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(54) **Title:** ADENO-ASSOCIATED VIRUS CAPSID VARIANTS AND METHODS OF USE THEREOF

(57) **Abstract:** The present disclosure provides recombinant adeno-associated virus virions with variant capsid protein, where the recombinant AAV (rAAV) virions exhibit one or more of increased ability to cross neuronal cell barriers, increased infectivity of a neural stem cell, increased infectivity of a neuronal cell, and reduced susceptibility to antibody neutralization, compared to a control AAV, and where the rAAV virions comprise a heterologous nucleic acid. The present disclosure provides methods of delivering a gene product to a neural stem cell or a neuronal cell in an individual. The present disclosure also provides methods of modifying a target nucleic acid present in a neural stem cell or neuronal cell.

ADENO-ASSOCIATED VIRUS CAPSID VARIANTS AND METHODS OF USE THEREOF

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/551,133, filed August 28, 2017, which application is incorporated herein by reference in its entirety.

INTRODUCTION

[0002] Adeno-associated virus (AAV) belongs to the *Parvoviridae* family and Dependovirus genus, whose members require co-infection with a helper virus such as adenovirus to promote replication, and AAV establishes a latent infection in the absence of a helper. Virions are composed of a 25 nm icosahedral capsid encompassing a 4.7 kb single-stranded DNA genome with two open reading frames: rep and cap. The non-structural rep gene encodes four regulatory proteins essential for viral replication, whereas cap encodes three structural proteins (VP1–3) that assemble into a 60-mer capsid shell. This viral capsid mediates the ability of AAV vectors to overcome many of the biological barriers of viral transduction–including cell surface receptor binding, endocytosis, intracellular trafficking, and unpackaging in the nucleus.

[0003] There is a need in the art for AAV virions with variant capsid proteins that confer increased ability to cross cell barriers and/or that confer increased ability to infect neural stem cells and/or that confer increased ability to infect a neuronal cell.

SUMMARY

[0004] The present disclosure provides recombinant adeno-associated virus virions with variant capsid protein, where the recombinant AAV (rAAV) virions exhibit one or more of increased ability to cross neuronal cell barriers, increased infectivity of a neural stem cell, increased infectivity of a neuronal cell, and reduced susceptibility to antibody neutralization, compared to a control AAV, and where the rAAV virions comprise a heterologous nucleic acid. The present disclosure provides methods of delivering a gene product to a neural stem cell or a neuronal cell in an individual. The present disclosure also provides methods of modifying a target nucleic acid present in a neural stem cell or neuronal cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[0005] **FIG. 1A-1F** provide a SCHEMA-guided design of a chimeric adeno-associated virus (AAV) library after applying the Recombination as a Shortest Path Problem (RASPP) method.

[0006] **FIG. 2** provides the primer sequences used to design constructs and amplify the AAV *cap* gene.

[0007] **FIG. 3** provides the primer sequences designed in j5 DNA assembly to amplify each sequence block for combinatorial golden gate assembly of the SCHEMA AAV library.

[0008] **FIG. 4** provides the polymerase chain reaction (PCR) reactions for combinatorial golden gate cloning of the SCHEMA AAV library.

[0009] **FIG. 5** provides the primers designed to incorporate silent mutations at block junctures to facilitate combinatorial golden gate cloning into the pBluescript vector backbone.

[0010] **FIG. 6A-6D** provide a depiction of a Cre-dependent selection strategy for AAV directed evolution.

[0011] **FIG. 7** depicts the levels of recombination during bacterial plasmid propagation in Sure2 recombinase deficient *E. coli*.

[0012] **FIG. 8** provides the amino acid sequence of SCH9 capsid.

[0013] **FIG. 9** provides the amino acid sequence of SCH2 capsid.

[0014] **FIG. 10** provides an amino acid alignment of the SCH9 and SCH2 AAV *cap* amino acid sequences with the parent AAV serotypes. SCH9 amino acid sequence: SEQ ID NO:1; SCH2 amino acid sequence: SEQ ID NO:2; AAV2 capsid amino acid sequence: SEQ ID NO:136; AAV6 capsid amino acid sequence: SEQ ID NO:11; AAV8 capsid amino acid sequence: SEQ ID NO:137; AAV9 capsid amino acid sequence: SEQ ID NO:138.

[0015] **FIG. 11A-11B** provide three-dimensional models of the SCH9 capsid.

[0016] **FIG. 12** depicts the viral genomic yield of recombinant self-complementary AAV vectors.

[0017] **FIG. 13A-13I** depict the effects of SCH9 on the transduction of neural stem cells in the subventricular zone (SVZ).

[0018] **FIG. 14A-14C** depict marker expression of SCH9 transduction in Purkinje cells of the cerebellum.

[0019] **FIG. 15** depicts GFP expression in the cerebellum three weeks after unilateral injection of recombinant AAV1 or SCH9 in the deep cerebellar nuclei.

[0020] **FIG. 16A-16C** depict the characterization of SCH9 glycan binding and resistance to neutralizing antibodies.

[0021] **FIG. 17** depicts the infectivity of SCH2 and SCH9, compared with AAV2, a control that is known to utilize AAVR.

[0022] **FIG. 18A-18F** provide amino acid sequences of *Streptococcus pyogenes* Cas9 polypeptide and variants.

[0023] **FIG. 19** provides an amino acid sequence of a *Staphylococcus aureus* Cas9 polypeptide.

[0024] **FIG. 20A-20C** provide amino acid sequences of various Cpf1 polypeptides.

[0025] **FIG. 21A-21C** provide an alignment of amino acid sequences of AAV capsid protein loop IV (GH loop) regions. Insertion sites are shown in bold and underlining.

DEFINITIONS

[0026] As used herein, the term "neural stem cell" (NSC) refers to an undifferentiated neural cell that can proliferate, self-renew, and differentiate into the main adult neural cells of the brain. NSCs are capable of self-maintenance (self-renewal), meaning that with each cell division, one daughter cell will also be a stem cell. The non-stem cell progeny of NSCs are termed neural progenitor cells. Neural progenitors cells generated from a single multipotent NSC are capable of differentiating into neurons, astrocytes (type I and type II), and oligodendrocytes. Hence, NSCs are "multipotent" because their progeny have multiple neural cell fates. Thus, NSCs can be functionally defined as a cell with the ability to: 1) proliferate, 2) self-renew, and 3) produce functional progeny that can differentiate into the three main cell types found in the central nervous system: neurons, astrocytes and oligodendrocytes. An NSC is generally negative for markers of mature neurons, mature glial cells, mature oligodendrocytes, and mature astrocytes.

[0027] As used herein, the terms "neural progenitor cell" or "neural precursor cell" refer to a cell that can generate progeny such as neuronal cells (e.g., neuronal precursors or mature neurons), glial precursors, mature astrocytes, or mature oligodendrocytes. Typically, the cells express some of the phenotypic markers that are characteristic of the neural lineage. A "neuronal progenitor cell" or "neuronal precursor cell" is a cell that can generate progeny that are mature neurons. These cells may or may not also have the capability to generate glial cells.

[0028] A "neuronal cell," as used herein, is used interchangeably with "neural cell" and refers to neurons and glia of the central nervous system or peripheral nervous system. The term "neuronal cell" includes cells such as astrocytes, oligodendrocytes, and Schwann cells. The term includes neuronal cells of any brain tissue (e.g., a brain tissue such as cerebral hemisphere, cerebral cortex, subcortex motor cortex, striatum, internal capsule, thalamus, hypothalamus, hippocampus, midbrain, brainstem, and cerebellum). A mature neuron can express one or more markers of a mature neuron, where such markers include, e.g., nestin, NeuroD1, neuron-specific enolase (NSE), neuron-specific nuclear protein (NeuN), neurofilament (NF), S100β, tau, microtubule-associated protein 2 (MAP2), tau, doublecortin (DCX), and the like.

[0029] "AAV" is an abbreviation for adeno-associated virus, and may be used to refer to the virus itself or derivatives thereof. The term covers all subtypes and both naturally occurring and recombinant forms, except where required otherwise. The abbreviation "rAAV" refers to recombinant adeno-associated virus, also referred to as a recombinant AAV vector (or "rAAV vector"). The term “AAV” includes AAV type 1 (AAV-1), AAV type 2 (AAV-2), AAV type 3 (AAV-3), AAV type 4 (AAV-4), AAV type 5 (AAV-5), AAV type 6 (AAV-6), AAV type 7 (AAV-7), AAV type 8 (AAV-8), AAV type 9 (AAV-9), AAV type 10 (AAV-10), avian AAV, bovine AAV, canine AAV, equine AAV, primate AAV, non-primate AAV, and ovine AAV. “Primate AAV” refers to AAV isolated from a primate, “non-primate AAV” refers to AAV isolated from a non-primate mammal, “bovine AAV” refers to AAV isolated from a bovine mammal (e.g., a cow), etc.

[0030] An "rAAV vector" as used herein refers to an AAV vector comprising a polynucleotide sequence not of AAV origin (i.e., a polynucleotide heterologous to AAV), typically a sequence of interest for introducing into a target cell. In general, the heterologous polynucleotide is flanked by at least one, and generally by two AAV inverted terminal repeat sequences (ITRs). The term rAAV vector encompasses both rAAV vector particles and rAAV vector plasmids.

[0031] An "AAV virus" or "AAV viral particle" or "rAAV vector particle" refers to a viral particle composed of at least one AAV capsid protein (typically by all of the capsid proteins of a wild-type AAV) and an encapsidated polynucleotide rAAV vector. If the particle comprises a heterologous polynucleotide (i.e. a polynucleotide other than a wild-type AAV genome, such as a transgene to be delivered to a mammalian cell), it is typically referred to as an "rAAV vector particle" or simply an "rAAV vector". Thus, production of rAAV particle necessarily includes production of rAAV vector, as such a vector is contained within an rAAV particle.

[0032] "Packaging" refers to a series of intracellular events that result in the assembly and encapsidation of an AAV particle.

[0033] AAV "rep" and "cap" genes refer to polynucleotide sequences encoding replication and encapsidation proteins of adeno-associated virus. AAV rep and cap are referred to herein as AAV "packaging genes."

[0034] A "helper virus" for AAV refers to a virus that allows AAV (e.g. wild-type AAV) to be replicated and packaged by a mammalian cell. A variety of such helper viruses for AAV are known in the art, including adenoviruses, herpesviruses and poxviruses such as vaccinia. The adenoviruses encompass a number of different subgroups, although Adenovirus type 5 of subgroup C is most commonly used. Numerous adenoviruses of human, non-human mammalian and avian origin are known and available from depositories such as the ATCC. Viruses of the

herpes family include, for example, herpes simplex viruses (HSV) and Epstein-Barr viruses (EBV), as well as cytomegaloviruses (CMV) and pseudorabies viruses (PRV); which are also available from depositories such as ATCC.

[0035] "Helper virus function(s)" refers to function(s) encoded in a helper virus genome which allow AAV replication and packaging (in conjunction with other requirements for replication and packaging described herein). As described herein, "helper virus function" may be provided in a number of ways, including by providing helper virus or providing, for example, polynucleotide sequences encoding the requisite function(s) to a producer cell in trans.

[0036] An "infectious" virus or viral particle is one that comprises a polynucleotide component which it is capable of delivering into a cell for which the viral species is tropic. The term does not necessarily imply any replication capacity of the virus. As used herein, an “infectious” virus or viral particle is one that can access a target cell, can infect a target cell, and can express a heterologous nucleic acid in a target cell. Thus, “infectivity” refers to the ability of a viral particle to access a target cell, infect a target cell, and express a heterologous nucleic acid in a target cell. Infectivity can refer to in vitro infectivity or *in vivo* infectivity. Assays for counting infectious viral particles are described elsewhere in this disclosure and in the art. Viral infectivity can be expressed as the ratio of infectious viral particles to total viral particles. Total viral particles can be expressed as the number of viral genome (vg) copies. The ability of a viral particle to express a heterologous nucleic acid in a cell can be referred to as “transduction.” The ability of a viral particle to express a heterologous nucleic acid in a cell can be assayed using a number of techniques, including assessment of a marker gene, such as a green fluorescent protein (GFP) assay (e.g., where the virus comprises a nucleotide sequence encoding GFP), where GFP is produced in a cell infected with the viral particle and is detected and/or measured; or the measurement of a produced protein, for example by an enzyme-linked immunosorbent assay (ELISA). Viral infectivity can be expressed as the ratio of infectious viral particles to total viral particles. Methods of determining the ratio of infectious viral particle to total viral particle are known in the art. See, e.g., Grainger et al. (2005) *Mol. Ther.* 11:S337 (describing a TCID50 infectious titer assay); and Zolotukhin et al. (1999) *Gene Ther.* 6:973.

[0037] A "replication-competent" virus (e.g. a replication-competent AAV) refers to a phenotypically wild-type virus that is infectious, and is also capable of being replicated in an infected cell (i.e. in the presence of a helper virus or helper virus functions). In the case of AAV, replication competence generally requires the presence of functional AAV packaging genes. In general, rAAV vectors as described herein are replication-incompetent in mammalian cells (especially in human cells) by virtue of the lack of one or more AAV packaging genes. Typically, such rAAV vectors lack any AAV packaging gene sequences in order to minimize the possibility that

replication competent AAV are generated by recombination between AAV packaging genes and an incoming rAAV vector. In general, rAAV vector preparations as described herein are those which contain few if any replication competent AAV (rcAAV, also referred to as RCA) (e.g., less than about 1 rcAAV per 10^2 rAAV particles, less than about 1 rcAAV per 10^4 rAAV particles, less than about 1 rcAAV per 10^8 rAAV particles, less than about 1 rcAAV per 10^{12} rAAV particles, or no rcAAV).

[0038] The term "polynucleotide" refers to a polymeric form of nucleotides of any length, including deoxyribonucleotides or ribonucleotides, or analogs thereof. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, and may be interrupted by non-nucleotide components. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The term polynucleotide, as used herein, refers interchangeably to double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

[0039] A polynucleotide or polypeptide has a certain percent "sequence identity" to another polynucleotide or polypeptide, meaning that, when aligned, that percentage of bases or amino acids are the same when comparing the two sequences. Sequence similarity can be determined in a number of different manners. To determine sequence identity, sequences can be aligned using the methods and computer programs, including BLAST, available over the world wide web at ncbi.nlm.nih.gov/BLAST/. Another alignment algorithm is FASTA, available in the Genetics Computing Group (GCG) package, from Madison, Wisconsin, USA, a wholly owned subsidiary of Oxford Molecular Group, Inc. Other techniques for alignment are described in Methods in Enzymology, vol. 266: Computer Methods for Macromolecular Sequence Analysis (1996), ed. Doolittle, Academic Press, Inc., a division of Harcourt Brace & Co., San Diego, California, USA. Of particular interest are alignment programs that permit gaps in the sequence. The Smith-Waterman is one type of algorithm that permits gaps in sequence alignments. See Meth. Mol. Biol. 70: 173-187 (1997). Also, the GAP program using the Needleman and Wunsch alignment method can be utilized to align sequences. See J. Mol. Biol. 48: 443-453 (1970)

[0040] Of interest is the BestFit program using the local homology algorithm of Smith Waterman (Advances in Applied Mathematics 2: 482-489 (1981) to determine sequence identity. The gap generation penalty will generally range from 1 to 5, usually 2 to 4 and in some cases will be 3. The gap extension penalty will generally range from about 0.01 to 0.20 and in many instances will be 0.10. The program has default parameters determined by the sequences inputted to be compared. Preferably, the sequence identity is determined using the default parameters

determined by the program. This program is available also from Genetics Computing Group (GCG) package, from Madison, Wisconsin, USA.

[0041] Another program of interest is the FastDB algorithm. FastDB is described in Current Methods in Sequence Comparison and Analysis, Macromolecule Sequencing and Synthesis, Selected Methods and Applications, pp. 127-149, 1988, Alan R. Liss, Inc. Percent sequence identity is calculated by FastDB based upon the following parameters:

[0042] Mismatch Penalty: 1.00;

[0043] Gap Penalty: 1.00;

[0044] Gap Size Penalty: 0.33; and

[0045] Joining Penalty: 30.0.

[0046] A "gene" refers to a polynucleotide containing at least one open reading frame that is capable of encoding a particular protein after being transcribed and translated.

[0047] The term “guide RNA”, as used herein, refers to an RNA that comprises: i) an “activator” nucleotide sequence that binds to a guide RNA-directed endonuclease (e.g., a class 2 CRISPR/Cas endonuclease such as a type II, type V, or type VI CRISPR/Cas endonuclease) and activates the RNA-directed endonuclease; and ii) a “targeter” nucleotide sequence that comprises a nucleotide sequence that hybridizes with a target nucleic acid. The “activator” nucleotide sequence and the “targeter” nucleotide sequence can be on separate RNA molecules (e.g., a “dual-guide RNA”); or can be on the same RNA molecule (a “single-guide RNA”).

[0048] A "small interfering" or "short interfering RNA" or siRNA is a RNA duplex of nucleotides that is targeted to a gene interest (a “target gene”). An "RNA duplex" refers to the structure formed by the complementary pairing between two regions of a RNA molecule. siRNA is "targeted" to a gene in that the nucleotide sequence of the duplex portion of the siRNA is complementary to a nucleotide sequence of the targeted gene. In some cases, the length of the duplex of siRNAs is less than 30 nucleotides. In some cases, the duplex can be 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11 or 10 nucleotides in length. In some cases, the length of the duplex is 19-25 nucleotides in length. The RNA duplex portion of the siRNA can be part of a hairpin structure. In addition to the duplex portion, the hairpin structure may contain a loop portion positioned between the two sequences that form the duplex. The loop can vary in length. In some cases, the loop is 5, 6, 7, 8, 9, 10, 11, 12 or 13 nucleotides in length. The hairpin structure can also contain 3' or 5' overhang portions. In some cases, the overhang is a 3' or a 5' overhang 0, 1, 2, 3, 4 or 5 nucleotides in length.

[0049] As used herein, the term "microRNA" refers to any type of interfering RNAs, including but not limited to, endogenous microRNAs and artificial microRNAs (e.g., synthetic miRNAs).

Endogenous microRNAs are small RNAs naturally encoded in the genome which are capable of modulating the productive utilization of mRNA. An artificial microRNA can be any type of RNA sequence, other than endogenous microRNA, which is capable of modulating the activity of an mRNA. A microRNA sequence can be an RNA molecule composed of any one or more of these sequences. MicroRNA (or "miRNA") sequences have been described in publications such as Lim, et al., 2003, Genes & Development, 17, 991-1008, Lim et al., 2003, Science, 299, 1540, Lee and Ambrose, 2001, Science, 294, 862, Lau et al., 2001, Science 294, 858-861, Lagos-Quintana et al., 2002, Current Biology, 12, 735-739, Lagos-Quintana et al., 2001, Science, 294, 853-857, and Lagos-Quintana et al., 2003, RNA, 9, 175-179. Examples of microRNAs include any RNA that is a fragment of a larger RNA or is a miRNA, siRNA, stRNA, sncRNA, tncRNA, snoRNA, smRNA, shRNA, snRNA, or other small non-coding RNA. See, e.g., US Patent Applications 20050272923, 20050266552, 20050142581, and 20050075492. A "microRNA precursor" (or "pre-miRNA") refers to a nucleic acid having a stem-loop structure with a microRNA sequence incorporated therein. A "mature microRNA" (or "mature miRNA") includes a microRNA that has been cleaved from a microRNA precursor (a "pre-miRNA"), or that has been synthesized (e.g., synthesized in a laboratory by cell-free synthesis), and has a length of from about 19 nucleotides to about 27 nucleotides, e.g., a mature microRNA can have a length of 19 nt, 20 nt, 21 nt, 22 nt, 23 nt, 24 nt, 25 nt, 26 nt, or 27 nt. A mature microRNA can bind to a target mRNA and inhibit translation of the target mRNA.

[0050] "Recombinant," as applied to a polynucleotide means that the polynucleotide is the product of various combinations of cloning, restriction or ligation steps, and other procedures that result in a construct that is distinct from a polynucleotide found in nature. A recombinant virus is a viral particle comprising a recombinant polynucleotide. The terms respectively include replicates of the original polynucleotide construct and progeny of the original virus construct.

[0051] A "control element" or "control sequence" is a nucleotide sequence involved in an interaction of molecules that contributes to the functional regulation of a polynucleotide, including replication, duplication, transcription, splicing, translation, or degradation of the polynucleotide. The regulation may affect the frequency, speed, or specificity of the process, and may be enhancing or inhibitory in nature. Control elements known in the art include, for example, transcriptional regulatory sequences such as promoters and enhancers. A promoter is a DNA region capable under certain conditions of binding RNA polymerase and initiating transcription of a coding region usually located downstream (in the 3' direction) from the promoter.

[0052] "Operatively linked" or "operably linked" refers to a juxtaposition of genetic elements, wherein the elements are in a relationship permitting them to operate in the expected manner. For instance, a promoter is operatively linked to a coding region if the promoter helps initiate

transcription of the coding sequence. There may be intervening residues between the promoter and coding region so long as this functional relationship is maintained.

[0053] An "expression vector" is a vector comprising a region which encodes a polypeptide of interest, and is used for effecting the expression of the protein in an intended target cell. An expression vector also comprises control elements operatively linked to the encoding region to facilitate expression of the protein in the target. The combination of control elements and a gene or genes to which they are operably linked for expression is sometimes referred to as an "expression cassette," a large number of which are known and available in the art or can be readily constructed from components that are available in the art.

[0054] "Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is being compared. For example, a polynucleotide introduced by genetic engineering techniques into a plasmid or vector derived from a different species is a heterologous polynucleotide. A promoter removed from its native coding sequence and operatively linked to a coding sequence with which it is not naturally found linked is a heterologous promoter. Thus, for example, an rAAV that includes a heterologous nucleic acid encoding a heterologous gene product is an rAAV that includes a nucleic acid not normally included in a naturally-occurring, wild-type AAV, and the encoded heterologous gene product is a gene product not normally encoded by a naturally-occurring, wild-type AAV. As another example, a variant AAV capsid protein that comprises a heterologous peptide inserted into the GH loop of the capsid protein is a variant AAV capsid protein that includes an insertion of a peptide not normally included in a naturally-occurring, wild-type AAV.

[0055] The terms "genetic alteration" and “genetic modification” (and grammatical variants thereof), are used interchangeably herein to refer to a process wherein a genetic element (e.g., a polynucleotide) is introduced into a cell other than by mitosis or meiosis. The element may be heterologous to the cell, or it may be an additional copy or improved version of an element already present in the cell. Genetic alteration may be effected, for example, by transfecting a cell with a recombinant plasmid or other polynucleotide through any process known in the art, such as electroporation, calcium phosphate precipitation, or contacting with a polynucleotide-liposome complex. Genetic alteration may also be effected, for example, by transduction or infection with a DNA or RNA virus or viral vector. Generally, the genetic element is introduced into a chromosome or mini-chromosome in the cell; but any alteration that changes the phenotype and/or genotype of the cell and its progeny is included in this term.

[0056] A cell is said to be "stably" altered, transduced, genetically modified, or transformed with a genetic sequence if the sequence is available to perform its function during extended culture of

the cell in vitro. Generally, such a cell is "heritably" altered (genetically modified) in that a genetic alteration is introduced which is also inheritable by progeny of the altered cell.

[0057] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, phosphorylation, or conjugation with a labeling component. Polypeptides such as anti-angiogenic polypeptides, neuroprotective polypeptides, and the like, when discussed in the context of delivering a gene product to a mammalian subject, and compositions therefor, refer to the respective intact polypeptide, or any fragment or genetically engineered derivative thereof, which retains the desired biochemical function of the intact protein. Similarly, references to nucleic acids encoding anti-angiogenic polypeptides, nucleic acids encoding neuroprotective polypeptides, and other such nucleic acids for use in delivery of a gene product to a mammalian subject (which may be referred to as "transgenes" to be delivered to a recipient cell), include polynucleotides encoding the intact polypeptide or any fragment or genetically engineered derivative possessing the desired biochemical function.

[0058] An "isolated" plasmid, nucleic acid, vector, virus, virion, host cell, or other substance refers to a preparation of the substance devoid of at least some of the other components that may also be present where the substance or a similar substance naturally occurs or is initially prepared from. Thus, for example, an isolated substance may be prepared by using a purification technique to enrich it from a source mixture. Enrichment can be measured on an absolute basis, such as weight per volume of solution, or it can be measured in relation to a second, potentially interfering substance present in the source mixture. Increasing enrichments of the embodiments of this invention are increasingly more isolated. An isolated plasmid, nucleic acid, vector, virus, host cell, or other substance is in some cases purified, e.g., from about 80% to about 90% pure, at least about 90% pure, at least about 95% pure, at least about 98% pure, or at least about 99%, or more, pure.

[0059] The terms "treatment", "treating", "treat" and the like are used herein to generally refer to obtaining a desired pharmacologic and/or physiologic effect. The effect can be prophylactic in terms of completely or partially preventing a disease or symptom(s) thereof and/or may be therapeutic in terms of a partial or complete stabilization or cure for a disease and/or adverse effect attributable to the disease. The term “treatment" encompasses any treatment of a disease in a mammal, particularly a human, and includes: (a) preventing the disease and/or symptom(s) from occurring in a subject who may be predisposed to the disease or symptom(s) but has not yet been diagnosed as having it; (b) inhibiting the disease and/or symptom(s), i.e., arresting development of a disease and/or the associated symptoms; or (c) relieving the disease and the

associated symptom(s), i.e., causing regression of the disease and/or symptom(s). Those in need of treatment can include those already afflicted (e.g., those with a neurological disorder) as well as those in which prevention is desired (e.g., those with increased susceptibility to a neurological disorder; those suspected of having a neurological disorder; those having one or more risk factors for a neurological disorder, etc.).

[0060] The terms “recipient”, “individual”, “subject”, “host”, and “patient”, are used interchangeably herein and refer to any mammalian subject for whom diagnosis, treatment, or therapy is desired, such as humans. "Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as non-human primates, dogs, horses, cats, cows, sheep, goats, pigs, camels, etc. In some cases, the mammal is a human.

[0061] A "therapeutically effective amount" or "efficacious amount" means the amount of a compound that, when administered to a mammal or other subject for treating a disease, is sufficient, in combination with another agent, or alone in one or more doses, to effect such treatment for the disease. The "therapeutically effective amount" will vary depending on the compound, the disease and its severity and the age, weight, etc., of the subject to be treated.

[0062] The terms “individual,” “host,” “subject,” and “patient” are used interchangeably herein, and refer to a mammal, including, but not limited to, human and non-human primates, including simians and humans; mammalian sport animals (e.g., horses, camels, etc.); mammalian farm animals (e.g., sheep, goats, cows, etc.); mammalian pets (dogs, cats, etc.); and rodents (e.g., mice, rats, etc.). In some cases, the individual is a human.

[0063] Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0064] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0065] Unless defined otherwise, all 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. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0066] It must be noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a neural stem cell” includes a plurality of such cells and reference to “the rAAV” includes reference to one or more rAAVs and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as “solely,” “only” and the like in connection with the recitation of claim elements, or use of a “negative” limitation.

[0067] It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All combinations of the embodiments pertaining to the invention are specifically embraced by the present invention and are disclosed herein just as if each and every combination was individually and explicitly disclosed. In addition, all sub-combinations of the various embodiments and elements thereof are also specifically embraced by the present invention and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

[0068] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DETAILED DESCRIPTION

[0069] The present disclosure provides recombinant adeno-associated virus virions with variant capsid protein, where the recombinant AAV (rAAV) virions exhibit one or more of increased ability to cross neuronal cell barriers, increased infectivity of a neural stem cell, increased infectivity of a

neuronal cell, and reduced susceptibility to antibody neutralization, compared to a control AAV, and where the rAAV virions comprise a heterologous nucleic acid. The present disclosure provides methods of delivering a gene product to a neural stem cell, a neuronal progenitor cell, or a neuronal cell in an individual. The present disclosure also provides methods of modifying a target nucleic acid present in a neural stem cell or neuronal cell. The present disclosure further provides methods of treating a neural disease.

RECOMBINANT AAV VIRIONS WITH VARIANT CAPSID POLYPEPTIDES

[0070] The present disclosure provides an infectious rAAV virion comprising: i) a variant AAV capsid polypeptide of the present disclosure; and ii) a heterologous nucleic acid comprising a nucleotide sequence encoding a heterologous polypeptide (i.e., a non-AAV polypeptide). In some cases, the variant AAV capsid protein comprises at least 5 segments from at least 3 different AAV serotypes, wherein each segment has a length of from about 50 amino acids to about 160 amino acids. The variant capsid protein confers one or more of the following properties: i) increased infectivity of a neural stem cell or neural progenitor cell, compared to the infectivity of the neural stem cell or neural progenitor cell by a control AAV virion comprising a corresponding parental AAV capsid protein or compared to a wild-type AAV virion, or compared to a control AAV virion comprising wild-type AAV capsid; ii) increased infectivity of a neuronal cell, compared to the infectivity of the neuronal cell by a control AAV virion comprising a corresponding parental AAV capsid protein or compared to a wild-type AAV virion, or compared to a control AAV virion comprising wild-type AAV capsid; iii) increased ability to cross a cellular barrier, compared to the ability of a control AAV virion comprising a corresponding parental AAV capsid protein or compared to the ability of a wild-type AAV virion to cross the cellular barrier, or compared to a control AAV virion comprising wild-type AAV capsid; iv) increased resistance to human AAV neutralizing antibodies, compared to the resistance exhibited by a control AAV virion comprising a corresponding parental AAV capsid protein or compared to a wild-type AAV virion, or compared to a control AAV virion comprising wild-type AAV capsid.

[0071] A control AAV virion can comprise a parental AAV capsid protein. A control AAV virion can be an AAV virion comprising wild-type AAV capsid, e.g., comprising only wild-type capsid (and not any variant AAV capsid of the present disclosure). For example, a control AAV virion can comprise wild-type AAV2 capsid. As another example, a control AAV virion can comprise wild-type AAV6 capsid. As another example, a control AAV virion can comprise wild-type AAV9 capsid.

Increased infectivity of a neural stem cell or neural progenitor cell

[0072] The present disclosure provides rAAV virions with a variant capsid protein, where rAAV virions exhibit increased infectivity of a neural stem cell (NSC) or a neural progenitor cell compared to the ability of a control, parental AAV not comprising the variant capsid protein, or compared to the ability of wild-type AAV, or compared to a control AAV virion comprising wild-type AAV capsid, to infect the NSC or neural progenitor cell; and where the rAAV virions comprise a heterologous nucleic acid.

[0073] In some cases, the NSC is a subventricular zone (SVZ) NSC. The SVZ is located along the ependymal cell layer, which separates the ventricular space from the SVZ. SVZ. NSCs can give rise to transit amplifying progenitors, which divide a few times before becoming neuroblasts. In some cases, the NSC is in the subgranular zone (SGZ) within the dentate gyrus of the hippocampus. Radial glia-like NSCs (RGLs) in the SGZ, at the border between the inner granule cell layer and the hilus, give rise to intermediate progenitor cells (IPCs), which exhibit limited rounds of proliferation before generating neuroblasts. Neural progenitor cells (NPCs) include transit amplifying cells, RGLs, IPCs, and neuroblasts. In some cases, the NSC is from the hippocampus, or is present in the hippocampus. In some cases, the NSC is present in the developing nervous system; e.g., the NSC is present in an embryo.

[0074] In some cases, an rAAV virion of the present disclosure exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of an NSC, compared to the infectivity of the NSC by an AAV virion comprising the corresponding parental AAV capsid protein, compared to the infectivity of the NSC by an AAV virion not comprising the variant capsid polypeptide, or compared to the infectivity of the NSC by a wild-type AAV virion (comprising a wild-type AAV capsid), or compared to the infectivity of the NSC by a control AAV virion comprising wild-type AAV capsid.

[0075] In some cases, a subject rAAV virion exhibits at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of an NSC, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection, compared to the infectivity of the NSC by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid, or compared to a control AAV virion comprising wild-type AAV capsid when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection.

[0076] In some cases, an rAAV virion of the present disclosure exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of an NPC, compared to the infectivity of the NPC by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid, compared to the infectivity of the NPC by an AAV virion not comprising the variant capsid polypeptide, or compared to the infectivity of the NPC by a wild-type AAV virion (comprising a wild-type AAV capsid), or compared to the infectivity of the NPC by a control AAV virion comprising wild-type AAV capsid.

[0077] In some cases, a subject rAAV virion exhibits at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of a neuroblast, compared to the infectivity of the neuroblast by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid, or compared to a control AAV virion comprising wild-type AAV capsid.

[0078] In some cases, a subject rAAV virion exhibits at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of a neuroblast, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection, compared to the infectivity of the neuroblast by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid, or compared to a control AAV virion comprising wild-type AAV capsid, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection.

[0079] In some cases, a subject rAAV virion exhibits at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of a transit amplifying cell, compared to the infectivity of the transit amplifying cell by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein, or compared to a control AAV virion comprising wild-type AAV capsid.

[0080] In some cases, a subject rAAV virion exhibits at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of a transit amplifying cell, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection, compared to the infectivity of the transit amplifying cell by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein, or compared to a control AAV virion comprising wild-type AAV capsid when administered via the same route of administration.

[0081] Whether a given rAAV virion exhibits increased infectivity of an NSC or a NPC can be determined *in vitro* or *in vivo*. For example, whether a given rAAV virion exhibits increased infectivity of an NSC can be determined by contacting the NSC *in vitro* with the rAAV virion, and detecting expression in the NSC of a heterologous gene product encoded by the rAAV virion. The heterologous gene product can provide a detectable signal, and the level of the detectable signal in the NSC can provide an indication as to whether a given rAAV virion exhibits increased infectivity of an NSC.

[0082] In some cases, an rAAV virion of the present disclosure that comprises: a) a variant capsid of the present disclosure comprising at least 5 segments from at least 3 different AAV serotypes, wherein each segment has a length of from about 50 amino acids to about 160 amino acids, as described below; and b) a heterologous nucleotide sequence encoding a heterologous gene product, when administered to an individual, results in a level of the heterologous gene product in a neural stem cell, that is at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, greater than the level of the gene product in the neural stem cell that results when a control rAAV virion that comprises: a) a control AAV (e.g., a wild-type AAV capsid); and b) heterologous nucleotide sequence encoding the heterologous gene product is administered to the individual. Administration can be via a number of routes, e.g., via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection.

[0083] Whether a given rAAV virion exhibits increased infectivity of an NSC can be determined by assessing a therapeutic effect of a therapeutic gene product encoded by the rAAV virion in an NSC. Therapeutic effects can include, e.g., a) an increase in neurogenesis; b) amelioration of a symptom of a neurological disease or disorder; etc. For example, an rAAV virion of the present disclosure that comprises: a) a variant capsid of the present disclosure; and b) a heterologous nucleotide sequence encoding a heterologous therapeutic gene product, when administered to an individual (e.g., via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection), results in a therapeutic effect of the therapeutic gene product in a neural stem cell, that is at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, greater than the therapeutic effect that results when a control rAAV virion that comprises: a) a control AAV capsid (e.g., a wild-type AAV capsid); and b) heterologous nucleotide sequence encoding the heterologous therapeutic gene product is administered via the same route of administration.

[0084] In some cases, a subject rAAV virion exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of an NSC, when administered via intracranial, intracerebroventicular, intrathecal,

intra-cisterna magna, or intravenous injection, compared to the infectivity of the NSC by an AAV virion comprising the corresponding parental AAV capsid protein, or compared to the infectivity of the NSC by a wild-type AAV virion (comprising a wild-type AAV capsid polypeptide), when administered via the same route of administration.

Increased infectivity of a neuronal cell

[0085] As noted above, in some cases, a variant capsid polypeptide present in an rAAV virion of the present disclosure confers increased infectivity of a neuronal cell on the rAAV virion, compared to the ability of a control parental AAV not comprising the variant capsid protein, or compared to a wild-type AAV to infect the neuronal cell.

[0086] In some cases, a subject rAAV virion exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of a neuronal cell, compared to the infectivity of the neuronal cell by an AAV virion comprising the corresponding parental AAV capsid protein, or compared to the infectivity of the neuronal cell by an AAV virion comprising wild-type AAV capsid polypeptide.

[0087] In some cases, a subject rAAV virion exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of a neuronal cell, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection, compared to the infectivity of the neuronal cell by an AAV virion comprising the corresponding parental AAV capsid protein, or compared to the infectivity of the neuronal cell by wild-type AAV, or compared to a control AAV virion comprising wild-type AAV capsid when administered via the same route of administration.

[0088] In some cases, a subject rAAV virion exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of a neuronal cell of the cerebral hemisphere, cerebral cortex, subcortex motor cortex, striatum, internal capsule, thalamus, hypothalamus, hippocampus, midbrain, brainstem, or the cerebellum, compared to the infectivity of the neuronal cell of the same tissue by an AAV virion comprising the corresponding parental AAV capsid protein, or compared to the infectivity of the neuronal cell by an AAV virion comprising wild-type AAV capsid polypeptide.

[0089] As one example, in some cases, a subject rAAV virion exhibits at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of a Purkinje cell, compared to the infectivity of the Purkinje cell by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein.

[0090] As one example, in some cases, a subject rAAV virion exhibits at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of a Purkinje cell, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection, compared to the infectivity of the Purkinje cell by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein, when administered via the same route of administration.

[0091] As one example, in some cases, a subject rAAV virion exhibits at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of a GABAergic cell, compared to the infectivity of the GABAergic cell by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein.

[0092] As one example, in some cases, a subject rAAV virion exhibits at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of a GABAergic cell, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection, compared to the infectivity of the GABAergic cell by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein, when administered via the same route of administration.

[0093] In some cases, a subject rAAV virion exhibits at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of a glial cell, compared to the infectivity of the glial cell by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein.

[0094] In some cases, a subject rAAV virion exhibits at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of a glial cell, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection, compared to the infectivity of the glial cell by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein, when administered via the same route of administration.

[0095] In some cases, a subject rAAV virion exhibits at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of an astrocyte, compared to the infectivity of the astrocyte by an AAV virion comprising the corresponding parental AAV capsid protein.

[0096] In some cases, a subject rAAV virion exhibits at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of an astrocyte, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection, compared to the infectivity of the astrocyte by an AAV virion comprising the corresponding parental AAV capsid protein, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection.

[0097] Whether a given rAAV virion exhibits increased infectivity of a neuronal cell can be determined *in vitro* or *in vivo*. For example, whether a given rAAV virion exhibits increased infectivity of a neuronal cell can be determined by contacting the neuronal cell *in vitro* with the rAAV virion, and detecting expression in the neuronal cell of a heterologous gene product encoded by the rAAV virion. The heterologous gene product can provide a detectable signal, and the level of the detectable signal in the neuronal cell can provide an indication as to whether a given rAAV virion exhibits increased infectivity of a neuronal cell.

[0098] Whether a given rAAV virion exhibits increased infectivity of a neuronal cell can be determined by detecting expression in a neuronal cell of a heterologous gene product encoded by the rAAV virion, following administration of the rAAV virion to an individual. Whether a given rAAV virion exhibits increased infectivity of a neuronal cell can be determined by detecting expression in a neuronal cell of a heterologous gene product encoded by the rAAV virion, following intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous administration of the rAAV virion. For example, an rAAV virion of the present disclosure that comprises: a) a variant capsid of the present disclosure comprising at least 5 segments from at least 3 different AAV serotypes, wherein each segment has a length of from about 50 amino acids to about 160 amino acids, as described above; and b) a heterologous nucleotide sequence encoding a heterologous gene product, when administered, results in a level of the heterologous gene product in a neuronal cell, that is at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, greater than the level of the gene product in the neuronal cell that results when a control rAAV virion that comprises: a) a control AAV capsid or a wild-type AAV capsid; and b) heterologous nucleotide sequence encoding the heterologous gene product is administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection.

[0099] Whether a given rAAV virion exhibits increased infectivity of a neuronal cell can be determined by assessing a therapeutic effect of a therapeutic gene product encoded by the rAAV virion in a neuronal cell. Therapeutic effects can include, e.g., a) an increase in neuronal cell function; b) amelioration of a symptom of a neurological disease or disorder; etc. For example, an rAAV virion of the present disclosure that comprises: a) a variant capsid of the present disclosure

comprising a peptide insert or a peptide replacement, as described above; and b) a heterologous nucleotide sequence encoding a heterologous therapeutic gene product, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection, results in a therapeutic effect of the therapeutic gene product in a neuronal cell, that is at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, greater than the therapeutic effect in the neuronal cell that results when a control rAAV virion that comprises: a) a control AAV capsid that does not comprises the peptide insert or the peptide replacement; and b) heterologous nucleotide sequence encoding the heterologous therapeutic gene product is administered via the same route of administration.

Crossing a cellular barrier

[00100] The present disclosure provides recombinant adeno-associated virus virions with variant capsid protein, where the rAAV virions exhibit increased ability to cross a cell barrier, i.e., a physiological barrier. For example, a cell barrier can comprise a layer of cells between a first compartment that does not include a neural stem cell and a second compartment that does include a neural stem cell. Such barriers include, e.g., the ependymal cell layer lining the lateral ventricles, the hypocellular layer, the astrocyte cell bodies layer, the blood-brain barrier, and the transition zone layer. Thus, the present disclosure provides an rAAV virion with a variant capsid protein, where the rAAV virion exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased ability to cross one or more of the ependymal cell layer, the hypocellular layer, the astrocyte cell bodies layer, and the transition zone layer, compared to the ability of a control AAV not comprising the variant capsid protein, or compared to the ability of a control AAV comprising wild-type AAV capsid protein, to cross the layer; and where the rAAV virions comprise a heterologous nucleic acid comprising a nucleotide sequence encoding a heterologous gene product.

[00101] In some cases, an rAAV virion of the present disclosure exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased ability to cross the ependymal cell layer lining the lateral ventricles, compared to the ability of a control rAAV virion comprising the corresponding parental AAV capsid, or comprising wild-type AAV capsid protein, to cross the ependymal cell layer.

[00102] In some cases, an rAAV virion of the present disclosure exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased ability to cross the blood-brain barrier, compared to the ability of a control rAAV virion comprising the corresponding parental AAV capsid, or comprising wild-type AAV capsid protein, to cross the blood-brain barrier.

[00103] In some cases, a subject rAAV virion exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased ability to cross the ependymal cell layer, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection, compared to the ability to cross the ependymal cell layer by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein, when administered via the same route of administration.

[00104] In some cases, an rAAV virion of the present disclosure exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased ability to cross the hypocellular layer, compared to the ability of a control rAAV virion comprising the corresponding parental AAV capsid, or comprising wild-type AAV capsid protein, to cross the hypocellular cell layer.

[00105] In some cases, a subject rAAV virion exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased ability to cross the hypocellular layer, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection, compared to the ability to cross the hypocellular layer by an AAV virion comprising the corresponding parental AAV capsid protein, when administered via the same route of administration.

[00106] In some cases, an rAAV virion of the present disclosure exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased ability to cross the astrocyte cell bodies layer, compared to the ability of a control rAAV virion comprising the corresponding parental AAV capsid, or comprising wild-type AAV capsid protein, to cross the astrocytes cell bodies layer.

[00107] In some cases, a subject rAAV virion exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased ability to cross the astrocyte cell bodies layer, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection, compared to the ability to cross the astrocyte cell bodies layer by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein, when administered via the same route of administration.

[00108] In some cases, an rAAV virion of the present disclosure exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased ability to cross the transition zone layer, compared to the ability of a

control rAAV virion comprising the corresponding parental AAV capsid, or comprising wild-type AAV capsid protein, to cross the transition zone layer.

[00109] In some cases, a subject rAAV virion exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased ability to cross the transition zone layer, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection, compared to the ability to cross the transition zone layer by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein, when administered via the same route of administration.

[00110] In some cases, an rAAV virion of the present disclosure exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased ability to cross the brain parenchyma, compared to the ability of a control rAAV virion comprising the corresponding parental AAV capsid, or comprising wild-type AAV capsid protein, to cross the brain parenchyma.

[00111] In some cases, a subject rAAV virion exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased ability to cross the brain parenchyma, when administered via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection, compared to the ability to cross the brain parenchyma by an AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein, when administered via the same route of administration.

[00112] In some cases, a subject rAAV virion, when injected via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous administration, exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased localization past the ependymal layer, compared to the extent of localization past the ependymal layer by a control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein, when injected via the same route of administration.

[00113] For example, in some cases, a subject rAAV virion, when injected via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous administration, exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased localization to the hypocellular layer, compared to the extent of localization to the hypocellular layer by a control AAV virion comprising the

corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein, when injected via the same route of administration.

[00114] As another example, in some cases, a subject rAAV virion, when injected by a via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous administration, exhibits at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased localization to the astrocyte cell bodies layer, compared to the extent of localization to the astrocyte cell bodies layer by a control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein, when injected via the same route of administration.

[00115] As another example, in some cases, a subject rAAV virion, when injected via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous administration, exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased localization to the transition zone layer, compared to the extent of localization to the transition zone layer by a control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein, when injected via the same route of administration.

[00116] As another example, in some cases, a subject rAAV virion, when injected via intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous administration, exhibits at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased localization to the brain parenchyma, compared to the extent of localization to the brain parenchyma by a control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid protein, when injected via the same route of administration.

Decreased susceptibility to neutralization by neutralizing antibodies

[00117] As noted above, in some cases, an rAAV virion of the present disclosure exhibits decreased binding to neutralizing antibodies, compared to the binding of the neutralizing antibodies to AAV comprising wild-type AAV capsid.

[00118] Decreased binding to neutralizing antibodies is advantageous. Neutralizing antibodies bind to wild-type capsid proteins. Binding of neutralizing antibodies to wild-type capsid proteins may have several effects, including limiting the residence time of an rAAV virions that comprises wild-type capsid proteins in the viral particle, preventing the virus from binding to the cell surface, aggregating the virus, induction of structural alterations in the capsid, and prevention of viral disassembly and uncoating (a step necessary to release the DNA). An rAAV particle that has decreased binding to neutralizing antibodies thus has increased capacity to infect

cells, and increased residence time in the body of an individual administered with the rAAV virion. Thus, the effective duration of delivery of gene product is increased.

[00119] In some case, an rAAV virion of the present disclosure exhibits increased resistance to neutralizing antibodies compared to wild-type AAV ("wt AAV") or AAV comprising a wild-type capsid protein. In some cases, an rAAV virion of the present disclosure has from about 1.5-fold to about 10-fold 10,000-fold greater resistance to neutralizing antibodies than wt AAV; e.g., in some cases, an rAAV virion of the present disclosure has from about 1.5-fold to about 2-fold, from about 2-fold to about 2.-5 fold, from about 2.5-fold to about 3-fold, from about 3-fold to about 4-fold, from about 4-fold to about 5-fold, from about 5-fold to about 6-fold, from about 6-fold to about 7-fold, from about 7-fold to about 8-fold, from about 8-fold to about 9-fold, or from about 9-fold to about 10-fold, greater resistance to neutralizing antibodies than wt AAV. In some cases, an rAAV virion of the present disclosure has from about 10-fold to about 10,000-fold greater resistance to neutralizing antibodies than wt AAV, e.g., an rAAV virion of the present disclosure has from about 10-fold to about 25-fold, from about 25-fold to about 50-fold, from about 50-fold to about 75-fold, from about 75-fold to about 100-fold, from about 100-fold to about 150-fold, from about 150-fold to about 200-fold, from about 200-fold to about 250-fold, from about 250-fold to about 300-fold, at least about 350-fold, at least about 400-fold, from about 400-fold to about 450-fold, from about 450-fold to about 500-fold, from about 500-fold to about 550-fold, from about 550-fold to about 600-fold, from about 600-fold to about 700-fold, from about 700-fold to about 800-fold, from about 800-fold to about 900-fold, from about 900-fold to about 1000-fold, from about 1,000-fold to about 2,000-fold, from about 2,000-fold to about 3,000-fold, from about 3,000-fold to about 4,000-fold, from about 4,000-fold to about 5,000-fold, from about 5,000-fold to about 6,000-fold, from about 6,000-fold to about 7,000-fold, from about 7,000-fold to about 8,000-fold, from about 8,000-fold to about 9,000-fold, or from about 9,000-fold to about 10,000-fold greater resistance to neutralizing antibodies than a wild-type AAV or an AAV comprising a wild-type capsid protein.

[00120] In some cases, an rAAV virion of the present disclosure exhibits decreased binding to a neutralizing antibody that binds a wild-type AAV capsid protein. For example, in some cases, an rAAV virion of the present disclosure exhibits from about 10-fold to about 10,000-fold reduced binding to a neutralizing antibody that binds a wild-type AAV capsid protein. For example, in some cases, an rAAV virion of the present disclosure exhibits from about 10-fold to about 25-fold, from about 25-fold to about 50-fold, from about 50-fold to about 75-fold, from about 75-fold to about 100-fold, from about 100-fold to about 150-fold, from about 150-fold to about 200-fold, from about 200-fold to about 250-fold, from about 250-fold to about 300-fold, at least about 350-fold, at least about 400-fold, from about 400-fold to about 450-fold, from about 450-

fold to about 500-fold, from about 500-fold to about 550-fold, from about 550-fold to about 600-fold, from about 600-fold to about 700-fold, from about 700-fold to about 800-fold, from about 800-fold to about 900-fold, from about 900-fold to about 1000-fold, from about 1,000-fold to about 2,000-fold, from about 2,000-fold to about 3,000-fold, from about 3,000-fold to about 4,000-fold, from about 4,000-fold to about 5,000-fold, from about 5,000-fold to about 6,000-fold, from about 6,000-fold to about 7,000-fold, from about 7,000-fold to about 8,000-fold, from about 8,000-fold to about 9,000-fold, or from about 9,000-fold to about 10,000-fold reduced binding (e.g., reduced affinity) to a neutralizing antibody that binds a wild-type capsid AAV protein, compared to the binding affinity of the antibody to wild-type AAV capsid protein.

[00121] In some cases, an anti-AAV neutralizing antibody binds to an rAAV virion of the present disclosure with an affinity of less than about 10^{-7} M, less than about 5 x 10^{-6} M, less than about 10^{-6} M, less than about 5 x 10^{-5} M, less than about 10^{-5} M, less than about 10^{-4} M, or lower.

[00122] In some cases, an rAAV virion of the present disclosure exhibits increased *in vivo* residence time compared to a wild-type AAV. For example, in some cases, an rAAV virion of the present disclosure exhibits a residence time that is at least about 10%, at least about 25%, at least about 50%, at least about 100%, at least about 3-fold, at least about 5-fold, at least about 10-fold, at least about 25-fold, at least about 50-fold, at least about 100-fold, or more, longer than the residence time of a wild-type AAV.

[00123] Whether a given rAAV of the present disclosure exhibits reduced binding to a neutralizing antibody can be determined using any of a variety of standard binding assays used to determine affinity.

<u>Selective infectivity</u>

[00124] In some cases, an rAAV virion of the present disclosure selectively infects a neuronal cell, e.g., an rAAV virion of the present disclosure infects a neural cell with 10-fold, 15-fold, 20-fold, 25-fold, 50-fold, or more than 50-fold, specificity than a non-neuronal cell.

[00125] In some cases, an rAAV virion of the present disclosure selectively infects a neural stem cell, e.g., a subject rAAV virion infects a neural stem cell with 10-fold, 15-fold, 20-fold, 25-fold, 50-fold, or more than 50-fold, specificity than a non-neural stem cell, e.g., a mesenchymal stem cell, a hematopoietic stem cell, etc.

<u>Variant capsid polypeptides</u>

[00126] As noted above, an rAAV virion of the present disclosure comprises a variant AAV capsid protein. In some cases, a variant AAV capsid protein present in an rAAV virion of the present disclosure comprises at least 5 segments from at least 3 different AAV serotypes, and each segment has a length of from about 50 amino acids to about 160 amino acids.

[00127] A variant AAV capsid protein of the present disclosure comprises segments from at least 3 different AAV serotypes. For example, in some cases, a variant AAV capsid protein variant AAV capsid protein of the present disclosure comprises a first segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 1-160 of a first AAV serotype; a second segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 51-320 of a second AAV serotype; a third segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 101-480 of a third AAV serotype; a fourth segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 151-640 of the second AAV serotype; and a fifth segment having a length of from about 50 amino acids to about 160 amino acids from amino acid 201 to the C-terminus of the second AAV serotype. In some cases, the first AAV serotype is AAV6, the second AAV serotype is AAV9, and the third AAV serotype is AAV8.

[00128] In some cases, a variant AAV capsid protein of the present disclosure comprises a first segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 1-160 of a first AAV serotype; a second segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 51-320 of a second AAV serotype; a third segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 101-480 of a third AAV serotype; a fourth segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 151-640 of the second AAV serotype; and a fifth segment having a length of from about 50 amino acids to about 160 amino acids from amino acid 201 to the C-terminus of a fourth AAV serotype. In some cases the first AAV serotype is AAV6, the second AAV serotype is AAV9, the third AAV serotype is AAV8, and the fourth AAV serotype is AAV2.

[00129] In some cases, a variant AAV capsid protein of the present disclosure comprises: i) a first segment having a length of from about 50 amino acids to about 160 amino acids and comprising an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, to a stretch of contiguous amino acids of amino acids 1-160 of the AAV6 capsid amino acid sequence depicted in FIG. 10; ii) a second segment having a length of from about 50 amino acids to about 160 amino acids and comprising an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, to a stretch of contiguous amino acids of amino acids 1-160 of the AAV9 capsid amino acid sequence depicted in FIG. 10; iii) a third segment having a length of from about 50 amino acids to about 160 amino acids and comprising an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, to a stretch of contiguous amino acids of amino acids 101-480 of the AAV8 capsid amino acid sequence depicted in FIG. 10; iv) a fourth segment having a

length of from about 50 amino acids to about 160 amino acids and comprising an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, to a stretch of contiguous amino acids of amino acids 151-640 of the AAV9 capsid amino acid sequence depicted in FIG. 10; v) a fifth segment having a length of from about 50 amino acids to about 160 amino acids and comprising an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, to a stretch of contiguous amino acids of amino acids 201 to the C-terminus of the AAV9 capsid amino acid sequence depicted in FIG. 10.

[00130] In some cases, a variant AAV capsid protein of the present disclosure comprises: i) a first segment having a length of from about 50 amino acids to about 160 amino acids and comprising an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, to a stretch of contiguous amino acids of amino acids 1-160 of the AAV6 capsid amino acid sequence depicted in FIG. 10; ii) a second segment having a length of from about 50 amino acids to about 160 amino acids and comprising an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, to a stretch of contiguous amino acids of amino acids 1-160 of the AAV9 capsid amino acid sequence depicted in FIG. 10; iii) a third segment having a length of from about 50 amino acids to about 160 amino acids and comprising an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, to a stretch of contiguous amino acids of amino acids 101-480 of the AAV8 capsid amino acid sequence depicted in FIG. 10; iv) a fourth segment having a length of from about 50 amino acids to about 160 amino acids and comprising an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, to a stretch of contiguous amino acids of amino acids 151-640 of the AAV9 capsid amino acid sequence depicted in FIG. 10; v) a fifth segment having a length of from about 50 amino acids to about 160 amino acids and comprising an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, to a stretch of contiguous amino acids of amino acids 201 to the C-terminus of the AAV2 capsid amino acid sequence depicted in FIG. 10.

[00131] In some cases, a variant AAV capsid protein of the present disclosure comprises a first segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 1-160 of a first AAV serotype, a second segment having a length of from about 50 amino acids to about 160 amino acids of a second AAV serotype, a third segment having a length of from about 50 amino acids to about 160 amino acids of a third AAV serotype, a fourth segment having a length of from about 50 amino acids to about 160 amino acids from the second AAV serotype, a fifth segment having a length of from about 50 amino acids to about 160 amino acids from the second AAV serotype, a sixth segment having a length of from about 50 amino acids to about 160 amino acids from a fourth AAV serotype, a seventh segment having a length of from

about 50 amino acids to about 160 amino acids from the second AAV serotype, and an eighth segment having a length of from about 50 amino acids to about 160 amino acids from the second AAV serotype. In some cases, the first AAV serotype is AAV6, the second AAV serotype is AAV9, the third AAV serotype is AAV8, and the fourth AAV serotype is AAV2.

[00132] In some cases, a variant AAV capsid protein of the present disclosure comprises an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, to the SCH9 amino acid sequence depicted in FIG. 10. In some cases, the variant capsid protein comprises the amino acid sequence of the SCH9 amino acid sequence depicted in FIG. 10.

[00133] In some cases, a variant AAV capsid protein of the present disclosure comprises a first segment having a length of from about 50 amino acids to about 160 amino acids from a first AAV serotype. a second segment having a length of from about 50 amino acids to about 160 amino acids from a second AAV serotype, a third segment having a length of from about 50 amino acids to about 160 amino acids from a third AAV serotype, a fourth segment having a length of from about 50 amino acids to about 160 amino acids from the second AAV serotype, a fifth segment having a length of from about 50 amino acids to about 160 amino acids from a fourth AAV serotype, a sixth segment having a length of from about 50 amino acids to about 160 amino acids from the fourth AAV serotype, a seventh segment having a length of from about 50 amino acids to about 160 amino acids from the second AAV serotype, and an eighth segment having a length of from about 50 amino acids to about 160 amino acids from the second AAV serotype. In some cases, the first serotype is AAV6, the second AAV serotype is AAV9, the third AAV serotype is AAV8, and the fourth AAV serotype is AAV2.

[00134] In some cases, a variant AAV capsid protein of the present disclosure comprises an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, to the SCH2 amino acid sequence depicted in FIG. 10. In some cases, the variant capsid protein comprises the amino acid sequence of the SCH2 amino acid sequence depicted in FIG. 10.

[00135] In some cases, a variant AAV capsid protein of the present disclosure comprises an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, to the SCH9 amino acid sequence depicted in FIG. 8. In some cases, a variant AAV capsid protein of the present disclosure comprises an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, to the SCH2 amino acid sequence depicted in FIG. 9.

Additional variations

[00136] In some cases, a variant capsid polypeptide of the present disclosure comprises one or more additional mutations (e.g., amino acid substitution; insertion of one or more amino acids; deletion of one or more amino acids).

[00137] For example, in some cases, a variant capsid polypeptide of the present disclosure comprises an insertion of from about 5 amino acids to about 20 amino acids (e.g., from 5 amino acids to 7 amino acids, from 7 amino acids to 10 amino acids, from 10 amino acids to 15 amino acids, or from 15 amino acids to 20 amino acids) in the capsid protein GH loop relative to a corresponding parental AAV capsid protein. The insertion site can be in the GH loop, or loop IV, of the AAV capsid protein, e.g., in a solvent-accessible portion of the GH loop, or loop IV, of the AAV capsid protein. For the GH loop/loop IV of AAV capsid, see, e.g., van Vliet et al. (2006) *Mol. Ther.* 14:809; Padron et al. (2005) *J. Virol.* 79:5047; and Shen et al. (2007) *Mol. Ther.* 15:1955.

[00138] In some cases, a heterologous peptide of from about 5 amino acids to about 20 amino acids (e.g., from 5 to 7, from 7 to 10, from 10 to 12, from 12 to 15, or from 15 to 20 amino acids) in length is inserted in an insertion site in the GH loop or loop IV of the capsid protein relative to a corresponding parental AAV capsid protein. For example, the insertion site can be within amino acids 411-650 of an AAV capsid protein, as depicted in FIG. 21A-21C, or a corresponding region of a variant AAV capsid protein of the present disclosure. Those skilled in the art, given the amino acid sequences depicted in FIG. 21A-21C, can readily determine a suitable insertion site in variant capsid of the present disclosure. For example, the insertion site can be between amino acids 587 and 588 of AAV2, or between amino acids 588 and 589 of AAV2, or the corresponding positions of the capsid subunit of another AAV serotype, or the corresponding positions of the capsid subunit of a variant AAV capsid of the present disclosure. It should be noted that the insertion site 587/588 is based on an AAV2 capsid protein. A heterologous peptide of 5 amino acids to about 20 amino acids (e.g., from 5 to 7, from 7 to 10, from 10 to 12, from 12 to 15, or from 15 to 20 amino acids) in length can be inserted in a corresponding site in an AAV serotype other than AAV2 (e.g., AAV8, AAV9, etc.), or the corresponding positions of the capsid subunit of a variant AAV capsid of the present disclosure. Those skilled in the art would know, based on a comparison of the amino acid sequences of capsid proteins of various AAV serotypes, where an insertion site “corresponding to amino acids 587-588 of AAV2” would be in a capsid protein of any given AAV serotype, or the corresponding positions of the capsid subunit of a variant AAV capsid of the present disclosure. See, e.g., GenBank Accession No. NP_049542 for AAV1; GenBank Accession No. NP_044927 for AAV4; GenBank Accession No. AAD13756 for AAV5; GenBank Accession No.

AAB95459 for AAV6; GenBank Accession No. YP_077178 for AAV7; GenBank Accession No. YP_077180 for AAV8; GenBank Accession No. AAS99264 for AAV9; GenBank Accession No. AAT46337 for AAV10; and GenBank Accession No. AAO88208 for AAVrh10. See, e.g., Santiago-Ortiz et al. (2015) *Gene Ther.* 22:934 for ancestral AAV capsid.

[00139] In some cases, a variant capsid polypeptide of the present disclosure comprises an insertion comprising an amino acid sequence selected from LGETTRP (SEQ ID NO:3), NETITRP (SEQ ID NO:4), KAGQANN (SEQ ID NO:5), KDPKTTN (SEQ ID NO:6), KDTDTTR (SEQ ID NO:7), RAGGSVG (SEQ ID NO:8), AVDTTKF (SEQ ID NO:9), and STGKVPN (SEQ ID NO:10).

[00140] In some cases, a variant capsid polypeptide of the present disclosure comprises an insertion comprising an amino acid sequence selected from LALIQDSMRA (SEQ ID NO:151); LANQEHVKNA (SEQ ID NO:152); TGVMRSTNSGLN (SEQ ID NO:153); TGEVDLAGGGLS (SEQ ID NO:154); TSPYSGSSDGLS (SEQ ID NO:155); TGGHDSSLDGLS (SEQ ID NO:156); TGDGGTTMNGLS (SEQ ID NO:157); TGGHGSAPDGLS (SEQ ID NO:158); TGMHVTMMAGLN (SEQ ID NO:159); TGASYLDNSGLS (SEQ ID NO:160); TVVSTQAGIGLS (SEQ ID NO:161); TGVMHSQASGLS (SEQ ID NO:162); TGDGSPAAPGLS (SEQ ID NO:163); TGSDMAHGTGLS (SEQ ID NO:164); TGLDATRDHGLSPVTGT (SEQ ID NO:165); TGSDGTRDHGLSPVTWT (SEQ ID NO:166); NGAVADYTRGLSPATGT (SEQ ID NO:167); TGGDPTRGTGLSPVTGA (SEQ ID NO:168); LQKNARPASTESVNFQ (SEQ ID NO:169); LQRGVRIPSVLEVNGQ (SEQ ID NO:170); LQRGNRPVTTADVNTQ (SEQ ID NO:171); and LQKADRQPGVVVVNCQ (SEQ ID NO:172). In some cases, the peptide insert is TGVMRSTNSGLN (SEQ ID NO:153). In some cases, the peptide insert is TGEVDLAGGGLS (SEQ ID NO:154). In some cases, the peptide insert is TSPYSGSSDGLS (SEQ ID NO:155). In some cases, the peptide insert is TGGHDSSLDGLS (SEQ ID NO:156). In some cases, the peptide insert is TGDGGTTMNGLS (SEQ ID NO:157). In some cases, the peptide insert is TGGHGSAPDGLS (SEQ ID NO:158). In some cases, the peptide insert is TGMHVTMMAGLN (SEQ ID NO:159). In some cases, the peptide insert is TGASYLDNSGLS (SEQ ID NO:160). In some cases, the peptide insert is TVVSTQAGIGLS (SEQ ID NO:161). In some cases, the peptide insert is TGVMHSQASGLS (SEQ ID NO:162). In some cases, the peptide insert is TGDGSPAAPGLS (SEQ ID NO:163). In some cases, the peptide insert is TGSDMAHGTGLS (SEQ ID NO:164). In some cases, the peptide insert is TGSDGTRDHGLSPVTWT (SEQ ID NO:166). In some cases, the peptide insert is NGAVADYTRGLSPATGT (SEQ ID NO:167). In some cases, the peptide insert is TGGDPTRGTGLSPVTGA (SEQ ID NO:168). In some cases, the peptide insert is

LQKNARPASTESVNFQ (SEQ ID NO:169). In some cases, the peptide insert is LQRGVRIPSVLEVNGQ (SEQ ID NO:170). In some cases, the peptide insert is LQRGNRPVTTADVNTQ (SEQ ID NO:171). In some cases, the peptide insert is LQKADRQPGVVVVNCQ (SEQ ID NO:172).

[00141] In some cases, the insertion site is between amino acids 587 and 588 of AAV2, between amino acids 590 and 591 of AAV1, between amino acids 575 and 576 of AAV5, between amino acids 590 and 591 of AAV6, between amino acids 589 and 590 of AAV7, between amino acids 590 and 591 of AAV8, between amino acids 588 and 589 of AAV9, or between amino acids 588 and 589 of AAV10.

[00142] As another example, in some cases, a variant capsid polypeptide of the present disclosure comprises an amino acid substitution compared to a parental AAV capsid protein. The amino acid substitution(s) can be located in a solvent accessible site in the capsid, e.g., a solvent-accessible loop. For example, the amino acid substitution(s) can be located in a GH loop in the AAV capsid protein. In some cases, the variant capsid protein comprises an amino acid substitution at amino acid 451 and/or 532, compared to the amino acid sequence of AAV6 capsid (SEQ ID NO:11), or the corresponding amino acid in a serotype other than AAV6. In some cases, the variant capsid protein comprises an amino acid substitution at amino acid 319 and/or 451 and/or 532 and/or 642, compared to the amino acid sequence of AAV6 capsid (SEQ ID NO:11), or the corresponding amino acid in a serotype other than AAV6. In some cases, the variant capsid protein comprises one or more of the following substitutions compared to the amino acid sequence of AAV6 capsid (SEQ ID NO:11): I319V, N451D, D532N, and H642N.

Heterologous gene products

[00143] As noted above, an rAAV virion of the present disclosure comprises a heterologous nucleic acid comprising a nucleotide sequence encoding one or more gene products (one or more heterologous gene products). In some cases, the gene product is a polypeptide. In some cases, the gene product is an RNA. In some cases, an rAAV virion of the present disclosure comprises a heterologous nucleotide sequence encoding both a heterologous nucleic acid gene product and a heterologous polypeptide gene product. Where the gene product is an RNA, in some cases, the RNA gene product encodes a polypeptide. Where the gene product is an RNA, in some cases, the RNA gene product does not encode a polypeptide. In some cases, an rAAV virion of the present disclosure comprises a single heterologous nucleic acid comprising a nucleotide sequence encoding a single heterologous gene product. In some cases, an rAAV virion of the present disclosure comprises a single heterologous nucleic acid comprising a nucleotide sequence encoding two heterologous gene products. Where the single heterologous nucleic acid encodes two heterologous gene products, in some cases, nucleotide sequences encoding the two

heterologous gene products are operably linked to the same promoter. Where the single heterologous nucleic acid encodes two heterologous gene products, in some cases, nucleotide sequences encoding the two heterologous gene products are operably linked to two different promoters. In some cases, an rAAV virion of the present disclosure comprises a single heterologous nucleic acid comprising a nucleotide sequence encoding three heterologous gene products. Where the single heterologous nucleic acid encodes three heterologous gene products, in some cases, nucleotide sequences encoding the three heterologous gene products are operably linked to the same promoter. Where the single heterologous nucleic acid encodes three heterologous gene products, in some cases, nucleotide sequences encoding the three heterologous gene products are operably linked to two or three different promoters. In some cases, an rAAV virion of the present disclosure comprises two heterologous nucleic acids, each comprising a nucleotide sequence encoding a heterologous gene product.

[00144] In some cases, the gene product is a polypeptide-encoding RNA. In some cases, the gene product is an interfering RNA. In some cases, the gene product is a microRNA (miRNA). In some cases, the gene product is an aptamer. In some cases, the gene product is a polypeptide. In some cases, the gene product is a therapeutic polypeptide, e.g., a polypeptide that provides clinical benefit. In some cases, the gene product is a site-specific nuclease that provide for site-specific knock-down of gene function. In some cases, the gene product is an RNA-guided endonuclease that provides for modification of a target nucleic acid. In some cases, the gene products are: i) an RNA-guided endonuclease that provides for modification of a target nucleic acid; and ii) a guide RNA that comprises a first segment that binds to a target sequence in a target nucleic acid and a second segment that binds to the RNA-guided endonuclease. In some cases, the gene products are: i) an RNA-guided endonuclease that provides for modification of a target nucleic acid; ii) a first guide RNA that comprises a first segment that binds to a first target sequence in a target nucleic acid and a second segment that binds to the RNA-guided endonuclease; and iii) a first guide RNA that comprises a first segment that binds to a second target sequence in the target nucleic acid and a second segment that binds to the RNA-guided endonuclease.

Nucleic acid gene products

[00145] Where the gene product is an interfering RNA (RNAi), suitable RNAi include RNAi that decrease the level of an apoptotic or angiogenic factor in a cell. For example, an RNAi can be an shRNA or siRNA that reduces the level of a gene product that induces or promotes apoptosis in a cell. Genes whose gene products induce or promote apoptosis are referred to herein as “pro-apoptotic genes” and the products of those genes (mRNA; protein) are referred to as “pro-

apoptotic gene products." Pro-apoptotic gene products include, e.g., Bax, Bid, Bak, and Bad gene products. See, e.g., U.S. Patent No. 7,846,730.

[00146] As one example, in some cases, an interfering RNA specifically reduces the level of an RNA and/or a polypeptide product of a defective allele. For example, in some cases, an RNAi specifically reduces the level of an RNA encoding Huntingtin and/or specifically reduces the level of a Huntingtin polypeptide.

[00147] As another example, in some cases, an miRNA specifically reduces the level of an RNA and/or a polypeptide product of a defective allele.

[00148] As another example, in some cases, an RNAi specifically reduces the level of an RNA encoding superoxide dismutase-1 (SOD1) RNA and/or specifically reduces the level of a SOD1 polypeptide, e.g., where the SOD1 RNA and polypeptide are encoded by a defective allele.

[00149] As another example, in some cases, an RNAi specifically reduces the level of an RNA encoding survival of motor neuron-1 (SMN1) RNA and/or specifically reduces the level of a SMN1 polypeptide, e.g., where the SMN1 RNA and polypeptide are encoded by a defective allele.

[00150] Interfering RNAs could also be against an angiogenic product, for example vascular endothelial growth factor (VEGF) (e.g., Cand5; see, e.g., U.S. Patent Publication No. 2011/0143400; U.S. Patent Publication No. 2008/0188437; and Reich et al. (2003) Mol. Vis. 9:210); VEGF receptor-1 (VEGFR1) (e.g., Sirna-027; see, e.g., Kaiser et al. (2010) Am. J. Ophthalmol. 150:33; and Shen et al. (2006) Gene Ther. 13:225); or VEGF receptor-2 (VEGFR2) (Kou et al. (2005) Biochem. 44:15064). See also, U.S. Patent Nos. 6,649,596, 6,399,586, 5,661,135, 5,639,872, and 5,639,736; and U.S. Patent Nos. 7,947,659 and 7,919,473.

[00151] Where the gene product is an aptamer, exemplary aptamers of interest include an aptamer against VEGF. See, e.g., Ng et al. (2006) Nat. Rev. Drug Discovery 5:123; and Lee et al. (2005) Proc. Natl. Acad. Sci. USA 102:18902. For example, a VEGF aptamer can comprise the nucleotide sequence 5'-cgcaaucagugaaugcuuauacauccg-3' (SEQ ID NO:12). Also suitable for use is a platelet-derived growth factor (PDGF)-specific aptamer, e.g., E10030; see, e.g., Ni and Hui (2009) Ophthalmologica 223:401; and Akiyama et al. (2006) J. Cell Physiol. 207:407).

Polypeptide gene products

[00152] Where the gene product is a polypeptide, in some cases, the polypeptide is a polypeptide that enhances function of a neural stem cell, a neural progenitor cell, or a neuronal cell.

[00153] In some cases, the gene product is a polypeptide that induces differentiation of a neural stem cell, e.g., induces the neural stem cell to differentiate into a neuron, a glial cell, an astrocyte, or an oligodendrocyte. Non-limiting examples of polypeptides that induce

differentiation of a neural stem cell include achaete-scute family basic helix-loop-helix transcription factor 1 (MASH1; Deng et al. (2010) *Biochem. Biophys. Res. Commun.* 392:548), paired like homeobox 2a (PHOX2A), neurogenin 1 (NGN1), paired box 6 (PAX6), sex determining region Y-box1 (SOX1), neurogenic differentiation 1 (NeuroD1), NeuroD-related factor (NDRF), oligodendrocyte transcription factor 2 (Olig2). See, e.g., Ohtsuka et al. (1998) *Cell Tissue Res.* 293:23; and Bond et al (2015) *Cell Stem Cell* 17:385.

[00154] Exemplary polypeptides include neuroprotective polypeptides (e.g., glial cell derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), neurotrophin-4 (NT4), nerve growth factor (NGF), and neurturin (NTN)); an aromatic L-amino acid decarboxylase; a glutamic acid decarboxylase; a tripeptidyl peptidase; an aspartoacylase; anti-angiogenic polypeptides (e.g., a soluble VEGF receptor; a VEGF-binding antibody; a VEGF-binding antibody fragment (e.g., a single chain anti-VEGF antibody); endostatin; tumstatin; angiostatin; a soluble Flt polypeptide (Lai et al. (2005) Mol. Ther. 12:659); an Fc fusion protein comprising a soluble Flt polypeptide (see, e.g., Pechan et al. (2009) Gene Ther. 16:10); ciliary neurotrophic factors; pituitary adenylate cyclase-activating polypeptides; tissue inhibitor of metalloproteinases-3 (TIMP-3); a transcription factor, e.g., neurogenic differentiation 1 (Neuro D1), oligodendrocyte transcription factor 1 (Olig1), oligodendrocyte transcription factor 2 (Olig2), Achaete-Scute Family BHLH Transcription Factor 1 (ASCii), DNA-protein inhibitor ID-1 (Id1), DNA-protein inhibitor ID-2 (Id2), neurogenin, signal transducer and activator of transcription 3, NK2 Transcription Factor-Like Protein B; and the like. Suitable polypeptides include, but are not limited to, glial derived neurotrophic factor (GDNF); fibroblast growth factor; fibroblast growth factor 2; neurturin (NTN); ciliary neurotrophic factor (CNTF); nerve growth factor (NGF); neurotrophin-4 (NT4); brain derived neurotrophic factor (BDNF); epidermal growth factor; X-linked inhibitor of apoptosis; and Sonic hedgehog.

Site-specific endonucleases

[00155] In some cases, a gene product of interest is a site-specific endonuclease that provides for site-specific knock-down of gene function, e.g., where the endonuclease knocks out an allele associated with a neural disease. For example, where a dominant allele encodes a defective copy of a gene that, when wild-type, is a neural structural protein and/or provides for normal neural function, a site-specific endonuclease can be targeted to the defective allele and knock out the defective allele. In some cases, a site-specific endonuclease is an RNA-guided endonuclease.

[00156] A site-specific nuclease can also be used to stimulate homologous recombination with a donor DNA that encodes a functional copy of the protein encoded by the defective allele. Thus, e.g., a subject rAAV virion can be used to deliver a site-specific endonuclease that knocks out a defective allele, and can be used to deliver a functional copy of the defective allele, resulting in

repair of the defective allele, thereby providing for production of a functional neural protein. In some cases, a subject rAAV virion comprises a heterologous nucleic acid comprising a nucleotide sequence that encodes a site-specific endonuclease; and a heterologous nucleotide sequence that encodes a functional copy of a defective allele, where the functional copy encodes a functional neural protein.

[00157] Examples of genes that can include mutations that are associated with or give rise to neurological diseases and disorders include, but are not limited to, hypoxanthine guanine phosphoriboxyltransferase (HPRT1), neurofibromatosis type II (NF2), ATP1A3 (encoding the α3 subunit of Na^{+}/K^{+}-ATPase), DYNC1H1 (encoding the heavy chain of cytoplasmic dynein-1), HTT (encoding huntingtin), SOD1, SMN1, ATX3 (encoding spinocerebellar ataxia-3), FXN/X25 (encoding frataxin), DMPK (encoding dystrophia myotonica protein kinase), ATXN2 (encoding ataxin-2), atrophin-1, NR4A2 (encoding nuclear receptor subfamily 4, Group A, member 2 protein), PINK1 (encoding PTEN induced putative kinase 1), LRRK2 (encoding leucine-rich repeat kinase 2), MeCP2 (encoding methyl-CpG-binding protein-2), and the like.

[00158] Site-specific endonucleases that are suitable for use include, e.g., zinc finger nucleases (ZFNs); meganucleases; and transcription activator-like effector nucleases (TALENs), where such site-specific endonucleases are non-naturally occurring and are modified to target a specific gene. Such site-specific nucleases can be engineered to cut specific locations within a genome, and non-homologous end joining can then repair the break while inserting or deleting several nucleotides. Such site-specific endonucleases (also referred to as "INDELs") then throw the protein out of frame and effectively knock out the gene. See, e.g., U.S. Patent Publication No. 2011/0301073. Suitable site-specific endonucleases include engineered meganuclease re-engineered homing endonucleases. Suitable endonucleases include an I-Tevl nuclease. Suitable meganucleases include I-Sce1 (see, e.g., Bellaiche et al. (1999) Genetics 152:1037); and I-Cre1 (see, e.g., Heath et al. (1997) Nature Structural Biology 4:468).

RNA-guided endonucleases

[00159] In some cases, the gene product is an RNA-guided endonuclease. In some cases, the gene product is an RNA comprising a nucleotide sequence encoding an RNA-guided endonuclease. In some cases, the gene product is a guide RNA, e.g., a single-guide RNA. In some cases, the gene products are: 1) a guide RNA; and 2) an RNA-guided endonuclease. The guide RNA can comprise: a) a protein-binding region that binds to the RNA-guided endonuclease; and b) a region that binds to a target nucleic acid. An RNA-guided endonuclease is also referred to herein as a "genome editing nuclease."

[00160] Examples of RNA-guided endonucleases are CRISPR/Cas endonucleases (e.g., class 2 CRISPR/Cas endonucleases such as a type II, type V, or type VI CRISPR/Cas endonucleases). A

suitable genome editing nuclease is a CRISPR/Cas endonuclease (e.g., a class 2 CRISPR/Cas endonuclease such as a type II, type V, or type VI CRISPR/Cas endonuclease). In some cases, a suitable RNA-guided endonuclease is a class 2 CRISPR/Cas endonuclease. In some cases, a suitable RNA-guided endonuclease is a class 2 type II CRISPR/Cas endonuclease (e.g., a Cas9 protein). In some cases, a genome targeting composition includes a class 2 type V CRISPR/Cas endonuclease (e.g., a Cpf1 protein, a C2c1 protein, or a C2c3 protein). In some cases, a suitable RNA-guided endonuclease is a class 2 type VI CRISPR/Cas endonuclease (e.g., a C2c2 protein; also referred to as a "Cas13a" protein). Also suitable for use is a CasX protein. Also suitable for use is a CasY protein.

[00161] In some cases, the genome-editing endonuclease is a Type II CRISPR/Cas endonuclease. In some cases, the genome-editing endonuclease is a Cas9 polypeptide. The Cas9 protein is guided to a target site (e.g., stabilized at a target site) within a target nucleic acid sequence (e.g., a chromosomal sequence or an extrachromosomal sequence, e.g., an episomal sequence, a minicircle sequence, a mitochondrial sequence, a chloroplast sequence, etc.) by virtue of its association with the protein-binding segment of the Cas9 guide RNA. In some cases, a Cas9 polypeptide comprises an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or more than 99%, amino acid sequence identity to the *Streptococcus pyogenes* Cas9 depicted in FIG. 18A. In some cases, the Cas9 polypeptide used in a composition or method of the present disclosure is a *Staphylococcus aureus* Cas9 (saCas9) polypeptide. In some cases, the saCas9 polypeptide comprises an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the saCas9 amino acid sequence depicted in FIG. 19.

[00162] In some cases, a suitable Cas9 polypeptide is a high-fidelity (HF) Cas9 polypeptide. Kleinstiver et al. (2016) *Nature* 529:490. For example, amino acids N497, R661, Q695, and Q926 of the amino acid sequence depicted in FIG. 18A are substituted, e.g., with alanine. For example, an HF Cas9 polypeptide can comprise an amino acid sequence having at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence depicted in FIG. 19A, where amino acids N497, R661, Q695, and Q926 are substituted, e.g., with alanine. A suitable Cas9 polypeptide comprises the amino acid sequence set forth in any one of FIG. 18A-18F.

[00163] In some cases, a suitable Cas9 polypeptide exhibits altered PAM specificity. See, e.g., Kleinstiver et al. (2015) *Nature* 523:481.

[00164] In some cases, the genome-editing endonuclease is a type V CRISPR/Cas endonuclease. In some cases a type V CRISPR/Cas endonuclease is a Cpf1 protein. In some cases, a Cpf1 protein comprises an amino acid sequence having at least 30%, at least 35%, at least 40%, at

least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 90%, or 100%, amino acid sequence identity to the Cpf1 amino acid sequence depicted in FIG. 20.

[00165] In some cases, the genome-editing endonuclease is a CasX or a CasY polypeptide. CasX and CasY polypeptides are described in Burstein et al. (2017) *Nature* 542:237.

RNA-guided endonucleases

[00166] An RNA-guided endonuclease is also referred to herein as a “genome editing nuclease.” Examples of suitable genome editing nucleases are CRISPR/Cas endonucleases (e.g., class 2 CRISPR/Cas endonucleases such as a type II, type V, or type VI CRISPR/Cas endonucleases). A suitable genome editing nuclease is a CRISPR/Cas endonuclease (e.g., a class 2 CRISPR/Cas endonuclease such as a type II, type V, or type VI CRISPR/Cas endonuclease). In some cases, a suitable genome editing nuclease is a class 2 CRISPR/Cas endonuclease. In some cases, a suitable genome editing nuclease a class 2 type II CRISPR/Cas endonuclease (e.g., a Cas9 protein). In some cases, a suitable genome editing nuclease a class 2 type V CRISPR/Cas endonuclease (e.g., a Cpf1 protein, a C2c1 protein, or a C2c3 protein). In some cases, a suitable genome editing nuclease is a class 2 type VI CRISPR/Cas endonuclease (e.g., a C2c2 protein; also referred to as a “Cas13a” protein). Also suitable for use is a CasX protein. Also suitable for use is a CasY protein.

[00167] In some cases, a genome editing nuclease is a fusion protein that is fused to a heterologous polypeptide (also referred to as a “fusion partner”). In some cases, a genome editing nuclease is fused to an amino acid sequence (a fusion partner) that provides for subcellular localization, i.e., the fusion partner is a subcellular localization sequence (e.g., one or more nuclear localization signals (NLSs) for targeting to the nucleus, two or more NLSs, three or more NLSs, etc.).

[00168] In some cases, the genome-editing endonuclease is a Type II CRISPR/Case endonuclease. In some cases, the genome-editing endonuclease is a Cas9 polypeptide. The Cas9 protein is guided to a target site (e.g., stabilized at a target site) within a target nucleic acid sequence (e.g., a chromosomal sequence or an extrachromosomal sequence, e.g., an episomal sequence, a minicircle sequence, a mitochondrial sequence, a chloroplast sequence, etc.) by virtue of its association with the protein-binding segment of the Cas9 guide RNA. In some cases, a Cas9 polypeptide comprises an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or more than 99%, amino acid sequence identity to the *Streptococcus pyogenes* Cas9 depicted in FIG. 18A. In some cases, the Cas9 polypeptide used in a composition or method of the present disclosure is a *Staphylococcus aureus* Cas9 (saCas9) polypeptide. In some cases, the saCas9 polypeptide

comprises an amino acid sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the saCas9 amino acid sequence depicted in FIG. 19.

[00169] In some cases, a suitable Cas9 polypeptide comprises an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or more than 99%, amino acid sequence identity to the *Streptococcus pyogenes* Cas9 depicted in FIG. 18A, but with K848A, K1003A, and R1060A substitutions. Slaymaker et al. (2016) *Science* 351: 84-88. In some cases, a suitable Cas9 polypeptide comprises the amino acid sequence depicted in FIG. 18E. A suitable Cas9 polypeptide comprises an amino acid sequence depicted in any one of FIG. 18A-18F.

[00170] In some cases, a suitable Cas9 polypeptide is a high-fidelity (HF) Cas9 polypeptide. Kleinstiver et al. (2016) *Nature* 529:490. For example, amino acids N497, R661, Q695, and Q926 of the amino acid sequence depicted in FIG. 18A are substituted, e.g., with alanine. For example, an HF Cas9 polypeptide can comprise an amino acid sequence having at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence depicted in FIG. 18A, where amino acids N497, R661, Q695, and Q926 are substituted, e.g., with alanine. In some cases, a suitable Cas9 polypeptide comprises the amino acid sequence depicted in FIG. 18F.

[00171] In some cases, a suitable Cas9 polypeptide exhibits altered PAM specificity. See, e.g., Kleinstiver et al. (2015) *Nature* 523:481.

[00172] In some cases, the genome-editing endonuclease is a type V CRISPR/Cas endonuclease. In some cases a type V CRISPR/Cas endonuclease is a Cpf1 protein. In some cases, a Cpf1 protein comprises an amino acid sequence having at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 90%, or 100%, amino acid sequence identity to the Cpf1 amino acid sequence depicted in FIG. 20A. In some cases, a Cpf1 protein comprises an amino acid sequence having at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 90%, or 100%, amino acid sequence identity to the Cpf1 amino acid sequence depicted in FIG. 20B. In some cases, a Cpf1 protein comprises an amino acid sequence having at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 90%, or 100%, amino acid sequence identity to the Cpf1 amino acid sequence depicted in FIG. 20C.

Enzymatically inactive RNA-guided endonucleases

[00173] Also suitable for use is an RNA-guided endonuclease with reduced enzymatic activity. Such an RNA-guided endonuclease is referred to as a "dead" RNA-guided endonuclease; for example, a Cas9 polypeptide that comprises certain amino acid substitutions such that it exhibits substantially no endonuclease activity, but such that it still binds to a target nucleic acid when complexed with a guide RNA, is referred to as a "dead" Cas9 or "dCas9." In some cases, a "dead" Cas9 protein has a reduced ability to cleave both the complementary and the non-complementary strands of a double stranded target nucleic acid. For example, a "nuclease defective" Cas9 lacks a functioning RuvC domain (i.e., does not cleave the non-complementary strand of a double stranded target DNA) and lacks a functioning HNH domain (i.e., does not cleave the complementary strand of a double stranded target DNA). As a non-limiting example, in some cases, the nuclease defective Cas9 protein harbors mutations at amino acid positions corresponding to residues D10 and H840 (e.g., D10A and H840A) of SEQ ID NO:40 (or the corresponding residues of a homolog of Cas9) such that the polypeptide has a reduced ability to cleave (e.g., does not cleave) both the complementary and the non-complementary strands of a target nucleic acid. Such a Cas9 protein has a reduced ability to cleave a target nucleic acid (e.g., a single stranded or double stranded target nucleic acid) but retains the ability to bind a target nucleic acid. A Cas9 protein that cannot cleave target nucleic acid (e.g., due to one or more mutations, e.g., in the catalytic domains of the RuvC and HNH domains) is referred to as a "nuclease defective Cas9", "dead Cas9" or simply "dCas9." Other residues can be mutated to achieve the above effects (i.e. inactivate one or the other nuclease portions). As non-limiting examples, residues D10, G12, G17, E762, H840, N854, N863, H982, H983, A984, D986, and/or A987 of *Streptococcus pyogenes* Cas9 (or the corresponding amino acids of a Cas9 homolog) can be altered (i.e., substituted). In some cases, two or more of D10, E762, H840, N854, N863, and D986 of *Streptococcus pyogenes* Cas9 (or the corresponding amino acids of a Cas9 homolog) are substituted. In some cases, D10 and N863 of *Streptococcus pyogenes* Cas9 (or the corresponding amino acids of a Cas9 homolog) are substituted with Ala. Also, mutations other than alanine substitutions are suitable.

[00174] In some cases, the genome-editing endonuclease is an RNA-guided endonuclease (and it corresponding guide RNA) known as Cas9-synergistic activation mediator (Cas9-SAM). The RNA-guided endonuclease (e.g., Cas9) of the Cas9-SAM system is a "dead" Cas9 fused to a transcriptional activation domain (wherein suitable transcriptional activation domains include, e.g., VP64, p65, MyoD1, HSF1, RTA, and SET7/9) or a transcriptional repressor domain (where suitable transcriptional repressor domains include, e.g., a KRAB domain, a NuE domain, an NcoR domain, a SID domain, and a SID4X domain). The guide RNA of the Cas9-SAM

system comprises a loop that binds an adapter protein fused to a transcriptional activator domain (e.g., VP64, p65, MyoD1, HSF1, RTA, or SET7/9) or a transcriptional repressor domain (e.g., a KRAB domain, a NuE domain, an NcoR domain, a SID domain, or a SID4X domain). For example, in some cases, the guide RNA is a single-guide RNA comprising an MS2 RNA aptamer inserted into one or two loops of the sgRNA; the dCas9 is a fusion polypeptide comprising dCas9 fused to VP64; and the adaptor/functional protein is a fusion polypeptide comprising: i) MS2; ii) p65; and iii) HSF1. See, e.g., U.S. Patent Publication No. 2016/0355797.

[00175] Also suitable for use is a chimeric polypeptide comprising: a) a dead RNA-guided endonuclease; and b) a heterologous fusion polypeptide. Examples of suitable heterologous fusion polypeptides include a polypeptide having, e.g., methylase activity, demethylase activity, transcription activation activity, transcription repression activity, transcription release factor activity, histone modification activity, RNA cleavage activity, DNA cleavage activity, DNA integration activity, or nucleic acid binding activity.

Guide RNA

[00176] A nucleic acid that binds to a class 2 CRISPR/Cas endonuclease (e.g., a Cas9 protein; a type V or type VI CRISPR/Cas protein; a Cpf1 protein; etc.) and targets the complex to a specific location within a target nucleic acid is referred to herein as a "guide RNA" or "CRISPR/Cas guide nucleic acid" or "CRISPR/Cas guide RNA." A guide RNA provides target specificity to the complex (the RNP complex) by including a targeting segment, which includes a guide sequence (also referred to herein as a targeting sequence), which is a nucleotide sequence that is complementary to a sequence of a target nucleic acid.

[00177] In some cases, a guide RNA includes two separate nucleic acid molecules: an "activator" and a "targeter" and is referred to herein as a "dual guide RNA", a "double-molecule guide RNA", a "two-molecule guide RNA", or a "dgRNA." In some cases, the guide RNA is one molecule (e.g., for some class 2 CRISPR/Cas proteins, the corresponding guide RNA is a single molecule; and in some cases, an activator and targeter are covalently linked to one another, e.g., via intervening nucleotides), and the guide RNA is referred to as a "single guide RNA", a "single-molecule guide RNA," a "one-molecule guide RNA", or simply "sgRNA."

[00178] Where the gene product is an RNA-guided endonuclease, or is both an RNA-guided endonuclease and a guide RNA, the gene product can modify a target nucleic acid. In some cases, e.g., where a target nucleic acid comprises a deleterious mutation in a defective allele (e.g., a deleterious mutation in a neural cell target nucleic acid), the RNA-guided endonuclease/guide RNA complex, together with a donor nucleic acid comprising a nucleotide sequence that corrects the deleterious mutation (e.g., a donor nucleic acid comprising a nucleotide sequence that encodes a functional copy of the protein encoded by the defective

allele), can be used to correct the deleterious mutation, e.g., via homology-directed repair (HDR).

[00179] In some cases, the gene products are an RNA-guided endonuclease and 2 separate sgRNAs, where the 2 separate sgRNAs provide for deletion of a target nucleic acid via non-homologous end joining (NHEJ).

[00180] In some cases, the gene products are: i) an RNA-guided endonuclease; and ii) one guide RNA. In some cases, the guide RNA is a single-molecule (or “single guide”) guide RNA (an “sgRNA”). In some cases, the guide RNA is a dual-molecule (or “dual-guide”) guide RNA (“dgRNA”).

[00181] In some cases, the gene products are: i) an RNA-guided endonuclease; and ii) 2 separate sgRNAs, where the 2 separate sgRNAs provide for deletion of a target nucleic acid via non-homologous end joining (NHEJ). In some cases, the guide RNAs are sgRNAs. In some cases, the guide RNAs are dgRNAs.

[00182] In some cases, the gene products are: i) a Cpf1 polypeptide; and ii) a guide RNA precursor; in these cases, the precursor can be cleaved by the Cpf1 polypeptide to generate 2 or more guide RNAs.

[00183] The present disclosure provides a method of modifying a target nucleic acid in a neuronal cell in an individual, where the target nucleic acid comprises a deleterious mutation, the method comprising administering to the individual an rAAV virion of the present disclosure, where the rAAV virion comprises a heterologous nucleic acid comprising: i) a nucleotide sequence encoding an RNA-guided endonuclease (e.g., a Cas9 endonuclease); ii) a nucleotide sequence encoding a sgRNA that comprises a nucleotide sequence that is complementary to the target nucleic acid; and iii) a nucleotide sequence encoding a donor DNA template that comprises a nucleotide sequence that corrects the deleterious mutation. Administration of the rAAV virion results in correction of the deleterious mutation in the target nucleic acid by HDR.

[00184] The present disclosure provides a method of modifying a target nucleic acid in a neuronal cell in an individual, where the target nucleic acid comprises a deleterious mutation, the method comprising administering to the individual an rAAV virion of the present disclosure, where the rAAV virion comprises a heterologous nucleic acid comprising: i) a nucleotide sequence encoding an RNA-guided endonuclease (e.g., a Cas9 endonuclease); ii) a nucleotide sequence encoding a first sgRNA that comprises a nucleotide sequence that is complementary to a first sequence in the target nucleic acid; and iii) a nucleotide sequence encoding a second sgRNA that comprises a nucleotide sequence that is complementary to a second sequence in the

target nucleic acid. Administration of the rAAV virion results in excision of the deleterious mutation in the target nucleic acid by NHEJ.

[00185] The present disclosure provides a method of modifying a target nucleic acid in a neural stem cell in an individual, where the target nucleic acid comprises a deleterious mutation, the method comprising administering to the individual an rAAV virion of the present disclosure, where the rAAV virion comprises a heterologous nucleic acid comprising: i) a nucleotide sequence encoding an RNA-guided endonuclease (e.g., a Cas9 endonuclease); ii) a nucleotide sequence encoding a sgRNA that comprises a nucleotide sequence that is complementary to the target nucleic acid; and iii) a nucleotide sequence encoding a donor DNA template that comprises a nucleotide sequence that corrects the deleterious mutation. Administration of the rAAV virion results in correction of the deleterious mutation in the target nucleic acid by HDR.

[00186] The present disclosure provides a method of modifying a target nucleic acid in a neural stem cell in an individual, where the target nucleic acid comprises a deleterious mutation, the method comprising administering to the individual an rAAV virion of the present disclosure, where the rAAV virion comprises a heterologous nucleic acid comprising: i) a nucleotide sequence encoding an RNA-guided endonuclease (e.g., a Cas9 endonuclease); ii) a nucleotide sequence encoding a first sgRNA that comprises a nucleotide sequence that is complementary to a first sequence in the target nucleic acid; and iii) a nucleotide sequence encoding a second sgRNA that comprises a nucleotide sequence that is complementary to a second sequence in the target nucleic acid. Administration of the rAAV virion results in excision of the deleterious mutation in the target nucleic acid by NHEJ.

PHARMACEUTICAL COMPOSITIONS

[00187] The present disclosure provides a pharmaceutical composition comprising: a) a subject rAAV virion, as described above; and b) a pharmaceutically acceptable carrier, diluent, excipient, or buffer. In some cases, the pharmaceutically acceptable carrier, diluent, excipient, or buffer is suitable for use in a human.

[00188] Such excipients, carriers, diluents, and buffers include any pharmaceutical agent that can be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. A wide variety of pharmaceutically acceptable excipients are known in the art and need not be discussed in detail herein. Pharmaceutically acceptable excipients have been amply

described in a variety of publications, including, for example, A. Gennaro (2000) "Remington: The Science and Practice of Pharmacy," 20th edition, Lippincott, Williams, & Wilkins; Pharmaceutical Dosage Forms and Drug Delivery Systems (1999) H.C. Ansel et al., eds., 7th ed., Lippincott, Williams, & Wilkins; and Handbook of Pharmaceutical Excipients (2000) A.H. Kibbe et al., eds., 3rd ed. Amer. Pharmaceutical Assoc.

METHODS

Methods of delivering a gene product

[00189] The present disclosure provides a method of delivering a gene product to a neuronal cell in an individual, the method comprising administering to the individual a subject rAAV virion as described above. The gene product can be a polypeptide or an interfering RNA (e.g., an shRNA, an siRNA, and the like), an aptamer, or a site-specific endonuclease (e.g., an RNA-guided endonuclease), as described above. Delivering a gene product to a neuronal cell can provide for treatment of a neural disease.

[00190] The present disclosure provides a method modifying a target nucleic acid in a neuronal cell, the method comprising contacting the neuronal cell with: 1) an rAAV virion of the present disclosure, wherein the rAAV virion comprises a heterologous nucleic acid comprising a nucleotide sequence encoding an RNA-guided endonuclease that binds a guide RNA; and 2) the guide RNA. The present disclosure provides a method modifying a target nucleic acid in a neuronal cell, the method comprising contacting the neuronal cell with an rAAV virion of the present disclosure, wherein the rAAV virion comprises a heterologous nucleic acid comprising a nucleotide sequence encoding: i) an RNA-guided endonuclease that binds a guide RNA; and ii) the guide RNA. In some cases, the method comprises contacting the neuronal cell with a donor DNA template. In some cases, the RNA-guided endonuclease is a Cas9 polypeptide. In some cases, the guide RNA is a single-guide RNA.

[00191] The present disclosure provides a method of delivering a gene product to an NSC cell in an individual, the method comprising administering to the individual a subject rAAV virion as described above. The gene product can be a polypeptide or an interfering RNA (e.g., an shRNA, an siRNA, and the like), an aptamer, or a site-specific endonuclease (e.g., an RNA-guided endonuclease), as described above. Delivering a gene product to an NSC can provide for treatment of a neural disease.

[00192] The present disclosure provides a method modifying a target nucleic acid in an NSC, the method comprising contacting the neural stem cell with: 1) an rAAV virion of the present disclosure, wherein the rAAV virion comprises a heterologous nucleic acid comprising a nucleotide sequence encoding an RNA-guided endonuclease that binds a guide RNA; and 2) the guide RNA. The present disclosure provides a method modifying a target nucleic acid in an

NSC, the method comprising contacting the NSC with an rAAV virion of the present disclosure, wherein the rAAV virion comprises a heterologous nucleic acid comprising a nucleotide sequence encoding: i) an RNA-guided endonuclease that binds a guide RNA; and ii) the guide RNA. In some cases, the method comprises contacting the NSC with a donor DNA template. In some cases, the RNA-guided endonuclease is a Cas9 polypeptide. In some cases, the guide RNA is a single-guide RNA.

[00193] The present disclosure provides a method of treating a neural disease (e.g., a neural disease), the method comprising administering to an individual in need thereof an effective amount of a subject rAAV virion as described above. A subject rAAV virion can be administered via intracranial injection, or by any other convenient mode or route of administration. Other convenient modes or routes of administration include, e.g., intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous etc.

[00194] A "therapeutically effective amount" will fall in a relatively broad range that can be determined through experimentation and/or clinical trials. For example, for *in vivo* injection, i.e., injection directly into the brain, a therapeutically effective dose will be on the order of from about 10^6 to about 10^{15} of the rAAV virions, e.g., from about 10^8 to 10^{12} rAAV virions. For in vitro transduction, an effective amount of rAAV virions to be delivered to cells will be on the order of from about 10^8 to about 10^{13} of the rAAV virions. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves.

[00195] In some cases, more than one administration (e.g., two, three, four or more administrations) may be employed to achieve the desired level of gene expression. In some cases, the more than one administration is administered at various intervals, e.g., daily, weekly, twice monthly, monthly, every 3 months, every 6 months, yearly, etc. In some cases, multiple administrations are administered over a period of time of from 1 month to 2 months, from 2 months to 4 months, from 4 months to 8 months, from 8 months to 12 months, from 1 year to 2 years, from 2 years to 5 years, or more than 5 years.

Methods of treating a neurological disease or disorder

[00196] Neurological diseases that can be treated using a subject method include neurological diseases and disorders of the central nervous system (CNS), and neurological diseases and disorders of the peripheral nervous system (PNS).

[00197] Neurological diseases and disorders include, but are not limited to, diffuse axonal injury, perinatal hypoxic-ischemic injury, traumatic brain injury, stroke, ischemic infarction, embolism, and hypertensive hemorrhage; exposure to CNS toxins, infections of the central nervous system,

such as bacterial meningitis; metabolic diseases such as those involving hypoxic-ischemic encephalopathy, peripheral neuropathy, and glycogen storage diseases; or from chronic neural injury or neurodegenerative disease, including but not limited to multiple sclerosis, Lewy Body dementia, Alzheimer's disease, Parkinson's disease, and Huntington's disease.

[00198] Neurological diseases and disorders include, but are not limited to, Parkinson's disease, Alzheimer's disease, Huntington's disease, Amyotrophic lateral sclerosis, Friedreich's ataxia, Lewy body disease, spinal muscular atrophy, multiple system atrophy, dementia, schizophrenia, paralysis, multiple sclerosis, spinal cord injuries, brain injuries, cranial nerve disorders, peripheral sensory neuropathies, epilepsy, prion disorders, Creutzfeldt-Jakob disease, Alper's disease, cerebellar/spinocerebellar degeneration, Batten disease, corticobasal degeneration, Bell's palsy, Guillain-Barre Syndrome, Pick's disease, Rett syndrome, frontotemporal dementia, and autism.

[00199] Neurological diseases and disorders of the PNS include, e.g., diabetic neuropathy; polyneuropathies; chronic inflammatory demyelinating polyneuropathy (CIPD); and the like.

[00200] The present disclosure provides methods of treating a neural disorder. In some cases, the methods comprise administering an rAAV virion of the present disclosure, or a composition comprising an rAAV virion of the present disclosure, to the brain of an individual in need thereof.

[00201] One of ordinary skill in the art can readily determine an effective amount of an rAAV virion by testing for an effect on one or more parameters, such as a symptom associated with a neurological disease or disorder. In some cases, administering an effective amount of an rAAV virion of the present disclosure results in a decrease in the rate of loss of brain function, anatomical integrity, or brain health, e.g. a 2-fold, 3-fold, 4-fold, or 5-fold or more decrease in the rate of loss and hence progression of disease, e.g. a 10-fold decrease or more in the rate of loss and hence progression of disease. In some cases, administering an effective amount of an rAAV virion of the present disclosure results in a gain in brain function, an improvement in brain anatomy or health, and/or a stabilization in brain function, e.g. a 2-fold, 3-fold, 4-fold or 5-fold improvement or more in brain function, brain anatomy or health, e.g. a 10-fold improvement or more in brain function, brain anatomy or health, and/or stability of the brain.

NUCLEIC ACIDS AND HOST CELLS

[00202] The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence that encodes a subject variant adeno-associated virus (AAV) capsid protein as described above.

[00203] A subject recombinant AAV vector can be used to generate a subject recombinant AAV virion, as described above. Thus, the present disclosure provides a recombinant AAV vector that, when introduced into a suitable cell, can provide for production of a subject recombinant AAV virion.

[00204] The present disclosure further provides host cells, e.g., isolated (genetically modified) host cells, comprising a subject nucleic acid. A subject host cell can be an isolated cell, e.g., a cell in in vitro culture. A subject host cell is useful for producing a subject rAAV virion, as described below. Where a subject host cell is used to produce a subject rAAV virion, it is referred to as a "packaging cell." In some cases, a subject host cell is stably genetically modified with a subject nucleic acid. In other cases, a subject host cell is transiently genetically modified with a subject nucleic acid.

[00205] A subject nucleic acid is introduced stably or transiently into a host cell, using established techniques, including, but not limited to, electroporation, calcium phosphate precipitation, liposome-mediated transfection, and the like. For stable transformation, a subject nucleic acid will generally further include a selectable marker, e.g., any of several well-known selectable markers such as neomycin resistance, and the like.

[00206] A subject host cell is generated by introducing a subject nucleic acid into any of a variety of cells, e.g., mammalian cells, including, e.g., murine cells, and primate cells (e.g., human cells). Suitable mammalian cells include, but are not limited to, primary cells and cell lines, where suitable cell lines include, but are not limited to, 293 cells, COS cells, HeLa cells, Vero cells, 3T3 mouse fibroblasts, C3H10T1/2 fibroblasts, CHO cells, and the like. Non-limiting examples of suitable host cells include, e.g., HeLa cells (e.g., American Type Culture Collection (ATCC) No. CCL-2), CHO cells (e.g., ATCC Nos. CRL9618, CCL61, CRL9096), 293 cells (e.g., ATCC No. CRL-1573), Vero cells, NIH 3T3 cells (e.g., ATCC No. CRL-1658), Huh-7 cells, BHK cells (e.g., ATCC No. CCL10), PC12 cells (ATCC No. CRL1721), COS cells, COS-7 cells (ATCC No. CRL1651), RAT1 cells, mouse L cells (ATCC No. CCLI.3), human embryonic kidney (HEK) cells (ATCC No. CRL1573), HLHepG2 cells, and the like. A subject host cell can also be made using a baculovirus to infect insect cells such as Sf9 cells, which produce AAV (see, e.g., U.S. Patent No. 7,271,002; US patent application 12/297,958).

[00207] In some cases, a subject genetically modified host cell includes, in addition to a nucleic acid comprising a nucleotide sequence encoding a variant AAV capsid protein, as described above, a nucleic acid that comprises a nucleotide sequence encoding one or more AAV rep proteins. In other cases, a subject host cell further comprises an rAAV vector. An rAAV virion can be generated using a subject host cell. Methods of generating an rAAV virion are described

in, e.g., U.S. Patent Publication No. 2005/0053922 and U.S. Patent Publication No. 2009/0202490.

Examples of Non-Limiting Aspects of the Disclosure

[00208] Aspects, including embodiments, of the present subject matter described above may be beneficial alone or in combination, with one or more other aspects or embodiments. Without limiting the foregoing description, certain non-limiting aspects of the disclosure numbered 1-65 are provided below. As will be apparent to those of skill in the art upon reading this disclosure, each of the individually numbered aspects may be used or combined with any of the preceding or following individually numbered aspects. This is intended to provide support for all such combinations of aspects and is not limited to combinations of aspects explicitly provided below:

[00209] Aspect 1. A recombinant adeno-associated virus (rAAV) virion comprising: a) a variant AAV capsid protein, wherein the variant AAV capsid protein comprises at least 5 segments from at least 3 different AAV serotypes, wherein each segment has a length of from about 50 amino acids to about 160 amino acids, and wherein the variant capsid protein confers one or more of the following properties: i) increased infectivity of a neural stem cell compared to the infectivity of the neural stem cell by a control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid; ii) increased infectivity of a neuron compared to the infectivity of the neuron by a control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid; and iii) increased resistance to human AAV neutralizing antibodies compared to the resistance exhibited by the control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid; and b) a heterologous nucleic acid comprising a nucleotide sequence encoding a heterologous gene product.

[00210] Aspect 2. The rAAV virion of aspect 1, wherein the variant AAV capsid protein comprises, in order from N-terminus to C-terminus: a first segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 1-160 of a first AAV serotype; a second segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 51-320 of a second AAV serotype; a third segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 101-480 of a third AAV serotype; a fourth segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 151-640 of the second AAV serotype; and a fifth segment having a length of from about 50 amino acids to about 160 amino acids from amino acid 201 to the C-terminus of the second AAV serotype.

[00211] Aspect 3. The rAAV virion of aspect 2, wherein the first AAV serotype is AAV6.

[00212] Aspect 4. The rAAV virion of aspect 2, wherein the second AAV serotype is AAV9.

[00213] Aspect 5. The rAAV virion of aspect 2, wherein the third AAV serotype is AAV8.

[00214] Aspect 6. The rAAV virion of aspect 1, wherein the variant AAV capsid protein comprises, in order from N-terminus to C-terminus: a first segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 1-160 of a first AAV serotype; a second segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 51-320 of a second AAV serotype; a third segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 101-480 of a third AAV serotype; a fourth segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 151-640 of the second AAV serotype; and a fifth segment having a length of from about 50 amino acids to about 160 amino acids from amino acid 201 to the C-terminus of a fourth AAV serotype.

[00215] Aspect 7. The rAAV virion of aspect 6, wherein the first AAV serotype is AAV6.

[00216] Aspect 8. The rAAV virion of aspect 6, wherein the second AAV serotype is AAV9.

[00217] Aspect 9. The rAAV virion of aspect 6, wherein the third AAV serotype is AAV8.

[00218] Aspect 10. The rAAV virion of aspect 6, wherein the fourth AAV serotype is AAV2.

[00219] Aspect 11. The rAAV virion of aspect 1, wherein the variant AAV capsid protein comprises, in order from N-terminus to C-terminus: a first segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 1-160 of a first AAV serotype; a second segment having a length of from about 50 amino acids to about 160 amino acids of a second AAV serotype; a third segment having a length of from about 50 amino acids to about 160 amino acids of a third AAV serotype; a fourth segment having a length of from about 50 amino acids to about 160 amino acids from the second AAV serotype; a fifth segment having a length of from about 50 amino acids to about 160 amino acids from the second AAV serotype; a sixth segment having a length of from about 50 amino acids to about 160 amino acids from a fourth AAV serotype; a seventh segment having a length of from about 50 amino acids to about 160 amino acids from the second AAV serotype; and an eighth segment having a length of from about 50 amino acids to about 160 amino acids from the second AAV serotype.

[00220] Aspect 12. The rAAV virion of aspect 11, wherein the first AAV serotype is AAV6.

[00221] Aspect 13. The rAAV virion of aspect 11, wherein the second AAV serotype is AAV9.

[00222] Aspect 14. The rAAV virion of aspect 11, wherein the third AAV serotype is AAV8.

[00223] Aspect 15. The rAAV virion of aspect 11, wherein the fourth AAV serotype is AAV2.

[00224] Aspect 16. The rAAV virion of aspect 1, wherein the variant AAV capsid protein comprises, in order from N-terminus to C-terminus: a first segment having a length of from

about 50 amino acids to about 160 amino acids from a first AAV serotype; a second segment having a length of from about 50 amino acids to about 160 amino acids from a second AAV serotype; a third segment having a length of from about 50 amino acids to about 160 amino acids from a third AAV serotype; a fourth segment having a length of from about 50 amino acids to about 160 amino acids from the second AAV serotype; a fifth segment having a length of from about 50 amino acids to about 160 amino acids from a fourth AAV serotype; a sixth segment having a length of from about 50 amino acids to about 160 amino acids from the fourth AAV serotype; a seventh segment having a length of from about 50 amino acids to about 160 amino acids from the second AAV serotype; and an eighth segment having a length of from about 50 amino acids to about 160 amino acids from the second AAV serotype.

[00225] Aspect 17. The rAAV virion of aspect 16, wherein the first AAV serotype is AAV6.

[00226] Aspect 18. The rAAV virion of aspect 16, wherein the second AAV serotype is AAV9.

[00227] Aspect 19. The rAAV virion of aspect 16, wherein the third AAV serotype is AAV8.

[00228] Aspect 20. The rAAV virion of aspect 16, wherein the fourth AAV serotype is AAV2.

[00229] Aspect 21. The rAAV virion of aspect 1, wherein the rAAV virion exhibits at least 5-fold increased infectivity of a neural stem cell compared to the infectivity of the neural stem cell by the control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid.

[00230] Aspect 22. The rAAV virion of aspect 21, wherein the control AAV virion is AAV9.

[00231] Aspect 23. The rAAV virion of aspect 21, wherein the control AAV virion is AAV2.

[00232] Aspect 24. The rAAV virion of aspect 1, wherein the rAAV virion exhibits at least 10-fold increased infectivity of a neural stem cell compared to the infectivity of the neural stem cell by the control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid.

[00233] Aspect 25. The rAAV virion of aspect 24, wherein the control AAV virion is AAV9.

[00234] Aspect 26. The rAAV virion of aspect 24, wherein the control AAV virion is AAV2.

[00235] Aspect 27. The rAAV virion of aspect 1, wherein the variant AAV capsid protein comprises an amino acid sequence having at least about 90% amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:1 (and depicted in FIG. 8).

[00236] Aspect 28. The rAAV virion of aspect 1, wherein the variant AAV capsid protein comprises an amino acid sequence having at least about 95% amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:1 (and depicted in FIG. 8).

[00237] Aspect 29. The rAAV virion of aspect 1, wherein the variant AAV capsid protein comprises the amino acid sequence set forth in SEQ ID NO:1 (and depicted in FIG. 8).

[00238] Aspect 30. The rAAV virion of aspect 1, wherein the variant AAV capsid protein comprises an amino acid sequence having at least about 90% amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:2 (and depicted in FIG. 9).

[00239] Aspect 31. The rAAV virion of aspect 1, wherein the variant AAV capsid protein comprises an amino acid sequence having at least about 95% amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:2 (and depicted in FIG. 9).

[00240] Aspect 32. The rAAV virion of aspect 1, wherein the variant AAV capsid protein comprises the amino acid sequence set forth in SEQ ID NO:2 (and depicted in FIG. 9).

[00241] Aspect 33. The rAAV virion of aspect 1, wherein the variant AAV capsid protein exhibits increased resistance to human AAV neutralizing antibodies compared to the resistance exhibited by the control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid.

[00242] Aspect 34. The rAAV virion of aspect 34, wherein the control AAV virion is AAV9.

[00243] Aspect 35. The rAAV virion of aspect 34, wherein the control AAV virion is AAV2.

[00244] Aspect 36. The rAAV virion of aspect 1, wherein the variant AAV capsid protein exhibits at least about 1.5-fold greater resistance to human AAV neutralizing antibodies compared to the resistance exhibited by the control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid.

[00245] Aspect 37. The rAAV virion of aspect 1, wherein the variant AAV capsid protein exhibits at least about 3-fold greater resistance to human AAV neutralizing antibodies compared to the resistance exhibited by the control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid.

[00246] Aspect 38. The rAAV virion of aspect 1, wherein the variant AAV capsid protein exhibits at least about 5-fold greater resistance to human AAV neutralizing antibodies compared to the resistance exhibited by the control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid.

[00247] Aspect 39. The rAAV virion of aspect 1, wherein the variant AAV capsid protein exhibits at least about 10-fold greater resistance to human AAV neutralizing antibodies compared to the resistance exhibited by the control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid.

[00248] Aspect 40. The rAAV virion of aspect 1, wherein the neural stem cell is from the subventricular zone.

[00249] Aspect 41. The rAAV virion of aspect 1, wherein the Purkinje cell is from the cerebellum.

[00250] Aspect 42. The rAAV virion of any one of aspects 1-41, wherein the gene product is an interfering RNA or an aptamer.

[00251] Aspect 43. The rAAV virion of any one of aspects 1-41, wherein the gene product is a polypeptide.

[00252] Aspect 44. The rAAV virion of aspect 43, wherein the polypeptide is a neuroprotective polypeptide, an anti-angiogenic polypeptide, a polypeptide that induces differentiation of a neural stem cell, or a polypeptide that enhances function of a neural stem cell.

[00253] Aspect 45. The rAAV virion of aspect 43, wherein the polypeptide is cerebrolysin, laminin-IKVAV, cripto, pituitary adenylate cyclase-activating polypeptide, nerve growth factor, brain derived neurotrophic factor, glial derived neurotrophic factor, fibroblast growth factor 2, neurturin, ciliary neurotrophic factor, epidermal growth factor, X-linked inhibitor of apoptosis, or Sonic hedgehog.

[00254] Aspect 46. The rAAV virion of aspect 43, wherein the polypeptide is a genome-editing enzyme.

[00255] Aspect 47. The rAAV virion of aspect 46, wherein the genome-editing enzyme is a Cas9 polypeptide, a zinc finger nuclease, a TALEN, or an enzymatically inactive type II CRISPR/Cas polypeptide.

[00256] Aspect 48. The rAAV virion of aspect 47, wherein the polypeptide is an RNA-guided endonuclease selected from a type II CRISPR/Cas polypeptide, a type V CRISPR/Cas polypeptide, and a type VI CRISPR/Cas polypeptide.

[00257] Aspect 49. The rAAV virion of any one of aspects 1-41, wherein the gene product is an RNA-guided endonuclease and a guide RNA.

[00258] Aspect 50. A pharmaceutical composition comprising: a) a recombinant adeno-associated virus virion of any one of aspects 1-49; and b) a pharmaceutically acceptable excipient.

[00259] Aspect 51. A method of delivering a gene product to a neural stem cell in an individual, the method comprising administering to the individual a recombinant adeno-associated virus (rAAV) virion according any one of aspects 1-49 or the composition of aspect 50.

[00260] Aspect 52. The method of aspect 51, wherein said administering is by intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection.

[00261] Aspect 53. The method of aspect 51, wherein the gene product is a short interfering RNA or an aptamer.

[00262] Aspect 54. The method of aspect 51, wherein the gene product is a polypeptide.

[00263] Aspect 55. The method of aspect 43, wherein the polypeptide is a neuroprotective polypeptide, an anti-angiogenic polypeptide, or a polypeptide that enhances function of a neural stem cell.

[00264] Aspect 56. The rAAV virion of aspect 44, wherein the polypeptide is cerebrolysin, laminin-IKVAV, cripto, pituitary adenylate cyclase-activating polypeptide, nerve growth factor, brain derived neurotrophic factor, glial derived neurotrophic factor, fibroblast growth factor 2, neurturin, ciliary neurotrophic factor, epidermal growth factor, X-linked inhibitor of apoptosis, aromatic L-amino acid decarboxylase, glutamic acid decarboxylase, tripeptidyl peptidase, aspartoacyclase, or Sonic hedgehog .

[00265] Aspect 57. The method of aspect 54, wherein the polypeptide is a genome-editing enzyme.

[00266] Aspect 58. The method of aspect 57, wherein the genome-editing enzyme is a Cas9 polypeptide, a zinc finger nuclease, a TALEN, or an enzymatically inactive type II CRISPR/Cas polypeptide.

[00267] Aspect 59. The method of aspect 57, wherein the polypeptide is an RNA-guided endonuclease selected from a type II CRISPR/Cas polypeptide, a type V CRISPR/Cas polypeptide, and a type VI CRISPR/Cas polypeptide.

[00268] Aspect 60. The method of aspect 51, wherein the gene product is an RNA-guided endonuclease and a guide RNA.

[00269] Aspect 61. A method of treating a neurological disorder, the method comprising administering to an individual in need thereof an effective amount of a recombinant adeno-associated virus (rAAV) virion according to any one of aspects 1-49 or the composition of aspect 50.

[00270] Aspect 62. The method of aspect 62, wherein the neurological disorder is spinocerebellar ataxia, Huntington's disease, Parkinson's disease, Alzheimer's disease, a lysosomal storage disorder, Friedreich's ataxia, glioblastoma, Rett syndrome, frontotemporal dementia, or epilepsy.

[00271] Aspect 63. An isolated nucleic acid comprising a nucleotide sequence that encodes a variant adeno-associated virus (AAV) capsid protein, wherein the variant AAV capsid protein comprises at least 5 segments from at least 3 different AAV serotypes, wherein each segment has a length of from about 50 amino acids to about 160 amino acids, and wherein the variant capsid protein confers one or more of the following properties: i) increased infectivity of a neural stem cell compared to the infectivity of the neural stem cell by a control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid; ii) increased infectivity of a neuron compared to the infectivity of the neuron by a control AAV virion

comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid; and iii) increased resistance to human AAV neutralizing antibodies compared to the resistance exhibited by the control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid.

[00272] Aspect 64. An isolated, genetically modified host cell comprising the nucleic acid of aspect 63.

[00273] Aspect 65. A variant adeno-associated virus (AAV) capsid protein, wherein the variant AAV capsid protein comprises at least 5 segments from at least 3 different AAV serotypes, wherein each segment has a length of from about 50 amino acids to about 160 amino acids, and wherein the variant capsid protein confers one or more of the following properties: i) increased infectivity of a neural stem cell compared to the infectivity of the neural stem cell by a control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid; ii) increased infectivity of a neuron compared to the infectivity of the neuron by a control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid; and iii) increased resistance to human AAV neutralizing antibodies compared to the resistance exhibited by the control AAV virion comprising the corresponding parental AAV capsid protein, or comprising wild-type AAV capsid.

EXAMPLES

[00274] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); kb, kilobase(s); bp, base pair(s); nt, nucleotide(s); i.m., intramuscular(ly); i.p., intraperitoneal(ly); s.c., subcutaneous(ly); and the like.

Example 1: SCHEMA-guided design of a chimeric AAV library

Materials and Methods

[00275] The following materials and methods generally apply to the results presented in the Examples described herein except where noted otherwise.

SCHEMA library design

[00276] A library of chimeric AAVs was designed using the SCHEMA scoring function and the RASPP algorithm, see, e.g., Voigt et al. (2002) *Nature Struct. Mol. Biol.* 6; and Endelman et al. (2004) *PEDS* 17 – that represented multiple phylogenetic clades, see, e.g., Gao et al. (2004) *J. Virol.* 78, had diverse receptor binding properties, see, e.g., Asokan et al. (2012) *Mol. Ther.* 20, and enjoyed some success in the clinic, see, e.g., Kotterman et al. (2015) *Annu. Rev. Biomed. Eng.* 17. The amino acid sequences of AAV2, 4, 5, 6, 8, and 9 were aligned using MUSCLE, see, e.g., Edgar et al. (2004) *Nucleic Acids Res.* 32, to generate the parent sequence alignment. SCHEMA was modified to consider both intra- and inter-subunit amino acid contacts in the multimeric AAV capsid, wherein a pair of residues is contacting if they contained nonhydrogen atoms within 4.5 angstroms. The crystal structures used for AAV2 (1LP3), AAV4 (2G8G), AAV5 (3NTT), AAV6 (3OAH), AAV8 (2QA0), and AAV9 (3UX1) to calculate contacting residue positions. The final contact map contained residue pairs that were contacting in at least 50% of these six parent structures. To achieve high library diversity, a library containing six crossovers should be designed within the crystallized region of the capsid and a seventh in the uncrystallized VP1 region (amino acids 1-216) at position 128 based on a previous example of successful recombination at that location. See, e.g., Excoffon et al. (2009) *Proc. Natl. Acad. Sci. U.S.A.*106. A library containing eight capsid protein blocks from six parent serotypes yields a theoretical library diversity of over 1.6 million (6^8) chimeric variants. A chimeric capsid's SCHEMA disruption $\langle E \rangle$ was the number of contacts that contain new amino acid combinations that were not present in any of the parent sequences. A chimeric capsid's $\langle m \rangle$ was the number of mutations from the closest parent sequence.

[00277] **FIG. 1**. The RASPP algorithm was used to design libraries that balanced the average structural disruption $\langle E \rangle$ and average sequence diversity $\langle m \rangle$. The SCHEMA scoring function was additionally modified to search for crossover locations that were amenable to combinatorial golden gate assembly for library construction, which required four nucleotide stretches that were conserved across all AAV parent sequences. In order to increase the number of possible crossovers sites and thereby probe a larger sequence space *in silico*, four nucleotide stretches were included that could be silently mutated during library assembly to be identical in all parent sequences. For the library design, a minimum allowed sequence block length of 20 amino acids and maximum length of 250 amino acids were considered. The final library was chosen based on its low $\langle E \rangle$, its uniform block size, and recombination of key capsid structural features.

SCHEMA library construction

[00278] **FIG. 2**. In order to facilitate combinatorial golden gate cloning with the type IIs restriction enzyme *Bsa*I, all *Bsa*I recognition sites found in pBluescript SK (+), AAV2, 4, 5, 6, 8, and 9 were silently mutated by QuikChange site-directed mutagenesis. **FIGS. 3 & 4.** The 48 DNA sequences corresponding to each shuffled block were PCR amplified from the parent *cap* genes using PCR primers designed in j5, a DNA assembly design automation software. See, e.g., Hillson et al. (2012) *ACS Synth. Biol.* 1. **FIG. 5**. Primers were designed to incorporate silent mutations at block junctures to facilitate golden gate cloning into the pBluescript vector backbone. The golden gate reaction was transformed into electrocompetent DH10B *E. coli* to achieve a library size greater than the theoretical diversity of 6^8 clones. The library was then subcloned from pBluescript to the AAV packaging plasmid pSub2FloxCap using the restriction enzymes *Hind*III and *Not*I.

[00279] The SCHEMA library, before and after packaging, was analyzed using Illumina sequencing. A 2.5-kb fragment containing the AAV *cap* gene was cut out of the pSub2FloxCap vector using the *Hind*III and *Not*I sites and gel extracted. These gel-extracted inserts were used as inputs to the Nextera XT DNA Sample Prep Kit (Illumina). Each sample was barcoded using a different index primer. The resulting libraries were quantified using a high-sensitivity Bioanalyzer chip (Agilent), a Qubit Assay Kit (Invitrogen), and finally quantitative PCR (Kapa Biosystems). The average sequence fragment was ~1,400 bp. The two libraries were pooled in equimolar proportions and sequenced using a MiSeq, version 3, 2 × 300 run with a 5% PhiX control spike-in. Sequencing reads were mapped to all AAV parents using Bowtie2, see, e.g., Langmead et al. (2009) *Genome Biol.* 10, and the specific sequence blocks present were determined considering the read position and sequence identity to the parents.

Design of AAV constructs for Cre-dependent selections

[00280] PCR primers used for construct design and amplification of *cap* are presented in FIG. 2. pSub2RepKO and pRepHelper were used. pSub2RepKO, a *rep* knockout in the AAV packaging plasmid pSub2, see, e.g., Maheshri et al. (2006) *Nature Biotechnol.* 24, was generated by digestion with *Sgra*I and *Bam*HI, Klenow reaction, and blunt-end ligation. pRepHelper, used to supply Rep *in trans* during AAV packaging, was created by sequential digestion of pAAV2/rh10 with *Pme*I and *Bsm*I, Klenow reaction, and blunt-end ligation. To insert the lox66 site 5' of *cap*, a unique *Bgl*II site was introduced into pSub2RepKO by site-directed mutagenesis using the primers BglIIFwd and BglIIRev. Oligonucleotides Lox66Fwd and Lox66Rev were annealed and ligated into the *Bgl*II and *Hind*III sites of pSub2RepKO to form pSub2Lox66. To insert the lox71 site 3' of *cap*, unique *Xho*I and *Kpn*I sites were introduced into pSub2Lox66 by site-directed mutagenesis with the primers XhoIFwd/XhoIRev and KpnIFwd/KpnIRev respectively.

Oligonucleotides SOELox71Fwd and SOELox71Rev were assembled by splice overlap extension and amplified with Lox71Fwd and Lox71Rev. The resulting fragment and pSub2Lox66 were digested with *Xho*I and *Kpn*I and ligated to create pSub2Flox. pSub2Flox and the AAV *cap* libraries used in this selection were digested with *Hind*III and *Not*I and ligated to generate pSub2FloxCap libraries for viral packaging.

AAV vector production

[00281] HEK293T cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37 °C and 5% CO_2. AAV libraries or self-complementary recombinant AAV vectors driving expression of green fluorescent protein (GFP) or Cre recombinase under the control of a cytomegalovirus early enhancer/chicken beta actin (CAG) promoter were packaged in HEK293T cells as previously described in Koerber et al. (2008) *Mol. Ther.* 16, and Maheshri et al. (2006) *Nature Biotechnol.* 24. Briefly, AAV vectors were produced by triple transient transfection, purified by iodixanol density centrifugation, and buffer exchanged into phosphate buffered saline (PBS) by Amicon filtration. DNase-resistant viral genomic titers were measured by quantitative real time PCR using a Biorad iCycler (Bio-Rad, Hercules, CA).

In vivo selections and characterization of SCHEMA AAV variants

[00282] Seven-week-old GFAP-Cre 73.12 (Jackson Laboratory Stock 012886), C57BL/6J (Jackson Laboratory Stock 000664), or Ai9 tdTomato mice (Jackson Laboratory Stock 007909) were anesthetized with isoflurane and placed in a stereotaxic apparatus. An incision was made to expose the skull and a hole was drilled for injection. For library selections, five microliters of an equimolar mixture of AAV libraries (1 x 1010 viral genomes/μl) was stereotaxically injected into the right lateral ventricle of GFAP-Cre mice (n = 3) at the coordinates 0.05 mm posterior and 1.0 mm lateral to the bregma at a depth of 2.5 mm using a Hamilton syringe as previously described 100. Injection coordinates were selected using a mouse brain atlas (Franklin and Paxinos, 2007) and adjusted after test injections with 0.1% FastGreen dye (Sigma). Injection accuracy throughout the study was confirmed by reporter expression in the choroid plexus and surrounding the contralateral ventricle. Mice were sacrificed three weeks after injection and brain tissue was harvested. The hemisphere contralateral to the injection site was homogenized on dry ice using a mortar and pestle. Homogenized tissue was digested in Hirt lysis buffer with proteinase K (New England Biolabs) and RNase A (ThermoFisher) at 55°C for 3 hours and extrachromosomal DNA was isolated using the Hirt method as previously described in Arad et al. (1998) *BioTechniques* 24. The PCR primers Cap_ISF and Cap_R were used to amplify inverted cap, while primers Cap_NSF and Cap_R specifically amplify non-inverted cap. The

primers Internal_Cap_ISF and Internal_Cap_R may be used for nested PCR if amplification of inverted cap is challenging. After three rounds of selection, capsid sequences were determined by Sanger sequencing (UC Berkeley DNA Sequencing Facility) and dominant variants were digested with HindIII and NotI and ligated into pXX2Not for recombinant AAV packaging.

[00283] To characterize SCH9 and AAV9 *in vivo*, five microliters of self-complementary recombinant vector (1×10^{10} viral genomes/μl) expressing GFP or Cre was stereotaxically injected into the right lateral ventricle of C57BL/6 or Ai9 tdTomato mice respectively at the coordinates 0.05 mm posterior and 1.0 mm lateral to the bregma at a depth of 2.5 mm using a Hamilton syringe. Ai9 mice received injections of 50 mg/kg BrdU (Sigma-Aldrich) for three consecutive days prior to injection of single-stranded SCH9 CAG-Cre. For injections of the deep cerebellar nuclei, four microliters of recombinant AAV vector (2×10^{9} viral genomes/μl) expressing GFP was stereotaxically injected into the right hemisphere with coordinates 6.0 mm posterior and 2.0 mm lateral to the bregma at a depth of 2.2 mm from the cerebellar surface using a Hamilton syringe. Animal procedures were approved by the UC Berkeley Laboratory Animal Care and Use Committee and conducted in accordance with NIH guidelines for animal care.

Immunohistochemistry

[00284] Mice were anesthetized by intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine and were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were post-fixed overnight in 4% paraformaldehyde at 4°C, washed in PBS, and stored in 30% sucrose until they sank. Serial coronal or sagittal sections were cut at 40 μm thickness on a Series 8000 sliding microtome (Bright) and stored in cryoprotectant at – 20°C until use. Free-floating sections were washed three times in PBS, incubated with blocking solution (10% donkey serum and 1% Triton X-100 in PBS) for 2 hours at room temperature, and stained with primary antibodies in blocking solution for 72 hours at 4°C. The following primary antibodies were used in this study: mouse anti-Calbindin (1:2000; Abcam, ab82812), rabbit anti-GFP (1:1000; Life Technologies, A-11122), goat anti-GFAP (1:750; Abcam, ab53554), guinea pig anti-DCX (1:1000, EMD Millipore, AB2253), rat anti-VCAM1 (1:50; EMD Millipore, MAB2627), chicken anti-GFAP (1:750; Abcam, ab4674), rat anti-BrdU (1:750; Abcam, ab6326), and rabbit anti-tdTomato (1:750, Rockland, 600-401-379). After three washes in PBS, sections were incubated with secondary antibodies for 2 hours at room temperature and stained with DAPI (Thermo Fisher) for ten minutes. Stained sections were washed three times in PBS and mounted onto slides using VectaShield HardSet Antifade Mounting Medium (Vector Laboratories).

Imaging and analysis

[00285] Images were acquired using a Zeiss Axio Scan.Z1 or a confocal Zeiss LSM 880 NLO AxioExaminer (UC Berkeley Molecular Imaging Center). All image analyses were conducted on original images acquired with equivalent settings. Data were presented as mean ± SEM and statistical significance was established by two-tailed Student's t-test.

[00286] The SVZ is composed of multiple cell types including ependymal cells, adult NSCs (B cells), transit amplifying cells (type C cells), neuroblasts (type A cells), and mature astrocytes. See, e.g., Lim et al. (2016) *Cold Spring Harb. Perspect. Biol.* 8. To evaluate the efficiency of NSC transduction in the SVZ, the molecular markers that were selectively expressed within NSCs were first assessed. Although most markers are expressed in multiple cell types in the SVZ, reflecting the continuum of gene expression during lineage progression, vascular cell adhesion molecule 1 (VCAM1) specifically localizes to the endfeet of NSCs that contact the ventricle. See, e.g., Kokovay et al. (2012) *Cell Stem Cell* 11.

[00287] To determine transduction volume in the SVZ, the surface area of GFP expression in the SVZ was quantified from thresholded images using CellProfiler, see, e.g., Carpenter et al. (2006) *Genome Biol.* 7, in six coronal sections spanning the SVZ from the anterior horn of the lateral ventricle to the anterior commissure with three mice per group. The total surface area was multiplied by the section thickness (40 μm) and the distance between sections to obtain the transduction volume. The same thresholded images were used for quantification of integrated intensity of GFP expression using CellProfiler.

[00288] To quantify the percentage of tdTomato positive neuroblasts in the rostral migratory stream the cell segmentation capabilities of CellProfiler were applied to threshold, segment, and score doublecortin and tdTomato positive cell bodies in the rostral migratory stream. Measurements were taken from two to five sagittal tissue sections containing the rostral migratory stream in each animal, with four to five mice in each group. To evaluate transduction of adult neural stem cells, the identities of all BrdU positive cells in the subventricular zone were scored by colocalization with tdTomato and GFAP or DCX. Counts were performed on confocal images of every fifth sagittal section spanning the SVZ in five mice with four to five sections per animal.

[00289] To calculate the percentage of calbindin stained area that is tdTomato positive, a CellProfiler pipeline was employed to generate a thresholded mask of the calbindin stain. This mask was applied to the thresholded tdTomato image and the tdTomato positive area was dividing by the total calbindin area. The integrated intensity of thresholded tdTomato within the

calbindin mask was also recorded. Measurements were taken from four to seven 40 µm sagittal tissue sections spanning the cerebellum, with four to five mice in each group.

In vitro characterization of SCHEMA AAV variants

[00290] Unless otherwise noted all cell lines were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37°C and 5% CO_2. The heparin affinity of SCH9, SCH2, and wild-type AAV2 were determined as previously described in Jang et al. (2011) *Mol. Ther.* 19. A 1 ml HiTrap heparin column (GE Healthcare Sciences) was equilibrated with 150 mM NaCl and 50 mM Tris at pH 7.5. 1 x 1011 purified viral genomic particles were loaded onto the column and eluted by 50 mM stepwise increases in NaCl up to a final concentration of 950 mM, followed by a 1M NaCl wash. A fraction of each elution was used to infect HEK293T cells, and the percentage of GFP positive cells was quantified 48 hours after infection using a Guava EasyCyte 6HT flow cytometer (EMD/Millipore) (UC Berkeley Stem Cell Center, Berkeley, CA).

[00291] AAV utilization of galactose and heparan sulfate proteoglycans for cell transduction was characterized as previously described in Shen et al. (2013) *J. Biol. Chem.* 288. CHO-Lec2 cells presenting terminal galactose residues on their surface were obtained from the tissue culture facility at the University of California, Berkeley and cultured in MEM α nucleosides (Gibco) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37°C and 5% CO_2. One day after seeding, cells were incubated at 4°C for 30 minutes followed by a complete media change into MEM with or without 100 µg/mL *Erythrina cristagalli* lectin (ECL) (Vector Labs). Self-complementary rAAV CAG-GFP virions were treated with soluble heparin (500 µg/mL) in PBS or mock-treated for 1 hour and then used to infect cells at a genomic MOI of 12,000 (n = 3). After a 1 hour incubation with virus, Lec2 cells were washed three times in cold PBS to remove unbound AAV, and the percentage of GFP-expressing cells was quantified 72 hours after infection by flow cytometry.

[00292] To analyze antibody evasion properties, SCH9, AAV2, AAV6, AAV8, and AAV9 were incubated at 37°C for 1 hour with serial dilutions of heat inactivated IVIG (Gammagard) and then used to infect HEK293T cells at a genomic MOI of 8,000 (n = 3) as previously described in Santiago-Ortiz et al. (2015) *Gene Ther.* 22. The percentage of GFP-expressing cells was quantified 48 hours after infection by flow cytometry. Neutralizing antibody titers were recorded as the first IVIG concentration at which a 50% or greater reduction in GFP expression was observed.

[00293] To study dependence on AAVR, wild type HeLa or AAVRKO cells (Clone KIAA0319L) were infected at a genomic MOI of 20,000 (n = 6) with SCH9, SCH2, or AAV2

carrying self-complementary CAG-GFP. The percentage of GFP-expressing cells was quantified 72 hours after infection by flow cytometry.

Results

[00294] A chimeric AAV library was designed that recombined six natural serotypes – AAV2, 4, 5, 6, 8, and 9. **FIG. 1.** After specifying the design parameters, the RASPP method (Recombination as a Shortest Path Problem), see, e.g., Endelman et al. (2004) *PEDS* 17, was applied to rapidly identify 160 of the least disruptive library designs (sets of seven crossover positions) over a range of mutation levels. For each of these designs, the average library disruption score $\langle E \rangle$ and number of amino acid mutations introduced $\langle m \rangle$ relative to the closest parent serotype were calculated (FIG. 1A), and the crossover locations of all RASPP designs were presented in FIG. 1B. A final design with an average disruption score $\langle E \rangle$ of 59 and average number of mutations $\langle m \rangle$ of 82 per subunit in the crystallized region of the capsid (FIG. 1A-C) was chosen for several reasons. First, this design was in a cluster of RASPP libraries (FIG. 1A) that represented a relative minimum in $\langle E \rangle$ at high mutation levels. Second, the selected design shuffled key capsid structural features, which included surface exposed loops and hypervariable regions that represented the most divergent regions in the evolution of natural AAV serotypes (FIG. 1C). Recombination within these contact rich regions resulted in greater disruption, but was also more likely to generate AAV chimeras with new and interesting functions. For example, significantly lower disruption scores could be achieved by combining blocks five and six, but doing so would generate capsids with surface exposed loop regions derived from a single parent sequence. Finally, this set of crossover positions was selected since it provided a relatively even distribution of block sizes. RASPP was programmed to consider a range of permissible block sizes from 20-250 amino acids. The majority of the lowest $\langle E \rangle$ designs contained two long blocks (> 175 amino acids for blocks 3 and 4) followed by a series of short blocks (< 30 amino acids for blocks 5-7) (FIG. 1B). In contrast, the chosen set of crossover positions (FIG. 1C) offered a more even distribution of block sizes, ensuring shuffling throughout the capsid as opposed to confining crossovers within a few regions that were of limited diversity in the parent sequences.

[00295] The selected library design was assembled by combinatorial golden gate cloning, see, e.g., Engler et al. (2013) *Methods Mol. Biol.* 1073, cloned in electrocompetent *E. coli* to yield over 5 x 10^6 transformants, and packaged into AAV virions. The frequency of parent serotypes at each block position was analyzed by deep sequencing before and after viral packaging (FIG. 1F). Each parent serotype sequence was well represented and distributed at each block location prior to viral packaging, but packaging presumably imposed a significant selective pressure for

stable capsids and thereby resulted in dramatic changes in library composition. For example, the frequency of AAV4 and AAV5 decreased by an average of 348 and 372-fold respectively across the packaged library, likely due to the low average amino acid sequence identity (AAV4: 60%, AAV5: 65%) of these serotypes with the other AAV parents used for library assembly. Changes in library composition upon packaging were also reflected in the decrease in the average disruption score $\langle E \rangle$ per crystallized subunit from 59 to 4 and in the average number of mutations $\langle m \rangle$ from 82 to 28. In agreement with prior applications of SCHEMA as described in Meyer et al. (2006) *PEDS* 19, and Otey et al. (2004) *Chem. & Biol.* 11, lower $\langle E \rangle$ chimeras were thus heavily enriched in the library. There was a preference for AAV2 at blocks five and six and AAV9 at block eight. These trends could be used in the future to guide rational capsid engineering.

Example 2: A Cre-dependent selection strategy for AAV directed evolution

[00296] To specifically target NSCs, an *in vivo* Cre-dependent directed evolution and selection strategy was designed to drive positive selection of AAV variants that infected NSCs in the SVZ. A conceptually analogous but distinct Cre-dependent system was reported during the course of this study. See Deverman et al. (2016) *Nature Biotechnol.* 34.

[00297] Over 300 transgenic mice that drove Cre expression under the control of a cell-type-specific promoter were developed. See, e.g., Heffner et al. (2012) *Nature Commun.* 3. **FIG. 6**. The cell-type-specificity of Cre expression was developed to mediate selective recovery of the AAV cap gene by flanking the cap gene with a pair of loxP sites. AAV infection of a Cre-expressing cell followed by second strand AAV genome synthesis led to the inversion of the floxed cap, and PCR primers that served as a forward and reverse pair only in the inverted gene template were used to selectively recover the Cre-inverted cap genes from the brain tissue (FIGS. 6A, B). Mutant loxP sites lox66 and lox71 40 were utilized to drive the equilibrium of Cre recombination towards unidirectional inversion. The loxP sites were initially inserted in the 3' UTR of cap, where they flanked short stuffer sequences containing the target sequence for the reverse primer used for Cre-dependent recovery. Recombination occurred at low levels during bacterial plasmid propagation, even in Sure2 recombinase deficient E. coli as depicted in **FIG. 7**. To prevent this undesired recovery of inverted cap during *in vivo* selections, the loxP sites were repositioned to flank cap such that artefactual inversion during bacterial propagation of the vector plasmid library would result in an inverted cap sequence that does not encode viral proteins and thus would not subsequently package in 293 cells, a provision not included in an alternate design. See, e.g., Deverman et al. (2016) *Nature Biotechnol.* 34. Note that insertion of

loxP sites flanking the cap gene altered the reading frame of the rep gene. The translation initiation codons of rep were thus removed, the viral promoter that drove cap expression was maintained (FIG. 6A), and rep was instead supplied in trans for viral packaging by transient transfection of a separate rep-encoding helper. These modifications to the viral packaging plasmids resulted in a high AAV viral genomic yield as quantified by qPCR (FIG. 6C).

[00298] Adult NSCs in the SVZ express glial markers including glial-fibrillary acidic protein (GFAP), see, e.g., Doetsch et al. (1999) *Cell* 97, glutamate aspartate transporter (GLAST), see, e.g., Platel et al. (2009) *Glia* 57, and brain lipid-binding protein (BLBP), see, e.g., Giachino et al. (2014) *Stem Cells* 32. To select for adult NSC transduction, the GFAP-Cre 73.12 mouse line was utilized in which Cre recombinase expression was controlled by the mouse GFAP promoter. Cre expression was observed in adult GFAP-expressing neural stem cells and mature astrocytes. See, e.g,. Garcia et al. (2004) *Nature Neurosci.* 7. Although Cre was expressed in astrocytes in addition to neural stem cells, the intracerebroventricular (ICV) route of administration resulted in preferential transduction of the SVZ where the neural stem cells resided, and GFAP served as an important marker of NSC identity. See, e.g., Doetsch et al. (1999) *Cell* 97. To validate Cre-dependent recovery of *cap*, AAV libraries containing floxed *cap* genes (pSub2FloxCap) were delivered to GFAP-Cre 73.12 or C57BL/6J control mice through an intracerebroventricular injection. Inverted *cap* could only be amplified from brain tissue of mice expressing Cre, while non-inverted *cap* was present in both groups (FIG.6D). For Cre recombination to occur, the AAV genome must be in double-stranded form, as required for expression of a therapeutic transgene. It was therefore likely that the non-inverted pool of *cap* genes amplified from the GFAP-Cre 73.12 mice represented capsids that failed to infect GFAP positive cells, were defective in some aspect of the viral life cycle (e.g. capsid uncoating, endosomal escape), or did not complete second strand synthesis. The Cre-dependent selection strategy thus exclusively recovered capsid variants that complete all steps necessary for robust transgene expression in the target cell type.

Example 3: *In vivo* library selections converge on a dominant SCHEMA AAV variant

[00299] After validating Cre-dependent recovery of *cap, in vivo* selections were initiated using an equimolar mixture of six AAV libraries, each containing 10^6 to 10^7 unique variants: (i) the new SCHEMA AAV, (ii) error-prone AAV9, (iii) ancestral AAV, see, e.g., Santiago-Ortiz et al. (2015) *Gene Ther.* 22, (iv) shuffled AAV generated by DNase I digestion and reassembly of AAV1, 2, 4, 5, 6, 8, and 9, see, e.g., Koerber et al. (2008) *Mol. Ther.* 16, (v) error-prone AAV2, see, e.g., Koerber et al. (2006) *Nature Protoc.* 1, and (vi) AAV2 7mer peptide insertion at amino acid 588, see, e.g., Muller et al. (2003) *Nature Biotechnol.* 21. Libraries iii-vi have previously

yielded highly infectious clones in our directed evolution selections and provided evolutionary competition for the SCHEMA library. See, e.g., Dalkara et al. (2013) *Sci. Transl. Med.* 5; Tervo et al. (2016) *Neuron*; Steines et al. (2016) *JCI Insight* 1; Koerber et al. (2008) *Mol. Ther.* 16; and Santiago-Ortiz et al. (2015) *Gene Ther.* 22. The libraries were combined and injected via intracerebroventricular administration into the right lateral ventricle of adult GFAP-Cre mice (n = 3) to transduce NSCs throughout the entire SVZ in both hemispheres. In contrast, direct SVZ injection is more disruptive to the local tissue and could require multiple injections to cover the same tissue volume.

[00300] Three weeks after injection the contralateral brain hemisphere was harvested, genomic DNA was extracted, and Cre-recombined AAV *cap* variants were recovered from GFAP expressing cells by PCR. The contralateral hemisphere was harvested to ensure that *cap* variants were not recovered from transduction associated with the injection tract through the cortex superior to the lateral ventricle. After three rounds of *in vivo* selection, Sanger sequencing analysis of 24 clones revealed convergence on two variants originating from the SCHEMA library. SCH9 (chimera 6, 9, 8, 9, 9, 2, 9, 9; $\langle E \rangle$ 9, $\langle m \rangle$ 49) represented 54% of the clones recovered, while SCH2 (chimera 6, 9, 8, 9, 2, 2, 9, 9; $\langle E \rangle$ 4, $\langle m \rangle$ 37) represented 33%. The remaining clones were derived from the AAV2 7mer insertion (8%) and ancestral libraries (4%). SCH9 differs from the closest parent, AAV9, by 58 total mutations (92% amino acid identity). Forty-nine of these ($\langle m \rangle$) are in the crystallized region of the capsid, and 9 are in the uncrystallized region. An amino acid alignment of sequences of SCH9, SCH2, and multiple parent AAV serotypes are presented in **FIGS. 8, 9, and 10**, respectively. The two SCHEMA variants differed only at block five, resulting in a difference of 18 amino acids. **FIG. 11**. A model of the three-dimensional structure of SCH9 depicted AAV9 at loop VR-IV on the capsid surface, AAV2 at loops V-VIII, and AAV8 at the fivefold pore structure. Based on these intriguing features, and its dominance of the selected pool, the *in vivo* characterization of SCH9.

<u>Example 4</u>: SCH9 efficiently transduces adult neural stem cells in the SVZ of adult mice

[00301] To assess the transduction profile of SCH9 in the SVZ, rAAV carrying a self-complementary CAG-GFP cassette was successfully packaged (recombinant AAV packaging yields are reported in **FIG. 12**) and delivered to the right lateral ventricle of adult C57BL/6J mice. SCH9 was benchmarked against AAV9 due to its broad use in the CNS and capacity to transduce the brain parenchyma from the cerebrospinal fluid (CSF) after intrathecal injection. See, e.g., Samaranch et al. (2012) *Hum. Gene Ther.* 23; and Schuster et al. (2014) *Front.*

Neuroanat. 8. Moreover, of the natural serotypes, AAV9 is the most closely related sequence to SCH9.

[00302] **FIG. 13.** Transduction of the contralateral hemisphere was analyzed four weeks after injection, and GFP expression was primarily associated with the region surrounding the ventricle, with greatest intensity in the subventricular zone (FIG. 13A). Transduction efficiency was evaluated by both the intensity of GFP expression and the total volume of the SVZ that was positive for GFP. The integrated GFP fluorescence intensity for SCH9 was 24-fold higher, and GFP was expressed in a 12-fold greater SVZ transduction volume, compared to AAV9 (FIGS. 13B, C). As an initial characterization, GFP/GFAP/VCAM1 positive adult neural stem cells were transduced by SCH9 in the subventricular zone (FIG.13D).

[00303] Recombinant AAV genomes were maintained episomally and were progressively lost during the cell divisions characteristic of adult neurogenesis in the SVZ. Specifically, lineage progression from a neural stem cell to an olfactory bulb interneuron involved over seven cell divisions. See, e.g., Ponti et al. (2013) *Cell Cycle* 12. As a result of the accompanying AAV genome dilution, at late time points after injection the majority of cells that continue to express transgene were slowly dividing NSCs or post-mitotic cells. Moreover, prior studies using integrating retroviral vectors indicated that the time required for neuroblasts to traverse the rostral migratory stream to the olfactory bulb was nine days, and that all transit amplifying cells and neuroblasts present in the SVZ at the time of injection differentiated and/or migrated to the olfactory bulb and established dendrites by 30 days post-injection. See, e.g., Petreanu et al. (2002) *J. Neurosci.* 22; and Lois et al. (1994) *Science* 264. These results indicated that neuroblasts present in the rostral migratory stream at late time points after injection were derived from NSCs, a conclusion that was previously used to establish lentiviral or non-viral transduction of NSCs in the SVZ. See, e.g., Consiglio et al. (2004) *Proc. Natl. Acad. Sci. U.S.A.* 101; and Barnabe-Heider et al. (2008) *Nature Methods* 5. A similar lineage analysis strategy was designed to determine the number of migrating neuroblasts expressing tdTomato 30 days post-injection as an indication of NSC transduction. Recombinant SCH9 or AAV9 encoding Cre recombinase was injected into the right lateral ventricle of adult Ai9 floxed STOP tdTomato mice, see, e.g., Madisen et al. (2010) *Nature Neurosci.* 13, within which Cre activity resulted in tdTomato expression in transduced cells and their progeny. The majority (injected right hemisphere 83.2 ± 3.6%, left hemisphere 50.3 ± 4.4%) of neuroblasts were positive for tdTomato in the rostral migratory stream 30 days post-injection of SCH9 expressing Cre (FIGS 13E, G), exceeding AAV9 transduction by over 4-fold. Furthermore, large numbers of tdTomato positive neuroblasts were observed migrating radially in the olfactory bulb and adopting the morphology of granule cell neurons (FIG.13F).

[00304] To further characterize NSC transduction, the thymidine analog BrdU (5-bromo-2'-deoxyuridine) was administered to label dividing cells in the SVZ prior to injection of single-stranded SCH9 CAG-Cre. After a wash-out period of two weeks, colocalization of tdTomato expression with BrdU incorporation into GFAP$^+$ NSCs was analyzed (FIG 13H). The percentage of adult NSCs (GFAP +, BrdU +, doublecortin -), transit amplifying cells (GFAP -, BrdU +, doublecortin -), and neuroblasts (GFAP -, BrdU +, doublecortin +) expressing tdTomato in the SVZ were quantified (FIG.13I). Approximately 60% of NSCs were transduced in both hemispheres, supporting the efficacy of SCH9 for gene delivery to NSCs using both single-stranded and self-complementary formats.

Example 5: SCH9 also displays tropism for Purkinje cells in the cerebellum

[00305] Capsid mutations that enhance infection of the target cell type can simultaneously improve transduction in other regions of the brain. **FIG. 14**. Although SCH9 transduction following intracerebroventricular injection was primarily associated with the SVZ, increased reporter expression was also observed in Purkinje cells of the cerebellum, a region of the brain directly accessible to vector circulating in the cerebrospinal fluid (FIG.14A). Purkinje cells are a key target of gene therapies for neurodegenerative diseases including spinocerebellar ataxias. See, e.g., Orr et al. (2012) *J. Cell Biol.* 197. Delivery of SCH9-Cre activated tdTomato reporter expression that was 12.2-fold more intense and covered 9.3-fold greater calbindin positive area than AAV9-Cre (FIGS.14B, C) as quantified by CellProfiler.

[00306] The success of SCH9 in transducing Purkinje cells from the cerebrospinal fluid suggested its potential as a gene delivery vector for the cerebellum. Cerebellar gene therapies have employed rAAV delivery to the deep cerebellar nuclei, a major hub in cerebellar circuitry that receives inhibitory inputs from Purkinje cells. See, e.g., Keiser et al. (2015) *Brain: J. Neurol.* 138; and Dodge et al. (2008) *Mol. Ther.* 16. By harnessing this circuitry, a single injection of rAAV into the deep cerebellar nuclei could transduce Purkinje cells throughout the cerebellar cortex through retrograde transport of the vector. **FIG. 15.** Transduction patterns of SCH9 with AAV1, the most commonly used serotype for gene delivery to the cerebellum, were compared after unilateral injection into the deep cerebellar nuclei of the right hemisphere. Both vectors supported strong transduction of Purkinje cells throughout the cerebellum in the ipsilateral hemisphere, indicating that SCH9 can be transported in the retrograde direction.

<u>Example 6</u>: SCH9 can utilize both heparan sulfate proteoglycans and galactose for cell transduction

[00307] Given the promising infectious properties of SCH9, its chimeric nature was next examined to determine whether it may have conferred a selective advantage to SCH9 by modulating the receptor binding capabilities of its multiple parent serotypes. Block six of SCH9 contained the heparin binding pocket of the AAV2 capsid. See, e.g., Kern et al. (2003) *J. Virol.* 77. In addition, blocks two and five contained the galactose binding residues D271, N272, N470, and Y446 of AAV9, while block six conserved residue W503. See, e.g., Bell et al. (2012) *J. Virol.* 86. In contrast, SCH2 lacked two of the key galactose binding residues due to substitution of AAV2 for AAV9 at block five.

[00308] **FIG. 16.** Chromatography was first employed to demonstrate that the heparin affinity of both SCHEMA variants was comparable to AAV2, indicating that the chimeric sequence context outside of the heparin pocket did not significantly influence binding affinity (FIG 16A). The potential for dual utilization of heparan sulfate proteoglycans (HSPG) and galactose was next evaluated by infecting CHO-Lec2 cells that express terminal galactose residues and HSPG on the cell surface. As previously described in Shen et al. (2013) *J. Biol. Chem.* 288, addition of *Erythrina cristagalli* lectin (ECL) blocked terminal galactose, whereas virus incubation with soluble heparin competitively inhibited AAV serotypes that utilized HSPG for cell entry. As expected, the AAV2 and AAV9 control vectors utilized HSPG and galactose, respectively. Interestingly, SCH2 was solely dependent on HSPG, while SCH9 was able to use both HSPG and galactose, and actually required that both be blocked to prevent cell transduction (FIG.16B). After characterizing the different glycan binding properties of SCH2 and SCH9, both variants were examined to determine whether they retained utilization of AAVR, a newly described protein receptor that was critical for AAV infection in natural AAV serotypes. See, e.g., Pillay et al. (2016) *Nature* 530. **FIG. 17.** SCH2, SCH9, and the AAV2 control were all clearly dependent on AAVR.

[00309] Finally, since DNA shuffling had been shown to disrupt neutralizing antibody epitopes, see, e.g., Maheshri et al. (2006) *Nature Biotechnol.* 24, and Grimm et al. (2008) *J. Virol.* 82, the resistance of SCH9 to human intravenous immunoglobulin (IVIG), a polyclonal mixture of antibodies against natural AAV serotypes, was quantified. The antibody titer required to neutralize SCH9 was two to ten-fold higher as presented herein in **Table 1**, than the parent sequences from which it was derived (FIG 16C). Notably, the greatest fold improvement was relative to AAV9, the most closely related parent sequence.

Table 1: Neutralizing IVIG titers of SCH9 and the parent serotypes from which they were derived. The neutralizing titers represent the first IVIG concentration at which 50% or greater reduction in GFP expression were observed.

Variant	Neutralizing IVIG Concentration (mg/mL)	SCH9 Fold Improvement
SCH9	0.20	N/A
AAV2	0.10	2
AAV6	0.10	2
AAV8	0.10	2
AAV9	0.02	10

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[00310] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

CLAIMS

What is claimed is:

1. A recombinant adeno-associated virus (rAAV) virion comprising:

a) a variant AAV capsid protein, wherein the variant AAV capsid protein comprises at least 5 segments from at least 3 different AAV serotypes, wherein each segment has a length of from about 50 amino acids to about 160 amino acids, and wherein the variant capsid protein confers one or more of the following properties:

i) increased infectivity of a neural stem cell, compared to the infectivity of the neural stem cell by a control AAV virion comprising a wild-type AAV virion;

ii) increased infectivity of a neuronal cell, compared to the infectivity of the neuronal cell by a control AAV virion comprising a wild-type AAV virion;

iii) increased ability to cross a cellular barrier, compared to the ability of a control AAV virion comprising a wild-type AAV capsid to cross the cellular barrier;

iv) increased resistance to human AAV neutralizing antibodies, compared to the resistance exhibited by a control AAV virion comprising a wild-type AAV capsid; and

b) a heterologous nucleic acid comprising a nucleotide sequence encoding a heterologous gene product.

2. The rAAV virion of claim 1, wherein the variant AAV capsid protein comprises, in order from N-terminus to C-terminus:

a) a first segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 1-160 of a first AAV serotype;
b) a second segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 51-320 of a second AAV serotype;
c) a third segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 101-480 of a third AAV serotype;
d) a fourth segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 151-640 of the second AAV serotype; and
e) a fifth segment having a length of from about 50 amino acids to about 160 amino acids from amino acid 201 to the C-terminus of the second AAV serotype.

3. The rAAV virion of claim 2, wherein the first AAV serotype is AAV6.

4. The rAAV virion of claim 2, wherein the second AAV serotype is AAV9.

5. The rAAV virion of claim 2, wherein the third AAV serotype is AAV8.

6. The rAAV virion of claim 1, wherein the variant AAV capsid protein comprises, in order from N-terminus to C-terminus:

a) a first segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 1-160 of a first AAV serotype;
b) a second segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 51-320 of a second AAV serotype;
c) a third segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 101-480 of a third AAV serotype;
d) a fourth segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 151-640 of the second AAV serotype; and
e) a fifth segment having a length of from about 50 amino acids to about 160 amino acids from amino acid 201 to the C-terminus of a fourth AAV serotype.

7. The rAAV virion of claim 6, wherein the first AAV serotype is AAV6.

8. The rAAV virion of claim 6, wherein the second AAV serotype is AAV9.

9. The rAAV virion of claim 6, wherein the third AAV serotype is AAV8.

10. The rAAV virion of claim 6, wherein the fourth AAV serotype is AAV2.

11. The rAAV virion of claim 1, wherein the variant AAV capsid protein comprises, in order from N-terminus to C-terminus:

a) a first segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 1-160 of a first AAV serotype;
b) a second segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 51-320 of a second AAV serotype;
c) a third segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 101-480 of a third AAV serotype;
d) a fourth segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 151-640 of the second AAV serotype; and

e) a fifth segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 201-800 of a fourth AAV serotype.

f) a sixth segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 251-960 of the fourth AAV serotype;

g) a seventh segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 301-1120 of the second AAV serotype; and

h) an eighth segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 351 to the C-terminus of the second AAV serotype.

12. The rAAV virion of claim 11, wherein the first AAV serotype is AAV6.

13. The rAAV virion of claim 11, wherein the second AAV serotype is AAV9.

14. The rAAV virion of claim 11, wherein the third AAV serotype is AAV8.

15. The rAAV virion of claim 11, wherein the fourth AAV serotype is AAV2.

16. The rAAV virion of claim 1, wherein the variant AAV capsid protein comprises, in order from N-terminus to C-terminus:

a) a first segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 1-160 of a first AAV serotype;

b) a second segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 51-320 of a second AAV serotype;

c) a third segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 101-480 of a third AAV serotype;

d) a fourth segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 151-640 of the second AAV serotype; and

e) a fifth segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 201-800 of a fourth AAV serotype.

f) a sixth segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 251-960 of the fourth AAV serotype;

g) a seventh segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 301-1120 of the second AAV serotype; and

h) an eighth segment having a length of from about 50 amino acids to about 160 amino acids from amino acids 351 to the C-terminus of the second AAV serotype.

17. The rAAV virion of claim 16, wherein the first AAV serotype is AAV6.

18. The rAAV virion of claim 16, wherein the second AAV serotype is AAV9.

19. The rAAV virion of claim 16, wherein the third AAV serotype is AAV8.

20. The rAAV virion of claim 16, wherein the fourth AAV serotype is AAV2.

21. The rAAV virion of claim 1, wherein the rAAV virion exhibits at least 5-fold increased infectivity of a neural stem cell compared to the infectivity of the neural stem cell by the control AAV virion comprising the corresponding parental AAV capsid protein.

22. The rAAV virion of claim 21, wherein the control AAV virion is AAV9.

23. The rAAV virion of claim 21, wherein the control AAV virion is AAV2.

24. The rAAV virion of claim 1, wherein the rAAV virion exhibits at least 10-fold increased infectivity of a neural stem cell compared to the infectivity of the neural stem cell by the control AAV virion comprising the corresponding parental AAV capsid protein.

25. The rAAV virion of claim 24, wherein the control AAV virion is AAV9.

26. The rAAV virion of claim 24, wherein the control AAV virion is AAV2.

27. The rAAV virion of claim 1, wherein the variant AAV capsid protein comprises an amino acid sequence having at least about 90% amino acid sequence identity to the amino acid sequence depicted in FIG. 8.

28. The rAAV virion of claim 1, wherein the variant AAV capsid protein comprises an amino acid sequence having at least about 95% amino acid sequence identity to the amino acid sequence depicted in FIG. 8.

29. The rAAV virion of claim 1, wherein the variant AAV capsid protein comprises the amino acid sequence depicted in FIG. 8.

30. The rAAV virion of claim 1, wherein the variant AAV capsid protein comprises an amino acid sequence having at least about 90% amino acid sequence identity to the amino acid sequence depicted in FIG. 9.

31. The rAAV virion of claim 1, wherein the variant AAV capsid protein comprises an amino acid sequence having at least about 95% amino acid sequence identity to the amino acid sequence depicted in FIG. 9.

32. The rAAV virion of claim 1, wherein the variant AAV capsid protein comprises the amino acid sequence depicted in FIG. 9.

33. The rAAV virion of claim 1, wherein the variant AAV capsid protein exhibits increased resistance to human AAV neutralizing antibodies compared to the resistance exhibited by a control AAV virion comprising a wild-type AAV capsid protein.

34. The rAAV virion of claim 33, wherein the control AAV virion is AAV9.

35. The rAAV virion of claim 33, wherein the control AAV virion is AAV2.

36. The rAAV virion of claim 1, wherein the variant AAV capsid protein exhibits at least about 1.5-fold greater resistance to human AAV neutralizing antibodies compared to the resistance exhibited by a control AAV virion comprising wild-type AAV capsid.

37. The rAAV virion of claim 1, wherein the variant AAV capsid protein exhibits at least about 3-fold greater resistance to human AAV neutralizing antibodies compared to the resistance exhibited by a control AAV virion comprising wild-type AAV capsid.

38. The rAAV virion of claim 1, wherein the variant AAV capsid protein exhibits at least about 5-fold greater resistance to human AAV neutralizing antibodies compared to the resistance exhibited by a control AAV virion comprising wild-type AAV capsid.

39. The rAAV virion of claim 1, wherein the variant AAV capsid protein exhibits at least about 10-fold greater resistance to human AAV neutralizing antibodies compared to the resistance exhibited by a control AAV virion comprising wild-type AAV capsid.

40. The rAAV virion of claim 1, wherein the neural stem cell is from the subventricular zone.

41. The rAAV virion of claim 1, wherein the neural stem cell is from the cerebellum.

42. The rAAV virion of any one of claims 1-41, wherein the gene product is an interfering RNA or an aptamer.

43. The rAAV virion of any one of claims 1-41, wherein the gene product is a polypeptide.

44. The rAAV virion of claim 43, wherein the polypeptide is a neuroprotective polypeptide, an anti-angiogenic polypeptide, a polypeptide that induces differentiation of a neural stem cell, or a polypeptide that enhances function of a neural stem cell.

45. The rAAV virion of claim 43, wherein the polypeptide is cerebrolysin, laminin-IKVAV, cripto, pituitary adenylate cyclase-activating polypeptide, nerve growth factor, brain derived neurotrophic factor, glial derived neurotrophic factor, fibroblast growth factor 2, neurturin, ciliary neurotrophic factor, epidermal growth factor, X-linked inhibitor of apoptosis, aromatic L-amino acid decarboxylase, glutamic acid decarboxylase, tripeptidyl peptidase, aspartoacyclase, or Sonic hedgehog.

46. The rAAV virion of claim 43, wherein the polypeptide is a genome-editing enzyme.

47. The rAAV virion of claim 46, wherein the genome-editing enzyme is a Cas9 polypeptide, a zinc finger nuclease, a TALEN, or an enzymatically inactive type II CRISPR/Cas polypeptide.

48. The rAAV virion of claim 47, wherein the polypeptide is an RNA-guided endonuclease selected from a type II CRISPR/Cas polypeptide, a type V CRISPR/Cas polypeptide, and a type VI CRISPR/Cas polypeptide.

49. The rAAV virion of any one of claims 1-41, wherein the gene product is an RNA-guided endonuclease and a guide RNA.

50. A pharmaceutical composition comprising:

a) a recombinant adeno-associated virus virion of any one of claims 1-49; and

b) a pharmaceutically acceptable excipient.

51. A method of delivering a gene product to a neural stem cell in an individual, the method comprising administering to the individual a recombinant adeno-associated virus (rAAV) virion according any one of claims 1-49 or the composition of claim 50.

52. The method of claim 51, wherein said administering is by intracranial, intracerebroventicular, intrathecal, intra-cisterna magna, or intravenous injection.

53. The method of claim 51, wherein the gene product is a short interfering RNA or an aptamer.

54. The method of claim 51, wherein the gene product is a polypeptide.

55. The method of claim 54, wherein the polypeptide is a neuroprotective polypeptide, an anti-angiogenic polypeptide, a polypeptide that induces differentiation of a neural stem cell, or a polypeptide that enhances function of a neural stem cell.

56. The method of claim 54, wherein the polypeptide is cerebrolysin, laminin-IKVAV, cripto, pituitary adenylate cyclase-activating polypeptide, nerve growth factor, brain derived neurotrophic factor, glial derived neurotrophic factor, fibroblast growth factor 2, neurturin, ciliary neurotrophic factor, epidermal growth factor, X-linked inhibitor of apoptosis, or Sonic hedgehog.

57. The method of claim 54, wherein the polypeptide is a genome-editing enzyme.

58. The method of claim 57, wherein the genome-editing enzyme is a Cas9 polypeptide, a zinc finger nuclease, a TALEN, or an enzymatically inactive type II CRISPR/Cas polypeptide.

59. The method of claim 57, wherein the polypeptide is an RNA-guided endonuclease selected from a type II CRISPR/Cas polypeptide, a type V CRISPR/Cas polypeptide, and a type VI CRISPR/Cas polypeptide.

60. The method of claim 51, wherein the gene product is an RNA-guided endonuclease and a guide RNA.

61. A method of treating a neurological disorder, the method comprising administering to an individual in need thereof an effective amount of a recombinant adeno-associated virus (rAAV) virion according to any one of claims 1-49 or the composition of claim 50.

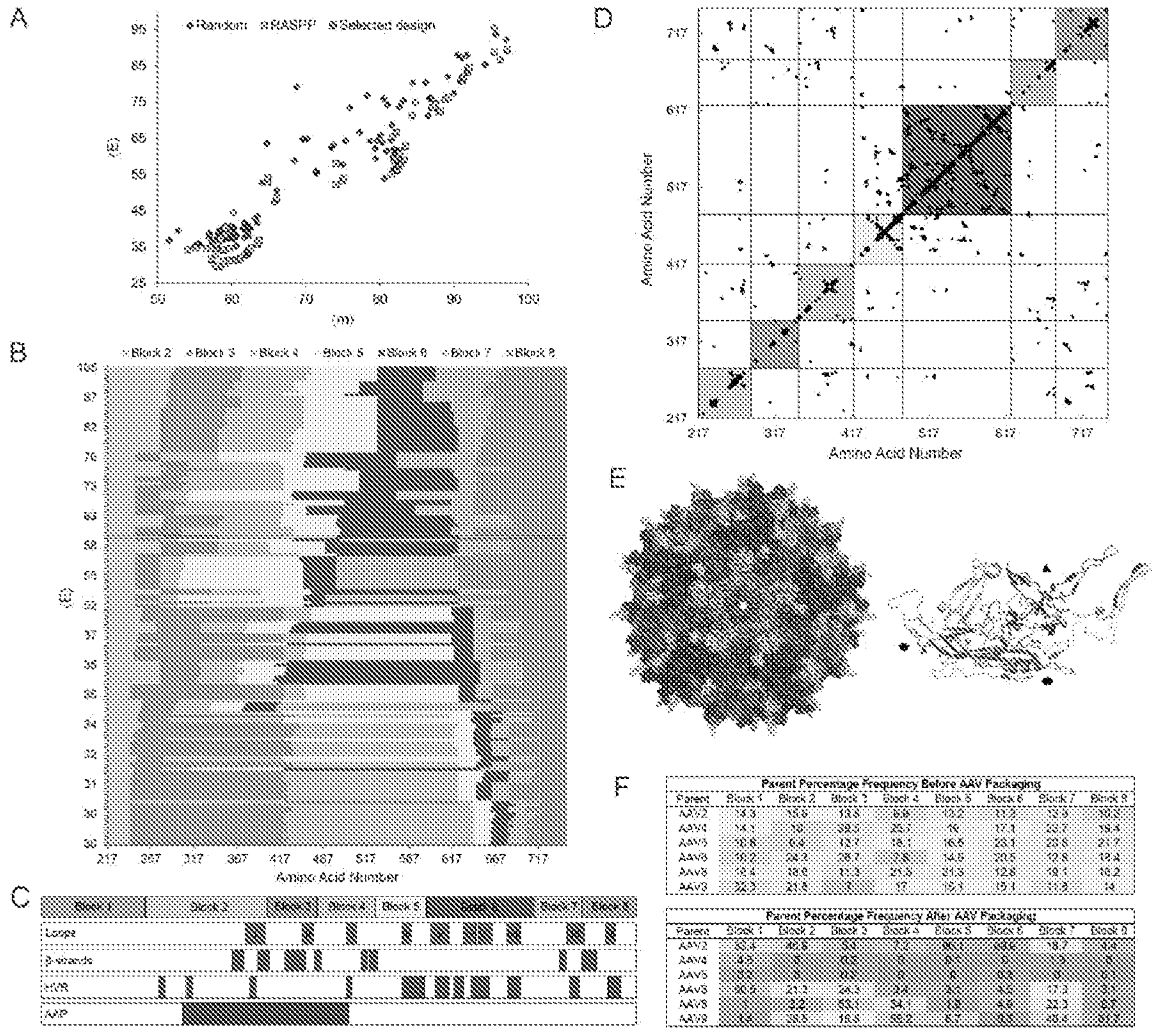
62. The method of claim 61, wherein the neurological disorder is spinocerebellar ataxia, Huntington's disease, Parkinson's disease, Alzheimer's disease, a lysosomal storage disorder, Friedreich's ataxia, glioblastoma, Rett syndrome, frontotemporal dementia, or epilepsy.

63. An isolated nucleic acid comprising a nucleotide sequence that encodes a variant adeno-associated virus (AAV) capsid protein, wherein the variant AAV capsid protein comprises at least 5 segments from at least 3 different AAV serotypes, wherein each segment has a length of from about 50 amino acids to about 160 amino acids, and wherein the variant capsid protein confers increased infectivity of a neural stem cell compared to the infectivity of the neural stem cell by a control AAV virion comprising wild-type AAV capsid.

64. An isolated, genetically modified host cell comprising the nucleic acid of claim 63.

65. A variant adeno-associated virus (AAV) capsid protein, wherein the variant AAV capsid protein comprises at least 5 segments from at least 3 different AAV serotypes, wherein each segment has a length of from about 50 amino acids to about 160 amino acids, and wherein the variant capsid protein confers increased infectivity of a neural stem cell compared to the infectivity of the neural stem cell by a control AAV virion comprising wild-type AAV capsid.

FIG. 1A-1F



Parent Percentage Frequency Before AAV Packaging								
Parent	Block 1	Block 2	Block 3	Block 4	Block 5	Block 6	Block 7	Block 8
AAV2	14.3	15.8	13.8	8.8	12.2	11.2	13.9	10.2
AAV4	14.1	16	32.5	25.7	16	17.1	23.7	18.4
AAV5	10.8	6.4	12.7	18.1	16.5	23.1	20.8	21.7
AAV6	19.2	24.2	28.7	7.8	14.5	20.5	12.8	18.4
AAV8	19.4	18.8	11.3	21.5	21.5	12.8	19.1	18.2
AAV9	22.3	21.8	[illegible]	17	19.1	15.1	11.9	14

Parent Percentage Frequency After AAV Packaging								
Parent	Block 1	Block 2	Block 3	Block 4	Block 5	Block 6	Block 7	Block 8
AAV2	32.4	40.8	[illegible]	7.2	[illegible]	[illegible]	18.7	[illegible]
AAV4	4.5	0	[illegible]	0	[illegible]	0	0	0
AAV5	[illegible]	0	[illegible]	0	0	[illegible]	0	[illegible]
AAV6	20.2	21.2	24.3	[illegible]	[illegible]	[illegible]	17.3	[illegible]
AAV8	[illegible]	2.2	53.1	34.1	[illegible]	[illegible]	22.3	[illegible]
AAV9	[illegible]	35.8	18.8	56.2	[illegible]	[illegible]	48.4	[illegible]

FIG. 2

Primer name	**Primer sequence (5'-3')**	**SEQ ID NO**
QC_pBluescript_Fwd	GCTGCAATGATACCGCGAAACCCACGCTC	13
QC_pBluescript_Rev	GAGCGTGGGTTTCGCGGTATCATTGCAGC	14
QC_AAV4_Fwd	GCTCCTGGAAAGAAGAGGCCGTTGATTGAATCCCC	15
QC_AAV4_Rev	GGGGATTCAATCAACGGCCTCTTCTTTCCAGGAGC	16
QC_AAV5_Fwd	GACCCGGAAACGGACTCGATCGAGGAG	17
QC_AAV5_Rev	CTCCTCGATCGAGTCCGTTTCCGGGTC	18
QC_AAV6_Fwd	GTTTAGCCGGGGCTCTCCAGCTGGC	19
QC_AAV6_Rev	GCCAGCTGGAGAGCCCCGGCTAAAC	20
QC_AAV8_Fwd	CTCCTGGAAAGAAGAGGCCGGTAGAGCCATCAC	21
QC_AAV8_Rev	GTGATGGCTCTACCGGCCTCTTCTTTCCAGGAG	22
BglIIFwd	CAAGCGGCCGCGTAAGCTTAGATCTCTGACGTCGATGG CTGCG	23
BglIIRev	CGCAGCCATCGACGTCAGAGATCTAAGCTTACGCGGCC GCTTG	24
Lox66Fwd	GATCTATAACTTCGTATAGCATACATTATACGAACGGTA CG	25
Lox66Rev	CGTACCGTTCGTATAATGTATGCTATACGAAGTTATTTC GA	26
XhoIFwd	CCGCTTGTTAATCAATAAACCGTTTAATTCGTTTCAGTT GACTCGAGGTCTCTGCGTATTTCTTTCT	27
XhoIRev	AGAAAGAAATACGCAGAGACCTCGAGTCAACTGAAAC GAATTAAACGGTTTATTGATTAACAAGCGG	28
KpnIFwd	CGTAGATAAGTAGCATGGCGGGTTAATCAGGTACCACA AGGAACCCCTAG	29
KpnIRev	CTAGGGGTTCCTTGTGGTACCTGATTAACCCGCCATGCT ACTTATCTACG	30
SOELox71Fwd	GTCAGCCTCGAGATAACTTCGTATAATGTATGCTATACG AACGGTACTGTGGTCGTCATTGGCAACTACACCTGTTCG	31
SOELox71Rev	CGTCACGGTACCTGTGGAATTGTGAGCGCTCACAATTCC ACAGCTAGCCTATTTACCGATACCACACGAACAGGTGT AGTTGCCAATGACG	32
Lox71Fwd	GTCAGCCTCGAGATAACTTCG	33
Lox71Rev	CGTCACGGTACCTGTGG	34
Cap_ISF	CATGGAAACTAGATAAGAAAGA	35
Cap_NSF	GGTACGAAGCTTCGATCAACTACGCAG	36
Cap_R	AGCTAGCCTATTTACCGATAC	37
Internal_Cap_ISF	AAGTTCAACTGAAACGAATTA	38
Internal_Cap_R	CACACGAACAGGTGTAGTT	39

FIG. 3

Primer name	Primer sequence (5'-3')	SEQ ID NO
DO_02_(Vector_Backbone)_forward	CACACCAGGTCTCATTGCGCGCTTGGCGTAATCATGG	50
DO_03_(Vector_Backbone)_reverse	CACACCAGGTCTCATTATAGTGAGTCGTATTACGCGC	51
DO_04_(AAV2_b1)_forward	CACACCAGGTCTCAATAAGGCGAATTGGGTACCG	52
DO_05_(AAV2_b1)_reverse	CACACCAGGTCTCAGTTCAAGAACCCTCTTTTTCGC	53
DO_06_(AAV2_b2)_forward	CACACCAGGTCTCAGAACCTCTGGGCCTGGTTGAG	54
DO_07_(AAV2_b2)_reverse	CACACCAGGTCTCAACCCCCAAGGGGTGCTGTAG	55
DO_08_(AAV2_b3)_forward	CACACCAGGTCTCAGGGTATTTTGACTTCAACAGATTCCACTGC	56
DO_09_(AAV2_b3)_reverse	CACACCAGGTCTCAAAAGACCTGAACCGTGCTGG	57
DO_10_(AAV2_b4)_forward	CACACCAGGTCTCACTTTACTGACTCGGAGTACCAGC	58
DO_11_(AAV2_b4)_reverse	CACACCAGGTCTCAGTAGCTGAAGGTAAAGTTGTTTCC	59
DO_12_(AAV2_b5)_forward	CACACCAGGTCTCACTACACTTTTGAGGACGTTCC	60
DO_13_(AAV2_b5)_reverse	CACACCAGGTCTCAAGTTCCTAGACTGGTCCCGAATG	61
DO_14_(AAV2_b6)_forward	CACACCAGGTCTCAAACTGGCTTCCTGGACCCTG	62
DO_15_(AAV2_b6)_reverse	CACACCAGGTCTCAGTCTCTGTCCTGCCAGACCATG	63
DO_16_(AAV2_b7)_forward	CACACCAGGTCTCAAGACGTGTACCTTCAGGGGC	64
DO_17_(AAV2_b7)_reverse	CACACCAGGTCTCAATGAAGGAAGCAAACTTTGCCG	65
DO_18_(AAV2_b8)_forward	CACACCAGGTCTCATCATCACACAGTACTCCACGG	66
DO_19_(AAV2_b8)_reverse	CACACCAGGTCTCAGCAAGCGCGCAATTAACCCTC	67
DO_20_(AAV4_b1)_reverse	CACACCAGGTCTCAGTTCAAGAACCCTCTTTTTGGC	68
DO_21_(AAV4_b2)_forward	CACACCAGGTCTCAGAACCTCTTGGTCTGGTTGAG	69
DO_22_(AAV4_b2)_reverse	CACACCAGGTCTCAACCCCCAGGGGGTGGAGAAT	70
DO_23_(AAV4_b3)_forward	CACACCAGGTCTCAGGGTATTTTGACTTCAACCGCTTCC	71
DO_24_(AAV4_b3)_reverse	CACACCAGGTCTCAAAAGATCTGAACCGTGCTGG	72
DO_25_(AAV4_b4)_forward	CACACCAGGTCTCACTTTGCGGACTCGTCGTACG	73
DO_26_(AAV4_b4)_reverse	CACACCAGGTCTCAGTAGGTAATTTCAAAGTTGTTGCC	74
DO_27_(AAV4_b5)_forward	CACACCAGGTCTCACTACAGTTTTGAGAAGGTGCCT	75
DO_28_(AAV4_b5)_reverse	CACACCAGGTCTCAAGTTCTTTTTAAAGTTGGAAAAGTTGGT	76
DO_29_(AAV4_b6)_forward	CACACCAGGTCTCAAACTGGCTGCCCGGGCCTTC	77
DO_30_(AAV4_b6)_reverse	CACACCAGGTCTCAGTCTCTGTTTTGCCAGACCATTC	78
DO_31_(AAV4_b7)_forward	CACACCAGGTCTCAAGACATTTACTACCAGGGTCCC	79
DO_32_(AAV4_b7)_reverse	CACACCAGGTCTCAATGAAGGAGTITACCGGAGTAGAG	80

FIG. 3

DO_33_(AAV4_b8)_forward	CACACCAGGTCTCATCATTACTCAGTACAGCACTGGC	81
DO_34_(AAV4_b8)_reverse	CACACCAGGTCTCAGCAAGCGCGCAATTAACCCTCACTAAAGG	82
DO_35_(AAV5_b1)_reverse	CACACCAGGTCTCAGTTCGAGAACCCTTTTCTTGGC	83
DO_36_(AAV5_b2)_forward	CACACCAGGTCTCAGAACCTTTTGGCCTGGTTGAA	84
DO_37_(AAV5_b2)_reverse	CACACCAGGTCTCAACCCCCAGGGGGTGCTGTAT	85
DO_38_(AAV5_b3)_forward	CACACCAGGTCTCAGGGTATTTTGACTTTAACCGCTTCC	86
DO_39_(AAV5_b3)_reverse	CACACCAGGTCTCAAAAGACTTGGACGGTGGAGG	87
DO_40_(AAV5_b4)_forward	CACACCAGGTCTCACTTTACGGACGACGACTACC	88
DO_41_(AAV5_b4)_reverse	CACACCAGGTCTCAGTAGGTAAACTCAAAGTTGTTGCC	89
DO_42_(AAV5_b5)_forward	CACACCAGGTCTCACTACAACTTTGAGGAGGTGCC	90
DO_43_(AAV5_b5)_reverse	CACACCAGGTCTCAAGTTTTTGTAGGTGTTGGCGTATCTCC	91
DO_44_(AAV5_b6)_forward	CACACCAGGTCTCAAACTGGTTCCCGGGGCCCAT	92
DO_45_(AAV5_b6)_reverse	CACACCAGGTCTCAGTCTCTCTCCATCCACACGC	93
DO_46_(AAV5_b7)_forward	CACACCAGGTCTCAAGACGTGTACCTCCAAGGACC	94
DO_47_(AAV5_b7)_reverse	CACACCAGGTCTCAATGAAGCTGCTGACGGGCAC	95
DO_48_(AAV5_b8)_forward	CACACCAGGTCTCATCATCACCCAGTACAGCACC	96
DO_49_(AAV6_b1)_reverse	CACACCAGGTCTCAGTTCGAGAACCCTCTTCTTGG	97
DO_50_(AAV6_b2)_forward	CACACCAGGTCTCAGAACCTTTTGGTCTGGTTGAGG	98
DO_51_(AAV6_b2)_reverse	CACACCAGGTCTCAACCCCCAGGGGGTGCTGTAGC	99
DO_52_(AAV6_b3)_forward	CACACCAGGTCTCAGGGTATTTTGATTTCAACAGATTCCACTGC	100
DO_53_(AAV6_b3)_reverse	CACACCAGGTCTCAAAAGACTTGAACCGTGCTGG	101
DO_54_(AAV6_b4)_forward	CACACCAGGTCTCACTTTTCGGACTCGGAGTACC	102
DO_55_(AAV6_b4)_reverse	CACACCAGGTCTCAGTAGCTGAAGGTAAAGTTATTGCC	103
DO_56_(AAV6_b5)_forward	CACACCAGGTCTCACTACACCTTCGAGGACGTGC	104
DO_57_(AAV6_b5)_reverse	CACACCAGGTCTCAAGTTTTTGGGCTGAACAGACATGC	105
DO_58_(AAV6_b6)_forward	CACACCAGGTCTCAAACTGGCTACCTGGACCCTG	106
DO_59_(AAV6_b6)_reverse	CACACCAGGTCTCAGTCTCTGTCTTGCCACACCATTC	107
DO_60_(AAV6_b7)_forward	CACACCAGGTCTCAAGACGTATACCTGCAGGGTCC	108
DO_61_(AAV6_b7)_reverse	CACACCAGGTCTCAATGAATGAAGCAAACTTTGTAGCC	109
DO_62_(AAV6_b8)_forward	CACACCAGGTCTCATCATCACCCAGTATTCCACAGG	110
DO_63_(AAV8_b1)_reverse	CACACCAGGTCTCAGTTCGAGAACCCGCTTCTTGG	111
DO_64_(AAV8_b2)_forward	CACACCAGGTCTCAGAACCTCTCGGTCTGGTTGAG	112
DO_65_(AAV8_b2)_reverse	CACACCAGGTCTCAACCCCCAGGGGGTGCTGTAG	113

FIG. 3

DO_66(AAV8_b3)_forward	CACACCAGGTCTCAGGGTATTTTGACTTTAACAGATTCCACTGC	114
DO_67_(AAV8_b3)_reverse	CACACCAGGTCTCAAAAGACCTGGATGGTGCTGG	115
DO_68_(AAV8_b4)_forward	CACACCAGGTCTCACTTTACGGACTCGGAGTACC	116
DO_69_(AAV8_b4)_reverse	CACACCAGGTCTCAGTAGGTAAACTGGAAGTTGTTGC	117
DO_70_(AAV8_b5)_reverse	CACACCAGGTCTCAAGTTCTTTGCCTGATTGGCC	118
DO_71_(AAV8_b6)_forward	CACACCAGGTCTCAAACTGGCTGCCAGGACCCTG	119
DO_72_(AAV8_b6)_reverse	CACACCAGGTCTCAGTCTCGGTTCTGCCAGACCATAC	120
DO_73_(AAV8_b7)_forward	CACACCAGGTCTCAAGACGTGTACCTGCAGGGTCC	121
DO_74_(AAV8_b7)_reverse	CACACCAGGTCTCAATGAAAGAGTTCAGCTTTGACTGG	122
DO_75_(AAV8_b8)_forward	CACACCAGGTCTCATCATCACGCAATACAGCACCG	123
DO_76_(AAV9_b1)_reverse	CACACCAGGTCTCAGTTCAAGAAGCCTCTTTTTGGC	124
DO_77_(AAV9_b2)_forward	CACACCAGGTCTCAGAACCTCTTGGTCTGGTTGAGG	125
DO_78_(AAV9_b3)_reverse	CACACCAGGTCTCAAAAGACCTGGACCGTGCTGG	126
DO_79_(AAV9_b4)_forward	CACACCAGGTCTCACTTTACGGACTCAGACTATCAGC	127
DO_80_(AAV9_b4)_reverse	CACACCAGGTCTCAGTAGCTGAACTGGAAGTTGTTACC	128
DO_81_(AAV9_b5)_forward	CACACCAGGTCTCACTACGAGTTTGAGAACGTACC	129
DO_82_(AAV9_b5)_reverse	CACACCAGGTCTCAAGTTTCTTCCCTGGACAGCC	130
DO_83_(AAV9_b6)_forward	CACACCAGGTCTCAAACTACATACCTGGACCCAGC	131
DO_84_(AAV9_b6)_reverse	CACACCAGGTCTCAGTCTCTGTCCTGCCAAACCATACC	132
DO_85_(AAV9_b7)_forward	CACACCAGGTCTCAAGACGTGTACCTGCAAGGAC	133
DO_86_(AAV9_b7)_reverse	CACACCAGGTCTCAATGAAAGAGTTCAGCTTGTCCTTG	134
DO_87_(AAV9_b8)_forward	CACACCAGGTCTCATCATCACCCAGTATTCTACTGGC	135

FIG 4

Block ID Number	Primary Template	Forward Primer	Reverse Primer	Amplicon length (bp)
0	pBluescript SK +	DO_02_(Vector_Backbone)_forward	DO_03_(Vector_Backbone)_reverse	2843
1	AAV2	DO_04_(AAV2_b1)_forward	DO_05_(AAV2_b1)_reverse	784
2	AAV2	DO_06_(AAV2_b2)_forward	DO_07_(AAV2_b2)_reverse	491
3	AAV2	DO_08_(AAV2_b3)_forward	DO_09_(AAV2_b3)_reverse	220
4	AAV2	DO_10_(AAV2_b4)_forward	DO_11_(AAV2_b4)_reverse	242
5	AAV2	DO_12_(AAV2_b5)_forward	DO_13_(AAV2_b5)_reverse	222
6	AAV2	DO_14_(AAV2_b6)_forward	DO_15_(AAV2_b6)_reverse	433
7	AAV2	DO_16_(AAV2_b7)_forward	DO_17_(AAV2_b7)_reverse	211
8	AAV2	DO_18_(AAV2_b8)_forward	DO_19_(AAV2_b8)_reverse	298
9	AAV4	DO_04_(AAV2_b1)_forward	DO_20_(AAV4_b1)_reverse	784
10	AAV4	DO_21_(AAV4_b2)_forward	DO_22_(AAV4_b2)_reverse	467
11	AAV4	DO_23_(AAV4_b3)_forward	DO_24_(AAV4_b3)_reverse	220
12	AAV4	DO_25_(AAV4_b4)_forward	DO_26_(AAV4_b4)_reverse	251
13	AAV4	DO_27_(AAV4_b5)_forward	DO_28_(AAV4_b5)_reverse	225
14	AAV4	DO_29_(AAV4_b6)_forward	DO_30_(AAV4_b6)_reverse	445
15	AAV4	DO_31_(AAV4_b7)_forward	DO_32_(AAV4_b7)_reverse	211
16	AAV4	DO_33_(AAV4_b8)_forward	DO_34_(AAV4_b8)_reverse	298
17	AAV5	DO_04_(AAV2_b1)_forward	DO_35_(AAV5_b1)_reverse	787
18	AAV5	DO_36_(AAV5_b2)_forward	DO_37_(AAV5_b2)_reverse	467
19	AAV5	DO_38_(AAV5_b3)_forward	DO_39_(AAV5_b3)_reverse	220
20	AAV5	DO_40_(AAV5_b4)_forward	DO_41_(AAV5_b4)_reverse	248
21	AAV5	DO_42_(AAV5_b5)_forward	DO_43_(AAV5_b5)_reverse	204
22	AAV5	DO_44_(AAV5_b6)_forward	DO_45_(AAV5_b6)_reverse	442
23	AAV5	DO_46_(AAV5_b7)_forward	DO_47_(AAV5_b7)_reverse	208
24	AAV5	DO_48_(AAV5_b8)_forward	DO_19_(AAV2_b8)_reverse	298
25	AAV6	DO_04_(AAV2_b1)_forward	DO_49_(AAV6_b1)_reverse	784
26	AAV6	DO_50_(AAV6_b2)_forward	DO_51_(AAV6_b2)_reverse	494
27	AAV6	DO_52_(AAV6_b3)_forward	DO_53_(AAV6_b3)_reverse	220
28	AAV6	DO_54_(AAV6_b4)_forward	DO_55_(AAV6_b4)_reverse	242
29	AAV6	DO_56_(AAV6_b5)_forward	DO_57_(AAV6_b5)_reverse	222
30	AAV6	DO_58_(AAV6_b6)_forward	DO_59_(AAV6_b6)_reverse	433
31	AAV6	DO_60_(AAV6_b7)_forward	DO_61_(AAV6_b7)_reverse	211
32	AAV6	DO_62_(AAV6_b8)_forward	DO_19_(AAV2_b8)_reverse	298
33	AAV8	DO_04_(AAV2_b1)_forward	DO_63_(AAV8_b1)_reverse	784
34	AAV8	DO_64_(AAV8_b2)_forward	DO_65_(AAV8_b2)_reverse	500
35	AAV8	DO_66_(AAV8_b3)_forward	DO_67_(AAV8_b3)_reverse	220
36	AAV8	DO_68_(AAV8_b4)_forward	DO_69_(AAV8_b4)_reverse	242
37	AAV8	DO_56_(AAV6_b5)_forward	DO_70_(AAV8_b5)_reverse	222
38	AAV8	DO_71_(AAV8_b6)_forward	DO_72_(AAV8_b6)_reverse	433
39	AAV8	DO_73_(AAV8_b7)_forward	DO_74_(AAV8_b7)_reverse	211
40	AAV8	DO_75_(AAV8_b8)_forward	DO_19_(AAV2_b8)_reverse	298
41	AAV9	DO_04_(AAV2_b1)_forward	DO_76_(AAV9_b1)_reverse	787
42	AAV9	DO_77_(AAV9_b2)_forward	DO_51_(AAV6_b2)_reverse	491
43	AAV9	DO_08_(AAV2_b3)_forward	DO_78_(AAV9_b3)_reverse	220
44	AAV9	DO_79_(AAV9_b4)_forward	DO_80_(AAV9_b4)_reverse	242
45	AAV9	DO_81_(AAV9_b5)_forward	DO_82_(AAV9_b5)_reverse	219
46	AAV9	DO_83_(AAV9_b6)_forward	DO_84_(AAV9_b6)_reverse	433
47	AAV9	DO_85_(AAV9_b7)_forward	DO_86_(AAV9_b7)_reverse	211
48	AAV9	DO_87_(AAV9_b8)_forward	DO_34_(AAV4_b8)_reverse	298

FIG 5

Block Identity	Overhang with Previous Block	Overhang with Next Block
(Vector_Backbone)	TTGC	ATAA
(AAV2_b1)	ATAA	GAAC
(AAV2_b2)	GAAC	GGGT
(AAV2_b3)	GGGT	CTTT
(AAV2_b4)	CTTT	CTAC
(AAV2_b5)	CTAC	AACT
(AAV2_b6)	AACT	AGAC
(AAV2_b7)	AGAC	TCAT
(AAV2_b8)	TCAT	TTGC
(AAV4_b1)	ATAA	GAAC
(AAV4_b2)	GAAC	GGGT
(AAV4_b3)	GGGT	CTTT
(AAV4_b4)	CTTT	CTAC
(AAV4_b5)	CTAC	AACT
(AAV4_b6)	AACT	AGAC
(AAV4_b7)	AGAC	TCAT
(AAV4_b8)	TCAT	TTGC
(AAV5_b1)	ATAA	GAAC
(AAV5_b2)	GAAC	GGGT
(AAV5_b3)	GGGT	CTTT
(AAV5_b4)	CTTT	CTAC
(AAV5_b5)	CTAC	AACT
(AAV5_b6)	AACT	AGAC
(AAV5_b7)	AGAC	TCAT
(AAV5_b8)	TCAT	TTGC
(AAV6_b1)	ATAA	GAAC
(AAV6_b2)	GAAC	GGGT
(AAV6_b3)	GGGT	CTTT
(AAV6_b4)	CTTT	CTAC
(AAV6_b5)	CTAC	AACT
(AAV6_b6)	AACT	AGAC
(AAV6_b7)	AGAC	TCAT
(AAV6_b8)	TCAT	TTGC
(AAV8_b1)	ATAA	GAAC
(AAV8_b2)	GAAC	GGGT
(AAV8_b3)	GGGT	CTTT
(AAV8_b4)	CTTT	CTAC
(AAV8_b5)	CTAC	AACT
(AAV8_b6)	AACT	AGAC
(AAV8_b7)	AGAC	TCAT
(AAV8_b8)	TCAT	TTGC
(AAV9_b1)	ATAA	GAAC
(AAV9_b2)	GAAC	GGGT
(AAV9_b3)	GGGT	CTTT
(AAV9_b4)	CTTT	CTAC
(AAV9_b5)	CTAC	AACT
(AAV9_b6)	AACT	AGAC
(AAV9_b7)	AGAC	TCAT
(AAV9_b8)	TCAT	TTGC

FIG. 6A-6D

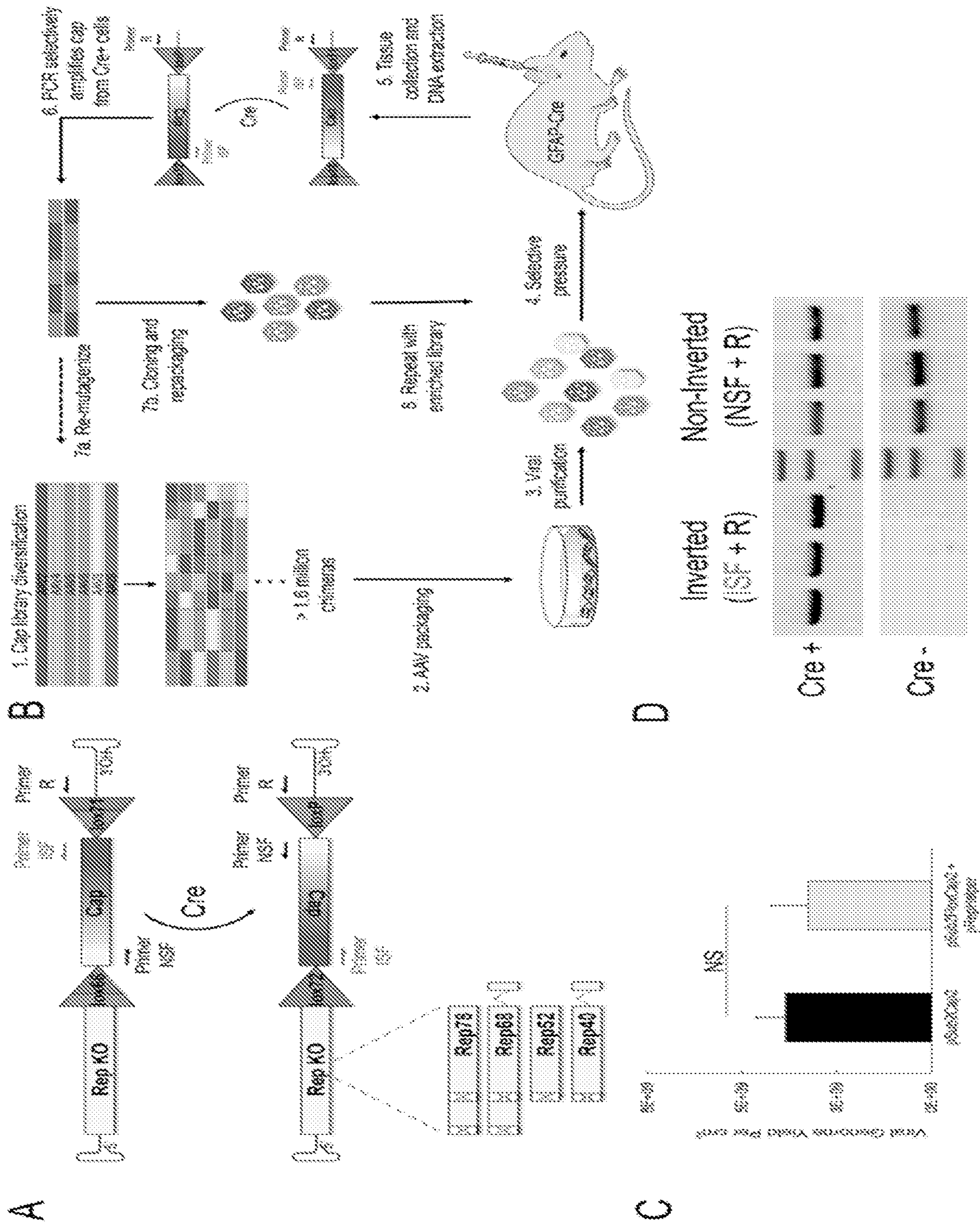
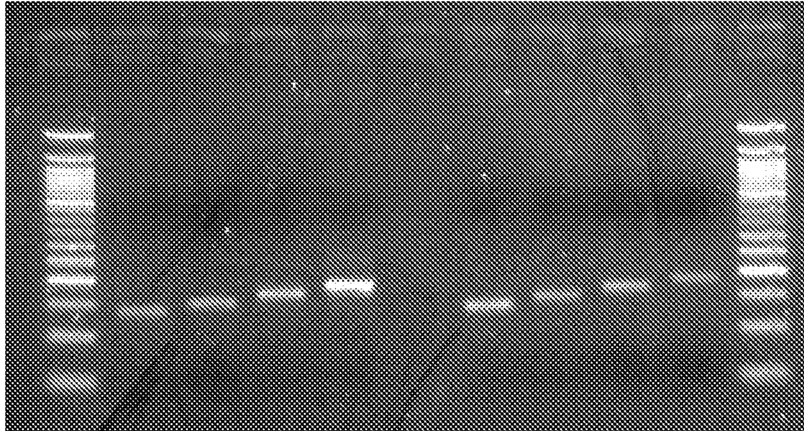


FIG. 7



Lane	1	2	3	4	5	6	7	8	9	10	11
Sample	50 bp DNA Ladder (NEB N3236L)	83 bp stuffer, Cre +	95 bp stuffer, Cre +	110 bp stuffer, Cre +	125 bp stuffer, Cre +		83 bp stuffer, recombinase deficient Sure2	95 bp stuffer, recombinase deficient Sure2	110 bp stuffer, recombinase deficient Sure2	125 bp stuffer, recombinase deficient Sure2	50 bp DNA Ladder (NEB N3236L)

FIG. 8

SCH9:

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLG
PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRVLEPLGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQP
AKKRLNFGQTGDTESVPDPQPIGEPPAAPSGVGSLTMASGGGAPVADNNEGADGVG
SSSGNWHCDSQWLGDRVITTSTRTWALPTYNNHLYKQISNSTSGGSSNDNAYFGYS
TPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLSFKLFNIQVKEVTQNEGTKTIA
NNLTSTIQVFTDSDYQLPYVLGSAHEGCLPPFPADVFMIPQYGYLTLNDGSQAVGRS
SFYCLEYFPSQMLRTGNNFQFSYEFENVPFHSSYAHSQSLDRLMNPLIDQYLYYLSKT
INGSGQNQQTLKFSVAGPSNMAVQGRNWLPGPCYRQQRVSKTSADNNNSEYSWTG
ATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKTNVDIEKVMITD
EEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGVLPGMVWQDRDVYLQGPI
WAKIPHTDGNFHPSPLMGGFGMKHPPPQILIKNTPVPADPPTAFNKDKLNSFITQYST
GQVSVEIEWELQKENSKRWNPEIQYTSNYYKSNNVEFAVNTEGVYSEPRPIGTRYLT
RNL (SEQ ID NO:1)

FIG. 9

SCH2:

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLG
PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRVLEPLGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQP
AKKRLNFGQTGDTESVPDPQPIGEPPAAPSGVGSLTMASGGGAPVADNNEGADGVG
SSSGNWHCDSQWLGDRVITTSTRTWALPTYNNHLYKQISNSTSGGSSNDNAYFGYS
TPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLSFKLFNIQVKEVTQNEGTKTIA
NNLTSTIQVFTDSDYQLPYVLGSAHEGCLPPFPADVFMIPQYGYLTLNDGSQAVGRS
SFYCLEYFPSQMLRTGNNFQFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRT
NTPSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEYSWTG
ATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKTNVDIEKVMITD
EEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGVLPGMVWQDRDVYLQGPI
WAKIPHTDGNFHPSPLMGGFGMKHPPPQILIKNTPVPADPPTAFNKDKLNSFITQYST
GQVSVEIEWELQKENSKRWNPEIQYTSNYYKSNNVEFAVNTEGVYSEPRPIGTRYLT
RNL (SEQ ID NO:2)

FIG. 10

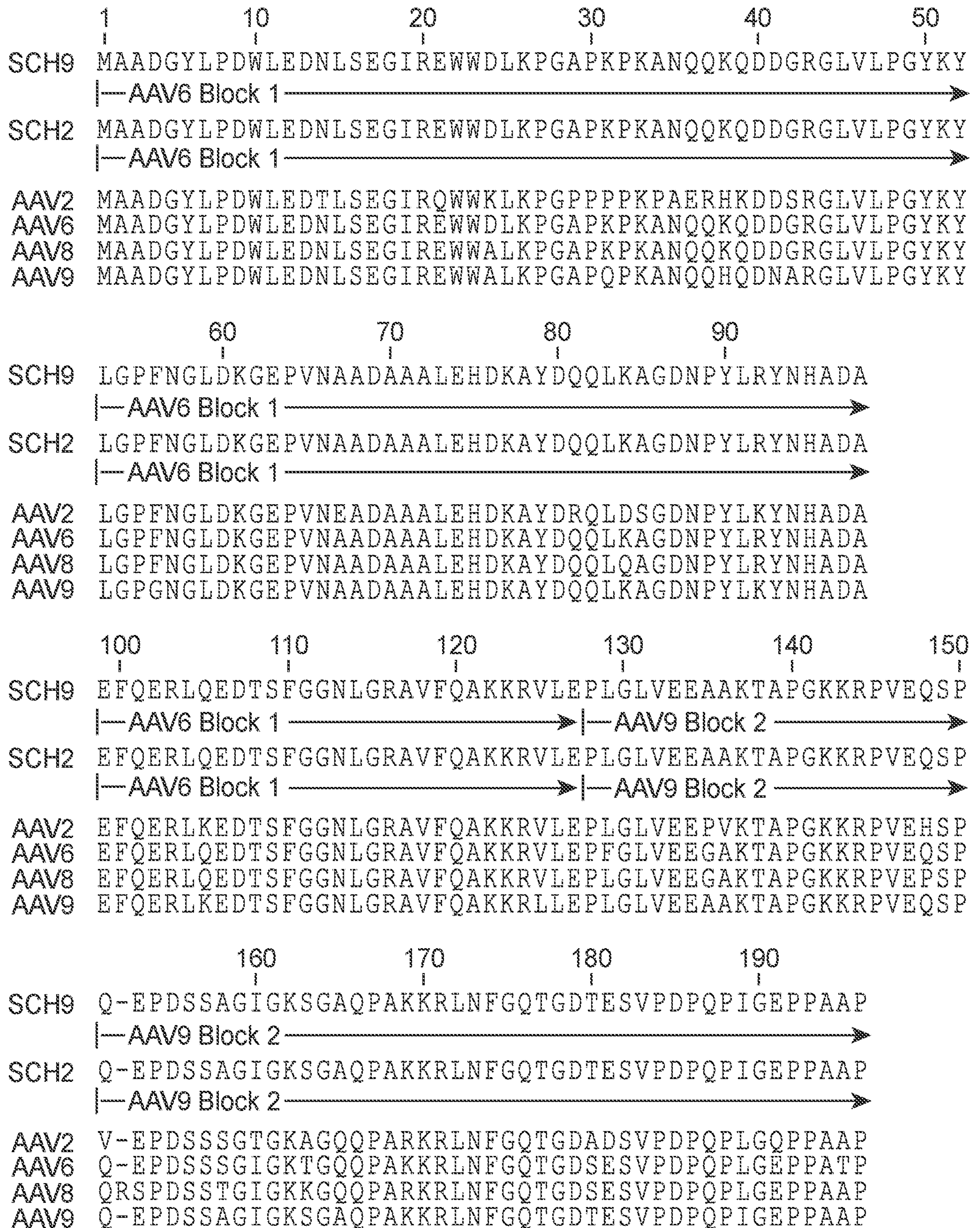


FIG. 10 (Cont.)

```
        200         210         220         230         240
SCH9  SGVGSLTMASGGGAPVADNNEGADGVGSSSGNWHCDSQWLGDRVITTSTRTW
      |—AAV9 Block 2 ————————————————————————————————————>
SCH2  SGVGSLTMASGGGAPVADNNEGADGVGSSSGNWHCDSQWLGDRVITTSTRTW
      |—AAV9 Block 2 ————————————————————————————————————>

AAV2  SGLGTNTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTW
AAV6  AAVGPTTMASGGGAPMADNNEGADGVGNASGNWHCDSTWLGDRVITTSTRTW
AAV8  SGVGPNTMAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRVITTSTRTW
AAV9  SGVGSLTMASGGGAPVADNNEGADGVGSSSGNWHCDSQWLGDRVITTSTRTW

      250         260         270         280         290
SCH9  ALPTYNNHLYKQISNSTSGGSSNDNAYFGYSTPWGYFDFNRFHCHF
      |—AAV9 Block 2 ———————————————————>|AAV8 Block 3>
SCH2  ALPTYNNHLYKQISNSTSGGSSNDNAYFGYSTPWGYFDFNRFHCHF
      |—AAV9 Block 2 ———————————————————>|AAV8 Block 3>

AAV2  ALPTYNNHLYKQISS--QSGASNDNHYFGYSTPWGYFDFNRFHCHF
AAV6  ALPTYNNHLYKQISSA-STGASNDNHYFGYSTPWGYFDFNRFHCHF
AAV8  ALPTYNNHLYKQISNGTSGGATNDNTYFGYSTPWGYFDFNRFHCHF
AAV9  ALPTYNNHLYKQISNSTSGGSSNDNAYFGYSTPWGYFDFNRFHCHF

          300         310         320         330         340
SCH9  SPRDWQRLINNNWGFRPKRLSFKLFNIQVKEVTQNEGTKTIANNLTSTIQVF
      |—AAV8 Block 3 ————————————————————————————————————>
SCH2  SPRDWQRLINNNWGFRPKRLSFKLFNIQVKEVTQNEGTKTIANNLTSTIQVF
      |—AAV8 Block 3 ————————————————————————————————————>

AAV2  SPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVF
AAV6  SPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTTNDGVTTIANNLTSTVQVF
AAV8  SPRDWQRLINNNWGFRPKRLSFKLFNIQVKEVTQNEGTKTIANNLTSTIQVF
AAV9  SPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTDNNGVKTIANNLTSTVQVF

        350         360         370         380         390
SCH9  TDSDYQLPYVLGSAHEGCLPPFPADVFMIPQYGYLTLNDGSQAVGR
      |—AAV9 Block 4 ———————————————————————————————>
SCH2  TDSDYQLPYVLGSAHEGCLPPFPADVFMIPQYGYLTLNDGSQAVGR
      |—AAV9 Block 4 ———————————————————————————————>

AAV2  TDSEYQLPYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGR
AAV6  SDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGR
AAV8  TDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGR
AAV9  TDSDYQLPYVLGSAHEGCLPPFPADVFMIPQYGYLTLNDGSQAVGR
```

FIG. 10 (Cont.)

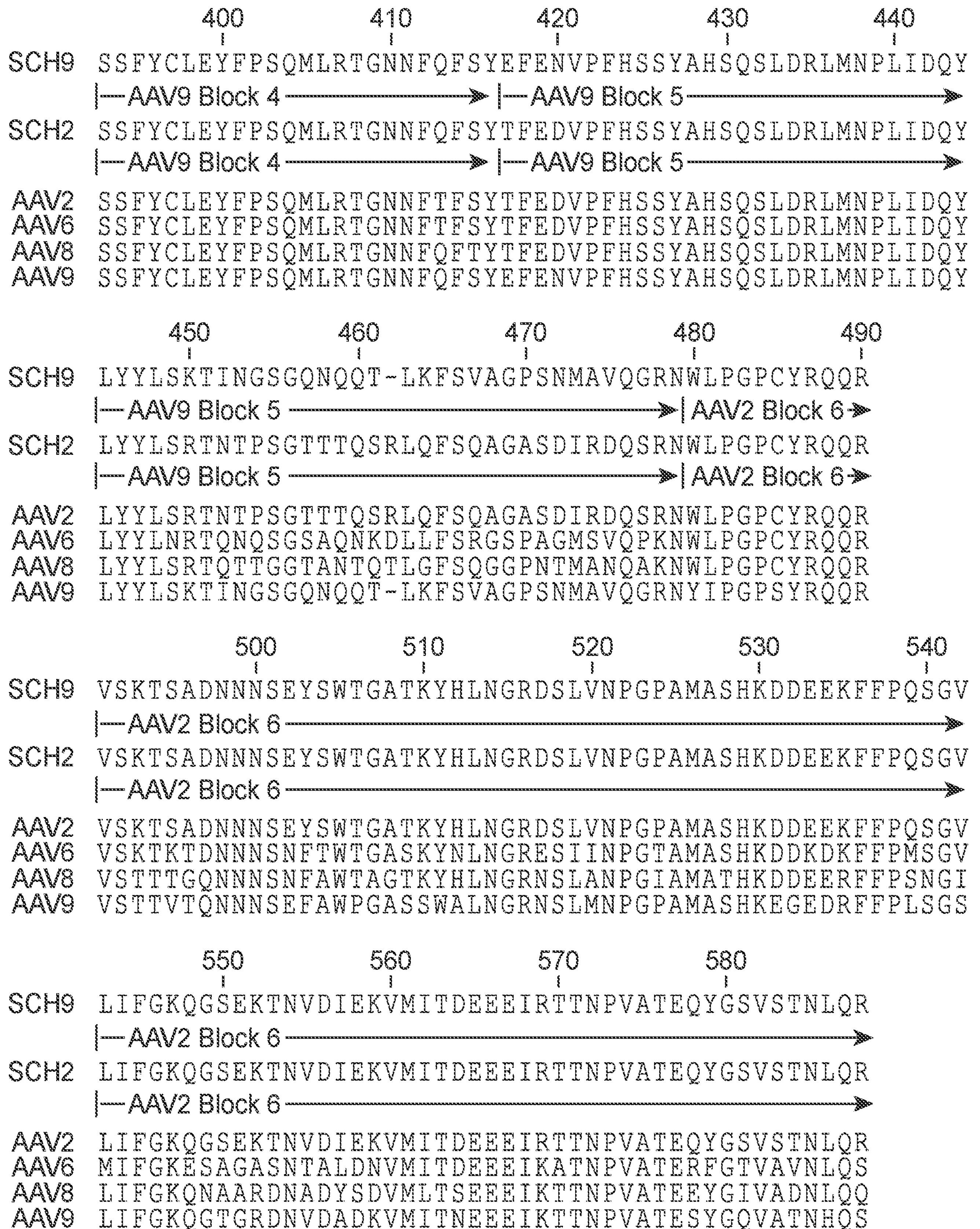


FIG. 10 (Cont.)

```
      590       600       610       620       630       640
SCH9  GNRQAATADVNTQGVLPGMVWQDRDVYLQGPIWAKIPHTDGNFHPSPLMGGF
      |—AAV2 Block 6 ——————————>|—AAV9 Block 7 ——————————>
SCH2  GNRQAATADVNTQGVLPGMVWQDRDVYLQGPIWAKIPHTDGNFHPSPLMGGF
      |—AAV2 Block 6 ——————————>|—AAV9 Block 7 ——————————>

AAV2  GNRQAATADVNTQGVLPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGF
AAV6  SSTDPATGDVHVMGALPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGF
AAV8  QNTAPQIGTVNSQGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGF
AAV9  AQAQAQTGWVQNQGILPGMVWQDRDVYLQGPIWAKIPHTDGNFHPSPLMGGF

                650       660       670       680
SCH9  GMKHPPPQILIKNTPVPADPPTAFNKDKLNSFITQYSTGQVSVEIE
      |—AAV9 Block 7 ———————————————>|—AAV9 Block 8 —>
SCH2  GMKHPPPQILIKNTPVPADPPTAFNKDKLNSFITQYSTGQVSVEIE
      |—AAV9 Block 7 ———————————————>|—AAV9 Block 8 —>

AAV2  GLKHPPPQILIKNTPVPANPSTTFSAAKFASFITQYSTGQVSVEIE
AAV6  GLKHPPPQILIKNTPVPANPPAEFSATKFASFITQYSTGQVSVEIE
AAV8  GLKHPPPQILIKNTPVPADPPTTFNQSKLNSFITQYSTGQVSVEIE
AAV9  GMKHPPPQILIKNTPVPADPPTAFNKDKLNSFITQYSTGQVSVEIE

        690       700       710       720       730    738
SCH9  WELQKENSKRWNPEIQYTSNYYKSNNVEFAVNTEGVYSEPRPIGTRYLTRNL
      |—AAV9 Block 8 ——————————————————————————————————————>
SCH2  WELQKENSKRWNPEIQYTSNYYKSNNVEFAVNTEGVYSEPRPIGTRYLTRNL
      |—AAV9 Block 8 ——————————————————————————————————————>

AAV2  WELQKENSKRWNPEIQYTSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL
AAV6  WELQKENSKRWNPEVQYTSNYAKSANVDFTVDNNGLYTEPRPIGTRYLTRPL
AAV8  WELQKENSKRWNPEIQYTSNYYKSTSVDFAVNTEGVYSEPRPIGTRYLTRNL
AAV9  WELQKENSKRWNPEIQYTSNYYKSNNVEFAVNTEGVYSEPRPIGTRYLTRNL
```

FIG. 11A-11B

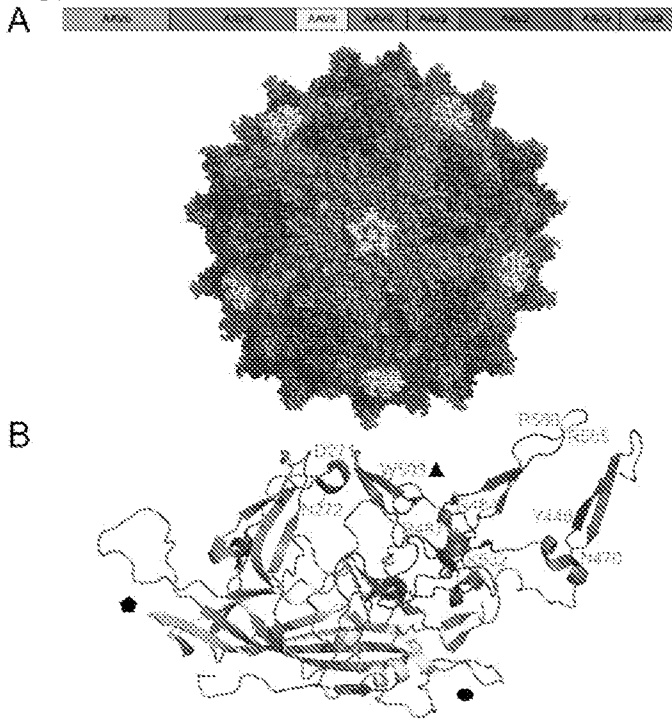


FIG. 12

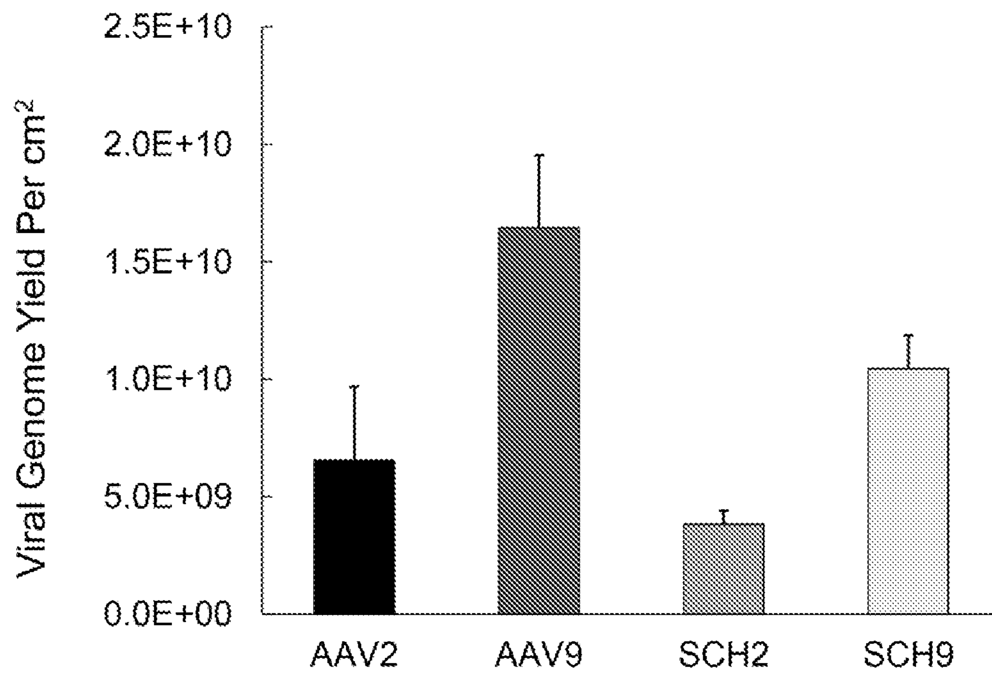


FIG. 13A-13I

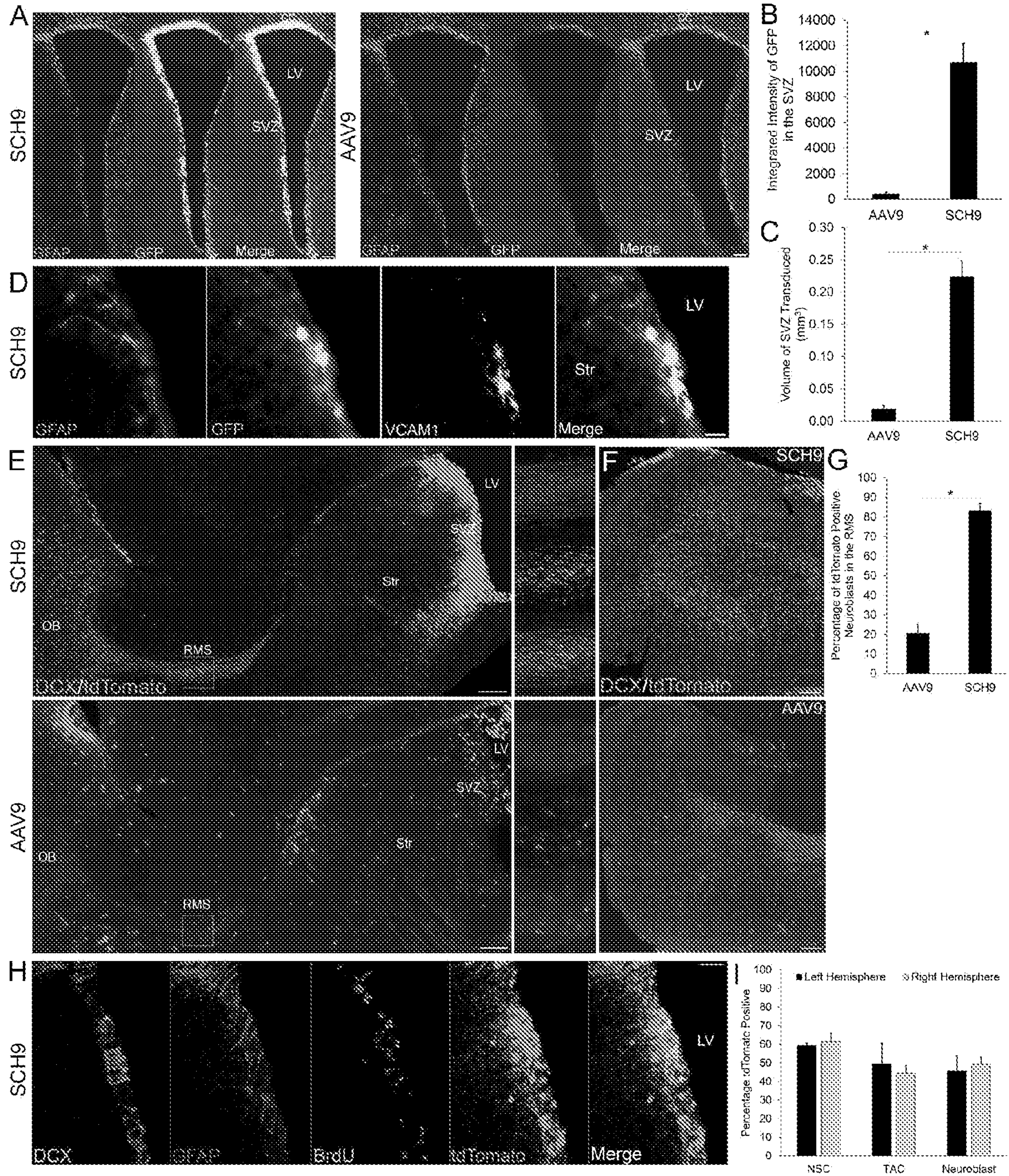


FIG. 14A-14C

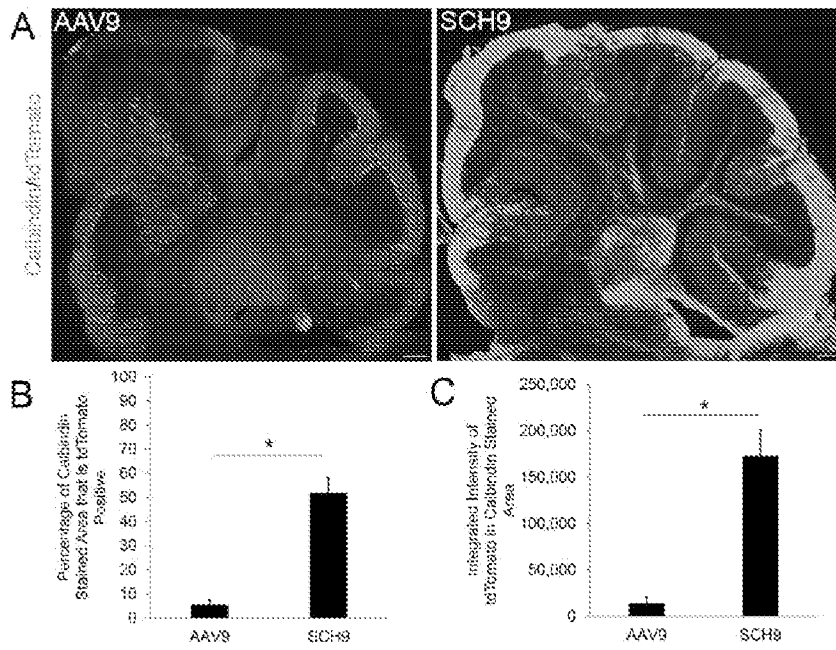


FIG. 15

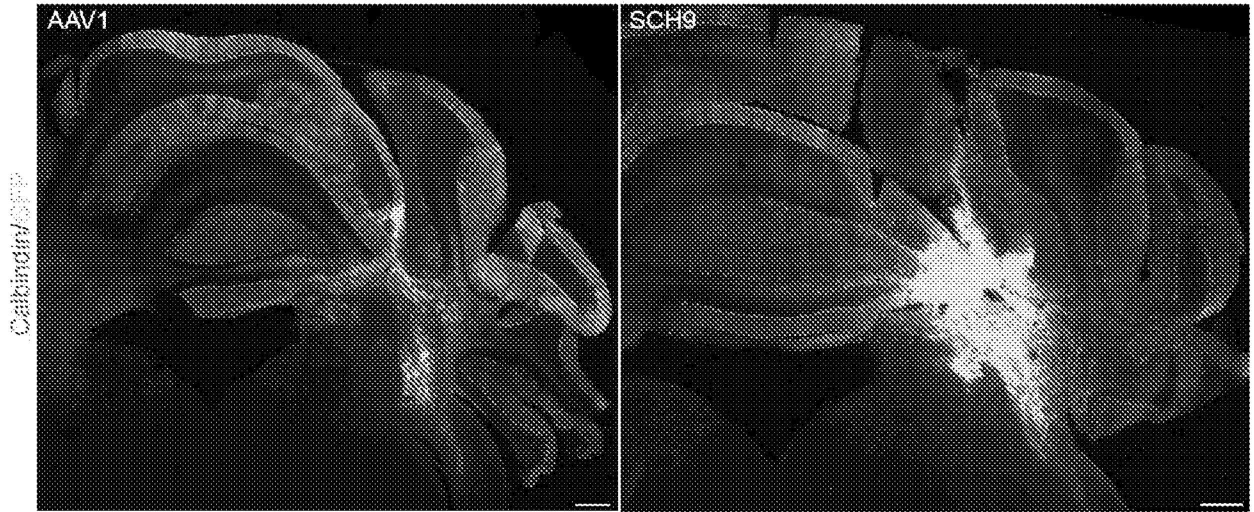


FIG. 16A-16C

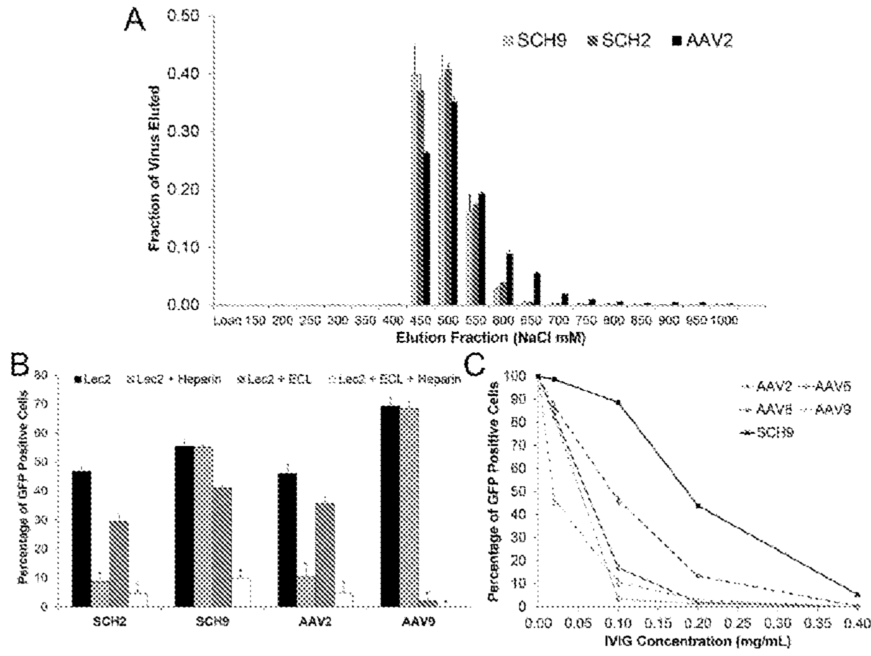


FIG. 17

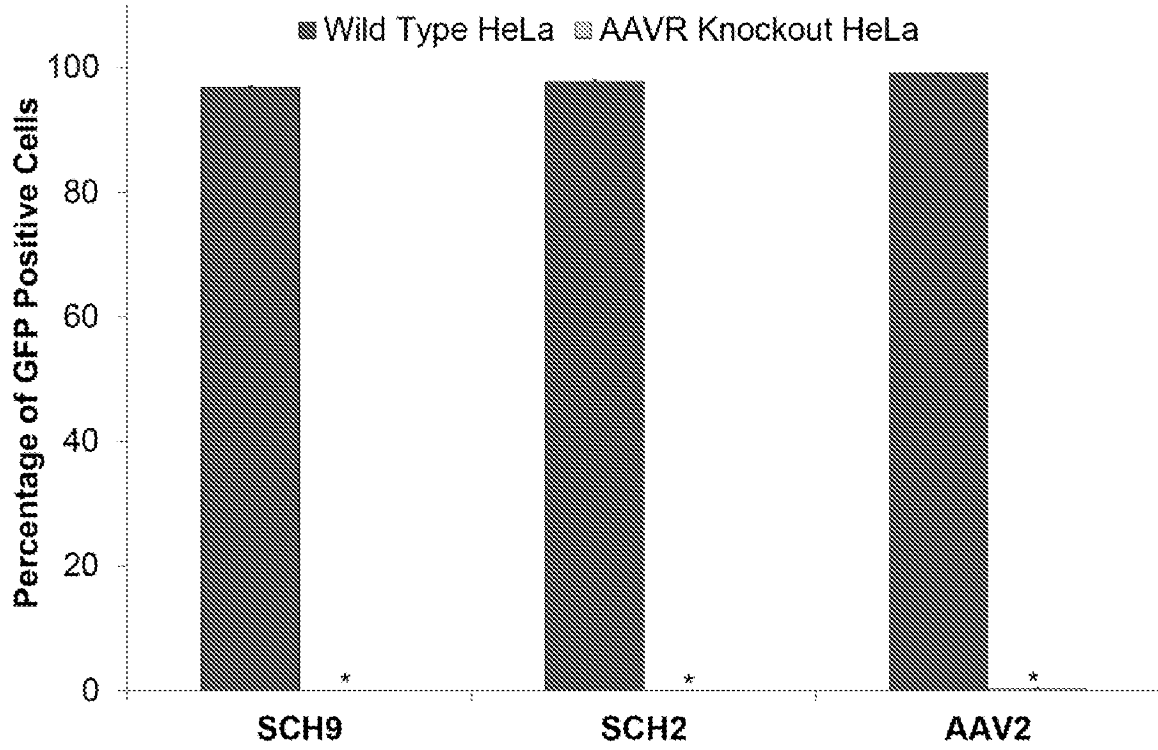


FIG. 18A

Streptococcus pyogenes Cas9

MDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYL
QEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFR
GHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLT
PNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDL
TLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIH
LGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTN
FDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEI
SGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLS
RKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDE
LVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQ
ELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGG
LSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLN
AVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVW
DKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKS
KKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYL
ASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPA
AFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGD (SEQ ID NO:40)

FIG. 18B

>nSpCas9 (SpCas9 D10A)
MDKKYSIGLAIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYL
QEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFR
GHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLT
PNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDL
TLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHL
GELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFD
KNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEIS
GVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLS
RKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDE
LVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQE
LDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGL
SELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLN
AVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIV
WDKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGK
SKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLY
LASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAP
AAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGD (SEQ ID NO:41)

FIG. 18C

SpCas9 (H840A)

MDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYL
QEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFR
GHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLT
PNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDL
TLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIH
LGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTN
FDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEI
SGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLS
RKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDE
LVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQ
ELDINRLSDYDVD**A**IVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGG
LSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLN
AVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVW
DKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKS
KKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYL
ASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPA
AFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGD (SEQ ID NO:42)

FIG. 18D

SpCas9 (D10A; H840A)
MDKKYSIGL**A**IGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYL
QEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFR
GHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLT
PNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDL
TLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIH
LGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTN
FDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEI
SGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLS
RKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDE
LVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQ
ELDINRLSDYDVD**A**IVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGG
LSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLN
AVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVW
DKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKS
KKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYL
ASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPA
AFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGD (SEQ ID NO:43)

FIG. 18E

enSpCas9 (nCas9 with K848A/K1003A/R1060A mutations

MDKKYSIGLAIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYL
QEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFR
GHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLT
PNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDL
TLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHL
GELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFD
KNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEIS
GVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLS
RKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDE
LVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQE
LDINRLSDYDVDHIVPQSFL**A**DDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGL
SELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLN
AVVGTALIKKYP**A**LESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRK**A**PLIETNGETGEIV
WDKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGK
SKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLY
LASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAP
AAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGD (SEQ ID NO:44)

FIG. 18F

nSpCas9-HF1

DKKYSIGLAIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQE
IFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGH
FLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNF
KSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLL
KALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGE
LHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTAFDKN
LPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGV
EDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGALSRK
LINGIRDKQSGKTILDFLKSDGFANRNFMALIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELV
KVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELD
INRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSE
LDKAGFIKRQLVETRAITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAV
VGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWD
KGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKSKK
LKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLAS
HYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAF
KYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGD (SEQ ID NO:45)

FIG. 19

Staphylococcus aureus Cas9

MKRNYILGLDIGITSVGYGIIDYETRDVIDAGVRLFKEANVENNEGRRSKRGARRLKRRRRHRIQRVKKLLFDYNLLTDHSEL
SGINPYEARVKGLSQKLSEEEFSAALLHLAKRRGVHNVNEVEEDTGNELSTKEQISRNSKALEEKYVAELQLERLKKDGEV
RGSINRFKTSDYVKEAKQLLKVQKAYHQLDQSFIDTYIDLLETRRTYYEGPGEGSPFGWKDIKEWYEMLMGHCTYFPEELR
SVKYAYNADLYNALNDLNNLVITRDENEKLEYYEKFQIIENVFKQKKKPTLKQIAKEILVNEEDIKGYRVTSTGKPEFTNLKVY
HDIKDITARKEIIENAELLDQIAKILTIYQSSEDIQEELTNLNSELTQEEIEQISNLKGYTGTHNLSLKAINLILDELWHTNDNQIAI
FNRLKLVPKKVDLSQQKEIPTTLVDDFILSPVVKRSFIQSIKVINAIIKKYGLPNDIIIELAREKNSKDAQKMINEMQKRNRQTN
ERIEEIIRTTGKENAKYLIEKIKLHDMQEGKCLYSLEAIPLEDLLNNPFNYEVDHIIPRSVSFDNSFNNKVLVKQEENSKKGNR
TPFQYLSSSDSKISYETFKKHILNLAKGKGRISKTKKEYLLEERDINRFSVQKDFINRNLVDTRYATRGLMNLLRSYFRVNNL
DVKVKSINGGFTSFLRRKWKFKKERNKGYKHHAEDALIIANADFIFKEWKKLDKAKKVMENQMFEEKQAESMPEIETEQEY
KEIFITPHQIKHIKDFKDYKYSHRVDKKPNRELINDTLYSTRKDDKGNTLIVNNLNGLYDKDNDKLKKLINKSPEKLLMYHHDP
QTYQKLKLIMEQYGDEKNPLYKYYEETGNYLTKYSKKDNGPVIKKIKYYGNKLNAHLDITDDYPNSRNKVVKLSLKPYRFDV
YLDNGVYKFVTVKNLDVIKKENYYEVNSKCYEEAKKLKKISNQAEFIASFYNNDLIKINGELYRVIGVNNDLLNRIEVNMIDITY
REYLENMNDKRPPRIIKTIASKTQSIKKYSTDILGNLYEVKSKKHPQIIKKG (SEQ ID NO:46)

FIG. 20A

Francisella tularensis Cpf1

```
   1 MSIYQEFVNK YSLSKTLRFE LIPQGKTLEN IKARGLILDD EKRAKDYKKA KQIIDKYHQF
  61 FIEEILSSVC ISEDLLQNYS DVYFKLKKSD DDNLQKDFKS AKDTIKKQIS EYIKDSEKFK
 121 NLFNQNLIDA KKGQESDLIL WLKQSKDNGI ELFKANSDIT DIDEALEIIK SFKGWTTYFK
 181 GFHENRKNVY SSNDIPTSII YRIVDDNLPK FLENKAKYES LKDKAPEAIN YEQIKKDLAE
 241 ELTFDIDYKT SEVNQRVFSL DEVFEIANFN NYLNQSGITK FNTIIGGKFV NGENTKRKGI
 301 NEYINLYSQQ INDKTLKKYK MSVLFKQILS DTESKSFVID KLEDDSDVVT TMQSFYEQIA
 361 AFKTVEEKSI KETLSLLFDD LKAQKLDLSK IYFKNDKSLT DLSQQVFDDY SVIGTAVLEY
 421 ITQQIAPKNL DNPSKKEQEL IAKKTEKAKY LSLETIKLAL EEFNKHRDID KQCRFEEILA
 481 NFAAIPMIFD EIAQNKDNLA QISIKYQNQG KKDLLQASAE DDVKAIKDLL DQTNNLLHKL
 541 KIFHISQSED KANILDKDEH FYLVFEECYF ELANIVPLYN KIRNYITQKP YSDEKFKLNF
 601 ENSTLANGWD KNKEPDNTAI LFIKDDKYYL GVMNKKNNKI FDDKAIKENK GEGYKKIVYK
 661 LLPGANKMLP KVFFSAKSIK FYNPSEDILR IRNHSTHTKN GSPQKGYEKF EFNIEDCRKF
 721 IDFYKQSISK HPEWKDFGFR FSDTQRYNSI DEFYREVENQ GYKLTFENIS ESYIDSVVNQ
 781 GKLYLFQIYN KDFSAYSKGR PNLHTLYWKA LFDERNLQDV VYKLNGEAEL FYRKQSIPKK
 841 ITHPAKEAIA NKNKDNPKKE SVFEYDLIKD KRFTEDKFFF HCPITINFKS SGANKFNDEI
 901 NLLLKEKAND VHILSIDRGE RHLAYYTLVD GKGNIIKQDT FNIIGNDRMK TNYHDKLAAI
 961 EKDRDSARKD WKKINNIKEM KEGYLSQVVH EIAKLVIEYN AIVVFEDLNF GFKRGRFKVE
1021 KQVYQKLEKM LIEKLNYLVF KDNEFDKTGG VLRAYQLTAP FETFKKMGKQ TGIIYYVPAG
1081 FTSKICPVTG FVNQLYPKYE SVSKSQEFFS KFDKICYNLD KGYFEFSFDY KNFGDKAAKG
1141 KWTIASFGSR LINFRNSDKN HNWDTREVYP TKELEKLLKD YSIEYGHGEC IKAAICGESD
1201 KKFFAKLTSV LNTILQMRNS KTGTELDYLI SPVADVNGNF FDSRQAPKNM PQDADANGAY
1261 HIGLKGLMLL GRIKNNQEGK KLNLVIKNEE YFEFVQNRNN (SEQ ID NO:47)
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FIG. 20B

Acidaminococcus sp. BV3L6 type V CRISPR-associated protein Cpf1

TQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKPIIDRIYKTYADQCLQLVQLDWENLSAAIDSYR
KEKTEETRNALIEEQATYRNAIHDYFIGRTDNLTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTT
YFSGFYENRKNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVSTSIEEVFSFPFYNQLLTQTQ
IDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAHIIASLPHRFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLR
NENVLETAEALFNELNSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLKHEDINLQEIISAA
GKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDSLLGLYHLLDWFAVDESNEVDPEFSARLTGIKLEMEPSL
SFYNKARNYATKKPYSVEKFKLNFQMPTLASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKTSEGFD
KMYYDYFPDAAKMIPKCSTQLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQTAYAKKTGDQKGYREALC
KWIDFTRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGKLYLFQIYNKDFAKGHHGKP
NLHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAHRLGEKMLNKKLKDQKTPIPDTLYQELYDYVNHRLSHDLS
DEARALLPNVITKEVSHEIIKDRRFTSDKFFFHVPITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVIDSTGKI
LEQRSLNTIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAVVVLENLNFGFKSKRTGIAE
KAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQLTDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIK
NHESRKHFLEGFDFLHYDVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRIVPVIENHRFTGR
YRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALIRSVLQMRNSNAATGEDYINSPVRDLNGVCFDSRFQN
PEWPMDADANGAYHIALKGQLLLNHLKESKDLKLQNGISNQDWLAYIQELRN (SEQ ID NO:48)

FIG. 20C

nCpf1 (AsCpf1 R1225A)

TQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKPIIDRIYKTYADQCLQLVQLDWENLSAAIDSYR
KEKTEETRNALIEEQATYRNAIHDYFIGRTDNLTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTT
YFSGFYENRKNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVSTSIEEVFSFPFYNQLLTQTQ
IDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAHIIASLPHRFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLR
NENVLETAEALFNELNSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLKHEDINLQEIISAA
GKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDSLLGLYHLLDWFAVDESNEVDPEFSARLTGIKLEMEPSL
SFYNKARNYATKKPYSVEKFKLNFQMPTLASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKTSEGFD
KMYYDYFPDAAKMIPKCSTQLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQTAYAKKTGDQKGYREALC
KWIDFTRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGKLYLFQIYNKDFAKGHHGKP
NLHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAHRLGEKMLNKKLKDQKTPIPDTLYQELYDYVNHRLSHDLS
DEARALLPNVITKEVSHEIIKDRRFTSDKFFFHVPITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVIDSTGKI
LEQRSLNTIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAVVVLENLNFGFKSKRTGIAE
KAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQLTDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIK
NHESRKHFLEGFDFLHYDVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRIVPVIENHRFTGR
YRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALIRSVLQM**A**NSNAATGEDYINSPVRDLNGVCFDSRFQN
PEWPMDADANGAYHIALKGQLLLNHLKESKDLKLQNGISNQDWLAYIQELRN (SEQ ID NO:49)

FIG. 21A

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AAV1        --TFSYTFEEVPFHSSYAHSQSLDRLMNPLIDQYLYYLNRTQ-NQSGSAQNKDLLFSRGS 467
AAV6        --TFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLNRTQ-NQSGSAQNKDLLFSRGS 467
AAV3        ---FSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLNRTQGTTSGTTNQSRLLFSQAG 467
AAV2        ---FSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTN-TPSGTTTQSRLQFSQAG 466
AAV8        NFQFTYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQTT-GGTANTQTLGFSQGG 469
AAV8.1      NFQFTYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQTT-GGTANTQTLGFSQGG 469
AAV8 rh8     FQFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLVRTQTTGTGGTQTLAFSQAGPS 469
AAV10       NFEFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQST-GGTQGTQQLLFSQAG 469
AAV7        -FEFSYSFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLARTQSNPGGTAGNRELQFYQGG 469
AAV9        -FQFSYEFENVPFHSSYAHSQSLDRLMNPLIDQYLYYLSKTI--NGSGQNQQTLKFSVAG 467
AAV9.1      -FQFSYEFENVPFHSSYAHSQSLDRLMNPLIDQYLYYLSKTI--NGSGQNQQTLKFSVAG 467
AAV5        NFEFTYNFEEVPFHSSFAPSQNLFKLANPLVDQYLYRFVSTN-------NTGGVQFNKNL 453
               *:* **:******:* **.* :* ***:***** :  *            : *

AAV1        PAGMSVQPKNWLPGPCYRQQRVSKTKTDNNNSNFTWTGASKYNLNGRESIINPGTAMASH 527
AAV6        PAGMSVQPKNWLPGPCYRQQRVSKTKTDNNNSNFTWTGASKYNLNGRESIINPGTAMASH 527
AAV3        PQSMSLQARNWLPGPCYRQQRLSKTANDNNNSNFPWTAASKYHLNGRDSLVNPGPAMASH 527
AAV2        ASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASH 526
AAV8        PNTMANQAKNWLPGPCYRQQRVSTTTGQNNNSNFAWTAGTKYHLNGRNSLANPGIAMATH 529
AAV8.1      PNTMANQAKNWLPGPCYRQQRVSTTTGQNNNSNFAWTAGTKYHLNGRNSLANPGIAMATH 529
AAV8 rh8    S--MANQARNWVPGPCYRQQRVSTTTNQNNNSNFAWTGAAKFKLNGRDSLMNPGVAMASH 527
AAV10       PANMSAQAKNWLPGPCYRQQRVSTTLSQNNNSNFAWTGATKYHLNGRDSLVNPGVAMATH 529
AAV7        PSTMAEQAKNWLPGPCFRQQRVSKTLDQNNNSNFAWTGATKYHLNGRNSLVNPGVAMATH 529
AAV9        PSNMAVQGRNYIPGPSYRQQRVSTTVTQNNNSEFAWPGASSWALNGRNSLMNPGPAMASH 527
AAV9.1      PSNMAVQGRNYIPGPSYRQQRVSTTVTQNNNSEFAWPGASSWALNGRNSLMNPGPAMASH 527
AAV5        AGRYANTYKNWFPGPMGRTQGWNLGSGVNRASVSAFATTNRMELEGASYQVPPQPNGMTN 513
            .       :*::***  * *   .      *. *  .:.   .    *:* .    *     ::
```

Figure 21B

```
AAV1        KDDEDKFFPMSGVMIFGK--ESAGASNTALD-NVMITDEEEIKATNPVATERFGTVAVNF 584
AAV6        KDDKDKFFPMSGVMIFGK--ESAGASNTALD-NVMITDEEEIKATNPVATERFGTVAVNL 584
AAV3        KDDEEKFFPMHGNLIFGK--EGTTASNAELD-NVMITDEEEIRTTNPVATEQYGTVANNL 584
AAV2        KDDEEKFFPQSGVLIFGK--QGSEKTNVDIE-KVMITDEEEIRTTNPVATEQYGSVSTNL 583
AAV8        KDDEERFFPSNGILIFGK--QNAARDNADYS-DVMLTSEEEIKTTNPVATEEYGIVADNL 586
AAV8.1      KDDEERFFPSNGILIFGK--QNAARDNADYS-DVMLTSEEEIKTTNPVATEEYGIVADNL 586
AAV8 rh8    KDDDDRFFPSSGVLIFGK--QGAGNDGVDYS-QVLITDEEEIKATNPVATEEYGAVAINN 584
AAV10       KDDEERFFPSSGVLMFGK--QGAGRDNVDYS-SVMLTSEEEIKTTNPVATEQYGVVADNL 586
AAV7        KDDEDRFFPSSGVLIFGK--TGAT-NKTTLE-NVLMTNEEEIRPTNPVATEEYGIVSSNL 585
AAV9        KEGEDRFFPLSGSLIFGK--QGTGRDNVDAD-KVMITNEEEIKTTNPVATESYGQVATNH 584
AAV9.1      KEGEDRFFPLSGSLIFGK--QGTGRDNVDAD-KVMITNEEEIKTTNPVATESYGQVATNH 584
AAV5        NLQGSNTYALENTMIFNSQPANPGTTATYLEGNMLITSESETQPVNRVAYNVGGQMATNN 573
            :   .. :.  . ::*..    ..     .  . .:::*.*.* :..* ** :   * :: *

AAV1        QSSSTDPATGDVHAMGALPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKNPP 644
AAV6        QSSSTDPATGDVHVMGALPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPP 644
AAV3        QSSNTAPTTGTVNHQGALPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPP 644
AAV2        QRGNRQAATADVNTQGVLPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPP 643
AAV8        QQQNTAPQIGTVNSQGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPP 646
AAV8.1      QGQRQAAQIGTVNSQGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPP 646
AAV8 rh8    QAANTQAQTGLVHNQGVIPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPP 644
AAV10       QQANTGPIVGNVNSQGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPP 646
AAV7        QAANTAAQTQVVNNQGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPP 645
AAV9        QSAQAQAQTGWVQNQGILPGMVWQDRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGMKHPP 644
AAV9.1      QSGQAQAATGWVQNQGILPGMVWQDRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGMKHPP 644
AAV5        QSSTTAPATGTYNLQEIVPGSVWMERDVYLQGPIWAKIPETGAHFHPSPAMGGFGLKHPP 633
            *     .     :    :** ** :**************.*..:***** *****:*:**
```

Figure 21C

```
AAV1          PQILIK- 650 (SEQ ID NO:139)
AAV6          PQILIK- 650 (SEQ ID NO:140)
AAV3          PQIMIK- 650 (SEQ ID NO:141)
AAV2          PQILIKN 650 (SEQ ID NO:142)
AAV8          PQILIKN 653 (SEQ ID NO:143)
AAV8.1        PQILIKN 653 (SEQ ID NO:144)
AAV8 rh8      PQILIKN 651 (SEQ ID NO:145)
AAV10         PQILIKN 653 (SEQ ID NO:146)
AAV7          PQILIKN 652 (SEQ ID NO:147)
AAV9          PQILIK- 650 (SEQ ID NO:148)
AAV9.1        PQILIK- 650 (SEQ ID NO:149)
AAV5          PMMLIKN 640 (SEQ ID NO:150)
              * ::**
```