1

- Presymptomatic GM1 subjects (≤6 months of age, with confirmed mutation and reduced serum β-gal activity) identified through prenatal screening or family history of an older sibling with a confirmed diagnosis of GM1 gangliosidosis with the same genotype. The sibling must have had symptom onset at ≤6 months of age.

- Symptomatic GM1 subjects (with confirmed mutation and reduced serum β-gal activity) must have medical record documentation of onset at ≤6 months of age, with hypotonia or any documented symptom consistent with GM1 gangliosidosis AND with at least 70% of age corrected expected motor development (BSID-III) at the time of dosing.

Type 2 (Late Infantile) GM1

- Symptomatic subjects with onset >6 months and ≤18 months of age with hypotonia or any documented symptoms consistent with GM1 gangliosidosis who have demonstrated a plateauing or delay in achieving further developmental milestones with at least 70% of age-corrected expected motor development (BSID-III).

Two doses of rAAVhu68.GLB1 are evaluated with staggered, sequential dosing of subjects. The rAAVhu68.GLB1 dose levels are determined based on data from the murine MED study and GLP NHP toxicology study and consist of a low dose (administered to Cohort 1) and a high dose (administered Cohort 2). The high dose is based on the maximum tolerated dose (MTD) in NHP toxicology study scaled to an equivalent human dose. A safety margin is applied so that the high dose selected for human subjects is one third to half of the equivalent human dose. The low dose typically is 2-3 fold less than the selected high dose provided it is a dose that exceeds the equivalent scaled MED in animal studies. This would ensure that both dose levels have the potential to confer therapeutic benefit, with the understanding that if tolerated, the higher dose would be expected to be advantageous. The sequential evaluation of the low dose followed by the high dose enables the identification of the maximum tolerated dose (MTD) of the two doses tested. Finally, an expansion cohort (Cohort 3) receive the MTD of rAAVhu68.GLB1. The 6 subjects in Cohort 3 (MTD) are enrolled simultaneously without staggered dosing. Cohort 3 may receive combination treatment with hematopoietic stem cell transplantation (HSCT) and rAAVhu68.GLB1. If tolerated, the higher dose would be expected to be advantageous.

The primary focus of this study is to evaluate the safety and tolerability of rAAVhu68.GLB1. NHP studies of ICM AAVhu68 delivery have demonstrated minimal to mild asymptomatic degeneration of DRG sensory neurons in some animals, thus detailed examinations are performed to evaluate sensory nerve toxicity, and sensory nerve conduction studies are employed in this trial to monitor for subclinical sensory neuron lesions. Of note, sensory neuron function loss (due to potential dorsal root ganglia toxicity) is evaluated by sensory nerve conduction studies conducted at 30 days, 3 months, 6 months, 12 months, 18

months, 24 months and at yearly intervals thereafter. Given that sensory neuron lesions appear within 2–4 weeks after AAV administration in non-clinical NHP studies, the more frequent assessments through 3 months post-treatment would enable evaluation of similar events in humans, allowing for potential variability in the toxicity kinetics. The follow up throughout the study would allow evaluation of late effects should the time course be different in humans, or in case clinical sequelae are observed, to evaluate how long they persist and whether they improve, stay stable or worsen over time.

Pharmacodynamic and efficacy endpoints are also evaluated in this study, and were chosen for their potential to demonstrate meaningful functional and clinical outcomes in this population. Endpoints are measured at 30 days, 90 days, 6 months, 12 months, 18 months, 24 months and then yearly up to the 5 year follow-up period, except for those that require sedation and/or LP. During the long-term follow up phase, measurement frequency decreases to once every 12 months. These time points were selected to facilitate thorough assessment of the safety and tolerability of rAAVhu68.GLB1. The early time points and 6 month interval were also selected in consideration of the rapid rate of disease progression in untreated infantile GM1 patients. This approach allows for thorough evaluation of pharmacodynamics and clinical efficacy measures in treated subjects over a period of follow up for which untreated comparator data exist and during which untreated patients are expected to show significant decline.

The secondary and exploratory efficacy endpoints include survival, feeding tube independence, seizure incidence and frequency, quality of life as measured by PedsQL and neurocognitive and behavioral development. The Bayley Scales of Infant Development and Vineland Scales are used to quantify the effects of rAAVhu68.GLB1 on development of and changes in adaptive behaviors, cognition, language, motor function, and health-related quality of life. Each measure was used either in the GM1 disease population or in a related population and are further refined based on input from parents and families to select the measures that are most meaningful and impactful to them. In order to standardize assessments, the sites participating in the trial are trained in the administration of the various scales by an experienced neuropsychologist.

Given the severity of disease in the target population, subjects may have achieved motor skills by enrollment, developed and subsequently lost other motor milestones, or not yet shown signs of motor milestone development. Assessments tracks age-at-achievement and age-at-loss for all milestones. Motor milestone achievement is defined for six gross milestones based on the WHO criteria.

Given that subjects with infantile GM1 gangliosidosis can develop symptoms within the months of life, and acquisition of the first WHO motor milestone (sitting without support) typically does not manifest before 4 months of age (median: 5.9 months of age), this endpoint may lack sensitivity to evaluate the extent of therapeutic benefit, especially in subjects who had more overt symptoms at the time of treatment. For this reason, assessment of age-appropriate developmental milestones that can be applied to infants are also be included (Scharf et al., 2016, Developmental Milestones. Pediatr Rev. 37(1):25-37; quiz 38, 47.). These data may be informative for summarizing retention, acquisition, or loss of developmental milestones over time relative to untreated children with infantile GM1 disease or the typical time of acquisition in neurotypical children.

As the disease progresses, children can develop seizures. The onset of seizure activity enables us to determine whether treatment with rAAVhu68.GLB1 can either prevent or delay onset of seizures or decrease the frequency of seizure events in this population. Parents are asked to keep seizure diaries, which tracks onset, frequency, length, and type of seizure. These entries are discussed with and interpreted by the clinician at each visit.

To assess the effect of rAAVhu68.GLB1 on the CNS manifestations of the disease volumetric changes are measured on MRI over time. The infantile phenotype of all gangliosidoses was shown to have a consistent pattern of macrocephaly and rapidly increasing intracranial MRI volume with both brain tissue volume (cerebral cortex and other smaller structures) and ventricular volume. Additionally, various smaller brain substructures including the corpus callosum, caudate and putamen as well as the cerebellar cortex generally decrease in size as the disease progresses (Regier et al., 2016, and Nestrasil et al., 2018, as cited herein). Treatment with rAAVhu68.GLB1 is expected to slow or cease the progression of CNS disease manifestations with evidence of stabilization in atrophy and volumetric changes. The exploratory endpoint assessing changes (normal/abnormal) in T1/T2 signal intensity in the thalamus and basal ganglia is based on reported evidence for changes in the thalamic structure in patients with GM1 and GM2 gangliosidosis (Kobayashi and Takashima, 1994, Thalamic hyperdensity on CT in infantile GM1-gangliosidosis. Brain and Development. 16(6):472-474).

Biomarkers for the trial include β-gal enzyme (GLB1) activity, which can be measured in CSF and serum, and brain MRI, which demonstrates consistent, rapid atrophy in infantile GM1 gangliosidosis (Regier et al., 2016b, as cited herein). Additional biomarkers are investigated in CSF and serum from collected samples.

A. Primary Objective:

- To assess the safety and tolerability of rAAVhu68.GLB1 through 2 years following administration of a single dose into the cisterna magna (ICM). adverse events, neurological exam, sensory nerve conduction studies, total Neuropathy Score-Nurse, hematology, serum chemistry, liver function tests, coagulation (PT, aPTT, INR), troponin-If, CSF anti-AAVhu68 nAbs, vector shedding, urinalysis, seizure diary, physical examination, vital signs, ECG, brain MRI, and CSF cytology and chemistry (cell counts, protein, glucose) will be assessed over 5 years.
- To assess the efficacy of rAAVhu68.GLB1 following administration of a single dose into the cisterna magna. Key Secondary Endpoint* will be assessed at 2 years and over 5 years:
  - Vineland Adaptive Behavior Scales, Second Edition
  - Other Secondary Endpoints will be assessed at 2 years and over 5 years:
  - Bayley Scale of Infant and Toddler Development, Third Edition
  - WHO Multicenter Growth Reference Study Motor
  - Development Milestones Assessment
  - Hammersmith Infant Neurodevelopment Examination
  - Clinician and Caregiver Global Impression of Severity and change
  - Exit interviews

*A fit-for-purpose clinical outcome assessment does not exist for GM1 gangliosidosis. Therefore, in parallel with the conduct of this study, the sponsor is working with subject matter experts to collect data from clinical experts and parents/caregivers to develop an outcomes measurement strategy including identification of a primary efficacy endpoint for cohort 3, and if needed, plans for a composite endpoint derived from the scales listed above, modification of an existing COA or developing supplementary patient-centric GM1-specific items or scales. Please see Statistical Analysis section for details.

B. Secondary Objectives:

- To assess the pharmacodynamics and biological activity of rAAVhu68.GLB1 over 24 months following a single dose into the cisterna magna. Assessments: CSF biomarkers: β-galactosidase activity, hexosaminidase activity, GM1 ganglioside levels; Serum biomarkers: β-galactosidase activity,

hexosaminidase activity; Urine Biomarker: keratan sulfate levels; All will be assessed at 30 days and over 5 years.

- To assess the effects of rAAVhu68.GLB1 following administration of a single dose into the cisternal magna on disease progression. Assessment: Total brain volume, brain substructure volume, and ventricle volume and T1/T2 signal intensity as measured by MRI; Skeletal abnormalities as measured by lateral spinal x-rays; Cardiomyopathy measured by cardiac echocardiogram; Hepatosplenomegaly measured by abdominal ultrasound; Cerebral function and diffuse slowing changes measured by serial EEG; Assessment of mechanical ventilation-free survival; Assessment of nutrition status by need of placement and use of feeding tubes; All will be assessed over 5 years.

To assess the effects of rAAVhu68.GLB1 following administration of a single dose into the cisternal magna on quality of life and healthcare resource utilization. Assessment: Quality of life: Pediatric Quality of Life Inventory/ Pediatric Quality of Life Inventory-Infant Scale; Healthcare resource utilization: chart review for hospital days, ER visits, ICU admissions, surgeries, need for hearing and visual aids; All will be assessed over 5 years.

C. Study Design:

Multicenter, open-label, single-arm dose escalation study of rAAVhu68.GLB1 (Table below). Up to a total of 12 pediatric subjects with infantile GM1 gangliosidosis are enrolled into 2 dose cohorts, and receive a single dose of rAAVhu68.GLB1 administered by ICM injection. Safety and tolerability are assessed through 2 years, and all subjects are followed through 5 years post-administration of rAAVhu68.GLB1 for the long-term evaluation of safety and tolerability, pharmacodynamics (durability of transgene expression) and durability of clinical outcomes.

Product Name:	AAVhu68.UbC.GLB1
Gene Inserts:	Artificial version of human *GLB1* gene encoding beta-galactosidase (beta-gal or β-gal)
Control Element:	Regulatory element derived from human ubiquitin C (UbC) promoter

Other elements:	Chimeric intron (CI)– a hybrid intron consisting of a human beta-globin splice donor and immunoglobulin G (IgG) splice acceptor elements A polyadenylation (PolyA) signal derived from Simian Virus 40 (SV40) late genes
AAV Serotype:	Hu68

The AAVhu68.UbC.GLB1 is supplied frozen (≤ -60°C) as a sterile solution in ITFFB (intrathecal final formulation buffer). Depending on the dose level and the age band of the subject, dilution of the AAVhu68.UbC.GLB1 DP in the ITFFBD01 (study drug diluent) may be required prior to administration. The AAVhu68.UbC.GLB1 DP and ITFFBD01 formulations are composed of 1 mM sodium phosphate, 150 mM sodium chloride, 3 mM potassium chloride, 1.4 mM calcium chloride, 0.8 mM magnesium chloride, 0.001% poloxamer 188, pH 7.2.

Potential subjects are screened from Days -35 to -1 prior to dosing to determine eligibility for the study. Up to 24 pediatric subjects with Type 1 (Infantile) and Type 2a (Late Infantile) GM1 gangliosidosis are enrolled into the study. Those subjects who meet the inclusion/exclusion criteria are admitted to the hospital on the morning of Day 1 or per institutional practice. Subjects receive a single ICM dose of rAAVhu68.GLB1 on Day 1 and remain in the hospital for at least 24 h after dosing for observation. Subsequent assessments are performed 7, 14 and 30 days after dosing, then every 60 days for the first year and every 90 days for the second year. The safety and tolerability of rAAVhu68.GLB1 are monitored through assessment of adverse events (AEs) and serious adverse events (SAEs), vital signs, physical examinations, sensory nerve conduction studies, and laboratory assessments (chemistry, hematology, coagulation studies, CSF analysis). Immunogenicity of the AAV and transgene product are also assessed. Efficacy assessments include survival, measurements of cognitive, motor and social development, changes in visual function and EEG, changes in liver and spleen volume, and biomarkers in CSF, serum, and urine.

The study consists of the following three cohorts administered rAAVhu68.GLB1 as a single ICM injection:

- Cohort 1 (Low Dose): Three eligible subjects (subjects #1 to #3) are enrolled and administered the low dose of rAAVhu68.GLB1 with a 4-week safety observation period between the first and second subject. If no safety review triggers

(SRTs) are observed, all available safety data is evaluated by an independent safety board 4 weeks after the third subject in Cohort 1 is administered rAAVhu68.GLB1.

- Cohort 2 (High Dose): If the decision is made to proceed, three eligible subjects (Subjects #4 to #6) are enrolled and administered the high dose of rAAVhu68.GLB1 with a 4-week safety observation period between the fourth and fifth subject. If no SRTs are observed, the independent safety board evaluates all available safety data, including safety data from subjects in Cohort 1, 4 weeks after the third subject Cohort 2 is administered rAAVhu68.GLB1.
- Cohort 3 (MTD): Pending a positive recommendation by the safety board, up to 6 additional subjects are enrolled and administered a single ICM dose of rAAVhu68.GLB1 at the MTD. Dosing for subjects in this cohort is not staggered with a 4-week safety observation period between subjects, and a safety board review is required following dosing of the first three subjects in this cohort.

D. Inclusion Criteria:

1. ≥1 month and <24 months of age at enrollment, with Type 1 (onset ≤6 months) or Type 2a (onset >6 and ≤18 months).

a. Type 1 Infantile GM1

- i. Presymptomatic subjects (≤6 months of age with confirmed mutation and reduced serum β-gal activity) identified through prenatal screening or family history of an older sibling with a confirmed diagnosis of GM1 gangliosidosis with the same genotype. Sibling must have had symptom onset at ≤6 months of age.

OR

- ii. Symptomatic subject (with confirmed mutation and reduced serum β-gal activity) must have a medical record documentation of onset at ≤6 months of age, with hypotonia or any documented symptom consistent with GM1 gangliosidosis AND with at least 70% of age corrected expected motor development (BSID-III), at the time of dosing.

b. Type 2a Late Infantile GM1:

i. Symptomatic subjects with onset >6 months and ≤18 months of age with hypotonia or any documented symptoms consistent with GM1 gangliosidosis who have demonstrated a plateauing or delay in achieving further developmental milestones with at least 70% of age corrected expected motor development (BSID-III).

2. Documentation that the subject is homozygous or compound heterozygous for GLB1 gene deletion or mutation AND decreased β-gal activity (≤20% of the lower normal value in leukocytes).

E. Exclusion Criteria:

1. Any clinically significant neurocognitive deficit not attributable to GM1 gangliosidosis or any other condition that may, in the opinion of the investigator, confound interpretation of study results.
2. If any subject had an acute illness requiring hospitalization within 30 days of enrollment, the history must be discussed with the sponsor's medical monitor before allowing the subject to be enrolled.
3. History of ventilation assisted respiratory support or a need for tracheostomy.
4. Intractable seizure or uncontrolled epilepsy defined as having had an episode of status epilepticus, or seizures requiring hospitalization within 30 days prior to dosing of investigational product.
5. Any contraindication to ICM administration procedure, including contraindications to fluoroscopic imaging and anesthesia.
6. Any contraindication to MRI or LP.
7. Prior gene therapy.
8. Use of miglustat within 48 hours prior to dosing of investigational product.
9. Use of enzyme replacement therapy or other investigational therapy within 5 half-lives prior to dosing of investigational product.
10. Any condition (e.g., history of any disease, evidence of any current disease, any finding upon physical examination, or any laboratory abnormality) that, in the opinion of the investigator, would put the subject at undue risk during procedure or would interfere with evaluation of the investigational product or interpretation of subject safety or study results. This includes:
    a. abnormal laboratory values considered clinically significant by the investigator
    b. Failure to thrive, defined as: Falling 20 percentiles (20/100) in body weight in the 3 months preceding Screening/Baseline
    c. Underlying defect in immune function

d. History of multiple and severe life-threatening infections

F. Route of Administration and Procedure

rAAVhu68.GLB1 as a single dose is administered on Day 1 to subjects via CT-guided sub-occipital injection into the cisterna magna.

On Day 1 the appropriate concentration of rAAVhu68.GLB1 is prepared by the Investigational Pharmacy associated with the study. A syringe containing 5.6 mL of rAAVhu68.GLB1 at the appropriate concentration is delivered to the procedure room. The following personnel are present for study drug administration: interventionalist performing the procedure; anesthesiologist and respiratory technician(s); nurses and physician assistants; CT (or operating room) technicians; site research coordinator.

Prior to study drug administration, a lumbar puncture is performed to remove a predetermined volume of CSF and then to inject iodinated contrast intrathecally (IT) to aid in visualization of relevant anatomy of the cisterna magna. Intravenous (IV) contrast may be administered prior to or during needle insertion as an alternative to the intrathecal contrast. The decision to used IV or IT contrast is at the discretion of the interventionalist. The subject is anesthetized, intubated, and positioned on the procedure table. The injection site is prepared and draped using sterile technique. A spinal needle (22-25 G) is advanced into the cisterna magna under fluoroscopic guidance. A larger introducer needle may be used to assist with needle placement. After confirmation of needle placement, the extension set is attached to the spinal needle and allowed to fill with CSF. At the discretion of the interventionalist, a syringe containing contrast material may be connected to the extension set and a small amount injected to confirm needle placement in the cisterna magna. After the needle placement is confirmed by CT guidance +/- contrast injection, a syringe containing 5.6 mL of rAAVhu68.GLB1 is connected to the extension set. The syringe contents are slowly injected over 1-2 minutes, delivering a volume of 5.0 mL. The needle is slowly removed from the subject.

A single dose into the cisterna magna (ICM) of rAAVhu68.GLB1 is safe and tolerable through 5 years following administration.

A single dose into the cisterna magna (ICM) of rAAVhu68.GLB1 improves survival, reduces probability of feeding tube dependence at 24 months of age, and/or reduces Disease progression as assessed by age at achievement, age at loss, and percentage of children maintaining or acquiring age-appropriate developmental and motor milestones.

Treatment slows of loss of neurocognitive function.

As prophylaxis for potential immune-mediated injury such as hepatotoxicity, subjects will receive systemic corticosteroids. Starting one day prior to rAAVhu68.GLB1 administration, systemic corticosteroids equivalent to oral prednisolone at 1 mg/kg of body weight per day will be administered for approximately 30 days (or until the scheduled Month 1 follow-up visit whichever occurs first). During this visit, clinical examination and laboratory testing should be performed per the schedule of assessments. For patients with unremarkable findings, the Investigator should taper the corticosteroid dose over the next 21 days per clinical judgement, starting at 0.75 mg/kg daily dose during Week 5, 0.5 mg/kg daily dose during Week 6 and then 0.25 mg/kg daily dose during Week 7. Consult expert(s) if patients do not respond adequately to the equivalent of 1 mg/kg/day regimen. If, in the opinion of the investigator, the subject develops clinical symptoms or clinical/laboratory signs of potential immune mediated toxicity, the dose, type and schedule of immunosuppression may be modified, and the study responsible physician should be notified. Routine vaccine schedules and local guidelines should be adhered to including recommendations to adjust timing of vaccines while the subject is under steroid treatment.

EXAMPLE 6: A Phase 1/2 Open-Label, Multi-Center Dose Escalation Study To Assess The Safety And Tolerability Of Single Doses Of rAAVhu68.GLB1 Delivered Into The Cisterna Magna (ICM) Of Pediatric Subjects With Infantile GM1 Gangliosidosis

GM1 subjects up to 24 months of age with symptom onset in the first 18 months are enrolled. This will include subjects with Type 1 (Infantile) and Type 2a (Late infantile) GM1. Type 1 (Infantile) subjects may show symptoms at birth. Therefore, treatment should start as early as possible to maximize potential benefit, and this study includes subjects who are at least one-month old. Another consideration in selecting the lower age limit is to ensure that the ICM procedure can be safely performed. The proposed ICM procedure includes pre-procedure MRI and MR angiogram of the brain and CT/CTA-guided ICM injection. There are no age-specific safety concerns with performing ICM administration in infants > 1 month of age.

ICM vector administration results in immediate vector distribution within the CNS compartment. Thus, clinical doses were determined by scaling according to brain mass, which provides an approximation of the size of the CNS compartment. Both efficacy and

toxicity are expected to be related to CNS vector exposure. Dose conversions will be based on a brain mass of 0.4 g for a juvenile-adult mouse, 90 g for juvenile and adult rhesus macaques (Herndon 1998) and a range of 370 g to 1080 g for human infants aged 0 to 30 months (Dekaban, 1978). Non-clinical and equivalent human doses are shown in the following table.

Comparison of Doses for Non-Clinical Studies

Dose (GC/g brain mass)	Juvenile Mouse MED Study (GC)	Juvenile Rhesus Macaque Toxicology Study (GC)
3.33×10^{11}	1.30×10^{11}	3.00×10^{13}
1.11×10^{11}	4.40×10^{10}	1.00×10^{13}
3.33×10^{10}	1.30×10^{10}	3.00×10^{12}
1.11×10^{10}	4.40×10^{9}	-

Abbreviations: GC, genome copies; MED, minimum effective dose; NHP, nonhuman primate.

Given that brain weights differ (e.g., there is an approximately 3-fold difference between a newborn and a 2-year old subject), a sliding scale will be used to determine the amount of drug product (in gene copies [GC]) to be administered to individual subjects in the FIH study based on published mean brain weights for infants and children up to 24 months of age. In this manner, subjects will be administered a volume of drug product that best approximates the intended dose in gene copies / estimated grams of brain weight.

Age	1 – 4 months	>4 – <8 months	≥8 – 12 months	≥12 months
Mean human brain weight (g)	488	610	780	960
Low dose 3.33×10^{10} GC/g	1.6×10^{13} GC	2.1×10^{13} GC	2.6×10^{13} GC	3.2×10^{13} GC

High dose 1.11 x 10^{11}GC/g	5.4 x 10^{13} GC	6.8 x 10^{13} GC	8.7 x 10^{13} GC	1.0 x 10^{14} GC
Maximum Feasible dose 3.33 x 10^{11}GC/g	1.6 x 10^{14} GC	2.1x10^{14} GC	2.6x10^{14} GC	3.2x10^{14} GC
Delivered Dosing Volume	3.0 mL	4.0 mL	5.0 mL	5.0 mL

1. Dose basis: 3.33e10 GC/gram of brain
2. Dose basis: 1.11e11 GC/gram of brain

GC: genome copies

Pediatric Administration Kit

Part	Description
BD™ Spinal Needles	25 G × 2 in. BD™ Spinal Needle with Quincke Bevel,
	25 G × 3 in. BD™ Spinal Needle with Quincke Bevel,
Baxter T-Connector Extension Set	T-Connector Extension Set with a Retractable T-Connector, an INTERLINK Injection Site, Female Luer Lock Adapter, non-DEHP, Approximate Volume 0.60 mL, Approximate Length 6.7" (17 cm)

Note: Multiple spinal needles are listed as options for pediatric administration to account for anatomical differences

Adult Administration Kit

Part	Description
BD™ Spinal Needles	22 G × 5 in. BD™ Spinal Needle with Quincke Bevel,
	18 G × 3 in. BD™ Spinal Needle with Quincke Bevel Introducer for the dosing 22G spinal needle,

Baxter T-Connector Extension Set	T-Connector Extension Set with a Retractable T-Connector, an INTERLINK Injection Site, Female Luer Lock Adapter, non-DEHP, Approximate Volume 0.60 mL, Approximate Length 6.7" (17 cm)

The study is a Phase 1/2, open-label, dose escalation study of AAVhu68.GLB1 to evaluate the safety, tolerability, and exploratory efficacy endpoints following a single dose of AAVhu68.GLB1 delivered into the cisterna magna (ICM) of pediatric subjection with the infantile form of GM1 (Type 1) or late infantile (Type 2a). This study enrolls up to 28 pediatric subjects and subjects receive a single dose of ICM-administered AAVhu68.GLB1.

Inclusion Criteria: This study will include infants with confirmed GLB1 mutations (homozygous or compound heterozygous for GLB1 gene deletion or mutation) AND have decreased β-gal activity (≤20% of the normal value in leukocytes) , ≥4 month and <24 months of age at enrollment, with either Type 1 (Infantile) GM1 characterized by early onset (≤6 months), predictive of rapid progression; or with Type 2a (Late Infantile) GM1 characterized by later onset presentation (>6 and ≤18 months), predictive of slower progression.

Type 1 (Infantile) GM1

- Presymptomatic subjects identified through (a) prenatal screening or family history of an older sibling with a confirmed diagnosis of GM1 with the same genotype and history of onset at <6 months; or (b) signs of prenatal GM1 disease, e.g., intrauterine growth retardation, hydrops fetalis, or placental vacuolization..
- Symptomatic subjects with medical record documentation of symptom onset at ≤6 months of age, with hypotonia and/or developmental delay and/or other signs consistent with GM1 (e.g., hepatosplenomegaly, skeletal dysplasia, cherry-red maculae, cardiomyopathy, and coarse facial features) AND must have at least one of the following remaining developmental skills within the past week confirmed/observed by the site examiner:.
  - Shows ability to move arms and legs intentionally.
  - Looks at an object of interest for at least 3 continuous seconds.

- When held upright against a caregiver's chest, can roll their head from one side to the other (e.g. if the child is laying with their left ear on the caregiver's shoulder, they can switch to laying with the right ear on the caregiver's shoulder without assistance or repositioning).
- Vocalizes a specific mood.
- Communicates with throaty, gurgling, or nasal sounds.
- - Fixes gaze on caregiver for at least 2 continuous seconds.

Type 2 (Late Infantile) GM1

- Presymptomatic subjects identified through (a) prenatal screening or family history of an older sibling with a confirmed diagnosis of GM1 with the same genotype and history of onset between 6-18 months of age; or (b) signs of prenatal GM1 disease including intrauterine growth retardation, hydrops fetalis, or placental vacuolization.
- Symptomatic subjects with onset >6 months and ≤18 months of age with hypotonia and/or plateauing or delay in achieving further developmental milestones and/or any other documented signs consistent with GM1 (e.g., hepatosplenomegaly, skeletal dysplasia, cherry-red maculae, cardiomyopathy, and coarse facial features) AND must meet age-dependent developmental criteria for symptomatic late infantile GM1 subjects below:
  - Symptomatic subjects < 12 months of age must have one of the age appropriate gross motor, fine motor, language/cognitive or social developmental milestones listed in the table below within the past week confirmed/observed by the site examiner.
  - Symptomatic subjects >12 and <24 months must have at least 2 of 4 developmental milestones for a child 50% of their age (see table below) within the past week confirmed/observed by the site examiner. For example, a 16-month-old child would have to have at least 2 developmental milestones for an 8-month old child.

Two doses of rAAVhu68.GLB1 are evaluated with staggered, sequential dosing of subjects. The rAAVhu68.GLB1 dose levels are determined based on data from the murine MED study and GLP NHP toxicology study and consist of a low dose (administered to Cohort 1 and 3) and a high dose (administered Cohort 2 and 4). The high dose is based on the maximum tolerated dose (MTD) in NHP toxicology study scaled to an equivalent human

dose. A safety margin is applied so that the high dose selected for human subjects is one third to half of the equivalent human dose. The low dose typically is 2-3 fold less than the selected high dose provided it is a dose that exceeds the equivalent scaled MED in animal studies. This would ensure that both dose levels have the potential to confer therapeutic benefit, with the understanding that if tolerated, the higher dose would be expected to be advantageous. The sequential evaluation of the low dose followed by the high dose enables the identification of the maximum tolerated dose (MTD) of the two doses tested.

Part 1		
Cohort	Patient	Assigned Intervention
Cohort 1	Late Onset Infantile GM1 Gangliosidosis (Type 2a)	rAAVhu68.GLB1 Dose 1: 3.3 x 10^{10} GC/g* Single dose of rAAVhu68.GLB1, via intra cisterna magna
Cohort 2	Late Onset Infantile GM1 Gangliosidosis (Type 2a)	rAAVhu68.GLB1 Dose 2: 1.1 x 10^{11} GC/g* Single dose of rAAVhu68.GLB1, via intra cisterna magna
Cohort 3	Early Onset Infantile GM1 Gangliosidosis (Type 1)	rAAVhu68.GLB1 Dose 1: 3.3 x 10^{10} GC/g* Single dose of rAAVhu68.GLB1, via intra cisterna magna
Cohort 4	Early Onset Infantile GM1 Gangliosidosis (Type 1)	rAAVhu68.GLB1 Dose 2: 1.1 x 10^{11} GC/g* Single dose of rAAVhu68.GLB1, via intra cisterna magna

*GC/g: gene copy per gram of estimated brain weight

Finally, an expansion cohort (Cohort 5 and 6) receive a single dose of rAAVhu68.GLB1 to confirm safety and efficacy of rAAVhu68.GLB1.

Part 2		
Cohort	Patient	Assigned Intervention
Cohort 5	Late Onset Infantile GM1 Gangliosidosis (Type 2a)	rAAVhu68.GLB1 Dose Single dose of rAAVhu68.GLB1 Dose, via intra cisterna magna Dose to be used for the confirmatory cohorts in Part 2 will be defined after a review of data from Part 1.

Cohort 6	Early Onset Infantile GM1 Gangliosidosis (Type 1)	rAAVhu68.GLB1 Single dose of rAAVhu68.GLB1, via intra cisterna magna Dose to be used for the confirmatory cohorts in Part 2 will be defined after a review of data from Part 1.

The primary focus of this study is to evaluate the safety and tolerability of rAAVhu68.GLB1. NHP studies of ICM AAVhu68 delivery have demonstrated minimal to mild asymptomatic degeneration of DRG sensory neurons in some animals, thus detailed examinations are performed to evaluate sensory nerve toxicity, and sensory nerve conduction studies are employed in this trial to monitor for subclinical sensory neuron lesions. Of note, sensory neuron function loss (due to potential dorsal root ganglia toxicity) is evaluated by sensory nerve conduction studies conducted at 30 days, 3 months, 6 months, 12 months, 18 months, 24 months and at yearly intervals thereafter. Given that sensory neuron lesions appear within 2–4 weeks after AAV administration in non-clinical NHP studies, the more frequent assessments through 3 months post-treatment would enable evaluation of similar events in humans, allowing for potential variability in the toxicity kinetics. The follow up throughout the study would allow evaluation of late effects should the time course be different in humans, or in case clinical sequelae are observed, to evaluate how long they persist and whether they improve, stay stable or worsen over time.

Pharmacodynamic and efficacy endpoints are also evaluated in this study, and were chosen for their potential to demonstrate meaningful functional and clinical outcomes in this population. Endpoints are measured at 30 days, 90 days, 6 months, 12 months, 18 months, 24 months and then yearly up to the 5 year follow-up period, except for those that require sedation and/or LP. During the long-term follow up phase, measurement frequency decreases to once every 12 months. These time points were selected to facilitate thorough assessment of the safety and tolerability of rAAVhu68.GLB1. The early time points and 6 month interval were also selected in consideration of the rapid rate of disease progression in untreated infantile GM1 patients. This approach allows for thorough evaluation of pharmacodynamics and clinical efficacy measures in treated subjects over a period of follow up for which untreated comparator data exist and during which untreated patients are expected to show significant decline.

The secondary and exploratory efficacy endpoints include survival, feeding tube independence, seizure incidence and frequency, quality of life as measured by PedsQL and

neurocognitive and behavioral development. The Bayley Scales of Infant Development and Vineland Scales are used to quantify the effects of rAAVhu68.GLB1 on development of and changes in adaptive behaviors, cognition, language, motor function, and health-related quality of life. Each measure was used either in the GM1 disease population or in a related population and are further refined based on input from parents and families to select the measures that are most meaningful and impactful to them. In order to standardize assessments, the sites participating in the trial are trained in the administration of the various scales by an experienced neuropsychologist.

Given the severity of disease in the target population, subjects may have achieved motor skills by enrollment, developed and subsequently lost other motor milestones, or not yet shown signs of motor milestone development. Assessments tracks age-at-achievement and age-at-loss for all milestones. Motor milestone achievement is defined for six gross milestones based on the WHO criteria.

Given that subjects with infantile GM1 gangliosidosis can develop symptoms within the months of life, and acquisition of the first WHO motor milestone (sitting without support) typically does not manifest before 4 months of age (median: 5.9 months of age), this endpoint may lack sensitivity to evaluate the extent of therapeutic benefit, especially in subjects who had more overt symptoms at the time of treatment. For this reason, assessment of age-appropriate developmental milestones that can be applied to infants are also be included (Scharf et al., 2016, Developmental Milestones. Pediatr Rev. 37(1):25-37; quiz 38, 47.). These data may be informative for summarizing retention, acquisition, or loss of developmental milestones over time relative to untreated children with infantile GM1 disease or the typical time of acquisition in neurotypical children.

As the disease progresses, children can develop seizures. The onset of seizure activity enables us to determine whether treatment with rAAVhu68.GLB1 can either prevent or delay onset of seizures or decrease the frequency of seizure events in this population. Parents are asked to keep seizure diaries, which tracks onset, frequency, length, and type of seizure. These entries are discussed with and interpreted by the clinician at each visit.

To assess the effect of rAAVhu68.GLB1 on the CNS manifestations of the disease volumetric changes are measured on MRI over time. The infantile phenotype of all gangliosidoses was shown to have a consistent pattern of macrocephaly and rapidly increasing intracranial MRI volume with both brain tissue volume (cerebral cortex and other smaller structures) and ventricular volume. Additionally, various smaller brain substructures including the corpus callosum, caudate and putamen as well as the cerebellar cortex

generally decrease in size as the disease progresses (Regier et al., 2016, and Nestrasil et al., 2018, as cited herein). Treatment with rAAVhu68.GLB1 is expected to slow or cease the progression of CNS disease manifestations with evidence of stabilization in atrophy and volumetric changes. The exploratory endpoint assessing changes (normal/abnormal) in T1/T2 signal intensity in the thalamus and basal ganglia is based on reported evidence for changes in the thalamic structure in patients with GM1 and GM2 gangliosidosis (Kobayashi and Takashima, 1994, Thalamic hyperdensity on CT in infantile GM1-gangliosidosis. Brain and Development. 16(6):472-474).

Biomarkers for the trial include β-gal enzyme (GLB1) activity, which can be measured in CSF and serum, and brain MRI, which demonstrates consistent, rapid atrophy in infantile GM1 gangliosidosis (Regier et al., 2016b, as cited herein). Additional biomarkers are investigated in CSF and serum from collected samples.

A. Primary Objective:

- To assess the safety and tolerability of rAAVhu68.GLB1 through 2 years following administration of a single dose into the cisterna magna (ICM). adverse events, neurological exam, sensory nerve conduction studies, total Neuropathy Score-Nurse, hematology, serum chemistry, liver function tests, coagulation (PT, aPTT, INR), troponin-If, CSF anti-AAVhu68 nAbs, vector shedding, urinalysis, seizure diary, physical examination, vital signs, ECG, brain MRI, and CSF cytology and chemistry (cell counts, protein, glucose) will be assessed over 5 years.
- To assess the efficacy of rAAVhu68.GLB1 following administration of a single dose into the cisterna magna. Key Secondary Endpoint* will be assessed at 2 years and over 5 years:
  - Vineland Adaptive Behavior Scales, Second Edition
  - Other Secondary Endpoints will be assessed at 2 years and over 5 years:
  - Bayley Scale of Infant and Toddler Development, Third Edition
  - WHO Multicenter Growth Reference Study Motor
  - Development Milestones Assessment
  - Hammersmith Infant Neurodevelopment Examination
  - Clinician and Caregiver Global Impression of Severity and change
  - Exit interviews

*A fit-for-purpose clinical outcome assessment does not exist for GM1 gangliosidosis. Therefore, in parallel with the conduct of this study, the sponsor is working with subject matter experts to collect data from clinical experts and parents/caregivers to develop an outcomes measurement strategy including identification of a primary efficacy endpoint for cohort 3, and if needed, plans for a composite endpoint derived from the scales listed above, modification of an existing COA or developing supplementary patient-centric GM1-specific items or scales. Please see Statistical Analysis section for details.

B. Secondary Objectives:

- To assess the pharmacodynamics and biological activity of rAAVhu68.GLB1 over 24 months following a single dose into the cisterna magna. Assessments: CSF biomarkers: β-galactosidase activity, hexosaminidase activity, GM1 ganglioside levels; Serum biomarkers: β-galactosidase activity, hexosaminidase activity; Urine Biomarker: keratan sulfate levels; All will be assessed at 30 days and over 5 years.
- To assess the effects of rAAVhu68.GLB1 following administration of a single dose into the cisternal magna on disease progression. Assessment: Total brain volume, brain substructure volume, and ventricle volume and T1/T2 signal intensity as measured by MRI; Skeletal abnormalities as measured by lateral spinal x-rays; Cardiomyopathy measured by cardiac echocardiogram; Hepatosplenomegaly measured by abdominal ultrasound; Cerebral function and diffuse slowing changes measured by serial EEG; Assessment of mechanical ventilation-free survival; Assessment of nutrition status by need of placement and use of feeding tubes; All will be assessed over 5 years.

To assess the effects of rAAVhu68.GLB1 following administration of a single dose into the cisternal magna on quality of life and healthcare resource utilization. Assessment: Quality of life: Pediatric Quality of Life Inventory/ Pediatric Quality of Life Inventory-Infant Scale; Healthcare resource utilization: chart review for hospital days, ER visits, ICU admissions, surgeries, need for hearing and visual aids; All will be assessed over 5 years.

C. Study Design:

Multicenter, open-label, single-arm dose escalation study of rAAVhu68.GLB1 (Table below). Up to a total of 28 pediatric subjects with infantile GM1 gangliosidosis are enrolled into 4 dose cohorts, and receive a single dose of

rAAVhu68.GLB1 administered by ICM injection. Safety and tolerability are assessed through 2 years, and all subjects are followed through 5 years post-administration of rAAVhu68.GLB1 for the long-term evaluation of safety and tolerability, pharmacodynamics (durability of transgene expression) and durability of clinical outcomes.

Product Name:	AAVhu68.UbC.GLB1
Gene Inserts:	Artificial version of human *GLB1* gene encoding beta-galactosidase (beta-gal or β-gal)
Control Element:	Regulatory element derived from human ubiquitin C (UbC) promoter
Other elements:	Chimeric intron (CI)– a hybrid intron consisting of a human beta-globin splice donor and immunoglobulin G (IgG) splice acceptor elements A polyadenylation (PolyA) signal derived from Simian Virus 40 (SV40) late genes
AAV Serotype:	Hu68

The AAVhu68.UbC.GLB1 is supplied frozen (≤ -60°C) as a sterile solution in ITFFB (intrathecal final formulation buffer). Depending on the dose level and the age band of the subject, dilution of the AAVhu68.UbC.GLB1 DP in the ITFFBD01 (study drug diluent) may be required prior to administration. The AAVhu68.UbC.GLB1 DP and ITFFBD01 formulations are composed of 1 mM sodium phosphate, 150 mM sodium chloride, 3 mM potassium chloride, 1.4 mM calcium chloride, 0.8 mM magnesium chloride, 0.001% poloxamer 188, pH 7.2.

Potential subjects are screened from Days -35 to -1 prior to dosing to determine eligibility for the study. Up to 28 pediatric subjects with Type 1 (Infantile) and Type 2a (Late Infantile) GM1 gangliosidosis are enrolled into the study. Those subjects who meet the inclusion/exclusion criteria are admitted to the hospital on the morning of Day 1 or per institutional practice. Subjects receive a single ICM dose of rAAVhu68.GLB1 on Day 1 and remain in the hospital for at least 24 h after dosing for observation. Subsequent assessments are performed 7, 14 and 30 days after dosing, then every 60 days for the first year and every 90 days for the second year. The safety and tolerability of rAAVhu68.GLB1 are monitored through assessment of adverse events (AEs) and serious adverse events (SAEs), vital signs, physical examinations, sensory nerve conduction studies, and laboratory assessments (chemistry, hematology, coagulation studies, CSF analysis). Immunogenicity of the AAV

and transgene product are also assessed. Efficacy assessments include survival, measurements of cognitive, motor and social development, changes in visual function and EEG, changes in liver and spleen volume, and biomarkers in CSF, serum, and urine.

The study consists of the following three cohorts administered rAAVhu68.GLB1 as a single ICM injection:

Part 1 (Dose Escalation Cohorts)		
Cohort	Patient	Assigned Intervention
Cohort 1	Late Onset Infantile GM1 Gangliosidosis (Type 2a)	rAAVhu68.GLB1 Dose 1: 3.3×10^{10} GC/g* Single dose of rAAVhu68.GLB1, via intra cisterna magna
Cohort 2	Late Onset Infantile GM1 Gangliosidosis (Type 2a)	rAAVhu68.GLB1 Dose 2: 1.1×10^{11} GC/g* Single dose of rAAVhu68.GLB1, via intra cisterna magna
Cohort 3	Early Onset Infantile GM1 Gangliosidosis (Type 1)	rAAVhu68.GLB1 Dose 1: 3.3×10^{10} GC/g* Single dose of rAAVhu68.GLB1, via intra cisterna magna
Cohort 4	Early Onset Infantile GM1 Gangliosidosis (Type 1)	rAAVhu68.GLB1 Dose 2: 1.1×10^{11} GC/g* Single dose of rAAVhu68.GLB1, via intra cisterna magna
Part 2 (Expansion Cohorts)		
Cohort 5	Late Onset Infantile GM1 Gangliosidosis (Type 2a)	rAAVhu68.GLB1 Dose Single dose of rAAVhu68.GLB1 Dose, via intra cisterna magna Dose to be used for the confirmatory cohorts in Part 2 will be defined after a review of data from Cohort 1-4.
Cohort 6	Early Onset Infantile GM1 Gangliosidosis (Type 1)	rAAVhu68.GLB1 Single dose of rAAVhu68.GLB1, via intra cisterna magna Dose to be used for the confirmatory cohorts in Part 2 will be defined after a review of data from Cohort 1-4.

*GC/g: gene copy per gram of estimated brain weight

D. Inclusion Criteria:

1. ≥4 month and <36 months of age at enrollment, with Type 1 (onset ≤6 months) or Type 2a (onset >6 and ≤18 months).

a. Type 1 Infantile GM1

- i. Presymptomatic subjects (≤6 months of age with confirmed mutation and reduced serum β-gal activity) identified through prenatal screening or family history of an older sibling with a confirmed diagnosis of GM1 gangliosidosis with the same genotype. Sibling must have had symptom onset at ≤6 months of age.

OR

- ii. Symptomatic subject (with confirmed mutation and reduced serum β-gal activity) must have a medical record documentation of onset at ≤6 months of age, with hypotonia or any documented symptom consistent with GM1 gangliosidosis AND with at least 70% of age corrected expected motor development (BSID-III), at the time of dosing.

b. Type 2a Late Infantile GM1:

i. Symptomatic subjects with onset >6 months and ≤18 months of age with hypotonia or any documented symptoms consistent with GM1 gangliosidosis who have demonstrated a plateauing or delay in achieving further developmental milestones with at least 70% of age corrected expected motor development (BSID-III).

2. Documentation that the subject is homozygous or compound heterozygous for GLB1 gene deletion or mutation AND decreased β-gal activity (≤20% of the lower normal value in leukocytes).

E. Exclusion Criteria:

1. Any clinically significant neurocognitive deficit not attributable to GM1 gangliosidosis or any other condition that may, in the opinion of the investigator, confound interpretation of study results.
2. If any subject had an acute illness requiring hospitalization within 30 days of enrollment, the history must be discussed with the sponsor's medical monitor before allowing the subject to be enrolled.
3. History of ventilation assisted respiratory support or a need for tracheostomy.

4. Intractable seizure or uncontrolled epilepsy defined as having had an episode of status epilepticus, or seizures requiring hospitalization within 30 days prior to dosing of investigational product.
5. Any contraindication to ICM administration procedure, including contraindications to fluoroscopic imaging and anesthesia.
6. Any contraindication to MRI or LP.
7. Prior gene therapy.
8. Use of miglustat within 48 hours prior to dosing of investigational product.
9. Use of enzyme replacement therapy or other investigational therapy within 5 half-lives prior to dosing of investigational product.
10. Any condition (e.g., history of any disease, evidence of any current disease, any finding upon physical examination, or any laboratory abnormality) that, in the opinion of the investigator, would put the subject at undue risk during procedure or would interfere with evaluation of the investigational product or interpretation of subject safety or study results. This includes:
    a. abnormal laboratory values considered clinically significant by the investigator
    b. Failure to thrive, defined as: Falling 20 percentiles (20/100) in body weight in the 3 months preceding Screening/Baseline
    c. Underlying defect in immune function
    d. History of multiple and severe life-threatening infections

F. Route of Administration and Procedure

rAAVhu68.GLB1 as a single dose is administered on Day 1 to subjects via CT-guided sub-occipital injection into the cisterna magna.

On Day 1 the appropriate concentration of rAAVhu68.GLB1 is prepared by the Investigational Pharmacy associated with the study. A syringe containing 5.6 mL of rAAVhu68.GLB1 at the appropriate concentration is delivered to the procedure room. The following personnel are present for study drug administration: interventionalist performing the procedure; anesthesiologist and respiratory technician(s); nurses and physician assistants; CT (or operating room) technicians; site research coordinator.

Prior to study drug administration, a lumbar puncture is performed to remove a predetermined volume of CSF and then to inject iodinated contrast intrathecally (IT) to aid in

visualization of relevant anatomy of the cisterna magna. Intravenous (IV) contrast may be administered prior to or during needle insertion as an alternative to the intrathecal contrast. The decision to used IV or IT contrast is at the discretion of the interventionalist. The subject is anesthetized, intubated, and positioned on the procedure table. The injection site is prepared and draped using sterile technique. A spinal needle (22-25 G) is advanced into the cisterna magna under fluoroscopic guidance. A larger introducer needle may be used to assist with needle placement. After confirmation of needle placement, the extension set is attached to the spinal needle and allowed to fill with CSF. At the discretion of the interventionalist, a syringe containing contrast material may be connected to the extension set and a small amount injected to confirm needle placement in the cisterna magna. After the needle placement is confirmed by CT guidance +/- contrast injection, a syringe containing 5.6 mL of rAAVhu68.GLB1 is connected to the extension set. The syringe contents are slowly injected over 1-2 minutes, delivering a volume of 5.0 mL. The needle is slowly removed from the subject.

A single dose into the cisterna magna (ICM) of rAAVhu68.GLB1 is safe and tolerable through 5 years following administration.

A single dose into the cisterna magna (ICM) of rAAVhu68.GLB1 improves survival, reduces probability of feeding tube dependence at 24 months of age, and/or reduces Disease progression as assessed by age at achievement, age at loss, and percentage of children maintaining or acquiring age-appropriate developmental and motor milestones.

Treatment slows of loss of neurocognitive function.

As prophylaxis for potential immune-mediated injury such as hepatotoxicity, subjects will receive systemic corticosteroids. Starting one day prior to rAAVhu68.GLB1 administration, systemic corticosteroids equivalent to oral prednisolone at 1 mg/kg of body weight per day will be administered for approximately 30 days (or until the scheduled Month 1 follow-up visit whichever occurs first). During this visit, clinical examination and laboratory testing should be performed per the schedule of assessments. For patients with unremarkable findings, the Investigator should taper the corticosteroid dose over the next 21 days per clinical judgement, starting at 0.75 mg/kg daily dose during Week 5, 0.5 mg/kg daily dose during Week 6 and then 0.25 mg/kg daily dose during Week 7, and the 0.25 mg/kg every other day during Week 8. Consult expert(s) if patients do not respond adequately to the equivalent of 1 mg/kg/day regimen. If, in the opinion of the investigator, the subject develops clinical symptoms or clinical/laboratory signs of potential immune mediated toxicity, the dose, type and schedule of immunosuppression may be modified, and

the study responsible physician should be notified. Routine vaccine schedules and local guidelines should be adhered to including recommendations to adjust timing of vaccines while the subject is under steroid treatment.

All documents cited in this specification are incorporated herein by reference, as is the Sequence Listing labeled "21-9595PCT_ST25.txt". Also incorporated herein by reference are US Provisional Patent Application No. 63/063,119, filed August 7, 2020, US Provisional Patent Application No. 63/007,297, filed April 8, 2020, and US Provisional Patent Application No 63/007,297, filed February 2, 2020. While the invention has been described with reference to particular embodiments, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

(Sequence Listing Free Text)

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11	<223> human elongation initiation factor 1 alpha promoter (EF1a)
12	<223> UbC.GLB1.SV40 vector genome
13	<223> EF1a.GLB1.SV40 vector genome
14	<223> UbC.GLB1.SV40 - 2
15	<223> UbC.GLB1.SV40 - 3
16	<223> Vector genome CB7.CI.GLB1.RBG <220> <221> repeat_region <222> (1)..(130) <223> 5" ITR from AAV2 <220> <221> repeat_region <222> (4232)..(4362) <223> 5" ITR from AAV2
17	<223> chicken beta-actin intron
18	<223> CB promoter
19	<223> CMV Immediate early Promoter
20	<223> Encoded AAV9 vp1 amino acid sequence

21	<223> Encoded AAVhu31 vp1 amino acid sequence
22	<223> Encoded AAVhu32 vp1 amino acid sequence
23	<223> AAV9 vp1 coding sequence
24	<223> AAVhu31 vp1 coding sequence
25	<223> AAVhu32 vp1 coding sequence

CLAIMS:

1. A therapeutic regimen useful for treatment of GM1 gangliosidosis in a human patient, wherein the regimen comprises administration of a recombinant adeno-associated virus (rAAV) vector having an AAV capsid and a vector genome comprising a sequence encoding human β-galactosidase under control of regulatory sequences that direct expression thereof in target cell, the administration comprising intra-cisterna magna (ICM) injection of a single dose comprising:

(i) about 1.6×10^{13} to about 1.6×10^{14} GC, wherein the patient is about 1 month to about 4 months of age;

(ii) about 2.1×10^{13} to about 2.1×10^{14} GC, wherein the patient is at least 4 months to under 8 months of age;

(iii) about 2.6×10^{13} to about 2.6×10^{14} GC, wherein the patient is at least 8 months up to 12 months of age; or

(iv) about 3.2×10^{13} to about 3.2×10^{14} GC, wherein the patient is at least 12 months of age.

2. The regimen according to claim 1, wherein the human β-galactosidase coding sequence comprises a nucleotide sequence set forth in SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 or a sequence at least 95% identical to any one of SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 that encodes the mature β-galactosidase of amino acids 24 to 677 of SEQ ID NO: 4.

3. The regimen according to claim 1 or 2, wherein the encoded human β-galactosidase has the sequence selected from:

(a) about amino acids 1 to 677 of SEQ ID NO: 4; and

(b) a synthetic human enzyme comprising a heterologous leader sequence fused to about amino acids 24 to 677 of SEQ ID NO: 4.

4. The regimen according to any one of claims 1 to 3, wherein the vector genome further comprises a 5' inverted terminal repeat (ITR) sequence, a regulatory element derived from the human ubiquitin C (UbC) promoter, a chimeric intron, a polyA signal, and/or a 3' ITR sequence.

5. The regimen according to any one of claims 1 to 4, wherein the patient has been identified as having type 1 (infantile) GM1 or type 2a (late infantile) GM1.

6. The regimen according to any one of claims 1 to 5, further comprising administration of at least one immunosuppressive co-therapy to the patient at least one day prior to or on the day of delivery of the rAAV.

7. The regimen according to claim 6, wherein the immunosuppressive co-therapy includes one or more corticosteroids.

8. The regimen according to claim 6 or 7, wherein the immunosuppressive co-therapy includes oral prednisolone.

9. The regimen according to claim 8, wherein the oral prednisolone is administered at about 1 mg/kg body weight.

10. The regimen according to any one of claims 5 to 9, wherein administration of the at least one immunosuppressive co-therapy continues for at least 3 to 4 weeks following administration of the rAAV.

11. The regimen according to any one of claims 1 to 10, wherein the efficacy of treatment is assessed by one or more of a delay in the onset of seizures, a decrease in the frequency of seizures, β-galactosidase in serum and/or cerebral spinal fluid, and volumetric changes in brain tissue as measured by magnetic resonance imaging (MRI).

12. A composition comprising a recombinant AAV(rAAV) vector comprising an AAV capsid and a vector genome comprising a human β-galactosidase coding sequence and expression control sequences that direct expression thereof in target cells, wherein the rAAV vector is formulated for intra-cisterna magna (ICM) injection to a human subject in need thereof to administer a dose of:

(i) about 1.6×10^{13} to about 1.6×10^{14} GC, wherein the patient is about 1 month to about 4 months of age;

(ii) about 2.1×10^{13} to about 2.1×10^{14} GC, wherein the patient is at least 4 months to under 8 months of age;

(iii) about 2.6×10^{13} to about 2.6×10^{14} GC, wherein the patient is at least 8 months up to 12 months of age; or

(iv) about 3.2×10^{13} to about 3.2×10^{14} GC, wherein the patient is at least 12 months of age.

13. The composition according to claim 12, wherein the human β-galactosidase coding sequence comprises a nucleotide sequence set forth in SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 or a sequence at least 95% identical to any one of SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 that encodes the mature β-galactosidase of amino acids 24 to 677 of SEQ ID NO: 4.

14. The composition according to claim 12 or 13, wherein the vector genome further comprises a 5' inverted terminal repeat (ITR) sequence, a regulatory element derived from the human ubiquitin C (UbC) promoter, a chimeric intron, a polyA signal, and/or a 3' ITR sequence.

15. The composition according to any one of claims 12 to 14, wherein the rAAV is formulated in a suspension to deliver 3.33×10^{10} GC per gram of brain mass to 3.33×10^{11} GC per gram of brain mass, optionally wherein the volume of the administered dose is about 3.0 mL to about 5.0 mL.

16. The composition according to any one of claims 12 to 15, wherein the rAAV is in a formulation buffer having a pH of 6 to 9, optionally wherein the pH is about 7.2.

17. The composition according to any one of claims 12 to 16 for use in a co-therapy comprising administration of at least one immunosuppressant to the patient at least one day prior to or on the day of delivery of the rAAV.

18. The composition according to claim 17, wherein the immunosuppressant is a corticosteroid, optionally orally delivered prednisolone.

19. A method of treating a patient with GM1 gangliosidosis, the method comprising administering a single dose of a recombinant adeno-associated virus (rAAV) to the patient by intracisternal magna (ICM) injection,

wherein the rAAV comprises an AAV capsid and a vector genome comprising a sequence encoding human β-galactosidase under control of regulatory sequences that direct expression thereof in a target cell, and

wherein the single dose is from $1x10^{10}$ GC to $3.4x10^{11}$ GC per gram of estimated brain mass of the patient.

20. The method of claim 19, wherein the patient has onset of a GM1 symptom at or before 18 months of age.

21. The method of claim 20, wherein the patient has onset of a GM1 symptom at 6 months of age or earlier.

22. The method of claim 20, wherein the patient has onset of a GM1 symptom at 6 to 18 months of age.

23. The method of any one of claims 19 to 21, wherein the patient has type1 (infantile) GM1.

24. The method of any one of claims 19, 20, or 22, wherein the patient has type2a (late infantile) GM1.

25. The method of any one of claims 19 to 24, wherein the patient has been diagnosed to have type 1 or type 2a GM1.

26. The method of any one of claims 19 to 25, wherein the subject is at least 4 months of age.

27. The method of claim 26, wherein the subject is 4 to 36 months of age.

28. The method of claim 26, wherein the subject is a human patient of 4 to 24 months of age.

29. The method of claim 26, wherein the patient is a human patient of 6 to 36 months of age.

30. The method of claim 26, wherein the patient is a human patient of 6 to 24 months of age.

31. The method of claim 26, wherein the patient is a human patient of 12 to 36 months of age.

32. The method of claim 26, wherein the patient is a human patient of 12 to 24 months of age.

33. The method of any one of claims 19 to 32, wherein the single dose is $3.3x10^{10}$ GC per gram of estimated brain mass of the patient.

34. The method of claim 33, wherein the single dose is $2.1x10^{13}$ to $2.5x10^{13}$ GC of the rAAV.

35. The method of claim 33, wherein the single dose is $2.6x10^{13}$ to $3.1x10^{13}$ GC of the rAAV.

36. The method of claim 33, wherein the single dose is $3.2x10^{13}$ to $4.5x10^{13}$ GC of the rAAV.

37. The method of any one of claims 19 to 32, wherein the single dose is $1.11x10^{11}$ GC per gram of estimated brain mass of the patient.

38. The method of claim 37, wherein the single dose is $6.8x10^{13}$ to $8.6x10^{13}$ GC of the rAAV.

39. The method of claim 37, wherein the single dose is $8.7x10^{13}$ to $0.9x10^{14}$ GC of the rAAV.

40. The method of claim 37, wherein the single dose is $1.0x10^{14}$ to $1.5x10^{14}$ GC of the rAAV.

41. The method of any one of claims 19 to 25, wherein the patient is 4 to 8 months of age, and the single dose is $2.1x10^{13}$ GC of the rAAV.

42. The method of any one of claims 19 to 25, wherein the patient is 4 to 8 months of age, and the single dose is $6.8x10^{13}$ GC of the rAAV.

43. The method of any one of claims 19 to 25, wherein the patient is 8 to 12 months of age, and the single dose is $2.6x10^{13}$ GC of the rAAV.

44. The method of any one of claims 19 to 25, wherein the patient is 8 to 12 months of age, and the single dose is $8.7x10^{13}$ GC of the rAAV.

45. The method of any one of claims 19 to 25, wherein the patient is at least 12 months of age, and the single dose is $3.2x10^{13}$ GC of the rAAV.

46. The method of any one of claims 19 to 25, wherein the patient is at least 12 months of age, and the single dose is $1.0x10^{14}$ GC of the rAAV.

47. The method of any one of claims 19 to 46, further comprising the step of hematopoietic stem cell transplantation.

48. The method of any one of claims 19 to 47, further comprising the step of administering a steroid to the patient.

49. The method of claim 48, wherein the steroid is a corticosteroid.

50. The method of claim 48 or 49, wherein the steroid is administered systematically daily for at least 21 days.

51. The method of claim 48 or 49, wherein the steroid is administered systematically daily for 30 days.

52. The method of any one of claims 19 to 51, wherein the sequence encoding human β-galactosidase comprises a nucleotide sequence set forth in SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 or a sequence at least 95% identical to any one of SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 that encodes the mature β-galactosidase of amino acids 24 to 677 of SEQ ID NO: 4.

53. The method of any one of claims 19 to 51, wherein the human β-galactosidase has an amino acid sequence of SEQ ID NO: 4 or a functional fragment thereof.

54. The method of any one of claims 19 to 51, wherein the vector genome has a sequence selected from SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15.

55. The method of any one of claims 19 to 51, wherein the vector genome has a sequence at least 95% identical to SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15.

56. The method of any one of claims 19 to 51, wherein the vector genome further comprises a 5' inverted terminal repeat (ITR) sequence, a regulatory element derived from the human ubiquitin C (UbC) promoter, a chimeric intron, a polyA signal, and/or a 3' ITR sequence.

57. A pharmaceutical composition in a unit dosage form, comprising:
1×10^{13} GC to 5×10^{14} of a recombinant adeno-associated virus (rAAV) vector in a buffer, wherein the rAAV comprises an AAV capsid and a vector genome comprising a sequence encoding human β-galactosidase under control of regulatory sequences that direct expression thereof in a target cell.

58. The pharmaceutical composition of claim 57, formulated for intracisternal magna (ICM) injection.

59. The pharmaceutical composition of claim 58, wherein the buffer comprises sodium phosphate, sodium chloride, potassium chloride, calcium chloride, magnesium chloride, and poloxamer 188.

60. The pharmaceutical composition of any one of claims 57 to 59, wherein the buffer comprises 1 mM sodium phosphate, 150 mM sodium chloride, 3 mM potassium

chloride, 1.4 mM calcium chloride, 0.8 mM magnesium chloride, and 0.001% poloxamer 188.

61. The pharmaceutical composition of any one of claims 57 to 60, in a liquid form.

62. The pharmaceutical composition of claim 61, having 3.0 mL, 4.0mL or 5.0 mL of volume.

63. The pharmaceutical composition of claim 57 to 62, comprising $2.1x10^{13}$ to $2.5x10^{13}$ GC of the rAAV.

64. The pharmaceutical composition of claim 57 to 62, comprising $2.6x10^{13}$ to $3.1x10^{13}$ GC of the rAAV.

65. The pharmaceutical composition of claim 57 to 62, comprising $3.2x10^{13}$ to $4.5x10^{13}$ GC of the rAAV.

66. The pharmaceutical composition of claim 57 to 62, comprising $6.8x10^{13}$ to $8.6x10^{13}$ GC of the rAAV.

67. The pharmaceutical composition of claim 57 to 62, comprising $8.7x10^{13}$ to $0.9x10^{14}$ GC of the rAAV.

68. The pharmaceutical composition of claim 57 to 62, comprising $1.0x10^{14}$ to $1.5x10^{14}$ GC of the rAAV.

69. The pharmaceutical composition of any one of claims 57 to 68, wherein the sequence encoding human β-galactosidase comprises a nucleotide sequence set forth in SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 or a sequence at least 95% identical to any one of SEQ ID NO: 8, SEQ ID NO: 7, SEQ ID NO: 6, or SEQ ID NO: 5 that encodes the mature β-galactosidase of amino acids 24 to 677 of SEQ ID NO: 4.

70. The pharmaceutical composition of any one of claims 57 to 68, wherein the human β-galactosidase has an amino acid sequence of SEQ ID NO: 4 or a functional fragment thereof.

71. The pharmaceutical composition of any one of claims 57 to 68, wherein the vector genome has a sequence selected from SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15.

72. The pharmaceutical composition of any one of claims 57 to 68, wherein the vector genome has a sequence at least 95% identical to SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15.

73. The pharmaceutical composition of any one of claims 57 to 68, wherein the vector genome further comprises a 5' inverted terminal repeat (ITR) sequence, a regulatory element derived from the human ubiquitin C (UbC) promoter, a chimeric intron, a polyA signal, and/or a 3' ITR sequence.