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(54) Title: GENE THERAPY FOR TREATMENT OF CANAVAN DISEASE

(57) Abstract: Compositions and methods for treatment of Canavan disease are provided. The compositions include recombinant adeno-associated virus (rAAV) vectors, lipid nanoparticles, a recombinant nucleic acid molecules having a nucleotide sequence encoding a functional aspartoacylase (ASPA). In one embodiment, the recombinant adeno-associated virus (rAAV) comprises an adeno-associated virus (AAV) capsid and a vector genome packaged therein, wherein the vector genome comprises an expression cassette comprising one or more regulatory sequences operably linked to a nucleotide sequence encoding a functional aspartoacylase (ASPA), and wherein the nucleotide sequence encoding the functional ASPA is SEQ ID NO: 1 or a nucleotide sequence at least 99% identical to SEQ ID NO: 1.

GENE THERAPY FOR TREATMENT OF CANAVAN DISEASE

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

The electronic sequence listing filed herewith named “22-9850PCT.xml” (29,558
5 bytes and created on December 10, 2024) is incorporated herein by reference in its
entirety.

BACKGROUND OF THE INVENTION

Canavan disease (CD) is a leukodystrophy caused by autosomal recessive
10 mutations in a gene for an enzyme called aspartoacylase (ASP), which breaks down N-
acetyl-l-aspartic acid (NAA). The disease is prevalent in, but not restricted to, Ashkenazi
Jewish individuals and typically begins in infancy. The resulting deficiency of ASPA
leads to accumulation of NAA in the brain and to oligodendrocyte dysfunction,
spongiform changes, and absence of myelin. However, the precise mechanisms causing
15 spongiform degeneration are uncertain. Diagnosis relies on clinical features,
neuroimaging, genetic testing, and exclusion of other leukodystrophies. Treatment is
supportive and the prognosis is poor with limited survival beyond the first decade.

What is needed are improved compositions and methods for treatment of Canavan
disease.

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SUMMARY OF THE INVENTION

In one aspect, provided herein is a recombinant adeno-associated virus (rAAV)
comprising an adeno-associated virus (AAV) capsid and a vector genome packaged
therein, wherein the vector genome comprises an expression cassette comprising one or
25 more regulator sequences operably linked to a nucleotide sequence encoding a functional
aspartoacylase (ASP), wherein the nucleotide sequence encoding the ASP is SEQ ID
NO: 1 or a nucleotide sequence at least 99% identical to SEQ ID NO: 1. In certain
embodiments, the functional ASP comprises the amino acid sequence of SEQ ID NO: 3
or an amino acid sequence at least 99% identical to SEQ ID NO: 3. In certain
30 embodiments, a pharmaceutical composition comprising the rAAV and a carrier, diluent,
and/or excipient is provided.

In another aspect, provided herein is a recombinant nucleic acid molecule comprising an AAV vector genome comprising: (a) an AAV 5' ITR sequence; (b) an expression cassette comprising one or more regulator sequences operably linked to a nucleotide sequence encoding a functional ASPA, wherein the nucleotide sequence encoding the ASPA is SEQ ID NO: 1 or a nucleotide sequence at least 99% identical to SEQ ID NO: 1; and (c) an AAV 3' ITR sequence. In certain embodiments, the functional ASPA comprises the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence at least 99% identical to SEQ ID NO: 3. In certain embodiments, the recombinant nucleic acid molecule is a plasmid. In certain embodiments, a packaging host cell comprising the recombinant nucleic acid molecule is provided.

In another aspect, provided herein is a method of treating Canavan disease in a subject in a need thereof, the method comprising administering to the subject a therapeutically effective amount of an rAAV, composition, or pharmaceutical composition described herein.

In another aspect, provided herein is a recombinant nucleic acid molecule that is a messenger RNA (mRNA) comprising a nucleotide sequence encoding a functional aspartoacylase (ASPA), wherein the nucleotide sequence encoding the functional ASPA is SEQ ID NO: 10 or a nucleotide sequence at least 99% identical to SEQ ID NO: 10. In certain embodiments, the functional ASPA comprises the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence at least 99% identical to SEQ ID NO: 3. In certain embodiments, the mRNA comprises a 5' cap, Kozak sequence, and/or polyA sequence. In certain embodiments, the mRNA is a nucleoside-modified mRNA. In certain embodiments, the recombinant nucleic acid molecule is encapsulated in a lipid nanoparticle (LNP).

In another aspect, provided herein is a method of treating Canavan disease in a subject in a need thereof, the method comprising administering to the subject a therapeutically effective amount of a recombinant nucleic acid molecule or composition described herein.

These and other advantages of the various embodiments described herein will be apparent from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a map of a vector genome for delivery of an engineered ASPA coding sequence.

FIG. 2 shows body weights of mice following ICV or IV administration of an AAV vector for delivery of ASPA.

FIG. 3 shows results of neurological and motor coordination assessments of mice following ICV or IV administration of an AAV vector for delivery of ASPA.

FIG. 4A and FIG. 4B show results from histological assessments of brain vacuolation following ICV or IV administration of an AAV vector for delivery of ASPA.

FIG. 5 shows representative immunofluorescence images for expression of ASPA in the CNS following administration of an AAV vector for delivery of ASPA.

FIG. 6 shows a study overview for assessing kinetics of LNP-ASPA mRNA delivery to the CNS of ASPAnur7 mice via ICV route of administration.

FIG. 7A – FIG. 7E shows representative IHC images for expression of ASPA in the CNS of ASPAnur7 mice following administration of LNP-ASPA mRNA on day 1 (D1), day 2 (D2), day 7 (D7), and day 14 (D14). Wildtype (WT) and ASPAnur7 baseline (BL) controls are shown in FIG. 7A.

FIG. 8A and FIG. 8B show representative immunofluorescence images for expression of ASPA in the CNS following administration of LNP-ASPA mRNA, including labeling of oligodendrocytes (Olig-2) and neurons (Neu-n).

FIG. 9 shows results from histological assessments of brain vacuolation following ICV or IV administration of LNP-ASPA mRNA.

FIG. 10 shows a comparison of vacuolation in ASPAnur7 (ASPA KO) mice that were administered LNP- ASPA mRNA or an AAV vector for delivery of ASPA.

FIG. 11 shows an overview of a study design to assess repeated administration of LNP-mRNA to ASPAnur7 mice.

DETAILED DESCRIPTION OF THE INVENTION

Provided herein are composition containing a nucleic acid having a sequence encoding aspartoacylase (ASPA). The compositions, as well as formulation and compositions containing these nucleic acids, are useful for treatment of Canavan disease in

patients in need thereof.

The terms “aspartoacylase,” “ASPA,” and “hASPA,” are used herein interchangeably and may refer to a gene or coding sequence, a nucleic acid sequence, or gene product.

5 “Patient” or “subject” or “individual” as used herein refers to a mammalian animal, including a human, a veterinary or farm animal, a domestic animal or pet, and animals normally used for clinical research. In certain embodiments, the subject is a human.

The words “comprise”, “comprises”, and “comprising” are to be interpreted inclusively rather than exclusively. The words “consist”, “consisting”, and its variants, are
10 to be interpreted exclusively, rather than inclusively. While various embodiments in the specification are presented using “comprising” language, under other circumstances, a related embodiment is also intended to be included and described using “consisting of” or “consisting essentially of” language. As used throughout this specification and the claims, the terms “comprising”, “containing”, “including”, and its variants are inclusive of other
15 components, elements, integers, steps and the like. Conversely, the term “consisting” and its variants are exclusive of other components, elements, integers, steps and the like.

It is to be noted that the term “a” or “an” refers to one or more. As such, the terms “a” (or “an”), “one or more,” and “at least one” are used interchangeably herein.

As used herein, the term “about” or “~” refers to a variant of $\pm 10\%$ from the
20 reference integer and values therebetween, unless otherwise specified. For example, “about” 500 μM includes ± 50 (i.e., 450 – 550, which includes the integers therebetween). For other values, particularly when reference is to a percentage (e.g., 90% of taste), the term “about” is inclusive of all values within the range including both the integer and fractions.

25 As described above, the term “about” when used to modify a numerical value means a variation of $\pm 10\%$, ($\pm 10\%$, e.g., ± 1 , ± 2 , ± 3 , ± 4 , ± 5 , ± 6 , ± 7 , ± 8 , ± 9 , ± 10 , or values therebetween) from the reference given, unless otherwise specified.

In certain instances, the term “E+#” or the term “e+#” is used to reference an exponent. For example, “5E10” or “5e10” is 5×10^{10} . These terms may be used
30 interchangeably.

With regard to the description of various embodiments herein, it is intended that

each of the compositions herein described, is useful, in another embodiment, in the methods of the invention. In addition, it is also intended that each of the compositions herein described as useful in the methods, is, in another embodiment, itself an embodiment of the invention.

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

10 *Nucleic Acids/Expression Cassettes*

In one aspect, provided herein are recombinant nucleic acid molecules that include an ASPA coding sequence. The nucleic acids are useful for generating vectors for therapeutic delivery of the ASPA transgene to target cells of a subject.

The term “nucleic acid” as used herein can be RNA, DNA, or a modification
15 thereof, and can be single or double stranded, and can be selected, for example, from a group including nucleic acid encoding a protein of interest, oligonucleotides, nucleic acid analogues, for example peptide-nucleic acid (PNA), pseudocomplementary PNA (pc-PNA), locked nucleic acid (LNA) etc. A nucleotide refers to a ribonucleotide,
20 deoxynucleotide or a modified form of either type of nucleotide (e.g., a peptide nucleic acid oligomer). One of ordinary skill will appreciate that functional variants of these nucleic acid molecules are also intended to be a part of the present invention. Functional variants are nucleic acid sequences that can be directly translated, using the standard genetic code, to provide an amino acid sequence identical to that translated from the parental nucleic acid molecules.

25 As used herein, the terms “nucleic acid sequence” and “nucleotide sequence” refer interchangeably to any molecule composed of or comprising monomeric nucleotides connected by phosphodiester linkages.

In certain embodiments, the recombinant nucleic acid molecules encoding a functional ASPA protein, and other constructs encompassed by the present disclosure, are
30 useful in generating expression cassettes and vector genomes and may be engineered for expression in yeast cells, insect cells, or mammalian cells, such as human cells. Methods

are known and have been described previously (e.g., WO 96/09378). A sequence is considered engineered if at least one non-preferred codon as compared to a wild type (WT) sequence is replaced by a codon that is more preferred. Herein, a non-preferred codon is a codon that is used less frequently in an organism than another codon coding for the same amino acid, and a codon that is more preferred is a codon that is used more frequently in an organism than a non-preferred codon. The frequency of codon usage for a specific organism can be found in codon frequency tables, such as in kazusa.jp/codon. Preferably more than one non-preferred codon, preferably most or all non-preferred codons, are replaced by codons that are more preferred. Preferably the most frequently used codons in an organism are used in an engineered sequence. Replacement by preferred codons generally leads to higher expression. It will also be understood by a skilled person that numerous different nucleic acid molecules can encode the same polypeptide as a result of the degeneracy of the genetic code. It is also understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the amino acid sequence encoded by the nucleic acid molecules to reflect the codon usage of any particular host organism in which the polypeptides are to be expressed. Therefore, unless otherwise specified, a “nucleic acid sequence encoding an amino acid sequence” includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleic acid sequences can be cloned using routine molecular biology techniques, or generated de novo by DNA synthesis, which can be performed using routine procedures by service companies having business in the field of DNA synthesis and/or molecular cloning (e.g., GeneArt, GenScript, Life Technologies, Eurofins).

By “engineered” is meant that a nucleic acid molecule that contains a sequence encoding an ASPA gene described herein in any suitable genetic element, e.g., naked DNA, phage, transposon, cosmid, episome, etc., which transfers the ASPA encoding sequence to a host cell, e.g., for generating non-viral delivery systems (e.g., RNA-based systems, naked DNA, or the like), or for generating viral vectors in a packaging host cell, and/or for delivery to target cells in a subject. The methods used to make such engineered constructs are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. See, e.g., Green and

Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (2012).

As used herein, the term “coding sequence” or refers to a nucleotide sequence that encodes a protein or polypeptide and denotes a sequence which is transcribed (in the case
5 of DNA) and translated (in the case of mRNA) into a polypeptide in vitro or in vivo when placed under the control of (operably linked to) one or more suitable regulatory sequences. Boundaries of a coding sequence are generally determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from prokaryotic or eukaryotic mRNA,
10 genomic DNA sequences from prokaryotic or eukaryotic DNA, and even synthetic DNA sequences.

As used herein, an “expression cassette” refers to a nucleic acid molecule comprising a biologically useful nucleic acid sequence (e.g., a gene cDNA encoding a protein, enzyme or other useful gene product, mRNA, etc.) and one or more regulatory
15 sequences operably linked thereto that direct or modulate transcription, translation, and/or expression of the nucleic acid sequence and its gene product. In certain embodiments, the term “expression cassette” refers to a nucleic acid molecule that includes an ASPA-encoding sequence and regulatory sequences therefore (e.g., promoter, enhancer, or polyA sequences), which expression cassette may be packaged into the capsid of a viral vector
20 (e.g., a viral particle). Typically, such an expression cassette for generating a viral vector contains a ASPA sequence described herein flanked by packaging signals of the viral genome and other expression control sequences such as those described herein. For example, for an AAV viral vector, the packaging signals are a 5' inverted terminal repeat (ITR) sequence and a 3' ITR sequence.

25 The term “expression” is used herein in its broadest meaning and comprises the production of RNA or of RNA and protein. Expression may be transient or may be stable.

In certain embodiments, provided herein is recombinant nucleic acid molecule that includes an expression cassette comprising: (i) a promoter sequence, (ii) a coding sequence for a functional ASPA protein, and (iii) a polyA sequence, optionally with spacer
30 sequences therebetween. In certain embodiments, the expression cassette comprises (i) a 5' AAV ITR sequence, (ii) a promoter sequence, (iii) a coding sequence for a function

ASPA protein, (iv) a polyA sequence, and (v) a 3' AAV ITR sequence, optionally with spacer sequences therebetween. In certain embodiments, the one or more of the promoter sequence, ASPA coding sequence, and polyA sequence are operably linked. In certain embodiments, the expression cassette includes additional regulatory elements (e.g., an
5 intron sequence).

A “vector” as used herein is a biological or chemical moiety comprising a nucleic acid sequence that can be introduced into an appropriate target cell for replication or expression of the nucleic acid sequence. Examples of a vector include but not limited to a recombinant virus, a plasmid, Lipoplexes, a Polymersome, Polyplexes, a dendrimer, a cell
10 penetrating peptide (CPP) conjugate, a magnetic particle, or a lipid nanoparticle (LNP). In certain embodiments, a vector is a nucleic acid molecule into which an engineered nucleic acid encoding a functional ASPA may be inserted, which can then be introduced into an appropriate target cell. Such vectors preferably have one or more origin of replication, and one or more site into which the recombinant DNA can be inserted. Vectors often have
15 means by which cells with vectors can be selected from those without, e.g., they encode drug resistance genes. Common vectors include plasmids, viral genomes, and “artificial chromosomes”. Conventional methods of generation, production, characterization or quantification of the vectors are available to one of skill in the art.

In certain embodiments, the vector described herein is a “replication-defective
20 virus” or a “viral vector” which refers to a synthetic or artificial viral particle in which an expression cassette containing a nucleic acid sequence encoding ASPA is packaged in a viral capsid or envelope, where any viral genomic sequences also packaged within the viral capsid or envelope are replication-deficient; i.e., they cannot generate progeny virions but retain the ability to infect target cells.

25 As used herein, a recombinant virus vector is an adeno-associated virus (AAV), an adenovirus, a bocavirus, a hybrid AAV/bocavirus, a herpes simplex virus, or a lentivirus.

In certain embodiments, a host cell having a nucleic acid including an ASPA encoding sequence or an expression cassette that includes an ASPA encoding sequence is provided. In certain embodiments, the host cell contains a plasmid having an ASPA
30 encoding sequence or an expression cassette that includes an ASPA encoding sequence as described herein.

As used herein, the term “host cell” may refer to a packaging cell line in which a vector (e.g., a recombinant AAV) is produced. A host cell may be a prokaryotic or eukaryotic cell (e.g., human, insect, or yeast) that contains exogenous or heterologous DNA that has been introduced into the cell by any means, e.g., electroporation, calcium phosphate precipitation, microinjection, transformation, viral infection, transfection, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion. Examples of host cells may include, but are not limited to an isolated cell, a cell culture, an *Escherichia coli* cell, a yeast cell, a human cell, a non-human cell, a mammalian cell, a non-mammalian cell, an insect cell, an HEK-293 cell, a liver cell, a kidney cell, a cell of the central nervous system, a neuron, a glial cell, or a stem cell.

As used herein, the term “target cell” refers to any cell in which expression of the functional ASPA is desired. In certain embodiments, the term “target cell” is intended to reference a cell of a subject being treated for Canavan disease that is intended to be transduced by a vector. Target cells include neuronal and non-neuronal cells. In certain embodiment, the target cell may be a central nervous system cell. In certain embodiments, the target cell is one or more of an excitatory neuron, an inhibitory neuron, a glial cell, a cortex cell, a frontal cortex cell, a cerebral cortex cell, a spinal cord cell. In certain embodiments, the target cells include oligodendrocytes of the CNS.

In certain embodiments, the compositions provided include an engineered hASPA coding sequence. The hASPA coding sequences utilized can be generated in vitro or synthetically, or by any other suitable method using techniques well known in the art. For example, the PCR-based accurate synthesis (PAS) of long DNA sequence method may be utilized, as described by Xiong et al, PCR-based accurate synthesis of long DNA sequences, *Nature Protocols* 1, 791 - 797 (2006). A method combining the dual asymmetrical PCR and overlap extension PCR methods is described by Young and Dong, Two-step total gene synthesis method, *Nucleic Acids Res.* 2004; 32(7): e59. See also, Gordeeva et al, *J Microbiol Methods*. Improved PCR-based gene synthesis method and its application to the *Citrobacter freundii* phytase gene codon modification. 2010 May;81(2):147-52. Epub 2010 Mar 10; see, also, the following patents on oligonucleotide synthesis and gene synthesis, *Gene Seq.* 2012 Apr;6(1):10-21; US 8008005; and US

7985565. Each of these documents is incorporated herein by reference. In addition, kits and protocols for generating DNA via PCR are available commercially. These include the use of polymerases including, without limitation, Taq polymerase; OneTaq® (New England Biolabs); Q5® High-Fidelity DNA Polymerase (New England Biolabs); and
5 GoTaq® G2 Polymerase (Promega). DNA may also be generated from cells transfected with plasmids containing the hOTC sequences described herein. Kits and protocols are known and commercially available and include, without limitation, QIAGEN plasmid kits; Chargeswitch® Pro Filter Plasmid Kits (Invitrogen); and GenElute™ Plasmid Kits (Sigma Aldrich). Other techniques useful herein include sequence-specific isothermal
10 amplification methods that eliminate the need for thermocycling. Instead of heat, these methods typically employ a strand-displacing DNA polymerase, like Bst DNA Polymerase, Large Fragment (New England Biolabs), to separate duplex DNA. DNA may also be generated from RNA molecules through amplification via the use of Reverse Transcriptases (RT), which are RNA-dependent DNA Polymerases. RTs polymerize a
15 strand of DNA that is complimentary to the original RNA template and is referred to as cDNA. This cDNA can then be further amplified through PCR or isothermal methods as outlined above. Custom DNA can also be generated commercially from companies including, without limitation, GenScript; GENEWIZ®; GeneArt® (Life Technologies); and Integrated DNA Technologies.

20 As used herein, the term “functional” refers to a biological molecule in a form in which it exhibits a property and/or activity by which it is characterized. A biological molecule may have two functions (i.e., bifunctional) or many functions (i.e., multifunctional).

By “functional ASPA,” is meant an encoded protein that provides at least about
25 50%, at least about 75%, at least about 80%, at least about 90%, or about the same, or greater than 100% of the biological activity level of the native ASPA protein, or a natural variant or polymorph thereof which is not associated with disease. A “functional ASPA” indicates that the protein provides biological function and/or activity including, but not limited to, the ability to convert NAA to acetate and aspartate. The biological activity of
30 ASPA, or a functional fragment thereof, also encompasses reversing or preventing the neurodegenerative phenotype associated with Canavan disease.

In certain embodiments, the amino acid sequence of the functional ASPA is that of SEQ ID NO: 3. In certain embodiments the amino acid sequence of the functional ASPA is SEQ ID NO: 3 or a sequence sharing at least 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 3.

5 In certain embodiments, a modified ASPA coding sequence is provided. In certain embodiments, the modified ASPA coding sequence is characterized by improved translation rate as compared to native hASPA following vector-mediated delivery. In certain embodiments, the modified ASPA coding sequence shares less than about 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%,
10 64%, 63%, 62%, 61% or less identity to the full-length native hASPA coding sequence. In certain embodiment, the modified hASPA coding sequence comprises SEQ ID NO: 1, or a sequence sharing at least 90%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 7. In certain embodiment, the modified hASPA coding sequence is a sequence sharing at least 90%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 1 that encodes the
15 amino acid sequence of SEQ ID NO: 3. In certain embodiments, the modified hASPA coding sequence is a sequence having up to 1, up to 2, up to 3, up to 4, up to 5, up to 6, up to 7, up to 8, up to 9, or up to 10 basepair mismatches when aligned to the nucleotide sequence of SEQ ID NO: 1, and expresses a functional ASPA.

In certain embodiments, provided herein is a recombinant nucleic acid molecule
20 that includes an expression cassette, wherein the expression cassette has the nucleic acid sequence of SEQ ID NO: 11, or a nucleic acid sequence sharing at least 90%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 11. In certain embodiments, the expression cassette has a nucleic acid sequence sharing at least 90%, 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 11, and includes one or more of the nucleotide sequence(s) set
25 forth in SEQ ID NOS: 1, 4, 5, 6, and 7. In certain embodiments, the expression cassette is flanked by a AAV 5' ITR sequence and a AAV 3' ITR sequence, optionally with additional sequences between the expression cassette and the flanking ITR sequences. In certain embodiments, provided herein is a recombinant nucleic acid molecule that includes the nucleotide sequence of SEQ ID NO: 2 or a nucleic acid sequence sharing at least 90%,
30 95%, 96%, 97%, 98%, or 99% identity with SEQ ID NO: 2. In certain embodiments, the expression cassette has a nucleic acid sequence sharing at least 90%, 95%, 96%, 97%,

98%, or 99% identity with SEQ ID NO: 2, and includes one or more of the nucleotide sequence(s) set forth in SEQ ID NOs: 1, 4, 5, 6, 7, 8, and 9.

The terms “percent (%) identity”, “sequence identity”, “percent sequence identity”, or “percent identical” in the context of nucleic acid sequences refers to the residues in the two sequences that are the same when aligned for correspondence. The length of sequence identity comparison may be over the full-length of the genome, the full-length of a gene coding sequence, or a fragment of at least about 500 to 5000 nucleotides, is desired.

However, identity among smaller fragments, e.g., of at least about nine nucleotides, usually at least about 20 to 24 nucleotides, at least about 28 to 32 nucleotides, at least about 36 or more nucleotides, may also be desired.

Percent identity may be readily determined for amino acid sequences over the full-length of a protein, polypeptide, about 32 amino acids, about 330 amino acids, or a peptide fragment thereof or the corresponding nucleic acid sequence coding sequences. A suitable amino acid fragment may be at least about 8 amino acids in length, and may be up to about 700 amino acids. Generally, when referring to “identity”, “homology”, or “similarity” between two different sequences, “identity”, “homology” or “similarity” is determined in reference to “aligned” sequences. “Aligned” sequences or “alignments” refer to multiple nucleic acid sequences or protein (amino acids) sequences, often containing corrections for missing or additional bases or amino acids as compared to a reference sequence.

Identity may be determined by preparing an alignment of the sequences and through the use of a variety of algorithms and/or computer programs known in the art or commercially available (e.g., BLAST, ExPASy; Clustal Omega; FASTA; using, e.g., Needleman-Wunsch algorithm, Smith-Waterman algorithm). Alignments are performed using any of a variety of publicly or commercially available Multiple Sequence Alignment Programs. Sequence alignment programs are available for amino acid sequences, e.g., the “Clustal Omega”, “Clustal X”, “MUSCLE”, “MAP”, “PIMA”, “MSA”, “BLOCKMAKER”, “MEME”, and “Match-Box” programs. Generally, any of these programs are used at default settings, although one of skill in the art can alter these settings as needed. Alternatively, one of skill in the art can utilize another algorithm or computer program which provides at least the level of identity or alignment as that provided by the referenced algorithms and programs. See, e.g., J. D. Thompson et al,

Nucl. Acids. Res., “A comprehensive comparison of multiple sequence alignments”, 27(13):2682-2690 (1999).

Multiple sequence alignment programs are also available for nucleic acid sequences. Examples of such programs include, “Clustal Omega”, “Clustal W”, “MUSCLE”, “CAP Sequence Assembly”, “BLAST”, “MAP”, and “MEME”, which are accessible through Web Servers on the internet. Other sources for such programs are known to those of skill in the art. Alternatively, Vector NTI utilities are also used. There are also a number of algorithms known in the art that can be used to measure nucleotide sequence identity, including those contained in the programs described above. As another example, polynucleotide sequences can be compared using Fasta™, a program in GCG Version 10.1. Fasta™ provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences. For instance, percent sequence identity between nucleic acid sequences can be determined using Fasta™ with its default parameters (a word size of 6 and the NOPAM factor for the scoring matrix) as provided in GCG Version 10.1, herein incorporated by reference.

As used herein, the term “regulatory sequence” or “expression control sequence” refers to nucleic acid sequences, such as initiator sequences, enhancer sequences, and promoter sequences, which induce, repress, or otherwise control the transcription of protein encoding nucleic acid sequences to which they are operably linked.

As used herein, “operably linked” sequences include both regulatory sequences that are contiguous or non-contiguous with an ASPA coding sequence and regulatory sequences that act in cis or trans with an ASPA coding sequence. Such regulatory sequences typically include, e.g., one or more of a promoter, an enhancer, an intron, a Kozak sequence, a polyadenylation sequence, and a TATA signal. The expression cassette may contain regulatory sequences upstream (5’ to) of the gene sequence, e.g., one or more of a promoter, an enhancer, an intron, etc., and one or more of an enhancer, or regulatory sequences downstream (3’ to) a gene sequence, e.g., 3’ untranslated region (3’ UTR) comprising a polyadenylation site, among other elements. In certain embodiments, the regulatory sequences are operably linked to the nucleic acid sequence of a gene product, wherein the regulatory sequences are separated from the nucleic acid sequence encoding the gene product by intervening nucleic acid sequences, i.e., 5’-untranslated regions (5’

UTR). In certain embodiments, the expression cassette comprises nucleic acid sequence of one or more of gene products. In some embodiments, the expression cassette can be a monocistronic or a bicistronic expression cassette.

As used herein, “encoded ASPA” or “encoded amino acid sequence” refers to the amino acid which is predicted based on the translation of a known DNA codon of a referenced nucleic acid sequence being translated to an amino acid.

In certain embodiments, the regulatory sequences include a promoter. In certain embodiments, the promoter is a chicken β -actin (also referred to as chicken beta-actin, CB, or CBA) promoter. In further embodiments, the promoter is a CB7 promoter (also referred to as hybrid CB7) comprising a cytomegalovirus immediate-early (CMV IE) enhancer and the chicken β -actin promoter sequences, optionally with spacer sequence, optionally with a chimeric intron comprising chicken beta actin intron and further comprising a chicken beta-actin splicing donor (including the exon sequence, chicken beta actin intron) and rabbit beta-globin splicing acceptor. See, e.g., cytomegalovirus (CMV) immediate early enhancer (260 bp, C4; GenBank # K03104.1). Chicken beta-actin promoter (281 bp; CB; GenBank # X00182.1).

In certain embodiments, still other promoters and/or enhancers may be selected. In another embodiment, a suitable promoter may include without limitation, an elongation factor 1 alpha (EF1 alpha) promoter (see, e.g., Kim DW et al, Use of the human elongation factor 1 alpha promoter as a versatile and efficient expression system. *Gene*. 1990 Jul 16;91(2):217-23), a human Synapsin 1 (hSyn) promoter (see, e.g., Kügler S et al, Human synapsin 1 gene promoter confers highly neuron-specific long-term transgene expression from an adenoviral vector in the adult rat brain depending on the transduced area. *Gene Ther*. 2003 Feb;10(4):337-47), a neuron-specific enolase (NSE) promoter (see, e.g., Kim J et al, Involvement of cholesterol-rich lipid rafts in interleukin-6-induced neuroendocrine differentiation of LNCaP prostate cancer cells. *Endocrinology*. 2004 Feb;145(2):613-9. Epub 2003 Oct 16), or a CB6 promoter (see, e.g., Large-Scale Production of Adeno-Associated Viral Vector Serotype-9 Carrying the Human Survival Motor Neuron Gene, *Mol Biotechnol*. 2016 Jan;58(1):30-6. doi: 10.1007/s12033-015-9899-5). In certain embodiments, the promoter is a JeT promoter (Tornøe J et al. *Gene*. 2002 Sep 4;297(1-2):21-32). In other embodiments, the promoter is a ubiquitin C (UbC) promoter. In other

embodiments, the UbC promoter is a human UbC promoter. See, e.g., WO 2001/091800. See, e.g., GenBank accession numbers AF232305 (rat), D63791 (human), NCBI Reference Sequence: NG_027722.2, and Schorpp et al. Nucleic Acids Res. 1996 May 1;24(9):1787-8. In still other embodiments, multiple enhancers and/or promoters may be included.

In certain embodiments, an additional or alternative promoter sequence may be included as part of the expression control sequences (regulatory sequences). Constitutive promoters, regulatable promoters [see, e.g., WO 2011/126808 and WO 2013/04943], tissue specific promoters, or a promoter responsive to physiologic cues may be utilized in the vectors described herein. The promoter(s) can be selected from different sources, e.g., human cytomegalovirus immediate-early (CMV IE) enhancer/promoter, the SV40 early enhancer/promoter, the JC polymovirus promoter, myelin basic protein (MBP) or glial fibrillary acidic protein (GFAP) promoters, herpes simplex virus (HSV-1) latency associated promoter (LAP), rouse sarcoma virus (RSV) long terminal repeat (LTR) promoter, neuron-specific promoter (NSE), platelet derived growth factor (PDGF) promoter, melanin-concentrating hormone (MCH) promoter, CBA, or matrix metalloprotein promoter (MPP), and the chicken beta-actin promoter. In certain embodiments, the expression cassette is designed for expression of ASPA in the central nervous system (CNS), including the cerebral spinal fluid and brain. In a further embodiment, the expression cassette is useful for expression in both the CNS and systemically.

In addition to a promoter, a vector may contain one or more other appropriate transcription initiation sequences, transcription termination sequences, enhancer sequences, efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA for example WPRE; sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product.

In certain embodiments, the regulatory sequences comprise an enhancer. In certain embodiments, the regulatory sequences comprise one enhancer. In another embodiment, the regulatory sequences contain two or more enhancers. These enhancers may be the

same or may be different. For example, an enhancer may include an alpha mic/bik enhancer or a CMV IE enhancer. The enhancer may be present in two copies which are located adjacent to one another in an expression cassette. Alternatively, the dual copies of the enhancer may be separated by one or more sequences.

5 In certain embodiments, the regulatory sequences comprise an intron. In certain embodiments, the intron is a chicken beta-actin intron. In one embodiment, the intron is 875 bp (GenBank # X00182.1). In certain embodiments, the intron is 973 bp intron from the chicken beta actin gene (GenBank # X00182.1). In certain embodiments, the intron is a chimeric intron comprising a chicken beta-actin splicing donor (including the exon
10 sequence), chicken beta actin intron, and rabbit beta globin splicing acceptor. In certain embodiments, the intron includes the nucleic acid sequence of SEQ ID NO: 6. In certain embodiments, the intron comprises a nucleotide sequence having up to 1, up to 2, up to 3, up to 4, up to 5, up to 6, up to 7, up to 8, up to 9, or up to 10 basepair mismatches when aligned to the nucleotide sequence of SEQ ID NO: 6. Other suitable introns include those
15 known in the art may be a human β -globulin intron, and/or a commercially available intron, and those described in WO 2011/126808.

 In certain embodiments, the regulatory sequences comprise a polyadenylation signal (polyA). Examples of suitable polyA sequences include, e.g., rabbit beta globin (RBG or rBG) poly A, SV40, SV50, bovine growth hormone (bGH), human growth
20 hormone, and synthetic polyAs. Optionally, one or more sequences may be selected to stabilize mRNA. In certain embodiments, the polyA is a rabbit beta globin poly A (rabbit globin polyA or rBG). See, e.g., WO 2014/151341. In certain embodiments, the rBG polyA comprises SEQ ID NO: 7. In certain embodiments, a human growth hormone (hGH) polyadenylation sequence, an SV40 polyA, or a synthetic polyA may be included
25 in an expression cassette. In certain embodiments, the SV40 polyA is selected.

 In certain embodiments, the expression cassette comprises at least one miRNA target sequence operably linked to a selected transgene, optionally in its 3' UTR and/or its 5' UTR. In certain embodiments, the mutant rAAV comprises a vector genome (comprising an expression cassette) which further comprises at least one miRNA seed,
30 binding site or full sequence. MicroRNAs (or miRNA or miR) are 19-25 nucleotide noncoding RNAs that bind to the sites of nucleic acid targets and down-regulate gene

expression either by reducing nucleic acid molecule stability or by inhibiting translation. In some embodiments, a microRNA sequence comprises a seed region, e.g., a sequence in the region of positions 2-8 of the mature microRNA, which has Watson-Crick sequence fully or partially complementarity to the miRNA target sequence of the nucleic acid. Such at least one miRNA may be used in combinations, including in an expression cassette or a vector genome also comprising a coding sequence for therapeutic protein, enzyme, or other moiety, and which is operably linked to the coding sequence. In certain embodiments, the vector genome may contain one miRNA to eight miRNA sequences, which are the same or different. Optionally, the vector genome does not contain therapeutic transgenes other than miRNA sequences.

In certain embodiments, the miRNA binding site is complementary to a miRNA expressed in a DRG (dorsal root ganglion) neuron, e.g., a miR183, and/or a miR182, binding site. In some embodiments, the miR binding site complementary to a miR expressed in expressed in a DRG neuron comprises a nucleotide sequence disclosed, e.g., in WO2020/132455, and in WO 2023/087019, the contents of which are incorporated by reference herein in its entirety.

In certain embodiments, the non-viral delivery system comprises an RNA molecule which is a messenger RNA (mRNA) molecule. In certain embodiments, the mRNA molecule is an in vitro transcribed (IVT) mRNA molecule. In certain embodiments, the IVT mRNA molecule is a nucleoside-modified mRNA molecule. In certain embodiments, the mRNA includes an ASPA coding sequence and a 5' untranslated region and/or a 3' untranslated region.

An mRNA may be a naturally or non-naturally occurring mRNA. An mRNA may include one or more modified nucleobases, nucleosides, or nucleotides. In some embodiments, the mRNA in the compositions comprise at least one modification which confers increased or enhanced stability to the nucleic acid, including, for example, improved resistance to nuclease digestion in vivo. An mRNA may include any number of base pairs, including tens, hundreds, or thousands of base pairs. Any number (e.g., all, some, or none) of nucleobases, nucleosides, or nucleotides may be an analog of a canonical species, substituted, modified, or otherwise non-naturally occurring. In certain embodiments, all of a particular nucleobase type may be modified. For example, all

cytosine in an mRNA may be 5-methylcytosine.

Recombinant Adeno-Associated Virus Vectors

In one aspect, provided herein are recombinant adeno-associated virus (rAAV)
5 vectors having capsid that contains an expression cassette that includes a nucleotide sequence encoding ASPA. The rAAV vectors are useful for treating Canavan disease in a subject in need thereof. Suitably, the AAV capsid selected targets cells to be treated.

As used herein, the terms “recombinant AAV”, “rAAV” and “artificial AAV” used interchangeably, mean, without limitation, a AAV comprising a capsid protein and a
10 vector genome packaged therein, wherein the vector genome comprising a nucleic acid heterologous to the AAV. In one embodiment, the capsid protein is a non-naturally occurring capsid. Such an artificial capsid may be generated by any suitable technique, using a selected AAV sequence (e.g., a fragment of a vp1 capsid protein) in combination with heterologous sequences which may be obtained from a different selected AAV, non-
15 contiguous portions of the same AAV, from a non-AAV viral source, or from a non-viral source. An artificial AAV may be, without limitation, a pseudotyped AAV, a chimeric AAV capsid, a recombinant AAV capsid, or a “humanized” AAV capsid. Pseudotyped vectors, wherein the capsid of one AAV is replaced with a heterologous capsid protein, are useful in the invention. In one embodiment, AAV2/5 and AAV2/8 are exemplary
20 pseudotyped vectors. The selected genetic element may be delivered by any suitable method, including transfection, electroporation, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion. The methods used to make such constructs are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic
25 techniques. See, e.g., Green and Sambrook, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY (2012).

As used herein, “rAAV.ASPA” or “rAAV.hASPA” refers to an rAAV having a vector genome that includes an ASPA coding sequence. A “rAAVhu68.ASPA” or
“rAAVhu68.hASPA” refers to an rAAV having an AAVhu68 capsid and a vector genome
30 that includes an ASPA coding sequence.

The rAAV vectors provided herein are not limited by the type of nucleic acid

molecule (e.g., vector genome) which is packaged in the mutant capsids. As used herein, a “vector genome” refers to the nucleic acid molecule packaged inside the rAAV capsid which forms a viral particle. Such a nucleic acid sequence contains AAV inverted terminal repeat sequences (ITRs). In the examples herein, a vector genome in a nucleic acid molecule useful in production contains, at a minimum, from 5' to 3', an AAV - 5' ITR, expression cassette comprising an ASPA coding sequence, and an AAV 3' ITR. In other embodiments, the orientation of the ITRs may change from the orientation presented in the vector genome of the nucleic acid used in production (e.g., a plasmid). Thus, in certain embodiments, the rAAV may comprise a vector genome flanked by 3' and 5' AAV ITRs, respectively. In certain embodiments, the rAAV may comprise a vector genome flanked by two 5' AAV ITRs. In certain embodiments, the rAAV may comprise a vector genome flanked by two 3' AAV ITRs. In other embodiments, an rAAV as provided herein may be partially truncated such that the 5' AAV ITR and/or the 3' AAV ITR is not detectable in the final rAAV product. In certain embodiments, the ITRs are from AAV2, a different source AAV than the capsid, or other than full-length ITRs may be selected. In certain embodiments, the ITRs are from the same AAV source as the AAV which provides the rep function during production or a trans-complementing AAV. Further, other ITRs, e.g., self-complementary (scAAV) ITRs, may be used. Both single-stranded AAV and self-complementary (sc) AAV are encompassed with the rAAV.

The term “AAV” as used herein refers to naturally occurring adeno-associated viruses, adeno-associated viruses available to one of skill in the art and/or in light of the composition(s) and method(s) described herein, as well as artificial AAVs. An adeno-associated virus (AAV) viral vector is an AAV DNase-resistant particle having an AAV protein capsid into which is packaged expression cassette flanked by AAV inverted terminal repeat sequences (ITRs) for delivery to target cells. An AAV capsid is composed of 60 capsid (cap) protein subunits, VP1, VP2, and VP3, that are arranged in an icosahedral symmetry in a ratio of approximately 1:1:10 to 1:1:20, depending upon the selected AAV. Various AAVs may be selected as sources for capsids of AAV viral vectors as identified above. See, e.g., US Published Patent Application No. 2007-0036760-A1; US Published Patent Application No. 2009-0197338-A1; EP 1310571. See also, WO 2003/042397 (AAV7 and other simian AAV), US Patent 7790449 and US

Patent 7282199 (AAV8), WO 2005/033321 and US 7,906,111 (AAV9), and WO 2006/110689, and WO 2003/042397 (rh.10). These documents also describe other AAV which may be selected for generating AAV and are incorporated by reference. Among the AAVs isolated or engineered from human or non-human primates (NHP) and well
5 characterized, human AAV2 is the first AAV that was developed as a gene transfer vector; it has been widely used for efficient gene transfer experiments in different target tissues and animal models. Unless otherwise specified, the AAV capsid, ITRs, and other selected AAV components described herein, may be readily selected from among any AAV, including, without limitation, the AAVs commonly identified as AAV1, AAV2, AAV3,
10 AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV8bp, AAV7M8 and AAVAnc80, AAVhu68, and variants of any of the known or mentioned AAVs or AAVs yet to be discovered or variants or mixtures thereof. An AAV9 capsid includes an rAAV having capsid proteins comprising an amino acid sequence which is 99% identical to AAS99264. See, also US7906111 and WO 2005/033321. Additional capsids are provided in WO
15 2022/119871 and WO 2022/226263, which are incorporated herein by reference. In certain embodiments, the capsid protein is designated by a number or a combination of numbers and letters following the term “AAV” in the name of the rAAV vector. See also PCT/US19/19804 and PCT/US19/19861, each entitled “Novel Adeno-Associated Virus (AAV) Vectors, AAV Vectors Having Reduced Capsid Deamidation And Uses Therefor”
20 and filed Feb 27, 2019, which are incorporated by reference herein in their entireties.

In certain embodiments, the AAV capsid is a Clade F capsid, such as AAV9 capsid, AAVhu68 capsid, hu31 capsid, hu32 capsid, or a variation thereof. See, e.g., WO 2005/033321 published April 14, 2015, WO 2018/160582, and US 2015/0079038, each of which is incorporated herein by reference in its entirety. In certain embodiments, the AAV
25 capsid is a non-clade F capsid, for example a Clade A, B, C, D, or E capsid. In certain embodiment, the non-Clade F capsid is an AAV1 or a variation thereof. In certain embodiment, the AAV capsid transduces a target cell other than the nervous system cells. In certain embodiments, the AAV capsid is a Clade A capsid (e.g., AAV1, AAV6, AAVrh91), a Clade B capsid (e.g., AAV 2), a Clade C capsid (e.g., hu53), a Clade D
30 capsid (e.g., AAV7), or a Clade E capsid (e.g., rh10).

In certain embodiments, the Clade F AAV capsid is an AAVhu68 capsid [See, e.g.,

US2020/0056159; PCT/US21/55436; SEQ ID NO: 8 for nucleic acid sequence; SEQ ID NO: 9 for amino acid sequence], an AAVhu95 capsid [See, e.g., US Provisional Application No. 63/251,599, filed October 2, 2021, International Patent Application No. PCT/US2022/077315, filed September 30, 2022]; an AAVhu96 capsid [See, e.g., US Provisional Application No. 63/251,599, filed October 2, 2021, and International Patent Application No. PCT/US2022/077315, filed September 30, 2022]; or an AAV9 capsid. In certain embodiments, the AAV capsid is a Clade A capsid, such as AAVrh91 capsid. See, PCT/US20/030266, filed April 29, 2020, now published WO2020/223231, and International Application No. PCT/US21/45945, filed August 13, 2021, which are incorporated herein by reference.

In certain embodiments, the AAV capsid for the compositions and methods described herein is chosen based on the target cell. In certain embodiment, the AAV capsid transduces a CNS cell and/or a PNS cell. In certain embodiments, another AAV capsid may be chosen. the AAV capsid is selected from a cy02 capsid, a rh43 capsid, an AAV8 capsid, a rh01 capsid, an AAV9 capsid, an rh8 capsid, a rh10 capsid, a bb01 capsid, a hu37 capsid, a rh02 capsid, a rh20 capsid, a rh39 capsid, a rh64 capsid, an AAV6 capsid, an AAV1 capsid, a hu44 capsid, a hu48 capsid, a cy05 capsid a hu11 capsid, a hu32 capsid, a pi2 capsid, or a variation thereof.

As used herein when used to refer to vp capsid proteins, the term “heterogeneous” or any grammatical variation thereof, refers to a population consisting of elements that are not the same, for example, having vp1, vp2, or vp3 (also referred to as VP1, VP2, VP3, or Vp1, Vp2, Vp3) monomers (proteins) with different modified amino acid sequences. The term “heterogeneous population” as used in connection with vp1, vp2 and vp3 proteins (alternatively termed isoforms), refers to differences in the amino acid sequence of the vp1, vp2 and vp3 proteins within a capsid. The AAV capsid contains subpopulations within the vp1 proteins, within the vp2 proteins and within the vp3 proteins which have modifications from the predicted amino acid residues. These subpopulations include, at a minimum, certain deamidated asparagine (N or Asn) residues. For example, certain subpopulations comprise at least one, two, three or four highly deamidated asparagines (N) positions in asparagine - glycine pairs and optionally further comprising other deamidated amino acids, wherein the deamidation results in an amino acid change and

other optional modifications.

As used herein, a “subpopulation” of vp proteins refers to a group of vp proteins which has at least one defined characteristic in common and which consists of at least one group member to less than all members of the reference group, unless otherwise specified.

5 For example, a “subpopulation” of vp1 proteins is at least one (1) vp1 protein and less than all vp1 proteins in an assembled AAV capsid, unless otherwise specified. A

“subpopulation” of vp3 proteins may be one (1) vp3 protein to less than all vp3 proteins in an assembled AAV capsid, unless otherwise specified. For example, vp1 proteins may be a subpopulation of vp proteins; vp2 proteins may be a separate subpopulation of vp

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

In certain embodiments, AAV capsids are provided which have a heterogeneous
15 population of AAV capsid isoforms (i.e., VP1, VP2, VP3) which contain multiple highly deamidated “NG” positions. In certain embodiments, the highly deamidated positions are in the locations identified below, with reference to the predicted full-length VP1 amino acid sequence. In other embodiments, the capsid gene is modified such that the referenced “NG” is ablated and a mutant “NG” is engineered into another position.

20 In certain embodiments, the rAAV provided herein is has an AAVhu68 capsid that includes AAVhu68 VP1 proteins, AAVhu68 VP2 proteins, and AAVhu68 VP3 proteins expressed from a nucleic acid sequence encoding SEQ ID NO: 13 (amino acids 1 to 736).

In certain embodiments, the AAVhu68 capsid comprises a heterogenous
population of AAVhu68 vp1 proteins, a heterogenous population of AAVhu68 vp2
25 proteins; and a heterogenous population of AAVhu68 vp3 proteins comprising amino acid residues 1 to 736 (vp1), amino acid residues 138 to 736 (vp2), and amino acid residues 203 to 736 (vp3) of SEQ ID NO: 13, respectively, with amino acid modifications, wherein the heterogenous AAVhu68 vp1 proteins, AAVhu68 vp2 proteins and AAVhu68 vp3 proteins contain amino acid modifications comprising 50% to 100% deamidation in at
30 least two asparagines (N) in asparagine - glycine pairs in SEQ ID NO: 13 in two or more of asparagines (N) at positions N57, N329, N452, and/or N512 based on the numbering of

the encoded AAVhu68 VP1 amino acid sequence (SEQ ID NO: 13) as determined using mass spectrometry and optionally further comprising subpopulations comprising other deamidated amino acids, wherein the deamidation results in an amino acid change, wherein the deamidated asparagines are deamidated to aspartic acid, isoaspartic acid, an interconverting aspartic acid/isoaspartic acid pair, or combinations thereof. In certain embodiments, the subpopulations of AAVhu68 VP1 proteins, AAVhu68 VP2 proteins and AAVhu68VP3 proteins further comprise one or more of: (a) at least 65% of asparagines (N) in position N57 of the vp1 proteins are deamidated, based on the numbering of SEQ ID NO: 13; (b) at least 75% of N in position N329 of the vp1, v2 and vp3 proteins are deamidated, based on the residue numbering of the amino acid sequence of SEQ ID NO: 13; (c) at least 50% of N in position N452 of the vp1, v2 and vp3 proteins are deamidated, based on the residue numbering of the amino acid sequence of SEQ ID NO: 13; and/or (d) at least 75% of N in N512 of the vp1, v2 and vp3 proteins are deamidated, based on the residue numbering of the amino acid sequence of SEQ ID NO: 13, wherein the heterogenous population of AAVhu68 vp1 proteins comprise glutamic acid at position 67 based on the numbering of SEQ ID NO: 13 and the heterogenous population of AAVhu68vp1 and AAVhu68 vp2 proteins comprising valine at position 157, based on the numbering of the vp1 capsid of SEQ ID NO: 13. In certain embodiments, the rAAVhu68 capsid comprises a subpopulation of vp1 in which 75% to 100 % of the N at position 57 of the vp1 proteins, based on the numbering of SEQ ID NO: 13, are deamidated as determined using mass spectrometry. In certain embodiments, the rAAVhu68 capsid comprises subpopulation of vp1 proteins, vp2 proteins, and/or vp3 proteins in which 75% to 100% of the N at position 329, based on the numbering of SEQ ID NO: 13, are deamidated as determined using mass spectrometry. In certain embodiments, the rAAVhu68 capsid comprises subpopulation of vp1 proteins, vp2 proteins, and/or vp3 proteins in which 75% to 100% of the N at position 452, based on the numbering of SEQ ID NO: 13, are deamidated as determined using mass spectrometry. In certain embodiments, the rAAVhu68 capsid comprises subpopulation of vp1 proteins, vp2 proteins, and/or vp3 proteins in which 75% to 100% of the N at position 512, based on the numbering of SEQ ID NO: 13, are deamidated. In certain embodiments, the nucleic acid sequence encoding the proteins is SEQ ID NO: 12, or a sequence at least 80% to at least

99% identical to SEQ ID NO: 12 which encodes the amino acid sequence of SEQ ID NO: 12. In certain embodiments, the nucleic acid sequence is at least 80% to 97% identical to SEQ ID NO: 12. In certain embodiments, the rAAVhu68 capsid comprises subpopulations with 50% to 100% deamidation in each of N57, N329, N452, and N512, and optionally
5 further comprise subpopulations of vp1, vp2 and/or vp3 proteins which further comprise 1% to about 40% deamidation in at least one or more of positions N94, N113, N252, N253, Q258, N270, N303, N304, N305, N319, N328, N336, N409, N410, N477, N515, N598, Q599, N628, N651, N663, N709, or combinations thereof, based on the amino acid numbering of SEQ ID NO: 13. In certain embodiments, the rAAVhu68 capsid vp1, vp2
10 and/or vp3 proteins further comprise one or more modifications selected from one or more modification in one or more of the following: acetylated lysine, phosphorylated serine and/or threonine, isomerized aspartic acid, oxidized tryptophan and/or methionine, or an amidated amino acid.

In certain embodiments, the AAVhu68 capsid comprises: a heterogenous
15 population of AAVhu68 vp1, AAVhu68vp2 and AAVhu68vp3 proteins produced by expression from a nucleic acid molecule having a nucleic acid sequence encoding the amino acid sequence of 1 to 736 of SEQ ID NO: 13, wherein the heterogenous population of AAVhu68 vp1 proteins, AAVhu68 vp2 proteins, and AAVhu68 vp3 proteins comprise amino acids 1 to 736 (vp1), amino acids 138 to 736 (vp2), and amino acids 203 to 736
20 (vp3) of SEQ ID NO: 13, respectively, with amino acid modifications comprising 50% to 100% deamidation in at least two asparagines (N)-glycine pairs in two or more of N57, N329, N452, and/or N512 of SEQ ID NO: 13 as determined using mass spectrometry and optionally further comprising subpopulations comprising other deamidated amino acids; wherein the heterogenous population of AAVhu68 vp1 proteins comprise glutamic acid at
25 position 67 based on the numbering of SEQ ID NO: 13 and the heterogenous population of AAVhu68vp1 and AAVhu68 vp2 proteins comprising valine at position 157, based on the numbering of the vp1 capsid of SEQ ID NO: 13. In certain embodiments, the VP1 proteins the nucleic acid sequence is SEQ ID NO: 12 or a sequence at least 70% identical to SEQ ID NO: 12 which encodes the amino acid sequence of SEQ ID NO: 13. In certain
30 embodiments, the nucleic acid sequence is SEQ ID NO: 12. In certain embodiments, the rAAVhu68 capsid comprises a subpopulation of AAVhu68 vp1 in which 75% to 100 % of

the N at position 57 of the vp1 proteins are deamidated, as determined using mass spectrometry. In certain embodiments, the rAAVhu68 capsid comprises subpopulation of AAVhu68 vp1 proteins, AAVhu68 vp2 proteins, and/or AAVhu68 vp3 proteins in which 75% to 100% of the N at position 329, based on the numbering of SEQ ID NO: 13 are deamidated as determined using mass spectrometry. In certain embodiments, the rAAVhu68 capsid comprises subpopulation of AAVhu68 vp1 proteins, AAVhu68 vp2 proteins, and/or AAVhu68 vp3 proteins in which 75% to 100% of the N at position 452, based on the numbering of SEQ ID NO: 13, are deamidated as determined using mass spectrometry. In certain embodiments, the rAAVhu68 capsid comprises subpopulation of AAVhu68 vp1 proteins, AAVhu68 vp2 proteins, and/or AAVhu68 vp3 proteins in which 75% to 100% of the N at position 512, based on the numbering of SEQ ID NO: 13, are deamidated. In certain embodiments, the rAAVhu68 capsid comprises subpopulations with 50% to 100% deamidation in each of N57, N329, N452, and N512, and optionally further comprise subpopulations of AAVhu68 vp1, AAVhu68 vp2 and/or AAVhu68 vp3 proteins which further comprise 1% to about 40% deamidation in at least one or more of positions N94, N113, N252, N253, Q258, N270, N303, N304, N305, N319, N328, N336, N409, N410, N477, N515, N598, Q599, N628, N651, N663, N709, or combinations thereof, based on the amino acid numbering of SEQ ID NO: 13.

In certain embodiments, the AAVhu68 capsid comprises a heterogenous population of AAVhu68 vp1 proteins, AAVhu68 vp2 proteins, and AAVhu68 vp3 proteins which comprise amino acids 1 to 736 (vp1), amino acids 138 to 736 (vp2), and amino acids 203 to 736 (vp3) of SEQ ID NO: 13, respectively, with amino acid modifications comprising 50% to 100% deamidation in at least two asparagines (N) in asparagine – glycine pairs in two or more of N57, N329, N452, and/or N512 of SEQ ID NO: 13 as determined using mass spectrometry and optionally further comprising subpopulations comprising other deamidated amino acids; wherein the AAVhu68 proteins contain amino acid modifications comprising 50% to 100% deamidation in two or more of asparagines (N) in position N57, N329, N452, and/or N512 based on the residue numbering of SEQ ID NO: 13 as determined using mass spectrometry and optionally further comprising subpopulations comprising other deamidated amino acids; and wherein the heterogenous population of AAVhu68 vp1 proteins comprise at least a subpopulation

having glutamic acid at position 67 based on the numbering of SEQ ID NO: 13, and the heterogenous population of AAVhu68vp1 and AAVhu68 vp2 proteins comprising at least subpopulations comprising valine at position 157 based on the numbering of SEQ ID NO: 13. In certain embodiments, the nucleic acid sequence is SEQ ID NO: 12 or a sequence at least 70% identical to SEQ ID NO: 12 which encodes the amino acid sequence of SEQ ID NO: 13. In certain embodiments, the nucleic acid sequence is SEQ ID NO: 12. In certain embodiments, the rAAVhu68 capsid comprises a subpopulation of AAVhu68 vp1 in which 75% to 100 % of the N at position 57 of the vp1 proteins are deamidated, as determined using mass spectrometry. In certain embodiments, the rAAVhu68 capsid comprises subpopulation of AAVhu68 vp1 proteins, AAVhu68 vp2 proteins, and/or AAVhu68 vp3 proteins in which 75% to 100% of the N at position 329, based on the numbering of SEQ ID NO: 13, are deamidated as determined using mass spectrometry. In certain embodiments, the rAAVhu68 capsid comprises subpopulation of AAVhu68 vp1 proteins, AAVhu68 vp2 proteins, and/or AAVhu68 vp3 proteins in which 75% to 100% of the N at position 452, based on the numbering of SEQ ID NO: 13, are deamidated as determined using mass spectrometry. In certain embodiments, the rAAVhu68 capsid comprises subpopulation of AAVhu68 vp1 proteins, AAVhu68 vp2 proteins, and/or AAVhu68 vp3 proteins in which 75% to 100% of the N at position 512, based on the numbering of SEQ ID NO: 13, are deamidated as determined using mass spectrometry. In certain embodiments, the rAAVhu68 capsid comprises subpopulations with 50% to 100% deamidation in each of N57, N329, N452, and N512, and optionally further comprise 1% to about 40% deamidation in at least one or more of positions N94, N113, N252, N253, Q258, N270, N303, N304, N305, N319, N328, N336, N409, N410, N477, N515, N598, Q599, N628, N651, N663, N709, or combinations thereof, based on the amino acid numbering of SEQ ID NO: 13.

In certain embodiments, the AAVhu68 capsid comprises AAVhu68 vp1 proteins, AAVhu68 vp2 proteins, and AAVhu68 vp3 proteins produced from a nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 13, wherein the AAVhu68vp1 proteins comprise a glutamic acid at position 67 and a valine at position 157 and the AAVhu68vp2 proteins comprise a valine at position 157 based on the numbering of SEQ ID NO: 13. In certain embodiments, AAVhu68 capsid comprises

subpopulations of AAVhu68 vp1, AAVhu68 vp2 and AAVhu68 vp3 proteins, wherein the subpopulations of the AAVhu68 vp1, AAVhu68 vp2 and AAV hu68 vp3 proteins comprise at least 50% to 100% deamidated asparagines (N) in asparagine - glycine pairs at each of positions 57, 329, 452, 512, relative to the amino acids in SEQ ID NO: 13,

5 wherein the deamidated asparagines are deamidated to aspartic acid, isoaspartic acid, an interconverting aspartic acid/isoaspartic acid pair, or combinations thereof, as determined using mass spectrometry. In certain embodiments, the nucleic acid sequence encoding the vp1 proteins is SEQ ID NO: 13, or a sequence at least 80% to at least 99% identical to SEQ ID NO: 12 which encodes the amino acid sequence of SEQ ID NO: 13; optionally
10 wherein the nucleic acid sequence is at least 80% to 97% identical to SEQ ID NO: 12.

In certain embodiments, AAVhu68 capsid comprises heterogenous populations of AAVhu68 vp1, AAVhu68 vp2 and AAVhu68 vp3 proteins, wherein the AAVhu68 vp1 proteins are amino acids 1 to 736 of SEQ ID NO: 13 (vp1) which comprise a glutamic acid at position 67 and a valine at position 157 and further comprise subpopulations of vp1
15 proteins comprising modified amino acids based on the amino acids positions in SEQ ID NO: 13, wherein the AAVhu68 vp2 proteins are amino acids 138 to 736 of SEQ ID NO: 13 (vp2) which comprise a valine at position 157 and further comprise subpopulations of vp2 proteins comprising modified amino acids based on the amino acid positions in SEQ ID NO: 13, and wherein the AAVhu68 vp3 proteins are amino acids 203 to 736 of SEQ ID
20 NO: 13 (vp3), which comprise subpopulations of vp3 proteins comprising modified amino acids based on the amino acid positions in SEQ ID NO: 13, wherein the subpopulations of the AAVhu68 vp1, AAVhu68 vp2 and AAV hu68 vp3 proteins comprise at least 50% to 100% deamidated asparagines (N) in asparagine - glycine pairs at each of positions 57, 329, 452, 512, relative to the amino acids in SEQ ID NO: 13, wherein the deamidated
25 asparagines are deamidated to aspartic acid, isoaspartic acid, an interconverting aspartic acid/isoaspartic acid pair, or combinations thereof, as determined using mass spectrometry. In certain embodiments, the subpopulations of AAVhu68 vp1, vp2 and vp3 proteins further comprise: (i) one or more modifications selected from: acetylated lysine, phosphorylated serine and/or threonine, isomerized aspartic acid, deamidated glutamines,
30 oxidated tryptophan and/or methionine, or an amidated amino acid as determined using mass spectrometry; and/or (ii) 1% to 40% deamidation of asparagines at one or more of

positions N94, N113, N252, N253, Q259, N270, N303, N304, N305, N319, N328, N336, N409, N410, N477, N515, N598, Q599, N628, N651, N663, N709, or combinations thereof, based on the numbering of SEQ ID NO: 13, as determined using mass

spectrometry. In certain embodiments, the AAVhu68 capsid subpopulations of AAVhu68

5 vp1, vp2 and vp3 capsid proteins further comprise (a) at least 65% of asparagines (N) in asparagine - glycine pairs located at positions 57 of the vp1 proteins are deamidated, as determined using mass spectrometry, based on the numbering of SEQ ID NO: 13; and/or (b) at least 75% of N in asparagine - glycine pairs in position 329 of the vp1, vp2 and vp3

10 proteins are deamidated, as determined using mass spectrometry, based on the residue numbering of the amino acid sequence of SEQ ID NO: 13; and/or (c) at least 50% of N in asparagine - glycine pairs in position 452 of the vp1, vp2 and vp3 proteins are deamidated, as determined using mass spectrometry, based on the residue numbering of the amino acid

15 spectrometry, based on the residue numbering of the amino acid sequence of SEQ ID NO: 13. In certain embodiments, the nucleic acid sequence encoding the vp1 proteins is SEQ ID NO: 12, or a sequence at least 80% to at least 99% identical to SEQ ID NO: 8 which encodes the amino acid sequence of SEQ ID NO: 13; optionally wherein the nucleic acid sequence is at least 80% to 97% identical to SEQ ID NO: 12. See also WO 2018/160582,

20 which is incorporated herein by reference in its entirety.

Additionally, provided herein, is an rAAV production system useful for producing a rAAV as described herein. The production system comprises a cell culture comprising (a) a nucleic acid sequence encoding an AAV capsid protein; (b) a vector genome; and (c) sufficient AAV rep functions and helper functions to permit packaging of the vector

25 genome into the AAV capsid. In certain embodiments, the cell culture is bacterial cell culture. In certain embodiments, the cell culture is mammalian cell culture. In certain embodiments, the cell culture is a human embryonic kidney 293 (HEK293) cell culture. In certain embodiments, the cell culture is a suspension cell culture. In certain embodiments, the AAV rep is from a different AAV. In certain embodiments, wherein the AAV rep is from AAV2. In certain embodiments, the AAV rep coding sequence and cap genes are on

30 the same nucleic acid molecule, wherein there is optionally a spacer between the rep

sequence and cap gene.

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

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

In certain embodiments, an expression cassettes described herein are engineered into a genetic element (e.g., a plasmid) which transfers the expression cassette carried
20 thereon into a packaging host cell for production of a viral vector. In one embodiment, the selected genetic element may be delivered to an AAV packaging cell by any suitable method, including transfection, electroporation, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion. Stable AAV packaging cells can also be made. The methods used to make such constructs are
25 known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. See, e.g., Molecular Cloning: A Laboratory Manual, ed. Green and Sambrook, Cold Spring Harbor Press, Cold Spring Harbor, NY (2012).

The term “AAV intermediate” or “AAV vector intermediate” refers to an
30 assembled rAAV capsid which lacks the desired genomic sequences packaged therein. These may also be termed an “empty” capsid. Such a capsid may contain no detectable

genomic sequences of an expression cassette, or only partially packaged genomic sequences which are insufficient to achieve expression of the gene product. These empty capsids are non-functional to transfer the gene of interest to a host cell.

The recombinant adeno-associated virus (AAV) described herein may be generated
5 using techniques which are known. See, e.g., WO 2003/042397; WO 2005/033321, WO 2006/110689; US 7588772 B2. Such a method involves culturing a host cell which contains a nucleic acid sequence encoding an AAV capsid protein; a functional rep gene; an expression cassette composed of, at a minimum, AAV inverted terminal repeats (ITRs) and a transgene; and sufficient helper functions to permit packaging of the expression
10 cassette into the AAV capsid protein. Methods of generating the capsid, coding sequences therefor, and methods for production of rAAV viral vectors have been described. See, e.g., Gao, et al, Proc. Natl. Acad. Sci. U.S.A. 100 (10), 6081-6086 (2003) and US 2013/0045186A1.

In one embodiment, a production cell culture useful for producing a recombinant
15 AAV having a capsid is provided. Such a cell culture contains a nucleic acid which expresses the AAV capsid protein in the host cell; a nucleic acid molecule suitable for packaging into the AAV capsid, e.g., a vector genome that contains AAV ITRs and a non-AAV nucleic acid sequence encoding a transgene (ASPA) operably linked to regulatory sequences that direct expression of the transgene in a host cell; and sufficient AAV rep
20 functions and adenovirus helper functions to permit packaging of the vector genome into the AAV capsid. In certain embodiments, the cell culture is composed of mammalian cells (e.g., human embryonic kidney 293 cells, among others) or insect cells (e.g., Spodoptera frugiperda (Sf9) cells). In certain embodiments, baculovirus provides the helper functions necessary for packaging the vector genome into the recombinant AAV capsid. Optionally
25 the rep functions are provided by an AAV other than AAV2, selected to complement the source of the ITRs.

Methods for manufacturing the gene therapy vectors described herein include methods well known in the art such as generation of plasmid DNA used for production of the gene therapy vectors, generation of the vectors, and purification of the vectors. In
30 some embodiments, the gene therapy vector is an AAV vector and the plasmids generated are an AAV cis-plasmid encoding the AAV vector genome and the gene of interest, an

AAV trans-plasmid containing AAV rep and cap genes, and an adenovirus helper plasmid. The vector generation process can include method steps such as initiation of cell culture, passage of cells, seeding of cells, transfection of cells with the plasmid DNA, post-transfection medium exchange to serum free medium, and the harvest of vector-containing cells and culture media. The harvested vector-containing cells and culture media are referred to herein as crude cell harvest. In yet another system, the gene therapy vectors are introduced into insect cells by infection with baculovirus-based vectors. For reviews on these production systems, see generally, e.g., Zhang et al., 2009, Adenovirus-adeno-associated virus hybrid for large-scale recombinant adeno-associated virus production, Human Gene Therapy 20:922-929, the contents of each of which is incorporated herein by reference in its entirety. Methods of making and using these and other AAV production systems are also described in the following US patents, the contents of each of which is incorporated herein by reference in its entirety: US Patent Nos. 5,139,941; 5,741,683; 6,057,152; 6,204,059; 6,268,213; 6,491,907; 6,660,514; 6,951,753; 7,094,604; 7,172,893; 7,201,898; 7,229,823; and 7,439,065.

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

A two-step affinity chromatography purification at high salt concentration followed anion exchange resin chromatography are used to purify the vector drug product and to remove empty capsids. These methods are described in more detail in International Patent Application No. PCT/US2016/065970, filed December 9, 2016, and US 11,098,286 B2, entitled "Scalable Purification Method for AAV9", which are incorporated by reference. Purification methods for AAV8, International Patent Application No. PCT/US2016/065976, filed December 9, 2016, and US 11,015,174 B2, entitled "Scalable Purification Method for AAV8", which are incorporated herein by reference. Purification methods for rh10, International Patent Application No. PCT/US16/066013, filed December 9, 2016, and US 11,028,372 B2, entitled "Scalable Purification Method for

AAVrh10”, which are incorporated herein by reference. Purification methods for AAV1, International Patent Application No. PCT/US2016/065974, filed December 9, 2016, and US 11,015,173 B2, entitled “Scalable Purification Method for AAV1”, which are incorporated herein by reference.

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

Generally, methods for assaying for empty capsids and AAV vector particles with packaged genomes have been known in the art. See, e.g., Grimm et al., Gene Therapy
15 (1999) 6:1322-1330; Sommer et al., Molec. Ther. (2003) 7:122-128. To test for denatured capsid, the methods include subjecting the treated AAV stock to SDS-polyacrylamide gel electrophoresis, consisting of any gel capable of separating the three capsid proteins, for example, a gradient gel containing 3-8% Tris-acetate in the buffer, then running the gel until sample material is separated, and blotting the gel onto nylon or nitrocellulose
20 membranes, preferably nylon. Anti-AAV capsid antibodies are then used as the primary antibodies that bind to denatured capsid proteins, preferably an anti-AAV capsid monoclonal antibody, most preferably the B1 anti-AAV-2 monoclonal antibody (Wobus et al., J. Virol. (2000) 74:9281-9293). A secondary antibody is then used, one that binds to the primary antibody and contains a means for detecting binding with the primary
25 antibody, more preferably an anti-IgG antibody containing a detection molecule covalently bound to it, most preferably a sheep anti-mouse IgG antibody covalently linked to horseradish peroxidase. A method for detecting binding is used to semi-quantitatively determine binding between the primary and secondary antibodies, preferably a detection method capable of detecting radioactive isotope emissions, electromagnetic radiation, or
30 colorimetric changes, most preferably a chemiluminescence detection kit. For example, for SDS-PAGE, samples from column fractions can be taken and heated in SDS-PAGE

loading buffer containing reducing agent (e.g., DTT), and capsid proteins were resolved on pre-cast gradient polyacrylamide gels (e.g., Novex). Silver staining may be performed using SilverXpress (Invitrogen, CA) according to the manufacturer's instructions or other suitable staining method, i.e., SYPRO ruby or coomassie stains. In one embodiment, the concentration of AAV vector genomes (vg) in column fractions can be measured by quantitative real time PCR (Q-PCR). Samples are diluted and digested with DNase I (or another suitable nuclease) to remove exogenous DNA. After inactivation of the nuclease, the samples are further diluted and amplified using primers and a TaqMan™ fluorogenic probe specific for the DNA sequence between the primers. The number of cycles required to reach a defined level of fluorescence (threshold cycle, Ct) is measured for each sample on an Applied Biosystems Prism 7700 Sequence Detection System. Plasmid DNA containing identical sequences to that contained in the AAV vector is employed to generate a standard curve in the Q-PCR reaction. The cycle threshold (Ct) values obtained from the samples are used to determine vector genome titer by normalizing it to the Ct value of the plasmid standard curve. End-point assays based on the digital PCR can also be used.

Additionally, another example of measuring empty to full particle ratio is also known in the art. Sedimentation velocity, as measured in an analytical ultracentrifuge (AUC) can detect aggregates, other minor components as well as providing good quantitation of relative amounts of different particle species based upon their different sedimentation coefficients. This is an absolute method based on fundamental units of length and time, requiring no standard molecules as references. Vector samples are loaded into cells with 2-channel charcoal-epon centerpieces with 12mm optical path length. The supplied dilution buffer is loaded into the reference channel of each cell. The loaded cells are then placed into an AN-60Ti analytical rotor and loaded into a Beckman-Coulter ProteomeLab XL-I analytical ultracentrifuge equipped with both absorbance and RI detectors. After full temperature equilibration at 20 °C the rotor is brought to the final run speed of 12,000 rpm. A280 scans are recorded approximately every 3 minutes for ~5.5 hours (110 total scans for each sample). The raw data is analyzed using the c(s) method and implemented in the analysis program SEDFIT. The resultant size distributions are graphed and the peaks integrated. The percentage values associated with each peak

represent the peak area fraction of the total area under all peaks and are based upon the raw data generated at 280nm; many labs use these values to calculate empty: full particle ratios. However, because empty and full particles have different extinction coefficients at this wavelength, the raw data can be adjusted accordingly. The ratio of the empty particle and full monomer peak values both before and after extinction coefficient-adjustment is
5 used to determine the empty-full particle ratio.

In one aspect, an optimized q-PCR method is used which utilizes a broad spectrum serine protease, e.g., proteinase K (such as is commercially available from Qiagen). More particularly, the optimized qPCR genome titer assay is similar to a standard assay, except
10 that after the DNase I digestion, samples are diluted with proteinase K buffer and treated with proteinase K followed by heat inactivation. Suitably samples are diluted with proteinase K buffer in an amount equal to the sample size. The proteinase K buffer may be concentrated to 2 -old or higher. Typically, proteinase K treatment is about 0.2 mg/mL, but may be varied from 0.1 mg/mL to about 1 mg/mL. The treatment step is generally
15 conducted at about 55 °C for about 15 minutes, but may be performed at a lower temperature (e.g., about 37 °C to about 50 °C) over a longer time period (e.g., about 20 minutes to about 30 minutes), or a higher temperature (e.g., up to about 60 °C) for a shorter time period (e.g., about 5 to 10 minutes). Similarly, heat inactivation is generally at about 95 °C for about 15 minutes, but the temperature may be lowered (e.g., about 70 to
20 about 90 °C) and the time extended (e.g., about 20 minutes to about 30 minutes). Samples are then diluted (e.g., 1000-fold) and subjected to TaqMan analysis as described in the standard assay.

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

In certain embodiments, the manufacturing process for rAAV as described herein involves method as described in US Provisional Patent Application No. 63/371,597, filed
30 August 16, 2022, and US Provisional Patent Application No. 63/371,592, filed August 16, 2022, which are incorporated herein by reference in their entirety.

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

The abbreviation “sc” refers to self-complementary. “Self-complementary AAV” refers a construct in which a coding region carried by a recombinant AAV nucleic acid sequence has been designed to form an intra-molecular double-stranded DNA template. Upon infection, rather than waiting for cell mediated synthesis of the second strand, the two complementary halves of scAAV will associate to form one double stranded DNA (dsDNA) unit that is ready for immediate replication and transcription. See, e.g., D M McCarty et al, “Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis”, *Gene Therapy*, (August 2001), Vol 8, Number 16, Pages 1248-1254. Self-complementary AAVs are described in, e.g., U.S. Patent Nos. 6,596,535; 7,125,717; and 7,456,683, each of which is incorporated herein by reference in its entirety.

As used herein, a “stock” of rAAV refers to a population of rAAV. Despite heterogeneity in their capsid proteins due to deamidation, rAAV in a stock are expected to share an identical vector genome. A stock can include rAAV having capsids with, for example, heterogeneous deamidation patterns characteristic of the selected AAV capsid proteins and a selected production system. The stock may be produced from a single

production system or pooled from multiple runs of the production system. A variety of production systems, including but not limited to those described herein, may be selected.

Nucleoside-Modified mRNA and Lipid Nanoparticles

5 In certain embodiments, the composition provided contains a nucleic acid molecule that is a nucleoside-modified mRNA encoding a function ASPA as described herein. Nucleoside-modified mRNA have particular advantages over non-modified mRNA, including for example, increased stability, low or absent innate immunogenicity, and enhanced translation. Nucleoside-modified mRNA useful in the present invention is
10 further described in U.S. Patent No. 8,278,036, which is incorporated by reference herein in its entirety.

 In certain embodiments, the nucleoside-modified RNA comprises the naturally occurring modified-nucleoside pseudouridine. In certain embodiments, inclusion of pseudouridine makes the mRNA more stable, non-immunogenic, and highly translatable
15 (Karikó et al., 2008, Mol Ther 16:1833-1840; Anderson et al., 2010, Nucleic Acids Res 38:5884-5892; Anderson et al., 2011, Nucleic Acids Research 39:9329-9338; Karikó et al., 2011, Nucleic Acids Research 39:e142; Karikó et al., 2012, Mol Ther 20:948-953; Karikó et al., 2005, Immunity 23:165-175).

 As used herein, the terms “modification” and “modified” as such terms relate to
20 the nucleic acids provided herein, include at least one alteration which preferably enhances stability and renders the mRNA more stable (e.g., resistant to nuclease digestion) than the wild-type or naturally occurring version of the mRNA. As used herein, the terms “stable” and “stability” as such terms relate to the nucleic acids of the present invention, and particularly with respect to the mRNA, refer to increased or enhanced resistance to
25 degradation by, for example nucleases (i.e., endonucleases or exonucleases) which are normally capable of degrading such mRNA. Increased stability can include, for example, less sensitivity to hydrolysis or other destruction by endogenous enzymes (e.g., endonucleases or exonucleases) or conditions within the target cell or tissue, thereby increasing or enhancing the residence of such mRNA in the target cell, tissue, subject
30 and/or cytoplasm. Also contemplated by the terms “modification” and “modified” as such terms related to the mRNA of the present invention are alterations which improve or

enhance translation of mRNA nucleic acids, including for example, the inclusion of sequences which function in the initiation of protein translation (e.g., the Kozak consensus sequence).

In certain embodiments, the non-viral delivery system comprises an RNA molecule which is a messenger RNA (mRNA) molecule. In certain embodiments, the mRNA molecule is an in vitro transcribed (IVT) mRNA molecule. In certain embodiments, the IVT mRNA molecule is a nucleoside-modified mRNA molecule. An mRNA may include a 5' untranslated region, a 3' untranslated region, and/or a coding or translating sequence.

An mRNA may be a naturally or non-naturally occurring mRNA. An mRNA may include one or more modified nucleobases, nucleosides, or nucleotides. In some embodiments, the mRNA in the compositions comprise at least one modification which confers increased or enhanced stability to the nucleic acid, including, for example, improved resistance to nuclease digestion in vivo. An mRNA may include any number of base pairs, including tens, hundreds, or thousands of base pairs. Any number (e.g., all, some, or none) of nucleobases, nucleosides, or nucleotides may be an analog of a canonical species, substituted, modified, or otherwise non-naturally occurring. In certain embodiments, all of a particular nucleobase type may be modified. For example, all cytosine in an mRNA may be 5-methylcytosine.

In certain embodiments, the nucleoside-modified mRNA of the invention is IVT mRNA. For example, in certain embodiments, the nucleoside-modified mRNA is synthesized by T7 phage RNA polymerase. In another embodiment, the nucleoside-modified mRNA is synthesized by SP6 phage RNA polymerase. In another embodiment, the nucleoside-modified RNA is synthesized by T3 phage RNA polymerase.

In certain embodiments, the modified nucleoside is m1acp3Ψ (1-methyl-3-(3-amino-3-carboxypropyl) pseudouridine). In another embodiment, the modified nucleoside is m1Ψ (1-methylpseudouridine). In another embodiment, the modified nucleoside is Ψm (2'-O-methylpseudouridine). In another embodiment, the modified nucleoside is m5D (5-methyldihydrouridine). In another embodiment, the modified nucleoside is m3Ψ (3-methylpseudouridine). In another embodiment, the modified nucleoside is a pseudouridine moiety that is not further modified. In another embodiment, the modified nucleoside is a monophosphate, diphosphate, or triphosphate of any of the above pseudouridines. In

another embodiment, the modified nucleoside is any other pseudouridine-like nucleoside known in the art.

In another embodiment, the modified nucleoside of the present invention is m5C (5-methylcytidine). In another embodiment, the modified nucleoside is m5U (5-methyluridine). In another embodiment, the modified nucleoside is m6A (N6-methyladenosine). In another embodiment, the modified nucleoside is s2U (2-thiouridine). In another embodiment, the modified nucleoside is Ψ (pseudouridine). In another embodiment, the modified nucleoside is Um (2'-O-methyluridine).

In other embodiments, the modified nucleoside is m1A (1-methyladenosine); m2A (2-methyladenosine); Am (2'-O-methyladenosine); ms2m6A (2-methylthio-N6-methyladenosine); i6A (N6-isopentenyladenosine); ms2i6A (2-methylthio-N6isopentenyladenosine); io6A (N6-(cis-hydroxyisopentenyl)adenosine); ms2io6A (2-methylthio-N6-(cis-hydroxyisopentenyl) adenosine); g6A (N6-glycinylocarbamoyladenosine); t6A (N6-threonylocarbamoyladenosine); ms2t6A (2-methylthio-N6-threonyl carbamoyladenosine); m6t6A (N6-methyl-N6-threonylocarbamoyladenosine); hn6A (N6-hydroxynorvalyl carbamoyladenosine); ms2hn6A (2-methylthio-N6-hydroxynorvalyl carbamoyladenosine); Ar(p) (2'-O-ribosyladenosine (phosphate)); I (inosine); m1I (1-methylinosine); m1Im (1,2'-O-dimethylinosine); m3C (3-methylcytidine); Cm (2'-O-methylcytidine); s2C (2-thiocytidine); ac4C (N4-acetylcytidine); f5C (5-formylcytidine); m5Cm (5,2'-O-dimethylcytidine); ac4Cm (N4-acetyl-2'-O-methylcytidine); k2C (lysidine); m1G (1-methylguanosine); m2G (N2-methylguanosine); m7G (7-methylguanosine); Gm (2'-O-methylguanosine); m22G (N2,N2-dimethylguanosine); m2Gm (N2,2'-O-dimethylguanosine); m22Gm (N2,N2,2'-O-trimethylguanosine); Gr(p) (2'-O-ribosylguanosine (phosphate)); yW (wybutosine); o2yW (peroxywybutosine); OHyW (hydroxywybutosine); OHyW* (undermodified hydroxywybutosine); imG (wyosine); mimG (methylwyosine); Q (queuosine); oQ (epoxyqueuosine); galQ (galactosyl-queuosine); manQ (mannosyl-queuosine); preQ0 (7-cyano-7-deazaguanosine); preQ1 (7-aminomethyl-7-deazaguanosine); G+ (archaeosine); D (dihydrouridine); m5Um (5,2'-O-dimethyluridine); s4U (4-thiouridine); m5s2U (5-methyl-2-thiouridine); s2Um (2-thio-2'-O-methyluridine); acp3U (3-(3-amino-3-carboxypropyl)uridine); ho5U (5-hydroxyuridine); mo5U (5-methoxyuridine); cmo5U

(uridine 5-oxyacetic acid); mcmo5U (uridine 5-oxyacetic acid methyl ester); chm5U (5-(carboxyhydroxymethyl)uridine); mchm5U (5-(carboxyhydroxymethyl)uridine methyl ester); mcm5U (5-methoxycarbonylmethyluridine); mcm5Um (5-methoxycarbonylmethyl-2'-O-methyluridine); mcm5s2U (5-methoxycarbonylmethyl-2-thiouridine); nm5s2U (5-aminomethyl-2-thiouridine); mnm5U (5-methylaminomethyluridine); mnm5s2U (5-methylaminomethyl-2-thiouridine); mnm5se2U (5-methylaminomethyl-2-selenouridine); ncm5U (5-carbamoylmethyluridine); ncm5Um (5-carbamoylmethyl-2'-O-methyluridine); cmnm5U (5-carboxymethylaminomethyluridine); cmnm5Um (5-carboxymethylaminomethyl-2'-O-methyluridine); cmnm5s2U (5-carboxymethylaminomethyl-2-thiouridine); m62A (N6,N6-dimethyladenosine); Im (2'-O-methylinosine); m4C (N4-methylcytidine); m4Cm (N4,2'-O-dimethylcytidine); hm5C (5-hydroxymethylcytidine); m3U (3-methyluridine); cm5U (5-carboxymethyluridine); m6Am (N6,2'-O-dimethyladenosine); m62Am (N6,N6,O-2'-trimethyladenosine); m2,7G (N2,7-dimethylguanosine); m2,2,7G (N2,N2,7-trimethylguanosine); m3Um (3,2'-O-dimethyluridine); m5D (5-methyldihydrouridine); f5Cm (5-formyl-2'-O-methylcytidine); m1Gm (1,2'-O-dimethylguanosine); m1Am (1,2'-O-dimethyladenosine); τ m5U (5-taurinomethyluridine); τ m5s2U (5-taurinomethyl-2-thiouridine); imG-14 (4-demethylwyosine); imG2 (isowyosine); or ac6A (N6-acetyladenosine).

In another embodiment, a nucleoside-modified mRNA comprises a combination of 2 or more of the above modifications. In another embodiment, the nucleoside-modified mRNA comprises a combination of 3 or more of the above modifications. In another embodiment, the nucleoside-modified RNA comprises a combination of more than 3 of the above modifications.

In another embodiment, between 0.1% and 100% of the residues in the nucleoside-modified RNA are modified (e.g., either by the presence of pseudouridine or a modified nucleoside base). In another embodiment, 0.1% of the residues are modified. In another embodiment, the fraction of modified residues is 0.2%. In another embodiment, the fraction is 0.3%. In another embodiment, the fraction is 0.4%. In another embodiment, the fraction is 0.5%. In another embodiment, the fraction is 0.6%. In another embodiment, the fraction is 0.8%. In another embodiment, the fraction is 1%. In another embodiment, the fraction is 1.5%. In another embodiment, the fraction is 2%. In another embodiment, the

fraction is 2.5%. In another embodiment, the fraction is 3%. In another embodiment, the fraction is 4%. In another embodiment, the fraction is 5%. In another embodiment, the fraction is 6%. In another embodiment, the fraction is 8%. In another embodiment, the fraction is 10%. In another embodiment, the fraction is 12%. In another embodiment, the fraction is 14%. In another embodiment, the fraction is 16%. In another embodiment, the fraction is 18%. In another embodiment, the fraction is 20%. In another embodiment, the fraction is 25%. In another embodiment, the fraction is 30%. In another embodiment, the fraction is 35%. In another embodiment, the fraction is 40%. In another embodiment, the fraction is 45%. In another embodiment, the fraction is 50%. In another embodiment, the fraction is 60%. In another embodiment, the fraction is 70%. In another embodiment, the fraction is 80%. In another embodiment, the fraction is 90%. In another embodiment, the fraction is 100%.

In certain embodiments, the mRNA includes a polyA sequence. A polyA sequence may be comprised entirely or mostly of adenine nucleotides or analogs or derivatives thereof. In certain embodiments, the polyA sequence is a tail located adjacent to a 3' untranslated region (3' UTR) of an mRNA.

Typically, a mature mRNA comprises a 5'-cap, optionally a 5'-UTR, an open reading frame, optionally a 3'-UTR and a poly(A) sequence. The term "UTR" refers to an "untranslated region" flanking the coding sequence of a recombinant nucleic acid as defined herein. In certain embodiments, a 5'-UTR starts with the transcriptional start site and ends one nucleotide before the start codon of the open reading frame. The 5'-UTR may comprise elements for controlling gene expression, also called "regulatory elements". Such regulatory elements may be, for example, ribosomal binding sites. The 5'-UTR may be post-transcriptionally modified, for example by addition of a 5' cap. Thus, 5'-UTRs may preferably correspond to the sequence of a nucleic acid, in particular a mature mRNA, which is located between the 5'-Cap and the start codon, and more specifically to a sequence, which extends from a nucleotide located 3' to the 5'-Cap, preferably from the nucleotide located immediately 3' to the 5'-Cap, to a nucleotide located 5' to the start codon of the protein coding sequence (transcriptional start site), preferably to the nucleotide located immediately 5' to the start codon of the protein coding sequence (transcriptional start site). The nucleotide located immediately 3' to the 5'-Cap of a mature

mRNA typically corresponds to the transcriptional start site. 5' UTRs typically have a length of less than 500, 400, 300, 250 or less than 200 nucleotides. In some embodiments its length may be in the range of at least 10, 20, 30 or 40, preferably up to 100 or 150, nucleotides. In certain embodiments, the at least one 5'-UTR element comprises of a nucleic acid sequence derived from the 5' UTR of a mammalian gene, preferably a human gene. A 3'-UTR corresponds to a sequence which is located between the stop codon of the protein coding sequence, preferably immediately 3' to the stop codon of the protein coding sequence, and the poly(A) sequence of the artificial nucleic acid molecule, e.g., RNA. In certain embodiments, the at least one 3'-UTR element comprises a nucleic acid sequence derived from the 3'-UTR of a mammalian gene, preferably a human gene.

In certain embodiments, the regulatory sequence of an mRNA transcript comprises of a cap structure at 5' end, an untranslated region at 5' end (5'UTR), an untranslated region at 3' end (3'UTR), and poly(A) tail at 3'end. In certain embodiments, the nucleic acid sequence of an mRNA transcript comprises modified nucleosides of 5-Methylcytosine, and/or pseudouridine. In certain embodiments, the 5' cap is 5' cap analog. See e.g., WO 2017/053297, which is incorporated herein by reference in its entirety. In certain embodiments, a 5' cap analog comprises cap analog comprises m7GpppN, optionally wherein the 5' cap analog is commercially available 5' cap, e.g., CleanCap® AU, CleanCap® AG, CleanCap® AG (3'OMe) (TriLink Biotechnologies, San Diego, CA, USA). In certain embodiments, the poly(A) tail comprises of at least 100 to at least 250 adenine nucleotides. In one embodiment, the poly(A) tail is at least 150 to at least 200 adenine nucleotides. In certain embodiments, the poly(A) tail is at least 120 adenine nucleotides.

In certain embodiments, the mRNA transcript sequence is 5'cap-ASPA coding sequence-3'poly(A)tail. In certain embodiments, the mRNA transcript sequence is 5'cap-5'UTR- ASPA coding sequence -3'UTR-3'poly(A)tail. In certain embodiments, the mRNA transcript sequence is 5'cap analog- ASPA coding sequence -3'poly(A)tail. In certain embodiments, the mRNA transcript sequence is 5'cap analog-5'UTR- ASPA coding sequence -3'UTR-3'poly(A)tail.

In certain embodiments, the mRNA transcript sequence is 5'cap- ASPA coding sequence (nucleoside-modified)-3'poly(A)tail. In certain embodiments, the mRNA

transcript sequence is 5' cap-5'UTR- ASPA coding sequence (nucleoside-modified)-
3'UTR-3'poly(A)tail. In certain embodiments, the mRNA transcript sequence is 5' cap
analog- ASPA coding sequence (nucleoside-modified -3'poly(A)tail. In certain
embodiments, the mRNA transcript sequence is 5' cap analog-5'UTR- ASPA coding
5 sequence (nucleoside-modified -3'UTR-3'poly(A)tail.

In one example, a non-viral vector genome comprising an mRNA transcript
comprising at a minimum, from 5' to 3', 5' cap, a 5' UTR, a nucleotide sequence encoding
a functional hASPA, a 3'UTR and a poly(A) tail.

In certain embodiments, the mRNA transcript comprises nucleic acid sequence of
10 SEQ ID NO: 10, further comprising a 5' cap which and a polyA tail. In certain
embodiments, the mRNA transcript comprises nucleic acid sequence of SEQ ID NO: 10,
further comprising a 5' cap which is a cap analog and a polyA tail comprising at least 100
adenine nucleotides. In certain embodiments, the mRNA transcript comprises nucleic acid
sequence of SEQ ID NO: 10, further comprising a 5' cap analog which is a CleanCap®
15 AG cap analog (TriLink Biotechnologies), and a polyA tail comprising at least 120
adenine nucleotides. In certain embodiments, the mRNA transcript comprises nucleic acid
sequence which is nucleoside modified nucleic acid sequence of SEQ ID NO: 10. In
certain embodiments, the mRNA transcript comprises nucleic acid sequence which is
nucleoside modified nucleic acid sequence of SEQ ID NO: 10, further comprising a 5' cap
20 and a polyA tail of at least 100 adenine nucleotides. In certain embodiments, the mRNA
transcript comprises nucleic acid sequence which is nucleoside modified nucleic acid
sequence of SEQ ID NO: 10, further comprising a 5' cap analog (e.g., CleanCap® AG cap
analog) and a polyA tail of at least 120 adenine nucleotides. In certain embodiments, the
mRNA transcript comprises nucleic acid sequence which is nucleoside modified nucleic
25 acid sequence of SEQ ID NO: 10, further comprising a 5' cap analog (e.g., CleanCap®
AG cap analog), a 5' UTR, a 3' UTR, and a polyA tail of at least 120 adenine nucleotides.

In certain embodiments, the compositions and methods provided herein involve a
nucleic acid molecule, which is an mRNA molecule encoding a functional ASPA, wherein
the mRNA molecule is encapsulated in an lipid nanoparticle (LNP) formulation.

30 As used herein, the term "lipid nanoparticle" or "LNP" refers to a transfer vehicle
comprising one or more lipids (e.g., cationic lipids, non- cationic lipids, and PEG-

modified lipids). Preferably, the lipid nanoparticles are formulated to deliver one or more mRNA to one or more target cells (e.g., liver and/or muscle). Examples of suitable lipids include, for example, the phosphatidyl compounds (e.g., phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides). Also contemplated is the use of polymers as transfer vehicles, whether alone or in combination with other transfer vehicles. Suitable polymers may include, for example, polyacrylates, polyalkycyanoacrylates, polylactide, polylactide-polyglycolide copolymers, polycaprolactones, dextran, albumin, gelatin, alginate, collagen, chitosan, cyclodextrins, dendrimers and polyethylenimine. In one embodiment, the transfer vehicle is selected based upon its ability to facilitate the transfection of a mRNA to a target cell. Useful lipid nanoparticles for mRNA comprise a cationic lipid to encapsulate and/or enhance the delivery of mRNA into the target cell that will act as a depot for protein production. As used herein, the phrase “cationic lipid” refers to any of a number of lipid species that carry a net positive charge at a selected pH, such as physiological pH. The contemplated lipid nanoparticles may be prepared by including multi-component lipid mixtures of varying ratios employing one or more cationic lipids, non-cationic lipids and PEG- modified lipids. Several cationic lipids have been described in the literature, many of which are commercially available. See, e.g., WO2014/089486, US 2018/0353616A1, and US 8,853,377B2, which are incorporated by reference. In certain embodiments, LNP formulation is performed using routine procedures comprising cholesterol, ionizable lipid, helper lipid, PEG-lipid and polymer forming a lipid bilayer around encapsulated mRNA (Kowalski et al., 2019, Mol. Ther. 27(4):710-728). In some embodiments, LNP comprises a cationic lipids (i.e. N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), or 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)) with helper lipid DOPE. In some embodiments, LNP comprises an ionizable lipid Dlin-MC3-DMA ionizable lipids, or diketopiperazine-based ionizable lipids (cKK-E12). In some embodiments, polymer comprises a polyethyleneimine (PEI), or a poly(β -amino)esters (PBAEs). See, e.g., WO2014/089486, US 2018/0353616A1, US2013/0037977A1, WO2015/074085A1, US9670152B2, WO 2013/182683, and US 8,853,377B2, which are incorporated by reference. In certain embodiments, other LNP formulations are used for mRNA encapsulation. See also, International Patent Application

No. PCT/US23/65720, filed April 13, 2023, US Provisional Patent Application No. 63/330,972, filed April 14, 2022 and US Provisional Patent Application No. 63/364,859, filed May 17, 2022 which are incorporated herein by reference. See, e.g., FIG 1B (B3). In certain embodiments, other non-LNP formulations are used for mRNA encapsulation, i.e.,
5 polymer-based, peptide-based, cationic nanoemulsion, dendritic cells-based and naked-mRNA. See also, Zeng, C., et al., "Formulation and Delivery Technologies for mRNA Vaccines", Current Topics in Microbiology and Immunology, 2020, 1-50, and Liang, Y., Front. Bioeng. Biotechnol., 2021, 9:718753, which are incorporated herein by reference.

In some embodiments, the mRNA is encapsulated to be delivered at an amount
10 greater than about 0.5 mg/kg (e.g., greater than about 1.0 mg/kg, 1.5 mg/kg, 2.0 mg/kg, 2.5 mg/kg, 3.0 mg/kg, 4.0 mg/kg, 5.0 mg/kg, 6.0 mg/kg, 7.0 mg/kg, 8.0 mg/kg, 9.0 mg/kg, or 10.0 mg/kg) body weight of mRNA per dose. In some embodiments, the mRNA is delivered at an amount ranging from about 0.1-100 mg/kg (e.g., about 0.1-90 mg/kg, 0.1-80 mg/kg, 0.1-70 mg/kg, 0.1-60 mg/kg, 0.1-50 mg/kg, 0.1-40 mg/kg, 0.1-30 mg/kg, 0.1-20
15 mg/kg, 0.1-10 mg/kg) body weight of mRNA per dose. In some embodiments, the mRNA is delivered at an amount of or greater than about 1 mg, 5 mg, 10 mg, 15 mg, 20 mg, 25 mg, 30 mg, 35 mg, 40 mg, 45 mg, 50 mg, 55 mg, 60 mg, 65 mg, 70 mg, 75 mg, 80 mg, 85 mg, 90 mg, 95 mg, 100 mg, 150 mg, 200 mg, 250 mg, 300 mg, 350 mg, 400 mg, 450 mg, or 500 mg per dose.

20 In certain embodiments, the mRNA transcript can be produced using commercially available kits and reagents, e.g., HiScribe™ T7 ARCA mRNA Kit, Invitrogen™ MEGAscript™ T7 Transcription Kit. In certain embodiments, the mRNA transcript can be produced using commercially available services, e.g., TriLink Biotechnologies (San Diego, CA).

25 *Compositions and Uses*

In certain embodiments, pharmaceutical compositions comprising the vectors described herein are provided. The pharmaceutical compositions include a nucleic acid having an ASPA coding sequence and is to be administered to a subject for treatment of
30 Canavan disease. In certain embodiments, the pharmaceutical composition includes a viral vector (e.g., rAAV.hASPA). In certain embodiments, the pharmaceutical composition

includes a nucleic acid molecule (e.g., mRNA) as described herein encapsulated in an LNP.

Provided herein is a pharmaceutical composition comprising an rAAV as described herein and an aqueous suspension media. In certain embodiments, the suspension is formulated for intravenous delivery, intrathecal administration, or intracerebroventricular administration. In certain embodiments, the composition contains at least one rAAV stock and an optional carrier, excipient and/or preservative.

In one embodiment, the composition includes a carrier, diluent, and/or excipient. Suitable carriers may be readily selected by one of skill in the art in view of the indication for which the transfer virus is directed. For example, one suitable carrier includes saline, which may be formulated with a variety of buffering solutions (e.g., phosphate buffered saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, and water. A suitable surfactant, or combination of surfactants, may be selected from among non-ionic surfactants that are nontoxic. In one embodiment, a difunctional block copolymer surfactant terminating in primary hydroxyl groups is selected, e.g., such as Poloxamer 188 (also known under the commercial names Pluronic® F68 [BASF], Lutrol® F68, Synperonic® F68, Kolliphor® P188) which has a neutral pH, has an average molecular weight of 8400. Other surfactants and other Poloxamers may be selected, i.e., nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)), Solutol HS 15 (Macrogol-15 Hydroxystearate), Labrasol (Polyoxy caprylic glyceride), polyoxy -oleyl ether, Tween (polyoxyethylene sorbitan fatty acid esters), ethanol and polyethylene glycol. In one embodiment, the formulation contains a poloxamer. These copolymers are commonly named with the letter "P" (for poloxamer) followed by three digits: the first two digits x 100 give the approximate molecular mass of the polyoxypropylene core, and the last digit x 10 gives the percentage polyoxyethylene content. In one embodiment Poloxamer 188 is selected. The surfactant may be present in an amount up to about 0.0005 % to about 0.001% of the suspension.

In one embodiment, the formulation buffer is phosphate-buffered saline (PBS) with total salt concentration of 200 mM, 0.001% (w/v) pluronic F68 (Final Formulation

Buffer, FFB). In another embodiment, the formulation buffer is phosphate buffer saline with 0.001% poloxamer 188 (137 mM Sodium Chloride, 2.7 mM Potassium Chloride, 10 mM Sodium Phosphate Dibasic, 1.8 mM Potassium Phosphate Monobasic, 0.001% Poloxamer 188, pH 7.3-7.5).

5 As used herein, “carrier” includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Supplementary active ingredients can also be incorporated into the compositions. The phrase “pharmaceutically-acceptable” refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a host. Delivery vehicles such as liposomes, nanocapsules, nanoparticles, lipid nanoparticle (LNP), microparticles, microspheres, lipid particles, vesicles, and the like, may be used for the introduction of the compositions of the present invention into suitable host cells. In particular, the rAAV vector delivered vector genomes may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like.

 In certain embodiments, the formulation may contain a buffered saline aqueous solution not comprising sodium bicarbonate. Such a formulation may contain a buffered saline aqueous solution comprising one or more of sodium phosphate, sodium chloride, potassium chloride, calcium chloride, magnesium chloride and mixtures thereof, in water, such as a Harvard’s buffer. In one embodiment, the buffer is PBS. In another embodiment, the buffer is an artificial cerebrospinal fluid (aCSF), e.g., Elliott’s formulation buffer; or Harvard apparatus perfusion fluid (an artificial CSF with final Ion Concentrations (in mM): Na 150; K 3.0; Ca 1.4; Mg 0.8; P 1.0; Cl 155). The aqueous solution may further contain Kolliphor® P188, a poloxamer which is commercially available from BASF which was formerly sold under the trade name Lutrol® F68. The aqueous solution may have a pH of 7.2.

 In another embodiment, the formulation may contain a buffered saline aqueous solution comprising 1 mM Sodium Phosphate (Na_3PO_4), 150 mM sodium chloride (NaCl), 3mM potassium chloride (KCl), 1.4 mM calcium chloride (CaCl_2), 0.8 mM magnesium

chloride (MgCl_2), and 0.001% poloxamer (e.g., Kolliphor®) 188, pH 7.2. See, e.g., harvardapparatus.com/harvard-apparatus-perfusion-fluid.html. In certain embodiments, Harvard's buffer is preferred due to better pH stability observed with Harvard's buffer.

In certain embodiments, the formulation buffer is artificial CSF with Pluronic F68.

5 In other embodiments, the formulation may contain one or more permeation enhancers. Examples of suitable permeation enhancers may include, e.g., mannitol, sodium glycocholate, sodium taurocholate, sodium deoxycholate, sodium salicylate, sodium caprylate, sodium caprate, sodium lauryl sulfate, polyoxyethylene-9-laurel ether, or EDTA.

10 In certain embodiments, the pharmaceutical composition is formulated at about 1×10^9 genome copies (GC)/mL to about 1×10^{14} GC/mL of a rAAV described herein. In other embodiments, the pharmaceutical composition is formulated at about 3×10^9 GC/mL to about 3×10^{13} GC/mL of the rAAV. In yet other embodiments, the pharmaceutical composition is formulated at about 1×10^9 GC/mL to about 1×10^{13} GC/mL of the rAAV.

15 In certain embodiments, the pharmaceutical composition is formulated at least 1×10^{11} GC/mL of the rAAV.

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

A suitable surfactant, or combination of surfactants, may be selected from among non-ionic surfactants that are nontoxic.

25 In certain embodiments, the composition is delivered at a pH in the range of 6 to 8, or 7.2 to 7.8, or 7.5 to 8. For intrathecal delivery, a pH above 7.5 may be desired, e.g., 7.5 to 8, or 7.8. For intravenous delivery, a pH of about 6.8 to about 7.2 may be desired.

Optionally, the compositions of the invention may contain other conventional pharmaceutical ingredients, such as preservatives, or chemical stabilizers. Suitable
30 exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur

dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

The compositions according to the present invention may comprise a pharmaceutically acceptable carrier, such as defined above. Suitably, the compositions described herein comprise an effective amount of a vector suspended in a pharmaceutically suitable carrier and/or admixed with suitable excipients designed for delivery to the subject via injection, osmotic pump, intrathecal catheter, or for delivery by another device or route.

In certain embodiments, a therapeutically effective amount of a vector is included in the pharmaceutical composition. The selection of the carrier is not a limitation of the present invention. Other conventional pharmaceutically acceptable carrier, such as preservatives, or chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

As used herein, the term “dosage” or “amount” can refer to the total dosage or amount delivered to the subject in the course of treatment, or the dosage or amount delivered in a single unit (or multiple unit or split dosage) administration.

In certain embodiments, the compositions are formulated in a dosage unit to contain an amount of rAAV that is in the range of about 1×10^9 genome copies (GC) per gram (g) of brain mass to about 1×10^{13} GC per g of brain mass, including all integers or fractional amounts within the range and the endpoints. In other embodiments, the dosage is 1×10^{10} GC per gram of brain mass to about 1×10^{13} GC per gram of brain mass. In specific embodiments, the dose of the rAAV administered to a patient is at least about 1.0×10^9 GC/g, about 1.5×10^9 GC/g, about 2.0×10^9 GC/g, about 2.5×10^9 GC/g, about 3.0×10^9 GC/g, about 3.5×10^9 GC/g, about 4.0×10^9 GC/g, about 4.5×10^9 GC/g, about 5.0×10^9 GC/g, about 5.5×10^9 GC/g, about 6.0×10^9 GC/g, about 6.5×10^9 GC/g, about 7.0×10^9 GC/g, about 7.5×10^9 GC/g, about 8.0×10^9 GC/g, about 8.5×10^9 GC/g, about 9.0×10^9 GC/g, about 9.5×10^9 GC/g, about 1.0×10^{10} GC/g, about 1.5×10^{10} GC/g, about 2.0×10^{10} GC/g, about 2.5×10^{10} GC/g, about 3.0×10^{10} GC/g, about 3.5×10^{10} GC/g, about 4.0×10^{10} GC/g, about 4.5×10^{10} GC/g, about 5.0×10^{10} GC/g, about 5.5×10^{10} GC/g, about

6.0 x 10¹⁰ GC/g, about 6.5 x 10¹⁰ GC/g, about 7.0 x 10¹⁰ GC/g, about 7.5 x 10¹⁰ GC/g, about 8.0 x 10¹⁰ GC/g, about 8.5 x 10¹⁰ GC/g, about 9.0 x 10¹⁰ GC/g, about 9.5 x 10¹⁰ GC/g, about 1.0 x 10¹¹ GC/g, about 1.5 x 10¹¹ GC/g, about 2.0 x 10¹¹ GC/g, about 2.5 x 10¹¹ GC/g, about 3.0 x 10¹¹ GC/g, about 3.5 x 10¹¹ GC/g, about 4.0 x 10¹¹ GC/g, about 4.5 x 10¹¹ GC/g, about 5.0 x 10¹¹ GC/g, about 5.5 x 10¹¹ GC/g, about 6.0 x 10¹¹ GC/g, about 6.5 x 10¹¹ GC/g, about 7.0 x 10¹¹ GC/g, about 7.5 x 10¹¹ GC/g, about 8.0 x 10¹¹ GC/g, about 8.5 x 10¹¹ GC/g, about 9.0 x 10¹¹ GC/g, about 9.5 x 10¹¹ GC/g, about 1.0 x 10¹² GC/g, about 1.5 x 10¹² GC/g, about 2.0 x 10¹² GC/g, about 2.5 x 10¹² GC/g, about 3.0 x 10¹² GC/g, about 3.5 x 10¹² GC/g, about 4.0 x 10¹² GC/g, about 4.5 x 10¹² GC/g, about 5.0 x 10¹² GC/g, about 5.5 x 10¹² GC/g, about 6.0 x 10¹² GC/g, about 6.5 x 10¹² GC/g, about 7.0 x 10¹² GC/g, about 7.5 x 10¹² GC/g, about 8.0 x 10¹² GC/g, about 8.5 x 10¹² GC/g, about 9.0 x 10¹² GC/g, about 9.5 x 10¹² GC/g, about 1.0 x 10¹³ GC/g, about 1.5 x 10¹³ GC/g, about 2.0 x 10¹³ GC/g, about 2.5 x 10¹³ GC/g, about 3.0 x 10¹³ GC/g, about 3.5 x 10¹³ GC/g, about 4.0 x 10¹³ GC/g, about 4.5 x 10¹³ GC/g, about 5.0 x 10¹³ GC/g, about 5.5 x 10¹³ GC/g, about 6.0 x 10¹³ GC/g, about 6.5 x 10¹³ GC/g, about 7.0 x 10¹³ GC/g, about 7.5 x 10¹³ GC/g, about 8.0 x 10¹³ GC/g, about 8.5 x 10¹³ GC/g, about 9.0 x 10¹³ GC/g, about 9.5 x 10¹³ GC/g, or about 1.0 x 10¹⁴ GC/g brain mass.

Further, compositions can be formulated in dosage units to contain an amount of replication-defective virus that is in the range of about 1.0 x 10⁹ GC to about 1.0 x 10¹⁶ GC of the rAAV (to treat an average subject of 70 kg in body weight) including all integers or fractional amounts within the range, and preferably 1.0 x 10¹² GC to 1.0 x 10¹⁴ GC for a human patient. In certain embodiments, the compositions are formulated to contain at least 1x10⁹, 2x10⁹, 3x10⁹, 4x10⁹, 5x10⁹, 6x10⁹, 7x10⁹, 8x10⁹, or 9x10⁹ GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1x10¹⁰, 2x10¹⁰, 3x10¹⁰, 4x10¹⁰, 5x10¹⁰, 6x10¹⁰, 7x10¹⁰, 8x10¹⁰, or 9x10¹⁰ GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1x10¹¹, 2x10¹¹, 3x10¹¹, 4x10¹¹, 5x10¹¹, 6x10¹¹, 7x10¹¹, 8x10¹¹, or 9x10¹¹ GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1x10¹², 2x10¹², 3x10¹², 4x10¹², 5x10¹², 6x10¹², 7x10¹², 8x10¹², or 9x10¹² GC per dose including all integers or fractional amounts

within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{13} , 2×10^{13} , 3×10^{13} , 4×10^{13} , 5×10^{13} , 6×10^{13} , 7×10^{13} , 8×10^{13} , or 9×10^{13} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{14} , 2×10^{14} , 3×10^{14} , 4×10^{14} , 5×10^{14} , 6×10^{14} , 7×10^{14} , 8×10^{14} , or 9×10^{14} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{15} , 2×10^{15} , 3×10^{15} , 4×10^{15} , 5×10^{15} , 6×10^{15} , 7×10^{15} , 8×10^{15} , or 9×10^{15} GC per dose including all integers or fractional amounts within the range. In certain embodiments, for human application the dose can range from 1×10^{10} to about 1×10^{12} GC per dose including all integers or fractional amounts within the range.

In certain embodiments, provided is a pharmaceutical composition comprising a non-viral carrier system (e.g., encapsulated in an LNP) mRNA (mRNA-LNP or LNP-mRNA) as described herein in a formulation buffer. In certain embodiments, the pharmaceutical composition comprising mRNA-LNP as described herein is administrable at a dose of about 0.01, 0.03, 0.25, 0.6, or 1 mg/kg (mg mRNA per kg of subject). In one embodiment, mRNA-LNP is dosed weekly. In one embodiment, mRNA-LNP is dosed twice a week. In one embodiment, mRNA-LNP is dosed biweekly.

Provided herein are also compositions containing at least one rAAV stock (e.g., an rAAV9.hASPA stock) and an optional carrier, excipient and/or preservative. An rAAV stock refers to a plurality of rAAV vectors which are the same, e.g., such as in the amounts described below in the discussion of concentrations and dosage units.

In some embodiments, the method comprises administering to a subject a suspension of a rAAV as described herein in a formulation buffer at a dose of 1×10^{11} to 1×10^{14} GC/kg. In certain embodiments, the method comprises intravenous administration at a dose ranging from about 1×10^{12} genome copies (GC)/kg of rAAV to about 1×10^{14} GC of rAAV per kg. In certain embodiments, a dose is about 1×10^{13} GC/kg to about 1×10^{14} GC of rAAV per patient, or about 3×10^{13} GC/kg. In certain embodiments, delivery via intravenous administration is contemplated with a dose of about 3×10^{12} GC/kg to about 1×10^{14} GC/kg, further including doses of about 3.0×10^{13} GC/kg and about 1.0×10^{13} GC/kg.

In one aspect, provided herein are methods of treating Canavan disease in a subject

in need thereof. The methods include administering a viral vector or non-viral vector as provided herein that contains an ASPA coding sequence, or a pharmaceutical composition comprising the viral vector or non-viral vector in an aqueous suspension.

In certain embodiments, the methods include treating a subject having one or more
5 symptoms of Canavan disease. Symptoms include intellectual disability, loss of previously acquired motor skills, feeding difficulties, abnormal muscle tone, macrocephaly, paralysis, and seizures. Life expectancy is generally limited to the first decade for children with the neonatal/infantile of CD. Individuals with the mild/juvenile form of CD may exhibit delayed development of speech and motor skills and have an average lifespan. In certain
10 embodiments, the subject is human and selected from the pediatric and adult population. In certain embodiments, the subject is younger than 18 years of age, younger than 10 years of age, or younger than 6 years of age. In certain embodiments, the subject is about 2 weeks, 1 month, 3 months, 6 months, 1 year, 2 years, 3 years, 4 years, or 5 years of age. In certain embodiments, treatment results in one or more of: 1) improved, increased and/or
15 enhanced balance, grip strength and/or motor coordination; 2) reduction, inhibition and/or neutralization of accumulation of NAA levels in vivo; 3) reduction, inhibition and/or neutralization of vacuole volume fraction in the thalamus; 4) reduction, inhibition and/or neutralization of vacuole volume fraction in the cerebellar white matter/pons; 5) improved, increased and/or enhanced number of oligodendrocytes in the thalamus; 6) improved,
20 increased and/or enhanced number of oligodendrocytes in the cortex; 7) improved, increased and/or enhanced number of neurons in the thalamus; 8) improved, increased and/or enhanced number of neurons in the cortex; or 9) improved, increased and/or enhanced cortical myelination.

In certain embodiments, the subject is delivered a therapeutically effective amount
25 of a composition described herein. As used herein, a “therapeutically effective amount” refers to the amount of the composition comprising the nucleic acid sequence encoding ASPA that delivers and expresses in the target cells an amount of the gene product sufficient to achieve efficacy. The dosage is adjusted to balance the therapeutic benefit against any side effects and such dosages may vary depending upon the therapeutic
30 application for which the recombinant vector is employed. The levels of expression of the transgene product can be monitored to determine the frequency of dosage.

As used herein, the terms “treatment” and “treating” refers to any method used to alleviate, delay onset, reduce severity or incidence, or yield prophylaxis of one or more symptoms or aspects of disease or condition. For the purposes of the present invention, treatment can be administered before, during, and/or after the onset of symptoms. In certain embodiments, the treating includes abrogating, substantially inhibiting, slowing, or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition, or substantially preventing the appearance of clinical or aesthetical symptoms of a condition, or decreasing the severity and/or frequency one or more symptoms resulting from the disease.

In certain embodiments, the compositions described herein are administered prophylactically (i.e., to prevent disease or disorder, e.g., a disease described herein) or therapeutically (i.e., to treat disease or disorder, such as a disease described herein), to subjects suffering from or at risk of (or susceptible to) developing the disease or disorder. Such subjects may be identified using standard clinical methods.

In the context of the present disclosure, prophylactic administration occurs prior to the manifestation of overt clinical symptoms of disease or disorder, such that the disease or disorder is prevented or alternatively delayed in its progression. In the context of the field of medicine, the term “prevent” encompasses any activity which reduces the burden of mortality or morbidity from a disease. Prevention can occur at primary, secondary and tertiary prevention levels. While primary prevention avoids the development of a disease, secondary and tertiary levels of prevention encompass activities aimed at preventing the progression of a disease and the emergence of symptoms as well as reducing the negative impact of an already established disease by restoring function and reducing disease-related complications.

Suitable, conventional, and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to a desired organ (e.g., brain, CSF), intranasal, intrathecal, intratracheal, intraarterial, intraocular, intravenous, intramuscular, subcutaneous, intradermal, intraparenchymal, intracerebroventricular, intrathecal, intracisterna magna (ICM), lumbar puncture and other parenteral routes of administration.

In certain embodiments, the composition is administered via intraparenchymal administration. In certain embodiments, the composition is administered via more than one route, for example intravenously and intrathecally.

As used herein, the term “administration” or any grammatical variations thereof
5 refers to delivery of a composition described herein to a subject.

Suitable volumes for delivery of these doses and concentrations may be determined by one of skill in the art. For example, volumes of about 1 μ L to 150 mL may be selected for CNS delivery, with the higher volumes being selected for adults. Typically, for newborn infants a suitable volume is about 0.5 mL to about 10 mL, for older infants, about
10 0.5 mL to about 15 mL may be selected. For toddlers, a volume of about 0.5 mL to about 20 mL may be selected. For children, volumes of up to about 30 mL may be selected. For pre-teens and teens, volumes up to about 50 mL may be selected. In still other embodiments, a patient may receive an intrathecal administration in a volume of about 5 mL to about 15 mL are selected, or about 7.5 mL to about 10 mL.

As used herein, the terms “intrathecal delivery” or “intrathecal administration”
15 refer to a route of administration via an injection into the spinal canal, more specifically into the subarachnoid space so that it reaches the cerebrospinal fluid (CSF). Intrathecal delivery may include lumbar puncture, intraventricular (including intracerebroventricular (ICV)), suboccipital/intracisternal, and/or C1-2 puncture. For example, material may be
20 introduced for diffusion throughout the subarachnoid space by means of lumbar puncture. In another example, injection may be into the cisterna magna. In certain embodiment, a rAAV vector, or composition as described herein is administered to a subject in need via the intrathecal administration. In certain embodiments, the intrathecal administration is performed as described in US Patent Publication No. 2018/0339065 A1, published
25 November 29, 2019, which is incorporated herein by reference in its entirety. In certain embodiments, the CNS administration is performed using Ommaya Reservoir (also referred to as Ommaya device or Ommaya system).

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

In certain embodiments, the method includes administering a co-therapy that is an immunosuppressant to the subject. Immunosuppressants for such co-therapy include, but are not limited to, a glucocorticoid, corticosteroids, antimetabolites, T-cell inhibitors, a macrolide (e.g., a rapamycin or rapalog), and cytostatic agents including an alkylating agent, an anti-metabolite, a cytotoxic antibiotic, an antibody, or an agent active on immunophilin. The immune suppressant may include a nitrogen mustard, nitrosourea, platinum compound, methotrexate, azathioprine, mercaptopurine, fluorouracil, dactinomycin, an anthracycline, mitomycin C, bleomycin, mithramycin, IL-2 receptor- (CD25-) or CD3-directed antibodies, anti-IL-2 antibodies, ciclosporin, tacrolimus, sirolimus, IFN- β , IFN- γ , an opioid, or TNF- α (tumor necrosis factor-alpha) binding agent.

In certain embodiments, the immunosuppressive therapy may be started 0, 1, 2, 7, or more days prior to administration of the rAAV vector. Such therapy may involve co-administration of two or more drugs, the (e.g., prednisone, micophenolate mofetil (MMF) and/or sirolimus (i.e., rapamycin)) on the same day. One or more of these drugs may be continued after gene therapy administration, at the same dose or an adjusted dose. Such therapy may be for about 1 week (7 days), about 60 days, or longer, as needed. In certain embodiments, a tacrolimus-free regimen is selected.

In certain embodiments, a combination regimen is provided for preventing off-target delivery rAAV, the regimen comprising (a) pretreating the patient by systemically administering a composition comprising anti-AAV capsid neutralizing antibodies directed against an AAV capsid in a recombinant AAV vector, and (b) administering the rAAV as described herein. See also, US Provisional Patent Application No. 63/328,227, filed April 6, 2022, which is incorporated herein by reference in its entirety.

As used herein, a “neutralizing antibody” or “NAb” binds specifically to a viral capsid or envelope and interferes with the infectivity of the virus or a recombinant viral vector having the viral capsid or envelope, thus preventing the recombinant viral vector from delivering effective amounts of a gene product encoded by an expression cassette in its vector genome. Various methods for assessing neutralizing antibodies in a patient’s sera may be utilized. The term method and assay may be used interchangeably. As used herein, the term “neutralization assay” and “serum virus neutralization assay” refers to a serological test to detect the presence of systemic antibodies that may prevent infectivity

of a virus. Such assays may also qualitatively or quantitatively discern the binding capacity (e.g., magnitude) or efficiency of the antibodies to neutralize a target.

Immunological assays may include enzyme immunoassay (EIA), radioimmunoassay (RIA), which uses radioactive isotopes, fluoroimmunoassay (FIA) which uses fluorescent materials, chemiluminescent immunoassay (CLIA) which uses chemiluminescent materials and counting immunoassay (CIA) which employs particle-counting techniques, other modified assays such as western blot, immunohistochemistry (IHC) and agglutination. One of the most common enzyme immunoassays is enzyme-linked immunosorbent assay (ELISA).

Example of suitable methods include those described, e.g., R Calcedo, et al, Journal Infectious Diseases, 2009, 199:381-290; GUO, et al., “Rapid AAV_Neutralizing Antibody Determination with a Cell-Binding Assay”, Molecular Therapy: Methods & Clinical Development Vol. 13 June 2019, T. Ito et al, “A convenient enzyme-linked immunosorbent assay for rapid screening of anti-adenovirus neutralizing antibodies”, Ann Clin Biochem 2009; 46: 508–510; US 2018/0356394A2 (Voyager Therapeutics). Additionally, commercial kits exist (see, e.g., Athena Diagnostics, Invitrogen, ThermoFisher.com; Covance).

The neutralization ability of an antibody is usually measured via the expression of a reporter gene such as luciferase or GFP. In order to determine and compare the activity of a neutralizing antibody, the antibody tested should display a neutralizing activity of 50% or more in one of the neutralization assays described herein. In some examples, neutralizing capacity is determined by measuring the activity of a reporter gene product (e.g., luciferase, GFP). The neutralizing capacity of an antibody to a specific viral vector may be at least 50%, e.g., at least 55%, 60%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99%.

As used herein, the term “NAb titer” a measurement of how much neutralizing antibody (e.g., anti-AAV Nab) is produced which neutralizes the physiologic effect of its targeted epitope (e.g., an AAV). Anti-AAV NAb titers may be measured as described in, e.g., Calcedo, R., et al., Worldwide Epidemiology of Neutralizing Antibodies to Adenovirus. Journal of Infectious Diseases, 2009. 199(3): p. 381-390, which is

incorporated by reference herein.

Still other co-therapeutics may include, e.g., anti-IgG enzymes, which have been described as being useful for depleting anti-AAV antibodies (and thus may permit administration to patients testing above a threshold level of antibody for the selected AAV capsid), and/or delivery of anti-FcRn antibodies and/or one or more of a) a steroid or combination of steroids and/or (b) an IgG-cleaving enzyme, (c) an inhibitor of Fc-IgE binding; (d) an inhibitor of Fc-IgM binding; (e) an inhibitor of Fc-IgA binding; and/or (f) gamma interferon. anti-FcRn antibodies include, e.g., rozanolixizumab (UCB7665) (UCB SA); IMVT-1401, RVT-1401 (HL161), HBM9161 (all form HanAll BioPharma Co. Ltd), Nipocalimab (M281) (Momenta Pharmaceuticals Inc), ARGX-113 (efgartigimod) (Argenx S.E.), orilanolimab (ALXN 1830, SYNT001, Alexion Pharmaceuticals Inc), SYNT002, ABY-039 (Affibody AB), or DX-2507 (Takeda Pharmaceutical Co. Ltd). In certain embodiments, a combination of anti-FcRn antibodies is administered. In certain embodiments, an anti-FcRn antibody is administered in combination with a suitable anti-FcRn ligand (i.e., a peptide or protein construct binding human FcRn so as to inhibit IgG binding).

In certain embodiments, an anti-FcRn ligand(s) (e.g., antibodies) is administered to a patient having neutralizing antibodies prior to and, optionally, concurrently with a selected viral vector. In certain embodiments, continued expression of an anti-FcRn ligand post administration of the gene therapy vector may be desired on a short-term (transient basis), e.g., until such time as the viral vector clears from the patient. In certain embodiments, persistent expression of an anti-FcRn ligand may be desired. Optionally, in this embodiment, the ligand may be delivered via a viral vector, including, e.g., in the viral vector expressing the therapeutic transgene. However, this embodiment is not desirable where the therapeutic gene being delivered is an antibody or antibody construct or another construct comprising an IgG chain. In such embodiments, where an antibody construct having an IgG chain is being delivered via a viral vector to a patient having pre-existing immunity, the anti-FcRn ligand is delivered or dosed transiently so that the amount of anti-FcRn ligand in the circulation is cleared from the sera before effective levels of vector-mediated transgene product are expressed.

In certain embodiments, the FcRn ligand is delivered one to seven days prior to

administration of the vector (e.g., rAAV). In certain embodiments, the FcRn ligand is delivered daily. In certain embodiments, the FcRn ligand (e.g., immunoglobulin construct(s)) is delivered on the same day as the vector is administered. In certain embodiments, the FcRn ligand (e.g., immunoglobulin construct(s)) is delivered at least
5 one day to four weeks post-rAAV administration. In certain embodiments, the ligand is delivered for four weeks to six months post-rAAV administration. In certain embodiments, the ligand is dosed via a different route of administration than the rAAV. In certain embodiments, the ligand is dosed orally, intravenously, or intraperitoneally. See also, International Patent Application No. PCT/US2021/037575, filed June 16, 2021, and
10 now published WO 2021/257668 A1, which is incorporated herein by reference in its entirety.

In certain embodiments, compositions are provided which comprise an LNP-encapsulated nucleic acid molecule (e.g., mRNA encoding ASPA) and one or more suspending agents, carriers, excipients, preservatives, or the like. Excipients and carriers
15 that can be used to prepare parenteral formulations comprising the proteins include, without limitation, aqueous solvents such as water, saline, physiological saline, buffered saline (e.g., phosphate-buffered saline), balanced salt solutions [e.g., Ringer's BSS] and aqueous dextrose solutions), isotonic/iso-osmotic agents (e.g., salts [e.g., NaCl, KCl and CaCl₂] and sugars [e.g., sucrose]), buffering agents and pH adjusters (e.g., sodium
20 dihydrogen phosphate [monobasic sodium phosphate]/disodium hydrogen phosphate [dibasic sodium phosphate], citric acid/sodium citrate and L-histidine/L-histidine HCl), non-solvents, or combinations of one or more aqueous and one or more non-aqueous solvents, and emulsifiers (e.g., non-ionic surfactants such as polysorbates [e.g., polysorbate 20 and 80] and poloxamers [e.g., poloxamer 188]). In certain embodiments,
25 the proteins may be delivered in a lipid particle, nanoparticle, in a liposome, micelle, an implant, hydrogel, or other suitable non-viral delivery or carrier system.

Suitable doses of an LNP-mRNA may include, e.g., 1 µg up to 100 mg/dose, but may vary based on the delivery system and route of administration. For example, intranasal delivery systems may deliver about 10 µg/ml to about 1 mg/ml, or about 100
30 µg/ml to about 1 to 10 mg/ml per dose. Higher doses may be delivered intravenously, or via other delivery routes. Dosing may be over period of 1 to 3 days, or daily over a week,

two weeks, three weeks, 4 weeks, 6 weeks, 2 months, 3 months, or longer. The doses may be higher in the beginning and taper. Optionally, doses may be delivered multiple times a day and/or days may be skipped between doses.

The therapeutic methods encompass the use of pharmaceutical compositions comprising at least one mRNA composition comprising an nucleic acid (e.g., an mRNA molecule encoding a functional ASPA described herein). The pharmaceutical compositions useful for practicing the methods may be administered to deliver a dose of from 0.001 ng/kg/day and 100 mg/kg/day based on the total weight of soluble ASPA mRNA. For example, in some embodiments, the pharmaceutical compositions useful for practicing the invention may be administered to deliver a dose of from 0.005 mg/kg/day and 5 mg/kg/day based on the total weight of soluble ASPA mRNA. In one embodiment, a dose provides a concentration of the LNP compositions from 10nM and 10 μ M in a mammal.

Typically, dosages which may be administered in a method to a mammal, preferably a human, range in amount from 0.01 μ g to about 50 mg per kilogram of body weight of the mammal (based on the weight of the mRNA) while the precise dosage administered will vary depending upon any number of factors, including but not limited to, the type of mammal and type of disease state being treated, the age of the mammal and the route of administration. Preferably, the dosage of the compound will vary from about 0.1 μ g to about 10 mg (based on the weight of the mRNA) per kilogram of body weight of the mammal. More preferably, the dosage will vary from about 1 μ g to about 5 mg (based on the weight of the mRNA) per kilogram of body weight of the mammal. For example, in some embodiments, the dosage will vary from about 0.005 mg to about 5 mg (based on the weight of the mRNA) per kilogram of body weight of the mammal.

In one embodiment, the dosage of the mRNA is administrable at about 0.01, 0.03, 0.25, 0.6, 1, or 2 mg/kg (based on the weight of the mRNA in the composition). In one embodiment, mRNA-LNP is dosed weekly. In one embodiment, mRNA is dosed twice a week. In one embodiment, mRNA is dosed biweekly.

The composition may be administered to a mammal as frequently as several times daily, or it may be administered less frequently, such as once a day, once a week, once every two weeks, once a month, or even less frequently, such as once every several months

or even once a year or less. The frequency of the dose will be readily apparent to the skilled artisan and will depend upon any number of factors, such as, but not limited to, the type and severity of the disease being treated, the type and age of the mammal, etc.

In certain embodiments, administration of a composition of the present invention
5 may be performed by single administration or boosted by multiple administrations.

EXAMPLES

The following examples are provided to illustrate certain aspects of the claimed invention. The invention is not limited to these examples.

10 Example 1: rAAV vector for delivery of ASPA

A recombinant vector genome containing an ASPA coding sequence was engineered for packaging in a AAVhu68 capsid. The vector genome and its components are depicted in FIG 1.

Description of the Vector Elements:

- 15 1. Inverted terminal repeats (ITR): AAV ITRs are sequences that are identical on both ends, but in opposite orientation. The AAV2 ITR sequences function as both the origin of vector DNA replication and the packaging signal of the vector genome, when AAV and adenovirus helper functions are provided in trans. As such, the ITR sequences represent the only cis sequences required for vector genome replication and packaging.
- 20 2. Promoter: CB7 promoter (a cytomegalovirus immediate-early (CMV IE) enhancer sequence (SEQ ID NO: 4) and a chicken β -actin promoter sequence (SEQ ID NO: 5))
3. Intron: chimeric chicken β -actin intron (SEQ ID NO: 6)
4. hASPA coding sequence: engineered ASPA coding sequence
- 25 (“ASPAcoV3”; SEQ ID NO: 1).
5. Polyadenylation Signal (polyA): The rabbit β -globin (rBG) polyadenylation (poly A) signal provides cis sequences for efficient polyadenylation of the antibody mRNA. This element functions as a signal for transcriptional termination, a specific cleavage event at the 3' end of the nascent transcript and addition of a long poly A tail.
- 30 (SEQ ID NO: 7).

AAVhu68 vectors were prepared using conventional triple transfection techniques in 293 cells as described [Mizukami, Hiroaki, et al. A Protocol for AAV vector production and purification. Diss. Division of Genetic Therapeutics, Center for Molecular Medicine, 1998; Lock, M., et al, Hum Gene Ther, 21: 1259-1271 (2010)].

- 5 trans plasmid: pAAV2/hu68n.KanR
 helper plasmid: pAdDeltaF6(Kan)

Example 2: rAAV-mediated delivery of ASPA

A study was conducted to evaluate efficacy of an AAVhu68.CB7.hASPA vector in the ASPAnur7 mouse model of Canavan disease. Mice were administered the vector at 1
10 month of age when they exhibited marked CNS vacuolation and wasting. Vector was delivered via intracerebroventricular (ICV) injection at 1×10^{11} GC total or intravenously (IV) at 5×10^{13} GC/kg. PBS-administered ASPAnur7 mice and wildtype mice were included as controls.

Body weights were monitored for the duration of the study (FIG. 2). Rapid body
15 weight gain or reversal of wasting was observed in the first two weeks for mice that received vector by ICV injection.

Mice were assigned multiparameter neurological assessments scores throughout the study (FIG. 3). Measures included a ledge test and assessment of gait. Overall improvements were observed in treated mice, with more rapid and better score correction
20 in ICV-treated mice compared to IV-treated mice.

Rotarod assessment indicated abnormal motor function at baseline. However, there was rapid normalization to WT performance in ICV-treated mice. In IV-treated mice, there was a stabilization and lack of progressive worsening.

Histology was performed to evaluate levels of vacuolation in untreated and treated
25 mice. Levels of vacuolation in tissue sections was assessed by automated analysis (FIG. 4A and FIG. 4B). Rapid reversal of brain vacuolation was observed following ICV administration at one month post-injection, with further amelioration at three months post-injection. IV injection at 3 months post-injection did not show a comparable level of improvement compared to ICV administration, despite a 10-fold higher total GC dose.

30 FIG. 5 shows immunofluorescent staining of brain sections. Despite ASPA not being secreted, the histological correction extends beyond the regions that are transduced.

Example 3: LNP-mediated delivery of ASPA

A study was performed to evaluate the kinetics of LNP-mRNA-mediated delivery of an ASPA coding mRNA (SEQ ID NO: 10) in the ASPAnur7 mouse model. The mRNA used in this study was synthesized based on the engineered ASPA coding sequence
5 provided in SEQ ID NO: 1. The mRNA constructs were synthesized by Trilink Biotechnologies and included a 5'UTR and Kozak sequence and a 3' polyA, as well as nucleoside modifications to improve stability and reduce immunogenicity.

Mice received a single administration of LNP via ICV injection at ~1 month of age. Wildtype (WT) PBS-injected mice and ASPAnur7 baseline mice were included as
10 controls. An overview of the study design is provided in FIG. 6.

Immunohistochemistry was performed to evaluate ASPA expression in brain sections (FIG. 7A – FIG. 7E). hASPA expression was highest on day 1 post-injection but was limited to the corpus callosum (CC) and rostral migratory stream (RMS). Expression of hASPA was detectable on day 14; however, it was limited to CC only.

15 Immunofluorescent staining revealed that the majority of hASPA signal co-localized with the oligodendrocyte marker Olig-2 and partially with the astrocyte marker GFAP (FIG. 8A and FIG. 8B). No colocalization with neuronal marker NeuN was observed.

FIG. 9 shows results of H&E analysis to assess levels of vacuolization. FIG. 10
20 shows a comparison of vacuolization in the thalamus of mice that received LNP-mRNA- or AAV-mediated delivery of ASPA.

FIG. 11 provides an overview of a study design to assess repeated administration of LNP-mRNA in the ASPAnur7 mouse model. LNP are administered every two weeks.

25 All documents cited in this specification are incorporated herein by reference. US Provisional Patent Application No. 63/610,490, filed December 15, 2024, is incorporated herein by reference. While the invention has been described with reference to particular embodiments, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of
30 the appended claims.

CLAIMS:

1. A recombinant adeno-associated virus (rAAV) comprising an adeno-associated virus (AAV) capsid and a vector genome packaged therein, wherein the vector genome comprises an expression cassette comprising one or more regulatory sequences operably linked to a nucleotide sequence encoding a functional aspartoacylase (ASPA), wherein the nucleotide sequence encoding the functional ASPA is SEQ ID NO: 1 or a nucleotide sequence at least 99% identical to SEQ ID NO: 1.

2. The rAAV of claim 1, wherein the functional ASPA comprises the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence at least 99% identical to SEQ ID NO: 3.

3. The rAAV of claim 1 or 2, wherein the vector genome comprises an AAV 5' inverted terminal repeat (ITR) sequence and/or an AAV 3' ITR sequence.

4. The recombinant nucleic acid molecule of any one of 1 to 3, wherein the AAV 5' ITR sequence and/or the AAV 3' ITR sequence are from AAV2, optionally wherein the AAV 5' ITR sequence comprises the nucleotide sequence of SEQ ID NO: 8 and/or the AAV 3' ITR sequence comprises the nucleotide sequence of SEQ ID NO: 9.

5. The rAAV of any one of claims 1 to 4, wherein the regulatory control sequences comprise a cytomegalovirus immediate early (CMV IE) enhancer and/or a chicken beta-actin promoter.

6. The rAAV of claim 5, wherein the enhancer comprises the nucleotide sequence of SEQ ID NO: 4.

7. The rAAV of claim 5 or 6, wherein the chicken beta-actin promoter comprises the nucleotide sequence of SEQ ID NO: 5.

8. The rAAV of any one of claims 1 to 7, wherein the regulatory control sequences comprise an intron.

9. The rAAV of claim 8, wherein the intron is a chimeric intron comprising chicken beta actin splicing donor including chicken beta actin intron and rabbit beta globin splicing acceptor.

10. The rAAV of claim 8 or 9, wherein the intron comprises the nucleotide sequence of SEQ ID NO: 6.

11. The rAAV of any one of claims 1 to 10, wherein the regulatory control sequences comprise a rabbit beta globin (rBG) polyadenylation (polyA) signal sequence.

12. The rAAV of claim 11, wherein the rBG polyA signal sequence comprises the nucleotide sequence of SEQ ID NO: 7.

13. The rAAV of any one of claims 1 to 12, wherein the vector genome comprises SEQ ID NO: 11 or a sequence at least 90%, 95%, or 99% identical to SEQ ID NO: 11.

14. The rAAV of any one of claims 1 to 13, wherein the vector genome comprises SEQ ID NO: 2 or a sequence at least 90%, 95%, or 99% identical to SEQ ID NO: 2.

15. The rAAV of any one of claims 1 to 14, wherein the AAV capsid is a clade F capsid, optionally an AAVhu68 capsid.

16. A composition comprising a stock of the rAAV of any one of claims 1 to 15 in an aqueous suspension.

17. A pharmaceutical composition comprising the rAAV of any one of claims 1 to 15 and a carrier, diluent, and/or excipient.

18. A recombinant nucleic acid molecule comprising an AAV vector genome comprising:

- (a) an AAV 5' ITR sequence;
- (b) an expression cassette comprising one or more regulator sequences operably linked to a nucleotide sequence encoding a functional ASPA, wherein the nucleotide sequence encoding the functional ASPA is SEQ ID NO: 1 or a nucleotide sequence at least 99% identical to SEQ ID NO: 1; and
- (c) an AAV 3' ITR sequence.

19. The recombinant nucleic acid molecule of claim 18, wherein the functional ASPA comprises the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence at least 99% identical to SEQ ID NO: 3.

20. The recombinant nucleic acid molecule of claim 18 or 19, wherein the vector genome comprises an AAV 5' inverted terminal repeat (ITR) sequence and/or an AAV 3' ITR sequence.

21. The recombinant nucleic acid molecule of any one of claims 18 to 20, wherein the AAV 5' ITR sequence and/or the AAV 3' ITR sequence are from AAV2, optionally wherein the AAV 5' ITR sequence comprises the nucleotide sequence of SEQ ID NO: 8 and/or the AAV 3' ITR sequence comprises the nucleotide sequence of SEQ ID NO: 9.

22. The recombinant nucleic acid molecule of any one of claims 18 to 21, wherein the regulatory control sequences comprise a cytomegalovirus immediate early (CMV IE) enhancer and/or a chicken beta-actin promoter.

23. The recombinant nucleic acid molecule of claim 22, wherein the enhancer comprises the nucleotide sequence of SEQ ID NO: 4.

24. The recombinant nucleic acid molecule of claim 22 or 23, wherein the chicken beta-actin promoter comprises the nucleotide sequence of SEQ ID NO: 5.

25. The recombinant nucleic acid molecule of any one of claims 18 to 24, wherein the regulatory control sequences comprise an intron.

26. The recombinant nucleic acid molecule of claim 25, wherein the intron is a chimeric intron comprising chicken beta actin splicing donor including chicken beta actin intron and rabbit beta globin splicing acceptor.

27. The recombinant nucleic acid molecule of claim 25 or 26, wherein the intron comprises the nucleotide sequence of SEQ ID NO: 6.

28. The recombinant nucleic acid molecule of any one of claims 18 to 27, wherein the regulatory control sequences comprise a rabbit beta globin (rBG) polyadenylation (polyA) signal sequence.

29. The recombinant nucleic acid molecule of claim 28, wherein the rBG polyA signal sequence comprises the nucleotide sequence of SEQ ID NO: 7.

30. The recombinant nucleic acid molecule of any one of claims 18 to 29, wherein the vector genome comprises SEQ ID NO: 11 or a sequence at least 90%, 95%, or 99% identical to SEQ ID NO: 11.

31. The recombinant nucleic acid molecule of any one of claims 18 to 30, wherein the vector genome comprises SEQ ID NO: 2 or a sequence at least 90%, 95%, or 99% identical to SEQ ID NO: 2.

32. The recombinant nucleic acid molecule of any one of claims 18 to 31, wherein the recombinant nucleic acid molecule is a plasmid.

33. A packaging host cell comprising the recombinant nucleic acid molecule of claim 32.

34. The packaging host cell of claim 33, which further comprises AAV rep coding sequences operably linked to sequences which express rep protein in the packaging host cell, an AAV capsid coding sequences operably linked to sequences which express AAV capsid proteins in the packaging host cell, and helper virus functions necessary to permit packaging of the vector genome in the AAV capsid.

35. The packaging host cell of claims 33 or 34, wherein the AAV capsid is a clade F capsid, optionally an AAVhu68 capsid.

36. A method of treating Canavan disease in a subject in a need thereof, the method comprising administering to the subject a therapeutically effective amount of the rAAV of any one of claims 1 to 15, the composition of claim 16, or the pharmaceutical composition of claim 17.

37. The method of claim 36, wherein the rAAV, composition, or pharmaceutical composition is administered intrathecally, optionally via intracerebroventricular (ICV) injection or intracisternal magna (ICM) injection.

38. A recombinant nucleic acid molecule that is a messenger RNA (mRNA) comprising a nucleotide sequence encoding a functional aspartoacylase (ASPA), wherein the nucleotide sequence encoding the functional ASPA is SEQ ID NO: 10 or a nucleotide sequence at least 99% identical to SEQ ID NO: 10.

39. The recombinant nucleic acid molecule of claim 38, wherein the functional ASPA comprises the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence at least 99% identical to SEQ ID NO: 3.

40. The recombinant nucleic acid molecule of claim 38 or 39, wherein the mRNA comprises a 5' cap, Kozak sequence, and/or polyA sequence.

41. The recombinant nucleic acid molecule of any one of claims 38 to 40, wherein the mRNA is a nucleoside-modified mRNA.

42. A composition comprising a lipid nanoparticle (LNP), wherein the recombinant nucleic acid molecule of any one of claims 38 to 41 is encapsulated in a lipid nanoparticle (LNP).

43. A method of treating Canavan disease in a subject in a need thereof, the method comprising administering to the subject a therapeutically effective amount of the recombinant nucleic acid molecule of any one or claims 38 to 41 or the composition of claim 42.

44. The method of claim 43, wherein the recombinant nucleic acid molecule or the composition is administered intrathecally, optionally via intracerebroventricular (ICV) injection or intracisternal magna (ICM) injection.

45. The rAAV of any one of claims 1 to 15, the recombinant nucleic acid molecule of any one of claims 18 to 32 or 38 to 41, or the composition of claims 42 for use in the preparation of a medicament for treating Canavan disease.

46. The rAAV of any one of claims 1 to 15, the recombinant nucleic acid molecule of any one of claims 18 to 32 or 38 to 41, or the composition of claims 42, for use in treating Canavan disease.

FIG. 1

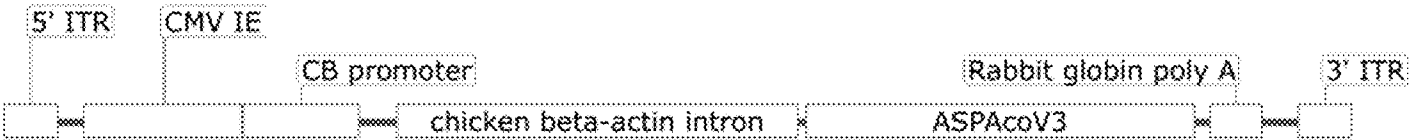


FIG. 2

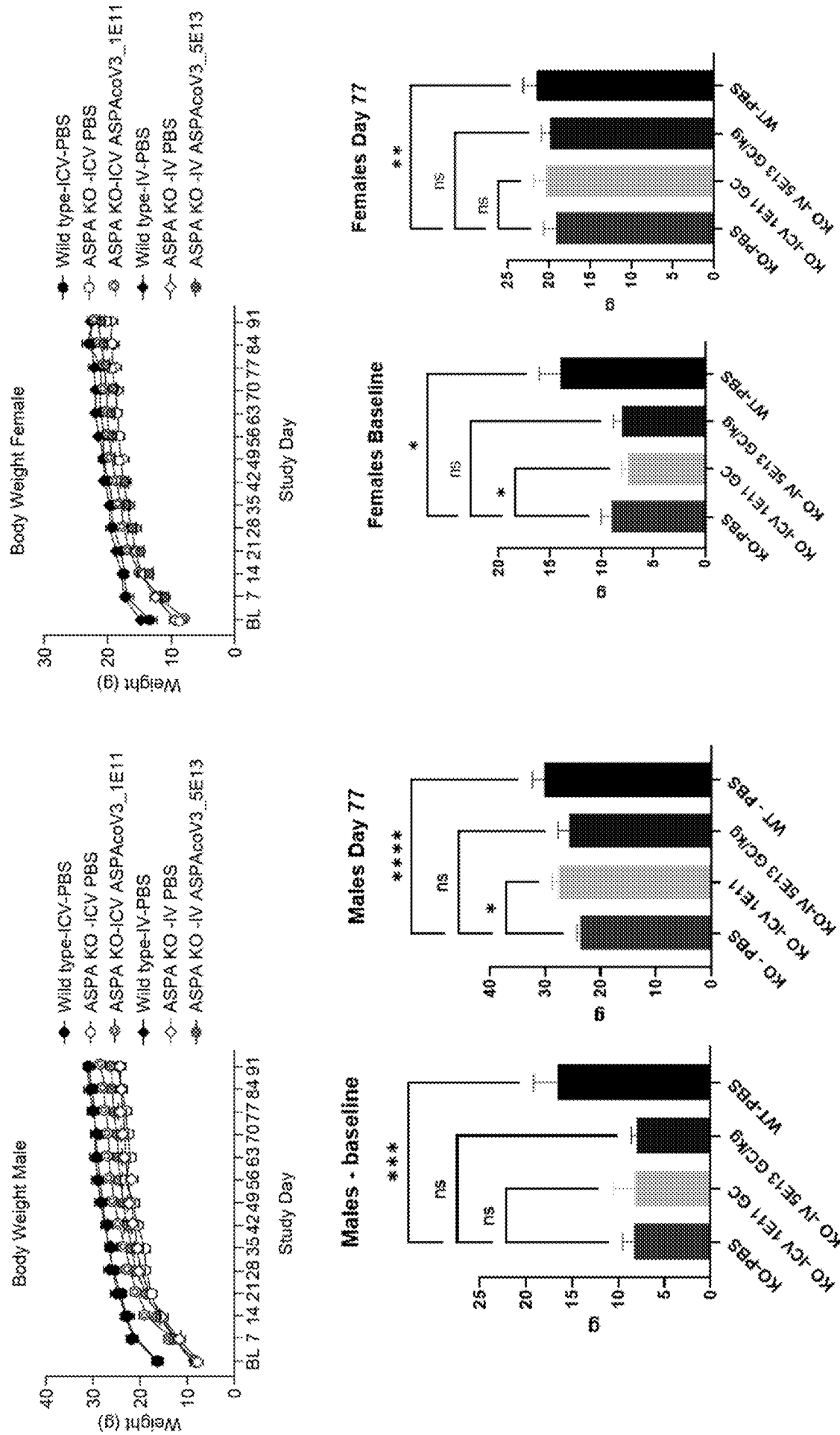


FIG. 3

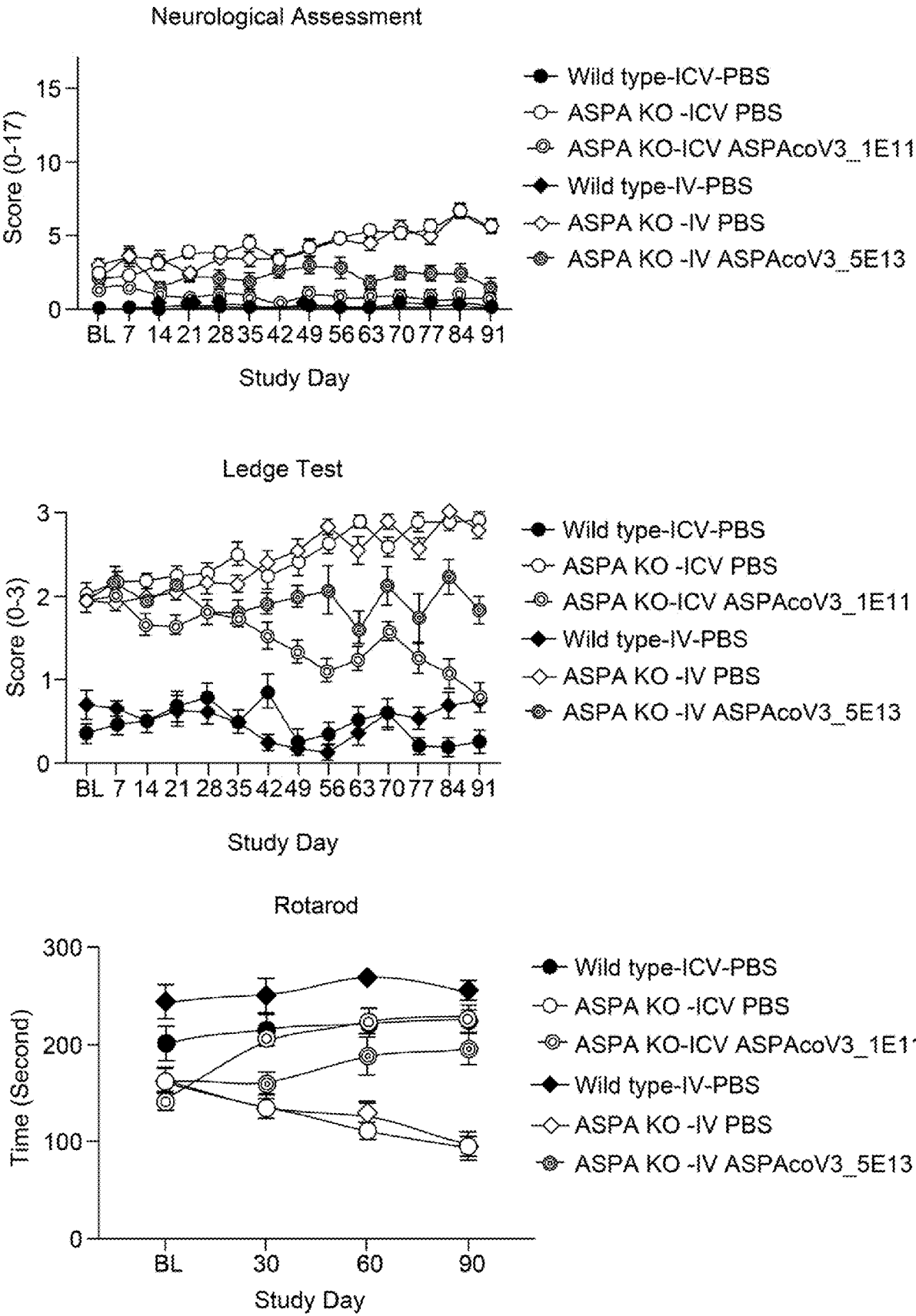


FIG. 4A

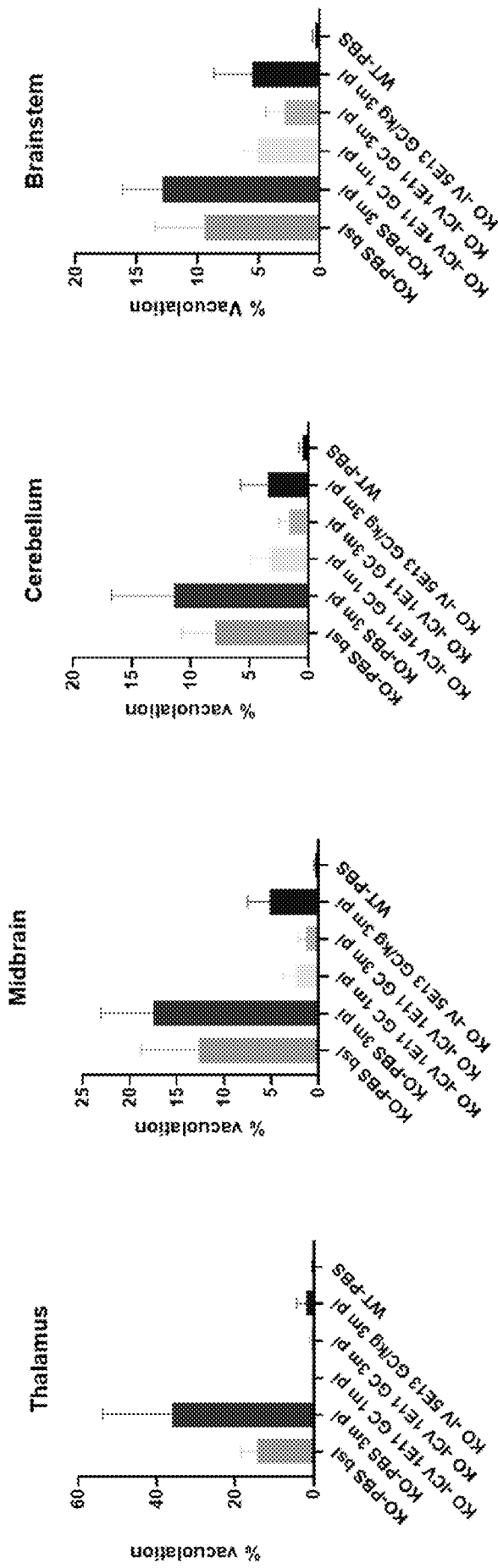


FIG. 4B

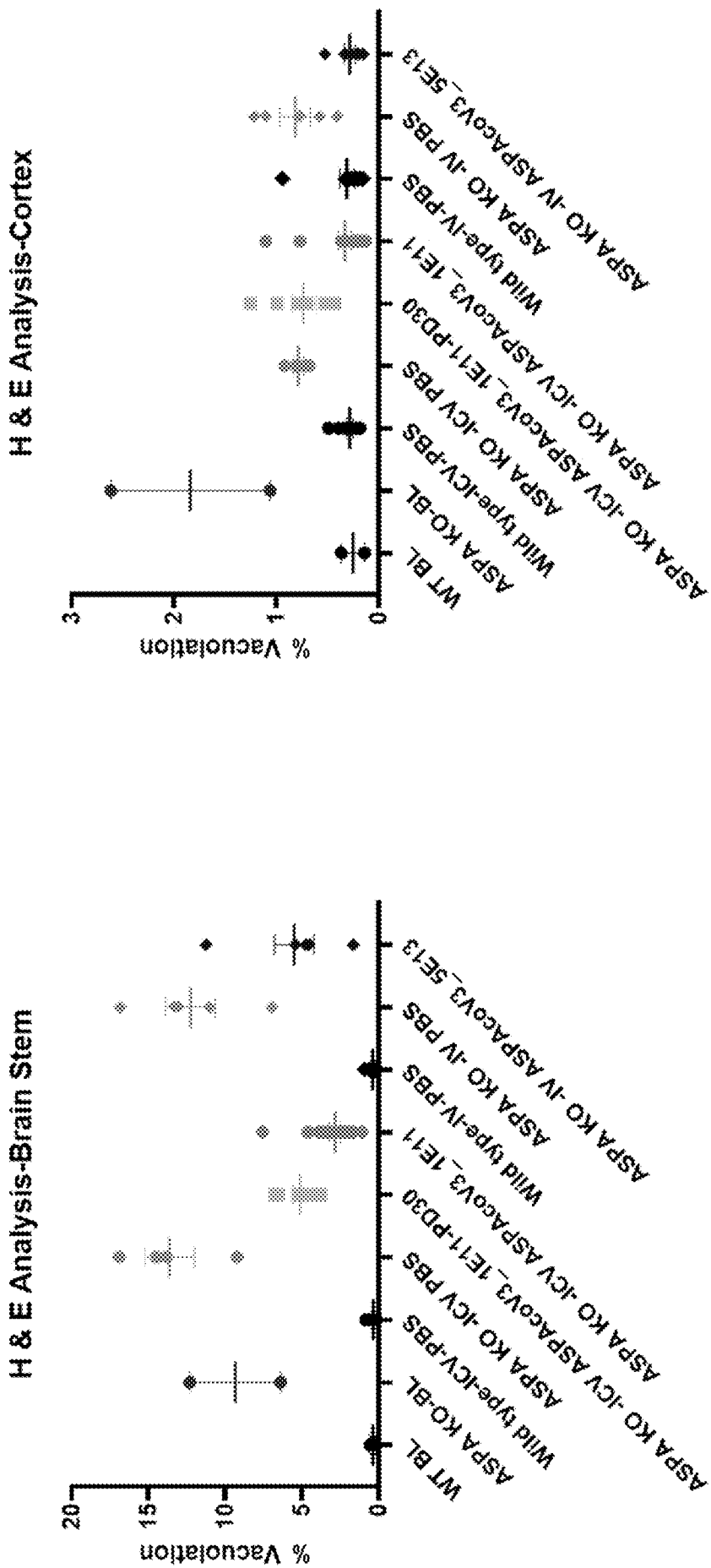


FIG. 5

GFAP; hASPA; DAPI



555-HOM-ICV-hASPAcoV3



FIG. 6

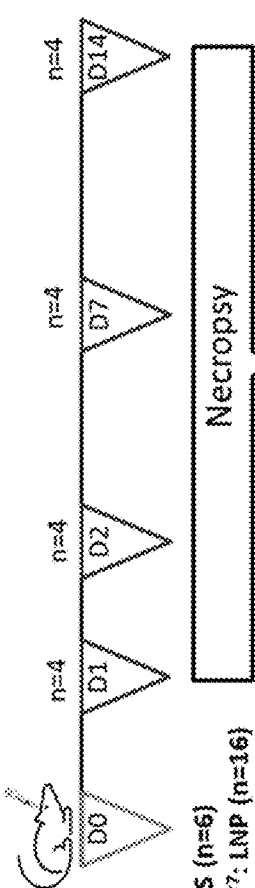
Study information and work plan task	Study Design	General updates
W3648 Pilot CD-LNP study Objective: Assess Kinetics of LNP-hASPAcoV3 mRNA in ASPA^{nu7} mice model	<u>ROA (Dose):</u> Unilateral ICV (5 µL) Control: Wildtype: (n=6; PBS-ICV) LNP: ASPA^{nu7} (n=16) Baseline: ASPA^{nu7} (n= 3) <u>Age at Enrollment:</u> 30 ± 5 days LNP: hAspAcoV3-mRNA (2900µg/mL). Endotoxin: 1.19 EU/mL Dose: 6 µg/ animal (12 µg/gram of brain) <u>Duration:</u> 14 days (Necropsy at BL, D1, D2, D7 and D14) <u>Readouts:</u> • H & E • hASPA IHC • IF: GFAP, Olig-2, NeuN, hASPA	In-life completed hASPA IHC completed IF Completed  WT: PBS (n=6) ASPA^{nu7}: LNP (n=16) ASPA^{nu7} = BL Necropsy (n=3) Necropsy Histopathology hASPA IHC H & E analysis

FIG. 7A

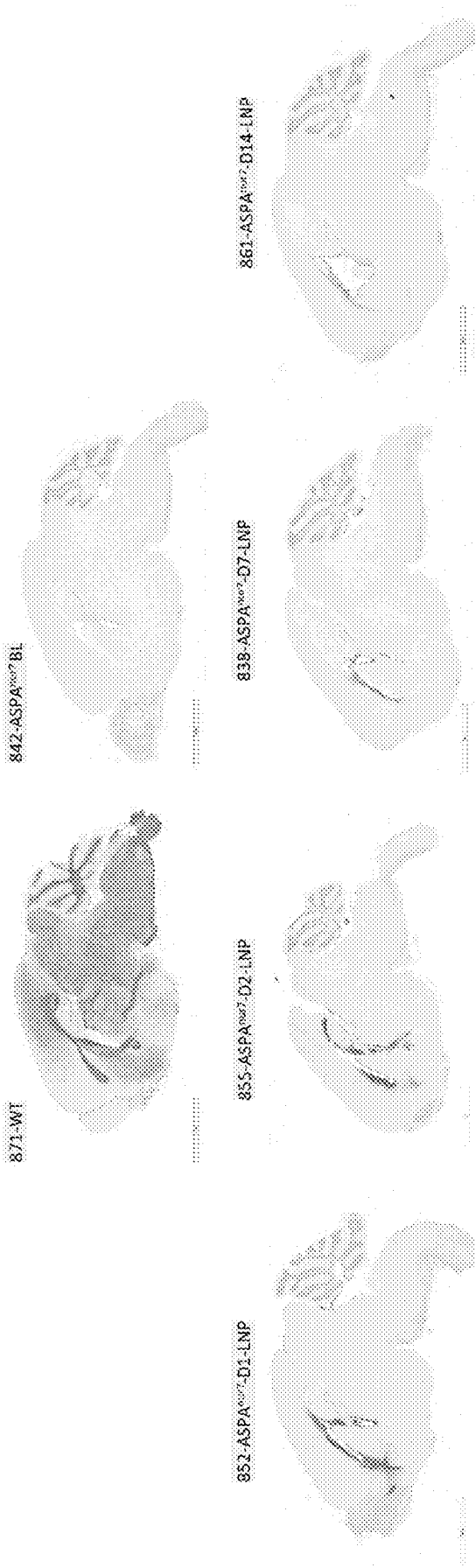


FIG. 7B

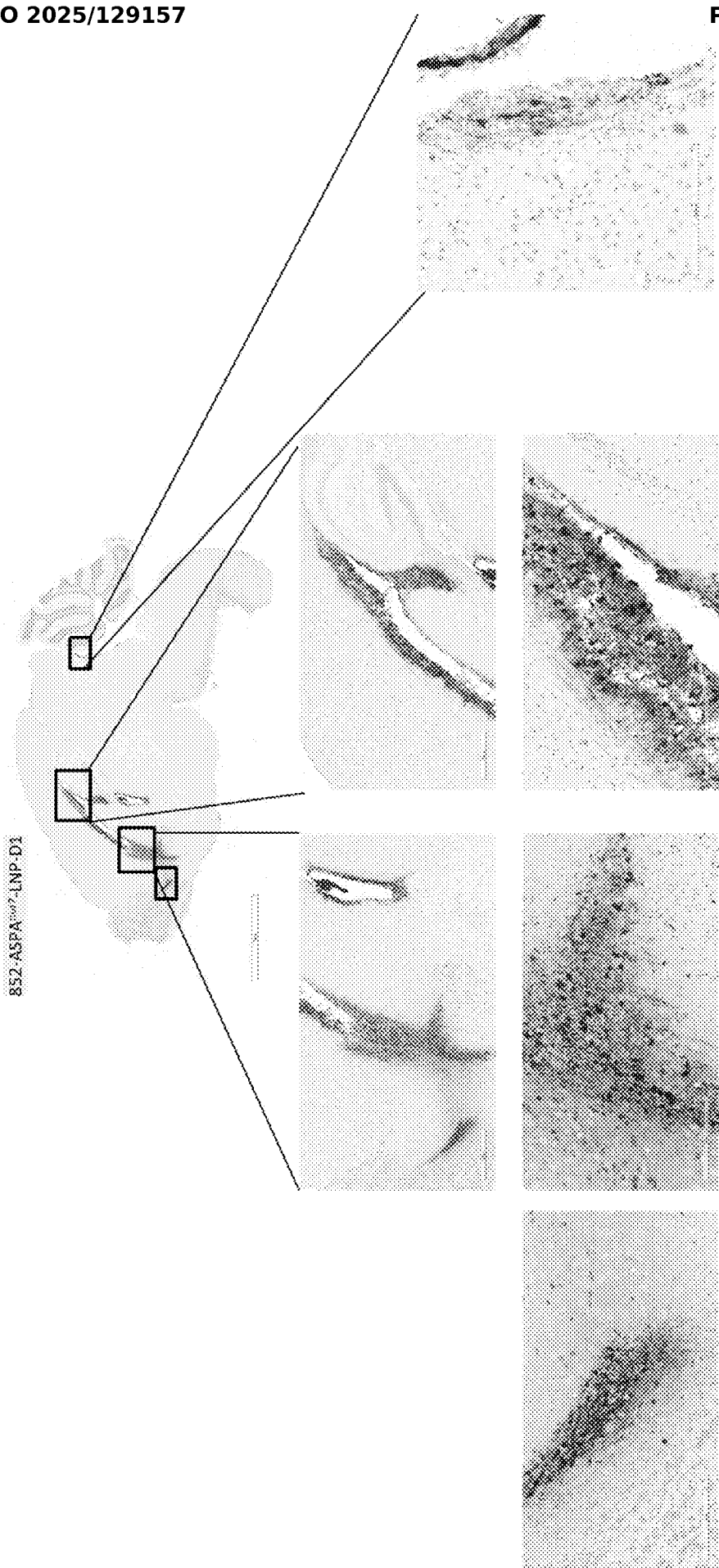


FIG. 7C

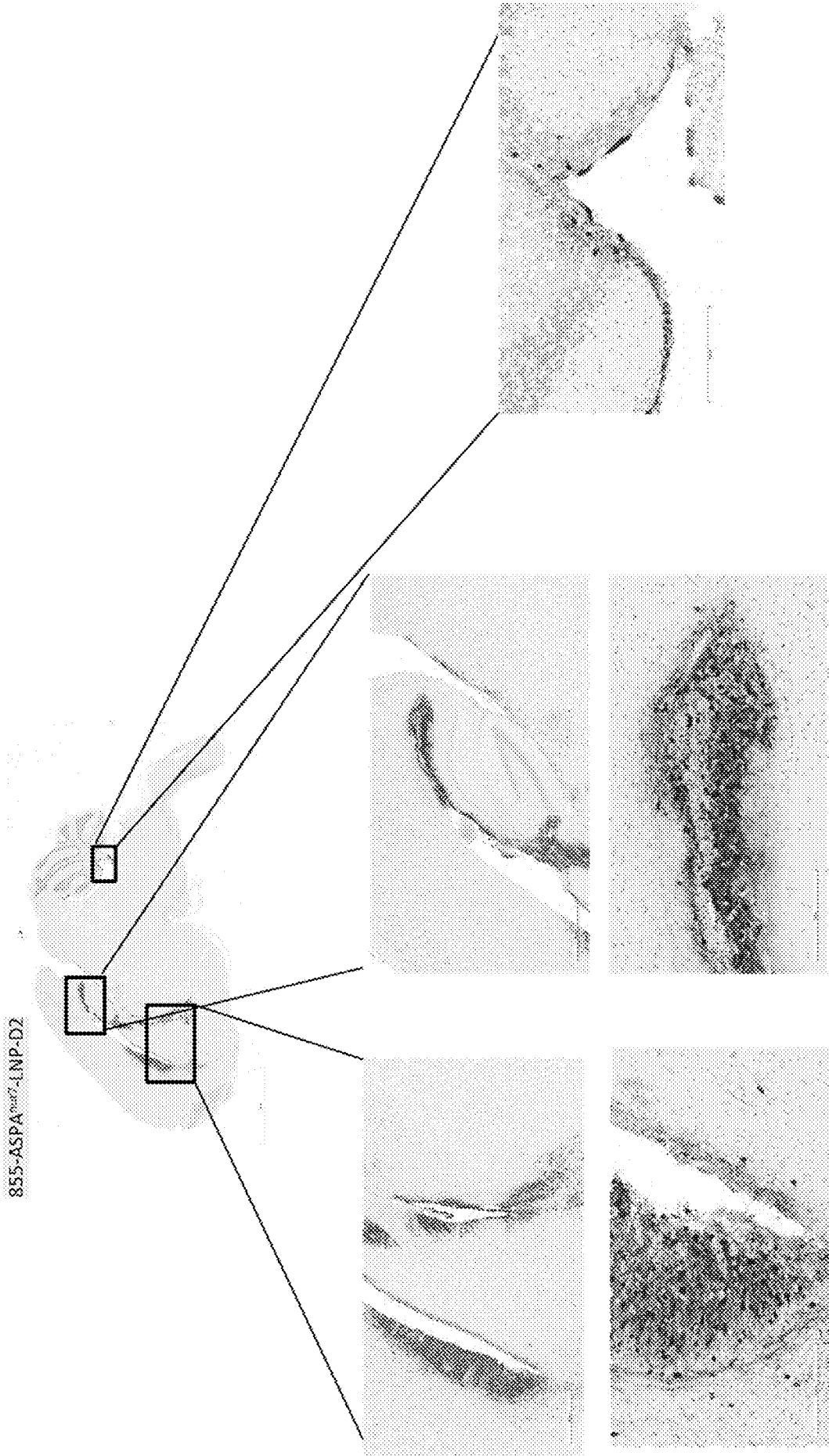


FIG. 7D

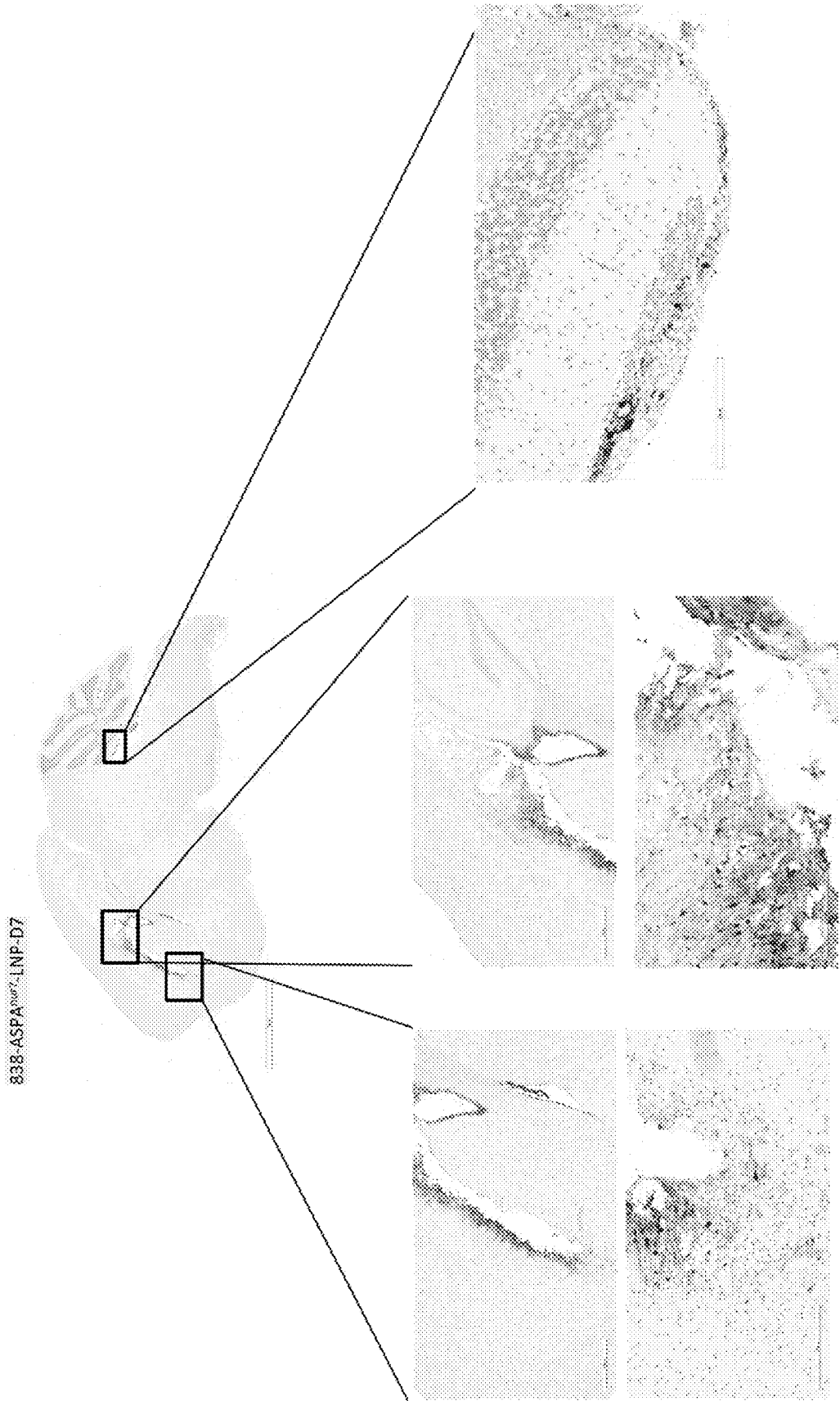


FIG. 7E

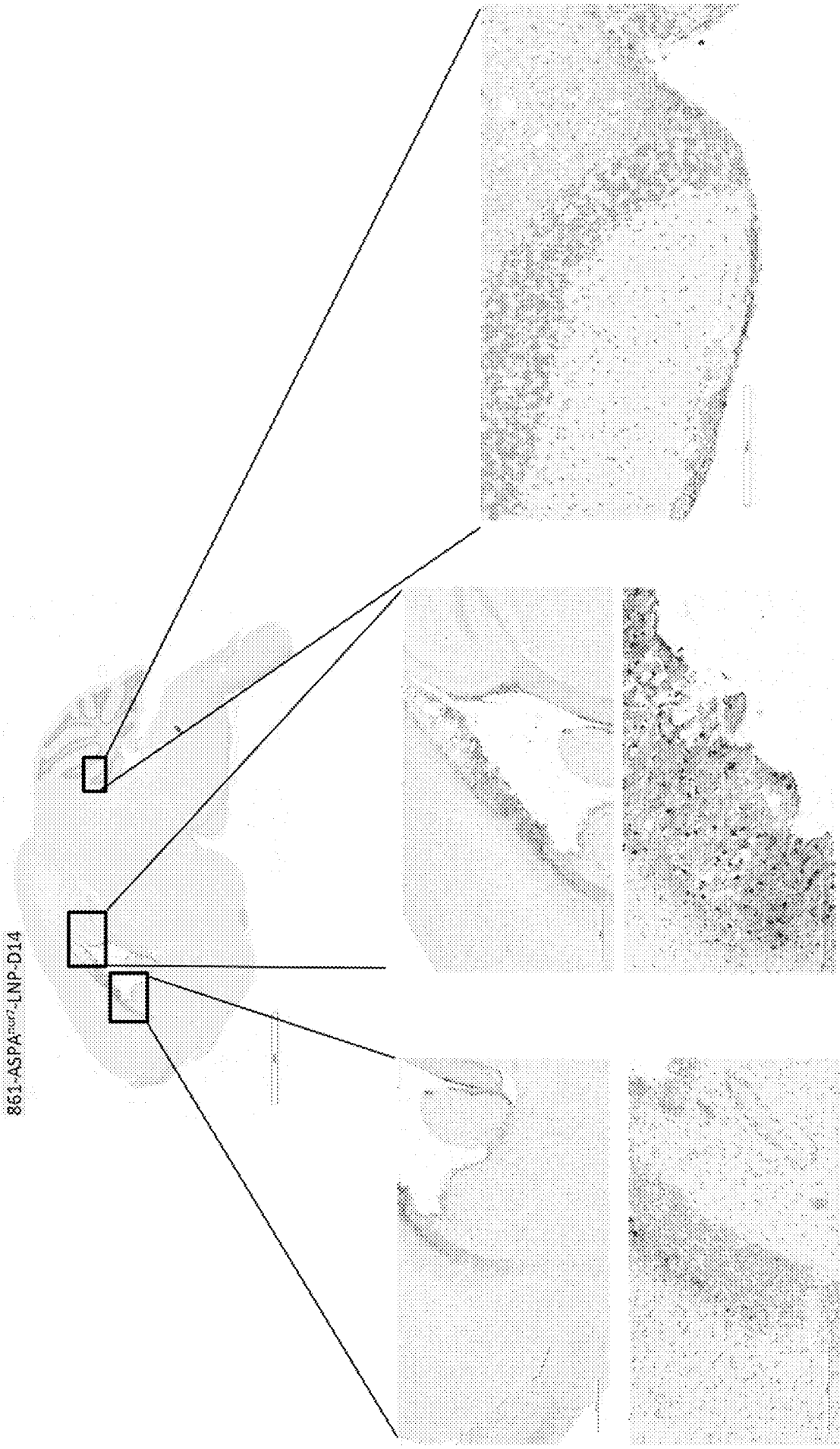
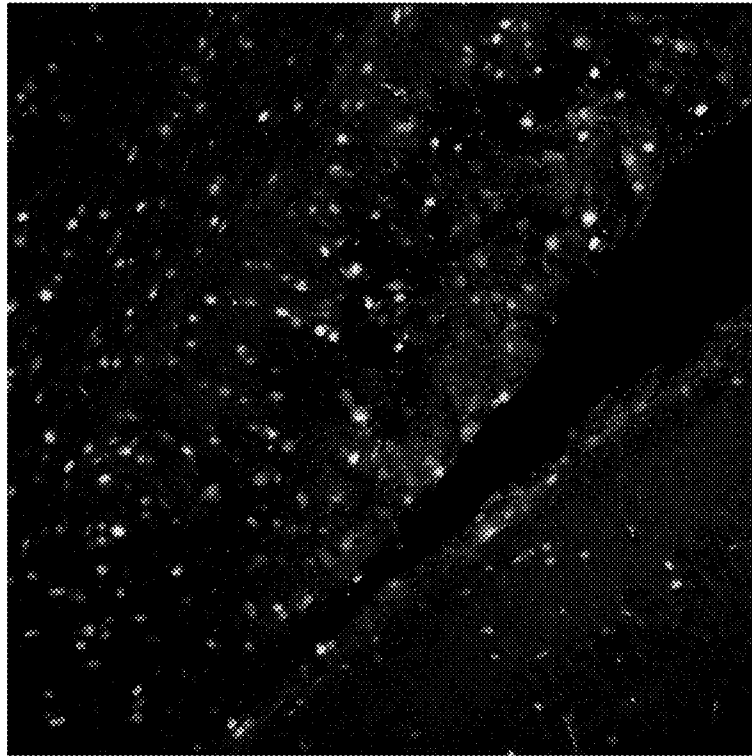


FIG. 8A

hASPA-Olig-2-DAPI



hASPA-NeuN-GFAP-DAPI

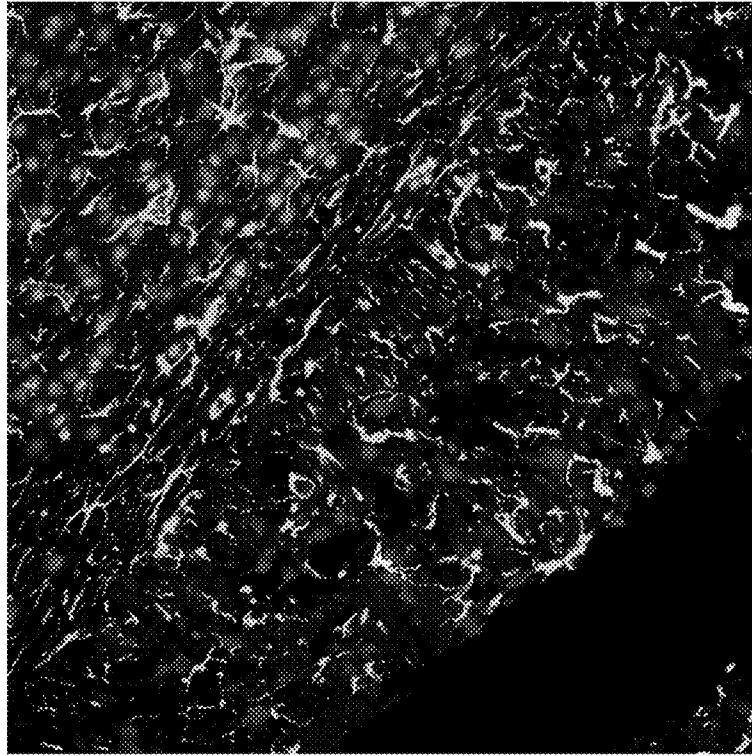
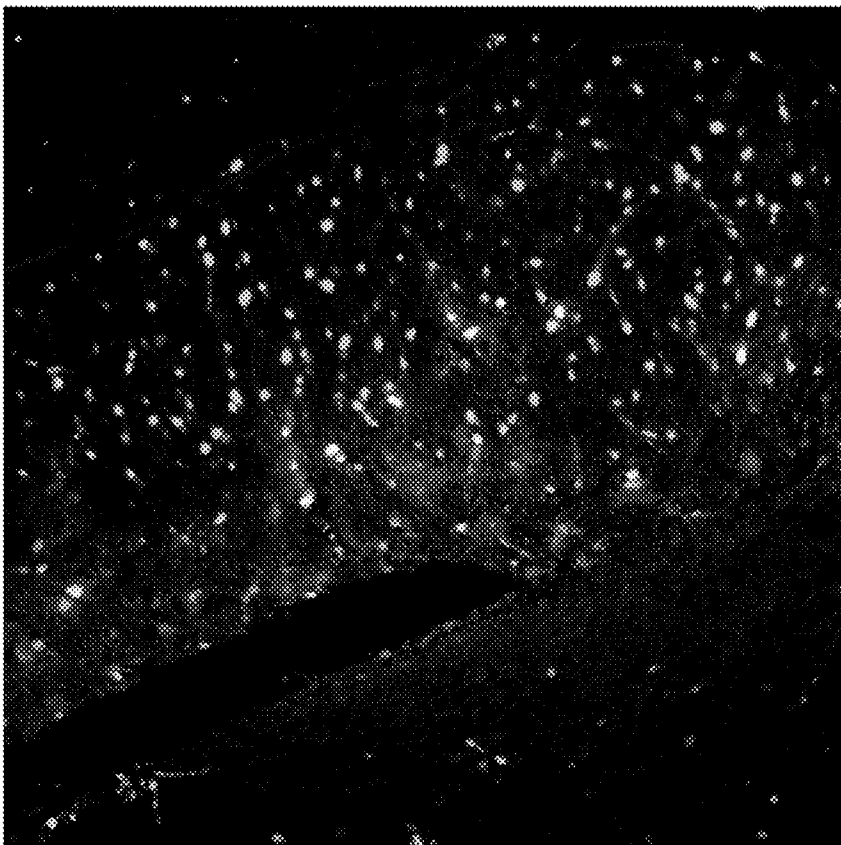


FIG. 8B

hASPA-Olig-2-DAPI



hASPA-Neu-n-GFAP-DAPI

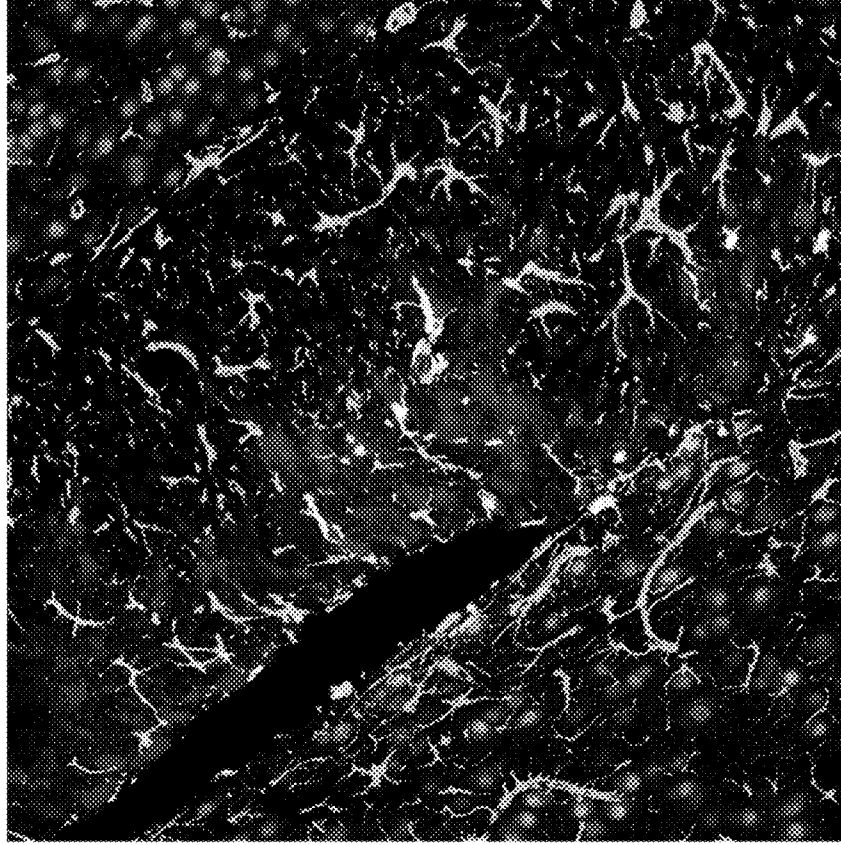


FIG. 9

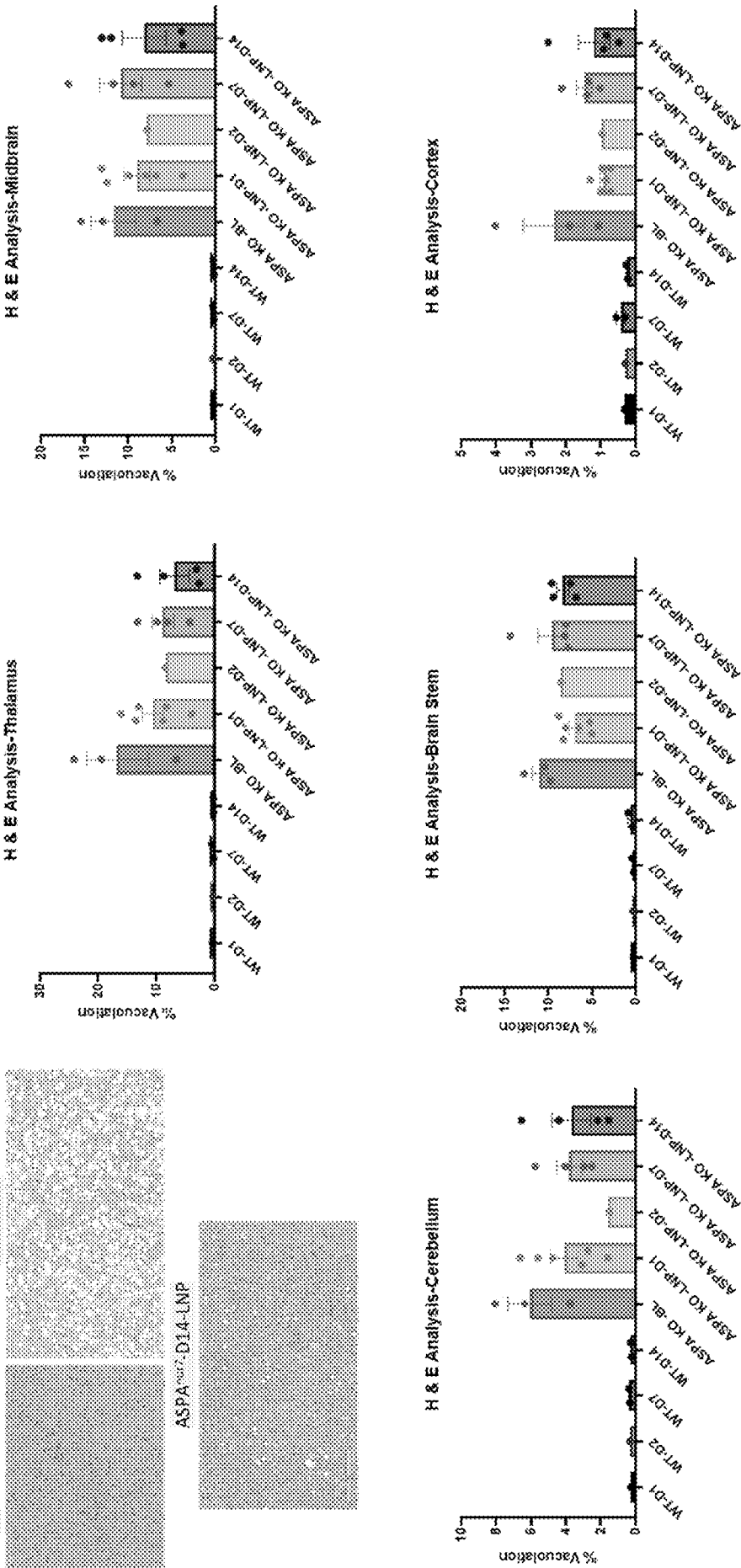
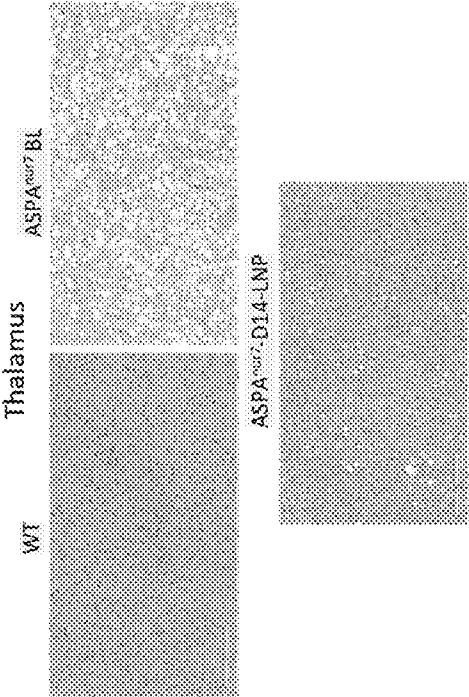


FIG. 10

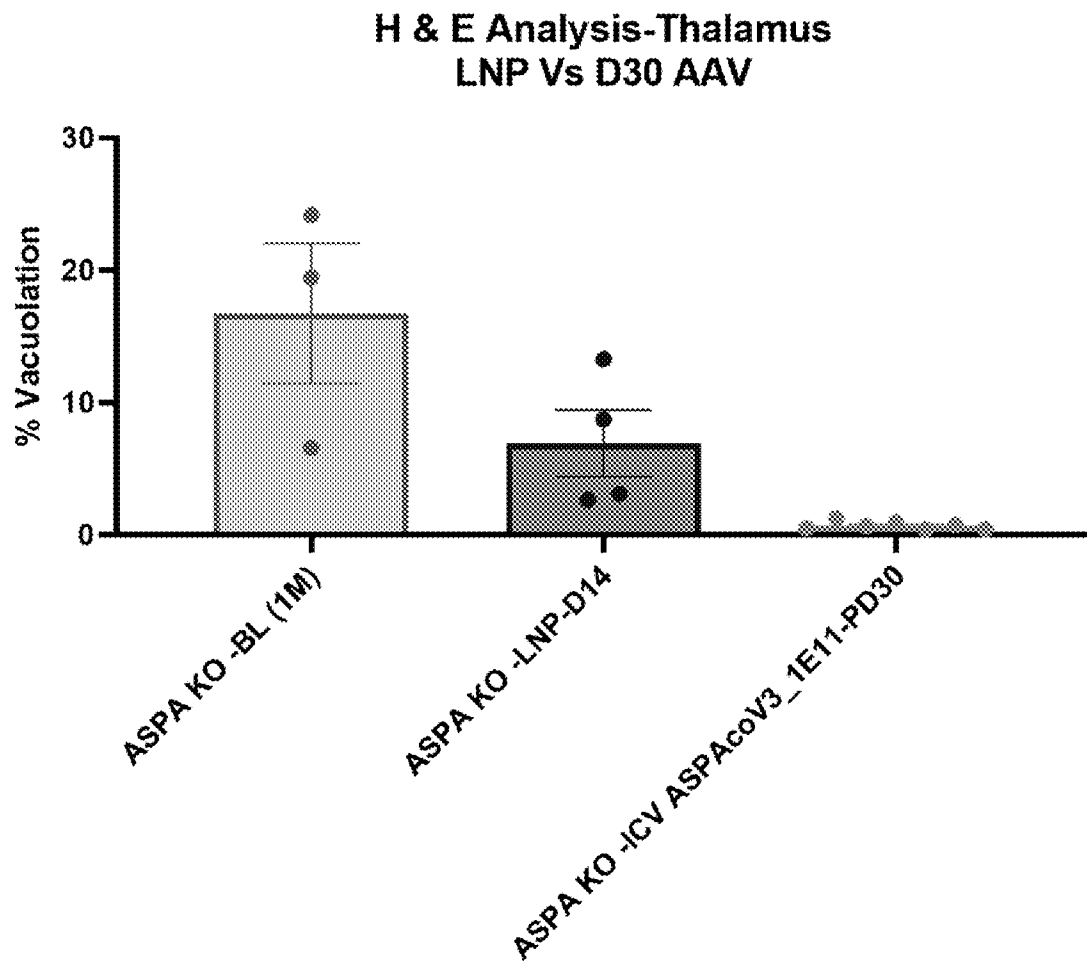
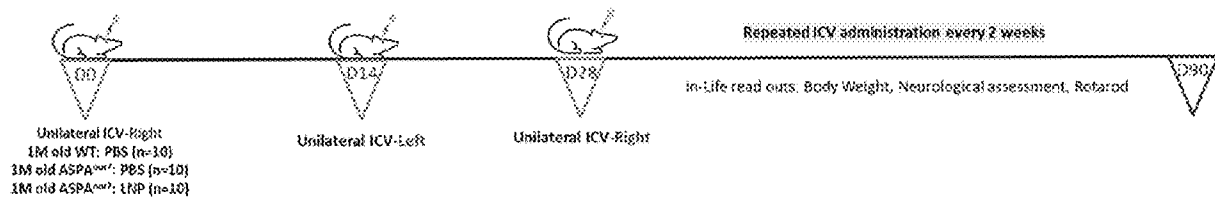


FIG. 11



INTERNATIONAL SEARCH REPORT

International application No

PCT/US2024/060265

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/86

ADD. A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2019/241633 A1 (FOTIN-MLECZEK MARIOLA [DE] ET AL) 8 August 2019 (2019-08-08) claims 1-3; sequences 13911, 854 -----	1 - 46
X	AU 2021 263 534 A1 (PFIZER) 19 January 2023 (2023-01-19) claims 1-62; sequence 4 -----	1 - 46



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 March 2025

Date of mailing of the international search report

19/03/2025

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2

NL - 2280 HV Rijswijk

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Fax: (+31-70) 340-3016

Authorized officer

Merk1, Philipp

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/060265

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2024/060265

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2019241633 A1	08-08-2019
		US 2022025369 A1	27-01-2022
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		EP 4142759 A1	08-03-2023
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		KR 20230003012 A	05-01-2023
		TW 202206599 A	16-02-2022
		US 2023165977 A1	01-06-2023
		WO 2021221995 A1	04-11-2021
