

METHODS FOR FUS-BASED DELIVERY OF VIRAL PARTICLES TO THE BRAIN**CROSS REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims the priority benefit of U.S. Provisional Application No. 63/625,206, filed January 25, 2024, which is incorporated by reference in its entirety.

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

[0002] The content of the electronic sequence listing (159792019040seqlist.xml; Size: 22,748 bytes; and Date of Creation: January 23, 2025) is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0003] The present disclosure relates to methods for treating neurodegenerative disorders in a patient in need thereof, the methods comprising administering to the patient a viral vector via focused ultrasonic delivery.

BACKGROUND

[0004] Gene therapy has demonstrated long-lasting therapeutic benefit for the treatment of neurological disorders affecting large parts of the central nervous system, such as spinal muscular atrophy(1). Recombinant adeno-associated virus (AAV) is the most advanced vector for gene delivery *in vivo*, and some AAV serotypes, such as AAV9, can cross the blood-brain barrier (BBB) after intravenous administration(2). However, BBB crossing of AAV9 requires high intravenous dosages of up to 2×10^{14} genome copies per kilogram (GC/kg) in children and more in adults where the ability of AAV9 to cross the BBB is limited(2, 3). The recent deaths of patients with X-linked myotubular myopathy receiving 3×10^{14} GC/kg AAV8 highlight the risks associated with intravenous AAV administration and the need to develop new strategies for AAV delivery to the central nervous system(4).

[0005] The permeability of the BBB can be increased transiently by the application of focused ultrasound combined with intravenous microbubbles (FUS-MB)(5). FUS induces an oscillation of the microbubbles, which decreases tight-junction proteins and increases

transecytosis across the endothelial cells(6, 7). The temporary increase in BBB permeability facilitates non-invasive delivery of intravenous AAV to FUS-targeted brain areas at dosages 50-100 times lower than needed for BBB crossing by AAV9 alone(8). Still, the delivery is limited to FUS-targeted brain areas, and while FUS can be targeted simultaneously to multiple brain regions it remains unsuitable for treating whole-brain diseases(9).

[0006] A challenge and unmet need of gene therapy is to deliver gene vectors to deep brain structures using a minimally invasive strategy. Safe methods for viral vector delivery to the whole brain at low dosage with low transduction to off-target peripheral organs are needed.

BRIEF SUMMARY

[0007] In some aspects, the disclosure provides methods of delivering viral particles to the brain of a mammal (e.g., a human patient), comprising administering the viral particles to the cerebral spinal fluid (CSF) of the patient and administering a plurality of microbubbles intravenously to the mammal, wherein one or more regions of the brain of the mammal are subjected to focused ultrasound (FUS). In some embodiments, the viral particles are delivered to the CSF of the mammal by injection of the viral particles into the cisterna magna of the patient. As shown herein, the combination of CSF administration and focused ultrasound with microbubbles facilitates gene delivery of the viral particles to the superficial and deep brain regions of the mammal that are difficult to reach. Specifically shown is that the combination of ICM administration of viral particles along with focused ultrasound-microbubble (FUS-MB) techniques enable viral particle delivery to both superficial brain areas and FUS-targeted deep brain structures.

[0008] In some embodiments, the viral particles are adeno-associated virus (AAV) particles. In some embodiments, the AAV particles is a recombinant adeno-associated virus (rAAV) particles.

[0009] In some embodiments, the administered viral particles comprise a dose level of from about 8×10^{11} to about 5×10^{13} genome copies per kilogram (GC/kg). In some embodiments, the administered viral particles comprise a dose level of from about 8×10^{11} to about 2×10^{13} genome copies per kilogram (GC/kg).

[0010] In some embodiments, methods comprising FUS-MB increases delivery of viral particles (e.g., AAV) administered via ICM to the striatum. In some embodiments, methods comprising FUS-MB increases delivery of viral particles administered via ICM to the striatum about 5-fold compared to delivery of viral particles administered via ICM alone. In some embodiments, methods comprising FUS-MB increases delivery of viral particles administered via ICM to the striatum about 2-fold, 3-fold, 4-fold, 5-fold, or more, compared to delivery of viral particles administered via ICM alone. In some embodiments, methods comprising FUS-MB increases delivery of viral particles (e.g., AAV) administered via ICM to the cerebral cortex. In some embodiments, methods comprising FUS-MB increases delivery of viral particles (e.g., AAV) administered via ICM to the thalamus.

[0011] Application of the FUS, administration of the microbubbles and administration of the viral particles can occur in any order. In some embodiments, the application of FUS occurs prior to the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs simultaneously with the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs less than one minute after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs at about 1 minute after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs at about 1, 2, 3, 4, 5, 10, 60, 120, 360 minutes, or more after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs at about 1, 2, 3, 4, 5, 10, 24 hours, or more after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs at about 1 or 2 hours after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs between about 1 minute to about 180 minutes after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs between about 10 minutes to about 180 minutes after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs between about 30 minutes to about 180 minutes after the administering of the viral particles and/or the plurality of microbubbles.

[0012] In some embodiments, provided herein are methods for treating neurological disorders requiring delivery of viral particles (e.g., AAV) to the brain of patients. In some embodiments, methods comprise CSF administration (e.g., intracisternal magna (ICM) administration) of a viral particle (e.g., adeno-associated virus (AAV)) for treating neurological disorders comprising beta-galactosidase-1 deficiency, Huntington's Disease, Batten disease, Alzheimer's disease, Parkinson's disease, multiple system atrophy, progressive supranuclear palsy, frontotemporal dementia, amyotrophic lateral sclerosis, or spinal muscular atrophy.

BRIEF DESCRIPTION OF DRAWINGS

FIGS. 1A-E show workflows and mechanisms for delivering a viral particle to the whole brain including both superficial and deep brain structures, in accordance with some embodiments. **FIG. 1A** shows intracisternal magna (ICM) administration of viral particles (e.g., AAV particles) may lead to widespread transduction, especially of superficial brain areas, but the transduction of deeper brain structures (e.g. striatum) is negligible. **FIG. 1B** shows a focused ultrasound (FUS) combined with intravenous (IV) microbubbles and intravenous viral particles delivers viral particles to any FUS-targeted brain region, including deep structures such as the striatum. **FIG. 1C** shows that the combination of FUS and IV microbubbles with viral particles administered ICM results in viral particle delivery widespread to superficial brain areas as well as to deep brain structures targeted with FUS. **FIG. 1D** shows a first possible mechanisms of action describing how FUS-MB may increase brain delivery of ICM-administered viral particles. **FIG. 1E** shows a second possible mechanisms of action describing how FUS-MB may increase brain delivery of ICM-administered viral particles.

[0013] **FIGS. 2A-K** show experimental design schematics and results for a pilot trial of adeno associated vector (AAV) dosing timepoints relative to focused ultrasound (FUS) applications, in accordance with some embodiments. To determine the diffusion of ICM-injected particles into the brain parenchyma (some of which could be via the perivascular space), the MR contrast agent gadolinium was injected ICM (**FIG. 2A**) and MR images were acquired after 45, 55, 70, and 80 minutes (**FIGS. 2B-E**). Five groups were tested for FUS-MB-mediated delivery of AAV and results are depicted in **FIGS. 2F-H**. AAV was injected intravenously during FUS application as a positive control as depicted in **FIG. 2F**. AAV was injected ICM either 60 minutes or 120 minutes prior to FUS-MB application or 10 minutes post FUS-MB

application as depicted in **FIG. 2G**. The injection of AAV after FUS application was used to determine whether distribution route #2 was possible. One group of animals received ICM injection without FUS-MB application as depicted in **FIG. 2H**. After 4 weeks, the animals were sacrificed, and one hemisphere was used for GFP immunohistochemistry (IHC) and the other for analysis of GFP mRNA expression as depicted in **FIG. 2I**. GFP protein expression was seen in the FUS-targeted spots in all animals except those not treated with FUS-MB where GFP expression was visible only in one area in the striatum close to the cortex (k10) as depicted in **FIG. 2J**. Results for the pilot study can be seen in **FIG. 2K**, where small group sizes (n=3-4 per group) showed GFP mRNA expression in the FUS-MB-targeted striatum with the highest expression in animals injected IV with AAV during FUS-MB followed by AAV injection ICM 120 minutes before FUS-MB. Injection of AAV ICM 60 minutes before FUS-MB and 10 minutes post FUS-MB both resulted in GFP mRNA levels trending towards a higher gene delivery than ICM injection without FUS-MB.

[0014] **FIGS. 3A-D** show results demonstrating AAV is significantly cleared from the cerebrospinal fluid into the blood, in accordance with some embodiments. AAV was found to be significantly cleared from the cerebrospinal fluid into the blood as depicted in **FIGS. 3A-D**. The distribution of AAV to peripheral organs following intravenous (IV) and ICM administration was measured by quantifying the GFP genome copies in the peripheral organs 4 weeks after AAV administration (**FIG. 3A**). There were no significant differences between the GFP genome copies in the peripheral organs with IV injection of the AAV compared to ICM injection (**FIG. 3B**). To determine the kinetics of AAV distribution in the blood following IV and ICM injection, GFP genome copies were quantified in blood samples taken 60 and 153 minutes post-ICM injection and 21 minutes post IV injection (**FIG. 3C**). GFP genome copies in the blood 60 minutes after ICM injection was not significantly higher than negative control samples (**FIG. 3D**). GFP genome copies in the blood 153 minutes after ICM injection was significantly higher than the 60 minutes time point and the negative control. The level of GFP genome copies was the highest of all groups 21 minutes after IV injection of the AAV. Bars represent mean +/- standard deviation. Statistical analysis was done using one-way ANOVA and post hoc Tukey's test, ****p < 0.0001, **p < 0.01, *p < 0.05, n= 4-6/group (n = 3 for negative control in **FIG. 3D**).

[0015] **FIGS. 4A-L** show results demonstrating FUS-mediated delivery of intracisternal magna (ICM) administered AAV to the striatum, in accordance with some embodiments. AAV was

injected intravenously (IV) during FUS-MB, ICM 120 minutes pre-FUS-MB or ICM without FUS-MB application. FUS was targeted bilaterally to the striatum. After 4 weeks the animals were sacrificed, one hemisphere was used for IHC and the other hemisphere which was dissected into brain regions for RNA extraction and qPCR analysis (**FIG. 4A**). IHC staining showed GFP expression in the FUS-targeted spots in the striatum (green, white arrows) in both animals injected IV and ICM, but not in animals injected ICM without FUS-MB application (**FIG. 4B**). Sectioning and staining of the dorsal root ganglions from the thoracic part of the spine showed sparse GFP expression that was similar between animals injected with AAV IV and ICM (**FIG. 4C**). GFP mRNA expression was quantified in multiple brain regions (**FIGS. 4C-K**). In the striatum, IV AAV + FUS-MB and ICM AAV + FUS-MB resulted in significantly higher GFP mRNA expression than ICM AAV without FUS-MB (**FIG. 4C**). There was no significant difference in GFP expression in the striatum between animals injected with AAV IV and ICM and treated with FUS-MB. ICM injection of AAV, both with and without FUS targeting the striatum, resulted in significantly higher GFP mRNA expression than IV injection in the thalamus, midbrain, cerebellum, hippocampus, and brainstem (**FIGS. 4C-H**). When targeting the striatum, the FUS also hits cortical structures (see **FIG. 4A, I-J**). AAV ICM injection did not result in higher GFP mRNA expression in the FUS-targeted cortical structures compared to animals injected with AAV IV and treated with FUS-MB. In cortical structures where FUS was not targeted, there was significantly higher GFP mRNA expression in animals injected ICM with AAV, both with and without FUS-MB application in the striatum, than in animals injected IV with AAV and FUS-MB (**FIG. 4K**). Data was not normally distributed and was therefore log₁₀-transformed, which provided normal distribution for the data from the striatum, thalamus, cerebellum, and cortex 3. Log₁₀-transformation did not achieve normal distribution for the data from the hippocampus, brainstem, midbrain, cortex1 and cortex2. These brain areas were statistically analyzed using the non-parametric Kruskal-Wallis test and post hoc Dunn's test.

[0016] **FIG. 5** shows accurate, large volume BBB opening achieved in both caudate nucleus and putamen using methods of the disclosure. Blood brain barrier opening was achieved throughout the left caudate (Cd) and putamen (Put) of all four animals. **FIG. 5A** shows axial MR images taken using a modified T2* relaxation map "T2*map" to detect minor perturbation of the BBB immediately following sonication. Animals 1001, 1002, 1003 and 1004 received between 1-3 sonication rounds per target depending on the extent of hypointense signal change observed.

Right (R) and left (L) sides of animals are denoted at top left of panel. **FIG. 5B** shows axial MR images taken following intravenous administration of gadolinium (Gd) contrast agent to indicate opened brain regions by showing as hyperintense contrast as compared to the same regions in the opposite, nontargeted hemisphere. Accurate opening throughout the targeted regions was demonstrated for all animals immediately after FUS. **FIG. 5C shows that** opening largely resolved in 1001 and 1003 by 2 days post-sonication, suggestive of excessive BBB opening in the other two animals. **FIGS. 5D and 5E** show axial, T2 weighted imaging hyperintensities two days after sonication, indicative of inflammation/edema that did not resolve, as this signal remained for the duration of the study, out to 20 days post-sonication.

[0017] **FIG. 6** shows enhancement of AAV vector genome biodistribution to the NHP striatum using MRIGFUS in combination with intra-CSF delivery of AAV. Vector genome biodistribution of AAV2HBKO and AAV.SAN006 was quantified 3 weeks following intra-CSF AAV administration and BBB opening. DNA was extracted from 49 gray matter punches encompassing 20 brain regions. Multiplex digital PCR was used with probes specific for each vector genome in combination with *TUBB1* used as the genomic normalizer to calculate vector copies/cell. **FIGS. 6A and 6B** show graphs of VG distribution for the indicated capsids in the untreated (“-FUS”) hemisphere vs sonicated (“+FUS”) hemisphere. Biodistribution in individual brain regions represent one to five punches averaged per animal. N=4 NHP. Data are mean +/- SEM. **FIGS. 6C and 6D** show VG data plotted to highlight spatial differences in BBB opening and resulting transduction within the targeted brain regions, VG data show transduction at the tissue punch level for both AAV2HBKO (**FIG. 6C**) and AAV.SAN006 (**FIG. 6D**) in the caudate and putamen of each animal. 1003 and 1004 are denoted by blue symbols. Data are mean +/- SEM. **FIG. 6E** shows quantification of AAV VG levels in the blood following intra-CSF administration of AAV. Genomic DNA was extracted from 200uL of whole blood drawn at the indicated timepoints to track the amount of AAV2HBKO-GFP and AAV.SAN006-mCherry in the blood before, during and 1 week after FUS. Values are VG per uL of blood from each animal with the average, predose background subtracted. Vector genome levels in spinal cord (SC) (**FIG. 6F**), dorsal root ganglia (DRG) (**FIG. 6G**) and liver and spleen (**FIG. 6H**). DNA was extracted from the indicated tissues from each animal, and multiplex dPCR used to quantitate GFP and mCherry VGs normalized to *TUBB1* to calculate VG/cell. Data are the mean +/-SEM. C, T and L refer to cervical, thoracic and lumbar regions of the spinal cord and DRGs.

[0018] **FIG. 7** shows enhancement of AAV-mediated transgene mRNA expression in the NHP striatum using MRIGFUS in combination with intra-CSF delivery of AAV. mRNA expression from AAV2HBKO (GFP) and AAV.SAN006 (mCherry) was quantified 3 weeks following intraCSF AAV administration and BBB opening. RNA was extracted from 49 gray matter punches encompassing 20 brain regions and multiplex RT-digital PCR was used to measure GFP, mCherry, *HPRT* and *RPP30* expression. Transgene expression was normalized to the mean of the housekeeper gene expression. **FIGS. 7A and 7B** show graphs of mRNA expression (GFP for AAV2HBKO, mCherry for AAV.SAN006) for the indicated capsids in the untreated (“- FUS”) hemisphere vs sonicated (“+FUS”) hemisphere. RNA expression in individual brain regions represents one to five punches averaged per animal (n=4 NHP). Data are mean +/- SEM. **FIGS. 7C and 7D** highlight potential spatial differences in BBB opening and resulting transduction within the targeted brain regions VG data are plotted to show transduction at the tissue punch level for both AAV2HBKO (**FIG. 7C**) and AAV.SAN006 (**FIG. 7D**) in the caudate and putamen of each animal. 1003 and 1004 are denoted by blue symbols. Data are mean +/-SEM.

[0019] **FIG. 8** shows Histology and imaging assessments. One coronal slab of brain tissue from each animal representing anterior caudate and putamen was excluded from frozen tissue analyses and instead fixed and embedded for exploratory histopathology and imaging. **FIGS. 8A and 8B** show representative images showing hematoxylin and eosin (H&E) staining of 5µm sections from animal 1003 indicative of cellular infiltrates and necrosis in the dorsal caudate of the FUS-treated, left hemisphere. These findings were consistently found in all animals but were restricted to dorsal caudate in each case. Putamen was spared in this animal and in all other animals. A1 and A2 insets are shown to detail findings in the caudate but not putamen. Scale bars are indicated in each panel. **FIGS. 8C and 8D** shows the evaluation of the pattern and extent of transduction in the anterior striatum in situ hybridization was performed on adjacent sections with probes specific to GFP or mCherry to visualize cells transduced by AAV2HBKO or AAV.SAN006 respectively. Tissue was counterstained with hematoxylin. High magnification images of the same region as shown in A2 and B2 are shown in C (GFP ISH) and D (mCherry ISH). Scale bars are indicated in each panel.

[0020] **FIG. 9** shows a table of Data comprising the individual and summed actual cavitation doses for caudate and putamen. Acoustic dose is provided in arbitrary units and is the dose

measured following the completion of each sonication round using ExAblate Neuro software. Due to the size of the region, ventral putamen was divided into two separate posterior (A) and anterior (B) regions within the same axial slice as indicated. Animals 1003 and 1004 were treated on 2 axial planes per structure to attempt more comprehensive tissue coverage. Total dose is shown as the sum of sonication rounds for each targeted region.

DETAILED DESCRIPTION

I. Introduction

[0021] Gene delivery via adeno-associated viral particles can provide lasting clinical benefits following a one-time treatment. Delivery throughout the brain is needed for the treatment of neurological disorders with widespread pathology, including Alzheimer and Parkinson diseases, and amyotrophic lateral sclerosis. Most gene vectors have poor diffusion in the brain tissue. Furthermore, it is only at high intravenous doses that gene vectors can overcome the blood-brain barrier. In contrast, relatively lower doses of gene vectors injected in the cerebrospinal fluid enable significant transduction of superficial brain regions. The remaining challenge and unmet need of gene therapy is to deliver gene vectors to deep brain structures using a minimally invasive strategy. Demonstrated herein is that non-invasive focused ultrasound blood-brain barrier modulation can increase the delivery of recombinant viruses significantly to deep brain structures following injection into the CSF (e.g., injection into the cisterna magna). Delivery of viral particles to the central nervous system, via administration in the cerebrospinal fluid, is being evaluated in several clinical trials for treating beta-galactosidase-1 deficiency, Batten disease, Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, and spinal muscular atrophy. The findings described herein show that the efficacy of gene therapies delivered in the cerebrospinal fluid can be enhanced by targeting brain areas of interest with focused ultrasound.

[0022] In some embodiments, focused ultrasound combined with intravenous microbubbles increases the delivery of the clinically relevant virus. In some embodiments, focused ultrasound combined with intravenous microbubbles (FUS-MB) increases the delivery of the clinically relevant virus (e.g., gene vector, or adeno-associated virus) to deep brain structures as compared to methods without FUS-MB.

[0023] In some embodiments, administration of viral vector (e.g., adeno-associated virus) in the cerebrospinal fluid (e.g., through the cisterna magna) is used to treat patients with neurological disorders. In some embodiments, focused ultrasound (e.g., FUS) combined with intravenous microbubbles (e.g., MB) increases the permeability of the blood-brain barrier in humans and enables delivery of intravenous adeno-associated virus in non-human primates. In some embodiments, the combination of these two clinically relevant gene delivery methods, CSF (e.g., intracisternal magna) administration and focused ultrasound with microbubbles, facilitate gene delivery, to superficial and deep brain structures. In some embodiments, intracisternal magna administration is less invasive than intracerebroventricular (IVC) administration. In some embodiments, methods may provide increased therapeutic efficacy of gene therapies. In some embodiments, methods may provide increased therapeutic efficacy of gene therapies particularly for disorders with brain regions that have remained difficult to reach.

[0024] In some aspects, provided herein are methods, compositions and kits for delivery of a viral vector to the brain for treatment of neurodegenerative disorders (e.g., disease). In some embodiments, the combination of intracisternal magna administration and focused ultrasound enables viral vector delivery to both superficial brain areas and focused ultrasound-targeted deep brain structures. The use of the proper viral vector (e.g., the proper AAV serotype) in conjunction with focused ultrasound microbubble-enhanced administration of the viral vector may also allow for delivery to the whole brain at dose levels low enough to allow for safe delivery to both children and adult human patients.

II. Definitions

[0025] A “vector,” as used herein, refers to a recombinant plasmid or virus that comprises a nucleic acid to be delivered into a host cell, either *in vitro* or *in vivo*.

[0026] The terms “polypeptide” and “protein” are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Such polymers of amino acid residues may contain natural or non-natural amino acid residues, and include, but are not limited to, peptides, oligopeptides, dimers, trimers, and multimers of amino acid residues. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include

post-expression modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, phosphorylation, and the like. Furthermore, for purposes of the present disclosure, a “polypeptide” refers to a protein which includes modifications, such as deletions, additions, and substitutions (generally conservative in nature), to the native sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

[0027] A “recombinant viral vector” refers to a recombinant polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of viral origin). In the case of recombinant AAV vectors, the recombinant nucleic acid is flanked by at least one and in some embodiments two, inverted terminal repeat sequences (ITRs).

[0028] A “recombinant AAV vector (rAAV vector)” refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of AAV origin) that are flanked by at least one, and in some embodiments two, AAV inverted terminal repeat sequences (ITRs). Such rAAV vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper virus (or that is expressing suitable helper functions) and that is expressing AAV rep and cap gene products (*i.e.* AAV Rep and Cap proteins). When a rAAV vector is incorporated into a larger polynucleotide (*e.g.*, in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the rAAV vector may be referred to as a “pro-vector” which can be “rescued” by replication and encapsidation in the presence of AAV packaging functions and suitable helper functions. A rAAV vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and encapsidated in a viral particle, particularly an AAV particle. A rAAV vector can be packaged into an AAV virus capsid to generate a “recombinant adeno-associated viral particle (rAAV particle)”.

[0029] “Heterologous” means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared or into which it is introduced or incorporated. For example, a polynucleotide introduced by genetic engineering techniques into a different cell type is a heterologous polynucleotide (and, when expressed, can encode a heterologous polypeptide).

Similarly, a cellular sequence (*e.g.*, a gene or portion thereof) that is incorporated into a viral vector is a heterologous nucleotide sequence with respect to the vector.

[0030] The term “transgene” refers to a polynucleotide that is introduced into a cell and is capable of being transcribed into RNA and optionally, translated and/or expressed under appropriate conditions. In aspects, it confers a desired property to a cell into which it was introduced, or otherwise leads to a desired therapeutic or diagnostic outcome.

[0031] “Chicken β -actin (CBA) promoter” refers to a polynucleotide sequence derived from a chicken β -actin gene (*e.g.*, *Gallus* beta actin, represented by GenBank Entrez Gene ID 396526). As used herein, “chicken β -actin promoter” may refer to a promoter containing a cytomegalovirus (CMV) early enhancer element, the promoter and first exon and intron of the chicken β -actin gene, and the splice acceptor of the rabbit beta-globin gene, such as the sequences described in Miyazaki, J. *et al.* (1989) *Gene* 79(2):269-77. As used herein, the term “CAG promoter” may be used interchangeably. As used herein, the term “CMV early enhancer/chicken beta actin (CAG) promoter” may be used interchangeably.

[0032] The terms “genome particles (gp),” “genome equivalents,” or “genome copies” as used in reference to a viral titer, refer to the number of virions containing the recombinant AAV DNA genome, regardless of infectivity or functionality. The number of genome particles in a particular vector preparation can be measured by procedures such as described in the Examples herein, or for example, in Clark *et al.* *Hum. Gene Ther.*, 10:1031-1039; Veldwijk *et al.* (2002) *Mol. Ther.*, 6:272-278.

[0033] The term “vector genome (vg)” as used herein may refer to one or more polynucleotides comprising a set of the polynucleotide sequences of a vector, *e.g.*, a viral vector. A vector genome may be encapsidated in a viral particle. Depending on the particular viral vector, a vector genome may comprise single-stranded DNA, double-stranded DNA, or single-stranded RNA, or double-stranded RNA. A vector genome may include endogenous sequences associated with a particular viral vector and/or any heterologous sequences inserted into a particular viral vector through recombinant techniques. For example, a recombinant AAV vector genome may include at least one ITR sequence flanking a promoter, a stuffer, a sequence of interest (*e.g.*, an RNAi), and a polyadenylation sequence. A complete vector genome may include a complete set of the polynucleotide sequences of a vector. In some embodiments, the

nucleic acid titer of a viral vector may be measured in terms of vg/mL. Methods suitable for measuring this titer are known in the art (*e.g.*, quantitative PCR).

[0034] The terms “infection unit (iu),” “infectious particle,” or “replication unit,” as used in reference to a viral titer, refer to the number of infectious and replication-competent recombinant AAV vector particles as measured by the infectious center assay, also known as replication center assay, as described, for example, in McLaughlin *et al.* (1988) *J. Virol.*, 62:1963-1973.

[0035] The term “transducing unit (tu)” as used in reference to a viral titer, refers to the number of infectious recombinant AAV vector particles that result in the production of a functional transgene product as measured in functional assays such as described in Examples herein, or for example, in Xiao *et al.* (1997) *Exp. Neurobiol.*, 144:113-124; or in Fisher *et al.* (1996) *J. Virol.*, 70:520-532 (LFU assay).

[0036] An “inverted terminal repeat” or “ITR” sequence is a term well understood in the art and refers to relatively short sequences found at the termini of viral genomes which are in opposite orientation.

[0037] An “AAV inverted terminal repeat (ITR)” sequence, a term well-understood in the art, is an approximately 145-nucleotide sequence that is present at both termini of the native single-stranded AAV genome. The outermost 125 nucleotides of the ITR can be present in either of two alternative orientations, leading to heterogeneity between different AAV genomes and between the two ends of a single AAV genome. The outermost 125 nucleotides also contains several shorter regions of self-complementarity (designated A, A', B, B', C, C' and D regions), allowing intrastrand base-pairing to occur within this portion of the ITR.

[0038] A “terminal resolution sequence” or “trs” is a sequence in the D region of the AAV ITR that is cleaved by AAV rep proteins during viral DNA replication. A mutant terminal resolution sequence is refractory to cleavage by AAV rep proteins.

[0039] “AAV helper functions” refer to functions that allow AAV to be replicated and packaged by a host cell. AAV helper functions can be provided in any of a number of forms, including, but not limited to, helper virus or helper virus genes which aid in AAV replication and packaging. Other AAV helper functions are known in the art such as genotoxic agents.

[0040] A “helper virus” for AAV refers to a virus that allows AAV (which is a defective parvovirus) to be replicated and packaged by a host cell. A helper virus provides “helper functions” which allow for the replication of AAV. A number of such helper viruses have been identified, including adenoviruses, herpesviruses and, poxviruses such as vaccinia and baculovirus. The adenoviruses encompass a number of different subgroups, although Adenovirus type 5 of subgroup C (Ad5) is most commonly used. Numerous adenoviruses of human, non-human mammalian and avian origin are known and are available from depositories such as the ATCC. Viruses of the herpes family, which are also available from depositories such as ATCC, include, for example, herpes simplex viruses (HSV), Epstein-Barr viruses (EBV), cytomegaloviruses (CMV) and pseudorabies viruses (PRV). Examples of adenovirus helper functions for the replication of AAV include E1A functions, E1B functions, E2A functions, VA functions and E4orf6 functions. Baculoviruses available from depositories include *Autographa californica* nuclear polyhedrosis virus.

[0041] A preparation of rAAV is said to be “substantially free” of helper virus if the ratio of infectious AAV particles to infectious helper virus particles is at least about 10^2 :1; at least about 10^4 :1, at least about 10^6 :1; or at least about 10^8 :1 or more. In some embodiments, preparations are also free of equivalent amounts of helper virus proteins (*i.e.*, proteins as would be present as a result of such a level of helper virus if the helper virus particle impurities noted above were present in disrupted form). Viral and/or cellular protein contamination can generally be observed as the presence of Coomassie staining bands on SDS gels (*e.g.*, the appearance of bands other than those corresponding to the AAV capsid proteins VP1, VP2 and VP3).

[0042] An “effective amount” is an amount sufficient to effect beneficial or desired results, including clinical results (*e.g.*, amelioration of symptoms, achievement of clinical endpoints, and the like). An effective amount can be administered in one or more administrations. In terms of a disease state, an effective amount is an amount sufficient to ameliorate, stabilize, or delay development of a disease.

[0043] An “individual” or “subject” is a mammal. Mammals include, but are not limited to, domesticated animals (*e.g.*, cows, sheep, cats, dogs, and horses), primates (*e.g.*, humans and non-human primates such as monkeys), rabbits, and rodents (*e.g.*, mice and rats). In certain embodiments, the individual or subject is a human.

[0044] As used herein, “treatment” is an approach for obtaining beneficial or desired clinical results. For purposes of this disclosure, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (e.g., not worsening) state of disease, preventing spread (e.g., metastasis) of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. “Treatment” can also mean prolonging survival as compared to expected survival if not receiving treatment.

[0045] As used herein, the term “prophylactic treatment” refers to treatment, wherein an individual is known or suspected to have or be at risk for having a disorder but has displayed no symptoms or minimal symptoms of the disorder. An individual undergoing prophylactic treatment may be treated prior to onset of symptoms.

[0046] Reference to “about” a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter *per se*. For example, description referring to “about X” includes description of “X.”

[0047] As used herein, the singular form of the articles “a,” “an,” and “the” includes plural references unless indicated otherwise.

[0048] It is understood that aspects and embodiments of the disclosure described herein include “comprising,” “consisting,” and/or “consisting essentially of” aspects and embodiments.

III. Delivery of viral particles to the CSF in combination with FUS-MB

[0049] In some aspects, provided herein are improved methods to deliver viral particles into the brain leveraging guided focused ultrasound (g-FUS). In some embodiments, g-FUS comprises ultrasound imaging-guided FUS (US-g-FUS). In some embodiments, g-FUS comprises magnetic resonance-guided focused ultrasound (MRg-FUS). In some embodiments, the guided FUS comprises imaging-guided FUS. In some embodiments, the imaging-guided FUS comprises US-g-FUS. In some embodiments, imaging-guided FUS comprises magnetic resonance imaging-guided FUS (MRI-g-FUS). Specific types of viral particles are discussed in Section IV, below. In some cases, an ultrasound transducer is noninvasively used to focus the ultrasound (FUS) within the brain to specific regions of interest. In some embodiments, FUS is

used in combination with gas-filled, microscopic bubbles that have been infused into the bloodstream in conjunction with the treatment molecule to be delivered to the brain. In some embodiments, a FUS signal excites the bubbles, temporarily permeabilizing the blood brain barrier in that location and allowing localized entry of viral particles to regions not normally accessible surgically but critical for treatment of neurologic diseases. In some embodiments, viral particles (e.g., AAV particles) are introduced via the CSF, allowing for much lower vector dose required and potential avoidance of peripheral immune responses, making clinical translation more realistic. Administration of viral particles to the CSF, e.g., via intracisternal magna (ICM) administration, mitigates problems for clinical translation of gene therapies via systemic administration, which generally require high doses of viral particles.

[0050] As depicted in **FIG. 1A**, intracisternal magna (ICM) administration of viral particles (e.g., AAV particles) **101** may lead to widespread transduction, especially of superficial brain areas, but the transduction of deeper brain structures (e.g. striatum) is negligible. As shown in **FIG. 1B**, focused ultrasound (FUS) **104** combined with intravenous (IV) microbubbles **105** and intravenous viral particles **101** delivers viral particles to any FUS-targeted brain region, including deep structures such as the striatum. However, in certain aspects, the transduction is only seen in the FUS spots **104**, as depicted in **FIG. 1B**. In accordance with the disclosure and as depicted in **FIG. 1C**, the combination of FUS and IV microbubbles with viral particles administered ICM results in viral particle delivery widespread to superficial brain areas as well as to deep brain structures targeted with FUS.

[0051] Without being bound by theory, two possible mechanisms of action describing how FUS-MB may increase brain delivery of ICM-administered viral particles have been identified. In one mechanism depicted in **FIG. 1D**, the viral particle **101** may travel in the cerebrospinal fluid (CSF) to the perivascular space and from there the interaction between FUS and intravenous MBs **105** (e.g., IV administered MBs) may create a pumping effect, which may increase the distribution of the viral particle from the CSF into the brain parenchyma. In a second mechanism depicted in **FIG. 1E**, viral particle (e.g. AAV) **101** injected via ICM may eventually be cleared from the CSF into the blood, where the viral particle can then enter the brain from the blood at FUS-targeted sites in a similar manner as when the viral particle is injected (e.g., administered) intravenously. In some embodiments, the first mechanism is active, and the second mechanism may not be. In some embodiments, the second mechanism is active,

and the first mechanism may not be. In some embodiments, the mechanism of action is related to distribution route #1, distribution route #2, or a combination of both.

[0052] In certain embodiments, methods may comprise injection directly into the cerebrospinal fluid through the cisterna magna as the route of administration for AAV for treatments of whole-brain diseases. In some embodiments, methods comprising FUS-MB may increase the delivery of intracisternal magna (ICM)-injected viral particles (e.g., AAV) to deep brain regions (e.g., thalamus) compared to methods without FUS-MB. In some embodiments, the combination of ICM administration and FUS-MB may mediate (e.g., enhance) viral particle delivery to both superficial brain areas and FUS-targeted deep brain structures (**FIG. 1C**).

[0053] In some embodiments, methods comprising FUS-MB may increase delivery of viral particles (e.g., AAV) administered via ICM to the striatum. The striatum is a deep brain structure where the viral particles (e.g., AAV) may not be efficiently transduced following ICM injection alone. The striatum controls both motor movements and emotional control/motivation and has been implicated in many neurological diseases, such as Huntington's disease. Several cell types of interest are located in the striatum, including without limitation spiny projection neurons (also known as medium spiny neurons), GABAergic interneurons, and cholinergic interneurons. Medium spiny neurons make up most of the striatal neurons. These neurons are GABAergic and express dopamine receptors. Each hemisphere of the brain contains a striatum.

[0054] In some embodiments, methods comprising FUS-MB may increase delivery of viral particles (e.g., AAV) administered via ICM to the cerebral cortex. The cerebral cortex is a deep brain structure where the viral particles (e.g., AAV) may not be efficiently transduced following ICM injection alone. In some embodiments, methods comprising FUS-MB may increase delivery of viral particles (e.g., AAV) administered via ICM to the striatum and the cerebral cortex of the brain of a human.

[0055] In some embodiments, a heterologous nucleic acid carried by the viral particle is expressed in one or more regions of interest in the CNS. For example, in some embodiments, the heterologous nucleic acid is expressed in at least the cerebral cortex and striatum.

[0056] In some embodiments, the heterologous nucleic acid is expressed in the frontal cortex, occipital cortex, and/or layer IV of the mammal. The cerebral cortex is known as the outer layer of the mammalian brain important for language, consciousness, memory, attention,

and awareness. The cerebral cortex is subdivided into a number of different components and regions due to its extensive anatomy and complex functions. In some embodiments, the cerebral cortex is divided into left and right hemispheres. In addition, it contains four gross lobes: frontal, parietal, temporal, and occipital. Frontal cortex may refer to the frontal lobe of the cortex and is known to provide a wide range of neurological functions related to non-task-based memory, social interactions, decision making, and other complex cognitive functions. Occipital cortex may refer to the occipital lobe of the cortex and is known to be involved in visual processing. Parietal cortex may refer to the parietal lobe of the cortex and is known to be involved in language processing, proprioception, and sensory inputs related to touch. Temporal cortex may refer to the temporal lobe of the cortex and is known to be involved in language, memory, and emotional association.

[0057] In addition, three general types of areas of the cortex are described: sensory, motor, and association. These may be divided into 5 functional subdivisions: primary motor cortex (involved in muscle control), premotor cortex (higher order motor areas that command primary motor areas), association areas (*e.g.*, parietal-temporal-occipital or prefrontal; these areas are involved in planning, memory, attention, and other higher cognitive tasks and assume the majority of the human cortex), higher order areas (sensory processing), and primary sensory areas (*e.g.*, auditory, visual, and somatosensory). In some embodiments, the heterologous nucleic acid is expressed in the prefrontal association cortical areas, the premotor cortex, the primary somatosensory cortical areas, sensory motor cortex, parietal cortex, occipital cortex, and/or primary motor cortex.

[0058] In addition, the cerebral cortex is divided into different cortical layers (moving from superficial to deep), each containing a characteristic pattern of neuronal connectivities and cell types. These layers are divided into supragranular layers (layers I-III), internal granular (IV), and infragranular (V and VI). Supragranular layers typically project to other cortical layers, whereas infragranular layers receive input from supragranular layers and send output to structures outside the cortex (*e.g.*, motor, sensory, and thalamic regions). Layer V contains pyramidal neurons with axons that connect to subcortical structures like the basal ganglia. Layer V neurons in the primary motor cortex also form the corticospinal tract that is critical for voluntary motor control. Layer IV receives inputs from the thalamus and connects to the rest of the column, thereby providing critical functions related to integration of the thalamus and cortex.

Characteristic cells of layer IV include stellate cells (*e.g.*, spiny stellate cells) and pyramidal neurons.

[0059] In some embodiments, the heterologous nucleic acid is further expressed in the thalamus, substantia nigra and/or hippocampus. In some embodiments, the heterologous nucleic acid is expressed in the thalamus, midbrain, cerebellum, hippocampus, and brainstem. The thalamus is between the cortex and midbrain, sends signals (*e.g.*, sensory and motor) to the cortex from subcortical areas, and plays a role in alertness and sleep. The thalamus also connects to the hippocampus, part of the limbic system and a critical mediator of long-term memory consolidation. Part of the basal ganglia, the substantia nigra contains many dopaminergic neurons and is important for movement and reward. CNS disorders like Parkinson's disease are associated with loss of dopaminergic neurons in the substantia nigra. It further provides dopamine to the striatum that is critical for proper striatal function.

[0060] In some embodiments, the heterologous nucleic acid is further expressed in the thalamus, subthalamic nucleus, globus pallidus, substantia nigra, putamen and/or hippocampus.

[0061] In some aspects, provided herein are various methods for delivering a viral particle (*e.g.*, AAV particle) **101** to the brain, comprising: a) administering: i) a viral particle; and ii) a plurality of microbubbles **105**; and b) applying focused ultrasound (FUS) to at least one region of interest (*e.g.*, deep brain structure or striatum), thereby causing entry of the viral particle to the region of interest of the brain. In some embodiments, the administering the viral particle **101** comprises a dose level of less than 1×10^{14} genome copies per kilogram (GC/kg) for a patient (*e.g.*, human patient).

[0062] In some embodiments, the administering the viral particle **101** comprises a dose level of at most 5×10^{13} genome copies per kilogram (GC/kg) for a patient (*e.g.*, human patient). In some embodiments, the administering the viral particle comprises a dose level of at most 1×10^{12} , 2×10^{12} , 3×10^{12} , 5×10^{12} , 8×10^{12} , 1×10^{13} , 2×10^{13} , 3×10^{13} , 4×10^{13} , 5×10^{13} , or 8×10^{13} genome copies per kilogram (GC/kg). In some embodiments, the administering the viral particle comprises a dose level of from about 1×10^{11} to about 8×10^{13} genome copies per kilogram (GC/kg). In some embodiments, the administering the viral particle comprises a dose level of from about 8×10^{11} to about 5×10^{13} genome copies per kilogram (GC/kg). In some embodiments, the administering the viral particle comprises a dose level of from about 8×10^{11}

to about 2×10^{13} genome copies per kilogram (GC/kg). In some embodiments, the administering the viral particle comprises a dose level of from about 1×10^{12} to about 1×10^{13} genome copies per kilogram (GC/kg). In some embodiments, the administering the viral particle comprises a dose level of from about 1×10^{12} to about 8×10^{12} genome copies per kilogram (GC/kg). In some embodiments, the administering the viral particle comprises a dose level of from about 2×10^{12} to about 6×10^{12} genome copies per kilogram (GC/kg).

[0063] In some embodiments, provided herein are methods comprising applying focused ultrasound in combination with administration of microbubbles (FUS-MB). In some embodiments, applying the FUS may occur after the administering of the viral particle and/or the plurality of microbubbles.

[0064] In some embodiments, the applying the FUS may occur prior to the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs simultaneously with the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs less than one minute after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs at about 1 minute after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs at about 1, 2, 3, 4, 5, 10, 60, 120, 360 minutes, or more after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs at about 1, 2, 3, 4, 5, 10, 24 hours, or more after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs at about 1 or 2 hours after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs between about 1 minute to about 180 minutes after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs between about 10 minutes to about 180 minutes after the administering of the viral particles and/or the plurality of microbubbles. In some embodiments, the applying of the FUS occurs between about 30 minutes to about 180 minutes after the administering of the viral particles and/or the plurality of microbubbles.

[0065] In some embodiments, the placement of the FUS spot is determined by imaging. In some embodiments, the placement of the FUS spot is determined by magnetic resonance imaging (MRI). In some embodiments, the administering further comprises administering a tracer. In some embodiments, the tracer may comprise an image contrast agent. In some embodiments, the image contrast agent may comprise an MRI contrast agent. In some embodiments, the MRI contrast agent may comprise Gadolinium. In some embodiments, the tracer may comprise Gadolinium.

[0066] In some embodiments, the applying the FUS comprises applying the FUS at a FUS frequency. In some embodiments, the FUS frequency may comprise about 0.58 MHz. In some embodiments, the FUS frequency may comprise 0.58 MHz. In some embodiments, the FUS frequency may comprise about 0.58 MHz or more. In some embodiments, the FUS frequency may comprise about 0.58 MHz or less. In some embodiments, the applying the FUS may comprise applying FUS via a 0.58 MHz spherically focused transducer. In some embodiments, the spherically focused transducer may comprise a 75 mm outer diameter, 26 mm inner diameter, and/or 60 mm radius of curvature.

[0067] In some embodiments, the plurality of microbubbles **105** is administered at about 0.2 mL/kg. In some embodiments, the plurality of microbubbles is administered at about 0.2 mL/kg or more. In some embodiments, the plurality of microbubbles is administered at about 0.2 mL/kg or less. In some embodiments, the plurality of microbubbles is administered at about 0.2 mL/kg. to about 0.4 mL/kg. In some embodiments, the plurality of microbubbles is administered at about 0.1 mL/kg. to about 0.2 mL/kg.

[0068] In some embodiments, the plurality of microbubbles **105** is administered at a fixed pressure of about 0.32 MPa. In some embodiments, the plurality of microbubbles is administered at a fixed pressure of about 0.32 MPa or more. In some embodiments, the plurality of microbubbles is administered at a fixed pressure of about 0.32 MPa or less.

[0069] In some embodiments, the plurality of microbubbles comprises a gas. In some embodiments, the gas may comprise octafluoropropane gas.

[0070] In some embodiments, each of the microbubbles of the plurality of microbubbles **105** may comprise a shell. In some embodiments, the shell may comprise a synthetic phospholipid shell.

[0071] In some embodiments, the plurality of microbubbles may comprise octafluoropropane gas encapsulated by a synthetic phospholipid shell. In some embodiments, the plurality of microbubbles may comprise DEFINITY® microbubbles.

[0072] In some embodiments, provided herein are methods for treating neurological disorders requiring delivery of viral particles (e.g., AAV) to the brain of patients. In some embodiments, methods may comprise intracisternal magna administration (e.g., ICM administration) of adeno-associated virus (e.g., AAV) for treating neurological disorders comprising beta-galactosidase-1 deficiency, Batten disease, Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, aromatic L-amino acid decarboxylase (AADC) deficiency, MSA, or spinal muscular atrophy.

[0073] In some embodiments, provided herein are methods for gene delivery to the entire brain for the treatment of neurological disorders causing widespread pathology such as monogenic disorders. In some aspects, provided herein are methods for gene delivery via adeno-associated viral particles configured to provide lasting clinical benefits following a one-time treatment. In some embodiments, a method may comprise gene delivery to the entire brain for the treatment of neurological disorders causing widespread pathology such as monogenic disorders or advanced disease stages of Alzheimer, Parkinson disease and amyotrophic lateral sclerosis.

[0074] In some embodiments, FUS-MB may increase the delivery of the clinically relevant viral particles to deep brain structures. In some embodiments, FUS-MB may increase the delivery of the clinically relevant viral particles to deep brain structures following injection in the cisterna magna. In some embodiments, FUS-MB increases the delivery of the clinically relevant viral particle (e.g., gene vector, or adeno-associated virus) by 2-fold, 3-fold, 4-fold, 5-fold, or more to deep brain structures following injection in the cisterna magna.

[0075] In some embodiments, the permeability of the BBB can be increased transiently by the application of FUS combined with intravenous microbubbles (FUS-MB). In some embodiments, FUS induces an oscillation of the microbubbles, thereby decreasing tight-junction proteins and increasing transcytosis across the endothelial cells. Such embodiments allow non-invasive delivery AAV via intravenous (IV) or ICM administration to FUS-targeted brain areas at dosages 50-100 times lower than needed for BBB crossing by AAV alone (e.g., without FUS

or without FUS-MB). In some embodiments, FUS-MB, may decrease the intravenous AAV dose needed for brain delivery.

[0076] Current methods of using FUS for AAV delivery to the brain involve intravenous (IV) infusion of high doses of AAV. However, as shown herein, the application of FUS and microbubbles increases brain delivery of AAVs administered in the CSF and lowers the dose (and by extension Cost of Goods and/or toxicity) of AAV required for transduction of deep brain structures with FUS while retaining the baseline footprint of transduction that CSF administration of AAV provides. In some embodiments, methods may comprise delivery comprising AAV in CSF while keeping microbubbles in blood to avoid the high material requirements needed for IV administration of AAV.

IV. Viral particles and methods of producing viral particles

[0077] The disclosure provides, *inter alia*, administering viral particles (e.g., recombinant viral particles) to the CSF in the brain of a patient. In some embodiments, the viral particles comprise an expression cassette comprising a transgene capable of encoding a disorder-related polypeptide. In other embodiments, the viral particles are capable of expressing an RNAi molecule capable of reducing or eliminating the expression of a particular polypeptide implicated in a disorder.

[0078] Nucleic acids can be delivered in any desired vector. These include adenovirus vectors, adeno-associated virus (AAV) vectors, retrovirus vectors, lentivirus vectors, and plasmid vectors. Exemplary types of viruses include HSV (herpes simplex virus), AAV (adeno associated virus), HIV (human immunodeficiency virus), BIV (bovine immunodeficiency virus), and MLV (murine leukemia virus).

AAV particles

[0079] In some embodiments, the viral particle to be administered to the CSF (e.g., via through the ICM) is a recombinant AAV particle comprising a nucleic acid comprising a transgene flanked by one or two ITRs. The nucleic acid is encapsidated in the AAV particle. The AAV particle also comprises capsid proteins. In some embodiments, the nucleic acid comprises the coding sequence(s) of interest, operatively linked components in the direction of

transcription, control sequences including transcription initiation and termination sequences, thereby forming an expression construct. The expression construct is flanked on the 5' and 3' end by at least one functional AAV ITR sequences. By “functional AAV ITR sequences” it is meant that the ITR sequences function as intended for the rescue, replication and packaging of the AAV virion. See Davidson *et al.*, *PNAS*, 2000, 97(7):3428-32; Passini *et al.*, *J. Virol.*, 2003, 77(12):7034-40; and Pechan *et al.*, *Gene Ther.*, 2009, 16:10-16, all of which are incorporated herein in their entirety by reference. For practicing some aspects of the disclosure, the recombinant vectors comprise at least all of the sequences of AAV essential for encapsidation and the physical structures for infection by the rAAV. AAV ITRs for use in the vectors of the disclosure need not have a wild-type nucleotide sequence (*e.g.*, as described in Kotin, *Hum. Gene Ther.*, 1994, 5:793-801), and may be altered by the insertion, deletion or substitution of nucleotides or the AAV ITRs may be derived from any of several AAV serotypes. More than 40 serotypes of AAV are currently known, and new serotypes and variants of existing serotypes continue to be identified. See Gao *et al.*, *PNAS*, 2002, 99(18): 11854-6; Gao *et al.*, *PNAS*, 2003, 100(10):6081-6; and Bossis *et al.*, *J. Virol.*, 2003, 77(12):6799-810. Use of any AAV serotype is considered within the scope of the present disclosure. In some embodiments, a rAAV vector is a vector derived from an AAV serotype, including without limitation, AAV ITRs are AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV2R471A, AAV DJ, a goat AAV, bovine AAV, or mouse AAV capsid serotype or the like. In some embodiments, the nucleic acid in the AAV comprises an ITR of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV2R471A, AAV DJ, a goat AAV, bovine AAV, or mouse AAV capsid serotype or the like. In some embodiments, the nucleic acid in the AAV further encodes a miRNA as described herein. In some embodiments the rAAV particle comprise an AAV1, an AAV2HBKO capsid (*e.g.*, as described in WO2015168666), an AAV9 capsid, a PHP.B capsid, a PHP.eB capsid, or an Olig001 capsid.

[0080] Different AAV serotypes are used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). A rAAV particle can comprise viral proteins and viral nucleic acids of the same serotype or a mixed serotype. For example, in some embodiments a rAAV particle can comprise AAV1 capsid proteins and at least one AAV2 ITR or it can comprise AAV2 capsid proteins and at least one

AAV1 ITR. Any combination of AAV serotypes for production of a rAAV particle is provided herein as if each combination had been expressly stated herein. In some embodiments, the invention provides rAAV particles comprising an AAV1 capsid and a rAAV vector of the present disclosure (*e.g.*, an expression construct comprising nucleic acid encoding a miRNA of the present disclosure), flanked by at least one AAV2 ITR. In some embodiments, the invention provides rAAV particles comprising an AAV2 capsid. In some embodiments the rAAV particle comprise an AAV1, an AAV2HBKO capsid (*e.g.*, as described in WO2015168666), an AAV9 capsid, a PHP.B capsid, a PHP.eB capsid, or an Olig001.

[0081] The capsid of an rAAV viral particle is known to include three capsid proteins: VP1, VP2, and VP3. These proteins contain significant amounts of overlapping amino acid sequence and unique N-terminal sequences. For instance, an AAV9 capsid includes 60 subunits arranged by icosahedral symmetry. AAV9 includes VP1 (SEQ ID NO: 6), VP2 (SEQ ID NO: 10), and VP3 (SEQ ID NO: 11), capsid proteins in a ratio of about 5:5:50. The VP proteins of AAV9 are products of the structural protein-encoding open reading frame of the genome, designated *cap*, VP1 (~82 kDa) and VP2 (~73 kDa), which are the minor capsid proteins, and VP3 (~61 kDa), the major capsid protein. Due to the utilization of both alternative splicing and leaky scanning, when expressed, the individual VPs share a C terminus that encompasses the entire VP3, while VP1 and VP2 are N-terminal VP3 extensions. VP1 and VP2 share a region of ~73 amino acids which is extended by an additional ~137 amino acids in VP1, designated the VP1 unique region (VP1u). See Penzes et al., (2021), Journal of Virology 95(19)e0084321.

[0082] In some embodiments, the rAAV particle can comprise a modified AAV9 capsid. In some embodiments of the modified AAV9 capsid proteins, a targeting peptide (*e.g.*, SEQ ID NO: 7) is incorporated into VP1. In some embodiments of a modified AAV9 capsid protein, a targeting peptide (*e.g.*, SEQ ID NO: 7) is incorporated into VP2. In some embodiments of the modified AAV9 capsid proteins disclosed herein, the targeting peptide (*e.g.*, SEQ ID NO: 7) is incorporated into VP3. In some embodiments of the modified AAV9 capsid proteins, the targeting peptide (*e.g.*, SEQ ID NO: 7) is incorporated into VP1, VP2 and VP3.

[0083] In particular embodiments, the targeting peptide of the modified AAV9 capsids are inserted after residue 588 of the AAV9 structural protein. In some embodiments, the targeting peptide has SEQ ID NO: 7. In some embodiments, the targeting peptide is flanked by linker

sequences on the N-terminal and the C-terminal end of the targeting peptide. In some embodiments, the linker sequence on the N-terminal side has the sequence AAA. In some embodiments, the linker sequence on the C-terminal side is AS. In some embodiments, the full sequence inserted after residue 588 of the AAV9 capsid structural protein has SEQ ID NO: 8. In some embodiments, the full modified AAV9 capsid structural protein has SEQ ID NO: 9. The modified AAV9 capsid structural protein having SEQ ID NO: 9 is also referred to herein as capsid SAN006. In some embodiments, the full modified AAV9 capsid structural protein that is at least 90% (e.g., at least 92%, at least 95%, at least 98%, at least 98.5%, at least 99%, at least 99.2%, at least 99.5%, or at least 99.8%) identical to SEQ ID NO: 9, wherein the modified AAV9 structural capsid comprises the targeting peptide of SEQ ID NO: 7.

[0084] In some embodiments, the transgene encoding a disorder-related polypeptide is codon-optimized. In some embodiments, the transgene encoding a disorder-related polypeptide is codon optimized for expression in a particular cell, such as a eukaryotic cell. In some embodiments, eukaryotic cells are those of or derived from a particular organism, such as a mammal, including but not limited to human, mouse, rat, rabbit, dog, or non-human primate. In general, codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon usage tables are readily available, for example, at the “Codon Usage Database”, and these tables can be adapted in a number of ways (see, e.g., Nakamura, Y. *et al.* (2000) *Nucleic Acids Res.* 28:292). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, Pa.), DNA2.0, GeneArt (GA) or Genscript (GS) and a GS algorithm combined with reduction in CpG content. In some embodiments, a transgene encoding the disorder-related polypeptide is codon optimized using the GA algorithm.

[0085] In some embodiments, the expression cassette further comprises an intron. A variety of introns for use in the disclosure are known to those of skill in the art, and include the MVM intron, the F IX truncated intron 1, the β -globin SD/immunoglobulin heavy chain SA, the adenovirus SD/immunoglobulin SA, the SV40 late SD/SA (19S/16S), and the hybrid adenovirus SD/IgG SA. (Wu *et al.* 2008, Kurachi *et al.*, 1995, Choi *et al.* 2014, Wong *et al.*, 1985, Yew *et*

al. 1997, Huang and Gorman (1990). In some embodiments, the intron is a chicken β -actin (CBA)/rabbit β -globin hybrid intron. In some embodiments, intron is a chicken β -actin (CBA)/rabbit β -globin hybrid promoter and intron where all the ATG sites are removed to minimize false translation start sites. In some embodiments the intron is an MVM intron, a F IX truncated intron 1, a β -globin SD/immunoglobulin heavy chain SA, an adenovirus SD/immunoglobulin SA, a SV40 late SD/SA (19S/16S), or a hybrid adenovirus SD/IgG SA. In some embodiments, the intron is a chicken β -actin (CBA)/rabbit β -globin hybrid intron.

[0086] In some embodiments, the expression cassette further comprises a polyadenylation signal. In some embodiments, the polyadenylation signal is a bovine growth hormone polyadenylation signal, an SV40 polyadenylation signal, or a HSV TK pA. In some embodiments, the polyadenylation signal is a synthetic polyadenylation signal as described in Levitt, N *et al.* (1989), *Genes Develop.* 3:1019-1025.

[0087] In some embodiments, the expression cassette comprises a stuffer nucleic acid. In some embodiments, the stuffer nucleic acid may comprise a sequence that encodes a reporter polypeptide. As will be appreciated by those of skill in the art, the stuffer nucleic acid may be located in a variety of regions within the nucleic acid and may be comprised of a continuous sequence (*e.g.*, a single stuffer nucleic acid in a single location) or multiple sequences (*e.g.*, more than one stuffer nucleic acid in more than one location (*e.g.*, 2 locations, 3 locations, *etc.*) within the nucleic acid. In some embodiments, the stuffer nucleic acid is located downstream of the transgene encoding the disorder-related polypeptide. In some embodiments, the stuffer nucleic acid is located upstream of the transgene encoding the disorder-related polypeptide (*e.g.*, between the promoter and the transgene). In some embodiments, a variety of nucleic acids are used as a stuffer nucleic acid. In some embodiments, the stuffer nucleic acid comprises all or a portion of a human alpha-1-antitrypsin (AAT) stuffer sequence or a C16 P1 chromosome 16 P1 clone (human C16) stuffer sequence. In some embodiments, the stuffer sequence comprises all or a portion of a gene. For example, the stuffer sequence may comprise a portion of the human AAT sequence. One skilled in the art would recognize that different portions of a gene (*e.g.*, the human AAT sequence) can be used as a stuffer fragment. For example, the stuffer fragment is from the 5' end of the gene, the 3' end of the gene, the middle of a gene, a non-coding portion of the gene (*e.g.*, an intron), a coding region of the gene (*e.g.*, an exon), or a mixture of non-coding and coding portions of a gene. One skilled in the art would also recognize that all or a portion of

stuffer sequence is used as a stuffer sequence. In some embodiments, the stuffer sequence is modified to remove internal ATG codons.

[0088] In some embodiments, the expression cassette is incorporated into a vector. In some embodiments, the expression cassette is incorporated into a viral vector. In some embodiments, the viral vector is a rAAV vector as described herein.

[0089] The expression cassette for expressing a disorder-related polypeptide or RNAi molecule is contained in a vector. In some embodiments, the present disclosure contemplates the use of a recombinant viral genome for introduction of nucleic acid sequences encoding the disorder-related polypeptide or RNAi molecule for packaging into a viral particle, *e.g.*, a viral particle described below. The recombinant viral genome may include any element to establish the expression of the disorder-related polypeptide, for example, a promoter, an ITR, a ribosome binding element, terminator, enhancer, selection marker, intron, polyA signal, and/or origin of replication.

[0090] In some embodiments, the disclosure provides viral particles comprising a single-stranded genome. In some aspects, the disclosure provides viral particles comprising a recombinant self-complementing genome. In some embodiments, the vector is a self-complementary vector. AAV viral particles with self-complementing genomes and methods of use of self-complementing AAV genomes are described in US Patent Nos. 6,596,535; 7,125,717; 7,765,583; 7,785,888; 7,790,154; 7,846,729; 8,093,054; and 8,361,457; and Wang Z., *et al.*, (2003) *Gene Ther* 10:2105-2111, each of which are incorporated herein by reference in its entirety. A rAAV comprising a self-complementing genome will quickly form a double stranded DNA molecule by virtue of its partially complementing sequences (*e.g.*, complementing coding and non-coding strands of a transgene). In some embodiments, the disclosure provides an AAV viral particle comprising an AAV genome, wherein the rAAV genome comprises a first heterologous polynucleotide sequence (*e.g.*, the coding strand of the disorder-related polypeptide of the disclosure) and a second heterologous polynucleotide sequence (*e.g.*, the noncoding or antisense strand of the disorder-related polypeptide or RNAi molecule) wherein the first heterologous polynucleotide sequence can form intrastrand base pairs with the second polynucleotide sequence along most or all of its length.

[0091] In some embodiments, the first heterologous polynucleotide sequence and a second heterologous polynucleotide sequence are linked by a sequence that facilitates intrastrand basepairing; *e.g.*, a hairpin DNA structure. Hairpin structures are known in the art, for example in siRNA molecules. In some embodiments, the first heterologous polynucleotide sequence and a second heterologous polynucleotide sequence are linked by a mutated ITR (*e.g.*, the right ITR). The mutated ITR may comprise a deletion of the D region comprising the terminal resolution sequence. In some embodiments, on replicating an AAV viral genome, the rep proteins may not cleave the viral genome at the mutated ITR and as such, a recombinant viral genome comprising the following in 5' to 3' order is packaged in a viral capsid: an AAV ITR, the first heterologous polynucleotide sequence including regulatory sequences, the mutated AAV ITR, the second heterologous polynucleotide in reverse orientation to the first heterologous polynucleotide and a third AAV ITR.

[0092] In some embodiments, the first heterologous nucleic acid sequence and a second heterologous nucleic acid sequence are linked by a mutated ITR (*e.g.*, the right ITR). In some embodiments, the ITR comprises the polynucleotide sequence 5'-CACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCACGCCCGGGCTTTGCCCCGGCG - 3' (SEQ ID NO: 12). The mutated ITR may comprise a deletion of the D region comprising the terminal resolution sequence. In some embodiments, as a result, on replicating an AAV viral genome, the rep proteins do not cleave the viral genome at the mutated ITR and as such, a recombinant viral genome comprising the following in 5' to 3' order is packaged in a viral capsid: an AAV ITR, the first heterologous polynucleotide sequence including regulatory sequences, the mutated AAV ITR, the second heterologous polynucleotide in reverse orientation to the first heterologous polynucleotide and a third AAV ITR.

[0093] In some embodiments of the above aspects and embodiments, the heterologous nucleic acid encodes a therapeutic polypeptide or therapeutic nucleic acid. In some embodiments, the heterologous nucleic acid encodes a therapeutic polypeptide. In some embodiments, the therapeutic polypeptide is an enzyme, a neurotrophic factor, a polypeptide that is deficient or mutated in an individual with a CNS-related disorder, an antioxidant, an anti-apoptotic factor, an anti-angiogenic factor, and an anti-inflammatory factor, alpha-synuclein, acid beta-glucosidase (GBA), beta-galactosidase-1 (GLB1), iduronate 2-sulfatase (IDS),

galactosylceramidase (GALC), a mannosidase, alpha-D-mannosidase (MAN2B1), beta-mannosidase (MANBA), pseudoarylsulfatase A (ARSA), N-acetylglucosamine-1-phosphotransferase (GNPTAB), acid sphingomyelinase (ASM), Niemann-Pick C protein (NPC1), acid alpha-1,4-glucosidase (GAA), hexosaminidase beta subunit, HEXB, N-sulfoglucosamine sulfohydrolase (MPS3A), N-alpha-acetylglucosaminidase (NAGLU), heparin acetyl-CoA, alpha-glucosaminidase N-acetyltransferase (MPS3C), N-acetylglucosamine-6-sulfatase (GNS), alpha-N-acetylgalactosaminidase (NAGA), beta-glucuronidase (GUSB), hexosaminidase alpha subunit (HEXA), huntingtin (HTT), lysosomal acid lipase (LIPA), Aspartylglucosaminidase, Alpha-galactosidase A, Palmitoyl protein thioesterase, Tripeptidyl peptidase, Lysosomal transmembrane protein, Cysteine transporter, Acid ceramidase, Acid alpha-L-fucosidase, cathepsin A, alpha-L-iduronidase, Arylsulfatase B, Arylsulfatase A, N-acetylgalactosamine-6-sulfate, Acid beta-galactosidase, or alpha-neuramidase. In other embodiments, the heterologous nucleic acid encodes a therapeutic nucleic acid. In some embodiments, the therapeutic nucleic acid is an siRNA, an shRNA, an RNAi, an miRNA, an antisense RNA, a ribozyme or a DNAzyme. In some embodiments, the therapeutic polypeptide or the therapeutic nucleic acid is used to treat a disorder of the CNS.

[0094] In some embodiments of the above aspects and embodiments, the disorder of the CNS is a lysosomal storage disease (LSD), Huntington's disease, epilepsy, Parkinson's disease, Alzheimer's disease, stroke, corticobasal degeneration (CBD), corticogasal ganglionic degeneration (CBGD), frontotemporal dementia (FTD), multiple system atrophy (MSA), progressive supranuclear palsy (PSP) or cancer of the brain. In some embodiments, the disorder is a lysosomal storage disease selected from the group consisting of Aspartylglucosaminuria, Fabry, Infantile Batten Disease (CNL1), Classic Late Infantile Batten Disease (CNL2), Juvenile Batten Disease (CNL3), Batten form CNL4, Batten form CNL5, Batten form CNL6, Batten form CNL7, Batten form CNL8, Cystinosis, Farber, Fucosidosis, Galactosidosis, Gaucher disease type 1, Gaucher disease type 2, Gaucher disease type 3, GM1 gangliosidosis, Hunter disease, Krabbe disease, α mannosidosis disease, β mannosidosis disease, Maroteaux-Lamy, metachromatic leukodystrophy disease, Morquio A, Morquio B, mucopolysaccharidosis II/III disease, Niemann-Pick A disease, Niemann-Pick B disease, Niemann-Pick C disease, Pompe disease, Sandhoff disease, Sanfillipo A disease, Sanfillipo B disease, Sanfillipo C disease, Sanfillipo D

disease, Schindler disease, Schindler-Kanzaki, sialidosis, Sly disease, Tay-Sachs disease, and Wolman disease.

[0095] In some embodiments of the above aspects and embodiments, the heterologous nucleic acid is operably linked to a promoter. In some embodiments, the promoter expresses the heterologous nucleic acid in a cell of the CNS. In some embodiments, the promoter expresses the heterologous nucleic acid in a brain cell. In some embodiments, the promoter expresses the heterologous nucleic acid in a neuron and/or a glial cell. In some embodiments, the neuron is a medium spiny neuron of the caudate nucleus, a medium spiny neuron of the putamen, a neuron of the cortex layer IV and/or a neuron of the cortex layer V. In some embodiments, the glial cell is an astrocyte. In some embodiments, the promoter is a CBA promoter, a minimum CBA promoter, a CMV promoter or a GUSB promoter. In other embodiments, the promoter is inducible. In further embodiments, the rAAV vector comprises one or more of an enhancer, a splice donor/splice acceptor pair, a matrix attachment site, or a polyadenylation signal. In some embodiments, the rAAV vector is a self-complementary rAAV vector. In some embodiments, the vector comprises a first nucleic acid sequence encoding the heterologous nucleic acid and a second nucleic acid sequence encoding a complement of the heterologous nucleic acid, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

Production of viral particles

[0096] Numerous methods are known in the art for production of rAAV vectors, including transfection, stable cell line production, and infectious hybrid virus production systems which include adenovirus-AAV hybrids, herpesvirus-AAV hybrids (Conway, JE *et al.*, (1997) J. Virology 71(11):8780-8789) and baculovirus-AAV hybrids (Urabe, M. *et al.*, (2002) *Human Gene Therapy* 13(16):1935-1943; Kotin, R. (2011) *Hum Mol Genet.* 20(R1): R2-R6). rAAV production cultures for the production of rAAV virus particles all require; 1) suitable host cells, 2) suitable helper virus function, 3) AAV rep and cap genes and gene products; 4) a nucleic acid (such as a therapeutic nucleic acid) flanked by at least one AAV ITR sequences (*e.g.*, an AAV

genome encoding a peptide of interest); and 5) suitable media and media components to support rAAV production. In some embodiments, the suitable host cell is a primate host cell. In some embodiments, the suitable host cell is a human-derived cell lines such as HeLa, A549, 293, or Perc.6 cells. In some embodiments, the suitable helper virus function is provided by wild-type or mutant adenovirus (such as temperature sensitive adenovirus), herpes virus (HSV), baculovirus, or a plasmid construct providing helper functions. In some embodiments, the AAV rep and cap gene products are from any AAV serotype. In general, but not obligatory, the AAV rep gene product is of the same serotype as the ITRs of the rAAV vector genome as long as the rep gene products may function to replicated and package the rAAV genome. In some embodiments, suitable media known in the art are used for the production of rAAV vectors. These media include, without limitation, media produced by Hyclone Laboratories and JRH including Modified Eagle Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), custom formulations such as those described in U.S. Patent No. 6,566,118, and Sf-900 II SFM media as described in U.S. Patent No. 6,723,551, each of which is incorporated herein by reference in its entirety, particularly with respect to custom media formulations for use in production of recombinant AAV vectors. In some embodiments, the AAV helper functions are provided by adenovirus or HSV. In some embodiments, the AAV helper functions are provided by baculovirus and the host cell is an insect cell (*e.g.*, *Spodoptera frugiperda* (Sf9) cells).

[0097] In certain aspects, one method for producing rAAV particles is the triple transfection method. In some embodiments, a plasmid containing a rep gene and a capsid gene, along with a helper adenoviral plasmid, are transfected (*e.g.*, using the calcium phosphate method) into a cell line (*e.g.*, HEK-293 cells), and virus are collected and optionally purified. As such, in some embodiments, the rAAV particle was produced by triple transfection of a nucleic acid encoding the rAAV vector, a nucleic acid encoding AAV rep and cap, and a nucleic acid encoding AAV helper virus functions into a host cell, wherein the transfection of the nucleic acids to the host cells generates a host cell capable of producing rAAV particles.

[0098] In some embodiments, rAAV particles are produced by a producer cell line method (*see* Martin *et al.*, (2013) *Human Gene Therapy Methods* 24:253-269; U.S. PG Pub. No. US2004/0224411). In some embodiments, a cell line (*e.g.*, a HeLa, 293, A549, or Perc.6 cell line) is stably transfected with a plasmid containing a rep gene, a capsid gene, and a vector genome comprising a promoter-heterologous nucleic acid sequence (*e.g.*, a disorder-related

polypeptide). In some embodiments, cell lines are screened to select a lead clone for rAAV production, which may then be expanded to a production bioreactor and infected with a helper virus (*e.g.*, an adenovirus or HSV) to initiate rAAV production. In some embodiments, viruses are subsequently be harvested, adenovirus are inactivated (*e.g.*, by heat) and/or removed, and the rAAV particles are purified. As such, in some embodiments, the rAAV particle is produced by a producer cell line comprising one or more of nucleic acid encoding the rAAV vector, a nucleic acid encoding AAV rep and cap, and a nucleic acid encoding AAV helper virus functions. As described herein, for some embodiments, the producer cell line method is advantageous for the production of rAAV particles with an oversized genome, as compared to the triple transfection method.

[0099] In some embodiments, the nucleic acid encoding AAV rep and cap genes and/or the rAAV genome are stably maintained in the producer cell line. In some embodiments, nucleic acid encoding AAV rep and cap genes and/or the rAAV genome is introduced on one or more plasmids into a cell line to generate a producer cell line. In some embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on the same plasmid. In other embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on different plasmids. In some embodiments, a cell line stably transfected with a plasmid maintains the plasmid for multiple passages of the cell line (*e.g.*, 5, 10, 20, 30, 40, 50 or more than 50 passages of the cell). For example, the plasmid(s) may replicate as the cell replicates, or the plasmid(s) may integrate into the cell genome. A variety of sequences that enable a plasmid to replicate autonomously in a cell (*e.g.*, a human cell) have been identified (*see, e.g.*, Krysan, P.J. *et al.* (1989) *Mol. Cell Biol.* 9:1026-1033). In some embodiments, the plasmid(s) may contain a selectable marker (*e.g.*, an antibiotic resistance marker) that allows for selection of cells maintaining the plasmid. Selectable markers commonly used in mammalian cells include without limitation blasticidin, G418, hygromycin B, zeocin, puromycin, and derivatives thereof. Methods for introducing nucleic acids into a cell are known in the art and include without limitation viral transduction, cationic transfection (*e.g.*, using a cationic polymer such as DEAE-dextran or a cationic lipid such as lipofectamine), calcium phosphate transfection, microinjection, particle bombardment, electroporation, and nanoparticle transfection (for more details, *see e.g.*, Kim, T.K. and Eberwine, J.H. (2010) *Anal. Bioanal. Chem.* 397:3173-3178).

[0100] In some embodiments, the nucleic acid encoding AAV rep and cap genes and/or the rAAV genome are stably integrated into the genome of the producer cell line. In some embodiments, nucleic acid encoding AAV rep and cap genes and/or the rAAV genome is introduced on one or more plasmids into a cell line to generate a producer cell line. In some embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on the same plasmid. In other embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on different plasmids. In some embodiments, the plasmid(s) may contain a selectable marker (*e.g.*, an antibiotic resistance marker) that allows for selection of cells maintaining the plasmid. Methods for stable integration of nucleic acids into a variety of host cell lines are known in the art. For example, repeated selection (*e.g.*, through use of a selectable marker) is used to select for cells that have integrated a nucleic acid containing a selectable marker (and AAV cap and rep genes and/or a rAAV genome). In other embodiments, nucleic acids are integrated in a site-specific manner into a cell line to generate a producer cell line. Several site-specific recombination systems are known in the art, such as FLP/FRT (*see, e.g.*, O’Gorman, S. *et al.* (1991) *Science* 251:1351-1355), Cre/loxP (*see, e.g.*, Sauer, B. and Henderson, N. (1988) *Proc. Natl. Acad. Sci.* 85:5166-5170), and phi C31-att (*see, e.g.*, Groth, A.C. *et al.* (2000) *Proc. Natl. Acad. Sci.* 97:5995-6000).

[0101] In some embodiments, the producer cell line is derived from a primate cell line (*e.g.*, a non-human primate cell line, such as a Vero or FRhL-2 cell line). In some embodiments, the cell line is derived from a human cell line. In some embodiments, the producer cell line is derived from HeLa, 293, A549, or PERC.6® (Cruce) cells. For example, prior to introduction and/or stable maintenance/integration of nucleic acid encoding AAV rep and cap genes and/or the oversized rAAV genome into a cell line to generate a producer cell line, the cell line is a HeLa, 293, A549, or PERC.6® (Cruce) cell line, or a derivative thereof.

[0102] In some embodiments, the producer cell line is adapted for growth in suspension. As is known in the art, anchorage-dependent cells are typically not able to grow in suspension without a substrate, such as microcarrier beads. Adapting a cell line to grow in suspension may include, for example, growing the cell line in a spinner culture with a stirring paddle, using a culture medium that lacks calcium and magnesium ions to prevent clumping (and optionally an antifoaming agent), using a culture vessel coated with a siliconizing compound, and selecting cells in the culture (rather than in large clumps or on the sides of the vessel) at each passage. For

further description, see, *e.g.*, ATCC frequently asked questions document (available at www.atcc.org/Global/FAQs/9/1/Adapting%20a%20monolayer%20cell%20line%20to%20suspension-40.aspx) and references cited therein.

[0103] In some aspects, a method is provided for producing any rAAV particle as disclosed herein comprising (a) culturing a host cell under a condition that rAAV particles are produced, wherein the host cell comprises (i) one or more AAV package genes, wherein each said AAV packaging gene encodes an AAV replication and/or encapsidation protein; (ii) a rAAV provector comprising a nucleic acid encoding a heterologous nucleic acid as described herein flanked by at least one AAV ITR, and (iii) an AAV helper function; and (b) recovering the rAAV particles produced by the host cell. In some embodiments, said at least one AAV ITR is selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, a goat AAV, bovine AAV, or mouse AAV serotype ITRs or the like. For example, in some embodiments, the AAV serotype is AAV1, AAV2, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, or AAVrh10. In certain embodiments, the nucleic acid in the AAV comprises an AAV2 ITR. In some embodiments, said encapsidation protein is selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAVSAN006, AAV10, AAVrh10, AAV11, AAV12, AAV2R471A, AAV2/2-7m8, AAV DJ, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV capsid, rAAV2/HBoV1 serotype, AAV-XL32, or AAV-XL32.1 capsid proteins or mutants thereof. In some embodiments, the encapsidation protein is an AAV8 capsid protein. In some embodiments, the rAAV particles comprise an AAV9 capsid and a recombinant genome comprising AAV2 ITRs, and nucleic acid encoding a therapeutic transgene/nucleic acid (*e.g.*, an expression cassette for expressing a disorder-related polypeptide). In some embodiments, the rAAV particles comprise an AAV.SAN006 capsid and a recombinant genome comprising AAV2 ITRs, and nucleic acid encoding a therapeutic transgene/nucleic acid (*e.g.*, an expression cassette for expressing a disorder-related polypeptide).

[0104] Suitable rAAV production culture media of the present disclosure is supplemented with serum or serum-derived recombinant proteins at a level of 0.5%-20% (v/v or w/v). In some embodiments, rAAV vectors are produced in serum-free conditions which may also be referred

to as media with no animal-derived products. One of ordinary skill in the art may appreciate that commercial or custom media designed to support production of rAAV vectors may also be supplemented with one or more cell culture components known in the art, including without limitation glucose, vitamins, amino acids, and or growth factors, in order to increase the titer of rAAV in production cultures.

[0105] rAAV production cultures can be grown under a variety of conditions (over a wide temperature range, for varying lengths of time, and the like) suitable to the particular host cell being utilized. As is known in the art, rAAV production cultures include attachment-dependent cultures which can be cultured in suitable attachment-dependent vessels such as, for example, roller bottles, hollow fiber filters, microcarriers, and packed-bed or fluidized-bed bioreactors. rAAV vector production cultures may also include suspension-adapted host cells such as HeLa, 293, and SF-9 cells which can be cultured in a variety of ways including, for example, spinner flasks, stirred tank bioreactors, and disposable systems such as the Wave bag system.

[0106] In some embodiments, rAAV vector particles of the disclosure are harvested from rAAV production cultures by lysis of the host cells of the production culture or by harvest of the spent media from the production culture, provided the cells are cultured under conditions known in the art to cause release of rAAV particles into the media from intact cells, as described more fully in U.S. Patent No. 6,566,118). Suitable methods of lysing cells are also known in the art and include for example multiple freeze/thaw cycles, sonication, microfluidization, and treatment with chemicals, such as detergents and/or proteases.

[0107] In a further embodiment, the rAAV particles are purified. The term “purified” as used herein includes a preparation of rAAV particles devoid of at least some of the other components that may also be present where the rAAV particles naturally occur or are initially prepared from. Thus, for example, isolated rAAV particles are prepared using a purification technique to enrich it from a source mixture, such as a culture lysate or production culture supernatant. Enrichment can be measured in a variety of ways, such as, for example, by the proportion of DNase-resistant particles (DRPs) or genome copies (gc) present in a solution, or by infectivity, or it can be measured in relation to a second, potentially interfering substance present in the source mixture, such as contaminants, including production culture contaminants or in-process contaminants, including helper virus, media components, and the like.

[0108] In some embodiments, the rAAV production culture harvest is clarified to remove host cell debris. In some embodiments, the production culture harvest is clarified by filtration through a series of depth filters including, for example, a grade DOHC Millipore Millistak+ HC Pod Filter, a grade A1HC Millipore Millistak+ HC Pod Filter, and a 0.2 μm Filter Opticap XL10 Millipore Express SHC Hydrophilic Membrane filter. Clarification can also be achieved by a variety of other standard techniques known in the art, such as, centrifugation or filtration through any cellulose acetate filter of 0.2 μm or greater pore size known in the art.

[0109] In some embodiments, the rAAV production culture harvest is further treated with Benzonase® to digest any high molecular weight DNA present in the production culture. In some embodiments, the Benzonase® digestion is performed under standard conditions known in the art including, for example, a final concentration of 1-2.5 units/ml of Benzonase® at a temperature ranging from ambient to 37°C for a period of 30 minutes to several hours.

[0110] In some embodiments, rAAV particles are isolated or purified using one or more of the following purification steps: equilibrium centrifugation; flow-through anionic exchange filtration; tangential flow filtration (TFF) for concentrating the rAAV particles; rAAV capture by apatite chromatography; heat inactivation of helper virus; rAAV capture by hydrophobic interaction chromatography; buffer exchange by size exclusion chromatography (SEC); nanofiltration; and rAAV capture by anionic exchange chromatography, cationic exchange chromatography, or affinity chromatography. For such embodiments, steps are used alone, in various combinations, or in different orders. In some embodiments, the method comprises all the steps in the order as described below. Methods to purify rAAV particles are found, for example, in Xiao *et al.*, (1998) Journal of Virology 72:2224-2232; US Patent Numbers 6,989,264 and 8,137,948; and WO 2010/148143.

VI. Methods of treatment

[0111] Certain aspects of the present disclosure relate to methods of treating various neurodegenerative diseases in an individual in need thereof. In some embodiments, the disclosure provides methods of treating a neurodegenerative disease by administering an effective amount of a viral particle of the disclosure for expressing a disorder-related polypeptide or RNAi molecule. In some embodiments, the viral particles are capable of expressing a

polypeptide associated with a neurodegenerative disorder. In other embodiments, the viral particles are capable of expressing an RNAi molecule capable of reducing or eliminating the expression of a particular polypeptide implicated in a neurodegenerative disorder as set forth herein. In some embodiments, the RNAi molecule is an artificial miRNA. In some embodiments, the RNAi molecule is a small interfering RNA (siRNA). In some embodiments, the RNAi molecule is a short hairpin RNA (shRNA).

[0112] The administration of the viral particle includes direct administration into the cerebrospinal fluid (CSF). In some embodiments, the viral particle is administered via direct injection into the spinal cord, via intrathecal injection, or via intracisternal injection. In some embodiments, the viral particle is administered to more than one location of the spinal cord or cisterna magna via ICM injection. In some embodiments, the viral particle is administered to the cisterna magna. In some embodiments, the viral particle is administered by intracerebroventricular injection (also referred to as intraventricular injection).

[0113] In some embodiments, an effective amount or therapeutically effective amount of viral particles are administered, depending on the objectives of treatment. For example, where a low percentage of transduction can achieve the desired therapeutic effect, then the objective of treatment is generally to meet or exceed this level of transduction. In some instances, this level of transduction can be achieved by transduction of only about 1 to 5% of the target cells of the desired tissue type, in some embodiments at least about 20% of the cells of the desired tissue type, in some embodiments at least about 50%, in some embodiments at least about 80%, in some embodiments at least about 95%, in some embodiments at least about 99% of the cells of the desired tissue type. In some embodiments, the viral particle composition is administered by one or more administrations, either during the same procedure or spaced apart by days, weeks, months, or years.

[0114] Methods to identify cells transduced by AAV viral particles are known in the art; for example, immunohistochemistry or the use of a marker such as enhanced green fluorescent protein can be used to detect transduction of viral particles; for example, viral particles comprising a rAAV capsid with one or more substitutions of amino acids.

[0115] In some embodiments, the disclosure provides a method for treating a human with a neurodegenerative disorder by administering an effective amount of a pharmaceutical

composition comprising a recombinant viral particle encoding a disorder-related polypeptide or RNAi molecule. In some embodiments, the pharmaceutical composition comprises one or more pharmaceutically acceptable excipients.

[0116] In some embodiments, the methods comprise administering an effective amount of a pharmaceutical composition comprising a recombinant viral vector encoding a disorder-related polypeptide of the present disclosure to treat a neurodegenerative disorder in an individual in need thereof. In some embodiments, the viral titer of the viral particles (*e.g.*, rAAV particles) is at least about any of 5×10^{12} , 6×10^{12} , 7×10^{12} , 8×10^{12} , 9×10^{12} , 10×10^{12} , 11×10^{12} , 15×10^{12} , 20×10^{12} , 25×10^{12} , 30×10^{12} , or 50×10^{12} genome copies/mL. In some embodiments, the viral titer of the viral particles (*e.g.*, rAAV particles) is about any of 5×10^{12} to 6×10^{12} , 6×10^{12} to 7×10^{12} , 7×10^{12} to 8×10^{12} , 8×10^{12} to 9×10^{12} , 9×10^{12} to 10×10^{12} , 10×10^{12} to 11×10^{12} , 11×10^{12} to 15×10^{12} , 15×10^{12} to 20×10^{12} , 20×10^{12} to 25×10^{12} , 25×10^{12} to 30×10^{12} , 30×10^{12} to 50×10^{12} , or 50×10^{12} to 100×10^{12} genome copies/mL. In some embodiments, the viral titer of the viral particles (*e.g.*, rAAV particles) is about any of 5×10^{12} to 10×10^{12} , 10×10^{12} to 25×10^{12} , or 25×10^{12} to 50×10^{12} genome copies/mL. In some embodiments, the viral titer of the viral particles (*e.g.*, rAAV particles) is at least about any of 5×10^9 , 6×10^9 , 7×10^9 , 8×10^9 , 9×10^9 , 10×10^9 , 11×10^9 , 15×10^9 , 20×10^9 , 25×10^9 , 30×10^9 , or 50×10^9 transducing units /mL. In some embodiments, the viral titer of the viral particles (*e.g.*, rAAV particles) is about any of 5×10^9 to 6×10^9 , 6×10^9 to 7×10^9 , 7×10^9 to 8×10^9 , 8×10^9 to 9×10^9 , 9×10^9 to 10×10^9 , 10×10^9 to 11×10^9 , 11×10^9 to 15×10^9 , 15×10^9 to 20×10^9 , 20×10^9 to 25×10^9 , 25×10^9 to 30×10^9 , 30×10^9 to 50×10^9 or 50×10^9 to 100×10^9 transducing units /mL. In some embodiments, the viral titer of the viral particles (*e.g.*, rAAV particles) is about any of 5×10^9 to 10×10^9 , 10×10^9 to 15×10^9 , 15×10^9 to 25×10^9 , or 25×10^9 to 50×10^9 transducing units /mL. In some embodiments, the viral titer of the viral particles (*e.g.*, rAAV particles) is at least any of about 5×10^{10} , 6×10^{10} , 7×10^{10} , 8×10^{10} , 9×10^{10} , 10×10^{10} , 11×10^{10} , 15×10^{10} , 20×10^{10} , 25×10^{10} , 30×10^{10} , 40×10^{10} , or 50×10^{10} infectious units/mL. In some embodiments, the viral titer of the viral particles (*e.g.*, rAAV particles) is at least any of about 5×10^{10} to 6×10^{10} , 6×10^{10} to 7×10^{10} , 7×10^{10} to 8×10^{10} , 8×10^{10} to 9×10^{10} , 9×10^{10} to 10×10^{10} , 10×10^{10} to 11×10^{10} , 11×10^{10} to 15×10^{10} , 15×10^{10} to 20×10^{10} , 20×10^{10} to 25×10^{10} , 25×10^{10} to 30×10^{10} , 30×10^{10} to 40×10^{10} , 40×10^{10} to 50×10^{10} , or 50×10^{10} to 100×10^{10} infectious units/mL. In some embodiments, the viral titer of the viral particles (*e.g.*, rAAV particles) is at

least any of about 5×10^{10} to 10×10^{10} , 10×10^{10} to 15×10^{10} , 15×10^{10} to 25×10^{10} , or 25×10^{10} to 50×10^{10} infectious units/mL. In some embodiments, the viral particles are rAAV particles. In some embodiments, the rAAV particles comprise an AAV.SAN006 capsid protein.

[0117] In some embodiments, the dose of viral particles administered to the individual is at least about any of 1×10^8 to about 6×10^{13} genome copies/kg of body weight. In some embodiments, the dose of viral particles administered to the individual is about any of 1×10^8 to about 6×10^{13} genome copies/kg of body weight. In some embodiments, the dose of viral particles administered to the individual is about any of 1×10^{10} , 2×10^{10} , 3×10^{10} , 4×10^{10} , 5×10^{10} , 6×10^{10} , 7×10^{10} , 8×10^{10} , 9×10^{10} , 1×10^{11} , 2×10^{11} , 3×10^{11} , 4×10^{11} , 5×10^{11} , 6×10^{11} , 7×10^{11} , 8×10^{11} , 9×10^{11} , 1×10^{12} , 2×10^{12} , 3×10^{12} , 4×10^{12} , 5×10^{12} , 6×10^{12} , 7×10^{12} , 8×10^{12} , 9×10^{12} , or 1×10^{13} genome copies/kg of body weight. In some embodiments, the dose of viral particles administered to the individual is about any of 0.5×10^{10} , 1.5×10^{10} , 2.5×10^{10} , 3.5×10^{10} , 4.5×10^{10} , 5.5×10^{10} , 6.5×10^{10} , 7.5×10^{10} , 8.5×10^{10} , 9.5×10^{10} , 10.5×10^{10} , 11.5×10^{10} , or 12.5×10^{10} genome copies/kg of body weight.

[0118] In some embodiments, the total amount of viral particles administered to the individual is at least about any of 1×10^9 to about 1×10^{14} genome copies. In some embodiments, the total amount of viral particles administered to the individual is about any of 1×10^9 to about 1×10^{14} genome copies. In some embodiments, the total amount of viral particles administered to the individual is about any of 1×10^{11} , 2×10^{11} , 3×10^{11} , 4×10^{11} , 5×10^{11} , 6×10^{11} , 7×10^{11} , 8×10^{11} , 9×10^{11} , 1×10^{12} , 2×10^{12} , 3×10^{12} , 4×10^{12} , 5×10^{12} , 6×10^{12} , 7×10^{12} , 8×10^{12} , 9×10^{12} , 1×10^{13} , 2×10^{13} , 3×10^{13} , 4×10^{13} , 5×10^{13} , 6×10^{13} , 7×10^{13} , 8×10^{13} , 9×10^{13} , or 1×10^{14} genome copies.

[0119] Compositions of the disclosure (*e.g.*, recombinant viral particles comprising a vector encoding a disorder-related polypeptide of the present disclosure) can be used either alone or in combination with one or more additional therapeutic agents for treating a neurodegenerative disorder. The interval between sequential administration can be in terms of at least (or, alternatively, less than) minutes, hours, or days.

[0120] In some embodiments, a viral particle composition of the present disclosure is used for administration to a human. In some embodiments, a viral particle composition of the present disclosure is used for pediatric administration. In some embodiments, an effective amount of

viral particles is administered to a patient that is less than one month, less than two months, less than three months, less than four months, less than five months, less than six months, less than seven months, less than eight months, less than nine months, less than ten months, less than eleven months, less than one year, less than 13 months, less than 14 months, less than 15 months, less than 16 months, less than 17 months, less than 18 months, less than 19 months, less than 20 months, less than 21 months, less than 22 months, less than two years, less than three years old, less than five years old or less than seven years old.

[0121] In some embodiments, a rAAV composition of the present disclosure is used for administration to a young adult. In some embodiments, an effective amount of viral particles is administered to a patient that is less than 12 years old, less than 13 years old, less than 14 years old, less than 15 years old, less than 16 years old, less than 17 years old, less than 18 years old, less than 19 years old, less than 20 years old, less than 21 years old, less than 22 years old, less than 23 years old, less than 24 years old, or less than 25 years old.

[0122] In some aspects, the invention provides rAAV vectors for use in methods of preventing or treating one or more gene defects (*e.g.*, heritable gene defects, somatic gene alterations, and the like) in a mammal, such as for example, a gene defect that results in a polypeptide deficiency or polypeptide excess in a subject, or for treating or reducing the severity or extent of deficiency in a subject manifesting a CNS-associated disorder linked to a deficiency in such polypeptides in cells and tissues. In some embodiments, methods involve administration of a rAAV vector that encodes one or more therapeutic peptides, polypeptides, functional RNAs, inhibitory nucleic acids, shRNAs, microRNAs, antisense nucleotides, *etc.* in a pharmaceutically-acceptable carrier to the subject in an amount and for a period of time sufficient to treat the CNS-associated disorder in the subject having or suspected of having such a disorder.

[0123] A rAAV vector may comprise as a transgene, a nucleic acid encoding a protein or functional RNA that modulates or treats a CNS-associated disorder. The following is a non-limiting list of genes associated with CNS-associated disorders: neuronal apoptosis inhibitory protein (NAIP), nerve growth factor (NGF), glial-derived growth factor (GDNF), brain-derived growth factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxylase (TH), GTP-cyclohydrolase (GTPCH), aspartoacylase (ASPA), superoxide dismutase (SOD1) and amino acid decarboxylase (AADC). For example, a useful transgene in the treatment of Parkinson's disease

encodes TH, which is a rate limiting enzyme in the synthesis of dopamine. A transgene encoding GTPCII, which generates the TII cofactor tetrahydrobiopterin, may also be used in the treatment of Parkinson's disease. A transgene encoding GDNF or BDNF, or AADC, which facilitates conversion of L-Dopa to DA, may also be used for the treatment of Parkinson's disease. For the treatment of ALS, a useful transgene may encode: GDNF, BDNF or CNTF. Also for the treatment of ALS, a useful transgene may encode a functional RNA, *e.g.*, shRNA, miRNA, that inhibits the expression of SOD1. For the treatment of ischemia a useful transgene may encode NAIP or NGF. In some embodiments, a transgene encoding Beta-glucuronidase (GUS) is useful for the treatment of certain lysosomal storage diseases (*e.g.*, Mucopolysaccharidosis type VII (MPS VII)). In some aspects, a transgene encoding a prodrug activation gene, *e.g.*, HSV-Thymidine kinase which converts ganciclovir to a toxic nucleotide which disrupts DNA synthesis and leads to cell death, is useful for treating certain cancers, *e.g.*, when administered in combination with the prodrug. In some examples, a transgene encoding an endogenous opioid, such as β -endorphin is useful for treating pain. In some examples, transgenes that are used in the rAAV vectors of the invention will be apparent to the skilled artisan (See, *e.g.*, Costantini LC, *et al.*, *Gene Therapy* (2000) 7, 93-109).

[0124] In some embodiments, the heterologous nucleic acid may encode a therapeutic nucleic acid. In some embodiments, a therapeutic nucleic acid may include without limitation an siRNA, an shRNA, an RNAi, an miRNA, an antisense RNA, a ribozyme or a DNAzyme. As such, a therapeutic nucleic acid may encode an RNA that when transcribed from the nucleic acids of the vector can treat a disorder of the invention (*e.g.*, a disorder of the CNS) by interfering with translation or transcription of an abnormal or excess protein associated with a disorder of the invention. For example, the nucleic acids of the invention may encode for an RNA which treats a disorder by highly specific elimination or reduction of mRNA encoding the abnormal and/or excess proteins. Therapeutic RNA sequences include RNAi, small inhibitory RNA (siRNA), micro RNA (miRNA), and/or ribozymes (such as hammerhead and hairpin ribozymes) that can treat disorders by highly specific elimination or reduction of mRNA encoding the abnormal and/or excess proteins.

[0125] In some embodiments, the heterologous nucleic acid may encode a therapeutic polypeptide. A therapeutic polypeptide may, *e.g.*, supply a polypeptide and/or enzymatic activity that is absent or present at a reduced level in a cell or organism. Alternatively, a

therapeutic polypeptide may supply a polypeptide and/or enzymatic activity that indirectly counteracts an imbalance in a cell or organism. For example, a therapeutic polypeptide for a disorder related to buildup of a metabolite caused by a deficiency in a metabolic enzyme or activity may supply a missing metabolic enzyme or activity, or it may supply an alternate metabolic enzyme or activity that leads to reduction of the metabolite. A therapeutic polypeptide may also be used to reduce the activity of a polypeptide (*e.g.*, one that is overexpressed, activated by a gain-of-function mutation, or whose activity is otherwise misregulated) by acting, *e.g.*, as a dominant-negative polypeptide.

[0126] In some embodiments, the therapeutic polypeptide or therapeutic nucleic acid is used to treat a disorder of the CNS. Without wishing to be bound to theory, it is thought that a therapeutic polypeptide or therapeutic nucleic acid may be used to reduce or eliminate the expression and/or activity of a polypeptide whose gain-of-function has been associated with a disorder, or to enhance the expression and/or activity of a polypeptide to complement a deficiency that has been associated with a disorder (*e.g.*, a mutation in a gene whose expression shows similar or related activity). Non-limiting examples of CNS disorders of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention (exemplary genes that may be targeted or supplied are provided in parenthesis for each disorder) comprise stroke (*e.g.*, *caspase-3*, *Beclin1*, *Ask1*, *PARI*, *HIF1 α* , *PUMA*, and/or any of the genes described in Fukuda, A.M. and Badaut, J. (2013) *Genes (Basel)* 4:435-456), Huntington's disease (mutant *HTT*), epilepsy (*e.g.*, *SCN1A*, *NMDAR*, *ADK*, and/or any of the genes described in Boison, D. (2010) *Epilepsia* 51:1659-1668), Parkinson's disease (alpha-synuclein), Lou Gehrig's disease (also known as amyotrophic lateral sclerosis; *SOD1*), Alzheimer's disease (tau, amyloid precursor protein), corticobasal degeneration or CBD (tau), corticogasal ganglionic degeneration or CBGD (tau), frontotemporal dementia or FTD (tau), progressive supranuclear palsy or PSP (tau), multiple system atrophy or MSA (alpha-synuclein), cancer of the brain (*e.g.*, a mutant or overexpressed oncogene implicated in brain cancer), and lysosomal storage diseases (LSD). Disorders of the invention may include those that involve large areas of the cortex, *e.g.*, more than one functional area of the cortex, more than one lobe of the cortex, and/or the entire cortex. Other non-limiting examples of disorders of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention comprise traumatic brain injury, enzymatic dysfunction disorders, psychiatric disorders (including post-traumatic stress

syndrome), neurodegenerative diseases, and cognitive disorders (including dementias, autism, and depression). Enzymatic dysfunction disorders include without limitation leukodystrophies (including Canavan's disease) and any of the lysosomal storage diseases described below.

[0127] In some embodiments, the therapeutic polypeptide or therapeutic nucleic acid is used to treat a lysosomal storage disease. As is commonly known in the art, lysosomal storage diseases are rare, inherited metabolic disorders characterized by defects in lysosomal function. Such disorders are often caused by a deficiency in an enzyme required for proper mucopolysaccharide, glycoprotein, and/or lipid metabolism, leading to a pathological accumulation of lysosomally stored cellular materials. Non-limiting examples of lysosomal storage diseases of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention (exemplary genes that may be targeted or supplied are provided in parenthesis for each disorder) include Gaucher disease type 2 or type 3 (acid beta-glucosidase, *GBA*), GM1 gangliosidosis (beta-galactosidase-1, *GLB1*), Hunter disease (iduronate 2-sulfatase, *IDS*), Krabbe disease (galactosylceramidase, *GALC*), a mannosidosis disease (a mannosidase, such as alpha-D-mannosidase, *MAN2B1*), β mannosidosis disease (beta-mannosidase, *MANBA*), metachromatic leukodystrophy disease (pseudoarylsulfatase A, *ARSA*), mucopolipidosisII/III disease (N-acetylglucosamine-1-phosphotransferase, *GNPTAB*), Niemann-Pick A disease (acid sphingomyelinase, *ASM*), Niemann-Pick C disease (Niemann-Pick C protein, *NPC1*), Pompe disease (acid alpha-1,4-glucosidase, *GAA*), Sandhoff disease (hexosaminidase beta subunit, *HEXB*), Sanfillipo A disease (N-sulfoglucosamine sulfohydrolase, *MPS3A*), Sanfillipo B disease (N-alpha-acetylglucosaminidase, *NAGLU*), Sanfillipo C disease (heparin acetyl-CoA:alpha-glucosaminidase N-acetyltransferase, *MPS3C*), Sanfillipo D disease (N-acetylglucosamine-6-sulfatase, *GNS*), Schindler disease (alpha-N-acetylgalactosaminidase, *NAGA*), Sly disease (beta-glucuronidase, *GUSB*), Tay-Sachs disease (hexosaminidase alpha subunit, *HEXA*), and Wolman disease (lysosomal acid lipase, *LIPA*).

[0128] As such, in some embodiments, the therapeutic polypeptide is caspase-3, Beclin1, Ask1, PAR1, HIF1 α , PUMA, SCN1A, NMDAR, ADK, alpha-synuclein, SOD1, acid beta-glucosidase (*GBA*), beta-galactosidase-1 (*GLB1*), iduronate 2-sulfatase (*IDS*), galactosylceramidase (*GALC*), a mannosidase, alpha-D-mannosidase (*MAN2B1*), beta-mannosidase (*MANBA*), pseudoarylsulfatase A (*ARSA*), N-acetylglucosamine-1-phosphotransferase (*GNPTAB*), acid sphingomyelinase (*ASM*), Niemann-Pick C protein

(NPC1), acid alpha-1,4-glucosidase (GAA), hexosaminidase beta subunit, HEXB, N-sulfoglucosamine sulfohydrolase (MPS3A), N-alpha-acetylglucosaminidase (NAGLU), heparin acetyl-CoA, alpha-glucosaminidase N-acetyltransferase (MPS3C), N-acetylglucosamine-6-sulfatase (GNS), alpha-N-acetylgalactosaminidase (NAGA), beta-glucuronidase (GUSB), hexosaminidase alpha subunit (HEXA), huntingtin (HTT), or lysosomal acid lipase (LIPA). The therapeutic polypeptide may increase or decrease the function of the target polypeptide in the subject (*e.g.*, it may supply the missing function in a lysosomal storage disease, or reduce the level of alpha-synuclein in MSA, such as by blocking its function or dysfunction). In some embodiments, the therapeutic nucleic acid is caspase-3, Beclin1, Ask1, PAR1, HIF1 α , PUMA, SCN1A, NMDAR, ADK, alpha-synuclein, SOD1, acid beta-glucosidase (GBA), beta-galactosidase-1 (GLB1), iduronate 2-sulfatase (IDS), galactosylceramidase (GALC), a mannosidase, alpha-D-mannosidase (MAN2B1), beta-mannosidase (MANBA), pseudoarylsulfatase A (ARSA), N-acetylglucosamine-1-phosphotransferase (GNPTAB), acid sphingomyelinase (ASM), Niemann-Pick C protein (NPC1), acid alpha-1,4-glucosidase (GAA), hexosaminidase beta subunit, HEXB, N-sulfoglucosamine sulfohydrolase (MPS3A), N-alpha-acetylglucosaminidase (NAGLU), heparin acetyl-CoA, alpha-glucosaminidase N-acetyltransferase (MPS3C), N-acetylglucosamine-6-sulfatase (GNS), alpha-N-acetylgalactosaminidase (NAGA), beta-glucuronidase (GUSB), hexosaminidase alpha subunit (HEXA), or lysosomal acid lipase (LIPA). The therapeutic nucleic acid may increase or decrease the function of the target polypeptide in the subject (*e.g.*, it may supply the missing function in a lysosomal storage disease or reduce the level of alpha-synuclein in MSA, such as by RNAi).

[0129] In some embodiments, an exemplary disease for which AAV expression in the cortex and striatum is useful is Huntington's disease (HD). Huntington's disease is caused by a CAG repeat expansion mutation that encodes an elongated polyglutamine (polyQ) repeat in the mutant huntingtin protein (mHTT). HD is a particularly attractive target for DNA- and RNA-based therapies as it is an autosomal dominant disease resulting from mutation on a single allele. AAV vectors provide an ideal delivery system for nucleic acid therapeutics and allow for long lasting and continuous expression of these huntingtin lowering molecules in the brain. To achieve maximal clinical efficacy in HD, delivery to both the striatum and cortex will likely be required. Postmortem analysis of HD patient brains revealed extensive medium spiny neuronal loss in the

striatum, in addition to loss of pyramidal neurons in the cerebral cortex and hippocampus. It was recently shown using conditional transgenic mouse models of HD that genetically reducing mHTT expression in neuronal populations in the striatum and cortex provides significantly more efficacy than reducing mHTT in either site alone (Wang *et al.*, (2014) *Nature medicine* 20:536-541). In some embodiments, delivery of gene therapy agents to both striatal and cortical regions is ideal for maximal therapeutic efficacy.

Methods for treating Parkinson's Disease and associated disorders

[0130] Of the most common neurodegenerative disorders are Parkinson's Disease (PD) and its associated disorders. PD and associated disorders are brain disorders that cause uncontrollable movement and reduced balance and coordination. PD and related movement disorders can include lewy body dementias (LBD) and multiple system atrophy (MSA). These disorders are characterized by neurodegeneration of basal ganglia, brainstem, cerebellum, and cerebral cortex. PD and its associated disorders are related to dysfunction of any of several disease associated genes, including, but not limited to: ATP13A2, PLA2G6, VPS35, DJ1, GBA1, SNCA, PINK1, PARKIN or LRRK2. For example, the ATP13A2 gene encodes a member of the P5 subfamily of ATPases which transports inorganic cations as well as other substrates. Mutations in this gene are associated with Kufor-Rakeb syndrome (KRS), also referred to as Parkinson disease 9. KRS is a very rare form of inherited juvenile-onset Parkinson's disease. As an additional example, the SNCA gene expresses Synucleins (e.g., small, soluble proteins) in the brain. Defects in the SNCA gene have been implicated in the pathogenesis of Parkinson disease.

[0131] Certain aspects of the present disclosure relate to methods of treating Parkinson's disease (PD) and associated disorders in an individual in need thereof. In some embodiments, the disclosure provides methods of treating PD and/or associated disorders by administering an effective amount of viral particles as disclosed herein. In some embodiments, the disclosure provides methods of treating PD and/or associated disorders administering an effective amount of viral particles comprising expression cassette for expressing a disorder-related polypeptide of the present disclosure. In some embodiments, the disorder-related polypeptide is a wild type disorder-related polypeptide.

[0132] In some embodiments, the method for treating PD and/or associated disorders may comprise delivering a viral particle expressing a transgene for modulating the function or levels of at least one target. In some embodiments, the method may comprise delivering an expression cassette expressing a transgene for modulating the function or levels of at least one target intracellularly or extracellularly. In some embodiments, wherein the method comprises delivering an expression cassette intracellularly, the method may comprise delivering a miRNA (e.g., artificial miRNA) targeting SNCA to lower expression. In some embodiments, the method may comprise intracellular delivery of a viral particle comprising an artificial miRNA configured to target SNCA and lower expression. In some embodiments, the method may comprise extracellular delivery of a viral particle comprising an expression cassette configured for expression of an antibody fragment for secretion and binding to a target of interest either intra- or extracellularly.

[0133] In some embodiments, expression of the disorder-related polypeptide is under control of a promoter. In some embodiments, the expression cassette is under the control of a promoter. In some embodiments, the transgene is under the control of a promoter. In some embodiments, the promoter may comprise a ubiquitous promoter or a neuron-specific promoter. In some embodiments, the ubiquitous promoter may comprise CBA, CAG, CAGG, CMV, H1, U6, or 7SK. In some embodiments, the neuron-specific promoter may comprise SYN1, NSE, CAMKII, or TUBB3. In some instances, the promoter (e.g., promoter activity) is regulated by an exogenous small molecule.

[0134] In some embodiments, the expression cassette may comprise at least one transgene. In some embodiments, at least one transgene (e.g., gene) may comprise ATP13A2, PLA2G6, VPS35, DJ1, GBA1, SNCA, PINK1, PARKIN or LRRK2. In some instances, transgene expression is regulated by an exogenous small molecule.

[0135] In some instances, the expression cassette may comprise at least one additional nucleotide sequence. In some embodiments, at least one additional nucleotide sequence is configured to improve RNA stability. In some embodiments, at least one additional nucleotide sequence configured to improve RNA stability may comprise WPRE. In some embodiments, at least one additional nucleotide sequence is configured to detarget expression from liver and/or DRG.

Methods for treating Alzheimer's Disease and associated disorders

[0136] Another common neurodegenerative disorder is Alzheimer's Disease (AD). In fact, AD and associated disorders comprise the most common form of dementia. Two main neuropathological lesions of AD are amyloid plaques and neurofibrillary tangles (NFTs). Amyloid plaques are composed of amyloid β (A β) peptides, which are cleaved from the Amyloid Precursor Protein (APP). NFTs, present in the brain of AD and related neurodegenerative disease patients, are constituted of tau proteins in hyperphosphorylated and aggregated form. Disorders associated with AD (e.g., related neurodegenerative disorders) are cerebral amyloid angiopathy (CAA), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), frontotemporal dementia with parkinsonism-17 (FTDP-17), Pick's Disease (PiD), argyrophilic grain disease (AGD), and globular glial tauopathy (GGT). AD and CAA are characterized by intracellular aggregation of hyperphosphorylated tau protein with extracellular aggregation of amyloid beta (A β) plaques. Whereas, the associated disorders including PSP, CBD, FTDP-17, PiD, GGT, and AGD are characterized by intracellular aggregation of hyperphosphorylated tau protein without extracellular aggregation of amyloid beta (A β) plaques. These pathological protein aggregates can cause synaptic dysfunction, neuroinflammation, and neurodegeneration in cerebral cortex, cerebellum, basal ganglia, midbrain, and brainstem. These processes can be related to dysfunction of any of several disease-associated genes, including, but not limited to: MAPT, BIN1, APP, PS1, APOE, CD33, and/or TREM2

[0137] Certain aspects of the present disclosure relate to methods of treating Alzheimer's Disease (AD) and/or associated disorders in an individual in need thereof. In some embodiments, the disclosure provides methods of treating AD and/or associated disorders by administering an effective amount of a viral particle of the disclosure. In some embodiments, the disclosure provides methods of treating AD and/or associated disorders by administering an effective amount of a viral particle for expressing a disorder-related polypeptide or RNAi molecule.

[0138] In some embodiments, the method comprises delivering a viral particle comprising an expression cassette configured to target tau pathology in AD and/or associated disorders. In some embodiments, the method comprises delivering a viral particle encoding an artificial

miRNA. In some embodiments, the method comprises delivering a viral particle encoding an antisense oligonucleotide (ASO). In some embodiments, the method may comprise delivering a viral particle comprising an expression cassette configured to target *MAPT* or *BIN1* mRNA. In some embodiments, the method may comprise delivering a viral particle comprising an expression cassette configured to target a vectorized antibody targeting tau or Bin1 protein. In some embodiments, the disorder-related polypeptide comprises the vectorized antibody targeting tau. In some embodiments, the disorder-related polypeptide comprises the vectorized antibody targeting the Bin1 protein.

[0139] In some embodiments, the expression cassette configured to target tau pathology in AD and/or associated disorders, is under control of a promoter. In some embodiments, the promoter may comprise a ubiquitous or neuron-specific promoter. In some embodiments, the ubiquitous promoter may comprise CBA, CAG, CAGG, CMV, H1, U6 or 7SK. In some embodiments, the neuron-specific promoter may comprise SYN1, NSE, CAMKII, or TUBB3. In some instances, the promoter is regulated by an exogenous small molecule.

[0140] In some embodiments, the method may comprise delivering a viral particle comprising an expression cassette configured to target A β pathology in AD and/or associated disorders. In some embodiments, the method comprises delivering a viral particle comprising an expression cassette (e.g., AAV genome) encoding an artificial miRNA. In some embodiments, the method comprises delivering a viral particle comprising an expression cassette (e.g., AAV genome) encoding an antisense oligonucleotide (ASO). In some embodiments, the method comprises delivering a viral particle comprising an expression cassette configured to target APP or PS1 gene. In some embodiments, the method comprises delivering a viral particle comprising an expression cassette configured to target a vectorized antibody targeting A β .

[0141] In some embodiments, the expression cassette configured to target A β pathology in AD and/or associated disorders, is under control of a promoter. In some embodiments, the promoter may comprise a ubiquitous or neuron-specific promoter. In some embodiments, the ubiquitous promoter may comprise CBA, CAG, CAGG, CMV, H1, U6 or 7SK. In some embodiments, the neuron-specific promoter may comprise SYN1, NSE, CAMKII, or TUBB3. In some instances, promoter is regulated by an exogenous small molecule.

[0142] In some embodiments, the method for treating AD and/or associated disorders, wherein patients comprise the APOE4 risk allele, the method may comprise delivering an expression cassette (e.g., AAV genome) encoding an ASO targeting APOE4 and/or encoding the protective allele APOE2.

[0143] In some embodiments, the method for treating AD and/or associated disorders is configured to target innate immune pathways contributing to neuroinflammation. In such methods, the method may comprise delivering an expression cassette (e.g., AAV genome) encoding an artificial miRNA or vectorized antibody targeting CD33 or encoding the soluble form of TREM2.

[0144] In some embodiments, wherein the method is configured to target innate immune pathways contributing to neuroinflammation, the expression cassette is under the control of a promoter. In such methods, AAV-encoded therapeutics are expressed under control of ubiquitous or neuron-specific promoters. In such embodiments, the ubiquitous promoter comprises CBA, CAG, CAGG, CMV, H1, U6, or 7SK. In such embodiments, the neuron-specific promoter comprises SYN1, NSE, CAMKII, or TUBB3.

[0145] In some instances, the viral particle encodes at least one nucleotide sequence. In some embodiments, at least one nucleotide sequence is configured to improve RNA stability. In some embodiments, wherein at least one nucleotide sequence is configured to improve RNA stability, at least one of the nucleotide sequences may comprise WPRE. In some embodiments, at least one nucleotide sequence is configured to target expression from liver and/or DRG.

[0146] In some embodiments, the expression cassette may comprise at least one transgene. In some embodiments, at least one transgene may comprise MAPT, BIN1, APP, PS1, APOE, CD33, TREM2, or a combination thereof. In some instances, the transgene expression is regulated by an exogenous small molecule.

Methods for treating Metachromatic Leukodystrophy

[0147] Metachromatic leukodystrophy (MLD) is an autosomal recessive neurodegenerative disorder caused by mutations in the enzyme arylsulfatase A (ARSA). Reduced levels of ARSA activity result in toxic accumulation of sulfatide characterized by degeneration of myelin-

forming cells (oligodendrocytes and Schwann cells) in the central and peripheral nervous system. This results in demyelination, dysfunction, degeneration of neurons and neuroinflammation (astrocytosis, microglial activation). Clinical manifestations are primarily in the nervous system, resulting in intellectual disability, emotional and behavioral problems, loss of motor skills (moving, speaking, swallowing), poor muscle function and paralysis, blindness, hearing loss and seizures. The current treatment for MLD involves autologous hematopoietic stem cell transplantation (HSCT) involving allogenic bone marrow transplant.

[0148] Certain aspects of the present disclosure relate to methods of treating MLD and/or increasing levels of an ARSA polypeptide in an individual in need thereof. In some embodiments, the disclosure provides methods of treating MLD by administering an effective amount of a viral particle of the disclosure for expressing an ARSA polypeptide. In some embodiments, the ARSA polypeptide is a wild type ARSA polypeptide.

[0149] In some embodiments, the method may provide for treating a human with MLD by administering an effective amount of a pharmaceutical composition comprising a recombinant viral vector encoding an ARSA polypeptide of the present disclosure. In some embodiments, the pharmaceutical composition comprises one or more pharmaceutically acceptable excipients.

Methods for treating Adrenoleukodystrophy and Adrenomyeloneuropathy

[0150] Certain aspects of the present disclosure relate to methods of treating Adrenoleukodystrophy (ALD) and Adrenomyeloneuropathy (ALM) in an individual in need thereof. In some embodiments, the disclosure provides methods of treating ALD and/or ALM by administering an effective amount of a viral particle of the disclosure. In some embodiments, the disclosure provides methods of treating a ALD and/or ALM by administering an effective amount of a viral particle comprising an expression cassette for expressing a disorder-related polypeptide or RNAi molecule. In some embodiments, the disorder-related polypeptide may comprise human ABCD1 polypeptide. In some embodiments, the expression cassette of the viral particle may comprise a transgene for encoding human ABCD1 polypeptide.

[0151] In some embodiments, expression of the disorder-related polypeptide is under control of a promoter. In some embodiments, the promoter may comprise a ubiquitous, neuron-specific, or astrocyte-specific promoter.

[0152] In some instances, the expression cassette of the viral particle may comprise at least one additional nucleotide sequence. In some embodiments, the at least one additional nucleotide sequence is for improving RNA stability. In some embodiments, the at least one additional nucleotide sequence is for improving RNA stability may comprise WPRE. In some embodiments, the at least one additional nucleotide sequence is for detargeting expression from liver and/or DRG.

[0153] In some instances, the expression cassette comprising the transgene encoding for the ABCD1 polypeptide is combined with an artificial miRNA. In alternative embodiments, two viral particle can be delivered to the patient, one encoding the ABCD1 polypeptide and the other encoding the artificial miRNA. In some embodiments, the artificial miRNA is configured for targeting ELOVL1. In some embodiments, the artificial miRNA is configured for targeting ELOVL1 to reduce expression of ELOVL1.

[0154] In some instances, promoter activity and/or transgene expression is/are regulated by an exogenous small molecule.

[0155] The expression cassette (e.g., expression cassette delivered in a rAAV particle) for expressing a disorder-related polypeptide is administered through various routes as provided in the present disclosure.

Methods for treating Amyotrophic Lateral Sclerosis and/or Frontotemporal Dementia

[0156] Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are two neurodegenerative diseases with considerable genetic overlap. ALS and FTD together are a heterogeneous group of disorders characterized by degeneration of the cerebral cortex and/or the spinal cord. This degeneration is related to dysfunction of one of several disease-causing genes, including, but not limited to: SOD1, TARDBP, C9orf72, FUS, GRN, VCP, TBK1, RIPK1, MAPT, NEK1, SQSTM1, CHCHD10. Studies involving these genes has led to the identification

of neurodegeneration pathways such as autophagy, RNA regulation, and vesicle and inclusion formation.

[0157] Certain aspects of the present disclosure relate to methods of treating Amyotrophic Lateral Sclerosis (ALS) and/or Frontotemporal Dementia (FTD) in an individual in need thereof. In some embodiments, the method of treating ALS and/or FTD comprises administering an effective amount of a viral particle of the disclosure. In some embodiments, the expression cassette of the viral particle may comprise a transgene. In some embodiments, the expression cassette is configured for encoding an artificial miRNA. In some embodiments, the expression cassette is configured for encoding a vectorized antibody. In some embodiments, the vectorized antibody is configured to target genes comprising SOD1, TARDBP, C9orf72 repeat expansion, FUS, RIPK1, or MAPT genes.

[0158] In some embodiments, the expression cassette is under control of a promoter. In some embodiments, the promoter may comprise a ubiquitous or neuron-specific promoter. In some embodiments, the ubiquitous promoter may comprise CBA, CAG, CAGG, CMV, H1, U6 or 7SK. In some embodiments, the neuron-specific promoter may comprise SYN1, NSE, CAMKII, or TUBB3. In some instances, promoter is regulated by an exogenous small molecule.

[0159] In some embodiments, the method may comprise regulating transgene expression by an exogenous small molecule.

[0160] In some instances, expression cassettes (e.g., AAV genomes) may encode for functional versions of a targeted gene.

[0161] In some instances, the expression cassette (e.g., AAV genome, genome, etc.) may encode artificial miRNA. In some embodiments, the artificial miRNA is configured to target endogenous, affected gene, as well as nucleotide sequences to improve RNA stability. In some embodiments, the artificial miRNA (e.g., at least one additional nucleotide sequence) may comprise WPRE. In some embodiments, at least one additional nucleotide sequence is configured to detarget expression from liver and/or DRG.

Methods for treating Neuropathic Pain

[0162] Neuropathic pain (NP) arises due to injury of the somatosensory nervous system. NP is characterized by dysfunction of dorsal root ganglia, spinal cord, and/or cerebral cortex. Often, NP is caused by nerve injury or trauma related to dysfunction of at least one of several genes, including, but not limited to: ADORA2A, CACNA1B, CACNA2D1, CACNA2D3, CACNA1H, CALCA, CALCB, GABBR1, GABBR2, GABRA1, GABRA2, GABRG1, GABRB3, GABRG2, GAD1, GAD2, HCN1, HCN2, HCN4, KCNA1, KCNA2, KCNB1, P2RX7, SCN9A, SCN10A, SCN11A, SLC12A2, SLC12A5, TRPA1, TRPV1. Patients with NP suffer not only from pain but also a high level of disability. Additionally, currently used therapeutics such as opioids can be highly addictive. Therefore, a cost-effective and non-addictive therapeutic is needed.

[0163] Certain aspects of the present disclosure relate to methods of treating Neuropathic Pain (NP) in an individual in need thereof. In some embodiments, the method of treating MP may comprise administering an effective amount of a viral particle of the disclosure. In some embodiments, the expression cassette may comprise a transgene. In some embodiments, the transgene (e.g., gene) may comprise ADORA2A, CACNA1B, CACNA2D1, CACNA2D3, CACNA1H, CALCA, CALCB, GABBR1, GABBR2, GABRA1, GABRA2, GABRG1, GABRB3, GABRG2, GAD1, GAD2, HCN1, HCN2, HCN4, KCNA1, KCNA2, KCNB1, P2RX7, SCN9A, SCN10A, SCN11A, SLC12A2, SLC12A5, TRPA1, or TRPV1.

[0164] In some embodiments, the expression cassette of the viral particle is configured for encoding an artificial miRNA. In some embodiments, the rAAV particle may comprise an artificial RNA. In some embodiments, the expression cassette of the viral particle is configured for encoding a target polypeptide. In some embodiments, the expression cassette of the viral particle is configured for encoding a target polypeptide (e.g., disorder-related polypeptide). In some embodiments, the expression cassette of the viral particle is configured for encoding the disorder-related polypeptide, wherein the disorder-related polypeptide may comprise ADORA2A, CACNA1B, CACNA2D1, CACNA2D3, CACNA1H, CALCA, CALCB, GABBR1, GABBR2, GABRA1, GABRA2, GABRG1, GABRB3, GABRG2, GAD1, GAD2, HCN1, HCN2, HCN4, KCNA1, KCNA2, KCNB1, P2RX7, SCN9A, SCN10A, SCN11A, SLC12A2, SLC12A5, TRPA1, TRPV1, or any combination thereof.

[0165] In some embodiments, the expression cassette of the viral particle may comprise at least one transgene for encoding at least one disorder-related polypeptide. In some embodiments, the expression cassette may comprise at least one transgene for encoding at least one polypeptide, wherein the at least one polypeptide may comprise ADORA2A, CACNA1B, CACNA2D1, CACNA2D3, CACNA1H, CALCA, CALCB, GABBR1, GABBR2, GABRA1, GABRA2, GABRG1, GABRB3, GABRG2, GAD1, GAD2, HCN1, HCN2, HCN4, KCNA1, KCNA2, KCNB1, P2RX7, SCN9A, SCN10A, SCN11A, SLC12A2, SLC12A5, TRPA1, TRPV1, or any combination thereof.

[0166] In some embodiments, the method may comprise delivering a viral particle encoding at least one artificial miRNA targeting ADORA2A, CACNA1B, CACNA2D1, CACNA2D3, CACNA1H, CALCA, CALCB, GABBR1, GABBR2, GABRA1, GABRA2, GABRG1, GABRB3, GABRG2, GAD1, GAD2, HCN1, HCN2, HCN4, KCNA1, KCNA2, KCNB1, P2RX7, SCN9A, SCN10A, SCN11A, SLC12A2, SLC12A5, TRPA1, TRPV1 or any combination thereof.

[0167] In some embodiments, the expression cassette is under control of a promoter. In some embodiments, the promoter may comprise a ubiquitous or neuron-specific promoter. In some embodiments, the ubiquitous promoter may comprise CBA, CAG, CAGG, CMV, H1, U6 or 7SK. In some embodiments, the neuron-specific promoter may comprise SYN1, NSE, CAMKII, or TUBB3. In some instances, promoter is regulated by an exogenous small molecule.

[0168] In some embodiments, the method may comprise regulating transgene expression by an exogenous small molecule.

[0169] In some instances, the expression cassette may also encode artificial miRNA. In some embodiments, the artificial miRNA is configured to target endogenous, affected gene, as well as nucleotide sequences to improve RNA stability. In some embodiments, the nucleotide sequences to improve RNA stability may comprise WPRE. In some embodiments, the expression cassette (e.g., the nucleotide sequences) is configured to detarget expression from liver and/or DRG.

Methods for treating Charcot-Marie-Tooth Diseases

[0170] Charcot-Marie-Tooth Diseases (CMT) is a neurodegenerative disorder that causes abnormalities in both sensory and motor nerves related to feet, legs, hands, and arms. CMT is a heterogenous group of peripheral neuropathies (e.g., affects nerves outside of the brain and spinal cord) affecting dorsal root ganglia and peripheral nerves. CMT is related to dysfunction of one of several genes, including, but not limited to: PMP22, GJB1, MFN2, TRPV4, NEFH, NEFL, DNM2, MPZ.

[0171] Certain aspects of the present disclosure relate to methods of treating Charcot-Marie-Tooth Diseases (CMT) in an individual in need thereof. In some embodiments, the method of treating CMT may comprise administering an effective amount of a viral particle of the disclosure. In some embodiments, the expression cassette of the viral particle may comprise a transgene. In some embodiments, the transgene may comprise PMP22, GJB1, MFN2, TRPV4, NEFH, NEFL, DNM2, or MPZ.

[0172] In some embodiments, the method for treating CMT may comprise delivering a viral particle comprising expression cassette encoding artificial miRNA. In some embodiments the artificial miRNA is configured to target PMP22 or TRPV4. In some embodiments, the expression cassette is configured to 'encode genes comprising GJB1 or MFN1'. In some embodiments, the expression cassette is configured to encode both an artificial miRNA targeting MFN2, NEFH, NEFL, DNM2, or MPZ along with targeted genes comprising GJB1 or MFN1.

[0173] In some embodiments, the expression cassette is under control of a ubiquitous promoter or a neuron-specific promoter. In some embodiments, the ubiquitous promoter may comprise CBA, CAG, CAGG, CMV, H1, U6, or 7SK. In some embodiments, the neuron-specific promoter may comprise SYN1, NSE, CAMKII, or TUBB3. In some embodiments, the promoter may comprise a Schwann cell-specific promoter. In some embodiments, the Schwann cell-specific promoter may comprise MBP, MPZ, or PMP22. In some embodiments, promoter activity is regulated by an exogenous small molecule.

[0174] In some instances, transgene expression is regulated by an exogenous small molecule.

[0175] In some instances, the expression cassette of the viral particle may comprise at least one additional nucleotide sequence. In some embodiments, at least one additional nucleotide

sequence is configured to improve RNA stability. In some embodiments, at least nucleotide sequence configured to improve RNA stability may comprise WPRE. In some embodiments, at least one additional nucleotide sequence may comprise a nucleotide sequence configured to detarget expression from the liver.

Methods for treating Fabry Disease

[0176] Fabry Disease (FD), otherwise known as alpha-galactosidase-A deficiency occurs when the enzyme alpha-galactosidase-A is unable to break down lipids. The buildup of lipid levels negatively affects function of the cerebral vasculature, heart, kidney, peripheral nervous system, and cerebral cortex. FD is caused by variants of the GLA gene that do not properly encode the Alpha-galactosidase-A enzyme.

[0177] Certain aspects of the present disclosure relate to methods of treating Fabry Disease (FD) in an individual in need thereof. In some embodiments, the method of treating FD may comprise administering an effective amount of a viral particle of the disclosure. In some embodiments, the expression cassette may comprise a transgene. In some embodiments, the transgene may comprise human GLA.

[0178] In some embodiments, the expression cassette of the viral particle is under control of a ubiquitous promoter or a neuron-specific promoter. In some embodiments, the ubiquitous promoter may comprise CBA, CAG, CAGG, CMV, H1, U6, or 7SK. In some embodiments, the Schwann cell-specific promoter may comprise MBP, MPZ, or PMP22. In some embodiments, promoter activity is regulated by an exogenous small molecule.

[0179] In some embodiments, the transgene expression is regulated by an exogenous small molecule.

[0180] In some instances, the human GLA gene is modified to include cell penetrating and/or signal peptides to enhance secretion and uptake.

[0181] In some instances, the expression cassette of the viral particle may comprise at least one additional nucleotide sequence. In some embodiments, at least one additional nucleotide sequence is configured to improve RNA stability. In some embodiments, at least nucleotide sequence configured to improve RNA stability may comprise WPRE. In some embodiments, the

at least one additional nucleotide sequence is configured to detarget expression from liver and/or DRG.

Methods for treating Angelman Syndrome

[0182] Angelman Syndrome (AS) is a neurodevelopmental disorder resulting in delayed development, problems with speech and balance, intellectual disability, and, sometimes, seizures. AS is caused by dysfunction of the UBE3A gene affecting the cerebral cortex. UBE3A encodes an E3 ubiquitin-protein ligase, which is part of the ubiquitin protein degradation system and is maternally expressed in the brain and spinal cord (central nervous system). AS is caused by materially inherited deletions of UBE3A.

[0183] Certain aspects of the present disclosure relate to methods of treating Angelman Syndrome (AS) in an individual in need thereof. In some embodiments, the method of treating AS may comprise administering an effective amount of a viral particle of the disclosure. In some embodiments, the expression cassette may comprise a transgene. In some embodiments, the transgene may comprise UBE3A. In some embodiments, the viral particle comprises an expression cassette encoding human UBE3A may also comprise an artificial miRNA. In some embodiments, the artificial miRNA is configured to target UBE3A-ATS.

[0184] In some embodiments, the expression cassette is under control of a promoter. In some embodiments, the expression cassette is under control of a ubiquitous promoter or a neuron-specific promoter. In some embodiments, the ubiquitous promoter may comprise CBA, CAG, CAGG, CMV, H1, U6, or 7SK. In some embodiments, the neuron-specific promoter may comprise SYN1, NSE, CAMKII, or TUBB3. In some embodiments, promoter activity is regulated by an exogenous small molecule.

[0185] In some embodiments, the method for treating AS may comprise expressing a transgene regulated by an exogenous small molecule.

[0186] In some instances, the expression cassette may comprise at least one additional nucleotide sequence. In some embodiments, the at least one additional nucleotide sequence is configured to improve RNA stability. In some embodiments, the at least nucleotide sequence

configured to improve RNA stability may comprise WPRE. In some embodiments, the at least one additional nucleotide sequence is configured to detarget expression from liver and/or DRG.

Methods for treating Fragile X Syndrome

[0187] Fragile X Syndrome (FXS) is a neurodevelopmental disorder caused by dysfunction of the FMR1 gene. FMR1 usually makes a protein called FMRP that is needed for brain development. People with FXS have the dysfunctional FMR1 gene, which results in developmental delays, learning disabilities and social and behavioral problems.

[0188] In some embodiments, the disclosure provides methods of treating Fragile X Syndrome (FXS) by administering an effective amount of a viral particle of the disclosure for expressing/encoding a disorder-related polypeptide of the present disclosure. In some embodiments, the disorder-related polypeptide may comprise human FMRP.

[0189] In some embodiments, the expression cassette is under control of a promoter. In some embodiments, the expression cassette is under control of a ubiquitous promoter or a neuron-specific promoter. In some embodiments, the ubiquitous promoter may comprise CBA, CAG, CAGG, CMV, H1, U6, or 7SK. In some embodiments, the neuron-specific promoter may comprise SYN1, NSE, CAMKII, or TUBB3. In some embodiments, promoter activity is regulated by an exogenous small molecule.

[0190] In some embodiments, the expression cassette may comprise a transgene. In some embodiments, the transgene may comprise FMR1. In some embodiments, the transgene expression is regulated by an exogenous small molecule.

[0191] In some embodiments, the expression cassette for expressing a disorder-related polypeptide is administered through various routes as provided herein.

[0192] In some instances, the expression cassette may comprise at least one additional nucleotide sequence. In some embodiments, the at least one additional nucleotide sequence is configured to improve RNA stability. In some embodiments, the at least nucleotide sequence configured to improve RNA stability may comprise WPRE. In some embodiments, the at least one additional nucleotide sequence is configured to detarget expression from liver and/or DRG.

Methods for treating Rett Syndrome

[0193] Rett Syndrome (RS) is a progressive neurodevelopmental disorder and a common cause of cognitive disability. The most common causes of RS are mutated variants of the MECP2 gene which affect the cerebral cortex. These gene mutations alter the structure of the MeCP2 protein or reduce the amount produced. The reduction of functional MeCP2 can impair the regulation of gene expression in brain cells and can disrupt alternative splicing of proteins that are important for communications between neurons.

[0194] In some embodiments, the disclosure provides methods of treating Rett Syndrome (RS) by administering an effective amount of a viral particle of the disclosure for expressing/encoding a disorder-related polypeptide of the present disclosure. In some embodiments, the disorder-related polypeptide is a wild type disorder-related polypeptide. In some embodiments, the disorder-related polypeptide may comprise methyl CpG binding protein 2 (MECP2).

[0195] In some embodiments, the expression cassette is under control of a promoter. In some embodiments, the expression cassette is under control of a ubiquitous promoter or a neuron-specific promoter. In some embodiments, the ubiquitous promoter may comprise CBA, CAG, CAGG, CMV, H1, U6, or 7SK. In some embodiments, the neuron-specific promoter may comprise SYN1, NSE, CAMKII, or TUBB3. In some embodiments, promoter activity is regulated by an exogenous small molecule.

[0196] In some embodiments, the expression cassette may comprise a transgene. In some embodiments, the transgene may comprise the transgene MECP2. In some embodiments, the transgene expression is regulated by an exogenous small molecule.

[0197] In some embodiments, the expression cassette for expressing a disorder-related polypeptide is administered through various routes as provided herein.

[0198] In some instances, the expression cassette may comprise at least one additional nucleotide sequence. In some embodiments, at least one additional nucleotide sequence is configured to improve RNA stability. In some embodiments, at least nucleotide sequence configured to improve RNA stability may comprise WPRE. In some embodiments, at least one additional nucleotide sequence is configured to detarget expression from liver and/or DRG.

Methods for treating Tuberous Sclerosis Complex

[0199] Tuberous Sclerosis Complex (TSC) is a rare genetic disorder caused by dysfunction of the *TSC1* or *TSC2* genes that results in spontaneous benign tumor growth in multiple organs throughout the body. *TSC1* is a tumor suppressor gene that encodes the growth inhibitory protein hamartin. *TSC2* is a tumor suppressor gene that encodes the growth inhibitory protein tuberin. Tuberin interacts with hamartin to form the TSC protein complex which functions in the control of cell growth. Dysfunction of *TSC1* or *TSC2* results in growth of tumors in tissues including but not limited to the brain, skin, heart, lungs, kidneys, and liver. Tumor growth in the cerebral cortex and ventricular space can cause seizure and hydrocephaly leading to neurodevelopmental delay, neurological disorders and in rare cases death.

[0200] In some embodiments, the disclosure provides methods of treating Tuberous Sclerosis Complex (TSC) by administering an effective amount of a viral particle of the disclosure for expressing/encoding a disorder-related polypeptide of the present disclosure. In some embodiments, the disorder-related polypeptide is a wild type disorder-related polypeptide. In some embodiments, the disorder-related polypeptide may comprise hamartin, tuberin, or a combination of both.

[0201] In some embodiments, the expression cassette is under control of a promoter. In some embodiments, the expression cassette is under control of a ubiquitous promoter or a neuron-specific promoter. In some embodiments, the ubiquitous promoter may comprise CBA, CAG, CAGG, CMV, H1, U6, or 7SK. In some embodiments, promoter activity is regulated by an exogenous small molecule.

[0202] In some embodiments, the expression cassette may comprise at least one transgene. In some embodiments, the at least one transgene may comprise the transgene *TSC1*, *TSC2*, or a combination of both. In some embodiments, the transgenic payload may comprise all or part of human *TSC1* and/or *TSC2* human cDNA. In some embodiments, the transgenic payload may comprise all or part of human *TSC1* and/or *TSC2* human cDNA that may include additional flexible linker sequence. In some embodiments, the transgene expression is regulated by an exogenous small molecule.

[0203] In some instances, the expression cassette is modified to include cell penetrating and/or signal peptides to enable secretion and uptake.

[0204] In some instances, the genetic payload may comprise at least one additional nucleotide sequence. In some embodiments, the at least one additional nucleotide sequence may comprise a nucleotide sequence to facilitate nuclear export. In some embodiments, the at least one additional nucleotide sequence may comprise a nucleotide sequence to improve RNA stability. In some embodiments, the nucleotide sequence to improve RNA stability may comprise a polyA tail. In some embodiments, the nucleotide sequence to improve RNA stability may comprise WPRE. In some embodiments, at least one additional nucleotide sequence is configured to detarget expression from liver and/or DRG.

[0205] In some instances, the expression cassette may comprise at least one tagging nucleotide sequence. In some embodiments, at least one tagging nucleotide sequence may comprise FLAG, His, Myc or any combination thereof.

[0206] In some embodiments, the viral particle for expressing a disorder-related polypeptide is administered through various routes as provided herein. In some embodiments, AAV administration is either intra-CSF (intrathecal, intra-cisterna magna, or intraventricular) or intravenous.

Methods for treating Neurofibromatosis type II

[0207] Neurofibromatosis type II (NF2) is a disorder characterized by nervous system and skin tumors and ocular abnormalities. Disruption in the function of the protein encoded by the NF2 gene has been implicated in tumorigenesis and metastasis. Dysfunction in the NF2 gene is caused by spontaneous genetic mutation.

[0208] In some embodiments, the disclosure provides methods of treating Neurofibromatosis type II (NF2) by administering an effective amount of a viral particle of the disclosure for expressing a disorder-related polypeptide. In some embodiments, the disorder-related polypeptide may comprise a polypeptide expressed by the gene NF2.

[0209] In some embodiments, the expression cassette of the viral particle is under control of a ubiquitous promoter, a neuron-specific promoter, or a Schwann cell-specific promoter. In some

embodiments, the ubiquitous promotor may comprise CBA, CAG, CAGG, CMV, H1, U6, or 7SK. In some embodiments, the neuron-specific promoter may comprise SYN1, NSE, CAMKII, or TUBB3. In some embodiments, the Schwann cell-specific promoter may comprise MBP, MPZ, or PMP22. In some embodiments, promoter activity is regulated by an exogenous small molecule.

[0210] In some embodiments, the expression cassette may comprise at least one transgene. In some instances, transgene expression is regulated by an exogenous small molecule. In some embodiments, the transgene may comprise human NF2. In some embodiments, the transgenic payload may comprise all or part of the human NF2 gene, an additional flexible linker sequence, or a combination of both.

[0211] In some instances, the expression cassette (e.g., AAV genome, genetic payload, etc.) is modified to include cell penetrating and/or signal peptides to enable secretion and uptake.

[0212] In some instances, the expression cassette may comprise at least one additional nucleotide sequence. In some embodiments, at least one additional nucleotide sequence may comprise a nucleotide sequence configured to detarget expression from liver and/or DRG.

[0213] In some instances, the expression cassette may comprise at least one tagging nucleotide sequence. In some embodiments, at least one tagging nucleotide sequence may comprise FLAG, His, Myc or any combination thereof.

[0214] In some embodiments, the viral particle for expressing a disorder-related polypeptide is administered through various routes as provided herein. In some embodiments, AAV administration is either intra-CSF (intrathecal, intra-cisterna magna, or intraventricular) or intravenous.

Methods for treating Pompe

[0215] Pompe is a neuromuscular disease with a caused by autosomal recessive mutations in the acid alpha-glucosidase (GAA) gene. The GAA gene encodes lysosomal alpha-glucosidase, which is essential for the degradation of glycogen to glucose in lysosomes. The hallmark pathology of Pompe is glycogen storage in the lysosomes of heart, skeletal muscles and central

nervous system, which lead to hypotonia, cardiomyopathy, respiratory deficiency, and neurological symptoms or even pre-mature death of patients with early onset.

[0216] In some embodiments, the disclosure provides methods of treating Pompe by administering an effective amount of a viral particle of the disclosure for expressing/encoding a disorder-related polypeptide of the present disclosure. In some embodiments, the disorder-related polypeptide comprises the lysosomal enzyme acid alpha-glucosidase (GAA). In some embodiments, the expression cassette is configured for encoding the human GAA polypeptide.

[0217] In some embodiments, the expression cassette is under control of a promoter. In some embodiments, the promoter may comprise a ubiquitous promoter, or a muscle-specific promoter. In some embodiments, the ubiquitous promoter may comprise CBA, CAG, CAGG, CMV, H1, U6, or 7SK. In some embodiments, the muscle-specific promoter may comprise Spc5-12, Desmin, or α MyHC. In some embodiments, promoter activity is regulated by an exogenous small molecule.

[0218] In some embodiments, the expression cassette may comprise at least one transgene. In some embodiments, at least one transgene may comprise the human GAA gene. In some instances, transgene expression is regulated by an exogenous small molecule.

[0219] In some instances, the human GAA gene is modified to comprise cell penetrating and/or signal peptides. In some instances, the human GAA gene may comprise cell penetrating and/or signal peptides to enable secretion and uptake.

[0220] In some instances, the expression cassette of the viral particle may comprise at least one additional nucleotide sequence. In some embodiments, at least one additional nucleotide sequence is configured to improve RNA stability. In some embodiments, at least one additional nucleotide sequence configured to improve RNA stability may comprise WPRE. In some embodiments, the at least one additional nucleotide sequence configured to detarget expression from liver and/or DRG.

[0221] In some embodiments, the expression cassette for expressing a disorder-related polypeptide is administered through various routes as provided herein. In some embodiments, the method for treating Pompe may comprise delivering the viral particle via IV and/or intra-CSF routes to target both the skeletal muscle and CNS components of the disease.

Methods for treating Globoid cell Leukodystrophy

[0222] Globoid cell leukodystrophy (GLD; Krabbe disease) an autosomal recessive neurodegenerative disorder caused by a defect in the GALC gene. The GALC gene encodes for the lysosomal enzyme, galactosylceramidase. Without the enzyme, buildup of cytotoxic galactosylsphingosine, a GALC substrate, drives death of oligodendrocytes and Schwann cells, leading to widespread myelin loss.

[0223] In some embodiments, the disclosure provides methods of treating Globoid cell leukodystrophy (GLD) by administering an effective amount of a viral particle of the disclosure for expressing/encoding a disorder-related polypeptide of the present disclosure. In some embodiments, the disorder-related polypeptide comprises the lysosomal enzyme, galactosylceramidase. In some embodiments, the method for treating GLD is configured to prevent buildup of cytotoxic galactosylsphingosine, a GALC substrate, which drives death of oligodendrocytes and Schwann cells, leading to widespread myelin loss.

[0224] In some embodiments, the expression cassette of the viral particle is under control of a ubiquitous promoter or a neuron-specific promoter. In some embodiments, the ubiquitous promoter may comprise CBA, CAG, CAGG, CMV, H1, U6, or 7SK. In some embodiments, the neuron-specific promoter may comprise SYN1, NSE, CAMKII, TUBB3. In some embodiments, the promoter may comprise a Schwann cell-specific promoter. In some embodiments, the Schwann cell-specific promoter may comprise MBP, MPZ, or PMP22. In some embodiments, promoter activity is regulated by an exogenous small molecule.

[0225] In some embodiments, the expression cassette may comprise at least one transgene. In some embodiments, at least one transgene may comprise the human GALC gene. In some instances, transgene expression is regulated by an exogenous small molecule.

[0226] In some instances, the human GALC gene is modified to comprise cell penetrating and/or signal peptides to enable secretion and uptake.

[0227] In some instances, the expression cassette may comprise at least one additional nucleotide sequence. In some embodiments, at least one additional nucleotide sequence is configured to improve RNA stability. In some embodiments, at least one additional nucleotide

sequence may comprise WPRE. In some embodiments, at least one additional nucleotide sequence is configured to detarget expression from liver and/or DRG.

[0228] In some embodiments, the viral particle is administered through various routes as provided herein. In some embodiments, the method for treating GLD may comprise delivering an expression cassette (e.g., AAV genome) via IV and/or intra-CSF routes to target both the PNS and CNS components of the disease.

Methods for treating Multiple Sclerosis

[0229] Globoid cell leukodystrophy (GLD; Krabbe disease) an autosomal recessive neurodegenerative disorder caused by a defect in the GALC gene. The GALC gene encodes for the lysosomal enzyme, galactosylceramidase. Without the enzyme, buildup of cytotoxic galactosylsphingosine, a GALC substrate, drives death of oligodendrocytes and Schwann cells, leading to widespread myelin loss.

[0230] In some embodiments, the disclosure provides methods of treating Multiple Sclerosis (MS) by administering an effective amount of a viral particle of the disclosure for expressing/encoding a disorder-related polypeptide. In some embodiments, the disorder-related polypeptide comprises a vectorized antibody against CD40L.

[0231] In some embodiments, the expression cassette of the viral particle is under control of a promoter. In some embodiments, the promoter comprises a ubiquitous promoter or a neuron-specific promoter. In some embodiments, the ubiquitous promoter may comprise CBA, CAG, CAGG, CMV, H1, U6, or 7SK. In some embodiments, the neuron-specific promoter may comprise SYN1, NSE, CAMKII, or TUBB3. In some embodiments, promoter activity is regulated by an exogenous small molecule.

[0232] In some embodiments, the expression cassette may comprise at least one transgene. In some embodiments, the at least one transgene may encode for a vectorized antibody against CD40L. In some instances, transgene expression is regulated by an exogenous small molecule.

[0233] In some instances, the vectorized antibody is modified to comprise cell penetrating and/or signal peptides to enable secretion and uptake.

[0234] In some instances, the expression cassette may comprise at least one additional nucleotide sequence. In some instances, the expression cassette (e.g., AAV genome) may comprise at least one additional nucleotide sequence configured to improve RNA stability. In some embodiments, at least one additional nucleotide sequence configured to improve RNA stability may comprise WPRE. In some embodiments, at least one additional nucleotide sequence configured to de-target expression from liver and/or DRG.

[0235] In some embodiments, the viral particle for expressing a disorder-related polypeptide is administered through various routes as provided herein.

Methods for treating Gaucher Disease

[0236] Gaucher Disease (GD) is an autosomal recessive lysosomal storage disorder caused by mutations in GBA1, the gene encoding glucocerebrosidase (GCase). Reduction or loss of GCase activity leads to the accumulation of toxic lipid substrates, disrupting cellular homeostasis. GD is further GD Type 1 (GD1) patients typically present with splenomegaly, hepatomegaly, and anemia or thrombocytopenia, while GD Type 3 (GD3) patients present with debilitating neurological symptoms and associated systemic manifestations of GD1. GD Type 2 (GD2) is the most severe form of the disease affecting infants before 6 months of age and in most cases causing early death by 2-3 years. Like GD3, GD2 also has CNS symptoms at a much higher severity. Current treatment landscape for GD includes enzyme replacement therapy for GD1 patients that alleviate symptoms but since ERTs fail to enter the brain, they do not help GD2 and GD3 patients that have CNS symptoms. Furthermore, GD1 patients have a lifelong burden of having to take bi-weekly infusion of the ERT.

[0237] In some embodiments, the disclosure provides methods of treating Gaucher Disease (GD) by administering an effective amount of a viral particle of the disclosure for expressing/encoding glucocerebrosidase (GCase). In some embodiments, the GCase (e.g., GCase peptide) is a wild type peptide. In some embodiments, the method may comprise restoring the loss of GCase activity. In some embodiments, given the disease symptoms, intra-CSF dosing for GD2 and GD3; IV dosing for GD1 of the same AAV gene therapy product would be ideal.

Methods for treating GBA-PD

[0238] About 5-10% of Parkinson's disease (PD) patients which is 0.5-1 million are carriers of GBA1 mutation in one of their alleles. GBA1 is an extremely well-credentialed target in GBA-PD. Just like with sporadic PD, there are no disease-modifying therapies yet. Current treatment options include Levodopa and/or dopamine agonists for symptom management but most patients notice the effects of these drugs wearing off over time.

[0239] In some embodiments, the disclosure provides methods of treating GBA-PD by administering an effective amount of an expression cassette (e.g., an expression cassette delivered in a rAAV particle) for expressing/encoding GBA1 polypeptide. In some embodiments, the method for treating GBA-PD may comprise a one-time administration of AAV-GBA1 delivered via intra-CSF administration to specifically target the CNS.

Methods for treating Huntington's Disease (HD)

[0240] HD is caused by a combination of hereditary and somatic expansion of trinucleotide repeats in the gene *HTT* that drives HD pathology in cerebral cortex, caudate, and putamen. Somatic instability of existing expansions is linked to the activity of DNA replication and repair components encoded by the genes including but not limited to *MSH2*, *MSH3*, *PMS1*, *PMS2*, *FAN1*, *RRM2B*, *LIG1*, *TCERG1*, and *MLH3*.

[0241] In some *embodiments*, the viral particles of the disclosure can be used for the treatment of HD. In some embodiments, treatment of HD involves delivery of an AAV genome encoding artificial microRNA, antisense oligonucleotide, linearized antibody, or nanobody targeting the *HTT* gene and/or *MSH2*, *MSH3*, *PMS1*, *PMS2*, *FAN1*, *RRM2B*, and *MLH3*, either alone or in combination, under control of ubiquitous (CBA, CAG, CAGG, CMV, H1, U6, 7SK, or others to be determined) or neuron-specific (*SYN1*, *NSE*, *CAMKII*, *TUBB3*, or others to be determined) promoters. In some instances, promoter activity and/or transgene expression is/are regulated by an exogenous small molecule. In other instances, treatment may encode biological machinery, including DNA binding protein, effector protein, and/or nucleotide guide molecule, required for editing to remove repeat expansions from the *HTT* gene.

VII. Kits or Articles of Manufacture

[0242] The expression cassettes (*e.g.*, an expression cassette for expressing a disorder-related polypeptide, such as a wild type human disorder-related polypeptide), rAAV vectors, particles, and/or pharmaceutical compositions as described herein is/are contained within a kit or article of manufacture, *e.g.*, designed for use in one of the methods of the disclosure as described herein.

[0243] Generally, the system comprises a cannula, one or more syringes (*e.g.*, 1, 2, 3, 4 or more), and one or more fluids (*e.g.*, 1, 2, 3, 4 or more) suitable for use in the methods of the disclosure.

[0244] In some embodiments, the syringe is any suitable syringe, provided it is capable of being connected to the cannula for delivery of a fluid. In some embodiments, the system has one syringe. In some embodiments, the system has two syringes. In some embodiments, the system has three syringes. In some embodiments, the system has four or more syringes. The fluids suitable for use in the methods of the disclosure include those described herein, for example, one or more fluids each comprising an effective amount of one or more vectors as described herein, and one or more fluids comprising one or more therapeutic agents.

[0245] In some embodiments, the kit comprises a single fluid (*e.g.*, a pharmaceutically acceptable fluid comprising an effective amount of the vector). In some embodiments, the kit comprises 2 fluids. In some embodiments, the kit comprises 3 fluids. In some embodiments, the kit comprises 4 or more fluids. A fluid may include a diluent, buffer, excipient, or any other liquid described herein or known in the art suitable for delivering, diluting, stabilizing, buffering, or otherwise transporting an expression cassette for expressing a disorder-related polypeptide or rAAV vector composition of the present disclosure. In some embodiments, the kit comprises one or more buffers, *e.g.*, an aqueous pH buffered solution. Examples of buffers may include without limitation phosphate, citrate, Tris, HEPES, and other organic acid buffers.

[0246] In some embodiments, the kit comprises a container. Suitable containers may include, *e.g.*, vials, bags, syringes, and bottles. In some embodiments, the container is made of one or more of a material such as glass, metal, or plastic. In some embodiments, the container is used to hold a rAAV composition of the present disclosure. In some embodiments, the container may also hold a fluid and/or other therapeutic agent.

[0247] In some embodiments, the kit comprises an additional therapeutic agent with a rAAV composition of the present disclosure. In some embodiments, the rAAV composition and the additional therapeutic agent are mixed. In some embodiments, the rAAV composition and the additional therapeutic agent are kept separate. In some embodiments, the rAAV composition and the additional therapeutic agent are in the same container. In some embodiments, the rAAV composition and the additional therapeutic agent are in different containers. In some embodiments, the rAAV composition and the additional therapeutic agent are administered simultaneously. In some embodiments, the rAAV composition and the additional therapeutic agent are administered on the same day. In some embodiments, the rAAV composition is administered within one day, two days, three days, four days, five days, six days, seven days, two weeks, three weeks, four weeks, two months, three months, four months, five months, or six months of administration of the additional therapeutic agent.

[0248] In some embodiments, the kit comprises a therapeutic agent to transiently suppress the immune system prior to AAV administration. In some embodiments, patients are transiently immune suppressed shortly before and after injection of the virus to inhibit the T cell response to the AAV particles (*e.g.*, see Ferreira *et al.*, *Hum. Gene Ther.* 25:180-188, 2014). In some embodiments, the kit further provides cyclosporine, mycophenolate mofetil, and/or methylprednisolone.

[0249] The rAAV particles and/or compositions of the disclosure may further be packaged into kits including instructions for use. In some embodiments, the kits further comprise a device for delivery (*e.g.*, any type of parenteral administration described herein) of compositions of rAAV particles. In some embodiments, the instructions for use include instructions according to one of the methods described herein. In some embodiments, the instructions are printed on a label provided with (*e.g.*, affixed to) a container. In some embodiments, the instructions for use include instructions for administering to an individual (*e.g.*, a human) an effective amount of rAAV particles, *e.g.*, for treating a neurodegenerative disease in an individual.

EXAMPLES

[0250] The invention will be more fully understood by reference to the following examples. They should not, however be construed as limiting the scope of the invention. It is understood

that the examples and embodiments described herein are for illustrative purposes only and that various modification or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended embodiments.

Example 1: Focused ultrasound increases gene delivery to deep brain structure following the administration of a recombinant adeno-associated virus in the cerebrospinal fluid

General Methods

Animals

[0251] Male Sprague Dawley rats (average 250 g) were ordered from Charles River either pre-cannulated in the cisterna magna or without cannula. The cannula was flushed to keep it open upon arrival with artificial cerebrospinal fluid (Bio-technie/Tocris, cat no 3525) in the volume indicated by the vendor as the void volume of the cannula. The experiments were conducted within 3 days of arrival of the animals to avoid clotting of the cannula prior to use. Animals were kept in a 12/12 light/dark cycle with food and water ad libitum and a temperature of 18° C - 22° C and humidity of 40%-60%. Animal work was performed according to the Canadian Council on Animals Care Policies & Guidelines and approved by the Sunnybrook Research Institute Animal Care Committee.

Adeno-associated virus

[0252] AAV2-HBKO encoding GFP under a CAG promoter was produced as previously described using polyethyleneimine transfection of HEK293T cells (J. A. Sullivan, et al., Rationally designed AAV2 and AAVrh8R capsids provide improved transduction in the retina and brain. *Gene Therapy* 25, 205–219 (2018); J. Naidoo, et al., Extensive Transduction and Enhanced Spread of a Modified AAV2 Capsid in the Non-human Primate CNS. *Molecular Therapy* 26, 1–13 (2018)). All animals, irrespective of administration route, were injected with 5.4×10^{11} GC AAV2-HBKO in 45 μ L sterile phosphate buffered saline (PBS) corresponding to a dose of 2.16×10^{12} GC/kg. Injection in intravenous catheters was followed by flushing with 0.2 mL 0.9% saline and injection in ICM cannulas was followed by flushing with artificial cerebrospinal fluid corresponding to the dead volume of the cannula as informed by the vendor.

ICM injection of gadolinium

[0253] Gadolinium (Gadodiamide MRI contrast agent, Omniscan, GE Healthcare Canada, Mississauga, ON, Canada) was injected in the ICM cannula followed by flushing with artificial cerebrospinal fluid corresponding to the dead volume in the cannula. To mimic the injections of AAV2-HBKO, gadolinium was injected in a volume of 45 μ L. MR images were acquired using a 7.0 T MRI instrument (BioSpin 7030; Bruker; Billerica, USA) at 45, 55, 70 and 80 minutes following injection.

FUS-MB treatment

[0254] For a detailed description of the FUS-MB treatment please see Z. Noroozian, et al., MRI-guided focused ultrasound for targeted delivery of rAAV to the brain. *Methods Mol Biol.* 1950, 177–197 (2019). Animals were anaesthetized with isoflurane and a tail-vein catheter was inserted followed by hair removal on the animal head to avoid trapping of air bubbles in the ultrasound gel. The animals were placed supine on an MR-compatible sled and T2-weighted MR images were acquired using a 7.0 T MRI instrument (BioSpin 7030; Bruker; Billerica, USA) and used for MR-guided targeting of the FUS. FUS was applied using a 0.58 MHz spherically focused transducer (75 mm outer diameter, 26 mm inner diameter, 60 mm radius of curvature) and an in-house manufactured system (prototype for LP100; FUS Instruments, Toronto, Canada). DEFINITY® microbubbles (0.2 mL/kg) were injected immediately upon FUS application at a fixed pressure of 0.32 MPa. For intravenous injection of AAV2-HBKO, the injection was performed immediately after injection of microbubbles followed by intravenous administration of 0.2 mL/kg gadolinium to visualize BBB permeability on T1-weighted MR images.

Tissue collection

[0255] Four weeks following AAV delivery, animals were deeply anaesthetized using 75 mg/kg ketamine and 10 mg/kg xylazine followed by transcardial perfusion with 0.9% saline. Brains were collected; one hemisphere was post-fixed for 16 h in 4% paraformaldehyde in 0.1 M PO_4 , followed by transfer to 30% sucrose, and the other hemisphere was dissected in brain

regions and flash frozen on dry ice. Organs and spines were collected and post-fixed for 16 h in 4% paraformaldehyde in 0.1 M PO₄ followed by transfer to PBS.

Immunohistochemistry and imaging

[0256] Brains were sectioned horizontally into 40 µm thick free-floating sections on a sliding microtome. Sections were washed three times for 10 minutes in PBS followed by antigen retrieval in 10 mM Tris base with 0.05% (v/v) Tween 20 and 1 mM EDTA (pH 9) at 70° C for 60 minutes. After allowing the sections to cool, washing in PBS wash repeated, followed by incubation for 120 minutes in blocking buffer (PBS with 0.3% Triton X-100, 3% (w/v) bovine serum albumin, and 10% (v/v) donkey serum). Sections were incubated with primary antibodies in blocking buffer overnight followed by washing in PBS and incubation overnight with secondary antibodies in blocking buffer. Staining with DAPI was performed by incubation for 10 minutes in PBS followed by washing in PBS, mounting on glass slides with polyvinyl alcohol medium and DABCO (Millipore, cat no 10981), and covering with glass coverslips. Primary antibodies include chicken anti-GFP 1:1000 (Abcam, ab13970) and guineapig anti-NeuN 1:500 (Millipore, ABN90). Secondary antibodies were purchased from Jackson ImmunoResearch and diluted 1:400.

[0257] Whole section images were acquired with a 10x objective using a Zeiss Axio Scan.Z1 slide scanner. Images used for quantifications were acquired with a 20x objective using a Leica Stellaris with white light laser.

RNA extraction and qPCR

[0258] Brain tissue was homogenized in 1 mL Trizol (Thermo Fisher, cat no 15596018) using a bead homogenizer followed by addition of 200 µL chloroform and 15 minutes centrifugation at 12,000xg. The supernatant was mixed 1:1 with 70% ethanol and RNA was extracted using PureLink RNA Mini Kit (Thermo Fisher cat no 12183018A) according to manufacturer's protocol. RNA concentrations were measured using a Nanodrop 2000 (Thermo Fisher). One µg RNA was used for cDNA preparation using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, cat no 4368814) according to manufacturer's protocol. cDNA was diluted 1:50 and 5 µL was added to each well of a 385-well plate together with 1 µL of each

reverse and forward primers (from a 10 μ M dilution), and 7 μ L SYBR Green qPCR master mix (Thermo Fisher, cat no 4472908). The following primers were used: GFP forward primer 5'-ACTACAACAGCCACAACGTCTATATCA-3' (SEQ ID NO: 13), GFP reverse primer 5'-GGCGGATCTTGAAGTTCACC-3' (SEQ ID NO: 14), and as reference genes Hprt1 forward primer 5'-TCCTCAGACCGCTTTTCCCGC-3' (SEQ ID NO: 15), Hprt1 reverse primer 5'-TCATCATCACTAATCACGACGCTGG-3' (SEQ ID NO: 16), Pgk1 forward primer 5'-ATGCAAAGACTGGCCAAGCTAC-3' (SEQ ID NO: 17), Pgk1 reverse primer 5'-AGCCACAGCCTCAGCATATTTTC-3' (SEQ ID NO: 18). Samples were run on a QuantStudio 6 Pro Real-Time PCR System (Applied Biosystem) and the program: hold 50° C for 2 min, 95° C for 10 min, 40 cycles of 95° C for 15 sec and 60° C for 1 min. Results were analyzed using the $2^{-\Delta\Delta C_T}$ method with the average of the reference genes.

DNA extraction and quantification of GFP genome copies

[0259] DNA was extracted from organs using QIAamp DNA FFPE Tissue Kit (Qiagen, cat no 56404) and from blood samples using DNeasy Blood and Tissue Kit (Qiagen, cat no 69504) according to manufacturer's protocol except for the final elution step being done with a low TE buffer instead of the elution buffer provided in the kit. Digital droplet PCR analysis was conducted as previously described in detail (*see* R. H. Kofoed, et al., The engineered AAV2-HBKO promotes non-invasive gene delivery to large brain regions beyond ultrasound targeted sites. *Molecular Therapy - Methods & Clinical Development* 27, 167–184 (2022)). GFP primers were 50-ACT ACA GCC ACA ACG TCT ATA TCA-30 (SEQ ID NO: 19) and reverse primer 50-GGCGGATCTTGAAGTTCACC-30 (SEQ ID NO: 20) (Invitrogen) and the probe was 50-6-FAM-CCG ACA AGC-ZENAGA AGA ACG GCA TCA-Iowa Black FQ-30 (Integrated DNA Technologies, Coralville, IA, USA). Rpp30 reference gene primers and probe were obtained as a ddPCR Copy Number Assay (Bio-Rad, Part number 10042961, Assay ID dRnoCNS421683336).

Spine analysis

[0260] Rat spines were excised by disconnecting the ribs and surrounding tissue. Subsequently, the spines were post-fixed overnight in a solution of 4% paraformaldehyde in 0.1 M PO₄. The spine underwent a 15-min wash in PBS and was then incubated for approximately

30 days in 10% EDTA in PBS at 37° C, pH 7.5 and on a rotatory shaker. The EDTA solution was changed twice daily, and the bone was regularly inspected until it reached the necessary softness for sectioning. After another 15-min PBS wash, the spine was post-fixed in 4% paraformaldehyde for 60 minutes at room temperature, followed by 15-min PBS wash. Spines were incubated in progressively increasing concentrations of sucrose in 0.1 M PO₄, starting from 10% and incrementally reaching 20% and 30% sucrose daily, all at 4° C, shaking. Subsequently, a section from the thoracic part of the spines were embedded in an 8x8 mm tissue embedding mold (Electron microscopy sciences, 70180) by freezing in Tissue-Tek OCT (Sakura, Torrance, USA) and sectioned into 12-µm-thick sections using a Leica CM3050 S cryostat. Sections were mounted onto Apex Superior Adhesive slides (Leica, 3800080E-144) and stored at -800C until staining. The spine sections were equilibrated to room temperature and washed 3 times 10 minutes in PBS, followed by incubation for 2 hours in blocking solution (PBS with 0.3% Triton X-100, 3% w/v bovine serum albumin, and 10% v/v donkey serum) in a humidify chamber. Sections were incubated overnight with primary antibodies in blocking solution at 4° C followed by three 10-min washes in PBS at room temperature. Subsequently, sections were incubated overnight at 4° C with secondary antibodies in blocking solution, followed by incubation in PBS with DAPI (1:10,000) (Sigma, D9542) for 10 min. Tissues were washed twice for 10 minutes in PBS at room temperature and once for 10 minutes in 0.1 M PO₄ before adding polyvinyl alcohol medium and DABCO (Millipore, 10,981), and covered with a glass coverslip. All solutions were directly applied to the mounted tissue. To prevent solutions from sliding off the slides a hydrophobic pen was utilized to outline the tissue's edges, ensuring the localization of reagents on tissue specimens.

Statistics

[0261] Normal distribution of qPCR data was determined using Shapiro-Wilk test. Non-transformed data from all brain regions was not normality distributed. Log10 transformation of results from the hippocampus, brainstem, midbrain, cortex1 and cortex2 did not result in normal distribution, and statistical analysis of qPCR data from these brain regions was therefore done using a non-parametric Kruskal-Wallis test with post hoc Dunn's test. All bars represent mean +/- standard deviation, ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05. N numbers are indicated in each figure legend.

Results

Kinetics of AAV distribution following ICM delivery and timing relative to FUS-MB application

[0262] To elucidate the ability of FUS-MB to increase brain delivery of ICM-administered AAV, first a series of pilot studies were performed to determine the optimal timepoint for AAV injection relative to FUS-MB. Distribution route #1 (**FIG. 1D**) depends on the AAV being present in the perivascular space at the time of FUS-MB application to take advantage of the putative pumping effect. To determine the kinetics of substances injected ICM in the rat model, a magnetic resonance imaging (MRI) contrast agent, gadolinium, was administered via ICM and MR imaging at 45, 55, 70 and 80 minutes post administration was performed (**FIGS. 2A-E**). Gadolinium enhancement (white) was clearly seen in brain areas proximal to the subarachnoid space containing cerebrospinal fluid (e.g., cortical areas, hippocampal formation, cerebellum, and ventral part of midbrain and brainstem) (**FIGS. 2B-E**). Gadolinium enhancement increased with time in brain structures further away from the subarachnoid space, such as the thalamus, when going from 45 minutes to 80 minutes post administration (gadolinium enhancement (white) increased in **FIG. 2E-e1** compared to **FIG. 2B-b1**). This suggests a gradual diffusion of the ICM-injected gadolinium into the brain parenchyma, perhaps via the perivascular space. The striatum remained without significant gadolinium enhancement at the time points investigated and it was chosen for FUS-targeting as a brain region with poor access to ICM-injected substances in this rat model. Because the diffusion of gadolinium into the brain parenchyma continued from 45 minutes to 80 minutes post-injection and AAV is larger (~26 nm and >3,500 kDa) than gadolinium (~0.5 kDa), 120 minutes was chosen as a possible timepoint when ICM-injected AAV could be present in the perivascular space. It remained possible that small amounts of gadolinium in the perivascular space may not have been visible on the MR images. To avoid the potential of losing a significant amount of AAV from the perivascular space into the blood before FUS-MB application, 60 minutes was also chosen as a timepoint for FUS-MB application following AAV injection ICM.

[0263] A pilot study was conducted to determine the feasibility of FUS-MB delivery of ICM-injected AAV to the brain using five treatment groups. Group 1: as a positive control of FUS-MB brain delivery animals were injected intravenously with AAV during FUS-MB

application as routinely done (**FIG. 2F**) (*see* R. H. Kofoed, et al., Efficacy of gene delivery to the brain using AAV and ultrasound depends on serotypes and brain areas. *Journal of Controlled Release* 351, 667–680 (2022); R. H. Kofoed, et al., The engineered AAV2-HBKO promotes non-invasive gene delivery to large brain regions beyond ultrasound targeted sites. *Molecular Therapy - Methods & Clinical Development* 27, 167–184 (2022); R. H. Kofoed, et al., Transgene distribution and immune response after ultrasound delivery of rAAV9 and PHP.B to the brain in a mouse model of amyloidosis. *Molecular Therapy - Methods & Clinical Development* 23, 390–405 (2021). Groups 2 and 3: AAV was injected 120 minutes and 60 minutes, respectively, before FUS-MB application to determine FUS-MB delivery to the brain of ICM-injected AAV (**FIG. 2G**). Group 4: to determine the relevance of distribution route #2 (**FIG. 1E**) a group of animals was injected with AAV 10 minutes post-FUS-MB application to investigate if AAV is delivered to the FUS-targeted site without potential perivascular pumping (i.e., no AAV in the perivascular space at the time of FUS-MB application) (**FIG. 2G**). Group 5: a group of animals was injected with AAV ICM without FUS-MB application (**FIG. 2H**). FUS was targeted bilaterally in two spots in the striatum (**FIGS. 2F-G**). Modified AAV2 (AAV2-HBKO) was utilized, which provided increased distribution in the brain parenchyma following intravenous injection and combined with FUS-MB targeting to the striatum, as well as a decreased uptake in the liver compared to AAV9 (*see* R. H. Kofoed, et al., The engineered AAV2-HBKO promotes non-invasive gene delivery to large brain regions beyond ultrasound targeted sites. *Molecular Therapy - Methods & Clinical Development* 27, 167–184 (2022)). AAV2-HBKO encoded green fluorescent protein (GFP) under control of the ubiquitous promoter CAG. AAV2-HBKO was administered at a dose of 2.16×10^{12} GC/kg irrespective of the administration route, which is within the range previously successfully used for FUS-MB-mediated AAV administration (e.g., delivery) to the brain following intravenous injection (1.67×10^{12} GC/kg – 1.67×10^{13} GC/kg) (*see* R. H. Kofoed, et al., The engineered AAV2-HBKO promotes non-invasive gene delivery to large brain regions beyond ultrasound targeted sites. *Molecular Therapy - Methods & Clinical Development* 27, 167–184 (2022)). Four weeks following AAV delivery and bilateral FUS-MB targeting to the striatum, the animals were sacrificed. One hemisphere was used for immunohistochemical (IHC) analysis of protein expression and the other hemisphere was used for reverse transcription quantitative polymerase chain reaction (qPCR) analysis of mRNA expression (**FIG. 2I**).

[0264] Images of sections stained with anti-GFP antibodies showed GFP expression in the FUS spots of all FUS-MB treated groups, irrespective of route of administration and injection timepoint, though with varying levels of expression (**FIG. 2J**). In animals injected intravenously with AAV, GFP expression was only visible in the FUS spots, whereas in animals injected ICM with AAV expression was visible in FUS spots as well as in cortical regions, hippocampus, and the midbrain (**FIG. 2J**). Analysis by qPCR of the FUS-targeted striatum showed the highest GFP mRNA levels in animals injected intravenously with AAV during FUS-MB, followed by animals injected ICM with AAV 120 minutes prior to FUS-MB treatment (**FIG. 2K**). Animals injected ICM with AAV 60 minutes prior to FUS-MB and 10 minutes post FUS-MB showed similar levels of GFP mRNA expression, trending towards a higher level of expression than animals injected ICM with AAV without FUS-MB (**FIG. 2K**). It was not possible to conduct a meaningful statistical analysis of the pilot study due to the variability between animals and the low number of animals per group (n=3-4). However, the IHC analysis confirms GFP expression in the FUS-targeted spots and hence the ability of FUS-MB to modulate brain delivery of AAV injected ICM. In addition, the observed GFP protein expression in the FUS spot in animals injected ICM with AAV 10 minutes post-FUS-MB suggests that distribution route #2 (**FIG. 1E**) is possible because in this group the AAV is not in the perivascular space when FUS-MB and the potential pumping effect is applied.

ICM-injected AAV is significantly cleared into the blood

[0265] To further investigate the influence of distribution route #2 (**FIG. 1E**), the levels of AAV that are cleared from the cerebrospinal fluid into the blood and reaching the peripheral organs following ICM administration of AAV and direct intravenous injection of AAV were compared.

[0266] Viral vector genome copies were quantified in peripheral organs 4 weeks following AAV administration (**FIG. 3A**). There were no significant differences between genome copies in the spleen, kidney, muscle, liver, heart, and lung measured by digital droplet PCR (ddPCR against GFP) analysis in animals injected intravenously with AAV compared to animals injected ICM (**FIG. 3B**). This suggests that most AAV-injected ICM was cleared from the cerebrospinal fluid into the blood, to transduce peripheral organs. This data also supports the hypothesis that at a given time point post-ICM administration the AAV concentration in the blood becomes high

enough to lead to a significant crossing from the blood into the brain at FUS-MB-targeted sites following ICM injection of AAV (**FIG. 3C**). To determine the level of AAV in the blood at the time points for FUS-MB treatment following intravenous (during FUS-MB) and ICM (60 minutes and 120 minutes prior to FUS-MB) injection of AAV, blood samples were obtained as soon as possible following FUS-MB treatments, resulting in blood samples from 60 minutes and 153 minutes post-ICM injection and 21 minutes post intravenous injection (**FIG. 3C**). Analysis of GFP genome copies (i.e., AAV) in the blood samples demonstrated a tendency to increased AAV levels 60 minutes after ICM injection, though this was not significantly different from negative control samples from animals not injected with AAV with the current sample size of $n = 3-6$ (**FIG. 3D**). Blood samples taken 153 minutes post ICM injection demonstrated a significant increase in GFP genome copies compared to 60 minutes after injection (**FIG. 3D**). The level of AAV in the blood 21 minutes post intravenous administration was approximately twice the level of AAV 153 minutes post ICM injection (**FIG. 3D**). Previous studies in mice have demonstrated that doubling the intravenous dosages of AAV2-HBKO do not significantly affect the percentage of transgene-positive cells obtained in the brain following FUS-MB delivery (*see* R. H. Kofoed, et al., The engineered AAV2-HBKO promotes non-invasive gene delivery to large brain regions beyond ultrasound targeted sites. *Molecular Therapy - Methods & Clinical Development* 27, 167–184 (2022)). Because of the significant clearance of AAV from the cerebrospinal fluid into the blood after 153 min, it is possible that distribution route #2 (**FIG. 1E**) is likely to be the primary delivery mechanism responsible for GFP protein and mRNA expression seen in animals injected ICM with AAV 120 minutes prior to FUS-MB (**FIGS. 2J-K**).

FUS-MB increases delivery of ICM-injected AAV to the striatum

[0267] The pilot study demonstrated a tendency towards higher GFP mRNA expression in the striatum when AAV was injected ICM 120 minutes prior to FUS-MB compared to 60 minutes prior to and 10 minutes post FUS-MB. In addition, the blood samples suggest a higher level of intravenous AAV 120 minutes compared to 60 minutes following ICM administration. In a larger cohort of animals, AAV delivery was therefore compared between animals injected intravenously with AAV during FUS-MB, animals injected ICM with AAV 120 minutes prior to FUS-MB, and animals injected ICM with AAV without FUS-MB application (**FIG. 4A**). Animals were sacrificed after 4 weeks and brains analyzed by IHC and qPCR (**FIG. 4A**). GFP

protein expression (green, white arrows) in the brain were seen in FUS-targeted brain areas in animals injected both intravenous and ICM with AAV, but not in animals injected ICM without FUS-MB application (**FIG. 4B**). ICM injection of AAV can lead to a high AAV uptake in dorsal root ganglions which can be neurotoxic (*see* J. Hordeaux, et al., Adeno-Associated Virus-Induced Dorsal Root Ganglion Pathology. Human Gene Therapy 31, 808–818 (2020)). Analysis of GFP mRNA expression in the FUS-targeted striatum showed increased levels of GFP mRNA expression in animals injected intravenously and ICM and treated with FUS-MB compared to animals injected ICM with AAV without FUS-MB application (**FIG. 4C**). There was no significant difference in GFP mRNA expression in the striatum in animal injected intravenously and ICM with AAV and treated with FUS-MB (**FIG. 4C**). The data in **FIG. 4C** was log10-transformed to obtain normal distribution, and the non-transformed data showed a 5.6-times increase in GFP mRNA expression in the striatum following FUS-MB application in animals injected ICM with AAV compared to animals without FUS-MB treatment. GFP mRNA levels were also measured in brain areas not targeted with FUS-MB, but where gadolinium enhancement in the pilot study suggested a significant delivery of ICM-injected particles (**FIGS. 2B-E**). ICM injection of AAV, with or without FUS-MB targeting the striatum, resulted in significantly increased levels of GFP mRNA expression in the thalamus, midbrain, cerebellum, hippocampus, and brainstem (**FIGS. 2D-H**). The depth of the FUS spots in the z-axis also targets brain structures located dorsally and ventrally relative to the striatum (**FIG. 4A**), (last diagram to the right, turquoise ovals). There was no significant difference in the cortical structures (e.g., Cortex 1 (CX1) and 2 (CX2)) that FUS spots partially covered (**FIGS. 4I-K**). However, in the cortical area (e.g., Cortex 3 (CX3)) which was not affected by FUS-MB, there was a significantly higher level of GFP mRNA in animals injected ICM with AAV compared to intravenously (**FIG. 4L**). The data presented in **FIGS. 4A-K** demonstrate that FUS-MB can increase delivery to deep brain structures, such as the striatum, even with ICM-injected AAV, which does not otherwise reach these structures efficiently. Importantly, ICM injection of AAV leads to significantly increased gene delivery to brain areas that are not targeted by FUS-MB compared to intravenous AAV.

Discussion

FUS-MB increases gene delivery to deep brain structures following ICM administration

[0268] The field of gene therapy is rapidly evolving. Monogenic disorders with a known underlying genetic cause are receiving attention as diseases with a high potential to be treated with gene therapy (*see* C. G. Limia, et al., Emerging Perspectives on Gene Therapy Delivery for Neurodegenerative and Neuromuscular Disorders. *J Pers Med* 12, 1979 (2022)). The first monogenic disease that was treated with a one-time gene therapy treatment was spinal muscular atrophy using an intravenous administration of AAV9 in a dose of 1.1×10^{14} GC/kg (final recommended dose) (*see* J. R. Mendell, et al., Five-Year Extension Results of the Phase 1 START Trial of Onasemnogene Apeparvovec in Spinal Muscular Atrophy. *JAMA Neurol* **78**, 834–841 (2021)). The ability of AAV9 to cross the BBB and blood-spinal cord barrier in infants ensures that the therapy can reach target cells in the central nervous system. However, high intravenous doses come with the risk of severe side effects and the decreased permeability of the BBB with age means that higher dosages are required to treat diseases that occur later in life (e.g., *see* K. D. Foust, et al., Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nature Biotechnology* **27**, 59–65 (2009) and *see* A. Philippidis, Fourth Boy Dies in Clinical Trial of Astellas' AT132. *Human Gene Therapy* 32, 1008–1010 (2021)). Direct delivery of AAV in the cerebrospinal fluid is emerging as a promising route of delivery to facilitate wide-spread gene delivery to the brain and spinal cord with higher efficiency than when using intravenous delivery. While administration in the cerebrospinal fluid through the cisterna magna results in gene delivery to multiple brain regions, delivery to deep brain structures, such as the striatum, is limited (*see* S. Nakamura, et al., Intra-cisterna magna delivery of an AAV vector with the GLUT1 promoter in a pig recapitulates the physiological expression of SLC2A1. *Gene Therapy* 28, 329–338 (2021)).

[0269] It was demonstrated that FUS-MB can increase the delivery of ICM-administered AAV2-HBKO to the striatum in rats. The delivery to the FUS-targeted striatum with ICM and IV AAV administration was not significantly different; however, the ICM route resulted in higher gene delivery to non-FUS-targeted areas than IV injection. Such results suggest that FUS-MB can be used to increase the transduction volume following ICM administration of AAV to include FUS-targeted deep brain structures, which cannot be reached efficiently by ICM injection alone. Increased delivery to deep brain structures by FUS-MB can enhance the

therapeutic efficacy of gene therapy treatments targeting diseases affecting the entire brain and ensure a better distribution of gene delivery across the brain.

Conclusion

[0270] This study demonstrates that FUS-MB can expand delivery of ICM-administered AAV vectors to deep brain structures that are poorly reached through ICM injection alone. Further studies are warranted to elucidate the underlying mechanism of action, which is important for the design of future studies and clinical translation. ICM administration is becoming a promising route of administration for AAV-based gene therapies in clinical trials (C. G. Limia, et al., Emerging Perspectives on Gene Therapy Delivery for Neurodegenerative and Neuromuscular Disorders. J Pers Med 12, 1979 (2022)). The ability of FUS-MB to expand the footprint of AAV biodistribution to deep brain structures following ICM administration has the potential to significantly improve therapeutic efficacy in neurological diseases.

Example 2: Non-human primate (NHP) study evaluating FUS following CSF-administered AAV

[0271] It is hypothesized that combining FUS with intra-CSF AAV administration will expand the transduction profile of AAV to targeted deep brain structures enabling translation of this technology for the treatment of neurodegenerative diseases where deep brain structures are affected. Proof of concept has been demonstrated in rat, therefore evaluating whether this approach can be scaled to the NHP brain is the next step. Proposed study groups are shown in Table 1.

Table 1: Proposed study groups

Group	Treatment and purpose	n	AAV	FUS (bilateral)	Study Duration
1	(Pilot) 1a) ICM GAD for kinetics of penetration into striatum, 1b) IV GAD + MB to confirm FUS coordinates/parameters for full bilateral caudate/putamen coverage	1	n/a	Caudate nucleus + putamen	3 weeks post dose

2	(Positive control) IV AAV + IV MB, FUS	2	I.V. AAV 2.5E ¹³ VG/kg (7.5E ¹³ -1E ¹⁴ VG total)	Caudate nucleus + putamen	
3	(noFUS control) ICM AAV no FUS	1	ICM AAV 2.5E ¹³ VG total	n/a	
4	ICM AAV + IV MB, FUS	3	ICM AAV 2.5E ¹³ VG total	Caudate nucleus + putamen	

[0272] The animals used in this study are 8 cynomolgus macaques (7 on study +1 spare if needed), age ~2-5 years. Prior to administration, prescreen sera from ~36 animals for levels of neutralizing antibodies to AAV2-HBKO and AAV-SAN006. The study duration will be 3 weeks post-FUS. The test articles are shown in Table 2.

Table 2:

AAV (infused as 1:1 mix)	Lot	Concentration	Dose
AAV2-HBKO-eGFP	TBD	1E13 VG/mL	Dependent on Route of administration –see below
AAV-SAN006-mCherry	TBD	1E13 VG/mL	

[0273] Microbubbles are DEFINITY® microbubbles infused IV at 4-16 uL/kg/5min.

[0274] Gadolinium is used as the tracer. Specifically Gadobutrol at 0.1mM/kg for IV administration and 2.5mL @ 5mg/mL for ICM administration.

[0275] CT scans are acquired prior to study start to determine skull morphometrics.

[0276] MRI scans are acquired pre-FUS (T2w and T1w) and pre-necropsy (T2w).

[0277] Test article dosing will be distributed across 4 groups shown in **Table 1**. Group 1: Pilot - two experimental procedures consisting of: Part a: Direct iCM infusion: gadobutrol solution (GAD) followed by MR imaging time course to confirm rate of GAD diffusion into parenchyma. T1 MR time course (pre-dose, 15min, 30min, 60 min, 90min, 120, 180 min); and

Part b: IV GAD with microbubbles (MB) to check the coordinates, parameters, timing for bilateral FUS to caudate and putamen. Group 2: Intravenous dosing of AAV and MB as follows: (1) intravenous AAV at 2.5×10^{13} total VG/kg ($5-8 \times 10^{13}$ VG/animal estimating 2-3kg); and (2) intravenous MB co-administered with AAV. Groups 3+4: Direct ICM infusion of AAV, with IV dosing of MB. Direct-CM: AAV at 3×10^{11} total VG/g brain (2.5×10^{13} VG/animal). Intravenous: Microbubbles (MB) delivered via IV administration ~2 hours post-ICM test article infusion.

[0278] Focused ultrasound: Bilateral striatum (putamen and caudate nucleus). FUS for group 1 will be done during microbubble infusion. FUS for group 2 will be done right after AAV and during microbubble infusion. FUS for group 4 will be done up to 2 hours after AAV dosing, during microbubble infusion.

[0279] As a viability check, animals will be checked twice daily for mortality/moribundity. Detailed clinical observation are carried out weekly. A physical examination and functional observational battery will be performed prior to dosing, 1 week post-dosing, and prior to necropsy.

[0280] Sample collection comprises collecting blood and cerebral spinal fluid (CSF). Blood sampling is performed predose, immediately after iCM injection of AAV, 15min, 30min, 60min, 6hours, 24 hours, 1 week post dosing, and at necropsy for all groups. CSF sample collection is performed pre-dose and at necropsy for groups 2, 3, 4.

[0281] Necropsy is performed on 8 animals. Gross necropsy is performed at 3 weeks post-FUS (+/- 2 days). Animals are perfused with nuclease-free phosphate-buffered saline (PBS). Brain processing comprises cutting into 4 mm coronal slice thickness with brain matrix. Following slicing, the brain is placed on a numbered mat (anterior side face down) in rostrocaudal order, hemisected and digital photographs are taken (photographs are included in the raw data). The slices are transferred to cassettes with the anterior surface of the brain placed face down in the cassettes to maintain left/right orientation. This right hemisphere is frozen for biodistribution and protein analysis. The left hemisphere is saved in 10% neutral buffered formalin (NBF) for histology, then transferred to PBS and stored refrigerated until shipment. The spinal cord processing consists of cutting into 29 segments, with alternate sections frozen for biodistribution and protein analysis. The remaining sections are saved in 10% NBF for histology, then transferred to PBS and stored refrigerated until shipment.

[0282] Dorsal Root Ganglia Processing consists of processing the left and right sides. The right side is frozen for biodistribution and protein analysis. The left is saved in 10% NBF for histology, then transferred to PBS and stored refrigerated until shipment.

[0283] Representative samples of peripheral tissues are frozen for biodistribution or fixed for histology.

[0284] Frozen tissue is analyzed via RT-dPCR and dPCR for transgene expression and biodistribution, respectively.

[0285] Fixed tissue is analyzed via IHC and in-situ hybridization (ISH) for transgene expression and biodistribution, respectively.

Example 3: Magnetic resonance imaging-guided focused-ultrasound (MRIGFUS) following CSF-administered adeno-associated viruses (AAV) in non-human primates (NHPs)

[0286] Intravenous injection of AAV for gene delivery to the brain requires high doses of vector, results in high peripheral exposure, and poor brain delivery due to inefficient blood-brain barrier crossing. Administration of AAV directly into the CSF is a clinically relevant route of administration and enables transduction of superficial brain regions at relatively low doses, however the distribution of AAV to deep brain structures remains limited.

[0287] MRI-guided, low-intensity focused ultrasound (MRIGFUS), combined with intravenously injected gas-filled microbubbles (MB), can safely and transiently open the blood brain barrier at specifically targeted locations, and is already in clinical trials for Alzheimer's Disease, Parkinson's Disease and ALS. It is also well established that this approach enables AAV vectors to cross from the blood into the brain, but only in the context of intravenous AAV injection, and only to those targeted regions.

[0288] It is hypothesized that combining FUS with intra-CSF AAV administration will expand the transduction profile of AAV to targeted deep brain structures enabling translation of this technology for the treatment of neurodegenerative diseases where deep brain structures are affected. Proof of concept has been demonstrated in rat, therefore evaluating whether this approach can be scaled to the NHP brain is the next step. Results on successful scaling of this concept to the NHP brain are presented below.

Methods

Plasmids, ITR vectors and AAV generation

[0289] To generate recombinant AAV.SAN006 or AAV2HBKO serotype vectors, transgene payload DNA (expressing either eGFP or mCherry) was cloned into a plasmid containing AAV2 inverted terminal repeats (ITRs) under control of the CAG promoter (human CMV enhancer, chicken β -actin promoter, and a chicken β -actin/rabbit β -globin hybrid intron). To generate AAV, adherent HEK293 cells were transfected using PEI (polyethyleneimine) with a 1:1:1 ratio of three plasmids (containing the ITR, AAV rep/cap and Ad helper). The Ad helper plasmid (pHelper) was obtained from Stratagene/Agilent Technologies (Santa Clara, CA). AAV purification was performed using cesium chloride ultracentrifugation, and virus was titered using ddPCR against the bGH polyA sequence and the concentration of each vector was adjusted accordingly prior to pooling for dose administration.

Animal use and care

[0290] All experiments were conducted in an AAALAC-accredited institution. All procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) in compliance with the Animal Welfare Act, as per guidelines specified by the Guide for the Care and Use of Laboratory Animals, NIH. Only purpose-bred naïve cynomolgus macaques (Cambodian males, 2-3 years old, 2-4kg) were used. NHPs were prescreened for AAV neutralizing antibodies to AAV2HBKO and AAV.SAN006, and seronegative animals were selected for the study. Prior to iCM administration and BBB opening, animals were sedated with ketamine (intramuscular at 0.10 mL/kg) and kept under anesthesia with isoflurane which was adjusted as needed for the duration of the dosing, sonication and MR imaging procedures. Standard buprenorphine (0.3mg/mL) was administered intramuscularly as needed and once prior to MR imaging. Prior to dosing the head was shaved and an intravenous catheter was placed. Body temperature was maintained using heat pads and/or air blankets.

Intra-cisterna magna (iCM) administration

[0291] Animals were placed in Trendelenburg position throughout dosing and for up to 15 minutes following dosing completion. Prior to dosing, approximately 1 mL of CSF was removed.

A 23G $\frac{3}{4}$ gauge needle was inserted into the cisterna magna under fluoroscopy guidance. Animals were infused with a total of 2.5E13 vector genomes (1E13 VG/mL in 2.5 mL) at 0.125 mL/min flow rate followed by a flush volume of 0.250 mL formulation buffer. The dosing needle was left in place for at least three minutes following dosing completion. Following the procedure, animals were transferred back to the MRI imaging suite for FUS treatment.

Low intensity FUS and MR imaging

[0292] FUS was performed with an Insightec ExAblate 4000 low frequency system (Insightec, Haifa, Israel) integrated with a 3T MR scanner (Seimens SKYRAFIT). The head of the animal was coupled to the transducer with degassed water and secured for the duration of the treatment. Baseline T1-weighted images were acquired and used to outline the left caudate and putamen of each NHP for targeting with Insightec ExAblate Neuro software using 1mm grid spacing for sonication spots. Care was taken to avoid placing spots near the lateral ventricle or to adjacent cortical regions. Microbubbles (DEFINITY) were prepared as per manufacturer's protocol and infused intravenously through a catheter at 5uL/kg/min prior to and for the duration of sonication. Ultrasound was applied at 230 kHz for 180sec at 0.5% duty cycle for each spot. Animals received between 1-3 sonication rounds per target (acoustic doses detailed in the table in **FIG. 9**), depending on the extent of hypointense signal change observed using a modified T2* relaxation map to detect subtle changes in the BBB following each round of sonication. Lack of signal change triggered additional rounds of sonication until signal changes were observed to ensure BBB opening. Animals were removed from the FUS system and repositioned in the MR scanner for follow-up imaging. BBB opening immediately after, and 2 days after sonication was evaluated by axial T1-weighted MR imaging following gadobenate dimeglumine (Bracco MultiHance) injected at 0.2 mmol/kg. T2-weighted scans were run for each animal at 2 days and 20 days post-FUS to monitor for signs of edema following FUS treatment.

NHP sample collection

[0293] Whole blood was collected into EDTA tubes prior to, 30min, 2-3 hours and 192 hours after AAV infusion for AAV vector genome quantification.

[0294] At necropsy, animals were perfused with chilled RNase/DNase free PBS pH 7.4, and their brains were cut in a brain matrix into 4mm coronal slices and hemisected. The left (FUS treated) and right (FUS untreated) hemispheres from all slabs were frozen on dry ice for biochemical analysis, except for one slab encompassing the anterior caudate nucleus and putamen. This slab was drop fixed in 10% neutral buffered formalin for 36-48 hours at room temperature before embedding in paraffin blocks for subsequent sectioning. Frozen tissue punches were collected from coronal brain slabs from the indicated regions (1-5 punches per region) using a 3mm biopsy punch and stored at -80C until subsequent tissue processing.

Tissue homogenization

[0295] Tissues were bead homogenized at 4°C in TE buffer (10mM Tris pH7.4, 1mM EDTA) using an Omni Beadruptor set for 20 second cycles 4.7 oscillation/sec., aliquoted, and stored at -80C until further use. Samples used for subsequent RNA analyses were first mixed with QIAzol lysis reagent prior to freezing.

DNA isolation and vector genome quantification

[0296] Automated gDNA isolation: TE homogenate aliquots were thawed and gDNA was isolated using QIAmp 96 DNA QIAcube HT kit (QIAGEN 51331) on the Qiacube HT according to manufacturer's instructions. DNA concentration and purity were assessed by measuring absorbance at A260/280 on a NanoDrop spectrophotometer (Thermo Fisher Scientific).

[0297] Vector genome copies were quantified from extracted gDNA by digital PCR using the QIAcuity Probe PCR Kit (QIAGEN 250102) with probes targeting AAV vector genome sequences specific for each AAV (mCherry for SAN006 and GFP for AAV2HBKO) and endogenous gDNA (*TUBB1* intron). Reactions were performed using the QIAcuity 8 digital PCR system (QIAGEN) with manufacturer's suggested thermocycling conditions. Vector genome and reference gene copies/ μ L were quantified using the QIAcuity Software Suite (QIAGEN), and 2x vector genome copies were divided by reference gene copies to calculate VG/cell.

RNA isolation and quantification

[0298] RNA was isolated from tissue homogenates using the RNeasy 96 QIAcube HT kit (QIAGEN, 74171) according to manufacturer's protocol. Briefly, tissue homogenates in QIAzol lysis reagent (QIAGEN, 79306) were thawed and mixed with chloroform (Fisher Scientific, C298-1). The mixture was centrifuged at 4°C and the aqueous phase was transferred to S block (QIAGEN, 19585) for further RNA isolation. The samples were placed into QIAcube HT instrument and RNA isolation was performed by following the steps from QIAcube HT Prep Software with on-column DNase (QIAGEN, 79256) treatment. After RNA isolation, concentration was measured with a NANODROP 8000 (Thermo Fisher Scientific). Reverse transcription was carried out by producing cDNA via QIAcuity OneStep advanced probe kit (QIAGEN, 250132) and using multiplexed primer-probe combinations specific to GFP, mCherry, *HPRT* and *RPP30*. Copies/uL of each transgene were normalized to the mean copies of *HPRT* and *RPP30* (Integrated DNA Technology).

Tissue sectioning and staining

[0299] Five µm-thick sections of brain tissue were cut with a microtome and mounted directly onto charged glass slides. Tissue sections were stained with either hematoxylin and eosin for histopathology assessment, or processed for ISH as follows. Target RNA was detected in tissue sections using automated ISH using a Leica Bond Rx stainer and standard RNAscope detection kits (red- RNAscope 2.5 LSx reagent kit- RED, ACD cat# 322750; brown- RNAscope 2.5 LSx reagent kit- BROWN, ACD cat# 322700) and protocols. ACD 2.5 LS Probes for target detection included: mCherry ACD cat# 431208 and GFP ACD cat# 400288. Probes were used as supplied and all standard RNAscope LSx protocols were used as pre-installed on Leica Bond Rx. Following staining completion, slides were removed from Leica Bond Rx, dehydrated in ethanol and xylene, and coverslip applied by Leica CV5030 coverslipper with Surgipath Micromount mounting medium (Leica, cat# 3801730). Images were collected using Leica Aperio AT2 brightfield slide scanner at 20X magnification.

Results

Intra-CSF administration of AAV followed by large volume BBB opening in the NHP caudate and putamen

[0300] To evaluate the feasibility of scaling the concept of intra-CSF AAV delivery combined with focused ultrasound (FUS) to the nonhuman primate (NHP) striatum, AAV was administered to four juvenile cynomolgus macaques via direct intra-cisterna magna (iCM) injection, followed by unilateral focused ultrasound targeting the caudate nucleus and putamen in the left hemisphere of each animal. Unilateral targeting was chosen to enable the contralateral hemisphere to be used as a control for iCM AAV without FUS. AAV was administered as a pooled, 1:1 mixture of two capsids, AAV2HBKO and AAV.SAN006, expressing eGFP and mCherry reporter proteins, respectively. Transcranial low intensity FUS was applied to the caudate and putamen using sonication spots distributed across 1 mm grids for each target. Due to the size of the region in some animals, ventral putamen was divided into two separate posterior (A) and anterior (B) sonication grids spread across the same axial plane. Additionally, two animals (1003 and 1004) were treated across two different axial planes (“slices”) per structure to yield more comprehensive coverage (summarized in the table in **FIG. 9**). A modified T2* mapping (T2*map) sequence was used to detect subtle changes in blood brain barrier (BBB) integrity immediately after each round of sonication, with the detection of signal hypointensity on the T2*map scan indicating sufficient BBB disruption and resulting in the end of sonication (**FIG. 5A**). BBB opening was confirmed in each animal with administration of intravenous gadolinium (Gd) followed by T1-weighted MR imaging to visualize extravasation of Gd. All animals displayed areas of Gd enhancement precisely overlapping with the targeted regions (**FIG. 5B**). Animals were allowed to recover, and MR scans were repeated two days later to evaluate the extent of BBB-closure (**FIG. 5C**). Opening largely resolved in animals 1001 and 1003, however the other two animals still had regions of Gd enhancement visible throughout the caudate and putamen, suggestive of excessive BBB opening. Additionally, T2-weighted images taken at two and 20 days post-FUS showed hyperintensities in the targeted regions, indicative of potential inflammation or edema post-treatment (**FIGS. 5D, 5E**). Apart from transient clinical signs in animal 1002 10 days after treatment (reduced activity, abnormal muscle tone, seizure), the combined intra-CSF AAV administration with FUS treatment was tolerated with no premature mortality or gross pathology findings at the time of necropsy three weeks later.

Enhancement of AAV vector genome biodistribution to the NHP striatum using MRIgFUS following iCM delivery of AAV

[0301] Brain tissue was collected for molecular and histopathological readouts three weeks following AAV administration. Brains were cut into 4mm coronal slabs that were flash frozen for subsequent DNA and RNA analyses, except for one brain slab per animal encompassing anterior caudate and putamen to be used for histology. DNA and RNA were extracted from 49 tissue punches per hemisphere from each NHP to evaluate AAV biodistribution and transgene expression. FUS had no effect on vector biodistribution to areas outside the caudate or putamen but significantly enhanced intra-CSF AAV2HBKO biodistribution for all animals in the targeted structures, necessarily encompassing the globus pallidus (**FIG. 6A**). When the data were viewed at the punch level to assess regional variability in biodistribution, FUS greatly enhanced transduction across the rostro-caudal axis of both caudate and putamen (**FIG. 6C**).

[0302] AAV.SAN006 exhibited robust transduction across the brain consistent with iCM injection, and FUS treatment did not alter transduction outside the targeted regions (**FIG. 6B**). Supporting the concept that different capsids may respond differently to FUS-mediated BBB opening (9), we observed a decrease in vector biodistribution when calculated as the average of tissue punch data pooled for each targeted region (**FIG. 6B**). However, when analyzed at the punch level it became clear that AAV.SAN006 biodistribution improved in several punches within these structures. Transduction of the putamen was improved for every animal, across 4/5 punch locations. In caudate, transduction was improved in most punch locations for two animals (1003 and 1004, denoted by circular symbols) (**FIG. 6D**). As depicted in **FIG. 6E**, AAV.SAN006 and AAV2HBKO VG levels were also quantified from DNA extracted from whole blood samples at baseline, 30min after infusion, after sonication was complete, and 7 days after AAV infusion. Both AAVs were present at high levels in the bloodstream throughout the period of time that the BBB was open following FUS. The kinetics of vector efflux from the CSF into the blood were similar, indicating that the differences in striatal transduction following FUS for the two vectors is unlikely to be due to differences in the amount of AAV present in the bloodstream. Vector genome levels were also measured in spinal cord (**FIG. 6F**), dorsal root ganglia (**FIG. 6G**), liver and spleen (**FIG. 6H**).

Enhancement of AAV transgene expression in the NHP striatum using MRIgFUS following iCM delivery of AAV

[0303] Total RNA was analyzed from the same punches as above to assess the level of mCherry and GFP reporter gene expression in each punch using multiplexed RT-dPCR. FUS did not alter transgene expression from either AAV in non-targeted brain regions, but greatly enhanced AAV2HBKO-mediated GFP expression across all punches taken from both the caudate and putamen, with enhancement approaching 1000-fold over the non-sonicated right hemisphere (**FIGS. 7A, 7C**). AAV.SAN006 displayed ubiquitous enhancement of mRNA expression in the FUS-targeted caudate and putamen for all animals exceeding 100-fold improvement in some regions of caudate and putamen (**FIGS. 7B, 7D**).

Histological confirmation of MR findings and of enhanced striatal transgene expression in the sonicated hemisphere

[0304] While most of the brain was processed and frozen exclusively for molecular analyses, one coronal slab of brain encompassing the anterior caudate and putamen was fixed for histological analyses. Gross examination revealed no notable differences between treated and untreated hemispheres. Upon microscopic evaluation of H&E-stained sections the treated putamen appeared normal for all animals. However, the dorsal caudate of the treated hemisphere for every animal showed findings indicative of tissue damage, including cell infiltration and necrosis, suggesting that BBB opening exceeded safe levels in this region (**FIG. 8A**). The untreated hemisphere appeared normal for caudate and putamen of all animals (**FIG. 8B**). To evaluate the spatial distribution and extent of transgene expression, *in situ* hybridization (ISH) was performed using probes specific for mCherry and GFP. GFP mRNA-positive cells were observed throughout both anterior caudate and putamen, indicating FUS treatment of these regions was sufficient to enhance transduction when combined with intra-CSF administration of AAV2HBKO (**FIG. 8C**). In contrast, mCherry positive cells were not detectable by chromogenic ISH in anterior caudate or putamen (**FIG. 8D**), likely reflecting the lower levels of mRNA expression (**FIG. 7D**) observed from AAV.SAN006-mCherry which was readily detectable using highly sensitive digital PCR methods, but likely below the threshold for detection via ISH.

REFERENCES

1. J. R. Mendell, *et al.*, Five-Year Extension Results of the Phase 1 START Trial of Onasemnogene Apeparvovec in Spinal Muscular Atrophy. *JAMA Neurol* **78**, 834–841 (2021).
2. K. D. Foust, *et al.*, Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nature Biotechnology* **27**, 59–65 (2009).
3. J. R. Mendell, *et al.*, Single-dose gene-replacement therapy for spinal muscular atrophy. *New England Journal of Medicine* **377**, 1713–1722 (2017).
4. A. Philippidis, Fourth Boy Dies in Clinical Trial of Astellas' AT132. *Human Gene Therapy* **32**, 1008–1010 (2021).
5. K. Hynynen, N. McDannold, N. Vykhodtseva, F. Jolesz, Noninvasive MR Imaging-guided Focal Opening of the Blood-Brain Barrier in Rabbits. *Radiology* **220**, 640–646 (2001).
6. N. Sheikov, *et al.*, Brain arterioles show more active vesicular transport of blood-borne tracer molecules than capillaries and venules after focused ultrasound-evoked opening of the blood-brain barrier. *Ultrasound in Medicine and Biology* **32**, 1399–1409 (2006).
7. N. Sheikov, N. McDannold, S. Sharma, K. Hynynen, Effect of Focused Ultrasound Applied With an Ultrasound Contrast Agent on the Tight Junctional Integrity of the Brain Microvascular Endothelium. *Ultrasound in Medicine and Biology* **34**, 1093–1104 (2008).
8. E. Thévenot, *et al.*, Targeted Delivery of Self-Complementary Adeno-Associated Virus Serotype 9 to the Brain, Using Magnetic Resonance Imaging-Guided Focused Ultrasound. *Human Gene Therapy* **23**, 1144–1155 (2012).
9. R. H. Kofoed, *et al.*, Efficacy of gene delivery to the brain using AAV and ultrasound depends on serotypes and brain areas. *Journal of Controlled Release* **351**, 667–680 (2022).
10. M. J. Castle, *et al.*, Postmortem analysis in a clinical trial of AAV2-NGF gene therapy for alzheimer's disease identifies a need for improved vector delivery. *Human Gene Therapy* **31**, 415–422 (2020).
11. Y. Chu, J. H. Kordower, Post-Mortem Studies of Neurturin Gene Therapy for Parkinson's Disease: Two Subjects with 10 Years CERE120 Delivery. *Mov Disord* **38**, 1728–1736 (2023).
12. C. G. Limia, *et al.*, Emerging Perspectives on Gene Therapy Delivery for Neurodegenerative and Neuromuscular Disorders. *J Pers Med* **12**, 1979 (2022).
13. P. M. Marchi, L. Marrone, M. Azzouz, Delivery of therapeutic AAV9 vectors via cisterna magna to treat neurological disorders. *Trends Mol Med* **28**, 79–80 (2022).

SEQUENCES

5' AAV2 ITR DNA Sequence

TTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCG
ACCTTTGGTCGCCCCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACTCCATCACTAG
GGGTTCT (SEQ ID NO: 1)

3' AAV2 ITR DNA Sequence in Flip Orientation

AGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGG
CAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGG
AGTGCCAA (SEQ ID NO: 2)

CMV Enhancer Element DNA Sequence (GenBank: K03104.1)

GACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGA
GTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCGCCATTGACG
TCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATT
TACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAA
TGACGGTAAATGGCCCGCCTGGCATTATGCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTAC
ATCTACGTATTAGTCATCGCTATTACCATGG (SEQ ID NO: 3)

Chicken-Actin Promoter DNA Sequence

TCGAGGTGAGCCCCACGTTCTGCTTCACTCTCCCCATCTCCCCCCCCTCCCAACCCCAATTTTGTATTAT
TTATTTTAAATTATTTTGTGCAGCGATGGGGCGGGGGGGGGGGGGGGCGCGCGCCAGGCGGGGC
GGGGCGGGGCGAGGGGCGGGGCGGGGCGAGGCGGAGAGGTGCGGCGGCAGCCAATCAGAGCGGCG
CGCTCCGAAAGTTTCCTTTATGGCGAGGCGGCGGCGGCGGCCCTATAAAAAGCGAAGCGCGCGG
CGGGCGGGAGTCGTCGCGCTGCCTTCGCCCCGTGCCCCGCTCCGCCGCC (SEQ ID NO: 4)

WPRE Element DNA Sequence

TTAATCAACCTCTGGATTACAAAATTTGTGAAAGATTGACTGGTATTCTTAACTATGTTGCTCCTTTACG
CTATGTGGATACGCTGCTTAAATGCCTTGTATCATGCTATTGCTTCCCGTATGGCTTTCATTTCTCCTCC
TTGTATAAATCCTGGTTGCTGTCTTTATGAGGAGTTGTGGCCGTTGTCAGGCAACGTGGCGTGGTGT
GCACTGTGTTTGCTGACGCAACCCCACTGGTTGGGGCATTGCCACCACCTGTCAGTCTCTTCCGGGAC
TTTCGCTTTCCTCCCTATTGCCACGGCGGAACTCATCGCCGCCTGCCTTGCCCGCTGCTGGACAGGG
GCTCGGCTGTTGGGCACTGACAATCCGTGGTGTGTCGGGGAAATCATCGTCCTTTCCTTGGCTGCTCG
CCTGTGTTGCCACCTGGATTCTGCGCGGGACGTCCTTCTGCTACGTCCCTTCGGCCCTCAATCCAGCGGA

CCTTCCTTCCCGCGGCCTGCTGCCGGCTCTGCCGGCCTTCCGCGTCTTCGCCTTCGCCCTCAGACGAGTC
GGATCTCCCTTTGGCCGCCTCCCCGCCTG (SEQ ID NO: 5)

AAV9 VP1 Capsid Amino Acid Sequence (Full Structural Protein)

Met	Ala	Asp	Gly	Tyr	Leu	Pro	Asp	Trp	Leu	Glu	Asp	Asn	Leu	Ser	1	5	10	15	
Glu	Gly	Ile	Arg	Glu	Trp	Trp	Ala	Leu	Lys	Pro	Gly	Ala	Pro	Gln	Pro	20	25	30	
Lys	Ala	Asn	Gln	Gln	His	Gln	Asp	Asn	Ala	Arg	Gly	Leu	Val	Leu	Pro	35	40	45	
Gly	Tyr	Lys	Tyr	Leu	Gly	Pro	Gly	Asn	Gly	Leu	Asp	Lys	Gly	Glu	Pro	50	55	60	
Val	Asn	Ala	Ala	Asp	Ala	Ala	Ala	Leu	Glu	His	Asp	Lys	Ala	Tyr	Asp	65	70	75	80
Gln	Gln	Leu	Lys	Ala	Gly	Asp	Asn	Pro	Tyr	Leu	Lys	Tyr	Asn	His	Ala	85	90	95	
Asp	Ala	Glu	Phe	Gln	Glu	Arg	Leu	Lys	Glu	Asp	Thr	Ser	Phe	Gly	Gly	100	105	110	
Asn	Leu	Gly	Arg	Ala	Val	Phe	Gln	Ala	Lys	Lys	Arg	Leu	Leu	Glu	Pro	115	120	125	
Leu	Gly	Leu	Val	Glu	Glu	Ala	Ala	Lys	Thr	Ala	Pro	Gly	Lys	Lys	Arg	130	135	140	
Pro	Val	Glu	Gln	Ser	Pro	Gln	Glu	Pro	Asp	Ser	Ser	Ala	Gly	Ile	Gly	145	150	155	160
Lys	Ser	Gly	Ala	Gln	Pro	Ala	Lys	Lys	Arg	Leu	Asn	Phe	Gly	Gln	Thr	165	170	175	
Gly	Asp	Thr	Glu	Ser	Val	Pro	Asp	Pro	Gln	Pro	Ile	Gly	Glu	Pro	Pro	180	185	190	

Ala Ala Pro Ser Gly Val Gly Ser Leu Thr Met Ala Ser Gly Gly Gly
 195 200 205

Ala Pro Val Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Ser Ser
 210 215 220

Ser Gly Asn Trp His Cys Asp Ser Gln Trp Leu Gly Asp Arg Val Ile
 225 230 235 240

Thr Thr Ser Thr Arg Thr Trp Ala Leu Pro Thr Tyr Asn Asn His Leu
 245 250 255

Tyr Lys Gln Ile Ser Asn Ser Thr Ser Gly Gly Ser Ser Asn Asp Asn
 260 265 270

Ala Tyr Phe Gly Tyr Ser Thr Pro Trp Gly Tyr Phe Asp Phe Asn Arg
 275 280 285

Phe His Cys His Phe Ser Pro Arg Asp Trp Gln Arg Leu Ile Asn Asn
 290 295 300

Asn Trp Gly Phe Arg Pro Lys Arg Leu Asn Phe Lys Leu Phe Asn Ile
 305 310 315 320

Gln Val Lys Glu Val Thr Asp Asn Asn Gly Val Lys Thr Ile Ala Asn
 325 330 335

Asn Leu Thr Ser Thr Val Gln Val Phe Thr Asp Ser Asp Tyr Gln Leu
 340 345 350

Pro Tyr Val Leu Gly Ser Ala His Glu Gly Cys Leu Pro Pro Phe Pro
 355 360 365

Ala Asp Val Phe Met Ile Pro Gln Tyr Gly Tyr Leu Thr Leu Asn Asp
 370 375 380

Gly Ser Gln Ala Val Gly Arg Ser Ser Phe Tyr Cys Leu Glu Tyr Phe
 385 390 395 400

Pro Ser Gln Met Leu Arg Thr Gly Asn Asn Phe Gln Phe Ser Tyr Glu
 405 410 415

Phe Glu Asn Val Pro Phe His Ser Ser Tyr Ala His Ser Gln Ser Leu

420 425 430
 Asp Arg Leu Met Asn Pro Leu Ile Asp Gln Tyr Leu Tyr Tyr Leu Ser
 435 440 445
 Lys Thr Ile Asn Gly Ser Gly Gln Asn Gln Gln Thr Leu Lys Phe Ser
 450 455 460
 Val Ala Gly Pro Ser Asn Met Ala Val Gln Gly Arg Asn Tyr Ile Pro
 465 470 475 480
 Gly Pro Ser Tyr Arg Gln Gln Arg Val Ser Thr Thr Val Thr Gln Asn
 485 490 495
 Asn Asn Ser Glu Phe Ala Trp Pro Gly Ala Ser Ser Trp Ala Leu Asn
 500 505 510
 Gly Arg Asn Ser Leu Met Asn Pro Gly Pro Ala Met Ala Ser His Lys
 515 520 525
 Glu Gly Glu Asp Arg Phe Phe Pro Leu Ser Gly Ser Leu Ile Phe Gly
 530 535 540
 Lys Gln Gly Thr Gly Arg Asp Asn Val Asp Ala Asp Lys Val Met Ile
 545 550 555 560
 Thr Asn Glu Glu Glu Ile Lys Thr Thr Asn Pro Val Ala Thr Glu Ser
 565 570 575
 Tyr Gly Gln Val Ala Thr Asn His Gln Ser Ala Gln Ala Gln Ala Gln
 580 585 590
 Thr Gly Trp Val Gln Asn Gln Gly Ile Leu Pro Gly Met Val Trp Gln
 595 600 605
 Asp Arg Asp Val Tyr Leu Gln Gly Pro Ile Trp Ala Lys Ile Pro His
 610 615 620
 Thr Asp Gly Asn Phe His Pro Ser Pro Leu Met Gly Gly Phe Gly Met
 625 630 635 640
 Lys His Pro Pro Pro Gln Ile Leu Ile Lys Asn Thr Pro Val Pro Ala
 645 650 655

Asp Pro Pro Thr Ala Phe Asn Lys Asp Lys Leu Asn Ser Phe Ile Thr
 660 665 670
 Gln Tyr Ser Thr Gly Gln Val Ser Val Glu Ile Glu Trp Glu Leu Gln
 675 680 685
 Lys Glu Asn Ser Lys Arg Trp Asn Pro Glu Ile Gln Tyr Thr Ser Asn
 690 695 700
 Tyr Tyr Lys Ser Asn Asn Val Glu Phe Ala Val Asn Thr Glu Gly Val
 705 710 715 720
 Tyr Ser Glu Pro Arg Pro Ile Gly Thr Arg Tyr Leu Thr Arg Asn Leu
 725 730 735
 (SEQ ID NO: 6)

Targeting Peptide Amino Acid Sequence

KGGGFHG (SEQ ID NO: 7)

Targeting Peptide Flanked by Linkers - Amino Acid Sequence

AAAKGGGFHGAS (SEQ ID NO: 8)

SAN0006 Capsid Amino Acid Sequence (Full Structural Protein)

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
 1 5 10 15
 Glu Gly Ile Arg Glu Trp Trp Ala Leu Lys Pro Gly Ala Pro Gln Pro
 20 25 30
 Lys Ala Asn Gln Gln His Gln Asp Asn Ala Arg Gly Leu Val Leu Pro
 35 40 45
 Gly Tyr Lys Tyr Leu Gly Pro Gly Asn Gly Leu Asp Lys Gly Glu Pro
 50 55 60
 Val Asn Ala Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
 65 70 75 80

Gln Gln Leu Lys Ala Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala
 85 90 95

Asp Ala Glu Phe Gln Glu Arg Leu Lys Glu Asp Thr Ser Phe Gly Gly
 100 105 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Leu Leu Glu Pro
 115 120 125

Leu Gly Leu Val Glu Glu Ala Ala Lys Thr Ala Pro Gly Lys Lys Arg
 130 135 140

Pro Val Glu Gln Ser Pro Gln Glu Pro Asp Ser Ser Ala Gly Ile Gly
 145 150 155 160

Lys Ser Gly Ala Gln Pro Ala Lys Lys Arg Leu Asn Phe Gly Gln Thr
 165 170 175

Gly Asp Thr Glu Ser Val Pro Asp Pro Gln Pro Ile Gly Glu Pro Pro
 180 185 190

Ala Ala Pro Ser Gly Val Gly Ser Leu Thr Met Ala Ser Gly Gly Gly
 195 200 205

Ala Pro Val Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Ser Ser
 210 215 220

Ser Gly Asn Trp His Cys Asp Ser Gln Trp Leu Gly Asp Arg Val Ile
 225 230 235 240

Thr Thr Ser Thr Arg Thr Trp Ala Leu Pro Thr Tyr Asn Asn His Leu
 245 250 255

Tyr Lys Gln Ile Ser Asn Ser Thr Ser Gly Gly Ser Ser Asn Asp Asn
 260 265 270

Ala Tyr Phe Gly Tyr Ser Thr Pro Trp Gly Tyr Phe Asp Phe Asn Arg
 275 280 285

Phe His Cys His Phe Ser Pro Arg Asp Trp Gln Arg Leu Ile Asn Asn
 290 295 300

Asn Trp Gly Phe Arg Pro Lys Arg Leu Asn Phe Lys Leu Phe Asn Ile
305 310 315 320

Gln Val Lys Glu Val Thr Asp Asn Asn Gly Val Lys Thr Ile Ala Asn
325 330 335

Asn Leu Thr Ser Thr Val Gln Val Phe Thr Asp Ser Asp Tyr Gln Leu
340 345 350

Pro Tyr Val Leu Gly Ser Ala His Glu Gly Cys Leu Pro Pro Phe Pro
355 360 365

Ala Asp Val Phe Met Ile Pro Gln Tyr Gly Tyr Leu Thr Leu Asn Asp
370 375 380

Gly Ser Gln Ala Val Gly Arg Ser Ser Phe Tyr Cys Leu Glu Tyr Phe
385 390 395 400

Pro Ser Gln Met Leu Arg Thr Gly Asn Asn Phe Gln Phe Ser Tyr Glu
405 410 415

Phe Glu Asn Val Pro Phe His Ser Ser Tyr Ala His Ser Gln Ser Leu
420 425 430

Asp Arg Leu Met Asn Pro Leu Ile Asp Gln Tyr Leu Tyr Tyr Leu Ser
435 440 445

Lys Thr Ile Asn Gly Ser Gly Gln Asn Gln Gln Thr Leu Lys Phe Ser
450 455 460

Val Ala Gly Pro Ser Asn Met Ala Val Gln Gly Arg Asn Tyr Ile Pro
465 470 475 480

Gly Pro Ser Tyr Arg Gln Gln Arg Val Ser Thr Thr Val Thr Gln Asn
485 490 495

Asn Asn Ser Glu Phe Ala Trp Pro Gly Ala Ser Ser Trp Ala Leu Asn
500 505 510

Gly Arg Asn Ser Leu Met Asn Pro Gly Pro Ala Met Ala Ser His Lys
515 520 525

Glu Gly Glu Asp Arg Phe Phe Pro Leu Ser Gly Ser Leu Ile Phe Gly
 530 535 540

Lys Gln Gly Thr Gly Arg Asp Asn Val Asp Ala Asp Lys Val Met Ile
 545 550 555 560

Thr Asn Glu Glu Glu Ile Lys Thr Thr Asn Pro Val Ala Thr Glu Ser
 565 570 575

Tyr Gly Gln Val Ala Thr Asn His Gln Ser Ala Gln Ala Ala Ala **Lys**
 580 585 590

Gly Gly Gly Phe His Gly Ala Ser Ala Gln Ala Gln Thr Gly Trp Val
 595 600 605

Gln Asn Gln Gly Ile Leu Pro Gly Met Val Trp Gln Asp Arg Asp Val
 610 615 620

Tyr Leu Gln Gly Pro Ile Trp Ala Lys Ile Pro His Thr Asp Gly Asn
 625 630 635 640

Phe His Pro Ser Pro Leu Met Gly Gly Phe Gly Met Lys His Pro Pro
 645 650 655

Pro Gln Ile Leu Ile Lys Asn Thr Pro Val Pro Ala Asp Pro Pro Thr
 660 665 670

Ala Phe Asn Lys Asp Lys Leu Asn Ser Phe Ile Thr Gln Tyr Ser Thr
 675 680 685

Gly Gln Val Ser Val Glu Ile Glu Trp Glu Leu Gln Lys Glu Asn Ser
 690 695 700

Lys Arg Trp Asn Pro Glu Ile Gln Tyr Thr Ser Asn Tyr Tyr Lys Ser
 705 710 715 720

Asn Asn Val Glu Phe Ala Val Asn Thr Glu Gly Val Tyr Ser Glu Pro
 725 730 735

Arg Pro Ile Gly Thr Arg Tyr Leu Thr Arg Asn Leu (SEQ ID NO: 9)
 740 745

AAV9 VP2 Capsid Amino Acid Sequence

Thr Ala Pro Gly Lys Lys Arg
 Pro Val Glu Gln Ser Pro Gln Glu Pro Asp Ser Ser Ala Gly Ile Gly
 Lys Ser Gly Ala Gln Pro Ala Lys Lys Arg Leu Asn Phe Gly Gln Thr
 Gly Asp Thr Glu Ser Val Pro Asp Pro Gln Pro Ile Gly Glu Pro Pro
 Ala Ala Pro Ser Gly Val Gly Ser Leu Thr Met Ala Ser Gly Gly Gly
 Ala Pro Val Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Ser Ser
 Ser Gly Asn Trp His Cys Asp Ser Gln Trp Leu Gly Asp Arg Val Ile
 Thr Thr Ser Thr Arg Thr Trp Ala Leu Pro Thr Tyr Asn Asn His Leu
 Tyr Lys Gln Ile Ser Asn Ser Thr Ser Gly Gly Ser Ser Asn Asp Asn
 Ala Tyr Phe Gly Tyr Ser Thr Pro Trp Gly Tyr Phe Asp Phe Asn Arg
 Phe His Cys His Phe Ser Pro Arg Asp Trp Gln Arg Leu Ile Asn Asn
 Asn Trp Gly Phe Arg Pro Lys Arg Leu Asn Phe Lys Leu Phe Asn Ile
 Gln Val Lys Glu Val Thr Asp Asn Asn Gly Val Lys Thr Ile Ala Asn
 Asn Leu Thr Ser Thr Val Gln Val Phe Thr Asp Ser Asp Tyr Gln Leu
 Pro Tyr Val Leu Gly Ser Ala His Glu Gly Cys Leu Pro Pro Phe Pro
 Ala Asp Val Phe Met Ile Pro Gln Tyr Gly Tyr Leu Thr Leu Asn Asp
 Gly Ser Gln Ala Val Gly Arg Ser Ser Phe Tyr Cys Leu Glu Tyr Phe
 Pro Ser Gln Met Leu Arg Thr Gly Asn Asn Phe Gln Phe Ser Tyr Glu
 Phe Glu Asn Val Pro Phe His Ser Ser Tyr Ala His Ser Gln Ser Leu
 Asp Arg Leu Met Asn Pro Leu Ile Asp Gln Tyr Leu Tyr Tyr Leu Ser
 Lys Thr Ile Asn Gly Ser Gly Gln Asn Gln Gln Thr Leu Lys Phe Ser
 Val Ala Gly Pro Ser Asn Met Ala Val Gln Gly Arg Asn Tyr Ile Pro
 Gly Pro Ser Tyr Arg Gln Gln Arg Val Ser Thr Thr Val Thr Gln Asn
 Asn Asn Ser Glu Phe Ala Trp Pro Gly Ala Ser Ser Trp Ala Leu Asn
 Gly Arg Asn Ser Leu Met Asn Pro Gly Pro Ala Met Ala Ser His Lys
 Glu Gly Glu Asp Arg Phe Phe Pro Leu Ser Gly Ser Leu Ile Phe Gly
 Lys Gln Gly Thr Gly Arg Asp Asn Val Asp Ala Asp Lys Val Met Ile
 Thr Asn Glu Glu Glu Ile Lys Thr Thr Asn Pro Val Ala Thr Glu Ser
 Tyr Gly Gln Val Ala Thr Asn His Gln Ser Ala Gln Ala Ala Ala Lys
 Gly Gly Gly Phe His Gly Ala Ser Ala Gln Ala Gln Thr Gly Trp Val
 Gln Asn Gln Gly Ile Leu Pro Gly Met Val Trp Gln Asp Arg Asp Val
 Tyr Leu Gln Gly Pro Ile Trp Ala Lys Ile Pro His Thr Asp Gly Asn
 Phe His Pro Ser Pro Leu Met Gly Gly Phe Gly Met Lys His Pro Pro
 Pro Gln Ile Leu Ile Lys Asn Thr Pro Val Pro Ala Asp Pro Pro Thr
 Ala Phe Asn Lys Asp Lys Leu Asn Ser Phe Ile Thr Gln Tyr Ser Thr
 Gly Gln Val Ser Val Glu Ile Glu Trp Glu Leu Gln Lys Glu Asn Ser
 Lys Arg Trp Asn Pro Glu Ile Gln Tyr Thr Ser Asn Tyr Tyr Lys Ser
 Asn Asn Val Glu Phe Ala Val Asn Thr Glu Gly Val Tyr Ser Glu Pro
 Arg Pro Ile Gly Thr Arg Tyr Leu Thr Arg Asn Leu (SEQ ID NO: 10)

AAV9 VP3 Capsid Amino Acid Sequence

Met Ala Ser Gly Gly Gly
 Ala Pro Val Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Ser Ser
 Ser Gly Asn Trp His Cys Asp Ser Gln Trp Leu Gly Asp Arg Val Ile
 Thr Thr Ser Thr Arg Thr Trp Ala Leu Pro Thr Tyr Asn Asn His Leu
 Tyr Lys Gln Ile Ser Asn Ser Thr Ser Gly Gly Ser Ser Asn Asp Asn
 Ala Tyr Phe Gly Tyr Ser Thr Pro Trp Gly Tyr Phe Asp Phe Asn Arg
 Phe His Cys His Phe Ser Pro Arg Asp Trp Gln Arg Leu Ile Asn Asn
 Asn Trp Gly Phe Arg Pro Lys Arg Leu Asn Phe Lys Leu Phe Asn Ile
 Gln Val Lys Glu Val Thr Asp Asn Asn Gly Val Lys Thr Ile Ala Asn
 Asn Leu Thr Ser Thr Val Gln Val Phe Thr Asp Ser Asp Tyr Gln Leu
 Pro Tyr Val Leu Gly Ser Ala His Glu Gly Cys Leu Pro Pro Phe Pro
 Ala Asp Val Phe Met Ile Pro Gln Tyr Gly Tyr Leu Thr Leu Asn Asp

Gly Ser Gln Ala Val Gly Arg Ser Ser Phe Tyr Cys Leu Glu Tyr Phe
Pro Ser Gln Met Leu Arg Thr Gly Asn Asn Phe Gln Phe Ser Tyr Glu
Phe Glu Asn Val Pro Phe His Ser Ser Tyr Ala His Ser Gln Ser Leu
Asp Arg Leu Met Asn Pro Leu Ile Asp Gln Tyr Leu Tyr Tyr Leu Ser
Lys Thr Ile Asn Gly Ser Gly Gln Asn Gln Gln Thr Leu Lys Phe Ser
Val Ala Gly Pro Ser Asn Met Ala Val Gln Gly Arg Asn Tyr Ile Pro
Gly Pro Ser Tyr Arg Gln Gln Arg Val Ser Thr Thr Val Thr Gln Asn
Asn Asn Ser Glu Phe Ala Trp Pro Gly Ala Ser Ser Trp Ala Leu Asn
Gly Arg Asn Ser Leu Met Asn Pro Gly Pro Ala Met Ala Ser His Lys
Glu Gly Glu Asp Arg Phe Phe Pro Leu Ser Gly Ser Leu Ile Phe Gly
Lys Gln Gly Thr Gly Arg Asp Asn Val Asp Ala Asp Lys Val Met Ile
Thr Asn Glu Glu Glu Ile Lys Thr Thr Asn Pro Val Ala Thr Glu Ser
Tyr Gly Gln Val Ala Thr Asn His Gln Ser Ala Gln Ala Gln Ala Gln
Thr Gly Trp Val Gln Asn Gln Gly Ile Leu Pro Gly Met Val Trp Gln
Asp Arg Asp Val Tyr Leu Gln Gly Pro Ile Trp Ala Lys Ile Pro His
Thr Asp Gly Asn Phe His Pro Ser Pro Leu Met Gly Gly Phe Gly Met
Lys His Pro Pro Pro Gln Ile Leu Ile Lys Asn Thr Pro Val Pro Ala
Asp Pro Pro Thr Ala Phe Asn Lys Asp Lys Leu Asn Ser Phe Ile Thr
Gln Tyr Ser Thr Gly Gln Val Ser Val Glu Ile Glu Trp Glu Leu Gln
Lys Glu Asn Ser Lys Arg Trp Asn Pro Glu Ile Gln Tyr Thr Ser Asn
Tyr Tyr Lys Ser Asn Asn Val Glu Phe Ala Val Asn Thr Glu Gly Val
Tyr Ser Glu Pro Arg Pro Ile Gly Thr Arg Tyr Leu Thr Arg Asn Leu
(SEQ ID NO: 11)

CLAIMS

1. A method of delivering a viral particle to the brain of a mammal comprising administering the viral particle to the cerebral spinal fluid (CSF) of the mammal and administering a plurality of microbubbles intravenously to the mammal, wherein one or more regions of the brain of the mammal are subjected to focused ultrasound (FUS).
2. The method of claim 1, wherein the FUS is MRI-guided focused ultrasound (MRIg-FUS).
3. The method of claim 1 or 2, wherein the mammal is a human.
4. The method of any one of claims 1-3, wherein the FUS is applied to the striatum of the mammal.
5. The method of any one of claims 1-3, wherein the FUS is applied to the caudate nucleus of the mammal.
6. The method of any one of claims 1-3, wherein the FUS is applied to the putamen of the mammal.
7. The method of any one of claims 1-3, wherein the FUS is applied to caudate nucleus and the putamen of the mammal.
8. The method of any one of claims 1-7, wherein the viral particle is administered to the brain via intracisternal magna (ICM) administration.
9. The method of any one of claims 1-8, wherein the plurality of microbubbles are administered at about 0.2 mL/kg.
10. The method of any one of claims 1-9, wherein administering the viral particle comprises a dose level of from about 1×10^{12} to about 8×10^{13} genome copies per kilogram (GC/kg).

11. The method of any one of claims 1-9, wherein administering the viral particle comprises a dose level of from about 1×10^{12} to about 8×10^{12} genome copies per kilogram (GC/kg).
12. The method of any one of claims 1-9, wherein administering the viral particle comprises a dose level of from about 2×10^{12} to about 6×10^{12} genome copies per kilogram (GC/kg).
13. The method of any one of claims 1-9, wherein administering the viral particle comprises a dose level of at most 1×10^{12} genome copies per kilogram (GC/kg).
14. The method of any one of claims 1-9, wherein administering the viral particle comprises a dose level of at most 2×10^{12} genome copies per kilogram (GC/kg).
15. The method of any one of claims 1-9, wherein administering the viral particle comprises a dose level of at most 8×10^{12} genome copies per kilogram (GC/kg).
16. The method of any one of claims 1-9, wherein administering the viral particle comprises a dose level of at most 1×10^{13} genome copies per kilogram (GC/kg).
17. The method of any one of claims 1-9, wherein administering the viral particle comprises a dose level of at most 2×10^{13} genome copies per kilogram (GC/kg).
18. The method of any one of claims 1-17, wherein the FUS is applied simultaneously with said administering of the viral particle.
19. The method of any one of claims 1-17, wherein the FUS is applied prior to said administering of the viral particle.
20. The method of any one of claims 1-17, wherein the FUS is applied after said administering of the viral particle.

21. The method of claim 20, wherein the FUS is applied at least five minutes after said administering of the viral particle.
22. The method of claim 20, wherein the FUS is applied from about 1 minute to about 180 minutes after said administering of the viral particle.
23. The method of claim 20, wherein the FUS is applied from about 30 minutes to about 180 minutes after said administering of the viral particle.
24. The method of any one of claims 1-23, wherein the viral particle comprises a viral vector encoding a heterologous nucleic acid.
25. The method of claim 24, wherein following the administration of the viral particle, the heterologous nucleic acid is expressed in the striatum of the mammal.
26. The method of claim 24, wherein following the administration of the viral particle, the heterologous nucleic acid is expressed in the cerebral cortex of the mammal.
27. The method of claim 24, wherein following the administration of the viral particle, the heterologous nucleic acid is expressed in the striatum and cerebral cortex of the mammal.
28. The method of any one of claims 25-27, wherein following the administration of the viral particle, the heterologous nucleic acid is expressed in the thalamus of the mammal.
29. The method of any one of claims 24-28, wherein following the administration of the viral particle, the heterologous nucleic acid is expressed in the superficial brain areas of the mammal.
30. The method of any one of claims 24-29, wherein the heterologous nucleic acid encodes a therapeutic polypeptide or therapeutic nucleic acid.

31. The method of any one of claims 24-29, wherein the heterologous nucleic acid encodes a therapeutic polypeptide.

32. The method of claim 31, wherein the therapeutic polypeptide is an enzyme, a neurotrophic factor, a polypeptide that is deficient or mutated in an individual with a CNS-related disorder, an antioxidant, an anti-apoptotic factor, an anti-angiogenic factor, and an anti-inflammatory factor, acid beta-glucosidase (GBA), beta-galactosidase-1 (GLB1), iduronate 2-sulfatase (IDS), galactosylceramidase (GALC), a mannosidase, alpha-D-mannosidase (MAN2B1), beta-mannosidase (MANBA), pseudoarylsulfatase A (ARSA), N-acetylglucosamine-1-phosphotransferase (GNPTAB), acid sphingomyelinase (ASM), Niemann-Pick C protein (NPC1), acid alpha-1,4-glucosidase (GAA), hexosaminidase beta subunit, HEXB, N-sulfoglucosamine sulfohydrolase (MPS3A), N-alpha-acetylglucosaminidase (NAGLU), heparin acetyl-CoA, alpha-glucosaminidase N-acetyltransferase (MPS3C), N-acetylglucosamine-6-sulfatase (GNS), alpha-N-acetylglactosaminidase (NAGA), beta-glucuronidase (GUSB), hexosaminidase alpha subunit (HEXA), lysosomal acid lipase (LIPA), Aspartylglucosaminidase, Alpha-galactosidase A, Palmitoyl protein thioesterase, Tripeptidyl peptidase, Lysosomal transmembrane protein, Cysteine transporter, Acid ceramidase, Acid alpha-L-fucosidase, cathepsin A, alpha-L-iduronidase, Arylsulfatase B, Arylsulfatase A, N-acetylglactosamine-6-sulfate, Acid beta-galactosidase, or alpha-neuramidase.

33. The method of any one of claims 24-29, wherein the heterologous nucleic acid encodes a therapeutic nucleic acid.

34. The method of claim 33, wherein the therapeutic nucleic acid is an siRNA, an shRNA, an RNAi, an miRNA, an antisense RNA, a ribozyme or a DNAzyme.

35. The method of any one of claims 31-34, wherein the therapeutic polypeptide or the therapeutic nucleic acid is used to treat a disorder of the CNS.

36. The method of claim 35, wherein the disorder of the CNS is a lysosomal storage disease (LSD), Huntington's disease, epilepsy, Parkinson's disease, Alzheimer's disease, stroke,

corticobasal degeneration (CBD), corticogasal ganglionic degeneration (CBGD), frontotemporal dementia (FTD), multiple system atrophy (MSA), progressive supranuclear palsy (PSP) or cancer of the brain.

37. The method of any one of claims 1-36, wherein the viral particle is a recombinant adeno-associated virus (rAAV).

38. The method of claim 37, wherein the rAAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV2R471A, AAV2/2-7m8, AAV DJ, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, a goat AAV, AAV1/AAV2 chimeric, bovine AAV, or mouse AAV capsid rAAV2/HBoV1 serotype capsid.

39. The method of claim 37, wherein the rAAV particle comprises an AAV serotype 2 (AAV2) capsid.

40. The method of claim 37, wherein the rAAV particle comprises an AAV-HBKO serotype 2 (AAV2) capsid.

41. The method of claim 37, wherein the rAAV particle comprises an AAV9 serotype capsid.

42. The method of claim 37, wherein the rAAV particle comprises a modified AAV9 serotype capsid.

43. The method of claim 42, wherein the rAAV particle comprises a modified AAV9 serotype capsid having SEQ ID NO: 9.

44. The method of any one of claims 37-43, wherein the rAAV vector comprises the heterologous nucleic acid flanked by one or more AAV inverted terminal repeat (ITR) sequences.

45. The method of claim 44, wherein the heterologous nucleic acid is flanked by two AAV ITRs.
46. The method of claim 44 or 45, wherein the AAV ITRs are AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV2R471A, AAV DJ, a goat AAV, bovine AAV, or mouse AAV serotype ITRs.
47. A method of treating a neurogenerative disorder, said method comprising administering a viral particle encoding a heterologous nucleic acid to the cerebral spinal fluid (CSF) of the brain of a mammal and administering a plurality of microbubbles intravenously to the mammal, wherein one or more regions of the brain of the mammal are subjected to focused ultrasound (FUS).
48. The method of claim 47, wherein the neurogenerative disorder is Alzheimer's disease.
49. The method of claim 47, wherein the neurogenerative disorder is Parkinson's disease.
50. The method of claim 47, wherein the neurogenerative disorder is amyotrophic lateral sclerosis.
51. The method of claim 47, wherein the neurogenerative disorder is spinal muscular atrophy.
52. The method of any one of claims 47-51, wherein the FUS is MRI-guided focused ultrasound (MRIg-FUS).
53. The method of any one of claims 47-52, wherein the mammal is a human.
54. The method of any one of claims 47-53, wherein the FUS is applied to the striatum of the mammal.
55. The method of any one of claims 47-53, wherein the FUS is applied to the caudate nucleus of the mammal.

56. The method of any one of claims 47-53, wherein the FUS is applied to the putamen of the mammal.
57. The method of any one of claims 47-53, wherein the FUS is applied to caudate nucleus and the putamen of the mammal.
58. The method of any one of claims 47-57, wherein the viral particle is administered to the brain via intracisternal magna (ICM) administration.
59. The method of any one of claims 47-58, wherein the plurality of microbubbles are administered at about 0.2 mL/kg.
60. The method of any one of claims 47-59, wherein administering the viral particle comprises a dose level of from about 1×10^{12} to about 8×10^{13} genome copies per kilogram (GC/kg).
61. The method of any one of claims 47-59, wherein administering the viral particle comprises a dose level of from about 1×10^{12} to about 8×10^{12} genome copies per kilogram (GC/kg).
62. The method of any one of claims 47-59, wherein administering the viral particle comprises a dose level of from about 2×10^{12} to about 6×10^{12} genome copies per kilogram (GC/kg).
63. The method of any one of claims 47-59, wherein administering the viral particle comprises a dose level of at most 1×10^{12} genome copies per kilogram (GC/kg).
64. The method of any one of claims 47-59, wherein administering the viral particle comprises a dose level of at most 2×10^{12} genome copies per kilogram (GC/kg).

65. The method of any one of claims 47-59, wherein administering the viral particle comprises a dose level of at most 3×10^{12} genome copies per kilogram (GC/kg).
66. The method of any one of claims 47-59, wherein administering the viral particle comprises a dose level of at most 8×10^{12} genome copies per kilogram (GC/kg).
67. The method of any one of claims 47-59, wherein administering the viral particle comprises a dose level of at most 1×10^{13} genome copies per kilogram (GC/kg).
68. The method of any one of claims 47-59, wherein administering the viral particle comprises a dose level of at most 2×10^{13} genome copies per kilogram (GC/kg).
69. The method of any one of claims 47-68, wherein the FUS is applied simultaneously with said administering of the viral particle.
70. The method of any one of claims 47-68, wherein the FUS is applied prior to said administering of the viral particle.
71. The method of any one of claims 47-68, wherein the FUS is applied after said administering of the viral particle.
72. The method of claim 71, wherein the FUS is applied at least five minutes after said administering of the viral particle.
73. The method of claim 71, wherein the FUS is applied from about 1 minute to about 180 minutes after said administering of the viral particle.
74. The method of claim 71, wherein the FUS is applied from about 30 minutes to about 180 minutes after said administering of the viral particle.

75. The method of any one of claims 47-74, wherein following the administration of the viral particle, the heterologous nucleic acid is expressed in the striatum of the mammal.
76. The method of any one of claims 47-74, wherein following the administration of the viral particle, the heterologous nucleic acid is expressed in the cerebral cortex of the mammal.
77. The method of any one of claims 47-74, wherein following the administration of the viral particle, the heterologous nucleic acid is expressed in the striatum and cerebral cortex of the mammal.
78. The method of any one of claims 75-77, wherein following the administration of the viral particle, the heterologous nucleic acid is expressed in the thalamus of the mammal.
79. The method of any one of claims 75-78, wherein following the administration of the viral particle, the heterologous nucleic acid is expressed in the superficial brain areas of the mammal.
80. The method of any one of claims 47-79, wherein the heterologous nucleic acid encodes a therapeutic polypeptide or therapeutic nucleic acid.
81. The method of any one of claims 47-79, wherein the heterologous nucleic acid encodes a therapeutic polypeptide.
82. The method of any one of claims 47-81 wherein the viral particle is a recombinant adeno-associated virus (rAAV).
83. The method of claim 82, wherein the rAAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV2R471A, AAV2/2-7m8, AAV DJ, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, a goat AAV, AAV1/AAV2 chimeric, bovine AAV, or mouse AAV capsid rAAV2/HBoV1 serotype capsid.

84. The method of claim 82, wherein the rAAV particle comprises an AAV serotype 2 (AAV2) capsid.
85. The method of claim 82, wherein the rAAV particle comprises an AAV-HBKO serotype 2 (AAV2) capsid.
86. The method of claim 82, wherein the rAAV particle comprises an AAV9 serotype capsid.
87. The method of claim 82, wherein the rAAV particle comprises a modified AAV9 serotype capsid.
88. The method of claim 87, wherein the rAAV particle comprises a modified AAV9 serotype capsid having SEQ ID NO: 9.
89. The method of any one of claims 82-88, wherein the rAAV vector comprises the heterologous nucleic acid flanked by one or more AAV inverted terminal repeat (ITR) sequences.
90. The method of claim 89, wherein the heterologous nucleic acid is flanked by two AAV ITRs.
91. The method of claim 89 or 90, wherein the AAV ITRs are AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV2R471A, AAV DJ, a goat AAV, bovine AAV, or mouse AAV serotype ITRs.
92. A method of delivering a viral particle to the brain of a mammal comprising administering the viral particle to the CSF of the mammal and administering MB intravenously to the mammal, wherein one or more regions of the brain of the mammal are subjected to FUS and administration of MB (FUS-MB), thereby allowing for increased delivery of the clinically relevant viral particle by at least 2-fold compared to delivery without FUS-MB.

93. A method of delivering a viral particle to the brain of a mammal comprising administering the viral particle to the CSF of the mammal and administering MB intravenously (IV) to the mammal, wherein one or more regions of the brain of the mammal are subjected to FUS-MB, thereby allowing for non-invasive delivery of AAV via IV or ICM administration to FUS-targeted brain areas at dosages at least 50 times lower than needed for blood-brain-barrier (BBB) crossing by AAV alone.

94. A method of delivering a viral particle to the brain of a mammal comprising administering the viral particle via ICM to the CSF of the mammal and administering MBs intravenously to the mammal, wherein one or more regions of the brain of the mammal are subjected to FUS-MB, and wherein expression of a viral vector of the viral particle in the brain is increased at least 5-fold compared to expression without FUS-MB.