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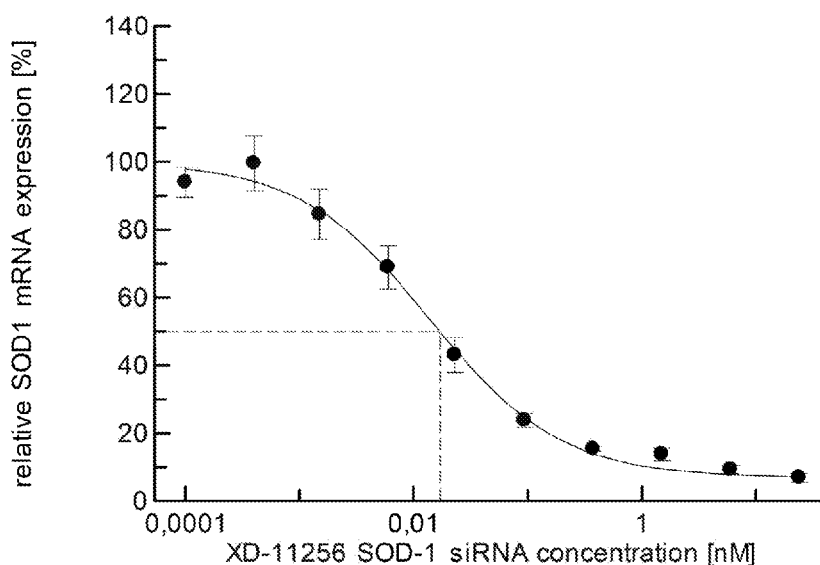
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(54) Title: TREATMENT OF AMYOTROPHIC LATERAL SCLEROSIS AND DISORDERS ASSOCIATED WITH THE SPINAL CORD

Figure 1



(57) Abstract: The present disclosure relates to AAVs encoding a SOD1 targeting polynucleotide which may be used to treat amyotrophic lateral sclerosis (ALS) and delivery methods for the treatment of spinal cord related disorders including ALS.

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**TREATMENT OF AMYOTROPHIC LATERAL SCLEROSIS AND DISORDERS
ASSOCIATED WITH THE SPINAL CORD**

REFERENCE TO RELEVANT APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/693,040, entitled "Treatment of ALS and disorders associated with the spinal cord", filed July 2, 2018, and U.S. Provisional Patent Application No. 62/746,104, entitled "Treatment of ALS and disorders associated with the spinal cord", filed October 16, 2018, the contents of each of which are herein incorporated by reference in their entirety.

REFERENCE TO THE SEQUENCE LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format as an ASCII text file. The Sequence Listing is provided as an ASCII text file entitled 20571070PCT_SEQLIST, created on July 2, 2019, which is 15,822 bytes in size. The Sequence Listing is incorporated herein by reference in its entirety.

FIELD OF THE DISCLOSURE

[0003] The present disclosure relates to compositions, methods and processes for the design, preparation, manufacture and/or formulation of polynucleotides, including AAV vectors, small interfering RNA (siRNA) duplexes, shRNA, microRNA or precursors thereof which target or encode molecules which target the superoxide dismutase 1 (SOD1) gene to interfere with SOD1 gene expression and/or SOD1 enzyme production. In some embodiments, polynucleotides are inserted into recombinant adeno-associated virus (AAV) vectors.

[0004] Methods for inhibiting SOD1 or altering the expression of any gene associated with a spinal cord related disease or disorder in a subject with a disease and/or other disorder associated with the spinal cord are also disclosed. The method includes the administration of the at least one polynucleotide into the subject with a disorder associated with the spinal cord (e.g., neurodegenerative disease) via at least the route of intraparenchymal delivery to the spinal cord. In these embodiments the disease is a motor neuron disease, and more specifically, the disease is amyotrophic lateral sclerosis (ALS).

BACKGROUND

[0005] Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a fatal progressive neurodegenerative disease, characterized by the predominant loss of upper and lower motor neurons (MNs) in primary motor cortex, the brainstem, and the spinal cord. Upper (e.g., cortical) and lower motor neurons (e.g., spinal cord) normally communicate

messages from the brain to the muscles to generate voluntary movement. When these neurons degenerate and/or die, the loss of the message to the muscles results in a gradual weakening and/or atrophy of the muscle and inability to initiate or control voluntary movements, until ultimately, an individual suffering from ALS loses muscle strength and the ability to move, speak, eat and even breathe. Most patients will require some form of breathing aid for survival, and even then, most ALS patients die as a result of respiratory failure within 2-5 years of diagnosis. During disease progression, some patients (e.g., FTD-ALS) may also develop frontotemporal dementia.

[0006] According to the ALS Association, approximately 5,600 people in the United States of America are diagnosed with ALS each year. The incidence of ALS is two per 100,000 people, and it is estimated that as many as 30,000 Americans may have the disease at any given time.

[0007] Two forms of ALS have been described: one is sporadic ALS (sALS), which is the most common form of ALS in the United States of America and accounts for 90 to 95% of all cases diagnosed; the other is familial ALS (fALS), which occurs in a family lineage mainly with a dominant inheritance and only accounts for about 5 to 10% of all cases in the United States of America. sALS and fALS are clinically indistinguishable.

[0008] Pathological studies have linked numerous cellular processes with disease pathogenesis such as increased ER stress, generation of free radicals (i.e., reactive oxygen species (ROS)), mitochondrial dysfunction, protein aggregation, apoptosis, inflammation and glutamate excitotoxicity, specifically in the motor neurons (MNs).

[0009] The causes of ALS are complicated and heterogeneous. In general, ALS is considered to be a complex genetic disorder in which multiple genes in combination with environmental exposures combine to render a person susceptible. More than a dozen genes associated with ALS have been discovered, including, SOD1 (Cu²⁺/Zn²⁺ superoxide dismutase), TDP-43 (TARDBP, TAR DNA binding protein-43), FUS (Fused in Sarcoma/Translocated in Sarcoma), ANG (Angiogenin), ATXN2 (Ataxin-2), valosin containing protein (VCP), OPTN (Optineurin) and an expansion of the noncoding GGGGCC hexanucleotide repeat in the chromosome 9, open reading frame 72 (C9ORF72). However, the exact mechanisms of motor neuron degeneration are still elusive.

[0010] Currently, there is no curative treatment for ALS. Until recently, the only FDA approved drug was Riluzole, which antagonizes the glutamate response to reduce the pathological development of ALS. However, only about a three-month life span expansion

for ALS patients in the early stages has been reported, and no therapeutic benefit for ALS patients in the late stages has been observed, indicating a lack of therapeutic options for this patient population (Bensimon G et al., *J Neurol.* 2002, 249, 609–615). In 2017, the FDA approved Radicava (edaravone) for the treatment of ALS, the first such approval in 22 years. Radicava is administered intravenously and serves as a free-radical scavenger, reducing oxidative stress in patients suffering from ALS and thereby slowing disease progression. In a clinical Phase 3 trial (NCT01492686) of 137 patients, Radicava slowed the decline in physical function as compared to those patients taking placebo and as determined by score on the ALS Functional Rating Scale-Revised (ALSFRS-R) (Writing group; Edaravone (MCI-186) ALS 19 Study Group *Lancet Neurol.* 2017 Jul;16(7):505-512). The approval of Radicava is considered an advance in terms of treatment of ALS, however it is still not a cure. New treatment strategies that can effectively prevent and/or significantly hinder the disease progression are still in demand.

[0011] Mutations in the gene of $\text{Cu}^{2+}/\text{Zn}^{2+}$ superoxide dismutase type I (SOD1) are the most common cause of fALS, accounting for about 20 to 30% of all fALS cases. Recent reports indicate that SOD1 mutations may also be linked to about 4% of all sALS cases (Robberecht and Philip, *Nat. Rev. Neurosci.*, 2013, 14, 248-264). SOD1-linked fALS is most likely not caused by loss of the normal SOD1 activity, but rather by a gain of a toxic function. One of the hypotheses for mutant SOD1-linked fALS toxicity proposes that an aberrant SOD1 enzyme causes small molecules such as peroxynitrite or hydrogen peroxide to produce damaging free radicals. Other hypotheses for mutant SOD1 neurotoxicity include inhibition of the proteasome activity, mitochondrial damage, disruption of RNA processing and formation of intracellular aggregates. Abnormal accumulation of mutant SOD1 variants and/or wild-type SOD1 in ALS forms insoluble fibrillar aggregates which are identified as pathological inclusions. Aggregated SOD1 protein can induce mitochondria stress (Vehvilainen P et al., *Front Cell Neurosci.*, 2014, 8, 126) and other toxicity to cells, particularly to motor neurons.

[0012] These findings indicate that SOD1 can be a potential therapeutic target for both familial and sporadic ALS. A therapy that can reduce the SOD1 protein, whether wildtype or mutant, produced in the central nervous system of ALS patients may ameliorate the symptoms of ALS in patients such as motor neuron degeneration and muscle weakness and atrophy. Agents and methods that aim to prevent the formation of wild type and/or mutant SOD1 protein aggregation may prevent disease progression and allow for amelioration of

ALS symptoms. RNA interfering (RNAi) mediated gene silencing has drawn researchers' interest in recent years. Small double stranded RNA (small interfering RNA) molecules that target the SOD1 gene have been taught in the art for their potential in treating ALS (See, e.g., U.S. Pat. No. 7,632,938 and U.S. Patent Publication No. 20060229268).

[0013] The present disclosure develops an RNA interference, or knock-down based approach to inhibit or prevent the expression of SOD1 gene in ALS patients for treatment of disease.

[0014] The present disclosure provides novel polynucleotides, including double stranded RNA (dsRNA) constructs and/or siRNA constructs, shRNA constructs and/or microRNA constructs and methods of their design. In addition, these siRNA constructs may be synthetic molecules encoded in an expression vector (one or both strands) for delivery into cells. Such vectors include, but are not limited to adeno-associated viral vectors such as vector genomes of any of the AAV serotypes or other viral delivery vehicles such as lentivirus, etc.

[0015] The present disclosure also provides novel methods for the delivery and/or transmission of the AAV vectors and viral genomes of the disclosure, which may be applied to other disorders associated with the spinal cord, such as, but not limited to, the larger family of motor neuron disorders, neuropathies, diseases of myelination, and proprioceptive, somatosensory and/or sensory disorders.

SUMMARY OF THE DISCLOSURE

[0016] The present disclosure provides AAV vectors encoding a SOD1 targeting polynucleotide to interfere with SOD1 gene expression and/or SOD1 protein production and methods of use thereof. Methods for treating diseases associated with motor neuron degeneration such as amyotrophic lateral sclerosis are also included in the present disclosure.

[0017] In certain embodiments, SOD1 is suppressed 30% in a subject treated with an AAV encoding a SOD1 targeting polynucleotide as compared to an untreated subject. The subject may be administered the AAV in an infusion or as a bolus at a pre-determined dose level. As a non-limiting example, the suppression is seen in the C1 to L7 ventral horn region.

[0018] The present disclosure relates to RNA molecule mediated gene specific interference with gene expression and protein production. Methods for treating diseases associated with motor neuron degeneration, such as amyotrophic lateral sclerosis are also included in the present disclosure. The siRNA included in the compositions featured herein encompass a dsRNA having an antisense strand (the antisense or guide strand) having a

region that is 30 nucleotides or less, generally 19-24 nucleotides in length, that is substantially complementary to at least part of an mRNA transcript of the SOD1 gene.

[0019] According to the present disclosure, each strand of the siRNA duplex targeting the SOD1 gene is about 19-25 nucleotides in length, preferably about 19 nucleotides, 20 nucleotides, 21 nucleotides, 22 nucleotides, 23 nucleotides, 24 nucleotides, or 25 nucleotides in length. In some aspects, the siRNAs may be unmodified RNA molecules.

[0020] In certain embodiments, an siRNA or dsRNA includes at least two sequences that are complementary to each other. The dsRNA includes a sense strand having a first sequence and an antisense strand having a second sequence. The antisense strand includes a nucleotide sequence that is substantially complementary to at least part of an mRNA encoding SOD1, and the region of complementarity is 30 nucleotides or less, and at least 15 nucleotides in length. Generally, the dsRNA is 19 to 24, e.g., 19 to 21 nucleotides in length. In some embodiments the dsRNA is from about 15 to about 25 nucleotides in length, and in other embodiments the dsRNA is from about 25 to about 30 nucleotides in length.

[0021] According to the present disclosure, AAV vectors comprising the nucleic acids encoding the siRNA duplexes, one strand of the siRNA duplex or the dsRNA targeting SOD1 or other neurodegenerative associated gene or spinal cord disease associated gene are produced, the AAV vector serotype may be AAV1, AAV2, AAV2G9, AAV3, AAV3a, AAV3b, AAV3-3, AAV4, AAV4-4, AAV5, AAV6, AAV6.1, AAV6.2, AAV6.1.2, AAV7, AAV7.2, AAV8, AAV9, AAV9.11, AAV9.13, AAV9.16, AAV9.24, AAV9.45, AAV9.47, AAV9.61, AAV9.68, AAV9.84, AAV9.9, AAV10, AAV11, AAV12, AAV16.3, AAV24.1, AAV27.3, AAV42.12, AAV42-1b, AAV42-2, AAV42-3a, AAV42-3b, AAV42-4, AAV42-5a, AAV42-5b, AAV42-6b, AAV42-8, AAV42-10, AAV42-11, AAV42-12, AAV42-13, AAV42-15, AAV42-aa, AAV43-1, AAV43-12, AAV43-20, AAV43-21, AAV43-23, AAV43-25, AAV43-5, AAV44.1, AAV44.2, AAV44.5, AAV223.1, AAV223.2, AAV223.4, AAV223.5, AAV223.6, AAV223.7, AAV1-7/rh.48, AAV1-8/rh.49, AAV2-15/rh.62, AAV2-3/rh.61, AAV2-4/rh.50, AAV2-5/rh.51, AAV3.1/hu.6, AAV3.1/hu.9, AAV3-9/rh.52, AAV3-11/rh.53, AAV4-8/rh.64, AAV4-9/rh.54, AAV4-19/rh.55, AAV5-3/rh.57, AAV5-22/rh.58, AAV7.3/hu.7, AAV16.8/hu.10, AAV16.12/hu.11, AAV29.3/bb.1, AAV29.5/bb.2, AAV106.1/hu.37, AAV114.3/hu.40, AAV127.2/hu.41, AAV127.5/hu.42, AAV128.3/hu.44, AAV130.4/hu.48, AAV145.1/hu.53, AAV145.5/hu.54, AAV145.6/hu.55, AAV161.10/hu.60, AAV161.6/hu.61, AAV33.12/hu.17, AAV33.4/hu.15, AAV33.8/hu.16, AAV52/hu.19, AAV52.1/hu.20, AAV58.2/hu.25, AAVA3.3, AAVA3.4, AAVA3.5, AAVA3.7, AAVC1,

AAVC2, AAVC5, AAV-DJ, AAV-DJ8, AAVF3, AAVF5, AAVH2, AAVrh.72, AAVhu.8, AAVrh.68, AAVrh.70, AAVpi.1, AAVpi.3, AAVpi.2, AAVrh.60, AAVrh.44, AAVrh.65, AAVrh.55, AAVrh.47, AAVrh.69, AAVrh.45, AAVrh.59, AAVhu.12, AAVH6, AAVLK03, AAVH-1/hu.1, AAVH-5/hu.3, AAVLG-10/rh.40, AAVLG-4/rh.38, AAVLG-9/hu.39, AAVN721-8/rh.43, AAVCh.5, AAVCh.5R1, AAVcy.2, AAVcy.3, AAVcy.4, AAVcy.5, AAVCy.5R1, AAVCy.5R2, AAVCy.5R3, AAVCy.5R4, AAVcy.6, AAVhu.1, AAVhu.2, AAVhu.3, AAVhu.4, AAVhu.5, AAVhu.6, AAVhu.7, AAVhu.9, AAVhu.10, AAVhu.11, AAVhu.13, AAVhu.15, AAVhu.16, AAVhu.17, AAVhu.18, AAVhu.20, AAVhu.21, AAVhu.22, AAVhu.23.2, AAVhu.24, AAVhu.25, AAVhu.27, AAVhu.28, AAVhu.29, AAVhu.29R, AAVhu.31, AAVhu.32, AAVhu.34, AAVhu.35, AAVhu.37, AAVhu.39, AAVhu.40, AAVhu.41, AAVhu.42, AAVhu.43, AAVhu.44, AAVhu.44R1, AAVhu.44R2, AAVhu.44R3, AAVhu.45, AAVhu.46, AAVhu.47, AAVhu.48, AAVhu.48R1, AAVhu.48R2, AAVhu.48R3, AAVhu.49, AAVhu.51, AAVhu.52, AAVhu.54, AAVhu.55, AAVhu.56, AAVhu.57, AAVhu.58, AAVhu.60, AAVhu.61, AAVhu.63, AAVhu.64, AAVhu.66, AAVhu.67, AAVhu.14/9, AAVhu.t 19, AAVrh.2, AAVrh.2R, AAVrh.8, AAVrh.8R, AAVrh.10, AAVrh.12, AAVrh.13, AAVrh.13R, AAVrh.14, AAVrh.17, AAVrh.18, AAVrh.19, AAVrh.20, AAVrh.21, AAVrh.22, AAVrh.23, AAVrh.24, AAVrh.25, AAVrh.31, AAVrh.32, AAVrh.33, AAVrh.34, AAVrh.35, AAVrh.36, AAVrh.37, AAVrh.37R2, AAVrh.38, AAVrh.39, AAVrh.40, AAVrh.46, AAVrh.48, AAVrh.48.1, AAVrh.48.1.2, AAVrh.48.2, AAVrh.49, AAVrh.51, AAVrh.52, AAVrh.53, AAVrh.54, AAVrh.56, AAVrh.57, AAVrh.58, AAVrh.61, AAVrh.64, AAVrh.64R1, AAVrh.64R2, AAVrh.67, AAVrh.73, AAVrh.74, AAVrh8R, AAVrh8R A586R mutant, AAVrh8R R533A mutant, AAAV, BAAV, caprine AAV, bovine AAV, AAVhE1.1, AAVhEr1.5, AAVhER1.14, AAVhEr1.8, AAVhEr1.16, AAVhEr1.18, AAVhEr1.35, AAVhEr1.7, AAVhEr1.36, AAVhEr2.29, AAVhEr2.4, AAVhEr2.16, AAVhEr2.30, AAVhEr2.31, AAVhEr2.36, AAVhER1.23, AAVhEr3.1, AAV2.5T, AAV-PAEC, AAV-LK01, AAV-LK02, AAV-LK03, AAV-LK04, AAV-LK05, AAV-LK06, AAV-LK07, AAV-LK08, AAV-LK09, AAV-LK10, AAV-LK11, AAV-LK12, AAV-LK13, AAV-LK14, AAV-LK15, AAV-LK16, AAV-LK17, AAV-LK18, AAV-LK19, AAV-PAEC2, AAV-PAEC4, AAV-PAEC6, AAV-PAEC7, AAV-PAEC8, AAV-PAEC11, AAV-PAEC12, AAV-2-pre-miRNA-101, AAV-8h, AAV-8b, AAV-h, AAV-b, AAV SM 10-2, AAV Shuffle 100-1, AAV Shuffle 100-3, AAV Shuffle 100-7, AAV Shuffle 10-2, AAV Shuffle 10-6, AAV Shuffle 10-8, AAV Shuffle 100-2, AAV SM 10-1, AAV SM 10-8, AAV SM 100-3, AAV

SM 100-10, BNP61 AAV, BNP62 AAV, BNP63 AAV, AAVrh.50, AAVrh.43, AAVrh.62, AAVrh.48, AAVhu.19, AAVhu.11, AAVhu.53, AAV4-8/rh.64, AAVLG-9/hu.39, AAV54.5/hu.23, AAV54.2/hu.22, AAV54.7/hu.24, AAV54.1/hu.21, AAV54.4R/hu.27, AAV46.2/hu.28, AAV46.6/hu.29, AAVI28.1/hu.43, true type AAV (ttAAV), UPENN AAV 10, Japanese AAV 10 serotypes, AAV CBr-7.1, AAV CBr-7.10, AAV CBr-7.2, AAV CBr-7.3, AAV CBr-7.4, AAV CBr-7.5, AAV CBr-7.7, AAV CBr-7.8, AAV CBr-B7.3, AAV CBr-B7.4, AAV CBr-E1, AAV CBr-E2, AAV CBr-E3, AAV CBr-E4, AAV CBr-E5, AAV CBr-e5, AAV CBr-E6, AAV CBr-E7, AAV CBr-E8, AAV CHt-1, AAV CHt-2, AAV CHt-3, AAV CHt-6.1, AAV CHt-6.10, AAV CHt-6.5, AAV CHt-6.6, AAV CHt-6.7, AAV CHt-6.8, AAV CHt-P1, AAV CHt-P2, AAV CHt-P5, AAV CHt-P6, AAV CHt-P8, AAV CHt-P9, AAV CKd-1, AAV CKd-10, AAV CKd-2, AAV CKd-3, AAV CKd-4, AAV CKd-6, AAV CKd-7, AAV CKd-8, AAV CKd-B1, AAV CKd-B2, AAV CKd-B3, AAV CKd-B4, AAV CKd-B5, AAV CKd-B6, AAV CKd-B7, AAV CKd-B8, AAV CKd-H1, AAV CKd-H2, AAV CKd-H3, AAV CKd-H4, AAV CKd-H5, AAV CKd-H6, AAV CKd-N3, AAV CKd-N4, AAV CKd-N9, AAV CLg-F1, AAV CLg-F2, AAV CLg-F3, AAV CLg-F4, AAV CLg-F5, AAV CLg-F6, AAV CLg-F7, AAV CLg-F8, AAV CLv-1, AAV CLv1-1, AAV Clv1-10, AAV CLv1-2, AAV CLv-12, AAV CLv1-3, AAV CLv-13, AAV CLv1-4, AAV Clv1-7, AAV Clv1-8, AAV Clv1-9, AAV CLv-2, AAV CLv-3, AAV CLv-4, AAV CLv-6, AAV CLv-8, AAV CLv-D1, AAV CLv-D2, AAV CLv-D3, AAV CLv-D4, AAV CLv-D5, AAV CLv-D6, AAV CLv-D7, AAV CLv-D8, AAV CLv-E1, AAV CLv-K1, AAV CLv-K3, AAV CLv-K6, AAV CLv-L4, AAV CLv-L5, AAV CLv-L6, AAV CLv-M1, AAV CLv-M11, AAV CLv-M2, AAV CLv-M5, AAV CLv-M6, AAV CLv-M7, AAV CLv-M8, AAV CLv-M9, AAV CLv-R1, AAV CLv-R2, AAV CLv-R3, AAV CLv-R4, AAV CLv-R5, AAV CLv-R6, AAV CLv-R7, AAV CLv-R8, AAV CLv-R9, AAV CSp-1, AAV CSp-10, AAV CSp-11, AAV CSp-2, AAV CSp-3, AAV CSp-4, AAV CSp-6, AAV CSp-7, AAV CSp-8, AAV CSp-8.10, AAV CSp-8.2, AAV CSp-8.4, AAV CSp-8.5, AAV CSp-8.6, AAV CSp-8.7, AAV CSp-8.8, AAV CSp-8.9, AAV CSp-9, AAV.hu.48R3, AAV.VR-355, AAV3B, AAV4, AAV5, AAVF1/HSC1, AAVF11/HSC11, AAVF12/HSC12, AAVF13/HSC13, AAVF14/HSC14, AAVF15/HSC15, AAVF16/HSC16, AAVF17/HSC17, AAVF2/HSC2, AAVF3/HSC3, AAVF4/HSC4, AAVF5/HSC5, AAVF6/HSC6, AAVF7/HSC7, AAVF8/HSC8, AAVF9/HSC9, AAV-PHP.B, AAV-PHP.A, G2B-26, G2B-13, TH1.1-32, TH1.1-35, AAVPHP.B2, AAVPHP.B3, AAVPHP.N/PHP.B-DGT, AAVPHP.B-EST, AAVPHP.B-GGT, AAVPHP.B-ATP, AAVPHP.B-ATT-T, AAVPHP.B-DGT-T,

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[0022] The present disclosure also provides pharmaceutical compositions comprising at least one siRNA duplex targeting the SOD1 gene and a pharmaceutically acceptable carrier. In some aspects, a nucleic acid sequence encoding the siRNA duplex is inserted into an AAV vector.

[0023] In some embodiments, the present disclosure provides methods for inhibiting/silencing of SOD1 gene expression in a cell. Accordingly, the siRNA duplexes or dsRNA can be used to substantially inhibit SOD1 gene expression in a cell, in particular in a motor neuron. In some aspects, the inhibition of SOD1 gene expression refers to an inhibition by at least about 20%, preferably by at least about 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95% and 100%. Accordingly, the protein product of the targeted gene may be inhibited by at least about 20%, preferably by at least about 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95% and 100%. The SOD1 gene can be either a wild type gene or a mutated SOD1 gene with at least one mutation. Accordingly, the SOD1 protein is either wild type protein or a mutated polypeptide with at least one mutation.

[0024] In some embodiments, the present disclosure provides methods for treating, or ameliorating amyotrophic lateral sclerosis associated with abnormal SOD1 gene and/or SOD1 protein in a subject in need of treatment, the method comprising administering to the subject a pharmaceutically effective amount of at least one siRNA duplex targeting the SOD1 gene, delivering said siRNA duplex into targeted cells, inhibiting SOD1 gene expression and protein production, and ameliorating symptoms of ALS in the subject.

[0025] In some embodiments, the AAV vector genome may include a promoter. In one aspect, the promoter may be HI. In some embodiments, the AAV vector genome may include a filler sequence. The filler sequence may be derived from a lentivirus. In some embodiments, the filler may be derived from a mammalian albumin gene. In some embodiments the mammalian albumin gene is the human albumin gene.

[0026] In some aspects, ALS is familial ALS linked to SOD1 mutations. In other aspects, ALS is sporadic ALS which is characterized by abnormal aggregation of SOD1 protein or

disruption of SOD1 protein function or localization, though not necessarily as a result of genetic mutation. The symptoms of ALS ameliorated by the present method may include motor neuron degeneration, muscle weakness, stiffness of muscles, slurred speech and /or difficulty in breathing.

[0027] In some embodiments, the siRNA duplexes or dsRNA targeting SOD1 gene or the AAV vectors comprising such siRNA-encoding molecules may be introduced directly into the central nervous system of the subject, for example, by intracranial injection.

[0028] In some embodiments, the pharmaceutical composition of the present disclosure is used as a solo therapy. In other embodiments, the pharmaceutical composition of the present disclosure is used in combination therapy. The combination therapy may be in combination with one or more neuroprotective agents such as small molecule compounds, growth factors and hormones which have been tested for their neuroprotective effect on motor neuron degeneration.

[0029] In some embodiments, the present disclosure provides methods for treating, or ameliorating amyotrophic lateral sclerosis by administering to a subject in need thereof a therapeutically effective amount of a plasmid or AAV vector described herein. The ALS may be familial ALS or sporadic ALS.

[0030] The methods may involve administering AAV particles to the subject intraparenchymally at one or more sites. The methods may involve administering AAV particles to the subject intraparenchymally into the spinal cord. In some aspects, the AAV particles may be administered to two sites within the spinal cord. In some embodiments, AAV particles may be administered at two sites within the cervical spinal cord. In some embodiments, AAV particles may be administered at levels C3 and C5 of the spinal cord. In certain embodiments, the volume of administration is from about 5 μ L to about 240 μ L at level C3 of the spinal cord and from about 5 μ L to about 240 μ L at level C5 of the spinal cord. In certain embodiments, the volume of administration may be from about 5 μ L to about 60 μ L at level C3 of the spinal cord and from about 5 μ L to about 60 μ L at level C5 of the spinal cord. In one aspect, the volume of administration may be from about 25 to about 40 μ L at level C3 of the spinal cord and from about 25 to about 40 μ L at level C5 of the spinal cord. The dose administered to the spinal cord may be from about 1×10^{10} vg to about 1×10^{12} vg at level C3 of the spinal cord and from about 1×10^{10} vg to about 1×10^{12} vg at level C5 of the spinal cord. In some aspects, the dose administered to the spinal cord may be from about 5×10^{11} vg to about 8×10^{11} vg at level C3 of the spinal cord and from about 5×10^{11} vg to about

8×10^{11} vg at level C5 of the spinal cord. In certain embodiments, the dose may be from about 2×10^{10} vg to about 7×10^{11} vg at level C3 of the spinal cord and from about 2×10^{10} vg to about 7×10^{12} vg at level C5 of the spinal cord. In one aspect, the injection rate may be 5 μ L/min.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] The foregoing and other objects, features and advantages will be apparent from the following description of particular embodiments of the disclosure, as illustrated in the accompanying drawings. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of various embodiments of the disclosure.

[0032] Figure 1 shows the dose response curve for human SOD1 mRNA expression with different nM concentrations of siRNA.

[0033] Figure 2 shows SOD1 mRNA knockdown in SK-RST cell line.

DETAILED DESCRIPTION

[0034] The present disclosure relates to SOD1 targeting polynucleotides as therapeutic agents. RNA interfering mediated gene silencing can specifically inhibit gene expression. The present disclosure therefore provides polynucleotides such as small double stranded RNA (dsRNA) molecules (small interfering RNA, siRNA), shRNA, microRNA and precursors thereof targeting SOD1 gene, pharmaceutical compositions encompassing such polynucleotides, as well as processes of their design. The present disclosure also provides methods of their use for inhibiting SOD1 gene expression and protein production, for treating disorders associated with the spinal cord and/or neurodegenerative disease, in particular, amyotrophic lateral sclerosis (ALS).

[0035] The details of one or more embodiments of the disclosure are set forth in the accompanying description below. Although any materials and methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred materials and methods are now described. Other features, objects and advantages of the invention will be apparent from the description. In the description, the singular forms also include the plural unless the context clearly dictates otherwise. 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. In the case of conflict, the present description will control.

Disorders associated with the spinal cord

[0036] The spinal cord is one of two components that together characterize the central nervous system (CNS; brain and spinal cord). The spinal cord connects the body to the brain,

servicing as a conduit for the messages and communications necessary for movement and sensation. The spinal cord is a fragile, thin, tubular bundle made up of nerve fibers and cell bodies, as well as support cells, housed within the vertebral column.

[0037] The motor neurons and pathways of the spinal cord are important for the initiation, execution, modification, and precision of movement. When these neurons and/or pathways are damaged in some manner, such as, but not limited to, trauma, tumorous growth, cardiovascular defects, inflammation, de-myelination, neuropathy, degeneration and/or cell death, the consequence is typically a defect in some form of movement. Similarly, sensory neurons and pathways of the spinal cord are critical for proprioception and sensation, and when damaged, can result in an inability to sense certain stimuli and/or pain syndromes.

[0038] Non-limiting examples of disorders such as those described above, which are associated with the spinal cord include, but are not limited to, motor neuron disease, amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease), progressive bulbar palsy, pseudobulbar palsy, primary lateral sclerosis, progressive muscular atrophy, spinal muscular atrophy, post-polio syndrome, bulbar palsy, Kennedy's disease, hereditary spastic paraplegia, Friedreich's ataxia, Charcot-Marie-Tooth disease, hereditary motor and sensory neuropathy, peroneal muscular atrophy, neuropathies, de-myelinating diseases, viral de-myelination, metabolic de-myelination, multiple sclerosis, neuromyelitis optica (Devic's disease), concentric sclerosis (Baló's sclerosis), ataxias, paraplegia, spinocerebellar ataxia, acute-disseminated encephalomyelitis, complex regional pain syndrome (CPRS I and CPRS II), ataxia telangiectasia, episodic ataxia, multiple system atrophy, sporadic ataxia, lipid storage diseases, Niemann-Pick disease, Fabry disease, Faber's disease, GM1 or GM2 gangliosidoses, Tay-Sachs disease, Sandhoff disease, Krabbe disease, metachromatic leukodystrophy, Machado-Joseph disease (spinocerebellar ataxia type 3), meningitis, myelitis, myopathy, mitochondrial myopathy, encephalomyopathy, Barth syndrome, Chronic progressive external ophthalmoplegia, Kearns-Sayre syndrome, Leigh syndrome, mitochondrial DNA depletion syndromes, myoclonus epilepsy with ragged red fibers, NARP (neuropathy, ataxia and retinitis pigmentosa), diseases of the neuromuscular junction, myasthenia gravis, myoclonus, neuropathic pain, neurodegenerative diseases, Parkinson's disease, Alzheimer's disease, Huntington's disease, Lewy body disease, Vitamin B12 deficiency, subacute combined degeneration of the spinal cord (Lichtheim's disease), tropical spastic paraparesis, distal hereditary motor neuronopathies, Morvan's syndrome, leukodystrophies, and/or Rett syndrome.

[0039] In certain embodiments, the compositions and methods of the present disclosure may be used to treat any disease of the central nervous system.

[0040] In certain embodiments, the compositions and methods of the present disclosure may be used to treat a disease associated with the spinal cord.

[0041] In certain embodiments, the compositions and methods of the present disclosure may be used for the treatment of a neurodegenerative disease.

[0042] In certain embodiments, the compositions and methods of the present disclosure may be used for the treatment of a motor neuron disease.

[0043] In certain embodiments, the compositions and methods of the present disclosure may be used for the treatment of amyotrophic lateral sclerosis (ALS).

Amyotrophic lateral sclerosis (ALS) and SOD1

[0044] Amyotrophic lateral sclerosis (ALS), an adult-onset neurodegenerative disorder, is a progressive and fatal disease characterized by the selective death of motor neurons in the motor cortex, brainstem and spinal cord. Patients diagnosed with ALS develop a progressive muscle phenotype characterized by spasticity, hyperreflexia or hyporeflexia, fasciculations, muscle atrophy and paralysis. These motor impairments are caused by the de-innervation of muscles due to the loss of motor neurons. The major pathological features of ALS include degeneration of the corticospinal tracts and extensive loss of lower motor neurons (LMNs) or anterior horn cells (Ghatak et al., *J Neuropathol Exp Neurol.*, 1986, 45, 385-395), degeneration and loss of Betz cells and other pyramidal cells in the primary motor cortex (Udaka et al., *Acta Neuropathol.*, 1986, 70, 289-295; Maekawa et al., *Brain.*, 2004, 127, 1237-1251) and reactive gliosis in the motor cortex and spinal cord (Kawamata et al., *Am J Pathol.*, 1992, 140, 691-707; and Schiffer et al., *J Neurol Sci.*, 1996, 139, 27-33). ALS is usually fatal within 3 to 5 years after the diagnosis due to respiratory defects and/or inflammation (Rowland LP and Shneibder NA, *N Engl. J. Med.*, 2001, 344, 1688-1700).

[0045] A cellular hallmark of ALS is the presence of proteinaceous, ubiquitinated, cytoplasmic inclusions in degenerating motor neurons and surrounding cells (e.g., astrocytes). Ubiquitinated inclusions (i.e., Lewy body-like inclusions or Skein-like inclusions) are the most common and specific type of inclusion in ALS and are found in LMNs of the spinal cord and brainstem, and in corticospinal upper motor neurons (UMNs) (Matsumoto et al., *J Neurol Sci.*, 1993, 115, 208-213; and Sasak and Maruyama, *Acta Neuropathol.*, 1994, 87, 578-585). A few proteins have been identified to be components of the inclusions, including ubiquitin, Cu/Zn superoxide dismutase 1 (SOD1), peripherin and Dornin. Neurofilamentous

inclusions are often found in hyaline conglomerate inclusions (HCIs) and axonal 'spheroids' in spinal cord motor neurons in ALS. Other types and less specific inclusions include Bunina bodies (cystatin C-containing inclusions) and Crescent shaped inclusions (SCIs) in upper layers of the cortex. Other neuropathological features seen in ALS include fragmentation of the Golgi apparatus, mitochondrial vacuolization and ultrastructural abnormalities of synaptic terminals (Fujita et al., *Acta Neuropathol.* 2002, 103, 243-247).

[0046] In addition, in frontotemporal dementia ALS (FTD-ALS), cortical atrophy (including the frontal and temporal lobes) is also observed, which may cause cognitive impairment in FTD-ALS patients.

[0047] ALS is a complex and multifactorial disease and multiple mechanisms hypothesized as responsible for ALS pathogenesis include dysfunction of protein degradation, glutamate excitotoxicity, mitochondrial dysfunction, apoptosis, oxidative stress, inflammation, protein misfolding and aggregation, aberrant RNA metabolism, and altered gene expression.

[0048] About 10% of ALS cases have family history of the disease, and these patients are referred to as familial ALS (fALS) or inherited patients, commonly with a Mendelian dominant mode of inheritance and high penetrance. The remainder (approximately 90%-95%) is classified as sporadic ALS (sALS), as they are not associated with a documented family history, which is thought to be due to other risk factors, including environmental factors, genetic polymorphisms, somatic mutations, and possibly gene-environmental interactions. In most cases, familial (or inherited) ALS is inherited as autosomal dominant disease, but pedigrees with autosomal recessive and X-linked inheritance and incomplete penetrance exist. Sporadic and familial forms are clinically indistinguishable, suggesting a common pathogenesis. The precise cause of the selective death of motor neurons in ALS remains elusive. Progress in understanding the genetic factors in fALS may shed light on both forms of the disease.

[0049] Recently, an explosion in research and understanding of genetic causes of ALS has led to the discovery of mutations in more than 10 different genes now known to cause fALS. The most common ones are found in the genes encoding Cu/Zn superoxide dismutase 1 (SOD1; ~ 20%) (Rosen DR et al., *Nature*, 1993, 362, 59-62), fused in sarcoma/translated in liposarcoma (FUS/TLS; 1-5%) and TDP-43 (TARDBP; 1-5%). Recently, a hexanucleotide repeat expansion (GGGGCC)_n in the C9orf72 gene was identified as the most frequent cause of fALS (~ 40%) in the Western population (reviewed by Renton et al., *Nat. Neurosci.*, 2014,

17, 17-23). Other genes mutated in ALS include alsin (ALS2), senataxin (SETX), vesicle-associated membrane protein (VAPB), angiogenin (ANG). fALS genes control different cellular mechanisms, suggesting that the pathogenesis of ALS is complicated and may be related to several different processes finally leading to motor neuron degeneration.

[0050] SOD1 is one of the three human superoxide dismutases identified and characterized in mammals: copper-zinc superoxide dismutase (Cu/ZnSOD or SOD1), manganese superoxide dismutase (MnSOD or SOD2), and extracellular superoxide dismutase (ECSOD or SOD3). SOD1 is a 32 kDa homodimer of a 153-residue polypeptide with one copper- and one zinc-binding site per subunit, which is encoded by SOD1 gene (GeneBank access No.: NM_000454.4) on human chromosome 21 (see Table 10). SOD1 catalyzes the reaction of superoxide anion ($O_2^{\cdot-}$) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) at a bound copper ion. The intracellular concentration of SOD1 is high (ranging from 10 to 100 μ M), accounting for 1% of the total protein content in the central nervous system (CNS). The protein is localized not only in the cytoplasm but also in the nucleus, lysosomes, peroxisomes, and mitochondrial intermembrane spaces in eukaryotic cells (Lindenau J et al., *Glia*, 2000, 29, 25–34).

[0051] Mutations in SOD1 gene are carried by 15–20% of fALS patients and by 1-2% of all ALS cases. Currently, at least 170 different mutations distributed throughout the 153-amino acid SOD1 polypeptide have been found to cause ALS, and an updated list can be found at the ALS online Genetic Database (ALSOD) (Wroe R et al., *Amyotroph Lateral Scler.*, 2008, 9, 249-250). Table 1 lists some examples of mutations in SOD1 in ALS. These mutations are predominantly single amino acid substitutions (i.e. missense mutations) although deletions, insertions, and C-terminal truncations also occur. Different SOD1 mutations display different geographic distribution patterns. For instance, about half of all Americans with ALS caused by SOD1 gene mutations have a particular mutation Ala4Val (or A4V). The A4V mutation is typically associated with more severe signs and symptoms. The H113T mutation is by far the most common mutation in the United Kingdom. The most prevalent mutation in Europe is D90A substitution.

Table 1. Examples of SOD1 mutations in ALS

	Mutations
Exon1 (220bp)	Q22L; E21K, G; F20C; N19S; G16A, S; V14M, S; G12R; G10G, V, R; L8Q, V; V7E; C6G, F; V5L; A4T, V, S
Exon2 (97bp)	T54R; E49K; H48R, Q; V47F, A; H46R; F45C; H43R; G41S, D; G37R; V29, insA
Exon3 (70bp)	D76Y, V; G72S, C; L67R, P66A; N65S; S59I, S
Exon4 (118bp)	D124G, V; V118L, InsAAAAC; L117V; T116T; R115G; G114A; H113T, F; I112M, T; G108V; L106V, F; S106L,

	delTCACTC; I104F; D101G, Y, H, N; E100G, K; I99V; V97L, M; D96N, V; A95T, V; G93S, V, A, C, R, D; D90V, A; A89T, V; T88delACTGCTGAC; V87A, M; N86I, S, D, K; G85R, S; L84V, F; H80R
Exon5 (461bp)	I151T, S; I149T; V148I, G; G147D, R; C146R, stop; A145T, G; L144F, S; G141E, stop; A140A, G; N139D, K, H, N; G138E; T137R; S134N; E133V, delGAA, insTT; E132insTT; G127R, InsTGGG; L126S, delITT, stop; D126, delITT

[0052] To investigate the mechanism of neuronal death associated with SOD1 gene defects, several rodent models of *SOD1*-linked ALS were developed in the art, which express the human SOD1 gene with different mutations, including missense mutations, small deletions or insertions. Some examples of ALS mouse models include SOD1^{G93A}, SOD1^{A4V}, SOD1^{G37R}, SOD1^{G85R}, SOD1^{D90A}, SOD1^{L84V}, SOD1^{I113T}, SOD1^{H36R/H48Q}, SOD1^{G127X}, SOD1^{L126X} and SOD1^{L126delITT}. There are two transgene rat models carrying two different human SOD1 mutations: SOD1^{H46R} and SOD1^{G93R}. These rodent ALS models can develop muscle weakness similar to human ALS patients and other pathogenic features that reflect several characteristics of the human disease, in particular, the selective death of spinal motor neurons, aggregation of protein inclusions in motor neurons and microglial activation. It is well known in the art that the transgenic rodents are good models of human *SOD1*-associated ALS disease and provide models for studying disease pathogenesis and developing disease treatment.

[0053] Studies in animal and cellular models showed that SOD1 pathogenic variants cause ALS by gain of function. That is to say, the superoxide dismutase enzyme gains new but harmful properties when altered by SOD1 mutations. For example, some SOD1 mutated variants in ALS increase oxidative stress (e.g., increased accumulation of toxic superoxide radicals) by disrupting redox cycle. Other studies also indicate that some SOD1 mutated variants in ALS might acquire toxic properties that are independent of its normal physiological function (such as abnormal aggregation of misfolded SOD1 variants). In the aberrant redox chemistry model, mutant SOD1 is unstable and through aberrant chemistry interacts with nonconventional substrates causing reactive oxygen species (ROS) overproduction. In the protein toxicity model, unstable, misfolded SOD1 aggregates into cytoplasmic inclusion bodies, sequestering proteins crucial for cellular processes. These two hypotheses are not mutually exclusive. It has been shown that oxidation of selected histidine residues that bind metals in the active site mediates SOD1 aggregation.

[0054] The aggregated mutant SOD1 protein may also induce mitochondrial dysfunction (Vehvilainen P et al., *Front Cell Neurosci.*, 2014, 8, 126), impairment of axonal transport,

aberrant RNA metabolism, glial cell pathology and glutamate excitotoxicity. In some sporadic ALS cases, misfolded wild-type SOD1 protein is found in diseased motor neurons which forms “toxic conformation” that is similar to familial ALS-linked SOD1 variants (Rotunno MS and Bosco DA, *Front Cell Neurosci.*, 2013, 16, 7, 253). Such evidence suggests that ALS is a protein misfolding disease analogous to other neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease.

[0055] Currently, no curative treatments are available for patients suffering from ALS. Until recently, the only FDA approved drug was Riluzole (also called Rilutek), an inhibitor of glutamate release, with a moderate effect on ALS, only extending survival by 2-3 months if it is taken for 18 months. Unfortunately, patients taking riluzole do not experience any slowing in disease progression or improvement in muscle function. Therefore, riluzole does not present a cure, or even an effective treatment. In 2017, the FDA approved Radicava (edaravone) for the treatment of ALS, the first such approval in 22 years. Administered intravenously and serving as a free-radical scavenger and anti-oxidant, Radicava has been shown to slow disease progression. In a clinical Phase 3 trial (NCT01492686) of 137 patients, Radicava slowed the decline in physical function as compared to those patients taking placebo and as determined by score on the ALS Functional Rating Scale-Revised (ALSFRS-R) (Writing group; Edaravone (MCI-186) ALS 19 Study Group *Lancet Neurol.* 2017 Jul;16(7):505-512). The approval of Radicava is considered an advance in terms of treatment of ALS, however it is still not a cure. Researchers continue to search for better therapeutic agents.

[0056] One approach to inhibit abnormal SOD1 protein aggregation is to silence/inhibit SOD1 gene expression in ALS. It has been reported that small interfering RNAs for specific gene silencing of the mutated allele is therapeutically beneficial for the treatment of fALS (e.g., Ralgh GS et al., *Nat. Medicine*, 2005, 11(4), 429-433; and Raoul C et al., *Nat. Medicine*, 2005, 11(4), 423-428; and Maxwell MM et al., *PNAS*, 2004, 101(9), 3178-3183; and Ding H et al., *Chinese Medical J.*, 2011, 124(1), 106-110; and Scharz DS et al., *Plos Genet.*, 2006, 2(9), e140; the content of each of which is incorporated herein by reference in their entirety).

[0057] Many other RNA therapeutic agents that target SOD1 gene and modulate SOD1 expression in ALS are taught in the art, such RNA based agents include antisense oligonucleotides and double stranded small interfering RNAs. See, e.g., Wang H et al., *J Biol. Chem.*, 2008, 283(23), 15845-15852; U.S. Pat. Nos. 7,498,316; 7,632,938; 7,678,895;

7,951,784; 7,977,314; 8,183,219; 8,309,533 and 8, 586, 554; and U.S. Patent publication Nos. 2006/0229268 and 2011/0263680; the content of each of which is herein incorporated by reference in their entirety.

[0058] The present disclosure employs viral vectors such as adeno-associated viral (AAV) vectors to deliver siRNA duplexes or SOD1 targeting polynucleotides into cells with high efficiency. The AAV vectors comprising RNAi molecules, e.g., siRNA molecules of the present disclosure may increase the delivery of active agents into motor neurons. SOD1 targeting polynucleotides may be able to inhibit SOD1 gene expression (e.g., mRNA level) significantly inside cells; therefore, ameliorating SOD1 expression induced stress inside the cells such as aggregation of protein and formation of inclusions, increased free radicals, mitochondrial dysfunction and RNA metabolism.

[0059] Such SOD1 targeting polynucleotides may be used for treating ALS. According to the present disclosure, methods for treating and/or ameliorating ALS in a patient comprises administering to the patient an effective amount of at least one SOD1 targeting polynucleotide encoding one or more siRNA duplexes into cells and allowing the inhibition/silence of SOD1 gene expression, are provided.

Compositions

Vectors

[0060] In some embodiments, the siRNA molecules described herein can be inserted into, or encoded by, vectors such as plasmids or viral vectors. Preferably, the siRNA molecules are inserted into, or encoded by, viral vectors.

[0061] Viral vectors may be Herpesvirus (HSV) vectors, retroviral vectors, adenoviral vectors, adeno-associated viral vectors, lentiviral vectors, and the like. In some specific embodiments, the viral vectors are AAV vectors.

Retroviral vectors

[0062] In some embodiments, the siRNA duplex targeting SOD1 gene may be encoded by a retroviral vector (See, e.g., U.S. Pat. Nos. 5,399,346; 5,124,263; 4,650,764 and 4,980,289; the content of each of which is incorporated herein by reference in their entirety).

Adenoviral vectors

[0063] Adenoviruses are eukaryotic DNA viruses that can be modified to efficiently deliver a nucleic acid to a variety of cell types *in vivo*, and have been used extensively in gene therapy protocols, including for targeting genes to neural cells. Various replication defective adenovirus and minimum adenovirus vectors have been described for nucleic acid

therapeutics (See, e.g., PCT Patent Publication Nos. WO199426914, WO 199502697, WO199428152, WO199412649, WO199502697 and WO199622378; the content of each of which is incorporated by reference in their entirety). Such adenoviral vectors may also be used to deliver siRNA molecules of the present disclosure to cells.

Adeno-associated viral (AAV) vectors

[0064] An AAV is a dependent parvovirus. Like other parvoviruses, AAV is a single stranded, non-enveloped DNA virus, having a genome of about 5000 nucleotides in length containing two open reading frames that encode the proteins responsible for replication (Rep) and the structural protein of the capsid (Cap). The open reading frames are flanked by two Inverted Terminal Repeat (ITR) sequences, which serve as the origin of replication of viral genome. Furthermore, the AAV genome contains a packaging sequence, allowing packaging of the viral genome into an AAV capsid. The AAV vector requires co-helper (e.g., adenovirus) to undergo a productive infection in infected cells. In the absence of such helper functions, the AAV virions essentially enter host cells and integrate into cells' genome.

[0065] AAV vectors have been investigated for siRNA delivery because of its several unique features. These features include (i) ability to infect both dividing and non-dividing cells; (ii) a broad host range for infectivity, including human cells; (iii) wild-type AAV has never been associated with any disease and cannot replicate in infected cells; (iv) lack of cell-mediated immune response against the vector and (v) ability to integrate into a host chromosome or persist episomally, thereby creating potential for long-term expression. Moreover, infection with AAV vectors has minimal influence on changing the pattern of cellular gene expression (Stilwell and Samulski et al., *Biotechniques*, 2003, 34, 148).

[0066] Typically, AAV vectors for siRNA delivery may be recombinant viral vectors which are replication defective because of lacking sequences encoding functional Rep and Cap proteins in viral genome. In some cases, the defective AAV vectors may lack most of all coding sequences and essentially only contains one or two AAV ITR sequences and a packaging sequence.

[0067] AAV vectors may also comprise self-complementary AAV vectors (scAAVs). scAAV vectors contain both DNA strands which anneal together to form double stranded DNA. By skipping second strand synthesis, scAAVs allow for rapid expression in the cell.

[0068] Methods for producing/ modifying AAV vectors are disclosed in the art such as pseudotyped AAV vectors (PCT Patent Publication Nos. WO200028004; WO200123001;

WO2004112727; WO 2005005610 and WO 2005072364, the content of each of which is incorporated herein by reference in their entirety).

[0069] AAV vectors for delivering siRNA molecules into mammalian cells, may be prepared or derived from various serotypes of AAVs, including, but not limited to, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV9.47, AAV9(hu14), AAV10, AAV11, AAV12, AAVrh8, AAVrh10, AAV-DJ8 and AAV-DJ. In some cases, different serotypes of AAVs may be mixed together or with other types of viruses to produce chimeric AAV vectors.

[0070] In certain embodiments, the AAV serotype is AAVrh10.

[0071] AAV vectors for siRNA delivery may be modified to enhance the efficiency of delivery. Such modified AAV vectors containing the siRNA expression cassette can be packaged efficiently and can be used to infect successfully the target cells at high frequency and with minimal toxicity.

[0072] In some embodiments, the AAV vector for delivering siRNA duplexes of the present disclosure may be a human serotype AAV vector. Such human AAV vector may be derived from any known serotype, e.g., from any one of serotypes AAV1-AAV11. As non-limiting examples, AAV vectors may be vectors comprising an AAV1-derived genome in an AAV1-derived capsid; vectors comprising an AAV2-derived genome in an AAV2-derived genome; vectors comprising an AAV4-derived genome in an AAV4 derived capsid; vectors comprising an AAV6-derived genome in an AAV6 derived capsid or vectors comprising an AAV9-derived genome in an AAV9 derived capsid.

[0073] In other embodiments, the AAV vector for delivering siRNA duplexes of the present disclosure may be a pseudotyped AAV vector which contains sequences and/or components originating from at least two different AAV serotypes. Pseudotyped AAV vectors may be vectors comprising an AAV genome derived from one AAV serotype and a Capsid protein derived at least in part from a different AAV serotype. As non-limiting examples, such pseudotyped AAV vectors may be vectors comprising an AAV2-derived genome in an AAV1-derived capsid; or vectors comprising an AAV2-derived genome in an AAV6-derived capsid; or vectors comprising an AAV2-derived genome in an AAV4-derived capsid; or an AAV2-derived genome in an AAV9-derived capsid.

[0074] In other embodiments, AAV vectors may be used for delivering siRNA molecules to the central nervous system (e.g., U.S. Pat. No. 6,180,613; the content of which is herein incorporated by reference in its entirety).

[0075] In some aspects, the AAV vector for delivering siRNA duplexes of the present disclosure may further comprise a modified capsid including peptides from non-viral origin. In other aspects, the AAV vector may contain a CNS specific chimeric capsid to facilitate the delivery of siRNA duplexes into the brain and the spinal cord. For example, an alignment of cap nucleotide sequences from AAV variants exhibiting CNS tropism may be constructed to identify variable region (VR) sequence and structure.

[0076] The present disclosure refers to structural capsid proteins (including VP1, VP2 and VP3) which are encoded by capsid (Cap) genes. These capsid proteins form an outer protein structural shell (i.e. capsid) of a viral vector such as AAV. VP capsid proteins synthesized from Cap polynucleotides generally include a methionine as the first amino acid in the peptide sequence (Met1), which is associated with the start codon (AUG or ATG) in the corresponding Cap nucleotide sequence. However, it is common for a first-methionine (Met1) residue or generally any first amino acid (AA1) to be cleaved off after or during polypeptide synthesis by protein processing enzymes such as Met-aminopeptidases. This “Met/AA-clipping” process often correlates with a corresponding acetylation of the second amino acid in the polypeptide sequence (e.g., alanine, valine, serine, threonine, etc.). Met-clipping commonly occurs with VP1 and VP3 capsid proteins but can also occur with VP2 capsid proteins.

[0077] Where the Met/AA-clipping is incomplete, a mixture of one or more (one, two or three) VP capsid proteins comprising the viral capsid may be produced, some of which may include a Met1/AA1 amino acid (Met+/AA+) and some of which may lack a Met1/AA1 amino acid as a result of Met/AA-clipping (Met-/AA-). For further discussion regarding Met/AA-clipping in capsid proteins, see Jin, et al. Direct Liquid Chromatography/Mass Spectrometry Analysis for Complete Characterization of Recombinant Adeno-Associated Virus Capsid Proteins. *Hum Gene Ther Methods*. 2017 Oct. 28(5):255-267; Hwang, et al. N-Terminal Acetylation of Cellular Proteins Creates Specific Degradation Signals. *Science*. 2010 February 19. 327(5968): 973--977; the contents of which are each incorporated herein by reference in its entirety.

[0078] According to the present disclosure, references to capsid proteins is not limited to either clipped (Met-/AA-) or unclipped (Met+/AA+) and may, in context, refer to independent capsid proteins, viral capsids comprised of a mixture of capsid proteins, and/or polynucleotide sequences (or fragments thereof) which encode, describe, produce or result in capsid proteins of the present disclosure. A direct reference to a “capsid protein” or “capsid

polypeptide” (such as VP1, VP2 or VP2) may also comprise VP capsid proteins which include a Met1/AA1 amino acid (Met+/AA+) as well as corresponding VP capsid proteins which lack the Met1/AA1 amino acid as a result of Met/AA-clipping (Met-/AA-).

[0079] Further according to the present disclosure, a reference to a specific SEQ ID NO: (whether a protein or nucleic acid) which comprises or encodes, respectively, one or more capsid proteins which include a Met1/AA1 amino acid (Met+/AA+) should be understood to teach the VP capsid proteins which lack the Met1/AA1 amino acid as upon review of the sequence, it is readily apparent any sequence which merely lacks the first listed amino acid (whether or not Met1/AA1).

[0080] As a non-limiting example, reference to a VP1 polypeptide sequence which is 736 amino acids in length and which includes a “Met1” amino acid (Met+) encoded by the AUG/ATG start codon may also be understood to teach a VP1 polypeptide sequence which is 735 amino acids in length and which does not include the “Met1” amino acid (Met-) of the 736 amino acid Met+ sequence. As a second non-limiting example, reference to a VP1 polypeptide sequence which is 736 amino acids in length and which includes an “AA1” amino acid (AA1+) encoded by any NNN initiator codon may also be understood to teach a VP1 polypeptide sequence which is 735 amino acids in length and which does not include the “AA1” amino acid (AA1-) of the 736 amino acid AA1+ sequence.

[0081] References to viral capsids formed from VP capsid proteins (such as reference to specific AAV capsid serotypes), can incorporate VP capsid proteins which include a Met1/AA1 amino acid (Met+/AA1+), corresponding VP capsid proteins which lack the Met1/AA1 amino acid as a result of Met/AA1-clipping (Met-/AA1-), and combinations thereof (Met+/AA1+ and Met-/AA1-).

[0082] As a non-limiting example, an AAV capsid serotype can include VP1 (Met+/AA1+), VP1 (Met-/AA1-), or a combination of VP1 (Met+/AA1+) and VP1 (Met-/AA1-). An AAV capsid serotype can also include VP3 (Met+/AA1+), VP3 (Met-/AA1-), or a combination of VP3 (Met+/AA1+) and VP3 (Met-/AA1-); and can also include similar optional combinations of VP2 (Met+/AA1) and VP2 (Met-/AA1-).

Viral Genome

[0083] In certain embodiments, as shown in an AAV particle comprises a viral genome with a payload region.

Viral Genome Size

[0084] In certain embodiments, the viral genome which comprises a payload described herein, may be single stranded or double stranded viral genome. The size of the viral genome may be small, medium, large or the maximum size. Additionally, the viral genome may comprise a promoter and a polyA tail.

[0085] In certain embodiments, the viral genome which comprises a payload described herein, may be a small single stranded viral genome. A small single stranded viral genome may be 2.7 to 3.5 kb in size such as about 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, and 3.5 kb in size. As a non-limiting example, the small single stranded viral genome may be 3.2 kb in size. Additionally, the viral genome may comprise a promoter and a polyA tail.

[0086] In certain embodiments, the viral genome which comprises a payload described herein, may be a small double stranded viral genome. A small double stranded viral genome may be 1.3 to 1.7 kb in size such as about 1.3, 1.4, 1.5, 1.6, and 1.7 kb in size. As a non-limiting example, the small double stranded viral genome may be 1.6 kb in size. Additionally, the viral genome may comprise a promoter and a polyA tail.

[0087] In certain embodiments, the viral genome which comprises a payload described herein, may be a medium single stranded viral genome. A medium single stranded viral genome may be 3.6 to 4.3 kb in size such as about 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2 and 4.3 kb in size. As a non-limiting example, the medium single stranded viral genome may be 4.0 kb in size. Additionally, the viral genome may comprise a promoter and a polyA tail.

[0088] In certain embodiments, the viral genome which comprises a payload described herein, may be a medium double stranded viral genome. A medium double stranded viral genome may be 1.8 to 2.1 kb in size such as about 1.8, 1.9, 2.0, and 2.1 kb in size. As a non-limiting example, the medium double stranded viral genome may be 2.0 kb in size. Additionally, the viral genome may comprise a promoter and a polyA tail.

[0089] In certain embodiments, the viral genome which comprises a payload described herein, may be a large single stranded viral genome. A large single stranded viral genome may be 4.4 to 6.0 kb in size such as about 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9 and 6.0 kb in size. As a non-limiting example, the large single stranded viral genome may be 4.7 kb in size. As another non-limiting example, the large single stranded viral genome may be 4.8 kb in size. As yet another non-limiting example, the large single stranded viral genome may be 6.0 kb in size. Additionally, the viral genome may comprise a promoter and a polyA tail.

[0090] In certain embodiments, the viral genome which comprises a payload described herein, may be a large double stranded viral genome. A large double stranded viral genome may be 2.2 to 3.0 kb in size such as about 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9 and 3.0 kb in size. As a non-limiting example, the large double stranded viral genome may be 2.4 kb in size. Additionally, the viral genome may comprise a promoter and a polyA tail.

Viral Genome Component: Inverted Terminal Repeats (ITRs)

[0091] The AAV particles of the present disclosure comprise a viral genome with at least one ITR region and a payload region. In certain embodiments the viral genome has two ITRs. These two ITRs flank the payload region at the 5' and 3' ends. The ITRs function as origins of replication comprising recognition sites for replication. ITRs comprise sequence regions which can be complementary and symmetrically arranged. ITRs incorporated into viral genomes of the disclosure may be comprised of naturally occurring polynucleotide sequences or recombinantly derived polynucleotide sequences.

[0092] The ITRs may be derived from the same serotype as the capsid, selected from any of the serotypes herein, or a derivative thereof. The ITR may be of a different serotype from the capsid. In certain embodiments the AAV particle has more than one ITR. In a non-limiting example, the AAV particle has a viral genome comprising two ITRs. In certain embodiments the ITRs are of the same serotype as one another. In another embodiment the ITRs are of different serotypes. Non-limiting examples include zero, one or both of the ITRs having the same serotype as the capsid. In certain embodiments both ITRs of the viral genome of the AAV particle are AAV2 ITRs.

[0093] Independently, each ITR may be about 100 to about 150 nucleotides in length. An ITR may be about 100-105 nucleotides in length, 106-110 nucleotides in length, 111-115 nucleotides in length, 116-120 nucleotides in length, 121-125 nucleotides in length, 126-130 nucleotides in length, 131-135 nucleotides in length, 136-140 nucleotides in length, 141-145 nucleotides in length or 146-150 nucleotides in length. In certain embodiments the ITRs are 140-142 nucleotides in length. Non limiting examples of ITR length are 102, 140, 141, 142, 145 nucleotides in length, and those having at least 95% identity thereto.

[0094] In certain embodiments, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule which may be located near the 5' end of the flip ITR in an expression vector. In another embodiment, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located near the 3' end of the flip ITR in an expression vector. In yet another embodiment, the AAV particle comprises a nucleic acid

sequence encoding an siRNA molecule may be located near the 5' end of the flop ITR in an expression vector. In yet another embodiment, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located near the 3' end of the flop ITR in an expression vector. In certain embodiments, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located between the 5' end of the flip ITR and the 3' end of the flop ITR in an expression vector. In certain embodiments, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located between (e.g., half-way between the 5' end of the flip ITR and 3' end of the flop ITR or the 3' end of the flop ITR and the 5' end of the flip ITR), the 3' end of the flip ITR and the 5' end of the flip ITR in an expression vector. As a non-limiting example, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more than 30 nucleotides downstream from the 5' or 3' end of an ITR (e.g., Flip or Flop ITR) in an expression vector. As a non-limiting example, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more than 30 nucleotides upstream from the 5' or 3' end of an ITR (e.g., Flip or Flop ITR) in an expression vector. As another non-limiting example, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located within 1-5, 1-10, 1-15, 1-20, 1-25, 1-30, 5-10, 5-15, 5-20, 5-25, 5-30, 10-15, 10-20, 10-25, 10-30, 15-20, 15-25, 15-30, 20-25, 20-30 or 25-30 nucleotides downstream from the 5' or 3' end of an ITR (e.g., Flip or Flop ITR) in an expression vector. As another non-limiting example, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located within 1-5, 1-10, 1-15, 1-20, 1-25, 1-30, 5-10, 5-15, 5-20, 5-25, 5-30, 10-15, 10-20, 10-25, 10-30, 15-20, 15-25, 15-30, 20-25, 20-30 or 25-30 upstream from the 5' or 3' end of an ITR (e.g., Flip or Flop ITR) in an expression vector. As a non-limiting example, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located within the first 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25% or more than 25% of the nucleotides upstream from the 5' or 3' end of an ITR (e.g., Flip or Flop ITR) in an expression vector. As another non-limiting example, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located with the first 1-5%, 1-10%, 1-15%, 1-20%, 1-25%, 5-10%, 5-15%, 5-20%, 5-25%, 10-15%, 10-20%, 10-25%, 15-20%, 15-25%, or 20-25% downstream from the 5' or 3' end of an ITR (e.g., Flip or Flop ITR) in an expression vector.

Viral Genome Component: Promoters

[0095] In certain embodiments, the payload region of the viral genome comprises at least one element to enhance the transgene target specificity and expression (See e.g., Powell et al. *Viral Expression Cassette Elements to Enhance Transgene Target Specificity and Expression in Gene Therapy*, 2015; the contents of which are herein incorporated by reference in its entirety). Non-limiting examples of elements to enhance the transgene target specificity and expression include promoters, endogenous miRNAs, post-transcriptional regulatory elements (PREs), polyadenylation (PolyA) signal sequences and upstream enhancers (USEs), CMV enhancers and introns.

[0096] A person skilled in the art may recognize that expression of the polypeptides of the disclosure in a target cell may require a specific promoter, including but not limited to, a promoter that is species specific, inducible, tissue-specific, or cell cycle-specific (Parr et al., *Nat. Med.* 3:1145-9 (1997); the contents of which are herein incorporated by reference in their entirety).

[0097] In certain embodiments, the promoter is deemed to be efficient when it drives expression of the polypeptide(s) encoded in the payload region of the viral genome of the AAV particle.

[0098] In certain embodiments, the promoter is a promoter deemed to be efficient to drive the expression of the modulatory polynucleotide.

[0099] In certain embodiments, the promoter is a promoter deemed to be efficient when it drives expression in the cell being targeted.

[0100] In certain embodiments, the promoter drives expression of the payload for a period of time in targeted tissues. Expression driven by a promoter may be for a period of 1 hour, 2, hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 2 weeks, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 3 weeks, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 31 days, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 1 year, 13 months, 14 months, 15 months, 16 months, 17 months, 18 months, 19 months, 20 months, 21 months, 22 months, 23

months, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years, 10 years or more than 10 years. Expression may be for 1-5 hours, 1-12 hours, 1-2 days, 1-5 days, 1-2 weeks, 1-3 weeks, 1-4 weeks, 1-2 months, 1-4 months, 1-6 months, 2-6 months, 3-6 months, 3-9 months, 4-8 months, 6-12 months, 1-2 years, 1-5 years, 2-5 years, 3-6 years, 3-8 years, 4-8 years or 5-10 years.

[0101] In certain embodiments, the promoter drives expression of the payload for at least 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 1 year, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years, 10 years, 11 years, 12 years, 13 years, 14 years, 15 years, 16 years, 17 years, 18 years, 19 years, 20 years, 21 years, 22 years, 23 years, 24 years, 25 years, 26 years, 27 years, 28 years, 29 years, 30 years, 31 years, 32 years, 33 years, 34 years, 35 years, 36 years, 37 years, 38 years, 39 years, 40 years, 41 years, 42 years, 43 years, 44 years, 45 years, 46 years, 47 years, 48 years, 49 years, 50 years, 55 years, 60 years, 65 years, or more than 65 years.

[0102] Promoters may be naturally occurring or non-naturally occurring. Non-limiting examples of promoters include viral promoters, plant promoters and mammalian promoters. In some embodiments, the promoters may be human promoters. In some embodiments, the promoter may be truncated.

[0103] Promoters which drive or promote expression in most tissues include, but are not limited to, human elongation factor 1 α -subunit (EF1 α), cytomegalovirus (CMV) immediate-early enhancer and/or promoter, chicken β -actin (CBA) and its derivative CAG, β glucuronidase (GUSB), or ubiquitin C (UBC). Tissue-specific expression elements can be used to restrict expression to certain cell types such as, but not limited to, muscle specific promoters, B cell promoters, monocyte promoters, leukocyte promoters, macrophage promoters, pancreatic acinar cell promoters, endothelial cell promoters, lung tissue promoters, astrocyte promoters, or nervous system promoters which can be used to restrict expression to neurons, astrocytes, or oligodendrocytes.

[0104] Non-limiting examples of muscle-specific promoters include mammalian muscle creatine kinase (MCK) promoter, mammalian desmin (DES) promoter, mammalian troponin I (TNNI2) promoter, and mammalian skeletal alpha-actin (ASKA) promoter (see, e.g. U.S. Patent Publication US 20110212529, the contents of which are herein incorporated by reference in their entirety)

[0105] Non-limiting examples of tissue-specific expression elements for neurons include neuron-specific enolase (NSE), platelet-derived growth factor (PDGF), platelet-derived

growth factor B-chain (PDGF- β), synapsin (Syn), methyl-CpG binding protein 2 (MeCP2), Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), metabotropic glutamate receptor 2 (mGluR2), neurofilament light (NFL) or heavy (NFH), β -globin minigene n β 2, preproenkephalin (PPE), enkephalin (Enk) and excitatory amino acid transporter 2 (EAAT2) promoters. Non-limiting examples of tissue-specific expression elements for astrocytes include glial fibrillary acidic protein (GFAP) and EAAT2 promoters. A non-limiting example of a tissue-specific expression element for oligodendrocytes includes the myelin basic protein (MBP) promoter.

[0106] In certain embodiments, the promoter may be less than 1 kb. The promoter may have a length of 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800 or more than 800 nucleotides. The promoter may have a length between 200-300, 200-400, 200-500, 200-600, 200-700, 200-800, 300-400, 300-500, 300-600, 300-700, 300-800, 400-500, 400-600, 400-700, 400-800, 500-600, 500-700, 500-800, 600-700, 600-800 or 700-800.

[0107] In certain embodiments, the promoter may be a combination of two or more components of the same or different starting or parental promoters such as, but not limited to, CMV and CBA. Each component may have a length of 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800 or more than 800. Each component may have a length between 200-300, 200-400, 200-500, 200-600, 200-700, 200-800, 300-400, 300-500, 300-600, 300-700, 300-800, 400-500, 400-600, 400-700, 400-800, 500-600, 500-700, 500-800, 600-700, 600-800 or 700-800. In certain embodiments, the promoter is a combination of a 382 nucleotide CMV-enhancer sequence and a 260 nucleotide CBA-promoter sequence.

[0108] In certain embodiments, the viral genome comprises a ubiquitous promoter. Non-limiting examples of ubiquitous promoters include CMV, CBA (including derivatives CAG, CBh, etc.), EF-1 α , PGK, UBC, GUSB (hGBp), and UCOE (promoter of HNRPA2B1-CBX3).

[0109] Yu et al. (Molecular Pain 2011, 7:63; the contents of which are herein incorporated by reference in their entirety) evaluated the expression of eGFP under the CAG, EFl α , PGK

and UBC promoters in rat DRG cells and primary DRG cells using lentiviral vectors and found that UBC showed weaker expression than the other 3 promoters and only 10-12% glial expression was seen for all promoters. Soderblom et al. (E. Neuro 2015; the contents of which are herein incorporated by reference in its entirety) evaluated the expression of eGFP in AAV8 with CMV and UBC promoters and AAV2 with the CMV promoter after injection in the motor cortex. Intranasal administration of a plasmid containing a UBC or EFl α promoter showed a sustained airway expression greater than the expression with the CMV promoter (See e.g., Gill et al., Gene Therapy 2001, Vol. 8, 1539-1546; the contents of which are herein incorporated by reference in their entirety). Husain et al. (Gene Therapy 2009; the contents of which are herein incorporated by reference in its entirety) evaluated an H β H construct with a hGUSB promoter, a HSV-ILAT promoter and an NSE promoter and found that the H β H construct showed weaker expression than NSE in mouse brain. Passini and Wolfe (J. Virol. 2001, 12382-12392, the contents of which are herein incorporated by reference in its entirety) evaluated the long term effects of the H β H vector following an intraventricular injection in neonatal mice and found that there was sustained expression for at least 1 year. Low expression in all brain regions was found by Xu et al. (Gene Therapy 2001, 8, 1323-1332; the contents of which are herein incorporated by reference in their entirety) when NFL and NFH promoters were used as compared to the CMV-lacZ, CMV-luc, EF, GFAP, hENK, nAChR, PPE, PPE + wpre, NSE (0.3 kb), NSE (1.8 kb) and NSE (1.8 kb + wpre). Xu et al. found that the promoter activity in descending order was NSE (1.8 kb), EF, NSE (0.3 kb), GFAP, CMV, hENK, PPE, NFL and NFH. NFL is a 650 nucleotide promoter and NFH is a 920 nucleotide promoter which are both absent in the liver but NFH is abundant in the sensory proprioceptive neurons, brain and spinal cord and NFL is present in the heart. Scn8a is a 470 nucleotide promoter which expresses throughout the DRG, spinal cord and brain with particularly high expression seen in the hippocampal neurons and cerebellar Purkinje cells, cortex, thalamus and hypothalamus (See e.g., Drews et al. *Identification of evolutionary conserved, functional noncoding elements in the promoter region of the sodium channel gene SCN8A*, Mamm Genome (2007) 18:723-731; and Raymond et al. *Expression of Alternatively Spliced Sodium Channel α -subunit genes*, Journal of Biological Chemistry (2004) 279(44) 46234-46241; the contents of each of which are herein incorporated by reference in their entireties).

[0110] Any of promoters taught by the aforementioned Yu, Soderblom, Gill, Husain, Passini, Xu, Drews or Raymond may be used in the present compositions.

- [0111] In certain embodiments, the promoter is not cell specific.
- [0112] In certain embodiments, the promoter is a ubiquitin c (UBC) promoter. The UBC promoter may have a size of 300-350 nucleotides. As a non-limiting example, the UBC promoter is 332 nucleotides.
- [0113] In certain embodiments, the promoter is a β -glucuronidase (GUSB) promoter. The GUSB promoter may have a size of 350-400 nucleotides. As a non-limiting example, the GUSB promoter is 378 nucleotides.
- [0114] In certain embodiments, the promoter is a neurofilament light (NFL) promoter. The NFL promoter may have a size of 600-700 nucleotides. As a non-limiting example, the NFL promoter is 650 nucleotides. As a non-limiting example, the construct may be AAV-promoter-CMV/globin intron-modulatory polynucleotide-RBG, where the AAV may be self-complementary and the AAV may be the DJ serotype.
- [0115] In certain embodiments, the promoter is a neurofilament heavy (NFH) promoter. The NFH promoter may have a size of 900-950 nucleotides. As a non-limiting example, the NFH promoter is 920 nucleotides. As a non-limiting example, the construct may be AAV-promoter-CMV/globin intron-modulatory polynucleotide-RBG, where the AAV may be self-complementary and the AAV may be the DJ serotype.
- [0116] In certain embodiments, the promoter is a scn8a promoter. The scn8a promoter may have a size of 450-500 nucleotides. As a non-limiting example, the scn8a promoter is 470 nucleotides. As a non-limiting example, the construct may be AAV-promoter-CMV/globin intron-modulatory polynucleotide-RBG, where the AAV may be self-complementary and the AAV may be the DJ serotype.
- [0117] In certain embodiments, the viral genome comprises a Pol III promoter.
- [0118] In certain embodiments, the viral genome comprises a P1 promoter.
- [0119] In certain embodiments, the viral genome comprises a FXN promoter.
- [0120] In certain embodiments, the promoter is a phosphoglycerate kinase 1 (PGK) promoter.
- [0121] In certain embodiments, the promoter is a chicken β -actin (CBA) promoter.
- [0122] In certain embodiments, the promoter is a CAG promoter which is a construct comprising the cytomegalovirus (CMV) enhancer fused to the chicken beta-actin (CBA) promoter.
- [0123] In certain embodiments, the promoter is a cytomegalovirus (CMV) promoter.
- [0124] In certain embodiments, the viral genome comprises a HI promoter.

[0125] In certain embodiments, the viral genome comprises a U6 promoter.

[0126] In certain embodiments, the promoter is a liver or a skeletal muscle promoter. Non-limiting examples of liver promoters include human α -1-antitrypsin (hAAT) and thyroxine binding globulin (TBG). Non-limiting examples of skeletal muscle promoters include Desmin, MCK or synthetic C5-12.

[0127] In certain embodiments, the promoter is a RNA pol III promoter. As a non-limiting example, the RNA pol III promoter is U6. As a non-limiting example, the RNA pol III promoter is H1.

[0128] In certain embodiments, the viral genome comprises two promoters. As a non-limiting example, the promoters are an EF1 α promoter and a CMV promoter.

[0129] In certain embodiments, the viral genome comprises an enhancer element, a promoter and/or a 5' UTR intron. The enhancer element, also referred to herein as an "enhancer," may be, but is not limited to, a CMV enhancer, the promoter may be, but is not limited to, a CMV, CBA, UBC, GUSB, NSE, Synapsin, MeCP2, and GFAP promoter and the 5' UTR/intron may be, but is not limited to, SV40, and CBA-MVM. As a non-limiting example, the enhancer, promoter and/or intron used in combination may be: (1) CMV enhancer, CMV promoter, SV40 5' UTR intron; (2) CMV enhancer, CBA promoter, SV 40 5' UTR intron; (3) CMV enhancer, CBA promoter, CBA-MVM 5' UTR intron; (4) UBC promoter; (5) GUSB promoter; (6) NSE promoter; (7) Synapsin promoter; (8) MeCP2 promoter, (9) GFAP promoter, (10) H1 promoter; and (11) U6 promoter.

[0130] In certain embodiments, the viral genome comprises an engineered promoter.

[0131] In another embodiment the viral genome comprises a promoter from a naturally expressed protein.

Viral Genome Component: Untranslated Regions (UTRs)

[0132] By definition, wild type untranslated regions (UTRs) of a gene are transcribed but not translated. Generally, the 5' UTR starts at the transcription start site and ends at the start codon and the 3' UTR starts immediately following the stop codon and continues until the termination signal for transcription.

[0133] Features typically found in abundantly expressed genes of specific target organs may be engineered into UTRs to enhance the stability and protein production. As a non-limiting example, a 5' UTR from mRNA normally expressed in the liver (e.g., albumin, serum amyloid A, Apolipoprotein A/B/E, transferrin, alpha fetoprotein, erythropoietin, or

Factor VIII) may be used in the viral genomes of the AAV particles of the disclosure to enhance expression in hepatic cell lines or liver.

[0134] While not wishing to be bound by theory, wild-type 5' untranslated regions (UTRs) include features which play roles in translation initiation. Kozak sequences, which are commonly known to be involved in the process by which the ribosome initiates translation of many genes, are usually included in 5' UTRs. Kozak sequences have the consensus CCR(A/G) CCAUGG, where R is a purine (adenine or guanine) three bases upstream of the start codon (ATG), which is followed by another 'G'.

[0135] In certain embodiments, the 5'UTR in the viral genome includes a Kozak sequence.

[0136] In certain embodiments, the 5'UTR in the viral genome does not include a Kozak sequence.

[0137] While not wishing to be bound by theory, wild-type 3' UTRs are known to have stretches of Adenosines and Uridines embedded therein. These AU rich signatures are particularly prevalent in genes with high rates of turnover. Based on their sequence features and functional properties, the AU rich elements (AREs) can be separated into three classes (Chen et al, 1995, the contents of which are herein incorporated by reference in its entirety): Class I AREs, such as, but not limited to, c-Myc and MyoD, contain several dispersed copies of an AUUUA motif within U-rich regions. Class II AREs, such as, but not limited to, GM-CSF and TNF- α , possess two or more overlapping UUAUUUA(U/A)(U/A) nonamers. Class III AREs, such as, but not limited to, c-Jun and Myogenin, are less well defined. These U rich regions do not contain an AUUUA motif. Most proteins binding to the AREs are known to destabilize the messenger, whereas members of the ELAV family, most notably HuR, have been documented to increase the stability of mRNA. HuR binds to AREs of all the three classes. Engineering the HuR specific binding sites into the 3' UTR of nucleic acid molecules will lead to HuR binding and thus, stabilization of the message *in vivo*.

[0138] Introduction, removal or modification of 3' UTR AU rich elements (AREs) can be used to modulate the stability of polynucleotides. When engineering specific polynucleotides, e.g., payload regions of viral genomes, one or more copies of an ARE can be introduced to make polynucleotides less stable and thereby curtail translation and decrease production of the resultant protein. Likewise, AREs can be identified and removed or mutated to increase the intracellular stability and thus increase translation and production of the resultant protein.

[0139] In certain embodiments, the 3' UTR of the viral genome may include an oligo(dT) sequence for templated addition of a poly-A tail.

[0140] In certain embodiments, the viral genome may include at least one miRNA seed, binding site or full sequence. microRNAs (or miRNA or miR) are 19-25 nucleotide noncoding RNAs that bind to the sites of nucleic acid targets and down-regulate gene expression either by reducing nucleic acid molecule stability or by inhibiting translation. A microRNA sequence comprises a "seed" region, i.e., a sequence in the region of positions 2-8 of the mature microRNA, which sequence has perfect Watson-Crick complementarity to the miRNA target sequence of the nucleic acid.

[0141] In certain embodiments, the viral genome may be engineered to include, alter or remove at least one miRNA binding site, sequence or seed region.

[0142] Any UTR from any gene known in the art may be incorporated into the viral genome of the AAV particle. These UTRs, or portions thereof, may be placed in the same orientation as in the gene from which they were selected or they may be altered in orientation or location. In certain embodiments, the UTR used in the viral genome of the AAV particle may be inverted, shortened, lengthened, made with one or more other 5' UTRs or 3' UTRs known in the art. As used herein, the term "altered" as it relates to a UTR, means that the UTR has been changed in some way in relation to a reference sequence. For example, a 3' or 5' UTR may be altered relative to a wild type or native UTR by the change in orientation or location as taught above or may be altered by the inclusion of additional nucleotides, deletion of nucleotides, swapping or transposition of nucleotides.

[0143] In certain embodiments, the viral genome of the AAV particle comprises at least one artificial UTRs which is not a variant of a wild type UTR.

[0144] In certain embodiments, the viral genome of the AAV particle comprises UTRs which have been selected from a family of transcripts whose proteins share a common function, structure, feature or property.

Viral Genome Component: Polyadenylation Sequence

[0145] In certain embodiments, the viral genome of the AAV particles of the present disclosure comprise at least one polyadenylation sequence. The viral genome of the AAV particle may comprise a polyadenylation sequence between the 3' end of the payload coding sequence and the 5' end of the 3' ITR.

[0146] In certain embodiments, the polyadenylation sequence or "polyA sequence" may range from absent to about 500 nucleotides in length. The polyadenylation sequence may be,

but is not limited to, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, and 500 nucleotides in length.

[0147] In certain embodiments, the polyadenylation sequence is 50-100 nucleotides in length.

[0148] In certain embodiments, the polyadenylation sequence is 50-150 nucleotides in length.

[0149] In certain embodiments, the polyadenylation sequence is 50-160 nucleotides in length.

- [0150] In certain embodiments, the polyadenylation sequence is 50-200 nucleotides in length.
- [0151] In certain embodiments, the polyadenylation sequence is 60-100 nucleotides in length.
- [0152] In certain embodiments, the polyadenylation sequence is 60-150 nucleotides in length.
- [0153] In certain embodiments, the polyadenylation sequence is 60-160 nucleotides in length.
- [0154] In certain embodiments, the polyadenylation sequence is 60-200 nucleotides in length.
- [0155] In certain embodiments, the polyadenylation sequence is 70-100 nucleotides in length.
- [0156] In certain embodiments, the polyadenylation sequence is 70-150 nucleotides in length.
- [0157] In certain embodiments, the polyadenylation sequence is 70-160 nucleotides in length.
- [0158] In certain embodiments, the polyadenylation sequence is 70-200 nucleotides in length.
- [0159] In certain embodiments, the polyadenylation sequence is 80-100 nucleotides in length.
- [0160] In certain embodiments, the polyadenylation sequence is 80-150 nucleotides in length.
- [0161] In certain embodiments, the polyadenylation sequence is 80-160 nucleotides in length.
- [0162] In certain embodiments, the polyadenylation sequence is 80-200 nucleotides in length.
- [0163] In certain embodiments, the polyadenylation sequence is 90-100 nucleotides in length.
- [0164] In certain embodiments, the polyadenylation sequence is 90-150 nucleotides in length.
- [0165] In certain embodiments, the polyadenylation sequence is 90-160 nucleotides in length.

[0166] In certain embodiments, the polyadenylation sequence is 90-200 nucleotides in length.

[0167] In certain embodiments, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located upstream of the polyadenylation sequence in an expression vector. Further, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located downstream of a promoter such as, but not limited to, CMV, U6, CAG, CBA or a CBA promoter with a SV40 intron or a human beta globin intron in an expression vector. As a non-limiting example, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more than 30 nucleotides downstream from the promoter and/or upstream of the polyadenylation sequence in an expression vector. As another non-limiting example, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located within 1-5, 1-10, 1-15, 1-20, 1-25, 1-30, 5-10, 5-15, 5-20, 5-25, 5-30, 10-15, 10-20, 10-25, 10-30, 15-20, 15-25, 15-30, 20-25, 20-30 or 25-30 nucleotides downstream from the promoter and/or upstream of the polyadenylation sequence in an expression vector. As a non-limiting example, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located within the first 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25% or more than 25% of the nucleotides downstream from the promoter and/or upstream of the polyadenylation sequence in an expression vector. As another non-limiting example, the AAV particle comprises a nucleic acid sequence encoding an siRNA molecule may be located with the first 1-5%, 1-10%, 1-15%, 1-20%, 1-25%, 5-10%, 5-15%, 5-20%, 5-25%, 10-15%, 10-20%, 10-25%, 15-20%, 15-25%, or 20-25% downstream from the promoter and/or upstream of the polyadenylation sequence in an expression vector.

[0168] In certain embodiments, the AAV particle comprises a rabbit globin polyadenylation (polyA) signal sequence (rBGpA).

[0169] In certain embodiments, the AAV particle comprises a human growth hormone polyadenylation (polyA) signal sequence.

Viral Genome Component: Introns

[0170] In certain embodiments, the payload region comprises at least one element to enhance the expression such as one or more introns or portions thereof. Non-limiting examples of introns include, MVM (67-97 bps), F.IX truncated intron 1 (300 bps), β -globin SD/immunoglobulin heavy chain splice acceptor (250 bps), adenovirus splice

donor/immunoglobulin splice acceptor (500 bps), SV40 late splice donor/splice acceptor (19S/16S) (180 bps) and hybrid adenovirus splice donor/IgG splice acceptor (230 bps).

[0171] In certain embodiments, the intron or intron portion may be 100-500 nucleotides in length. The intron may have a length of 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490 or 500. The intron may have a length between 80-100, 80-120, 80-140, 80-160, 80-180, 80-200, 80-250, 80-300, 80-350, 80-400, 80-450, 80-500, 200-300, 200-400, 200-500, 300-400, 300-500, or 400-500.

[0172] In certain embodiments, the AAV viral genome may comprise a promoter such as, but not limited to, CMV or U6. As a non-limiting example, the promoter for the AAV comprising the nucleic acid sequence for the siRNA molecules of the present disclosure is a CMV promoter. As another non-limiting example, the promoter for the AAV comprising the nucleic acid sequence for the siRNA molecules of the present disclosure is a U6 promoter.

[0173] In certain embodiments, the AAV viral genome may comprise a CMV promoter.

[0174] In certain embodiments, the AAV viral genome may comprise a U6 promoter.

[0175] In certain embodiments, the AAV viral genome may comprise a CMV and a U6 promoter.

[0176] In certain embodiments, the AAV viral genome may comprise a HI promoter.

[0177] In certain embodiments, the AAV viral genome may comprise a CBA promoter.

[0178] In certain embodiments, the encoded siRNA molecule may be located downstream of a promoter in an expression vector such as, but not limited to, CMV, U6, HI, CBA, CAG, or a CBA promoter with an intron such as SV40 or others known in the art. Further, the encoded siRNA molecule may also be located upstream of the polyadenylation sequence in an expression vector. As a non-limiting example, the encoded siRNA molecule may be located within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more than 30 nucleotides downstream from the promoter and/or upstream of the polyadenylation sequence in an expression vector. As another non-limiting example, the encoded siRNA molecule may be located within 1-5, 1-10, 1-15, 1-20, 1-25, 1-30, 5-10, 5-15, 5-20, 5-25, 5-30, 10-15, 10-20, 10-25, 10-30, 15-20, 15-25, 15-30, 20-25, 20-30 or 25-30 nucleotides downstream from the promoter and/or upstream of the polyadenylation sequence in an expression vector. As a non-limiting example, the encoded siRNA molecule may be located within the first 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%,

10%, 15%, 20%, 25% or more than 25% of the nucleotides downstream from the promoter and/or upstream of the polyadenylation sequence in an expression vector. As another non-limiting example, the encoded siRNA molecule may be located with the first 1-5%, 1-10%, 1-15%, 1-20%, 1-25%, 5-10%, 5-15%, 5-20%, 5-25%, 10-15%, 10-20%, 10-25%, 15-20%, 15-25%, or 20-25% downstream from the promoter and/or upstream of the polyadenylation sequence in an expression vector.

Viral Genome Component: Filler Sequence

[0179] In certain embodiments, the viral genome comprises one or more filler sequences.

[0180] In certain embodiments, the viral genome comprises one or more filler sequences in order to have the length of the viral genome be the optimal size for packaging. As a non-limiting example, the viral genome comprises at least one filler sequence in order to have the length of the viral genome be about 2.3 kb. As a non-limiting example, the viral genome comprises at least one filler sequence in order to have the length of the viral genome be about 4.6 kb.

[0181] In certain embodiments, the viral genome comprises one or more filler sequences in order to reduce the likelihood that a hairpin structure of the vector genome (e.g., a modulatory polynucleotide described herein) may be read as an inverted terminal repeat (ITR) during expression and/or packaging. As a non-limiting example, the viral genome comprises at least one filler sequence in order to have the length of the viral genome be about 2.3 kb. As a non-limiting example, the viral genome comprises at least one filler sequence in order to have the length of the viral genome be about 4.6 kb.

[0182] In certain embodiments, the viral genome is a single stranded (ss) viral genome and comprises one or more filler sequences which have a length about between 0.1 kb - 3.8 kb, such as, but not limited to, 0.1 kb, 0.2 kb, 0.3 kb, 0.4 kb, 0.5 kb, 0.6 kb, 0.7 kb, 0.8 kb, 0.9 kb, 1 kb, 1.1 kb, 1.2 kb, 1.3 kb, 1.4 kb, 1.5 kb, 1.6 kb, 1.7 kb, 1.8 kb, 1.9 kb, 2 kb, 2.1 kb, 2.2 kb, 2.3 kb, 2.4 kb, 2.5 kb, 2.6 kb, 2.7 kb, 2.8 kb, 2.9 kb, 3 kb, 3.1 kb, 3.2 kb, 3.3 kb, 3.4 kb, 3.5 kb, 3.6 kb, 3.7 kb, or 3.8 kb. As a non-limiting example, the total length filler sequence in the vector genome is 3.1 kb. As a non-limiting example, the total length filler sequence in the vector genome is 2.7 kb. As a non-limiting example, the total length filler sequence in the vector genome is 0.8 kb. As a non-limiting example, the total length filler sequence in the vector genome is 0.4 kb. As a non-limiting example, the length of each filler sequence in the vector genome is 0.8 kb. As a non-limiting example, the length of each filler sequence in the vector genome is 0.4 kb.

[0183] In certain embodiments, the viral genome is a self-complementary (sc) viral genome and comprises one or more filler sequences which have a length about between 0.1 kb – 1.5 kb, such as, but not limited to, 0.1 kb, 0.2 kb, 0.3 kb, 0.4 kb, 0.5 kb, 0.6 kb, 0.7 kb, 0.8 kb, 0.9 kb, 1 kb, 1.1 kb, 1.2 kb, 1.3 kb, 1.4 kb, or 1.5 kb. As a non-limiting example, the total length filler sequence in the vector genome is 0.8 kb. As a non-limiting example, the total length filler sequence in the vector genome is 0.4 kb. As a non-limiting example, the length of each filler sequence in the vector genome is 0.8 kb. As a non-limiting example, the length of each filler sequence in the vector genome is 0.4 kb

[0184] In certain embodiments, the viral genome comprises any portion of a filler sequence. The viral genome may comprise 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% of a filler sequence.

[0185] In certain embodiments, the viral genome is a single stranded (ss) viral genome and comprises one or more filler sequences in order to have the length of the viral genome be about 4.6 kb. As a non-limiting example, the viral genome comprises at least one filler sequence and the filler sequence is located 3' to the 5' ITR sequence. As a non-limiting example, the viral genome comprises at least one filler sequence and the filler sequence is located 5' to a promoter sequence. As a non-limiting example, the viral genome comprises at least one filler sequence and the filler sequence is located 3' to the polyadenylation signal sequence. As a non-limiting example, the viral genome comprises at least one filler sequence and the filler sequence is located 5' to the 3' ITR sequence. As a non-limiting example, the viral genome comprises at least one filler sequence, and the filler sequence is located between two intron sequences. As a non-limiting example, the viral genome comprises at least one filler sequence, and the filler sequence is located within an intron sequence. As a non-limiting example, the viral genome comprises two filler sequences, and the first filler sequence is located 3' to the 5' ITR sequence and the second filler sequence is located 3' to the polyadenylation signal sequence. As a non-limiting example, the viral genome comprises two filler sequences, and the first filler sequence is located 5' to a promoter sequence and the second filler sequence is located 3' to the polyadenylation signal sequence. As a non-limiting example, the viral genome comprises two filler sequences, and the first filler sequence is located 3' to the 5' ITR sequence and the second filler sequence is located 5' to the 5' ITR sequence.

[0186] In certain embodiments, the viral genome is a self-complementary (sc) viral genome and comprises one or more filler sequences in order to have the length of the viral genome be about 2.3 kb. As a non-limiting example, the viral genome comprises at least one filler sequence and the filler sequence is located 3' to the 5' ITR sequence. As a non-limiting example, the viral genome comprises at least one filler sequence and the filler sequence is located 5' to a promoter sequence. As a non-limiting example, the viral genome comprises at least one filler sequence and the filler sequence is located 3' to the polyadenylation signal sequence. As a non-limiting example, the viral genome comprises at least one filler sequence and the filler sequence is located 5' to the 3' ITR sequence. As a non-limiting example, the viral genome comprises at least one filler sequence, and the filler sequence is located between two intron sequences. As a non-limiting example, the viral genome comprises at least one filler sequence, and the filler sequence is located within an intron sequence. As a non-limiting example, the viral genome comprises two filler sequences, and the first filler sequence is located 3' to the 5' ITR sequence and the second filler sequence is located 3' to the polyadenylation signal sequence. As a non-limiting example, the viral genome comprises two filler sequences, and the first filler sequence is located 5' to a promoter sequence and the second filler sequence is located 3' to the polyadenylation signal sequence. As a non-limiting example, the viral genome comprises two filler sequences, and the first filler sequence is located 3' to the 5' ITR sequence and the second filler sequence is located 5' to the 5' ITR sequence.

[0187] In certain embodiments, the viral genome may comprise one or more filler sequences between one of more regions of the viral genome. In certain embodiments, the filler region may be located before a region such as, but not limited to, a payload region, an inverted terminal repeat (ITR), a promoter region, an intron region, an enhancer region, a polyadenylation signal sequence region, and/or an exon region. In certain embodiments, the filler region may be located after a region such as, but not limited to, a payload region, an inverted terminal repeat (ITR), a promoter region, an intron region, an enhancer region, a polyadenylation signal sequence region, and/or an exon region. In certain embodiments, the filler region may be located before and after a region such as, but not limited to, a payload region, an inverted terminal repeat (ITR), a promoter region, an intron region, an enhancer region, a polyadenylation signal sequence region, and/or an exon region.

[0188] In certain embodiments, the viral genome may comprise one or more filler sequences which bifurcates at least one region of the viral genome. The bifurcated region of

the viral genome may comprise 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% of the of the region to the 5' of the filler sequence region. As a non-limiting example, the filler sequence may bifurcate at least one region so that 10% of the region is located 5' to the filler sequence and 90% of the region is located 3' to the filler sequence. As a non-limiting example, the filler sequence may bifurcate at least one region so that 20% of the region is located 5' to the filler sequence and 80% of the region is located 3' to the filler sequence. As a non-limiting example, the filler sequence may bifurcate at least one region so that 30% of the region is located 5' to the filler sequence and 70% of the region is located 3' to the filler sequence. As a non-limiting example, the filler sequence may bifurcate at least one region so that 40% of the region is located 5' to the filler sequence and 60% of the region is located 3' to the filler sequence. As a non-limiting example, the filler sequence may bifurcate at least one region so that 50% of the region is located 5' to the filler sequence and 50% of the region is located 3' to the filler sequence. As a non-limiting example, the filler sequence may bifurcate at least one region so that 60% of the region is located 5' to the filler sequence and 40% of the region is located 3' to the filler sequence. As a non-limiting example, the filler sequence may bifurcate at least one region so that 70% of the region is located 5' to the filler sequence and 30% of the region is located 3' to the filler sequence. As a non-limiting example, the filler sequence may bifurcate at least one region so that 80% of the region is located 5' to the filler sequence and 20% of the region is located 3' to the filler sequence. As a non-limiting example, the filler sequence may bifurcate at least one region so that 90% of the region is located 5' to the filler sequence and 10% of the region is located 3' to the filler sequence.

[0189] In certain embodiments, the viral genome comprises a filler sequence after the 5' ITR.

[0190] In certain embodiments, the viral genome comprises a filler sequence after the promoter region. In certain embodiments, the viral genome comprises a filler sequence after the payload region. In certain embodiments, the viral genome comprises a filler sequence after the intron region. In certain embodiments, the viral genome comprises a filler sequence after the enhancer region. In certain embodiments, the viral genome comprises a filler sequence after the polyadenylation signal sequence region. In certain embodiments, the viral genome comprises a filler sequence after the exon region.

[0191] In certain embodiments, the viral genome comprises a filler sequence before the promoter region. In certain embodiments, the viral genome comprises a filler sequence before the payload region. In certain embodiments, the viral genome comprises a filler sequence before the intron region. In certain embodiments, the viral genome comprises a filler sequence before the enhancer region. In certain embodiments, the viral genome comprises a filler sequence before the polyadenylation signal sequence region. In certain embodiments, the viral genome comprises a filler sequence before the exon region.

[0192] In certain embodiments, the viral genome comprises a filler sequence before the 3' ITR.

[0193] In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the 5' ITR and the promoter region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the 5' ITR and the payload region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the 5' ITR and the intron region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the 5' ITR and the enhancer region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the 5' ITR and the polyadenylation signal sequence region.

[0194] In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the 5' ITR and the exon region.

[0195] In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the promoter region and the payload region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the promoter region and the intron region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the promoter region and the enhancer region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the promoter region and the polyadenylation signal sequence region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the promoter region and the exon region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the promoter region and the 3' ITR.

[0196] In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the payload region and the intron region. In certain embodiments,

a filler sequence may be located between two regions, such as, but not limited to, the payload region and the enhancer region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the payload region and the polyadenylation signal sequence region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the payload region and the exon region.

[0197] In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the payload region and the 3' ITR.

[0198] In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the intron region and the enhancer region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the intron region and the polyadenylation signal sequence region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the intron region and the exon region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the intron region and the 3' ITR. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the enhancer region and the polyadenylation signal sequence region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the enhancer region and the exon region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the enhancer region and the 3' ITR.

[0199] In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the polyadenylation signal sequence region and the exon region. In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the polyadenylation signal sequence region and the 3' ITR.

[0200] In certain embodiments, a filler sequence may be located between two regions, such as, but not limited to, the exon region and the 3' ITR.

[0201] In certain embodiments, the filler sequence may be derived from a region or a portion of a lentivirus.

[0202] In some embodiments, the filler sequence may be derived from a region or a portion of the albumin gene. In certain embodiments, the filler sequence may be derived from a region or a portion of the human albumin gene (NCBI Reference Sequence: NG_009291.1).

Payloads

[0203] The AAV particles of the present disclosure comprise at least one payload region. As used herein, "payload" or "payload region" refers to one or more polynucleotides or

polynucleotide regions encoded by or within a viral genome or an expression product of such polynucleotide or polynucleotide region, e.g., a transgene, a polynucleotide encoding a polypeptide or multi-polypeptide or a modulatory nucleic acid or regulatory nucleic acid. Payloads of the present disclosure typically encode modulatory polynucleotides or fragments or variants thereof.

[0204] The payload region may be constructed in such a way as to reflect a region similar to or mirroring the natural organization of an mRNA.

[0205] The payload region may comprise a combination of coding and non-coding nucleic acid sequences.

[0206] In some embodiments, the AAV payload region may encode a coding or non-coding RNA.

[0207] In certain embodiments, the AAV particle comprises a viral genome with a payload region comprising nucleic acid sequences encoding a siRNA, miRNA or other RNAi agent. In such an embodiment, a viral genome encoding more than one polypeptide may be replicated and packaged into a viral particle. A target cell transduced with a viral particle may express the encoded siRNA, miRNA or other RNAi agent inside a single cell.

Modulatory Polynucleotides

[0208] In certain embodiments, modulatory polynucleotides, e.g., RNA or DNA molecules, may be used to treat neurodegenerative disease, in particular, amyotrophic lateral sclerosis (ALS). As used herein, a "modulatory polynucleotide" is any nucleic acid sequence(s) which functions to modulate (either increase or decrease) the level or amount of a target gene, e.g., mRNA or protein levels.

[0209] In certain embodiments, the modulatory polynucleotides may comprise at least one nucleic acid sequence encoding at least one siRNA molecule. The nucleic acids may, independently if there is more than one, encode 1, 2, 3, 4, 5, 6, 7, 8, 9, or more than 9 siRNA molecules.

[0210] In certain embodiments, the molecular scaffold may be located downstream of a CMV promoter, fragment or variant thereof.

[0211] In certain embodiments, the molecular scaffold may be located downstream of a CBA promoter, fragment or variant thereof.

[0212] In certain embodiments, the molecular scaffold may be a natural pri-miRNA scaffold located downstream of a CMV promoter. As a non-limiting example, the natural pri-miRNA scaffold is derived from the human miR155 scaffold.

[0213] In certain embodiments, the molecular scaffold may be a natural pri-miRNA scaffold located downstream of a CBA promoter.

[0214] In certain embodiments, the selection of a molecular scaffold and modulatory polynucleotide is determined by a method of comparing modulatory polynucleotides in pri-miRNA (see e.g., the method described by Miniarikova et al. *Design, Characterization, and Lead Selection of Therapeutic miRNAs Targeting Huntingtin for Development of Gene Therapy for Huntington's Disease*. *Molecular Therapy-Nucleic Acids* (2016) 5, e297 and International Publication No. WO2016102664; the contents of each of which are herein incorporated by reference in their entireties). To evaluate the activities of the modulatory polynucleotides, the molecular scaffold used which may be used is a human pri-miRNA scaffold (e.g., miR155 scaffold) and the promoter may be CMV. The activity may be determined *in vitro* using HEK293T cells and a reporter (e.g., Luciferase).

[0215] In order to evaluate the optimal molecular scaffold for the modulatory polynucleotide, the modulatory polynucleotide is used in pri-miRNA scaffolds with a CAG promoter. The constructs are co-transfected with a reporter (e.g., luciferase reporter) at 50 ng. Constructs with greater than 80% knockdown at 50 ng co-transfection are considered efficient. In one aspect, the constructs with strong guide-strand activity are preferred. The molecular scaffolds can be processed in HEK293T cells by NGS to determine guide-passenger ratios, and processing variability.

[0216] To evaluate the molecular scaffolds and modulatory polynucleotides *in vivo* the molecular scaffolds comprising the modulatory polynucleotides are packaged in AAV (e.g., the serotype may be AAV5 (see e.g., the method and constructs described in WO2015060722, the contents of which are herein incorporated by reference in their entirety)) and administered to an *in vivo* model and the guide-passenger ratios, 5' and 3' end processing, ratio of guide to passenger strands, and knockdown can be determined in different areas of the model (e.g., tissue regions).

[0217] In certain embodiments, the selection of a molecular scaffold and modulatory polynucleotide is determined by a method of comparing modulatory polynucleotides in natural pri-miRNA and synthetic pri-miRNA. The modulatory polynucleotide may, but it not limited to, targeting an exon other than exon 1. To evaluate the activities of the modulatory polynucleotides, the molecular scaffold is used with a CBA promoter. In one aspect, the activity may be determined *in vitro* using HEK293T cells, HeLa cell and a reporter (e.g., Luciferase) and knockdown efficient modulatory polynucleotides showed SOD1 knockdown

of at least 80% in the cell tested. Additionally, the modulatory polynucleotides which are considered most efficient showed low to no significant passenger strand (p-strand) activity. In another aspect, the endogenous SOD1 knockdown efficacy is evaluated by transfection *in vitro* using HEK293T cells, HeLa cell and a reporter. Efficient modulatory polynucleotides show greater than 50% endogenous SOD1 knockdown. In yet another aspect, the endogenous SOD1 knockdown efficacy is evaluated in different cell types (e.g., HEK293, HeLa, primary astrocytes, U251 astrocytes, SH-SY5Y neuron cells and fibroblasts from ALS patients) by infection (e.g., AAV2). Efficient modulatory polynucleotides show greater than 60% endogenous SOD1 knockdown.

[0218] To evaluate the molecular scaffolds and modulatory polynucleotides *in vivo* the molecular scaffolds comprising the modulatory polynucleotides are packaged in AAV and administered to an *in vivo* model and the guide-passenger ratios, 5' and 3' end processing, ratio of guide to passenger strands, and knockdown can be determined in different areas of the model (e.g., tissue regions). The molecular scaffolds can be processed from *in vivo* samples by NGS to determine guide-passenger ratios, and processing variability.

[0219] In certain embodiments, the modulatory polynucleotide is designed using at least one of the following properties: loop variant, seed mismatch/bulge/wobble variant, stem mismatch, loop variant and vassal stem mismatch variant, seed mismatch and basal stem mismatch variant, stem mismatch and basal stem mismatch variant, seed wobble and basal stem wobble variant, or a stem sequence variant.

[0220] The present disclosure relates, in part, to RNA interfering (RNAi) induced inhibition of gene expression for treating neurodegenerative disorders. Provided are siRNA duplexes or dsRNA that target SOD1 gene. Such siRNA duplexes or dsRNA can silence SOD1 gene expression in cells, for example, motor neurons, therefore, ameliorating symptoms of ALS such as motor death and muscle atrophy. The SOD1 siRNA may be encoded in polynucleotides of a recombinant AAV vector.

[0221] siRNA duplexes or dsRNA targeting a specific mRNA may be designed and synthesized as part of a target SOD1 targeting polynucleotide *in vitro* and introduced into cells for activating RNAi process.

siRNA Molecules

[0222] The present disclosure relates to RNA interference (RNAi) induced inhibition of gene expression for treating neurodegenerative disorders. Provided herein are siRNA

duplexes or encoded dsRNA that target the gene of interest (referred to herein collectively as "siRNA molecules"). Such siRNA duplexes or encoded dsRNA can reduce or silence gene expression in cells, such as but not limited to, medium spiny neurons, cortical neurons and/or astrocytes.

[0223] RNAi (also known as post-transcriptional gene silencing (PTGS), quelling, or co-suppression) is a post-transcriptional gene silencing process in which RNA molecules, in a sequence specific manner, inhibit gene expression, typically by causing the destruction of specific mRNA molecules. The active components of RNAi are short/small double stranded RNAs (dsRNAs), called small interfering RNAs (siRNAs), that typically contain 15-30 nucleotides (e.g., 19 to 25, 19 to 24 or 19-21 nucleotides) and 2 nucleotide 3' overhangs and that match the nucleic acid sequence of the target gene. These short RNA species may be naturally produced *in vivo* by Dicer-mediated cleavage of larger dsRNAs and they are functional in mammalian cells.

[0224] Naturally expressed small RNA molecules, named microRNAs (miRNAs), elicit gene silencing by regulating the expression of mRNAs. The miRNAs containing RNA Induced Silencing Complex (RISC) targets mRNAs presenting a perfect sequence complementarity with nucleotides 2-7 in the 5' region of the miRNA which is called the seed region, and other base pairs with its 3' region. miRNA mediated down regulation of gene expression may be caused by cleavage of the target mRNAs, translational inhibition of the target mRNAs, or mRNA decay. miRNA targeting sequences are usually located in the 3'-UTR of the target mRNAs. A single miRNA may target more than 100 transcripts from various genes, and one mRNA may be targeted by different miRNAs.

[0225] siRNA duplexes or dsRNA targeting a specific mRNA may be designed and synthesized *in vitro* and introduced into cells for activating RNAi processes. Elbashir et al. demonstrated that 21-nucleotide siRNA duplexes (termed small interfering RNAs) were capable of effecting potent and specific gene knockdown without inducing immune response in mammalian cells (Elbashir SM et al., *Nature*, 2001, 411, 494-498). Since this initial report, post-transcriptional gene silencing by siRNAs quickly emerged as a powerful tool for genetic analysis in mammalian cells and has the potential to produce novel therapeutics.

[0226] RNAi molecules which were designed to target against a nucleic acid sequence that encodes poly-glutamine repeat proteins which cause poly-glutamine expansion diseases such as Huntington's Disease, are described in US Patent No. 9,169,483 and 9,181,544 and International Patent Publication No. WO2015179525, the content of each of which is herein

incorporated by reference in their entirety. US Patent Nos. 9,169,483 and 9,181,544 and International Patent Publication No. WO2015179525 each provide isolated RNA duplexes comprising a first strand of RNA (e.g., 15 contiguous nucleotides) and second strand of RNA (e.g., complementary to at least 12 contiguous nucleotides of the first strand) where the RNA duplex is about 15 to 30 base pairs in length. The first strand of RNA and second strand of RNA may be operably linked by an RNA loop (~4 to 50 nucleotides) to form a hairpin structure which may be inserted into an expression cassette. Non-limiting examples of loop portions include SEQ ID NO: 9-14 of US Patent No. 9,169,483, the content of which is herein incorporated by reference in its entirety. Non-limiting examples of strands of RNA which may be used, either full sequence or part of the sequence, to form RNA duplexes include SEQ ID NO: 1-8 of US Patent No. 9,169,483 and SEQ ID NO: 1-11, 33-59, 208-210, 213-215 and 218-221 of US Patent No. 9,181,544, the contents of each of which is herein incorporated by reference in its entirety. Non-limiting examples of RNAi molecules include SEQ ID NOs: 1-8 of US Patent No. 9,169,483, SEQ ID NOs: 1-11, 33-59, 208-210, 213-215 and 218-221 of US Patent No. 9,181,544 and SEQ ID NOs: 1, 6, 7, and 35-38 of International Patent Publication No. WO2015179525, the contents of each of which is herein incorporated by reference in their entirety.

[0227] *In vitro* synthesized siRNA molecules may be introduced into cells in order to activate RNAi. An exogenous siRNA duplex, when it is introduced into cells, similar to the endogenous dsRNAs, can be assembled to form the RNA Induced Silencing Complex (RISC), a multiunit complex that interacts with RNA sequences that are complementary to one of the two strands of the siRNA duplex (i.e., the antisense strand). During the process, the sense strand (or passenger strand) of the siRNA is lost from the complex, while the antisense strand (or guide strand) of the siRNA is matched with its complementary RNA. In particular, the targets of siRNA containing RISC complexes are mRNAs presenting a perfect sequence complementarity. Then, siRNA mediated gene silencing occurs by cleaving, releasing and degrading the target.

[0228] The siRNA duplex comprised of a sense strand homologous to the target mRNA and an antisense strand that is complementary to the target mRNA offers much more advantage in terms of efficiency for target RNA destruction compared to the use of the single strand (ss)-siRNAs (e.g. antisense strand RNA or antisense oligonucleotides). In many cases, it requires higher concentration of the ss-siRNA to achieve the effective gene silencing potency of the corresponding duplex.

[0229] Any of the foregoing molecules may be encoded by a viral genome.

Design and Sequences of siRNA duplexes targeting gene of interest

[0230] The present disclosure provides small interfering RNA (siRNA) duplexes (and modulatory polynucleotides encoding them) that target mRNA to interfere with gene expression and/or protein production.

[0231] The encoded siRNA duplex of the present disclosure contains an antisense strand and a sense strand hybridized together forming a duplex structure, wherein the antisense strand is complementary to the nucleic acid sequence of the targeted gene, and wherein the sense strand is homologous to the nucleic acid sequence of the targeted gene. In some aspects, the 5' end of the antisense strand has a 5' phosphate group and the 3' end of the sense strand contains a 3' hydroxyl group. In other aspects, there are none, one or 2 nucleotide overhangs at the 3' end of each strand.

[0232] Some guidelines for designing siRNAs have been proposed in the art. These guidelines generally recommend generating a 19-nucleotide duplexed region, symmetric 2-3 nucleotide 3' overhangs, 5' phosphate and 3' hydroxyl groups targeting a region in the gene to be silenced. Other rules that may govern siRNA sequence preference include, but are not limited to, (i) A/U at the 5' end of the antisense strand; (ii) G/C at the 5' end of the sense strand; (iii) at least five A/U residues in the 5' terminal one-third of the antisense strand; and (iv) the absence of any GC stretch of more than 9 nucleotides in length. In accordance with such consideration, together with the specific sequence of a target gene, highly effective siRNA molecules essential for suppressing mammalian target gene expression may be readily designed.

[0233] According to the present disclosure, siRNA molecules (e.g., siRNA duplexes or encoded dsRNA) that target the gene of interest are designed. Such siRNA molecules can specifically, suppress gene expression and protein production. In some aspects, the siRNA molecules are designed and used to selectively "knock out" gene variants in cells, i.e., mutated transcripts. In some aspects, the siRNA molecules are designed and used to selectively "knock down" gene variants in cells. In other aspects, the siRNA molecules are able to inhibit or suppress both the wild type and mutated version of the gene of interest.

[0234] In certain embodiments, an siRNA molecule of the present disclosure comprises a sense strand and a complementary antisense strand in which both strands are hybridized together to form a duplex structure. The antisense strand has sufficient complementarity to the target mRNA sequence to direct target-specific RNAi, i.e., the siRNA molecule has a

sequence sufficient to trigger the destruction of the target mRNA by the RNAi machinery or process.

[0235] In certain embodiments, an siRNA molecule of the present disclosure comprises a sense strand and a complementary antisense strand in which both strands are hybridized together to form a duplex structure and where the start site of the hybridization to the mRNA is between nucleotide 10 and 1000 on the target mRNA sequence. As a non-limiting example, the start site may be between nucleotide 10-20, 20-30, 30-40, 40-50, 60-70, 70-80, 80-90, 90-100, 100-150, 150-200, 200-250, 250-300, 300-350, 350-400, 400-450, 450-500, 500-550, 550-600, 600-650, 650-700, 700-700, 750-800, 800-850, 850-900, 900-950, 950-1000, on the target mRNA sequence. As yet another non-limiting example, the start site may be nucleotide 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448,

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[0236] In some embodiments, the antisense strand and target mRNA sequences have 100% complementarity. The antisense strand may be complementary to any part of the target mRNA sequence.

[0237] In other embodiments, the antisense strand and target mRNA sequences comprise at least one mismatch. As a non-limiting example, the antisense strand and the target mRNA sequence have at least 30%, 40%, 50%, 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-99%, 30-40%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-99%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-99%, 50-60%, 50-70%, 50-80%, 50-90%, 50-95%, 50-99%, 60-70%, 60-80%, 60-90%, 60-95%, 60-99%, 70-80%, 70-90%, 70-95%, 70-99%, 80-90%, 80-95%, 80-99%, 90-95%, 90-99% or 95-99% complementarity.

[0238] In certain embodiments, an siRNA or dsRNA includes at least two sequences that are complementary to each other.

[0239] According to the present disclosure, the siRNA molecule has a length from about 10-50 or more nucleotides, i.e., each strand comprising 10-50 nucleotides (or nucleotide analogs). Preferably, the siRNA molecule has a length from about 15-30, e.g., 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in each strand, wherein one of the strands is sufficiently complementary to a target region. In certain embodiments, each strand of the siRNA molecule has a length from about 19 to 25, 19 to 24 or 19 to 21 nucleotides. In certain embodiments, at least one strand of the siRNA molecule is 19 nucleotides in length. In certain embodiments, at least one strand of the siRNA molecule is 20 nucleotides in length. In certain embodiments, at least one strand of the siRNA molecule is 21 nucleotides in length. In certain embodiments, at least one strand of the siRNA molecule is 22 nucleotides in length. In certain embodiments, at least one strand of the siRNA molecule is 23 nucleotides in length. In certain embodiments, at least one strand of the siRNA molecule is 24 nucleotides in length. In certain embodiments, at least one strand of the siRNA molecule is 25 nucleotides in length.

[0240] In some embodiments, the siRNA molecules of the present disclosure can be synthetic RNA duplexes comprising about 19 nucleotides to about 25 nucleotides, and two overhanging nucleotides at the 3'-end. In some aspects, the siRNA molecules may be unmodified RNA molecules. In other aspects, the siRNA molecules may contain at least one modified nucleotide, such as base, sugar or backbone modifications.

[0241] In certain embodiments, the siRNA molecules of the present disclosure may comprise an antisense sequence and a sense sequence, or a fragment or variant thereof. As a non-limiting example, the antisense sequence and the sense sequence have at least 30%, 40%, 50%, 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-99%, 30-40%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-99%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-99%, 50-60%, 50-70%, 50-80%, 50-90%, 50-95%, 50-99%, 60-70%, 60-80%, 60-90%, 60-95%, 60-99%, 70-80%, 70-90%, 70-95%, 70-99%, 80-90%, 80-95%, 80-99%, 90-95%, 90-99% or 95-99% complementarity.

[0242] In other embodiments, the siRNA molecules of the present disclosure can be encoded in plasmid vectors, AAV particles, viral genome or other nucleic acid expression vectors for delivery to a cell.

[0243] DNA expression plasmids can be used to stably express the siRNA duplexes or dsRNA of the present disclosure in cells and achieve long-term inhibition of the target gene expression. In one aspect, the sense and antisense strands of a siRNA duplex are typically linked by a short spacer sequence leading to the expression of a stem-loop structure termed short hairpin RNA (shRNA). The hairpin is recognized and cleaved by Dicer, thus generating mature siRNA molecules.

[0244] According to the present disclosure, AAV particles comprising the nucleic acids encoding the siRNA molecules targeting the mRNA are produced, the AAV serotypes may be any of the serotypes listed herein. Non-limiting examples of the AAV serotypes include, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV9.47, AAV9(hu14), AAV10, AAV11, AAV12, AAVrh8, AAVrh10, AAV-DJ8, AAV-DJ, AAV-PHP.A, AAV-PHP.B, AAVPHP.B2, AAVPHP.B3, AAVPHP.N/PHP.B-DGT, AAVPHP.B-EST, AAVPHP.B-GGT, AAVPHP.B-ATP, AAVPHP.B-ATT-T, AAVPHP.B-DGT-T, AAVPHP.B-GGT-T, AAVPHP.B-SGS, AAVPHP.B-AQP, AAVPHP.B-QQP, AAVPHP.B-SNP(3), AAVPHP.B-SNP, AAVPHP.B-QGT, AAVPHP.B-NQT, AAVPHP.B-EGS, AAVPHP.B-SGN, AAVPHP.B-EGT, AAVPHP.B-DST, AAVPHP.B-DST, AAVPHP.B-STP, AAVPHP.B-PQP, AAVPHP.B-SQP, AAVPHP.B-QLP, AAVPHP.B-TMP, AAVPHP.B-TTP, AAVPHP.S/G2A12, AAVG2A15/G2A3, AAVG2B4, AAVG2B5, and variants thereof.

[0245] In some embodiments, the siRNA duplexes or encoded dsRNA of the present disclosure suppress (or degrade) the target mRNA. Accordingly, the siRNA duplexes or encoded dsRNA can be used to substantially inhibit the gene expression in a cell, for example a neuron. In some aspects, the inhibition of the gene expression refers to an inhibition by at least about 20%, preferably by at least about 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 90%, 95%, 99% and 100%, or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-100%, 30-40%, 30-45%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-100%, 35-45%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-100%, 45-50%, 45-55%, 50-60%, 50-70%, 50-75%, 50-80%, 50-90%, 50-95%, 50-100%, 55-65%, 57-68%, 60-70%, 60-80%, 60-90%, 60-95%, 60-100%, 70-80%, 70-85%, 70-90%, 70-95%, 70-100%, 80-90%, 80-95%, 80-100%, 85-99%, 90-95%, 90-100% or 95-100%. Accordingly, the protein product of the targeted gene may be inhibited by at least about 20%, preferably by at least about 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 90%, 95%, 99% and 100%, or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-100%, 30-40%, 30-45%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-100%, 35-45%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-100%, 45-50%, 45-55%, 50-60%, 50-70%, 50-75%, 50-80%, 50-90%, 50-95%, 50-100%, 55-65%, 57-68%, 60-70%, 60-80%, 60-90%, 60-95%, 60-100%, 70-80%, 70-85%, 70-90%, 70-95%, 70-100%, 80-90%, 80-95%, 80-100%, 85-99%, 90-95%, 90-100% or 95-100%. As a non-limiting example, the inhibition may be 30-40%. As a non-limiting example, the inhibition may be 30-45%. As a non-limiting example, the inhibition may be 35-45%. As a non-limiting example, the inhibition may be greater than 50%. As a non-limiting example, the inhibition may be 50-60%. As a non-limiting example, the inhibition may be greater than 60%. As a non-limiting example, the inhibition may be 50-75%. As a non-limiting example, the inhibition may be 55-65%. As a non-limiting example, the inhibition may be 57-68%. As a non-limiting example, the inhibition may be 70-80%. As a non-limiting example, the inhibition may be 70-85%. As a non-limiting example, the inhibition may be 85-99%. As a non-limiting

example, the inhibition may be 35%. As a non-limiting example, the inhibition may be 36%. As a non-limiting example, the inhibition may be 40%. As a non-limiting example, the inhibition may be 41%. As a non-limiting example, the inhibition may be 43%. As a non-limiting example, the inhibition may be 45%. As a non-limiting example, the inhibition may be 49%. As a non-limiting example, the inhibition may be 62%. As a non-limiting example, the inhibition may be 64%. As a non-limiting example, the inhibition may be 74%. As a non-limiting example, the inhibition may be 77%. As a non-limiting example, the inhibition may be 84%. As a non-limiting example, the inhibition may be 87%. As a non-limiting example, the inhibition may be 95%. As a non-limiting example, the inhibition may be 99%. As a non-limiting example, the inhibition may be 100%.

[0246] In certain embodiments, the siRNA duplexes or encoded dsRNA of the present disclosure suppress (or degrade) the target mRNA in spinal cord motor neurons. In some aspects, the inhibition of the gene expression refers to suppression of at least about 20%, preferably by at least about 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 90%, 95%, 99% and 100%, or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-100%, 30-40%, 30-45%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-100%, 35-45%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-100%, 45-50%, 45-55%, 50-60%, 50-70%, 50-75%, 50-80%, 50-90%, 50-95%, 50-100%, 55-65%, 57-68%, 60-70%, 60-80%, 60-90%, 60-95%, 60-100%, 70-80%, 70-85%, 70-90%, 70-95%, 70-100%, 80-90%, 80-95%, 80-100%, 85-99%, 90-95%, 90-100% or 95-100%. Accordingly, the protein product of the targeted gene may be inhibited by at least about 20%, preferably by at least about 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 90%, 95%, 99% and 100%, or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-100%, 30-40%, 30-45%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-100%, 35-45%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-100%, 45-50%, 45-55%, 50-60%, 50-70%, 50-75%, 50-80%, 50-90%, 50-95%, 50-100%, 55-65%, 57-68%, 60-70%, 60-80%, 60-90%, 60-95%, 60-100%, 70-80%, 70-85%, 70-90%, 70-95%, 70-100%, 80-90%, 80-95%,

80-100%, 85-99%, 90-95%, 90-100% or 95-100%. As a non-limiting example, the suppression may be 30-45%. As a non-limiting example, the suppression may be 35-45%. As a non-limiting example, the suppression may be greater than 50%. As a non-limiting example, the suppression may be greater than 60%. As a non-limiting example, the suppression may be 50-60%. As a non-limiting example, the suppression may be 55-65%. As a non-limiting example, the suppression may be 50-75%. As a non-limiting example, the suppression may be 57-68%. As a non-limiting example, the suppression may be 70-80%. As a non-limiting example, the suppression may be 70-85%. As a non-limiting example, the suppression may be 85-99%. As a non-limiting example, the suppression may be 35%. As a non-limiting example, the suppression may be 36%. As a non-limiting example, the suppression may be 40%. As a non-limiting example, the suppression may be 41%. As a non-limiting example, the suppression may be 43%. As a non-limiting example, the suppression may be 45%. As a non-limiting example, the suppression may be 49%. As a non-limiting example, the suppression may be 62%. As a non-limiting example, the suppression may be 64%. As a non-limiting example, the suppression may be 74%. As a non-limiting example, the suppression may be 77%. As a non-limiting example, the suppression may be 84%. As a non-limiting example, the suppression may be 87%. As a non-limiting example, the suppression may be 95%. As a non-limiting example, the suppression may be 99%. As a non-limiting example, the suppression may be 100%.

[0247] In certain embodiments, the siRNA duplexes or encoded dsRNA of the present disclosure suppress (or degrade) the target mRNA in spinal cord motor neurons by 78%.

[0248] In certain embodiments, the siRNA duplexes or encoded dsRNA of the present disclosure suppress (or degrade) the target mRNA in spinal cord motor neurons by 45-55%.

[0249] In certain embodiments, the siRNA duplexes or encoded dsRNA of the present disclosure suppress (or degrade) the target mRNA in vg+ cells of motor neuron morphology. In some aspects, the inhibition of the gene expression refers to an inhibition by at least about 20%, preferably by at least about 30%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 90%, 95% and 100%, or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-100%, 30-40%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-100%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-100%, 45-50%, 45-55%, 50-60%, 50-70%, 50-80%, 50-90%, 50-95%, 50-100%, 60-70%, 60-80%, 60-90%, 60-95%, 60-100%, 70-80%, 70-90%,

70-95%, 70-100%, 80-90%, 80-95%, 80-100%, 90-95%, 90-100% or 95-100%. Accordingly, the protein product of the targeted gene may be inhibited by at least about 20%, preferably by at least about 30%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 90%, 95% and 100%, or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-100%, 30-40%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-100%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-100%, 45-50%, 45-55%, 50-60%, 50-70%, 50-80%, 50-90%, 50-95%, 50-100%, 60-70%, 60-80%, 60-90%, 60-95%, 60-100%, 70-80%, 70-90%, 70-95%, 70-100%, 80-90%, 80-95%, 80-100%, 90-95%, 90-100% or 95-100%.

[0250] In certain embodiments, the siRNA duplexes or encoded dsRNA of the present disclosure suppress (or degrade) the target mRNA in vg+ cells of motor neuron morphology by 53%.

[0251] In certain embodiments, the siRNA molecules comprise a miRNA seed match for the target located in the guide strand. In another embodiment, the siRNA molecules comprise a miRNA seed match for the target located in the passenger strand. In yet another embodiment, the siRNA duplexes or encoded dsRNA targeting the gene of interest do not comprise a seed match for the target located in the guide or passenger strand.

[0252] In certain embodiments, the siRNA duplexes or encoded dsRNA targeting the gene of interest may have almost no significant full-length off target effects for the guide strand. In another embodiment, the siRNA duplexes or encoded dsRNA targeting the gene of interest may have almost no significant full-length off target effects for the passenger strand. The siRNA duplexes or encoded dsRNA targeting the gene of interest may have less than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 1-5%, 2-6%, 3-7%, 4-8%, 5-9%, 5-10%, 6-10%, 5-15%, 5-20%, 5-25% 5-30%, 10-20%, 10-30%, 10-40%, 10-50%, 15-30%, 15-40%, 15-45%, 20-40%, 20-50%, 25-50%, 30-40%, 30-50%, 35-50%, 40-50%, 45-50% full-length off target effects for the passenger strand. In yet another embodiment, the siRNA duplexes or encoded dsRNA targeting the gene of interest may have almost no significant full-length off target effects for the guide strand or the passenger strand. The siRNA duplexes or encoded dsRNA targeting the gene of interest may have less than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 1-5%, 2-6%, 3-7%, 4-8%, 5-9%, 5-10%, 6-10%, 5-15%, 5-20%, 5-25% 5-30%, 10-20%, 10-30%, 10-40%, 10-50%, 15-

30%, 15-40%, 15-45%, 20-40%, 20-50%, 25-50%, 30-40%, 30-50%, 35-50%, 40-50%, 45-50% full-length off target effects for the guide or passenger strand.

[0253] In certain embodiments, the siRNA duplexes or encoded dsRNA targeting the gene of interest may have high activity *in vitro*. In another embodiment, the siRNA molecules may have low activity *in vitro*. In yet another embodiment, the siRNA duplexes or dsRNA targeting the gene of interest may have high guide strand activity and low passenger strand activity *in vitro*.

[0254] In certain embodiments, the siRNA molecules have a high guide strand activity and low passenger strand activity *in vitro*. The target knock-down (KD) by the guide strand may be at least 40%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, 99.5% or 100%. The target knock-down by the guide strand may be 40-50%, 45-50%, 50-55%, 50-60%, 60-65%, 60-70%, 60-75%, 60-80%, 60-85%, 60-90%, 60-95%, 60-99%, 60-99.5%, 60-100%, 65-70%, 65-75%, 65-80%, 65-85%, 65-90%, 65-95%, 65-99%, 65-99.5%, 65-100%, 70-75%, 70-80%, 70-85%, 70-90%, 70-95%, 70-99%, 70-99.5%, 70-100%, 75-80%, 75-85%, 75-90%, 75-95%, 75-99%, 75-99.5%, 75-100%, 80-85%, 80-90%, 80-95%, 80-99%, 80-99.5%, 80-100%, 85-90%, 85-95%, 85-99%, 85-99.5%, 85-100%, 90-95%, 90-99%, 90-99.5%, 90-100%, 95-99%, 95-99.5%, 95-100%, 99-99.5%, 99-100% or 99.5-100%. As a non-limiting example, the target knock-down (KD) by the guide strand is greater than 70%. As a non-limiting example, the target knock-down (KD) by the guide strand is greater than 60%.

[0255] In certain embodiments, the highest knock-down from delivery of the siRNA molecules is seen around the injection site(s).

[0256] In certain embodiments, knock-down is seen in the ventral horn and around the injection site(s) after delivery of the siRNA molecules.

[0257] In certain embodiments, the siRNA duplex is designed so there is no miRNA seed match for the sense or antisense sequence to the non-gene of interest sequence.

[0258] In certain embodiments, the IC_{50} of the guide strand for the nearest off target is greater than 100 multiplied by the IC_{50} of the guide strand for the on-target gene. As a non-limiting example, if the IC_{50} of the guide strand for the nearest off target is greater than 100 multiplied by the IC_{50} of the guide strand for the target then the siRNA molecule is said to have high guide strand selectivity for inhibiting the gene of interest *in vitro*.

[0259] In certain embodiments, the 5' processing of the guide strand has a correct start (n) at the 5' end at least 75%, 80%, 85%, 90%, 95%, 99% or 100% of the time *in vitro* or *in vivo*.

As a non-limiting example, the 5' processing of the guide strand is precise and has a correct start (n) at the 5' end at least 99% of the time *in vitro*. As a non-limiting example, the 5' processing of the guide strand is precise and has a correct start (n) at the 5' end at least 99% of the time *in vivo*. As a non-limiting example, the 5' processing of the guide strand is precise and has a correct start (n) at the 5' end at least 90% of the time *in vitro*. As a non-limiting example, the 5' processing of the guide strand is precise and has a correct start (n) at the 5' end at least 90% of the time *in vivo*. As a non-limiting example, the 5' processing of the guide strand is precise and has a correct start (n) at the 5' end at least 85% of the time *in vitro*. As a non-limiting example, the 5' processing of the guide strand is precise and has a correct start (n) at the 5' end at least 85% of the time *in vivo*.

[0260] In certain embodiments, the guide to passenger (G:P) (also referred to as the antisense to sense) strand ratio expressed is 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:10, 2:9, 2:8, 2:7, 2:6, 2:5, 2:4, 2:3, 2:2, 2:1, 3:10, 3:9, 3:8, 3:7, 3:6, 3:5, 3:4, 3:3, 3:2, 3:1, 4:10, 4:9, 4:8, 4:7, 4:6, 4:5, 4:4, 4:3, 4:2, 4:1, 5:10, 5:9, 5:8, 5:7, 5:6, 5:5, 5:4, 5:3, 5:2, 5:1, 6:10, 6:9, 6:8, 6:7, 6:6, 6:5, 6:4, 6:3, 6:2, 6:1, 7:10, 7:9, 7:8, 7:7, 7:6, 7:5, 7:4, 7:3, 7:2, 7:1, 8:10, 8:9, 8:8, 8:7, 8:6, 8:5, 8:4, 8:3, 8:2, 8:1, 9:10, 9:9, 9:8, 9:7, 9:6, 9:5, 9:4, 9:3, 9:2, 9:1, 10:10, 10:9, 10:8, 10:7, 10:6, 10:5, 10:4, 10:3, 10:2, 10:1, 1:99, 5:95, 10:90, 15:85, 20:80, 25:75, 30:70, 35:65, 40:60, 45:55, 50:50, 55:45, 60:40, 65:35, 70:30, 75:25, 80:20, 85:15, 90:10, 95:5, or 99:1 *in vitro* or *in vivo*. The guide to passenger ratio refers to the ratio of the guide strands to the passenger strands after intracellular processing of the pri-microRNA. For example, a 80:20 guide-to-passenger ratio would have 8 guide strands to every 2 passenger strands processed from the precursor. As a non-limiting example, the guide-to-passenger strand ratio is 8:2 *in vitro*. As a non-limiting example, the guide-to-passenger strand ratio is 8:2 *in vivo*. As a non-limiting example, the guide-to-passenger strand ratio is 9:1 *in vitro*. As a non-limiting example, the guide-to-passenger strand ratio is 9:1 *in vivo*.

[0261] In certain embodiments, the guide to passenger (G:P) strand ratio is in a range of 1-99, 1.3-99, 5-99, 10-99, 15-99, 20-99, 25-99, 30-99, 35-99, 40-99, 45-99, 50-99, 55-99, 60-99, 65-99, 70-99, 75-99, 80-99, 85-99, 90-99, 95-99, 1-10, 1-15, 1-20, 1-25, 1-30, 1-35, 1-40, 1-45, 1-50, 1-55, 1-60, 1-65, 1-70, 1-75, 1-80, 1-85, 1-90, 1-95, 5-10, 5-15, 5-20, 5-25, 5-30, 5-35, 5-40, 5-45, 5-50, 5-55, 5-60, 5-65, 5-70, 5-75, 5-80, 5-85, 5-90, 5-95, 10-15, 10-20, 10-25, 10-30, 10-35, 10-40, 10-45, 10-50, 10-55, 10-60, 10-65, 10-70, 10-75, 10-80, 10-85, 10-90, 10-95, 15-20, 15-25, 15-30, 15-35, 15-40, 15-45, 15-50, 15-55, 15-60, 15-65, 15-70, 15-75, 15-80, 15-85, 15-90, 15-95, 20-25, 20-30, 20-35, 20-40, 20-45, 20-50, 20-55, 20-60, 20-

65, 20-70, 20-75, 20-80, 20-85, 20-90, 20-95, 25-30, 25-35, 25-40, 25-45, 25-50, 25-55, 25-60, 25-65, 25-70, 25-75, 25-80, 25-85, 25-90, 25-95, 30-35, 30-40, 30-45, 30-50, 30-55, 30-60, 30-65, 30-70, 30-75, 30-80, 30-85, 30-90, 30-95, 35-40, 35-45, 35-50, 35-55, 35-60, 35-65, 35-70, 35-75, 35-80, 35-85, 35-90, 35-95, 40-45, 40-50, 40-55, 40-60, 40-65, 40-70, 40-75, 40-80, 40-85, 40-90, 40-95, 45-50, 45-55, 45-60, 45-65, 45-70, 45-75, 45-80, 45-85, 45-90, 45-95, 50-55, 50-60, 50-65, 50-70, 50-75, 50-80, 50-85, 50-90, 50-95, 55-60, 55-65, 55-70, 55-75, 55-80, 55-85, 55-90, 55-95, 60-65, 60-70, 60-75, 60-80, 60-85, 60-90, 60-95, 65-70, 65-75, 65-80, 65-85, 65-90, 65-95, 70-75, 70-80, 70-85, 70-90, 70-95, 75-80, 75-85, 75-90, 75-95, 80-85, 80-90, 80-95, 85-90, 85-95, or 90-95. As a non-limiting example, the guide to passenger ratio is a range of 1.3 to 99. As a non-limiting example, the guide to passenger ratio is a range of 10 to 99.

[0262] In certain embodiments, the guide to passenger (G:P) strand ratio is 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16, 16.5, 17, 17.5, 18, 18.5, 19, 19.5, 20, 20.5, 21, 21.5, 22, 22.5, 23, 23.5, 24, 24.5, 25, 25.5, 26, 26.5, 27, 27.5, 28, 28.5, 29, 29.5, 30, 30.5, 31, 31.5, 32, 32.5, 33, 33.5, 34, 34.5, 35, 35.5, 36, 36.5, 37, 37.5, 38, 38.5, 39, 39.5, 40, 40.5, 41, 41.5, 42, 42.5, 43, 43.5, 44, 44.5, 45, 45.5, 46, 46.5, 47, 47.5, 48, 48.5, 49, 49.5, 50, 50.5, 51, 51.5, 52, 52.5, 53, 53.5, 54, 54.5, 55, 55.5, 56, 56.5, 57, 57.5, 58, 58.5, 59, 59.5, 60, 60.5, 61, 61.5, 62, 62.5, 63, 63.5, 64, 64.5, 65, 65.5, 66, 66.5, 67, 67.5, 68, 68.5, 69, 69.5, 70, 70.5, 71, 71.5, 72, 72.5, 73, 73.5, 74, 74.5, 75, 75.5, 76, 76.5, 77, 77.5, 78, 78.5, 79, 79.5, 80, 80.5, 81, 81.5, 82, 82.5, 83, 83.5, 84, 84.5, 85, 85.5, 86, 86.5, 87, 87.5, 88, 88.5, 89, 89.5, 90, 90.5, 91, 91.5, 92, 92.5, 93, 93.5, 94, 94.5, 95, 95.5, 96, 96.5, 97, 97.5, 98, 98.5, or 99. As a non-limiting example, the guide to passenger (G:P) strand ratio is 11.5. As a non-limiting example, the guide to passenger (G:P) strand ratio is 99.

[0263] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is greater than 1.

[0264] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is greater than 2.

[0265] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is greater than 5.

[0266] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is greater than 10.

[0267] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is greater than 20.

[0268] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is greater than 50.

[0269] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is greater than 300.

[0270] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is 314.

[0271] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is greater than 400.

[0272] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is 434.

[0273] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is at least 3:1.

[0274] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is at least 5:1.

[0275] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is at least 10:1.

[0276] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is at least 20:1.

[0277] In certain embodiments, the guide to passenger (G: P) (also referred to as the antisense to sense) strand ratio expressed is at least 50:1.

[0278] In certain embodiments, the passenger to guide (P:G) (also referred to as the sense to antisense) strand ratio expressed is 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:10, 2:9, 2:8, 2:7, 2:6, 2:5, 2:4, 2:3, 2:2, 2:1, 3:10, 3:9, 3:8, 3:7, 3:6, 3:5, 3:4, 3:3, 3:2, 3:1, 4:10, 4:9, 4:8, 4:7, 4:6, 4:5, 4:4, 4:3, 4:2, 4:1, 5:10, 5:9, 5:8, 5:7, 5:6, 5:5, 5:4, 5:3, 5:2, 5:1, 6:10, 6:9, 6:8, 6:7, 6:6, 6:5, 6:4, 6:3, 6:2, 6:1, 7:10, 7:9, 7:8, 7:7, 7:6, 7:5, 7:4, 7:3, 7:2, 7:1, 8:10, 8:9, 8:8, 8:7, 8:6, 8:5, 8:4, 8:3, 8:2, 8:1, 9:10, 9:9, 9:8, 9:7, 9:6, 9:5, 9:4, 9:3, 9:2, 9:1, 10:10, 10:9, 10:8, 10:7, 10:6, 10:5, 10:4, 10:3, 10:2, 10:1, 1:99, 5:95, 10:90, 15:85, 20:80, 25:75, 30:70, 35:65, 40:60, 45:55, 50:50, 55:45, 60:40, 65:35, 70:30, 75:25, 80:20, 85:15, 90:10, 95:5, or 99:1 *in vitro* or *in vivo*. The passenger to guide ratio refers to the ratio of the passenger strands to the guide strands after the intracellular processing of the pri-microRNA. For example, an 80:20 of passenger-to-guide ratio would have 8 passenger strands to every 2 guide strands processed from the precursor. As a non-limiting example, the passenger-to-guide strand ratio is 80:20 *in vitro*. As a non-limiting example, the passenger-to-guide strand

ratio is 80:20 *in vivo*. As a non-limiting example, the passenger-to-guide strand ratio is 8:2 *in vitro*. As a non-limiting example, the passenger-to-guide strand ratio is 8:2 *in vivo*. As a non-limiting example, the passenger-to-guide strand ratio is 9:1 *in vitro*. As a non-limiting example, the passenger-to-guide strand ratio is 9:1 *in vivo*.

[0279] In certain embodiments, the passenger to guide (P:G) strand ratio is in a range of 1-99, 1.3-99, 5-99, 10-99, 15-99, 20-99, 25-99, 30-99, 35-99, 40-99, 45-99, 50-99, 55-99, 60-99, 65-99, 70-99, 75-99, 80-99, 85-99, 90-99, 95-99, 1-10, 1-15, 1-20, 1-25, 1-30, 1-35, 1-40, 1-45, 1-50, 1-55, 1-60, 1-65, 1-70, 1-75, 1-80, 1-85, 1-90, 1-95, 5-10, 5-15, 5-20, 5-25, 5-30, 5-35, 5-40, 5-45, 5-50, 5-55, 5-60, 5-65, 5-70, 5-75, 5-80, 5-85, 5-90, 5-95, 10-15, 10-20, 10-25, 10-30, 10-35, 10-40, 10-45, 10-50, 10-55, 10-60, 10-65, 10-70, 10-75, 10-80, 10-85, 10-90, 10-95, 15-20, 15-25, 15-30, 15-35, 15-40, 15-45, 15-50, 15-55, 15-60, 15-65, 15-70, 15-75, 15-80, 15-85, 15-90, 15-95, 20-25, 20-30, 20-35, 20-40, 20-45, 20-50, 20-55, 20-60, 20-65, 20-70, 20-75, 20-80, 20-85, 20-90, 20-95, 25-30, 25-35, 25-40, 25-45, 25-50, 25-55, 25-60, 25-65, 25-70, 25-75, 25-80, 25-85, 25-90, 25-95, 30-35, 30-40, 30-45, 30-50, 30-55, 30-60, 30-65, 30-70, 30-75, 30-80, 30-85, 30-90, 30-95, 35-40, 35-45, 35-50, 35-55, 35-60, 35-65, 35-70, 35-75, 35-80, 35-85, 35-90, 35-95, 40-45, 40-50, 40-55, 40-60, 40-65, 40-70, 40-75, 40-80, 40-85, 40-90, 40-95, 45-50, 45-55, 45-60, 45-65, 45-70, 45-75, 45-80, 45-85, 45-90, 45-95, 50-55, 50-60, 50-65, 50-70, 50-75, 50-80, 50-85, 50-90, 50-95, 55-60, 55-65, 55-70, 55-75, 55-80, 55-85, 55-90, 55-95, 60-65, 60-70, 60-75, 60-80, 60-85, 60-90, 60-95, 65-70, 65-75, 65-80, 65-85, 65-90, 65-95, 70-75, 70-80, 70-85, 70-90, 70-95, 75-80, 75-85, 75-90, 75-95, 80-85, 80-90, 80-95, 85-90, 85-95, or 90-95.

[0280] In certain embodiments, the passenger to guide (P:G) strand ratio is 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16, 16.5, 17, 17.5, 18, 18.5, 19, 19.5, 20, 20.5, 21, 21.5, 22, 22.5, 23, 23.5, 24, 24.5, 25, 25.5, 26, 26.5, 27, 27.5, 28, 28.5, 29, 29.5, 30, 30.5, 31, 31.5, 32, 32.5, 33, 33.5, 34, 34.5, 35, 35.5, 36, 36.5, 37, 37.5, 38, 38.5, 39, 39.5, 40, 40.5, 41, 41.5, 42, 42.5, 43, 43.5, 44, 44.5, 45, 45.5, 46, 46.5, 47, 47.5, 48, 48.5, 49, 49.5, 50, 50.5, 51, 51.5, 52, 52.5, 53, 53.5, 54, 54.5, 55, 55.5, 56, 56.5, 57, 57.5, 58, 58.5, 59, 59.5, 60, 60.5, 61, 61.5, 62, 62.5, 63, 63.5, 64, 64.5, 65, 65.5, 66, 66.5, 67, 67.5, 68, 68.5, 69, 69.5, 70, 70.5, 71, 71.5, 72, 72.5, 73, 73.5, 74, 74.5, 75, 75.5, 76, 76.5, 77, 77.5, 78, 78.5, 79, 79.5, 80, 80.5, 81, 81.5, 82, 82.5, 83, 83.5, 84, 84.5, 85, 85.5, 86, 86.5, 87, 87.5, 88, 88.5, 89, 89.5, 90, 90.5, 91, 91.5, 92, 92.5, 93, 93.5, 94, 94.5, 95, 95.5, 96, 96.5, 97, 97.5, 98, 98.5, or 99.

[0281] In certain embodiments, the passenger to guide (P:G) (also referred to as the sense to antisense) strand ratio expressed is greater than 1.

[0282] In certain embodiments, the passenger to guide (P: G) (also referred to as the sense to antisense) strand ratio expressed is greater than 2.

[0283] In certain embodiments, the passenger to guide (P: G) (also referred to as the sense to antisense) strand ratio expressed is greater than 5.

[0284] In certain embodiments, the passenger to guide (P: G) (also referred to as the sense to antisense) strand ratio expressed is greater than 10.

[0285] In certain embodiments, the passenger to guide (P: G) (also referred to as the sense to antisense) strand ratio expressed is greater than 20.

[0286] In certain embodiments, the passenger to guide (P: G) (also referred to as the sense to antisense) strand ratio expressed is greater than 50.

[0287] In certain embodiments, the passenger to guide (P: G) (also referred to as the sense to antisense) strand ratio expressed is at least 3:1.

[0288] In certain embodiments, the passenger to guide (P: G) (also referred to as the sense to antisense) strand ratio expressed is at least 5:1.

[0289] In certain embodiments, the passenger to guide (P: G) (also referred to as the sense to antisense) strand ratio expressed is at least 10:1.

[0290] In certain embodiments, the passenger to guide (P: G) (also referred to as the sense to antisense) strand ratio expressed is at least 20:1.

[0291] In certain embodiments, the passenger to guide (P: G) (also referred to as the sense to antisense) strand ratio expressed is at least 50:1.

[0292] In certain embodiments, a passenger-guide strand duplex is considered effective when the pri- or pre-microRNAs demonstrate, but methods known in the art and described herein, greater than 2-fold guide to passenger strand ratio when processing is measured. As a non-limiting examples, the pri- or pre-microRNAs demonstrate great than 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, or 2 to 5-fold, 2 to 10-fold, 2 to 15-fold, 3 to 5-fold, 3 to 10-fold, 3 to 15-fold, 4 to 5-fold, 4 to 10-fold, 4 to 15-fold, 5 to 10-fold, 5 to 15-fold, 6 to 10-fold, 6 to 15-fold, 7 to 10-fold, 7 to 15-fold, 8 to 10-fold, 8 to 15-fold, 9 to 10-fold, 9 to 15-fold, 10 to 15-fold, 11 to 15-fold, 12 to 15-fold, 13 to 15-fold, or 14 to 15-fold guide to passenger strand ratio when processing is measured.

[0293] In certain embodiments, the vector genome encoding the dsRNA comprises a sequence which is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more than

99% of the full length of the construct. As a non-limiting example, the vector genome comprises a sequence which is at least 80% of the full-length sequence of the construct.

[0294] In certain embodiments, the siRNA molecules may be used to silence wild type or mutant version of the gene of interest by targeting at least one exon on the gene of interest sequence. The exon may be exon 1, exon 2, exon 3, exon 4, exon 5, exon 6, exon 7, exon 8, exon 9, exon 10, exon 11, exon 12, exon 13, exon 14, exon 15, exon 16, exon 17, exon 18, exon 19, exon 20, exon 21, exon 22, exon 23, exon 24, exon 25, exon 26, exon 27, exon 28, exon 29, exon 30, exon 31, exon 32, exon 33, exon 34, exon 35, exon 36, exon 37, exon 38, exon 39, exon 40, exon 41, exon 42, exon 43, exon 44, exon 45, exon 46, exon 47, exon 48, exon 49, exon 50, exon 51, exon 52, exon 53, exon 54, exon 55, exon 56, exon 57, exon 58, exon 59, exon 60, exon 61, exon 62, exon 63, exon 64, exon 65, exon 66, and/or exon 67.

Design and Sequences of siRNA duplexes targeting SOD1 gene

[0295] The present disclosure provides small interfering RNA (siRNA) duplexes (and modulatory polynucleotides encoding them) that target SOD1 mRNA to interfere with SOD1 gene expression and/or SOD1 protein production.

[0296] The encoded siRNA duplex of the present disclosure contains an antisense strand and a sense strand hybridized together forming a duplex structure, wherein the antisense strand is complementary to the nucleic acid sequence of the targeted SOD1 gene, and wherein the sense strand is homologous to the nucleic acid sequence of the targeted SOD1 gene. In some aspects, the 5' end of the antisense strand has a 5' phosphate group and the 3' end of the sense strand contains a 3' hydroxyl group. In other aspects, there are none, one or 2 nucleotide overhangs at the 3' end of each strand.

[0297] Some guidelines for designing siRNAs have been proposed in the art. These guidelines generally recommend generating a 19-nucleotide duplexed region, symmetric 2-3 nucleotide 3' overhangs, 5' - phosphate and 3' - hydroxyl groups targeting a region in the gene to be silenced. Other rules that may govern siRNA sequence preference include, but are not limited to, (i) A/U at the 5' end of the antisense strand; (ii) G/C at the 5' end of the sense strand; (iii) at least five A/U residues in the 5' terminal one-third of the antisense strand; and (iv) the absence of any GC stretch of more than 9 nucleotides in length. In accordance with such consideration, together with the specific sequence of a target gene, highly effective siRNA molecules essential for suppressing the SOD1 gene expression may be readily designed.

[0298] According to the present disclosure, siRNA molecules (e.g., siRNA duplexes or encoded dsRNA) that target the SOD1 gene are designed. Such siRNA molecules can specifically, suppress SOD1 gene expression and protein production. In some aspects, the siRNA molecules are designed and used to selectively “knock out” SOD1 gene variants in cells, i.e., mutated SOD1 transcripts that are identified in patients with ALS disease. In some aspects, the siRNA molecules are designed and used to selectively “knock down” SOD1 gene variants in cells. In other aspects, the siRNA molecules are able to inhibit or suppress both the wild type and mutated SOD1 gene.

[0299] In certain embodiments, an siRNA molecule of the present disclosure comprises a sense strand and a complementary antisense strand in which both strands are hybridized together to form a duplex structure. The antisense strand has sufficient complementarity to the SOD1 mRNA sequence to direct target-specific RNAi, i.e., the siRNA molecule has a sequence sufficient to trigger the destruction of the target mRNA by the RNAi machinery or process.

[0300] In certain embodiments, an siRNA molecule of the present disclosure comprises a sense strand and a complementary antisense strand in which both strands are hybridized together to form a duplex structure and where the start site of the hybridization to the SOD1 mRNA is between nucleotide 15 and 1000 on the SOD1 mRNA sequence. As a non-limiting example, the start site may be between nucleotide 15-25, 15-50, 15-75, 15-100, 100-150, 150-200, 200-250, 250-300, 300-350, 350-400, 400-450, 450-500, 500-550, 550-600, 600-650, 650-700, 700-70, 750-800, 800-850, 850-900, 900-950, and 950-1000 on the SOD1 mRNA sequence. As yet another non-limiting example, the start site may be nucleotide 26, 27, 28, 29, 30, 32, 33, 34, 35, 36, 37, 74, 76, 77, 78, 149, 153, 157, 160, 177, 192, 193, 195, 196, 197, 198, 199, 206, 209, 210, 239, 241, 261, 263, 264, 268, 269, 276, 278, 281, 284, 290, 291, 295, 296, 316, 317, 329, 330, 337, 350, 351, 352, 354, 357, 358, 364, 375, 378, 383, 384, 390, 392, 395, 404, 406, 417, 418, 469, 470, 475, 476, 480, 487, 494, 496, 497, 501, 504, 515, 518, 522, 523, 524, 552, 554, 555, 562, 576, 577, 578, 579, 581, 583, 584, 585, 587, 588, 589, 593, 594, 595, 596, 597, 598, 599, 602, 607, 608, 609, 610, 611, 612, 613, 616, 621, 633, 635, 636, 639, 640, 641, 642, 643, 644, 645, 654, 660, 661, 666, 667, 668, 669, 673, 677, 692, 698, 699, 700, 701, 706, 749, 770, 772, 775, 781, 800, 804, 819, 829, 832, 833, 851, 854, 855, 857, 858, 859, 861, 869, 891, 892, 906, 907, 912, 913, 934, 944, and 947 on the SOD1 mRNA sequence.

[0301] In some embodiments, the antisense strand and target SOD1 mRNA sequences have 100% complementarity. The antisense strand may be complementary to any part of the target SOD1 mRNA sequence.

[0302] In other embodiments, the antisense strand and target SOD1 mRNA sequences comprise at least one mismatch. As a non-limiting example, the antisense strand and the target SOD1 mRNA sequence have at least 30%, 40%, 50%, 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-99%, 30-40%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-99%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-99%, 50-60%, 50-70%, 50-80%, 50-90%, 50-95%, 50-99%, 60-70%, 60-80%, 60-90%, 60-95%, 60-99%, 70-80%, 70-90%, 70-95%, 70-99%, 80-90%, 80-95%, 80-99%, 90-95%, 90-99% or 95-99% complementarity.

[0303] In certain embodiments, an siRNA or dsRNA targeting SOD1 includes at least two sequences that are complementary to each other.

[0304] According to the present disclosure, the siRNA molecule targeting SOD1 has a length from about 10-50 or more nucleotides, i.e., each strand comprising 10-50 nucleotides (or nucleotide analogs). Preferably, the siRNA molecule has a length from about 15-30, e.g., 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in each strand, wherein one of the strands is sufficiently complementary to a target region. In certain embodiments, each strand of the siRNA molecule has a length from about 19 to 25, 19 to 24 or 19 to 21 nucleotides. In certain embodiments, at least one strand of the siRNA molecule is 19 nucleotides in length. In certain embodiments, at least one strand of the siRNA molecule is 20 nucleotides in length. In certain embodiments, at least one strand of the siRNA molecule is 21 nucleotides in length. In certain embodiments, at least one strand of the siRNA molecule is 22 nucleotides in length. In certain embodiments, at least one strand of the siRNA molecule is 23 nucleotides in length. In certain embodiments, at least one strand of the siRNA molecule is 24 nucleotides in length. In certain embodiments, at least one strand of the siRNA molecule is 25 nucleotides in length.

[0305] In some embodiments, the siRNA molecules of the present disclosure targeting SOD1 can be synthetic RNA duplexes comprising about 19 nucleotides to about 25 nucleotides, and two overhanging nucleotides at the 3'-end. In some aspects, the siRNA molecules may be unmodified RNA molecules. In other aspects, the siRNA molecules may contain at least one modified nucleotide, such as base, sugar or backbone modifications.

[0306] In certain embodiments, the siRNA molecules of the present disclosure targeting SOD1 may comprise a nucleotide sequence such as, but not limited to, the antisense (guide) sequences in Table 2 or a fragment or variant thereof. As a non-limiting example, the antisense sequence used in the siRNA molecule of the present disclosure is at least 30%, 40%, 50%, 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-99%, 30-40%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-99%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-99%, 50-60%, 50-70%, 50-80%, 50-90%, 50-95%, 50-99%, 60-70%, 60-80%, 60-90%, 60-95%, 60-99%, 70-80%, 70-90%, 70-95%, 70-99%, 80-90%, 80-95%, 80-99%, 90-95%, 90-99% or 95-99% of a nucleotide sequence in Table 2. As another non-limiting example, the antisense sequence used in the siRNA molecule of the present disclosure comprises at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or more than 21 consecutive nucleotides of a nucleotide sequence in Table 2. As yet another non-limiting example, the antisense sequence used in the siRNA molecule of the present disclosure comprises nucleotides 1 to 22, 1 to 21, 1 to 20, 1 to 19, 1 to 18, 1 to 17, 1 to 16, 1 to 15, 1 to 14, 1 to 13, 1 to 12, 1 to 11, 1 to 10, 1 to 9, 1 to 8, 2 to 22, 2 to 21, 2 to 20, 2 to 19, 2 to 18, 2 to 17, 2 to 16, 2 to 15, 2 to 14, 2 to 13, 2 to 12, 2 to 11, 2 to 10, 2 to 9, 2 to 8, 3 to 22, 3 to 21, 3 to 20, 3 to 19, 3 to 18, 3 to 17, 3 to 16, 3 to 15, 3 to 14, 3 to 13, 3 to 12, 3 to 11, 3 to 10, 3 to 9, 3 to 8, 4 to 22, 4 to 21, 4 to 20, 4 to 19, 4 to 18, 4 to 17, 4 to 16, 4 to 15, 4 to 14, 4 to 13, 4 to 12, 4 to 11, 4 to 10, 4 to 9, 4 to 8, 5 to 22, 5 to 21, 5 to 20, 5 to 19, 5 to 18, 5 to 17, 5 to 16, 5 to 15, 5 to 14, 5 to 13, 5 to 12, 5 to 11, 5 to 10, 5 to 9, 5 to 8, 6 to 22, 6 to 21, 6 to 20, 6 to 19, 6 to 18, 6 to 17, 6 to 16, 6 to 15, 6 to 14, 6 to 13, 6 to 12, 6 to 11, 6 to 10, 7 to 22, 7 to 21, 7 to 20, 7 to 19, 7 to 18, 7 to 17, 7 to 16, 7 to 15, 7 to 14, 7 to 13, 7 to 12, 8 to 22, 8 to 21, 8 to 20, 8 to 19, 8 to 18, 8 to 17, 8 to 16, 8 to 15, 8 to 14, 8 to 13, 8 to 12, 9 to 22, 9 to 21, 9 to 20, 9 to 19, 9 to 18, 9 to 17, 9 to 16, 9 to 15, 9 to 14, 10 to 22, 10 to 21, 10 to 20, 10 to 19, 10 to 18, 10 to 17, 10 to 16, 10 to 15, 10 to 14, 11 to 22, 11 to 21, 11 to 20, 11 to 19, 11 to 18, 11 to 17, 11 to 16, 11 to 15, 11 to 14, 12 to 22, 12 to 21, 12 to 20, 12 to 19, 12 to 18, 12 to 17, 12 to 16, 13 to 22, 13 to 21, 13 to 20, 13 to 19, 13 to 18, 13 to 17, 13 to 16, 14 to 22, 14 to 21, 14 to 20, 14 to 19, 14 to 18, 14 to 17, 15 to 22, 15 to 21, 15 to 20, 15 to 19, 15 to 18, 16 to 22, 16 to 21, 16 to 20, 17 to 22, 17 to 21, or 18 to 22 of the sequences in Table 2.

Table 2. Antisense Sequences

Antisense ID	Sequence	SEQ ID NO
A-4002	UAUUAAAGUGAGGACCUUCUU	1

[0307] In certain embodiments, the siRNA molecules of the present disclosure targeting SOD1 may comprise a nucleotide sequence such as, but not limited to, the sense (passenger) sequences in Table 3 or a fragment or variant thereof. As a non-limiting example, the sense sequence used in the siRNA molecule of the present disclosure is at least 30%, 40%, 50%, 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-99%, 30-40%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-99%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-99%, 50-60%, 50-70%, 50-80%, 50-90%, 50-95%, 50-99%, 60-70%, 60-80%, 60-90%, 60-95%, 60-99%, 70-80%, 70-90%, 70-95%, 70-99%, 80-90%, 80-95%, 80-99%, 90-95%, 90-99% or 95-99% of a nucleotide sequence in Table 3. As another non-limiting example, the sense sequence used in the siRNA molecule of the present disclosure comprises at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or more than 21 consecutive nucleotides of a nucleotide sequence in Table 3. As yet another non-limiting example, the sense sequence used in the siRNA molecule of the present disclosure comprises nucleotides 1 to 22, 1 to 21, 1 to 20, 1 to 19, 1 to 18, 1 to 17, 1 to 16, 1 to 15, 1 to 14, 1 to 13, 1 to 12, 1 to 11, 1 to 10, 1 to 9, 1 to 8, 2 to 22, 2 to 21, 2 to 20, 2 to 19, 2 to 18, 2 to 17, 2 to 16, 2 to 15, 2 to 14, 2 to 13, 2 to 12, 2 to 11, 2 to 10, 2 to 9, 2 to 8, 3 to 22, 3 to 21, 3 to 20, 3 to 19, 3 to 18, 3 to 17, 3 to 16, 3 to 15, 3 to 14, 3 to 13, 3 to 12, 3 to 11, 3 to 10, 3 to 9, 3 to 8, 4 to 22, 4 to 21, 4 to 20, 4 to 19, 4 to 18, 4 to 17, 4 to 16, 4 to 15, 4 to 14, 4 to 13, 4 to 12, 4 to 11, 4 to 10, 4 to 9, 4 to 8, 5 to 22, 5 to 21, 5 to 20, 5 to 19, 5 to 18, 5 to 17, 5 to 16, 5 to 15, 5 to 14, 5 to 13, 5 to 12, 5 to 11, 5 to 10, 5 to 9, 5 to 8, 6 to 22, 6 to 21, 6 to 20, 6 to 19, 6 to 18, 6 to 17, 6 to 16, 6 to 15, 6 to 14, 6 to 13, 6 to 12, 6 to 11, 6 to 10, 7 to 22, 7 to 21, 7 to 20, 7 to 19, 7 to 18, 7 to 17, 7 to 16, 7 to 15, 7 to 14, 7 to 13, 7 to 12, 8 to 22, 8 to 21, 8 to 20, 8 to 19, 8 to 18, 8 to 17, 8 to 16, 8 to 15, 8 to 14, 8 to 13, 8 to 12, 9 to 22, 9 to 21, 9 to 20, 9 to 19, 9 to 18, 9 to 17, 9 to 16, 9 to 15, 9 to 14, 10 to 22, 10 to 21, 10 to 20, 10 to 19, 10 to 18, 10 to 17, 10 to 16, 10 to 15, 10 to 14, 11 to 22, 11 to 21, 11 to 20, 11 to 19, 11 to 18, 11 to 17, 11 to 16, 11 to 15, 11 to 14, 12 to 22, 12 to 21, 12 to 20, 12 to 19, 12 to 18, 12 to 17, 12 to 16, 13 to 22, 13 to 21, 13 to 20, 13 to 19, 13 to 18, 13 to 17, 13 to 16, 14 to 22, 14 to 21, 14 to 20, 14 to 19, 14 to 18, 14 to 17, 15 to

22, 15 to 21, 15 to 20, 15 to 19, 15 to 18, 16 to 22, 16 to 21, 16 to 20, 17 to 22, 17 to 21, or 18 to 22 of the sequences in Table 3.

Table 3. Sense Sequences

Sense ID	Sequence	SEQ ID NO
S-4003	GCAGGUCCUCACUUUAAUGCU	2

[0308] In certain embodiments, the siRNA molecules of the present disclosure targeting SOD1 may comprise an antisense sequence from Table 2 and a sense sequence from Table 3, or a fragment or variant thereof. As a non-limiting example, the antisense sequence and the sense sequence have at least 30%, 40%, 50%, 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-99%, 30-40%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-99%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-99%, 50-60%, 50-70%, 50-80%, 50-90%, 50-95%, 50-99%, 60-70%, 60-80%, 60-90%, 60-95%, 60-99%, 70-80%, 70-90%, 70-95%, 70-99%, 80-90%, 80-95%, 80-99%, 90-95%, 90-99% or 95-99% complementarity.

[0309] In certain embodiments, the siRNA molecules of the present disclosure targeting SOD1 may comprise the sense and antisense siRNA duplex as described in Table 4. As a non-limiting example, these siRNA duplexes may be tested for *in vitro* inhibitory activity on endogenous SOD1 gene expression.

Table 4. Sense and antisense strand sequences of SOD1 dsRNA

siRNA Duplex ID	SS ID	Sense Strand Sequence (5'-3')	SS SEQ ID	AS ID	Antisense Strand Sequence (5'-3')	AS SEQ ID
D-4012	S-4003	GCAGGUCCUCACUUUAAUGCU	2	A-4002	UAUUAAAGUGA GGACCUGCUU	1

[0310] In other embodiments, the siRNA molecules of the present disclosure targeting SOD1 can be encoded in plasmid vectors, AAV particles, viral genome or other nucleic acid expression vectors for delivery to a cell.

[0311] DNA expression plasmids can be used to stably express the siRNA duplexes or dsRNA of the present disclosure targeting SOD1 in cells and achieve long-term inhibition of the target gene expression. In one aspect, the sense and antisense strands of a siRNA duplex are typically linked by a short spacer sequence leading to the expression of a stem-loop structure termed short hairpin RNA (shRNA). The hairpin is recognized and cleaved by Dicer, thus generating mature siRNA molecules.

[0312] According to the present disclosure, AAV particles comprising the nucleic acids encoding the siRNA molecules targeting SOD1 mRNA are produced, the AAV serotypes may be any of the serotypes listed herein. Non-limiting examples of the AAV serotypes include, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV9.47, AAV9(hu14), AAV10, AAV11, AAV12, AAVrh8, AAVrh10, AAV-DJ8, AAV-DJ, AAV-PHP.A, and/or AAV-PHP.B, AAVPHP.B2, AAVPHP.B3, AAVPHP.N/PHP.B-DGT, AAVPHP.B-EST, AAVPHP.B-GGT, AAVPHP.B-ATP, AAVPHP.B-ATT-T, AAVPHP.B-DGT-T, AAVPHP.B-GGT-T, AAVPHP.B-SGS, AAVPHP.B-AQP, AAVPHP.B-QQP, AAVPHP.B-SNP(3), AAVPHP.B-SNP, AAVPHP.B-QGT, AAVPHP.B-NQT, AAVPHP.B-EGS, AAVPHP.B-SGN, AAVPHP.B-EGT, AAVPHP.B-DST, AAVPHP.B-DST, AAVPHP.B-STP, AAVPHP.B-PQP, AAVPHP.B-SQP, AAVPHP.B-QLP, AAVPHP.B-TMP, AAVPHP.B-TTP, AAVPHP.S/G2A12, AAVG2A15/G2A3, AAVG2B4, AAVG2B5, and variants thereof.

[0313] In some embodiments, the siRNA duplexes or encoded dsRNA of the present disclosure suppress (or degrade) SOD1 mRNA. Accordingly, the siRNA duplexes or encoded dsRNA can be used to substantially inhibit SOD1 gene expression in a cell. In some aspects, the inhibition of SOD1 gene expression refers to an inhibition by at least about 20%, preferably by at least about 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95% and 100%, or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-100%, 30-40%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-100%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-100%, 50-60%, 50-70%, 50-80%, 50-90%, 50-95%, 50-100%, 60-70%, 60-80%, 60-90%, 60-95%, 60-100%, 70-80%, 70-90%, 70-95%, 70-100%, 80-90%, 80-95%, 80-100%, 90-95%, 90-100% or 95-100%. Accordingly, the protein product of the targeted gene may be inhibited by at least about 20%, preferably by at least about 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95% and 100%, or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-100%, 30-40%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-100%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-100%, 50-60%, 50-70%, 50-80%, 50-90%, 50-95%, 50-100%, 60-70%, 60-80%, 60-90%, 60-95%, 60-100%, 70-80%, 70-90%, 70-95%, 70-100%, 80-90%, 80-95%, 80-100%, 90-95%, 90-100% or 95-100%.

[0314] According to the present disclosure, the siRNA molecules are designed and tested for their ability in reducing SOD1 mRNA levels in cultured cells. Such siRNA molecules

may form a duplex such as, but not limited to, include those listed in Table 4. As a non-limiting example, the siRNA duplexes may be siRNA duplex ID D-4012.

[0315] In certain embodiments, the siRNA molecules comprise a miRNA seed match for SOD1 located in the guide strand. In another embodiment, the siRNA molecules comprise a miRNA seed match for SOD1 located in the passenger strand. In yet another embodiment, the siRNA duplexes or encoded dsRNA targeting SOD1 gene do not comprise a seed match for SOD1 located in the guide or passenger strand.

[0316] In certain embodiments, the siRNA duplexes or encoded dsRNA targeting SOD1 gene may have almost no significant full-length off target effects for the guide strand. In another embodiment, the siRNA duplexes or encoded dsRNA targeting SOD1 gene may have almost no significant full-length off target effects for the passenger strand. The siRNA duplexes or encoded dsRNA targeting SOD1 gene may have less than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 1-5%, 2-6%, 3-7%, 4-8%, 5-9%, 5-10%, 6-10%, 5-15%, 5-20%, 5-25%, 5-30%, 10-20%, 10-30%, 10-40%, 10-50%, 15-30%, 15-40%, 15-45%, 20-40%, 20-50%, 25-50%, 30-40%, 30-50%, 35-50%, 40-50%, 45-50% full-length off target effects for the passenger strand. In yet another embodiment, the siRNA duplexes or encoded dsRNA targeting SOD1 gene may have almost no significant full-length off target effects for the guide strand or the passenger strand. The siRNA duplexes or encoded dsRNA targeting SOD1 gene may have less than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 1-5%, 2-6%, 3-7%, 4-8%, 5-9%, 5-10%, 6-10%, 5-15%, 5-20%, 5-25%, 5-30%, 10-20%, 10-30%, 10-40%, 10-50%, 15-30%, 15-40%, 15-45%, 20-40%, 20-50%, 25-50%, 30-40%, 30-50%, 35-50%, 40-50%, 45-50% full-length off target effects for the guide or passenger strand.

[0317] In certain embodiments, the siRNA duplexes or encoded dsRNA targeting SOD1 gene may have high activity *in vitro*. In another embodiment, the siRNA molecules may have low activity *in vitro*. In yet another embodiment, the siRNA duplexes or dsRNA targeting the SOD1 gene may have high guide strand activity and low passenger strand activity *in vitro*.

[0318] In certain embodiments, the siRNA molecules targeting SOD1 have a high guide strand activity and low passenger strand activity *in vitro*. The target knock-down (KD) by the guide strand may be at least 40%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, 99.5% or 100%. The target knock-down by the guide strand may be 40-50%, 45-50%, 50-55%, 50-60%, 60-65%, 60-70%, 60-75%, 60-80%, 60-85%, 60-90%, 60-95%, 60-99%, 60-

99.5%, 60-100%, 65-70%, 65-75%, 65-80%, 65-85%, 65-90%, 65-95%, 65-99%, 65-99.5%, 65-100%, 70-75%, 70-80%, 70-85%, 70-90%, 70-95%, 70-99%, 70-99.5%, 70-100%, 75-80%, 75-85%, 75-90%, 75-95%, 75-99%, 75-99.5%, 75-100%, 80-85%, 80-90%, 80-95%, 80-99%, 80-99.5%, 80-100%, 85-90%, 85-95%, 85-99%, 85-99.5%, 85-100%, 90-95%, 90-99%, 90-99.5%, 90-100%, 95-99%, 95-99.5%, 95-100%, 99-99.5%, 99-100% or 99.5-100%. As a non-limiting example, the target knock-down (KD) by the guide strand is greater than 70%. As a non-limiting example, the target knock-down (KD) by the guide strand is greater than 60%.

[0319] In certain embodiments, the siRNA duplex target SOD1 is designed so there is no miRNA seed match for the sense or antisense sequence to the non-SOD1 sequence.

[0320] In certain embodiments, the IC_{50} of the guide strand in the siRNA duplex targeting SOD1 for the nearest off target is greater than 100 multiplied by the IC_{50} of the guide strand for the on-target gene, SOD1. As a non-limiting example, if the IC_{50} of the guide strand for the nearest off target is greater than 100 multiplied by the IC_{50} of the guide strand for the target then the siRNA molecules are said to have high guide strand selectivity for inhibiting SOD1 *in vitro*.

[0321] In certain embodiments, the 5' processing of the guide strand of the siRNA duplex targeting SOD1 has a correct start (n) at the 5' end at least 75%, 80%, 85%, 90%, 95%, 99% or 100% of the time *in vitro* or *in vivo*. As a non-limiting example, the 5' processing of the guide strand is precise and has a correct start (n) at the 5' end at least 99% of the time *in vitro*. As a non-limiting example, the 5' processing of the guide strand is precise and has a correct start (n) at the 5' end at least 99% of the time *in vivo*. As a non-limiting example, the 5' processing of the guide strand is precise and has a correct start (n) at the 5' end at least 90% of the time *in vitro*. As a non-limiting example, the 5' processing of the guide strand is precise and has a correct start (n) at the 5' end at least 90% of the time *in vivo*. As a non-limiting example, the 5' processing of the guide strand is precise and has a correct start (n) at the 5' end at least 85% of the time *in vitro*. As a non-limiting example, the 5' processing of the guide strand is precise and has a correct start (n) at the 5' end at least 85% of the time *in vivo*.

[0322] In certain embodiments, the 5' processing of the guide strand of the siRNA duplex targeting SOD1 has a correct start (n) at the 5' end in a range of 75-95%, 75-90%, 75-85%, 75-80%, 80-95%, 80-90%, 80-85%, 85-95%, 85-90%, or 90-95%. As a non-limiting

example, the 5' processing of the guide strand of the siRNA duplex targeting SOD1 has a correct start (n) at the 5' end in a range of 75-95%.

[0323] In certain embodiments, the 5' processing of the guide strand of the siRNA duplex targeting SOD1 has a correct start (n) at the 5' end for 75%, 75.1%, 75.2%, 75.3%, 75.4%, 75.5%, 75.6%, 75.7%, 75.8%, 75.9%, 76%, 76.1%, 76.2%, 76.3%, 76.4%, 76.5%, 76.6%, 76.7%, 76.8%, 76.9%, 77%, 77.1%, 77.2%, 77.3%, 77.4%, 77.5%, 77.6%, 77.7%, 77.8%, 77.9%, 78%, 78.1%, 78.2%, 78.3%, 78.4%, 78.5%, 78.6%, 78.7%, 78.8%, 78.9%, 79%, 79.1%, 79.2%, 79.3%, 79.4%, 79.5%, 79.6%, 79.7%, 79.8%, 79.9%, 80%, 80.1%, 80.2%, 80.3%, 80.4%, 80.5%, 80.6%, 80.7%, 80.8%, 80.9%, 81%, 81.1%, 81.2%, 81.3%, 81.4%, 81.5%, 81.6%, 81.7%, 81.8%, 81.9%, 82%, 82.1%, 82.2%, 82.3%, 82.4%, 82.5%, 82.6%, 82.7%, 82.8%, 82.9%, 83%, 83.1%, 83.2%, 83.3%, 83.4%, 83.5%, 83.6%, 83.7%, 83.8%, 83.9%, 84%, 84.1%, 84.2%, 84.3%, 84.4%, 84.5%, 84.6%, 84.7%, 84.8%, 84.9%, 85%, 85.1%, 85.2%, 85.3%, 85.4%, 85.5%, 85.6%, 85.7%, 85.8%, 85.9%, 86%, 86.1%, 86.2%, 86.3%, 86.4%, 86.5%, 86.6%, 86.7%, 86.8%, 86.9%, 87%, 87.1%, 87.2%, 87.3%, 87.4%, 87.5%, 87.6%, 87.7%, 87.8%, 87.9%, 88%, 88.1%, 88.2%, 88.3%, 88.4%, 88.5%, 88.6%, 88.7%, 88.8%, 88.9%, 89%, 89.1%, 89.2%, 89.3%, 89.4%, 89.5%, 89.6%, 89.7%, 89.8%, 89.9%, 90%, 90.1%, 90.2%, 90.3%, 90.4%, 90.5%, 90.6%, 90.7%, 90.8%, 90.9%, 91%, 91.1%, 91.2%, 91.3%, 91.4%, 91.5%, 91.6%, 91.7%, 91.8%, 91.9%, 92%, 92.1%, 92.2%, 92.3%, 92.4%, 92.5%, 92.6%, 92.7%, 92.8%, 92.9%, 93%, 93.1%, 93.2%, 93.3%, 93.4%, 93.5%, 93.6%, 93.7%, 93.8%, 93.9%, 94%, 94.1%, 94.2%, 94.3%, 94.4%, 94.5%, 94.6%, 94.7%, 94.8%, 94.9%, or 95% of the constructs expressed. As a non-limiting example, the 5' processing of the guide strand of the siRNA duplex targeting SOD1 has a correct start (n) at the 5' end for 81% of the constructs expressed. As a non-limiting example, the 5' processing of the guide strand of the siRNA duplex targeting SOD1 has a correct start (n) at the 5' end for 90 % of the constructs expressed.

[0324] In certain embodiments, a passenger-guide strand duplex for SOD1 is considered effective when the pri- or pre-microRNAs demonstrate, by methods known in the art and described herein, greater than 2-fold guide to passenger strand ratio when processing is measured. As a non-limiting examples, the pri- or pre-microRNAs demonstrate great than 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, or 2 to 5-fold, 2 to 10-fold, 2 to 15-fold, 3 to 5-fold, 3 to 10-fold, 3 to 15-fold, 4 to 5-fold, 4 to 10-fold, 4 to 15-fold, 5 to 10-fold, 5 to 15-fold, 6 to 10-fold, 6 to 15-fold, 7 to 10-fold, 7 to 15-fold, 8 to 10-fold, 8 to 15-fold, 9 to 10-fold, 9 to 15-fold, 10 to 15-

fold, 11 to 15-fold, 12 to 15-fold, 13 to 15-fold, or 14 to 15-fold guide to passenger strand ratio when processing is measured.

[0325] In certain embodiments, the siRNA molecules may be used to silence wild type or mutant SOD1 by targeting at least one exon on the SOD1 sequence. The exon may be exon 1, exon 2, exon 3, exon 4, exon 5, exon 6, exon 7, exon 8, exon 9, exon 10, exon 11, exon 12, exon 13, exon 14, exon 15, exon 16, exon 17, exon 18, exon 19, exon 20, exon 21, exon 22, exon 23, exon 24, exon 25, exon 26, exon 27, exon 28, exon 29, exon 30, exon 31, exon 32, exon 33, exon 34, exon 35, exon 36, exon 37, exon 38, exon 39, exon 40, exon 41, exon 42, exon 43, exon 44, exon 45, exon 46, exon 47, exon 48, exon 49, exon 50, exon 51, exon 52, exon 53, exon 54, exon 55, exon 56, exon 57, exon 58, exon 59, exon 60, exon 61, exon 62, exon 63, exon 64, exon 65, exon 66, and/or exon 67.

[0326] In certain embodiments, the range of guide strands to the total endogenous pool of miRNAs is 0.001-0.6%, 0.005-0.6%, 0.01-0.6%, 0.015-0.6%, 0.02-0.6%, 0.025-0.6%, 0.03-0.6%, 0.035-0.6%, 0.04-0.6%, 0.045-0.6%, 0.05-0.6%, 0.055-0.6%, 0.06-0.6%, 0.065-0.6%, 0.07-0.6%, 0.075-0.6%, 0.08-0.6%, 0.085-0.6%, 0.09-0.6%, 0.095-0.6%, 0.1-0.6%, 0.15-0.6%, 0.2-0.6%, 0.25-0.6%, 0.3-0.6%, 0.35-0.6%, 0.4-0.6%, 0.45-0.6%, 0.5-0.6%, 0.55-0.6%, 0.001-0.5%, 0.005-0.5%, 0.01-0.5%, 0.015-0.5%, 0.02-0.5%, 0.025-0.5%, 0.03-0.5%, 0.035-0.5%, 0.04-0.5%, 0.045-0.5%, 0.05-0.5%, 0.055-0.5%, 0.06-0.5%, 0.065-0.5%, 0.07-0.5%, 0.075-0.5%, 0.08-0.5%, 0.085-0.5%, 0.09-0.5%, 0.095-0.5%, 0.1-0.5%, 0.15-0.5%, 0.2-0.5%, 0.25-0.5%, 0.3-0.5%, 0.35-0.5%, 0.4-0.5%, 0.45-0.5%, 0.001-0.4%, 0.005-0.4%, 0.01-0.4%, 0.015-0.4%, 0.02-0.4%, 0.025-0.4%, 0.03-0.4%, 0.035-0.4%, 0.04-0.4%, 0.045-0.4%, 0.05-0.4%, 0.055-0.4%, 0.06-0.4%, 0.065-0.4%, 0.07-0.4%, 0.075-0.4%, 0.08-0.4%, 0.085-0.4%, 0.09-0.4%, 0.095-0.4%, 0.1-0.4%, 0.15-0.4%, 0.2-0.4%, 0.25-0.4%, 0.3-0.4%, 0.35-0.4%, 0.001-0.3%, 0.005-0.3%, 0.01-0.3%, 0.015-0.3%, 0.02-0.3%, 0.025-0.3%, 0.03-0.3%, 0.035-0.3%, 0.04-0.3%, 0.045-0.3%, 0.05-0.3%, 0.055-0.3%, 0.06-0.3%, 0.065-0.3%, 0.07-0.3%, 0.075-0.3%, 0.08-0.3%, 0.085-0.3%, 0.09-0.3%, 0.095-0.3%, 0.1-0.3%, 0.15-0.3%, 0.2-0.3%, 0.25-0.3%, 0.001-0.2%, 0.005-0.2%, 0.01-0.2%, 0.015-0.2%, 0.02-0.2%, 0.025-0.2%, 0.03-0.2%, 0.035-0.2%, 0.04-0.2%, 0.045-0.2%, 0.05-0.2%, 0.055-0.2%, 0.06-0.2%, 0.065-0.2%, 0.07-0.2%, 0.075-0.2%, 0.08-0.2%, 0.085-0.2%, 0.09-0.2%, 0.095-0.2%, 0.1-0.2%, 0.15-0.2%, 0.001-0.1%, 0.005-0.1%, 0.01-0.1%, 0.015-0.1%, 0.02-0.1%, 0.025-0.1%, 0.03-0.1%, 0.035-0.1%, 0.04-0.1%, 0.045-0.1%, 0.05-0.1%, 0.055-0.1%, 0.06-0.1%, 0.065-0.1%, 0.07-0.1%, 0.075-0.1%, 0.08-0.1%, 0.085-0.1%, 0.09-0.1%, or 0.095-0.1%. As a

non-limiting example, the range is 0.06-0.6%. As a non-limiting example, the range is 0.4-0.5%.

[0327] In certain embodiments, the percent of guide strands to the total endogenous pool of miRNAs is 0.001%, 0.002%, 0.003%, 0.004%, 0.005%, 0.006%, 0.007%, 0.008%, 0.009%, 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, or 0.6%. As a non-limiting example, the percent is 0.06%. As a non-limiting example, the percent is 0.4%. As a non-limiting example, the percent is 0.5%.

siRNA modification

[0328] In some embodiments, the siRNA molecules of the present disclosure, when not delivered as a precursor or DNA, may be chemically modified to modulate some features of RNA molecules, such as, but not limited to, increasing the stability of siRNAs *in vivo*. The chemically modified siRNA molecules can be used in human therapeutic applications, and are improved without compromising the RNAi activity of the siRNA molecules. As a non-limiting example, the siRNA molecules modified at both the 3' and the 5' end of both the sense strand and the antisense strand.

[0329] In some aspects, the siRNA duplexes of the present disclosure may contain one or more modified nucleotides such as, but not limited to, sugar modified nucleotides, nucleobase modifications and/or backbone modifications. In some aspects, the siRNA molecule may contain combined modifications, for example, combined nucleobase and backbone modifications.

[0330] In certain embodiments, the modified nucleotide may be a sugar-modified nucleotide. Sugar modified nucleotides include, but are not limited to 2'-fluoro, 2'-amino and 2'-thio modified ribonucleotides, e.g. 2'-fluoro modified ribonucleotides. Modified nucleotides may be modified on the sugar moiety, as well as nucleotides having sugars or analogs thereof that are not ribosyl. For example, the sugar moieties may be, or be based on, mannoses, arabinoses, glucopyranoses, galactopyranoses, 4'-thioribose, and other sugars, heterocycles, or carbocycles.

[0331] In certain embodiments, the modified nucleotide may be a nucleobase-modified nucleotide.

[0332] In certain embodiments, the modified nucleotide may be a backbone-modified nucleotide. In some embodiments, the siRNA duplexes of the present disclosure may further comprise other modifications on the backbone. A normal "backbone", as used herein, refers to the repeating alternating sugar-phosphate sequences in a DNA or RNA molecule. The

deoxyribose/ribose sugars are joined at both the 3'-hydroxyl and 5'-hydroxyl groups to phosphate groups in ester links, also known as "phosphodiester" bonds/linker (PO linkage). The PO backbones may be modified as "phosphorothioate backbone (PS linkage). In some cases, the natural phosphodiester bonds may be replaced by amide bonds but the four atoms between two sugar units are kept. Such amide modifications can facilitate the solid phase synthesis of oligonucleotides and increase the thermodynamic stability of a duplex formed with siRNA complement. See e.g. Mesmacker et al., *Pure & Appl. Chem.*, 1997, 3, 437-440; the content of which is incorporated herein by reference in its entirety.

[0333] Modified bases refer to nucleotide bases such as, for example, adenine, guanine, cytosine, thymine, uracil, xanthine, inosine, and queuosine that have been modified by the replacement or addition of one or more atoms or groups. Some examples of modifications on the nucleobase moieties include, but are not limited to, alkylated, halogenated, thiolated, aminated, amidated, or acetylated bases, individually or in combination. More specific examples include, for example, 5-propynyluridine, 5-propynylcytidine, 6-methyladenine, 6-methylguanine, N,N,-dimethyladenine, 2-propyladenine, 2-propylguanine, 2-aminoadenine, 1-methylinosine, 3-methyluridine, 5-methyleytidine, 5-methyluridine and other nucleotides having a modification at the 5 position, 5-(2-amino)propyl uridine, 5-halocytidine, 5-halouridine, 4-acetylcytidine, 1-methyladenosine, 2-methyladenosine, 3-methylcytidine, 6-methyluridine, 2-methylguanosine, 7-methylguanosine, 2,2-dimethylguanosine, 5-methylaminoethyluridine, 5-methyloxyuridine, deazanucleotides such as 7-deaza-adenosine, 6-azouridine, 6-azocytidine, 6-azothymidine, 5-methyl-2-thiouridine, other thio bases such as 2-thiouridine and 4-thiouridine and 2-thiocytidine, dihydrouridine, pseudouridine, queuosine, archaeosine, naphthyl and substituted naphthyl groups, any O- and N-alkylated purines and pyrimidines such as N6-methyladenosine, 5-methylcarbonylmethyluridine, uridine 5-oxyacetic acid, pyridine-4-one, pyridine-2-one, phenyl and modified phenyl groups such as aminophenol or 2,4,6-trimethoxy benzene, modified cytosines that act as G-clamp nucleotides, 8-substituted adenines and guanines, 5-substituted uracils and thymines, azapyrimidines, carboxyhydroxyalkyl nucleotides, carboxyalkylaminoalkyl nucleotides, and alkylcarbonylalkylated nucleotides.

[0334] In certain embodiments, the modified nucleotides may be on just the sense strand.

[0335] In another embodiment, the modified nucleotides may be on just the antisense strand.

[0336] In some embodiments, the modified nucleotides may be in both the sense and antisense strands.

[0337] In some embodiments, the chemically modified nucleotide does not affect the ability of the antisense strand to pair with the target mRNA sequence.

[0338] In certain embodiments, the AAV particle comprising a nucleic acid sequence encoding the siRNA molecules of the present disclosure may encode siRNA molecules which are polycistronic molecules. The siRNA molecules may additionally comprise one or more linkers between regions of the siRNA molecules.

Molecular Scaffold

[0339] In certain embodiments, the siRNA molecules may be encoded in a modulatory polynucleotide which also comprises a molecular scaffold. As used herein a “molecular scaffold” is a framework or starting molecule that forms the sequence or structural basis against which to design or make a subsequent molecule.

[0340] In certain embodiments, the molecular scaffold comprises at least one 5' flanking region. As a non-limiting example, the 5' flanking region may comprise a 5' flanking sequence which may be of any length and may be derived in whole or in part from wild type microRNA sequence or be a completely artificial sequence.

[0341] In some embodiments, one or both of the 5' and 3' flanking sequences are absent.

[0342] In some embodiments the 5' and 3' flanking sequences are the same length.

[0343] In some embodiments the 5' flanking sequence is from 1-10 nucleotides in length, from 5-15 nucleotides in length, from 10-30 nucleotides in length, from 20-50 nucleotides in length, greater than 40 nucleotides in length, greater than 50 nucleotides in length, greater than 100 nucleotides in length or greater than 200 nucleotides in length.

[0344] In some embodiments, the 5' flanking sequence may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197,

198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, or 500 nucleotides in length.

[0345] In some embodiments the 3' flanking sequence is from 1-10 nucleotides in length, from 5-15 nucleotides in length, from 10-30 nucleotides in length, from 20-50 nucleotides in length, greater than 40 nucleotides in length, greater than 50 nucleotides in length, greater than 100 nucleotides in length or greater than 200 nucleotides in length.

[0346] In some embodiments, the 3' flanking sequence may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215,

216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, or 500 nucleotides in length.

[0347] In some embodiments the 5' and 3' flanking sequences are the same sequence. In some embodiments they differ by 2%, 3%, 4%, 5%, 10%, 20% or more than 30% when aligned to each other.

[0348] In certain embodiments, the molecular scaffold comprises at least one 3' flanking region. As a non-limiting example, the 3' flanking region may comprise a 3' flanking sequence which may be of any length and may be derived in whole or in part from wild type microRNA sequence or be a completely artificial sequence.

[0349] In certain embodiments, the molecular scaffold comprises at least one loop motif region. As a non-limiting example, the loop motif region may comprise a sequence which may be of any length.

[0350] In certain embodiments, the molecular scaffold comprises a 5' flanking region, a loop motif region and/or a 3' flanking region.

[0351] In certain embodiments, at least one siRNA, miRNA or other RNAi agent described herein, may be encoded by a modulatory polynucleotide which may also comprise at least one molecular scaffold. The molecular scaffold may comprise a 5' flanking sequence which may be of any length and may be derived in whole or in part from wild type

microRNA sequence or be completely artificial. The 3' flanking sequence may mirror the 5' flanking sequence and/or a 3' flanking sequence in size and origin. Either flanking sequence may be absent. The 3' flanking sequence may optionally contain one or more CNNC motifs, where "N" represents any nucleotide.

[0352] Forming the stem of a stem loop structure is a minimum of the modulatory polynucleotide encoding at least one siRNA, miRNA or other RNAi agent described herein. In some embodiments, the siRNA, miRNA or other RNAi agent described herein comprises at least one nucleic acid sequence which is in part complementary or will hybridize to a target sequence. In some embodiments the payload is an siRNA molecule or fragment of an siRNA molecule.

[0353] In some embodiments, the 5' arm of the stem loop structure of the modulatory polynucleotide comprises a nucleic acid sequence encoding a sense sequence. Non-limiting examples of sense sequences, or fragments or variants thereof, which may be encoded by the modulatory polynucleotide are described in Table 3.

[0354] In some embodiments, the 3' arm of the stem loop of the modulatory polynucleotide comprises a nucleic acid sequence encoding an antisense sequence. The antisense sequence, in some instances, comprises a "G" nucleotide at the 5' most end. Non-limiting examples of antisense sequences, or fragments or variants thereof, which may be encoded by the modulatory polynucleotide are described in Table 2.

[0355] In other embodiments, the sense sequence may reside on the 3' arm while the antisense sequence resides on the 5' arm of the stem of the stem loop structure of the modulatory polynucleotide. Non-limiting examples of sense and antisense sequences which may be encoded by the modulatory polynucleotide are described in Tables 2 and 3.

[0356] In certain embodiments, the sense and antisense sequences may be completely complementary across a substantial portion of their length. In other embodiments the sense sequence and antisense sequence may be at least 70, 80, 90, 95 or 99% complementarity across independently at least 50, 60, 70, 80, 85, 90, 95, or 99 % of the length of the strands.

[0357] Neither the identity of the sense sequence nor the homology of the antisense sequence need to be 100% complementarity to the target sequence.

[0358] In certain embodiments, separating the sense and antisense sequence of the stem loop structure of the modulatory polynucleotide is a loop sequence (also known as a loop motif, linker or linker motif). The loop sequence may be of any length, between 4-30 nucleotides, between 4-20 nucleotides, between 4-15 nucleotides, between 5-15 nucleotides,

between 6-12 nucleotides, 6 nucleotides, 7 nucleotides, 8 nucleotides, 9 nucleotides, 10 nucleotides, 11 nucleotides, 12 nucleotides, 13 nucleotides, 14 nucleotides, and/or 15 nucleotides.

[0359] In some embodiments, the loop sequence comprises a nucleic acid sequence encoding at least one UGUG motif. In some embodiments, the nucleic acid sequence encoding the UGUG motif is located at the 5' terminus of the loop sequence.

[0360] In certain embodiments, spacer regions may be present in the modulatory polynucleotide to separate one or more modules (e.g., 5' flanking region, loop motif region, 3' flanking region, sense sequence, antisense sequence) from one another. There may be one or more such spacer regions present.

[0361] In certain embodiments, a spacer region of between 8-20, i.e., 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides may be present between the sense sequence and a flanking region sequence.

[0362] In certain embodiments, the length of the spacer region is 13 nucleotides and is located between the 5' terminus of the sense sequence and the 3' terminus of the flanking sequence. In certain embodiments, a spacer is of sufficient length to form approximately one helical turn of the sequence.

[0363] In certain embodiments, a spacer region of between 8-20, i.e., 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides may be present between the antisense sequence and a flanking sequence.

[0364] In certain embodiments, the spacer sequence is between 10-13, i.e., 10, 11, 12 or 13 nucleotides and is located between the 3' terminus of the antisense sequence and the 5' terminus of a flanking sequence. In certain embodiments, a spacer is of sufficient length to form approximately one helical turn of the sequence.

[0365] In certain embodiments, the molecular scaffold of the modulatory polynucleotide comprises in the 5' to 3' direction, a 5' flanking sequence, a 5' arm, a loop motif, a 3' arm and a 3' flanking sequence. As a non-limiting example, the 5' arm may comprise a nucleic acid sequence encoding a sense sequence and the 3' arm comprises a nucleic acid sequence encoding the antisense sequence. In another non-limiting example, the 5' arm comprises a nucleic acid sequence encoding the antisense sequence and the 3' arm comprises a nucleic acid sequence encoding the sense sequence.

[0366] In certain embodiments, the 5' arm, sense and/or antisense sequence, loop motif and/or 3' arm sequence may be altered (e.g., substituting 1 or more nucleotides, adding

nucleotides and/or deleting nucleotides). The alteration may cause a beneficial change in the function of the construct (e.g., increase knock-down of the target sequence, reduce degradation of the construct, reduce off target effect, increase efficiency of the payload, and reduce degradation of the payload).

[0367] In certain embodiments, the molecular scaffold of the modulatory polynucleotides is aligned in order to have the rate of excision of the guide strand (also referred to herein as the antisense strand) be greater than the rate of excision of the passenger strand (also referred to herein as the sense strand). The rate of excision of the guide or passenger strand may be, independently, 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more than 99%. As a non-limiting example, the rate of excision of the guide strand is at least 80%. As another non-limiting example, the rate of excision of the guide strand is at least 90%.

[0368] In certain embodiments, the rate of excision of the guide strand is greater than the rate of excision of the passenger strand. In one aspect, the rate of excision of the guide strand may be at least 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more than 99% greater than the passenger strand.

[0369] In certain embodiments, the efficiency of excision of the guide strand is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more than 99%. As a non-limiting example, the efficiency of the excision of the guide strand is greater than 80%.

[0370] In certain embodiments, the efficiency of the excision of the guide strand is greater than the excision of the passenger strand from the molecular scaffold. The excision of the guide strand may be 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 times more efficient than the excision of the passenger strand from the molecular scaffold.

[0371] In certain embodiments, the molecular scaffold comprises a dual-function targeting modulatory polynucleotide. As used herein, a “dual-function targeting” modulatory polynucleotide is a polynucleotide where both the guide and passenger strands knock down the same target or the guide and passenger strands knock down different targets.

[0372] In certain embodiments, the molecular scaffold of the modulatory polynucleotides described herein may comprise a 5' flanking region, a loop motif region and a 3' flanking region. Non-limiting examples of the sequences for the 5' flanking region, loop motif region (may also be referred to as a linker region) and the 3' flanking region which may be used, or

fragments thereof used, in the modulatory polynucleotides described herein are shown in Tables 5 – 7.

Table 5. 5' Flanking Regions for Molecular Scaffold

5' Flanking Region Name	5' Flanking Region Sequence	5' Flanking Region SEQ ID
5F1	CTCCCGCAGAACACCATGCGCTCCACGGAA	3
5F2	GTGCTGGGCGGGGGGGCGGCGGGCCCTCCCGC AGAACACCATGCGCTCTTCGGAA	13
5F3	GTGCTGGGCGGGGGGGCGGCGGGCCCTCCCGC AGAACACCATGCGCTCCACGGAA	14

Table 6. Loop Motif Regions for Molecular Scaffold

Loop Motif Region Name	Loop Motif Region Sequence	Loop Motif Region SEQ ID
L1	GTGGCCACTGAGAAG	4
L2	TGTGATTTGG	15
L3	GTGGCCACTGAGAAG	16

Table 7. 3' Flanking Regions for Molecular Scaffold

3' Flanking Region Name	3' Flanking Region Sequence	3' Flanking Region SEQ ID
3F1	CTGAGGAGCGCCTTGACAGCAGCCATGGGAG GGCC	5
3F2	TGGCCGTGTAGTGCTACCCAGCGCTGGCTGCC TCCTCAGCATTGCAATTCCTCTCCCATCTGGG CACCAGTCAGCTACCCTGGTGGGAATCTGGGT AGCC	17
3F3	CTGTGGAGCGCCTTGACAGCAGCCATGGGAG GGCCGCCCCCTACCTCAGTGA	18

[0373] In certain embodiments, the molecular scaffold may comprise at least one 5' flanking region, fragment or variant thereof listed in Table 5. As a non-limiting example, the 5' flanking region may be 5F1.

[0374] In certain embodiments, the molecular scaffold may comprise at least one 5F1 flanking region.

[0375] In certain embodiments, the molecular scaffold may comprise at least one loop motif region, fragment or variant thereof listed in Table 6. As a non-limiting example, the loop motif region may be L1.

[0376] In certain embodiments, the molecular scaffold may comprise at least one L1 loop motif region.

[0377] In certain embodiments, the molecular scaffold may comprise at least one 3' flanking region, fragment or variant thereof listed in Table 7. As a non-limiting example, the 3' flanking region may be 3F1.

[0378] In certain embodiments, the molecular scaffold may comprise at least one 3F1 flanking region.

[0379] In certain embodiments, the molecular scaffold may comprise at least one 5' flanking region, fragment or variant thereof, and at least one loop motif region, fragment or variant thereof, as described in Tables 5 and 6. As a non-limiting example, the 5' flanking region and the loop motif region may be 5F1 and L1.

[0380] In certain embodiments, the molecular scaffold may comprise at least one 3' flanking region, fragment or variant thereof, and at least one motif region, fragment or variant thereof, as described in Tables 6 and 7. As a non-limiting example, the 3' flanking region and the loop motif region may be 3F1 and L1.

[0381] In certain embodiments, the molecular scaffold may comprise at least one 5' flanking region, fragment or variant thereof, and at least one 3' flanking region, fragment or variant thereof, as described in Tables 5 and 7. As a non-limiting example, the flanking regions may be 5F1 and 3F1.

[0382] In certain embodiments, the molecular scaffold may comprise at least one 5' flanking region, fragment or variant thereof, at least one loop motif region, fragment or variant thereof, and at least one 3' flanking region as described in Tables 5 – 7. As a non-limiting example, the flanking and loop motif regions may be 5F1, L1 and 3F1.

[0383] In certain embodiments, the molecular scaffold may be a natural pri-miRNA scaffold. As a non-limiting example, the molecular scaffold may be a scaffold derived from the human miR155 scaffold.

[0384] In certain embodiments, the molecular scaffold may comprise one or more linkers known in the art. The linkers may separate regions or one molecular scaffold from another. As a non-limiting example, the molecular scaffold may be polycistronic.

Modulatory Polynucleotide Comprising Molecular Scaffold and siRNA Molecules Targeting SOD1

[0385] In certain embodiments, the modulatory polynucleotide may comprise 5' and 3' flanking regions, loop motif region, and nucleic acid sequences encoding sense sequence and antisense sequence as described in Table 8. In Table 8, the DNA sequence identifier for the passenger and guide strands are described as well as the 5' and 3' Flanking Regions and the

Loop region (also referred to as the linker region). In Table 8, the “miR” component of the name of the sequence does not necessarily correspond to the sequence numbering of miRNA genes (e.g., VOYSOD1miR-102 is the name of the sequence and does not necessarily mean that miR-102 is part of the sequence).

Table 8. SOD1 Modulatory Polynucleotide Sequence Regions (5’ to 3’)

Modulatory Polynucleotide Construct Name	5’ Flanking to 3’ Flanking SEQ ID NO	5’ Flanking SEQ ID NO	Passenger SEQ ID NO	Loop SEQ ID NO	Guide SEQ ID NO	3’ Flanking SEQ ID NO
VOYSOD1miR104-788.2	6	3	7	4	8	5
VOYSOD1miR127-860	19	13	20	15	21	17
VOYSOD1miR114-861	22	14	23	16	24	18

AAV Particles Comprising Modulatory Polynucleotides

[0386] In certain embodiments, the AAV particle comprises a viral genome with a payload region comprising a modulatory polynucleotide sequence. In such an embodiment, a viral genome encoding more than one polypeptide may be replicated and packaged into a viral particle. A target cell transduced with a viral particle comprising a modulatory polynucleotide may express the encoded sense and/or antisense sequences in a single cell.

[0387] In some embodiments, the AAV particles are useful in the field of medicine for the treatment, prophylaxis, palliation or amelioration of neurological diseases and/or disorders.

[0388] In certain embodiments, the AAV particles comprising modulatory polynucleotide sequence which comprises a nucleic acid sequence encoding at least one siRNA molecule may be introduced into mammalian cells.

[0389] Where the AAV particle payload region comprises a modulatory polynucleotide, the modulatory polynucleotide may comprise sense and/or antisense sequences to knock down a target gene. The AAV viral genomes encoding modulatory polynucleotides described herein may be useful in the fields of human disease, viruses, infections veterinary applications and a variety of *in vivo* and *in vitro* settings.

[0390] In certain embodiments, the AAV particle viral genome may comprise at least one inverted terminal repeat (ITR) region. The ITR region(s) may, independently, have a length such as, but not limited to, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148,

149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, and 175 nucleotides. The length of the ITR region for the viral genome may be 75-80, 75-85, 75-100, 80-85, 80-90, 80-105, 85-90, 85-95, 85-110, 90-95, 90-100, 90-115, 95-100, 95-105, 95-120, 100-105, 100-110, 100-125, 105-110, 105-115, 105-130, 110-115, 110-120, 110-135, 115-120, 115-125, 115-140, 120-125, 120-130, 120-145, 125-130, 125-135, 125-150, 130-135, 130-140, 130-155, 135-140, 135-145, 135-160, 140-145, 140-150, 140-165, 145-150, 145-155, 145-170, 150-155, 150-160, 150-175, 155-160, 155-165, 160-165, 160-170, 165-170, 165-175, and 170-175 nucleotides. As a non-limiting example, the viral genome comprises an ITR that is about 105 nucleotides in length. As a non-limiting example, the viral genome comprises an ITR that is about 141 nucleotides in length. As a non-limiting example, the viral genome comprises an ITR that is about 130 nucleotides in length.

[0391] In certain embodiments, the AAV particle viral genome may comprises two inverted terminal repeat (ITR) regions. Each of the ITR regions may independently have a length such as, but not limited to, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, and 175 nucleotides. The length of the ITR regions for the viral genome may be 75-80, 75-85, 75-100, 80-85, 80-90, 80-105, 85-90, 85-95, 85-110, 90-95, 90-100, 90-115, 95-100, 95-105, 95-120, 100-105, 100-110, 100-125, 105-110, 105-115, 105-130, 110-115, 110-120, 110-135, 115-120, 115-125, 115-140, 120-125, 120-130, 120-145, 125-130, 125-135, 125-150, 130-135, 130-140, 130-155, 135-140, 135-145, 135-160, 140-145, 140-150, 140-165, 145-150, 145-155, 145-170, 150-155, 150-160, 150-175, 155-160, 155-165, 160-165, 160-170, 165-170, 165-175, and 170-175 nucleotides. As a non-limiting example, the viral genome comprises an ITR that is about 105 nucleotides in length and 141 nucleotides in length. As a non-limiting example, the viral genome comprises an ITR that is about 105 nucleotides in length and 130 nucleotides in length. As a non-limiting example, the viral genome comprises an ITR that is about 130 nucleotides in length and 141 nucleotides in length.

[0392] In certain embodiments, the AAV particle viral genome comprises two ITR sequence regions.

[0393] In certain embodiments, the AAV particle viral genome may comprise at least one multiple filler sequence region. The filler region(s) may, independently, have a length such as, but not limited to, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 585, 586, 587, 588, 589, 590, 591, 592, 593, 594, 595, 596, 597, 598, 599, 600, 601, 602, 603, 604, 605, 606, 607, 608, 609, 610, 611, 612, 613, 614, 615, 616, 617,

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region that is about 2403 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 2405 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 3013 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 3021 nucleotides in length.

[0394] In certain embodiments, the AAV particle viral genome may comprise at least one multiple filler sequence region. The filler region(s) may, independently, have a length such as, but not limited to, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527,

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2586, 2587, 2588, 2589, 2590, 2591, 2592, 2593, 2594, 2595, 2596, 2597, 2598, 2599, 2600, 2601, 2602, 2603, 2604, 2605, 2606, 2607, 2608, 2609, 2610, 2611, 2612, 2613, 2614, 2615, 2616, 2617, 2618, 2619, 2620, 2621, 2622, 2623, 2624, 2625, 2626, 2627, 2628, 2629, 2630, 2631, 2632, 2633, 2634, 2635, 2636, 2637, 2638, 2639, 2640, 2641, 2642, 2643, 2644, 2645, 2646, 2647, 2648, 2649, 2650, 2651, 2652, 2653, 2654, 2655, 2656, 2657, 2658, 2659, 2660, 2661, 2662, 2663, 2664, 2665, 2666, 2667, 2668, 2669, 2670, 2671, 2672, 2673, 2674, 2675, 2676, 2677, 2678, 2679, 2680, 2681, 2682, 2683, 2684, 2685, 2686, 2687, 2688, 2689, 2690, 2691, 2692, 2693, 2694, 2695, 2696, 2697, 2698, 2699, 2700, 2701, 2702, 2703, 2704, 2705, 2706, 2707, 2708, 2709, 2710, 2711, 2712, 2713, 2714, 2715, 2716, 2717, 2718, 2719, 2720, 2721, 2722, 2723, 2724, 2725, 2726, 2727, 2728, 2729, 2730, 2731, 2732, 2733, 2734, 2735, 2736, 2737, 2738, 2739, 2740, 2741, 2742, 2743, 2744, 2745, 2746, 2747, 2748, 2749, 2750, 2751, 2752, 2753, 2754, 2755, 2756, 2757, 2758, 2759, 2760, 2761, 2762, 2763, 2764, 2765, 2766, 2767, 2768, 2769, 2770, 2771, 2772, 2773, 2774, 2775, 2776, 2777, 2778, 2779, 2780, 2781, 2782, 2783, 2784, 2785, 2786, 2787, 2788, 2789, 2790, 2791, 2792, 2793, 2794, 2795, 2796, 2797, 2798, 2799, 2800, 2801, 2802, 2803, 2804, 2805, 2806, 2807, 2808, 2809, 2810, 2811, 2812, 2813, 2814, 2815, 2816, 2817, 2818, 2819, 2820, 2821, 2822, 2823, 2824, 2825, 2826, 2827, 2828, 2829, 2830, 2831, 2832, 2833, 2834, 2835, 2836, 2837, 2838, 2839, 2840, 2841, 2842, 2843, 2844, 2845, 2846, 2847, 2848, 2849, 2850, 2851, 2852, 2853, 2854, 2855, 2856, 2857, 2858, 2859, 2860, 2861, 2862, 2863, 2864, 2865, 2866, 2867, 2868, 2869, 2870, 2871, 2872, 2873, 2874, 2875, 2876, 2877, 2878, 2879, 2880, 2881, 2882, 2883, 2884, 2885, 2886, 2887, 2888, 2889, 2890, 2891, 2892, 2893, 2894, 2895, 2896, 2897, 2898, 2899, 2900, 2901, 2902, 2903, 2904, 2905, 2906, 2907, 2908, 2909, 2910, 2911, 2912, 2913, 2914, 2915, 2916, 2917, 2918, 2919, 2920, 2921, 2922, 2923, 2924, 2925, 2926, 2927, 2928, 2929, 2930, 2931, 2932, 2933, 2934, 2935, 2936, 2937, 2938, 2939, 2940, 2941, 2942, 2943, 2944, 2945, 2946, 2947, 2948, 2949, 2950, 2951, 2952, 2953, 2954, 2955, 2956, 2957, 2958, 2959, 2960, 2961, 2962, 2963, 2964, 2965, 2966, 2967, 2968, 2969, 2970, 2971, 2972, 2973, 2974, 2975, 2976, 2977, 2978, 2979, 2980, 2981, 2982, 2983, 2984, 2985, 2986, 2987, 2988, 2989, 2990, 2991, 2992, 2993, 2994, 2995, 2996, 2997, 2998, 2999, 3000, 3001, 3002, 3003, 3004, 3005, 3006, 3007, 3008, 3009, 3010, 3011, 3012, 3013, 3014, 3015, 3016, 3017, 3018, 3019, 3020, 3021, 3022, 3023, 3024, 3025, 3026, 3027, 3028, 3029, 3030, 3031, 3032, 3033, 3034, 3035, 3036, 3037, 3038, 3039, 3040, 3041, 3042, 3043, 3044, 3045, 3046, 3047, 3048, 3049, 3050, 3051, 3052, 3053, 3054, 3055, 3056, 3057, 3058, 3059, 3060, 3061, 3062, 3063, 3064, 3065, 3066, 3067, 3068, 3069, 3070, 3071, 3072, 3073, 3074, 3075, 3076, 3077, 3078, 3079, 3080,

3081, 3082, 3083, 3084, 3085, 3086, 3087, 3088, 3089, 3090, 3091, 3092, 3093, 3094, 3095, 3096, 3097, 3098, 3099, 3100, 3101, 3102, 3103, 3104, 3105, 3106, 3107, 3108, 3109, 3110, 3111, 3112, 3113, 3114, 3115, 3116, 3117, 3118, 3119, 3120, 3121, 3122, 3123, 3124, 3125, 3126, 3127, 3128, 3129, 3130, 3131, 3132, 3133, 3134, 3135, 3136, 3137, 3138, 3139, 3140, 3141, 3142, 3143, 3144, 3145, 3146, 3147, 3148, 3149, 3150, 3151, 3152, 3153, 3154, 3155, 3156, 3157, 3158, 3159, 3160, 3161, 3162, 3163, 3164, 3165, 3166, 3167, 3168, 3169, 3170, 3171, 3172, 3173, 3174, 3175, 3176, 3177, 3178, 3179, 3180, 3181, 3182, 3183, 3184, 3185, 3186, 3187, 3188, 3189, 3190, 3191, 3192, 3193, 3194, 3195, 3196, 3197, 3198, 3199, 3200, 3201, 3202, 3203, 3204, 3205, 3206, 3207, 3208, 3209, 3210, 3211, 3212, 3213, 3214, 3215, 3216, 3217, 3218, 3219, 3220, 3221, 3222, 3223, 3224, 3225, 3226, 3227, 3228, 3229, 3230, 3231, 3232, 3233, 3234, 3235, 3236, 3237, 3238, 3239, 3240, 3241, 3242, 3243, 3244, 3245, 3246, 3247, 3248, 3249, and 3250 nucleotides. The length of any filler region for the viral genome may be 50-100, 100-150, 150-200, 200-250, 250-300, 300-350, 350-400, 400-450, 450-500, 500-550, 550-600, 600-650, 650-700, 700-750, 750-800, 800-850, 850-900, 900-950, 950-1000, 1000-1050, 1050-1100, 1100-1150, 1150-1200, 1200-1250, 1250-1300, 1300-1350, 1350-1400, 1400-1450, 1450-1500, 1500-1550, 1550-1600, 1600-1650, 1650-1700, 1700-1750, 1750-1800, 1800-1850, 1850-1900, 1900-1950, 1950-2000, 2000-2050, 2050-2100, 2100-2150, 2150-2200, 2200-2250, 2250-2300, 2300-2350, 2350-2400, 2400-2450, 2450-2500, 2500-2550, 2550-2600, 2600-2650, 2650-2700, 2700-2750, 2750-2800, 2800-2850, 2850-2900, 2900-2950, 2950-3000, 3000-3050, 3050-3100, 3100-3150, 3150-3200, and 3200-3250 nucleotides. As a non-limiting example, the viral genome comprises a filler region that is about 55 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 56 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 97 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 103 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 105 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 357 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 363 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 712 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 714 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 1203 nucleotides in length. As a non-limiting example, the viral genome

comprises a filler region that is about 1209 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 1512 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 1519 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 2395 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 2403 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 2405 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 3013 nucleotides in length. As a non-limiting example, the viral genome comprises a filler region that is about 3021 nucleotides in length.

[0395] In certain embodiments, the AAV particle viral genome may comprise at least one enhancer sequence region. The enhancer sequence region(s) may, independently, have a length such as, but not limited to, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, and 400 nucleotides. The length of the enhancer region for the viral genome may be 300-310, 300-325, 305-315, 310-320, 315-325, 320-330, 325-335, 325-350, 330-340, 335-345, 340-350, 345-355, 350-360, 350-375, 355-365, 360-370, 365-375, 370-380, 375-385, 375-400, 380-390, 385-395, and 390-400 nucleotides. As a non-limiting example, the viral genome comprises an enhancer region that is about 303 nucleotides in length. As a non-limiting example, the viral genome comprises an enhancer region that is about 382 nucleotides in length.

[0396] In certain embodiments, the AAV particle viral genome may comprise at least one promoter sequence region. The promoter sequence region(s) may, independently, have a length such as, but not limited to, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133,

134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 585, 586, 587, 588, 589, 590, 591, 592, 593, 594, 595, 596, 597, 598, 599, and 600 nucleotides. The length of the promoter region for the viral genome may be 4-10, 10-20, 10-50, 20-30, 30-40, 40-50, 50-60, 50-100, 60-70, 70-80, 80-90, 90-100, 100-110, 100-150, 110-120, 120-130, 130-140, 140-150, 150-160, 150-200, 160-170, 170-180, 180-190, 190-200, 200-210, 200-250, 210-220, 220-230, 230-240, 240-250, 250-260, 250-300, 260-270, 270-280, 280-290, 290-300, 300-310, 300-350, 310-320, 320-330, 330-340, 340-350, 350-360, 350-400, 360-370, 370-380, 380-390, 390-400, 400-410, 400-450, 410-420, 420-430, 430-440, 440-450, 450-460, 450-500, 460-470, 470-480, 480-490, 490-500, 500-510, 500-550,

510-520, 520-530, 530-540, 540-550, 550-560, 550-600, 560-570, 570-580, 580-590, and 590-600 nucleotides. As a non-limiting example, the viral genome comprises a promoter region that is about 4 nucleotides in length. As a non-limiting example, the viral genome comprises a promoter region that is about 17 nucleotides in length. As a non-limiting example, the viral genome comprises a promoter region that is about 204 nucleotides in length. As a non-limiting example, the viral genome comprises a promoter region that is about 219 nucleotides in length. As a non-limiting example, the viral genome comprises a promoter region that is about 260 nucleotides in length. As a non-limiting example, the viral genome comprises a promoter region that is about 303 nucleotides in length. As a non-limiting example, the viral genome comprises a promoter region that is about 382 nucleotides in length. As a non-limiting example, the viral genome comprises a promoter region that is about 588 nucleotides in length.

[0397] In certain embodiments, the AAV particle viral genome may comprise at least one exon sequence region. The exon region(s) may, independently, have a length such as, but not limited to, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, and 150 nucleotides. The length of the exon region for the viral genome may be 2-10, 5-10, 5-15, 10-20, 10-30, 10-40, 15-20, 15-25, 20-30, 20-40, 20-50, 25-30, 25-35, 30-40, 30-50, 30-60, 35-40, 35-45, 40-50, 40-60, 40-70, 45-50, 45-55, 50-60, 50-70, 50-80, 55-60, 55-65, 60-70, 60-80, 60-90, 65-70, 65-75, 70-80, 70-90, 70-100, 75-80, 75-85, 80-90, 80-100, 80-110, 85-90, 85-95, 90-100, 90-110, 90-120, 95-100, 95-105, 100-110, 100-120, 100-130, 105-110, 105-115, 110-120, 110-130, 110-140, 115-120, 115-125, 120-130, 120-140, 120-150, 125-130, 125-135, 130-140, 130-150, 135-140, 135-145, 140-150, and 145-150 nucleotides. As a non-limiting example, the viral genome comprises an exon region that is about 53 nucleotides in length. As a non-limiting example, the viral genome comprises an exon region that is about 134 nucleotides in length.

[0398] In certain embodiments, the AAV particle viral genome may comprise at least one intron sequence region. The intron region(s) may, independently, have a length such as, but

not limited to, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, and 350 nucleotides. The length of the intron region for the viral genome may be 25-35, 25-50, 35-45, 45-55, 50-75, 55-65, 65-75, 75-85, 75-100, 85-95, 95-105, 100-125, 105-115, 115-125, 125-135, 125-150, 135-145, 145-155, 150-175, 155-165, 165-175, 175-185, 175-200, 185-195, 195-205, 200-225, 205-215, 215-225, 225-235, 225-250, 235-245, 245-255, 250-275, 255-265, 265-275, 275-285, 275-300, 285-295, 295-305, 300-325, 305-315, 315-325, 325-335, 325-350, and 335-345 nucleotides. As a non-limiting example, the viral genome comprises an intron region that is about 32 nucleotides in length. As a non-limiting example, the viral genome comprises an intron region that is about 172 nucleotides in length. As a non-limiting example, the viral genome comprises an intron region that is about 201 nucleotides in length. As a non-limiting example, the viral genome comprises an intron region that is about 347 nucleotides in length.

[0399] In certain embodiments, the AAV particle viral genome may comprise at least one polyadenylation signal sequence region. The polyadenylation signal region sequence region(s) may, independently, have a length such as, but not limited to, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61,

62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 585, 586, 587, 588, 589, 590, 591, 592, 593, 594, 595, 596, 597, 598, 599, and 600 nucleotides. The length of the polyadenylation signal sequence region for the viral genome may be 4-10, 10-20, 10-50, 20-30, 30-40, 40-50, 50-60, 50-100, 60-70, 70-80, 80-90, 90-100, 100-110, 100-150, 110-120, 120-130, 130-140, 140-150, 150-160, 150-200, 160-170, 170-180, 180-190, 190-200, 200-210, 200-250, 210-220,

220-230, 230-240, 240-250, 250-260, 250-300, 260-270, 270-280, 280-290, 290-300, 300-310, 300-350, 310-320, 320-330, 330-340, 340-350, 350-360, 350-400, 360-370, 370-380, 380-390, 390-400, 400-410, 400-450, 410-420, 420-430, 430-440, 440-450, 450-460, 450-500, 460-470, 470-480, 480-490, 490-500, 500-510, 500-550, 510-520, 520-530, 530-540, 540-550, 550-560, 550-600, 560-570, 570-580, 580-590, and 590-600 nucleotides. As a non-limiting example, the viral genome comprises a polyadenylation signal sequence region that is about 127 nucleotides in length. As a non-limiting example, the viral genome comprises a polyadenylation signal sequence region that is about 225 nucleotides in length. As a non-limiting example, the viral genome comprises a polyadenylation signal sequence region that is about 476 nucleotides in length. As a non-limiting example, the viral genome comprises a polyadenylation signal sequence region that is about 477 nucleotides in length.

[0400] In certain embodiments, the AAV particle viral genome comprises more than one polyA signal sequence region.

[0401] Non-limiting examples of ITR to ITR sequences of AAV particles comprising a viral genome with a payload region comprising a modulatory polynucleotide sequence are described in Table 9A. Table 9A also provides an alternate name for the ITR to ITR construct indicated by the "VOYSOD" identifier.

Table 9A. ITR to ITR Sequences of AAV Particles, H1.mir.104-788.2 (with lentivirus derived filler) comprising Modulatory Polynucleotides

ITR to ITR Construct Name	ITR to ITR SEQ ID NO	Modulatory Polynucleotide SEQ ID NO
H1.mir.104-788.2 with lentivirus derived filler (VOYSOD16)	9	6

[0402] Table 9B provides ITR to ITR sequence of H1.mir104-788.2 with albumin derived filler. Also provided in Table 9B are the components that comprise the ITR to ITR sequence. In some embodiments, the components may be separated from each other by vector backbone sequence.

Table 9B. ITR to ITR of AAV Particles, H1.mir104-788.2 (with albumin derived filler) comprising Modulatory Polynucleotides and its components

Description	SEQ ID NO.
ITR to ITR of H1.mir104-788.2 with albumin derived filler	25

ITR-ITR Components of H1.mir104-788.2 (with albumin derived filler)	
5'ITR	26
Albumin derived filler	27
H1 promoter	28
Modulatory Polynucleotide (SOD1-miR104-788.2)	6
rBGpA	29
3'ITR	30

[0403] In certain embodiments, the AAV particle comprises a viral genome which comprises a sequence which has a percent identity to SEQ ID NO: 9. The viral genome may have 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or 100% identity to SEQ ID NO: 9. The viral genome may have 1-10%, 10-20%, 30-40%, 50-60%, 50-70%, 50-80%, 50-90%, 50-99%, 50-100%, 60-70%, 60-80%, 60-90%, 60-99%, 60-100%, 70-80%, 70-90%, 70-99%, 70-100%, 80-85%, 80-90%, 80-95%, 80-99%, 80-100%, 90-95%, 90-99%, or 90-100% to SEQ ID NO: 9. As a non-limiting example, the viral genome comprises a sequence which as 80% identity to SEQ ID NO: 9. As another non-limiting example, the viral genome comprises a sequence which as 85% identity to SEQ ID NO: 9. As another non-limiting example, the viral genome comprises a sequence which as 90% identity to SEQ ID NO: 9. As another non-limiting example, the viral genome comprises a sequence which as 95% identity to SEQ ID NO: 9. As another non-limiting example, the viral genome comprises a sequence which as 99% identity to SEQ ID NO: 9.

[0404] In certain embodiments, the AAV particle comprises a viral genome which comprises a sequence which has a percent identity to SEQ ID NO: 25. The viral genome may have 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or 100% identity to SEQ ID NO: 25. The viral genome may have 1-10%, 10-20%, 30-40%, 50-60%, 50-70%, 50-80%, 50-90%, 50-99%, 50-100%, 60-70%, 60-80%, 60-90%, 60-99%, 60-100%, 70-80%, 70-90%, 70-99%, 70-100%, 80-85%, 80-90%, 80-95%, 80-99%, 80-100%, 90-95%, 90-99%, or 90-100% to SEQ ID NO: 25. As a non-limiting example, the viral genome comprises a sequence which as 80% identity to SEQ ID NO: 25. As another non-limiting example, the viral genome comprises a sequence which as 85% identity to SEQ ID NO: 25. As another non-limiting example, the viral genome comprises a sequence which as 90% identity to SEQ ID NO: 25. As another non-limiting example, the viral genome comprises a sequence which as 95%

identity to SEQ ID NO: 25. As another non-limiting example, the viral genome comprises a sequence which has 99% identity to SEQ ID NO: 25.

[0405] AAV particles may be modified to enhance the efficiency of delivery. Such modified AAV particles comprising the nucleic acid sequence encoding the siRNA molecules of the present disclosure can be packaged efficiently and can be used to successfully infect the target cells at high frequency and with minimal toxicity.

[0406] In some embodiments, the AAV particle comprising a nucleic acid sequence encoding the siRNA molecules of the present disclosure may be a human serotype AAV particle. Such human AAV particle may be derived from any known serotype, e.g., from any one of serotypes AAV1-AAV11. As non-limiting examples, AAV particles may be vectors comprising an AAV1-derived genome in an AAV1-derived capsid; vectors comprising an AAV2-derived genome in an AAV2-derived capsid; vectors comprising an AAV4-derived genome in an AAV4 derived capsid; vectors comprising an AAV6-derived genome in an AAV6 derived capsid or vectors comprising an AAV9-derived genome in an AAV9 derived capsid.

[0407] In other embodiments, the AAV particle comprising a nucleic acid sequence for encoding siRNA molecules of the present disclosure may be a pseudotyped hybrid or chimeric AAV particle which contains sequences and/or components originating from at least two different AAV serotypes. Pseudotyped AAV particles may be vectors comprising an AAV genome derived from one AAV serotype and a capsid protein derived at least in part from a different AAV serotype. As non-limiting examples, such pseudotyped AAV particles may be vectors comprising an AAV2-derived genome in an AAV1-derived capsid; or vectors comprising an AAV2-derived genome in an AAV6-derived capsid; or vectors comprising an AAV2-derived genome in an AAV4-derived capsid; or an AAV2-derived genome in an AAV9-derived capsid. In like fashion, the present disclosure contemplates any hybrid or chimeric AAV particle.

[0408] In other embodiments, AAV particles comprising a nucleic acid sequence encoding the siRNA molecules of the present disclosure may be used to deliver siRNA molecules to the central nervous system (e.g., U.S. Pat. No. 6,180,613; the contents of which is herein incorporated by reference in its entirety).

[0409] In some aspects, the AAV particles comprising a nucleic acid sequence encoding the siRNA molecules of the present disclosure may further comprise a modified capsid including peptides from non-viral origin. In other aspects, the AAV particle may contain a

CNS specific chimeric capsid to facilitate the delivery of encoded siRNA duplexes into the brain and the spinal cord. For example, an alignment of cap nucleotide sequences from AAV variants exhibiting CNS tropism may be constructed to identify variable region (VR) sequence and structure.

[0410] In other embodiments, the siRNA molecules of the present disclosure can be encoded in plasmid vectors, viral vectors (e.g., AAV vectors), genome or other nucleic acid expression vectors for delivery to a cell.

[0411] DNA expression plasmids can be used to stably express the siRNA duplexes or dsRNA of the present disclosure in cells and achieve long-term inhibition of target gene.

[0412] In one aspect, the sense and antisense strands of a siRNA duplex encoded by a SOD1 targeting polynucleotide are typically linked by a short spacer sequence leading to the expression of a stem-loop structure termed short hairpin RNA (shRNA). The hairpin is recognized and cleaved by Dicer, thus generating mature siRNA molecules.

[0413] According to the present disclosure, AAV vectors comprising the nucleic acids of the siRNA molecules targeting SOD1 mRNA are produced, the AAV vectors may be AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV9.47, AAV9(hu14), AAV10, AAV11, AAV12, AAVrh8, AAVrh10, AAV-DJ8 and AAV-DJ, and variants thereof.

[0414] In some embodiments, the siRNA duplexes or dsRNA of the present disclosure when expressed suppress (or degrade) target mRNA (i.e. SOD1). Accordingly, the siRNA duplexes or dsRNA encoded by a SOD1 targeting polynucleotide can be used to substantially inhibit SOD1 gene expression in a cell, for example a motor neuron. In some aspects, the inhibition of SOD1 gene expression refers to an inhibition by at least about 20%, preferably by at least about 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95% and 100%. Accordingly, the protein product of the targeted gene may be inhibited by at least about 20%, preferably by at least about 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95% and 100%. The SOD1 gene can be either a wild type gene or a mutated SOD1 gene with at least one mutation. Accordingly, the protein is either wild type protein or a mutated polypeptide with at least one mutation.

Viral production

[0415] The present disclosure provides a method for the generation of parvoviral particles, e.g. AAV particles, by viral genome replication in a viral replication cell comprising contacting the viral replication cell with an AAV polynucleotide or AAV genome.

[0416] The present disclosure provides a method for producing an AAV particle having enhanced (increased, improved) transduction efficiency comprising the steps of: 1) co-transfecting competent bacterial cells with a bacmid vector and either a viral construct vector and/or AAV payload construct vector, 2) isolating the resultant viral construct expression vector and AAV payload construct expression vector and separately transfecting viral replication cells, 3) isolating and purifying resultant payload and viral construct particles comprising viral construct expression vector or AAV payload construct expression vector, 4) co-infecting a viral replication cell with both the AAV payload and viral construct particles comprising viral construct expression vector or AAV payload construct expression vector, and 5) harvesting and purifying the viral particle comprising a parvoviral genome.

[0417] In certain embodiments, the present disclosure provides a method for producing an AAV particle comprising the steps of 1) simultaneously co-transfecting mammalian cells, such as, but not limited to HEK293 cells, with a payload region, a construct expressing rep and cap genes and a helper construct, 2) harvesting and purifying the AAV particle comprising a viral genome.

Cells

[0418] The present disclosure provides a cell comprising an AAV polynucleotide and/or AAV genome.

[0419] Viral production disclosed herein describes processes and methods for producing AAV particles that contact a target cell to deliver a payload construct, *e.g.* a recombinant viral construct, which comprises a polynucleotide sequence encoding a payload molecule.

[0420] In certain embodiments, the AAV particles may be produced in a viral replication cell that comprises an insect cell.

[0421] Growing conditions for insect cells in culture, and production of heterologous products in insect cells in culture are well-known in the art, *see* U.S. Pat. No. 6,204,059, the contents of which are herein incorporated by reference in their entirety.

[0422] Any insect cell which allows for replication of parvovirus and which can be maintained in culture can be used in accordance with the present disclosure. Cell lines may be used from *Spodoptera frugiperda*, including, but not limited to the Sf9 or Sf21 cell lines, *Drosophila* cell lines, or mosquito cell lines, such as *Aedes albopictus* derived cell lines. Use of insect cells for expression of heterologous proteins is well documented, as are methods of introducing nucleic acids, such as vectors, *e.g.*, insect-cell compatible vectors, into such cells and methods of maintaining such cells in culture. *See*, for example, *Methods in Molecular*

Biology, ed. Richard, Humana Press, NJ (1995); O'Reilly *et al.*, *Baculovirus Expression Vectors, A Laboratory Manual*, Oxford Univ. Press (1994); Samulski *et al.*, *J. Vir.* 63:3822-8 (1989); Kajigaya *et al.*, *Proc. Nat'l. Acad. Sci. USA* 88: 4646-50 (1991); Ruffing *et al.*, *J. Vir.* 66:6922-30 (1992); Kimbauer *et al.*, *Vir.* 219:37-44 (1996); Zhao *et al.*, *Vir.* 272:382-93 (2000); and Samulski *et al.*, U.S. Pat. No. 6,204,059, the contents of each of which is herein incorporated by reference in its entirety.

[0423] The viral replication cell may be selected from any biological organism, including prokaryotic (*e.g.*, bacterial) cells, and eukaryotic cells, including, insect cells, yeast cells and mammalian cells. Viral replication cells may comprise mammalian cells such as A549, WEH1, 3T3, 10T1/2, BHK, MDCK, COS 1, COS 7, BSC 1, BSC 40, BMT 10, VERO, WI38, HeLa, HEK293, Saos, C2C12, L cells, HT1080, HepG2 and primary fibroblast, hepatocyte and myoblast cells derived from mammals. Viral replication cells comprise cells derived from mammalian species including, but not limited to, human, monkey, mouse, rat, rabbit, and hamster or cell type, including but not limited to fibroblast, hepatocyte, tumor cell, cell line transformed cell, etc.

Mammalian cell (small scale) production of AAV Particles

[0424] Viral production disclosed herein describes processes and methods for producing AAV particles that contact a target cell to deliver a payload, *e.g.* a recombinant viral construct, which comprises a polynucleotide sequence encoding a payload.

[0425] In certain embodiments, the AAV particles may be produced in a viral replication cell that comprises a mammalian cell.

[0426] Viral replication cells commonly used for production of recombinant AAV particles include, but are not limited to 293 cells, COS cells, HeLa cells, KB cells, and other mammalian cell lines as described in U.S. Pat. Nos. 6,156,303, 5,387,484, 5,741,683, 5,691,176, and 5,688,676; U.S. patent application 2002/0081721, and International Patent Applications WO 00/47757, WO 00/24916, and WO 96/17947, the contents of each of which are herein incorporated by reference in their entireties.

[0427] In certain embodiments, AAV particles are produced in mammalian-cells wherein all three VP proteins are expressed at a stoichiometry approaching 1:1:10 (VP1:VP2:VP3). The regulatory mechanisms that allow this controlled level of expression include the production of two mRNAs, one for VP1, and the other for VP2 and VP3, produced by differential splicing.

[0428] In another embodiment, AAV particles are produced in mammalian cells using a triple transfection method wherein a payload construct, parvoviral Rep and parvoviral Cap and a helper construct are comprised within three different constructs. The triple transfection method of the three components of AAV particle production may be utilized to produce small lots of virus for assays including transduction efficiency, target tissue (tropism) evaluation, and stability.

[0429] AAV particles described herein may be produced by triple transfection or baculovirus mediated virus production, or any other method known in the art. Any suitable permissive or packaging cell known in the art may be employed to produce the vectors. Mammalian cells are often preferred. Also preferred are trans-complementing packaging cell lines that provide functions deleted from a replication-defective helper virus, e.g., 293 cells or other E1a trans-complementing cells.

[0430] The gene cassette may contain some or all of the parvovirus (e.g., AAV) cap and rep genes. Preferably, however, some or all of the cap and rep functions are provided in trans by introducing a packaging vector(s) encoding the capsid and/or Rep proteins into the cell. Most preferably, the gene cassette does not encode the capsid or Rep proteins. Alternatively, a packaging cell line is used that is stably transformed to express the cap and/or rep genes.

[0431] Recombinant AAV virus particles are, in some cases, produced and purified from culture supernatants according to the procedure as described in US20160032254, the contents of which are incorporated by reference. Production may also involve methods known in the art including those using 293T cells, sf9 insect cells, triple transfection or any suitable production method.

[0432] In some cases, 293T cells (adhesion/suspension) are transfected with polyethyleneimine (PEI) with plasmids required for production of AAV, i.e., AAV2 rep, an adenoviral helper construct and a ITR flanked transgene cassette. The AAV2 rep plasmid also contains the cap sequence of the particular virus being studied. Twenty-four hours after transfection (no medium changes for suspension), which occurs in DMEM/F17 with/without serum, the medium is replaced with fresh medium with or without serum. Three (3) days after transfection, a sample is taken from the culture medium of the 293 adherent cells. Subsequently cells are scraped, or suspension cells are pelleted, and transferred into a receptacle. For adhesion cells, after centrifugation to remove cellular pellet, a second sample is taken from the supernatant after scraping. Next, cell lysis is achieved by three consecutive freeze-thaw cycles (-80C to 37C) or adding detergent triton. Cellular debris is removed by

centrifugation or depth filtration and sample 3 is taken from the medium. The samples are quantified for AAV particles by DNase resistant genome titration by DNA qPCR. The total production yield from such a transfection is equal to the particle concentration from sample 3.

[0433] AAV particle titers are measured according to genome copy number (genome particles per milliliter). Genome particle concentrations are based on DNA quantitative PCR of the vector DNA as previously reported (Clark et al. (1999) *Hum. Gene Ther.*, 10:1031-1039; Veldwijk et al. (2002) *Mol. Ther.*, 6:272-278).

Baculovirus

[0434] Particle production disclosed herein describes processes and methods for producing AAV particles that contact a target cell to deliver a payload construct which comprises a polynucleotide sequence encoding a payload.

[0435] Briefly, the viral construct vector and the AAV payload construct vector are each incorporated by a transposon donor/acceptor system into a bacmid, also known as a baculovirus plasmid, by standard molecular biology techniques known and performed by a person skilled in the art. Transfection of separate viral replication cell populations produces two baculoviruses, one that comprises the viral construct expression vector, and another that comprises the AAV payload construct expression vector. The two baculoviruses may be used to infect a single viral replication cell population for production of AAV particles.

[0436] Baculovirus expression vectors for producing viral particles in insect cells, including but not limited to *Spodoptera frugiperda* (Sf9) cells, provide high titers of viral particle product. Recombinant baculovirus encoding the viral construct expression vector and AAV payload construct expression vector initiates a productive infection of viral replicating cells. Infectious baculovirus particles released from the primary infection secondarily infect additional cells in the culture, exponentially infecting the entire cell culture population in a number of infection cycles that is a function of the initial multiplicity of infection, *see* Urabe, M. *et al.*, *J Virol.* 2006 Feb; 80 (4):1874-85, the contents of which are herein incorporated by reference in their entirety.

[0437] Production of AAV particles with baculovirus in an insect cell system may address known baculovirus genetic and physical instability. In certain embodiments, the production system addresses baculovirus instability over multiple passages by utilizing a titerless infected-cells preservation and scale-up system. Small scale seed cultures of viral producing cells are transfected with viral expression constructs encoding the structural, non-structural, components of the viral particle. Baculovirus-infected viral producing cells are harvested into

aliquots that may be cryopreserved in liquid nitrogen; the aliquots retain viability and infectivity for infection of large scale viral producing cell culture Wasilko DJ *et al.*, Protein Expr Purif. 2009 Jun; 65(2):122-32, the contents of which are herein incorporated by reference in their entirety.

[0438] A genetically stable baculovirus may be used to produce source of the one or more of the components for producing AAV particles in invertebrate cells. In certain embodiments, defective baculovirus expression vectors may be maintained episomally in insect cells. In such an embodiment the bacmid vector is engineered with replication control elements, including but not limited to promoters, enhancers, and/or cell-cycle regulated replication elements.

[0439] In certain embodiments, baculoviruses may be engineered with a (non-) selectable marker for recombination into the chitinase/cathepsin locus. The chia/v-cath locus is non-essential for propagating baculovirus in tissue culture, and the V-cath (EC 3.4.22.50) is a cysteine endoprotease that is most active on Arg-Arg dipeptide containing substrates. The Arg-Arg dipeptide is present in densovirus and parvovirus capsid structural proteins but infrequently occurs in dependovirus VP1.

[0440] In certain embodiments, stable viral replication cells permissive for baculovirus infection are engineered with at least one stable integrated copy of any of the elements necessary for AAV replication and viral particle production including, but not limited to, the entire AAV genome, Rep and Cap genes, Rep genes, Cap genes, each Rep protein as a separate transcription cassette, each VP protein as a separate transcription cassette, the AAP (assembly activation protein), or at least one of the baculovirus helper genes with native or non-native promoters.

Large-scale production

[0441] In some embodiments, AAV particle production may be modified to increase the scale of production. Large scale viral production methods according to the present disclosure may include any of those taught in US Patent Nos. 5,756,283, 6,258,595, 6,261,551, 6,270,996, 6,281,010, 6,365,394, 6,475,769, 6,482,634, 6,485,966, 6,943,019, 6,953,690, 7,022,519, 7,238,526, 7,291,498 and 7,491,508 or International Publication Nos. WO1996039530, WO1998010088, WO1999014354, WO1999015685, WO1999047691, WO2000055342, WO2000075353 and WO2001023597, the contents of each of which are herein incorporated by reference in their entirety. Methods of increasing viral particle production scale typically comprise increasing the number of viral replication cells. In some

embodiments, viral replication cells comprise adherent cells. To increase the scale of viral particle production by adherent viral replication cells, larger cell culture surfaces are required. In some cases, large-scale production methods comprise the use of roller bottles to increase cell culture surfaces. Other cell culture substrates with increased surface areas are known in the art. Examples of additional adherent cell culture products with increased surface areas include, but are not limited to CELLSTACK[®], CELLCUBE[®] (Corning Corp., Corning, NY) and NUNC[™] CELL FACTORY[™] (Thermo Scientific, Waltham, MA.) In some cases, large-scale adherent cell surfaces may comprise from about 1,000 cm² to about 100,000 cm². In some cases, large-scale adherent cell cultures may comprise from about 10⁷ to about 10⁹ cells, from about 10⁸ to about 10¹⁰ cells, from about 10⁹ to about 10¹² cells or at least 10¹² cells. In some cases, large-scale adherent cultures may produce from about 10⁹ to about 10¹², from about 10¹⁰ to about 10¹³, from about 10¹¹ to about 10¹⁴, from about 10¹² to about 10¹⁵ or at least 10¹⁵ viral particles.

[0442] In some embodiments, large-scale viral production methods of the present disclosure may comprise the use of suspension cell cultures. Suspension cell culture allows for significantly increased numbers of cells. Typically, the number of adherent cells that can be grown on about 10-50 cm² of surface area can be grown in about 1 cm³ volume in suspension.

[0443] Transfection of replication cells in large-scale culture formats may be carried out according to any methods known in the art. For large-scale adherent cell cultures, transfection methods may include, but are not limited to the use of inorganic compounds (*e.g.* calcium phosphate), organic compounds [*e.g.* polyethyleneimine (PEI)] or the use of non-chemical methods (*e.g.* electroporation.) With cells grown in suspension, transfection methods may include, but are not limited to the use of calcium phosphate and the use of PEI. In some cases, transfection of large scale suspension cultures may be carried out according to the section entitled "Transfection Procedure" described in Feng, L. *et al.*, 2008. *Biotechnol Appl. Biochem.* 50:121-32, the contents of which are herein incorporated by reference in their entirety. According to such embodiments, PEI-DNA complexes may be formed for introduction of plasmids to be transfected. In some cases, cells being transfected with PEI-DNA complexes may be 'shocked' prior to transfection. This comprises lowering cell culture temperatures to 4°C for a period of about 1 hour. In some cases, cell cultures may be shocked for a period of from about 10 minutes to about 5 hours. In some cases, cell cultures may be shocked at a temperature of from about 0°C to about 20°C.

[0444] In some cases, transfections may include one or more vectors for expression of an RNA effector molecule to reduce expression of nucleic acids from one or more AAV payload construct. Such methods may enhance the production of viral particles by reducing cellular resources wasted on expressing payload constructs. In some cases, such methods may be carried according to those taught in US Publication No. US2014/0099666, the contents of which are herein incorporated by reference in their entirety.

Bioreactors

[0445] In some embodiments, cell culture bioreactors may be used for large scale viral production. In some cases, bioreactors comprise stirred tank reactors. Such reactors generally comprise a vessel, typically cylindrical in shape, with a stirrer (*e.g.* impeller.) In some embodiments, such bioreactor vessels may be placed within a water jacket to control vessel temperature and/or to minimize effects from ambient temperature changes. Bioreactor vessel volume may range in size from about 500 ml to about 2 L, from about 1 L to about 5 L, from about 2.5 L to about 20 L, from about 10 L to about 50 L, from about 25 L to about 100 L, from about 75 L to about 500 L, from about 250 L to about 2,000 L, from about 1,000 L to about 10,000 L, from about 5,000 L to about 50,000 L or at least 50,000 L. Vessel bottoms may be rounded or flat. In some cases, animal cell cultures may be maintained in bioreactors with rounded vessel bottoms.

[0446] In some cases, bioreactor vessels may be warmed through the use of a thermocirculator. Thermocirculators pump heated water around water jackets. In some cases, heated water may be pumped through pipes (*e.g.* coiled pipes) that are present within bioreactor vessels. In some cases, warm air may be circulated around bioreactors, including, but not limited to air space directly above culture medium. Additionally, pH and CO₂ levels may be maintained to optimize cell viability.

[0447] In some cases, bioreactors may comprise hollow-fiber reactors. Hollow-fiber bioreactors may support the culture of both anchorage dependent and anchorage independent cells. Further bioreactors may include, but are not limited to packed-bed or fixed-bed bioreactors. Such bioreactors may comprise vessels with glass beads for adherent cell attachment. Further packed-bed reactors may comprise ceramic beads.

[0448] In some cases, viral particles are produced through the use of a disposable bioreactor. In some embodiments, such bioreactors may include WAVE™ disposable bioreactors.

[0449] In some embodiments, AAV particle production in animal cell bioreactor cultures may be carried out according to the methods taught in US Patent Nos. 5,064,764, 6,194,191, 6,566,118, 8,137,948 or US Patent Application No. US2011/0229971, the contents of each of which are herein incorporated by reference in their entirety.

Cell Lysis

[0450] Cells of the disclosure, including, but not limited to viral production cells, may be subjected to cell lysis according to any methods known in the art. Cell lysis may be carried out to obtain one or more agents (e.g. viral particles) present within any cells described herein. In some embodiments, cell lysis may be carried out according to any of the methods listed in US Patent Nos. 7,326,555, 7,579,181, 7,048,920, 6,410,300, 6,436,394, 7,732,129, 7,510,875, 7,445,930, 6,726,907, 6,194,191, 7,125,706, 6,995,006, 6,676,935, 7,968,333, 5,756,283, 6,258,595, 6,261,551, 6,270,996, 6,281,010, 6,365,394, 6,475,769, 6,482,634, 6,485,966, 6,943,019, 6,953,690, 7,022,519, 7,238,526, 7,291,498 and 7,491,508 or International Publication Nos. WO1996039530, WO1998010088, WO1999014354, WO1999015685, WO1999047691, WO2000055342, WO2000075353 and WO2001023597, the contents of each of which are herein incorporated by reference in their entirety. Cell lysis methods may be chemical or mechanical. Chemical cell lysis typically comprises contacting one or more cells with one or more lysis agent. Mechanical lysis typically comprises subjecting one or more cells to one or more lysis condition and/or one or more lysis force.

[0451] In some embodiments, chemical lysis may be used to lyse cells. As used herein, the term "lysis agent" refers to any agent that may aid in the disruption of a cell. In some cases, lysis agents are introduced in solutions, termed lysis solutions or lysis buffers. As used herein, the term "lysis solution" refers to a solution (typically aqueous) comprising one or more lysis agent. In addition to lysis agents, lysis solutions may include one or more buffering agents, solubilizing agents, surfactants, preservatives, cryoprotectants, enzymes, enzyme inhibitors and/or chelators. Lysis buffers are lysis solutions comprising one or more buffering agent. Additional components of lysis solutions may include one or more solubilizing agent. As used herein, the term "solubilizing agent" refers to a compound that enhances the solubility of one or more components of a solution and/or the solubility of one or more entities to which solutions are applied. In some cases, solubilizing agents enhance protein solubility. In some cases, solubilizing agents are selected based on their ability to enhance protein solubility while maintaining protein conformation and/or activity.

[0452] Exemplary lysis agents may include any of those described in US Patent Nos. 8,685,734, 7,901,921, 7,732,129, 7,223,585, 7,125,706, 8,236,495, 8,110,351, 7,419,956, 7,300,797, 6,699,706 and 6,143,567, the contents of each of which are herein incorporated by reference in their entirety. In some cases, lysis agents may be selected from lysis salts, amphoteric agents, cationic agents, ionic detergents and non-ionic detergents. Lysis salts may include, but are not limited to, sodium chloride (NaCl) and potassium chloride (KCl.) Further lysis salts may include any of those described in US Patent Nos. 8,614,101, 7,326,555, 7,579,181, 7,048,920, 6,410,300, 6,436,394, 7,732,129, 7,510,875, 7,445,930, 6,726,907, 6,194,191, 7,125,706, 6,995,006, 6,676,935 and 7,968,333, the contents of each of which are herein incorporated by reference in their entirety. Concentrations of salts may be increased or decreased to obtain an effective concentration for rupture of cell membranes. Amphoteric agents, as referred to herein, are compounds capable of reacting as an acid or a base. Amphoteric agents may include, but are not limited to lysophosphatidylcholine, 3-((3-Cholamidopropyl) dimethylammonium)-1-propanesulfonate (CHAPS), ZWITTERGENT® and the like. Cationic agents may include, but are not limited to, cetyltrimethylammonium bromide (C (16) TAB) and Benzalkonium chloride. Lysis agents comprising detergents may include ionic detergents or non-ionic detergents. Detergents may function to break apart or dissolve cell structures including, but not limited to cell membranes, cell walls, lipids, carbohydrates, lipoproteins and glycoproteins. Exemplary ionic detergents include any of those taught in US Patent Nos. 7,625,570 and 6,593,123 or US Publication No. US2014/0087361, the contents of each of which are herein incorporated by reference in their entirety. Some ionic detergents may include, but are not limited to, sodium dodecyl sulfate (SDS), cholate and deoxycholate. In some cases, ionic detergents may be included in lysis solutions as a solubilizing agent. Non-ionic detergents may include, but are not limited to octylglucoside, digitonin, lubrol, C12E8, TWEEN®-20, TWEEN®-80, Triton X-100 and Nonidet P-40. Non-ionic detergents are typically weaker lysis agents but may be included as solubilizing agents for solubilizing cellular and/or viral proteins. Further lysis agents may include enzymes and urea. In some cases, one or more lysis agents may be combined in a lysis solution in order to enhance one or more of cell lysis and protein solubility. In some cases, enzyme inhibitors may be included in lysis solutions in order to prevent proteolysis that may be triggered by cell membrane disruption.

[0453] In some embodiments, mechanical cell lysis is carried out. Mechanical cell lysis methods may include the use of one or more lysis condition and/or one or more lysis force.

As used herein, the term “lysis condition” refers to a state or circumstance that promotes cellular disruption. Lysis conditions may comprise certain temperatures, pressures, osmotic purity, salinity and the like. In some cases, lysis conditions comprise increased or decreased temperatures. According to some embodiments, lysis conditions comprise changes in temperature to promote cellular disruption. Cell lysis carried out according to such embodiments may include freeze-thaw lysis. As used herein, the term “freeze-thaw lysis” refers to cellular lysis in which a cell solution is subjected to one or more freeze-thaw cycle. According to freeze-thaw lysis methods, cells in solution are frozen to induce a mechanical disruption of cellular membranes caused by the formation and expansion of ice crystals. Cell solutions used according freeze-thaw lysis methods, may further comprise one or more lysis agents, solubilizing agents, buffering agents, cryoprotectants, surfactants, preservatives, enzymes, enzyme inhibitors and/or chelators. Once cell solutions subjected to freezing are thawed, such components may enhance the recovery of desired cellular products. In some cases, one or more cryoprotectants are included in cell solutions undergoing freeze-thaw lysis. As used herein, the term “cryoprotectant” refers to an agent used to protect one or more substance from damage due to freezing. Cryoprotectants may include any of those taught in US Publication No. US2013/0323302 or US Patent Nos. 6,503,888, 6,180,613, 7,888,096, 7,091,030, the contents of each of which are herein incorporated by reference in their entirety. In some cases, cryoprotectants may include, but are not limited to dimethyl sulfoxide, 1,2-propanediol, 2,3-butanediol, formamide, glycerol, ethylene glycol, 1,3-propanediol and n-dimethyl formamide, polyvinylpyrrolidone, hydroxyethyl starch, agarose, dextrans, inositol, glucose, hydroxyethylstarch, lactose, sorbitol, methyl glucose, sucrose and urea. In some embodiments, freeze-thaw lysis may be carried out according to any of the methods described in US Patent No. 7,704,721, the contents of which are herein incorporated by reference in their entirety.

[0454] As used herein, the term “lysis force” refers to a physical activity used to disrupt a cell. Lysis forces may include, but are not limited to mechanical forces, sonic forces, gravitational forces, optical forces, electrical forces and the like. Cell lysis carried out by mechanical force is referred to herein as “mechanical lysis.” Mechanical forces that may be used according to mechanical lysis may include high shear fluid forces. According to such methods of mechanical lysis, a microfluidizer may be used. Microfluidizers typically comprise an inlet reservoir where cell solutions may be applied. Cell solutions may then be pumped into an interaction chamber via a pump (e.g. high-pressure pump) at high speed

and/or pressure to produce shear fluid forces. Resulting lysates may then be collected in one or more output reservoir. Pump speed and/or pressure may be adjusted to modulate cell lysis and enhance recovery of products (*e.g.* viral particles.) Other mechanical lysis methods may include physical disruption of cells by scraping.

[0455] Cell lysis methods may be selected based on the cell culture format of cells to be lysed. For example, with adherent cell cultures, some chemical and mechanical lysis methods may be used. Such mechanical lysis methods may include freeze-thaw lysis or scraping. In another example, chemical lysis of adherent cell cultures may be carried out through incubation with lysis solutions comprising surfactant, such as Triton-X-100. In some cases, cell lysates generated from adherent cell cultures may be treated with one more nuclease to lower the viscosity of the lysates caused by liberated DNA.

[0456] In certain embodiments, a method for harvesting AAV particles without lysis may be used for efficient and scalable AAV particle production. In a non-limiting example, AAV particles may be produced by culturing an AAV particle lacking a heparin binding site, thereby allowing the AAV particle to pass into the supernatant, in a cell culture, collecting supernatant from the culture; and isolating the AAV particle from the supernatant, as described in US Patent Application 20090275107, the contents of which are incorporated herein by reference in their entirety.

Clarification

[0457] Cell lysates comprising viral particles may be subjected to clarification. Clarification refers to initial steps taken in purification of viral particles from cell lysates. Clarification serves to prepare lysates for further purification by removing larger, insoluble debris. Clarification steps may include, but are not limited to centrifugation and filtration. During clarification, centrifugation may be carried out at low speeds to remove larger debris only. Similarly, filtration may be carried out using filters with larger pore sizes so that only larger debris is removed. In some cases, tangential flow filtration may be used during clarification. Objectives of viral clarification include high throughput processing of cell lysates and to optimize ultimate viral recovery. Advantages of including a clarification step include scalability for processing of larger volumes of lysate. In some embodiments, clarification may be carried out according to any of the methods presented in US Patent Nos. 8,524,446, 5,756,283, 6,258,595, 6,261,551, 6,270,996, 6,281,010, 6,365,394, 6,475,769, 6,482,634, 6,485,966, 6,943,019, 6,953,690, 7,022,519, 7,238,526, 7,291,498, 7,491,508, US Publication Nos. US2013/0045186, US2011/0263027, US2011/0151434, US2003/0138772,

and International Publication Nos. WO2002012455, WO1996039530, WO1998010088, WO1999014354, WO1999015685, WO1999047691, WO2000055342, WO2000075353 and WO2001023597, the contents of each of which are herein incorporated by reference in their entirety.

[0458] Methods of cell lysate clarification by filtration are well understood in the art and may be carried out according to a variety of available methods including, but not limited to passive filtration and flow filtration. Filters used may comprise a variety of materials and pore sizes. For example, cell lysate filters may comprise pore sizes of from about 1 μM to about 5 μM , from about 0.5 μM to about 2 μM , from about 0.1 μM to about 1 μM , from about 0.05 μM to about 0.05 μM and from about 0.001 μM to about 0.1 μM . Exemplary pore sizes for cell lysate filters may include, but are not limited to, 2.0, 1.9, 1.8, 1.7, 1.6, 1.5, 1.4, 1.3, 1.2, 1.1, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.95, 0.9, 0.85, 0.8, 0.75, 0.7, 0.65, 0.6, 0.55, 0.5, 0.45, 0.4, 0.35, 0.3, 0.25, 0.2, 0.15, 0.1, 0.05, 0.22, 0.21, 0.20, 0.19, 0.18, 0.17, 0.16, 0.15, 0.14, 0.13, 0.12, 0.11, 0.1, 0.09, 0.08, 0.07, 0.06, 0.05, 0.04, 0.03, 0.02, 0.01, 0.02, 0.019, 0.018, 0.017, 0.016, 0.015, 0.014, 0.013, 0.012, 0.011, 0.01, 0.009, 0.008, 0.007, 0.006, 0.005, 0.004, 0.003, 0.002, 0.001 and 0.001 μM . In certain embodiments, clarification may comprise filtration through a filter with 2.0 μM pore size to remove large debris, followed by passage through a filter with 0.45 μM pore size to remove intact cells.

[0459] Filter materials may be composed of a variety of materials. Such materials may include, but are not limited to polymeric materials and metal materials (e.g. sintered metal and pored aluminum.) Exemplary materials may include, but are not limited to nylon, cellulose materials (e.g. cellulose acetate), polyvinylidene fluoride (PVDF), polyethersulfone, polyamide, polysulfone, polypropylene, and polyethylene terephthalate. In some cases, filters useful for clarification of cell lysates may include, but are not limited to ULTIPLATE PROFILE™ filters (Pall Corporation, Port Washington, NY), SUPOR™ membrane filters (Pall Corporation, Port Washington, NY)

[0460] In some cases, flow filtration may be carried out to increase filtration speed and/or effectiveness. In some cases, flow filtration may comprise vacuum filtration. According to such methods, a vacuum is created on the side of the filter opposite that of cell lysate to be filtered. In some cases, cell lysates may be passed through filters by centrifugal forces. In some cases, a pump is used to force cell lysate through clarification filters. Flow rate of cell lysate through one or more filters may be modulated by adjusting one of channel size and/or fluid pressure.

[0461] According to some embodiments, cell lysates may be clarified by centrifugation. Centrifugation may be used to pellet insoluble particles in the lysate. During clarification, centrifugation strength [expressed in terms of gravitational units (g), which represents multiples of standard gravitational force] may be lower than in subsequent purification steps. In some cases, centrifugation may be carried out on cell lysates at from about 200 g to about 800 g, from about 500 g to about 1500 g, from about 1000 g to about 5000 g, from about 1200 g to about 10000 g or from about 8000 g to about 15000 g. In some embodiments, cell lysate centrifugation is carried out at 8000 g for 15 minutes. In some cases, density gradient centrifugation may be carried out in order to partition particulates in the cell lysate by sedimentation rate. Gradients used according to methods of the present disclosure may include, but are not limited to cesium chloride gradients and iodixanol step gradients.

Purification: Chromatography

[0462] In some cases, AAV particles may be purified from clarified cell lysates by one or more methods of chromatography. Chromatography refers to any number of methods known in the art for separating out one or more elements from a mixture. Such methods may include, but are not limited to ion exchange chromatography (*e.g.* cation exchange chromatography and anion exchange chromatography), immunoaffinity chromatography and size-exclusion chromatography. In some embodiments, methods of viral chromatography may include any of those taught in US Patent Nos. 5,756,283, 6,258,595, 6,261,551, 6,270,996, 6,281,010, 6,365,394, 6,475,769, 6,482,634, 6,485,966, 6,943,019, 6,953,690, 7,022,519, 7,238,526, 7,291,498 and 7,491,508 or International Publication Nos. WO1996039530, WO1998010088, WO1999014354, WO1999015685, WO1999047691, WO2000055342, WO2000075353 and WO2001023597, the contents of each of which are herein incorporated by reference in their entirety.

[0463] In some embodiments, ion exchange chromatography may be used to isolate viral particles. Ion exchange chromatography is used to bind viral particles based on charge-charge interactions between capsid proteins and charged sites present on a stationary phase, typically a column through which viral preparations (*e.g.* clarified lysates) are passed. After application of viral preparations, bound viral particles may then be eluted by applying an elution solution to disrupt the charge-charge interactions. Elution solutions may be optimized by adjusting salt concentration and/or pH to enhance recovery of bound viral particles. Depending on the charge of viral capsids being isolated, cation or anion exchange chromatography methods may be selected. Methods of ion exchange chromatography may

include, but are not limited to any of those taught in US Patent Nos. 7,419,817, 6,143,548, 7,094,604, 6,593,123, 7,015,026 and 8,137,948, the contents of each of which are herein incorporated by reference in their entirety.

[0464] In some embodiments, immunoaffinity chromatography may be used. Immunoaffinity chromatography is a form of chromatography that utilizes one or more immune compounds (*e.g.* antibodies or antibody-related structures) to retain viral particles. Immune compounds may bind specifically to one or more structures on viral particle surfaces, including, but not limited to one or more viral coat protein. In some cases, immune compounds may be specific for a particular viral variant. In some cases, immune compounds may bind to multiple viral variants. In some embodiments, immune compounds may include recombinant single-chain antibodies. Such recombinant single chain antibodies may include those described in Smith, R.H. *et al.*, 2009. *Mol. Ther.* 17(11):1888-96, the contents of which are herein incorporated by reference in their entirety. Such immune compounds are capable of binding to several AAV capsid variants, including, but not limited to AAV1, AAV2, AAV6 and AAV8.

[0465] In some embodiments, size-exclusion chromatography (SEC) may be used. SEC may comprise the use of a gel to separate particles according to size. In viral particle purification, SEC filtration is sometimes referred to as “polishing.” In some cases, SEC may be carried out to generate a final product that is near-homogenous. Such final products may in some cases be used in pre-clinical studies and/or clinical studies (Kotin, R.M. 2011. *Human Molecular Genetics.* 20(1):R2-R6, the contents of which are herein incorporated by reference in their entirety.) In some cases, SEC may be carried out according to any of the methods taught in US Patent Nos. 6,143,548, 7,015,026, 8,476,418, 6,410,300, 8,476,418, 7,419,817, 7,094,604, 6,593,123, and 8,137,948, the contents of each of which are herein incorporated by reference in their entirety.

[0466] In certain embodiments, the compositions comprising at least one AAV particle may be isolated or purified using the methods described in US Patent No. US 6146874, the contents of which are herein incorporated by reference in its entirety.

[0467] In certain embodiments, the compositions comprising at least one AAV particle may be isolated or purified using the methods described in US Patent No. US 6660514, the contents of which are herein incorporated by reference in its entirety.

[0468] In certain embodiments, the compositions comprising at least one AAV particle may be isolated or purified using the methods described in US Patent No. US 8283151, the contents of which are herein incorporated by reference in its entirety.

[0469] In certain embodiments, the compositions comprising at least one AAV particle may be isolated or purified using the methods described in US Patent No. US 8524446, the contents of which are herein incorporated by reference in its entirety.

Introduction into cells

[0470] To ensure the chemical and biological stability of siRNA duplexes, it is important to deliver polynucleotides encoding the siRNAs inside the target cells. The polynucleotides of the present disclosure may be introduced into cells using any of a variety of approaches.

[0471] In some embodiments, the polynucleotide of the present disclosure is introduced into a cell by contacting the cell with the polynucleotide. In some embodiments, the polynucleotide is introduced into a cell by contacting the cell with a composition comprising the polynucleotide and a lipophilic carrier. In other embodiments, the polynucleotide is introduced into a cell by transfecting or infecting the cell with a vector comprising nucleic acid sequences capable of producing the siRNA duplex when transcribed in the cell.

[0472] In some embodiments, the siRNA duplex is introduced into a cell by injecting into the cell a vector comprising nucleic acid sequences capable of producing the siRNA duplex when transcribed in the cell.

[0473] In other embodiments, the polynucleotides of the present disclosure may be delivered into cells by electroporation (e.g. U.S. Patent Publication No. 20050014264; the content of which is herein incorporated by reference in its entirety).

[0474] In addition, the siRNA molecules inserted into viral vectors (e.g. AAV vectors) may be delivered into cells by viral infection. These viral vectors are engineered and optimized to facilitate the entry of siRNA molecule into cells that are not readily amenable to transfection. Also, some synthetic viral vectors possess an ability to integrate the shRNA into the cell genome, thereby leading to stable siRNA expression and long-term knockdown of a target gene. In this manner, viral vectors are engineered as vehicles for specific delivery while lacking the deleterious replication and/or integration features found in wild-type virus.

[0475] In some embodiments, the cells may include, but are not limited to, cells of mammalian origin, cells of human origins, embryonic stem cells, induced pluripotent stem cells, neural stem cells, and neural progenitor cells.

Pharmaceutical compositions and formulation

[0476] In addition to the pharmaceutical compositions, e.g., siRNA duplexes (including the encoding plasmids or expression vectors, such as viruses, e.g., AAV) to be delivered, provided herein are principally directed to pharmaceutical compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to any other animal, e.g., to non-human animals, e.g. non-human mammals. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and/or perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions is contemplated include, but are not limited to, humans and/or other primates; mammals, including commercially relevant mammals such as cattle, pigs, horses, sheep, cats, dogs, mice, and/or rats; and/or birds, including commercially relevant birds such as poultry, chickens, ducks, geese, and/or turkeys.

[0477] In some embodiments, compositions are administered to humans, human patients or subjects. For the purposes of the present disclosure, the phrase "active ingredient" generally refers either to synthetic siRNA duplexes or to the viral vector carrying the siRNA duplexes, or to the siRNA molecule delivered by a viral vector as described herein.

[0478] Formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with an excipient and/or one or more other accessory ingredients, and then, if necessary and/or desirable, dividing, shaping and/or packaging the product into a desired single- or multi-dose unit.

[0479] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the disclosure will vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered.

[0480] The siRNA duplexes or viral vectors encoding them can be formulated using one or more excipients to: (1) increase stability; (2) increase cell transfection or transduction; (3)

permit the sustained or delayed release; or (4) alter the biodistribution (e.g., target the viral vector to specific tissues or cell types such as brain and motor neurons).

[0481] Formulations of the present disclosure can include, without limitation, saline, lipidoids, liposomes, lipid nanoparticles, polymers, lipoplexes, core-shell nanoparticles, peptides, proteins, cells transfected with viral vectors (e.g., for transplantation into a subject), nanoparticle mimics and combinations thereof. Further, the viral vectors of the present disclosure may be formulated using self-assembled nucleic acid nanoparticles.

[0482] Formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of associating the active ingredient with an excipient and/or one or more other accessory ingredients.

[0483] A pharmaceutical composition in accordance with the present disclosure may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a "unit dose" refers to a discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

[0484] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the present disclosure may vary, depending upon the identity, size, and/or condition of the subject being treated and further depending upon the route by which the composition is to be administered. For example, the composition may comprise between 0.1% and 99% (w/w) of the active ingredient. By way of example, the composition may comprise between 0.1% and 100%, e.g., between .5 and 50%, between 1-30%, between 5-80%, at least 80% (w/w) active ingredient.

[0485] In some embodiments, the formulations described herein may contain at least one SOD1 targeting polynucleotide. As a non-limiting example, the formulations may contain 1, 2, 3, 4 or 5 polynucleotide that target SOD1 gene at different sites.

[0486] In some embodiments, a pharmaceutically acceptable excipient may be at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% pure. In some embodiments, an excipient is approved for use for humans and for veterinary use. In some embodiments, an excipient may be approved by United States Food and Drug Administration.

In some embodiments, an excipient may be of pharmaceutical grade. In some embodiments, an excipient may meet the standards of the United States Pharmacopoeia (USP), the European Pharmacopoeia (EP), the British Pharmacopoeia, and/or the International Pharmacopoeia.

[0487] Excipients, which, as used herein, includes, but is not limited to, any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, and the like, as suited to the particular dosage form desired. Various excipients for formulating pharmaceutical compositions and techniques for preparing the composition are known in the art (see Remington: The Science and Practice of Pharmacy, 21st Edition, A. R. Gennaro, Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference in its entirety). The use of a conventional excipient medium may be contemplated within the scope of the present disclosure, except insofar as any conventional excipient medium may be incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition.

[0488] Exemplary diluents include, but are not limited to, calcium carbonate, sodium carbonate, calcium phosphate, dicalcium phosphate, calcium sulfate, calcium hydrogen phosphate, sodium phosphate lactose, sucrose, cellulose, microcrystalline cellulose, kaolin, mannitol, sorbitol, inositol, sodium chloride, dry starch, cornstarch, powdered sugar, *etc.*, and/or combinations thereof.

[0489] In some embodiments, the formulations may comprise at least one inactive ingredient. As used herein, the term “inactive ingredient” refers to one or more inactive agents included in formulations. In some embodiments, all, none or some of the inactive ingredients which may be used in the formulations of the present disclosure may be approved by the US Food and Drug Administration (FDA).

[0490] Formulations of viral vectors carrying SOD1 targeting polynucleotides disclosed herein may include cations or anions. In certain embodiments, the formulations include metal cations such as, but not limited to, Zn^{2+} , Ca^{2+} , Cu^{2+} , Mg^{+} and combinations thereof.

[0491] As used herein, “pharmaceutically acceptable salts” refers to derivatives of the disclosed compounds wherein the parent compound is modified by converting an existing acid or base moiety to its salt form (e.g., by reacting the free base group with a suitable organic acid). Examples of pharmaceutically acceptable salts include, but are not limited to,

mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. Representative acid addition salts include acetate, acetic acid, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzene sulfonic acid, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like, as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. The pharmaceutically acceptable salts of the present disclosure include the conventional non-toxic salts of the parent compound formed, for example, from non-toxic inorganic or organic acids. The pharmaceutically acceptable salts of the present disclosure can be synthesized from the parent compound which contains a basic or acidic moiety by conventional chemical methods. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with a stoichiometric amount of the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, nonaqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred. Lists of suitable salts are found in *Remington's Pharmaceutical Sciences*, 17th ed., Mack Publishing Company, Easton, Pa., 1985, p. 1418, *Pharmaceutical Salts: Properties, Selection, and Use*, P.H. Stahl and C.G. Wermuth (eds.), Wiley-VCH, 2008, and Berge et al., *Journal of Pharmaceutical Science*, 66, 1-19 (1977); the content of each of which is incorporated herein by reference in their entirety.

[0492] The term "pharmaceutically acceptable solvate," as used herein, means a compound of the disclosure wherein molecules of a suitable solvent are incorporated in the crystal lattice. A suitable solvent is physiologically tolerable at the dosage administered. For example, solvates may be prepared by crystallization, recrystallization, or precipitation from a solution that includes organic solvents, water, or a mixture thereof. Examples of suitable solvents are ethanol, water (for example, mono-, di-, and tri-hydrates), *N*-

methylpyrrolidinone (NMP), dimethyl sulfoxide (DMSO), *N,N'*-dimethylformamide (DMF), *N,N'*-dimethylacetamide (DMAC), 1,3-dimethyl-2-imidazolidinone (DMEU), 1,3-dimethyl-3,4,5,6-tetrahydro-2-(1H)-pyrimidinone (DMPU), acetonitrile (ACN), propylene glycol, ethyl acetate, benzyl alcohol, 2-pyrrolidone, benzyl benzoate, and the like. When water is the solvent, the solvate is referred to as a "hydrate."

[0493] According to the present disclosure, the SOD1 targeting polynucleotides, or AAV vectors comprising the same, may be formulated for CNS delivery. Agents that cross the brain blood barrier may be used. For example, some cell penetrating peptides that can target siRNA molecules to the brain blood barrier endothelium may be used to formulate the siRNA duplexes targeting SOD1 gene (e.g., Mathupala, *Expert Opin Ther Pat.*, 2009, 19, 137-140; the content of which is incorporated herein by reference in its entirety).

[0494] In certain embodiments, the AAV particles of the disclosure may be formulated in PBS, in combination with an ethylene oxide/propylene oxide copolymer (also known as pluronic or poloxamer).

[0495] In certain embodiments, the AAV particles of the disclosure may be formulated in PBS with 0.001% pluronic acid (F-68) (poloxamer 188) at a pH of about 7.0.

[0496] In certain embodiments, the AAV particles of the disclosure may be formulated in PBS with 0.001% pluronic acid (F-68) (poloxamer 188) at a pH of about 7.3.

[0497] In certain embodiments, the AAV particles of the disclosure may be formulated in PBS with 0.001% pluronic acid (F-68) (poloxamer 188) at a pH of about 7.4.

[0498] In certain embodiments, the AAV particles of the disclosure may be formulated in a solution comprising sodium chloride, sodium phosphate and an ethylene oxide/propylene oxide copolymer.

[0499] In certain embodiments, the AAV particles of the disclosure may be formulated in a solution comprising sodium chloride, sodium phosphate dibasic, sodium phosphate monobasic and poloxamer 188/pluronic acid (F-68).

Administration

[0500] The SOD1 targeting polynucleotides of the present disclosure may be administered by any route which results in a therapeutically effective outcome. These include, but are not limited to intraparenchymal (into brain tissue), intraparenchymal (spinal cord), intraparenchymal (CNS), enteral (into the intestine), gastroenteral, epidural (into the dura matter), oral (by way of the mouth), transdermal, peridural, intracerebral (into the cerebrum), intracerebroventricular (into the cerebral ventricles), epicutaneous (application onto the skin),

intradermal, (into the skin itself), subcutaneous (under the skin), nasal administration (through the nose), intravenous (into a vein), intravenous bolus, intravenous drip, intraarterial (into an artery), intramuscular (into a muscle), intracardiac (into the heart), intraosseous infusion (into the bone marrow), intrathecal (into the spinal canal), intraperitoneal, (infusion or injection into the peritoneum), intravesical infusion, intravitreal, (through the eye), intracavernous injection (into a pathologic cavity) intracavitary (into the base of the penis), intravaginal administration, intrauterine, extra-amniotic administration, transdermal (diffusion through the intact skin for systemic distribution), transmucosal (diffusion through a mucous membrane), transvaginal, insufflation (snorting), sublingual, sublabial, enema, eye drops (onto the conjunctiva), in ear drops, auricular (in or by way of the ear), buccal (directed toward the cheek), conjunctival, cutaneous, dental (to a tooth or teeth), electro-osmosis, endocervical, endosinusal, endotracheal, extracorporeal, hemodialysis, infiltration, interstitial, intra-abdominal, intra-amniotic, intra-articular, intrabiliary, intrabronchial, intrabursal, intracartilaginous (within a cartilage), intracaudal (within the cauda equine), intracisternal (within the cisterna magna cerebellomedularis), intracorneal (within the cornea), dental intracornal, intracoronary (within the coronary arteries), intracorporus cavernosum (within the dilatable spaces of the corporus cavernosa of the penis), intradiscal (within a disc), intraductal (within a duct of a gland), intraduodenal (within the duodenum), intradural (within or beneath the dura), intraepidermal (to the epidermis), intraesophageal (to the esophagus), intragastric (within the stomach), intragingival (within the gingivae), intraileal (within the distal portion of the small intestine), intralesional (within or introduced directly to a localized lesion), intraluminal (within a lumen of a tube), intralymphatic (within the lymph), intramedullary (within the marrow cavity of a bone), intrameningeal (within the meninges), intraocular (within the eye), intraovarian (within the ovary), intrapericardial (within the pericardium), intrapleural (within the pleura), intraprostatic (within the prostate gland), intrapulmonary (within the lungs or its bronchi), intrasinal (within the nasal or periorbital sinuses), intraspinal (within the vertebral column), intrasynovial (within the synovial cavity of a joint), intratendinous (within a tendon), intratesticular (within the testicle), intrathecal (within the cerebrospinal fluid at any level of the cerebrospinal axis), intrathoracic (within the thorax), intratubular (within the tubules of an organ), intratumor (within a tumor), intratympanic (within the aurus media), intravascular (within a vessel or vessels), intraventricular (within a ventricle), iontophoresis (by means of electric current where ions of soluble salts migrate into the tissues of the body), irrigation (to bathe or flush

open wounds or body cavities), laryngeal (directly upon the larynx), nasogastric (through the nose and into the stomach), occlusive dressing technique (topical route administration which is then covered by a dressing which occludes the area), ophthalmic (to the external eye), oropharyngeal (directly to the mouth and pharynx), parenteral, percutaneous, periarticular, peridural, perineural, periodontal, rectal, respiratory (within the respiratory tract by inhaling orally or nasally for local or systemic effect), retrobulbar (behind the pons or behind the eyeball), soft tissue, subarachnoid, subconjunctival, submucosal, topical, transplacental (through or across the placenta), transtracheal (through the wall of the trachea), transtympanic (across or through the tympanic cavity), ureteral (to the ureter), urethral (to the urethra), vaginal, caudal block, diagnostic, nerve block, biliary perfusion, cardiac perfusion, photopheresis, intrastriatal (within the striatum) infusion or spinal.

[0501] In specific embodiments, compositions including AAV vectors comprising at least one SOD1 targeting polynucleotide may be administered in a way which allows them to enter the central nervous system and penetrate into motor neurons.

[0502] In some embodiments, the therapeutics of the present disclosure may be administered by muscular injection. Rizvanov et al. demonstrated for the first time that siRNA molecules, targeting mutant human SOD1 mRNA, is taken up by the sciatic nerve, retrogradely transported to the perikarya of motor neurons, and inhibits mutant SOD1 mRNA in SOD1^{G93A} transgenic ALS mice (Rizvanov AA et al., *Exp. Brain Res.*, 2009, 195(1), 1-4; the content of which is incorporated herein by reference in its entirety). Another study also demonstrated that muscle delivery of AAV expressing small hairpin RNAs (shRNAs) against the mutant SOD1 gene, led to significant mutant SOD1 knockdown in the muscle as well as innervating motor neurons (Towne C et al., *Mol Ther.*, 2011; 19(2): 274-283; the content of which is incorporated herein by reference in its entirety).

[0503] In some embodiments, AAV vectors that express siRNA duplexes of the present disclosure may be administered to a subject by peripheral injections and/or intranasal delivery. It was disclosed in the art that the peripheral administration of AAV vectors for siRNA duplexes can be transported to the central nervous system, for example, to the motor neurons (e.g., U. S. Patent Publication Nos. 20100240739; and 20100130594; the content of each of which is incorporated herein by reference in their entirety).

[0504] In other embodiments, compositions comprising at least one siRNA duplex of the disclosure may be administered to a subject by intracranial delivery (See, e.g., U. S. Pat. No. 8,119,611; the content of which is incorporated herein by reference in its entirety).

[0505] The SOD1 targeting polynucleotides of the present disclosure may be administered in any suitable forms, either as a liquid solution or suspension, as a solid form suitable for liquid solution or suspension in a liquid solution. They may be formulated with any appropriate and pharmaceutically acceptable excipient.

[0506] The SOD1 targeting polynucleotides of the present disclosure may be administered in a “therapeutically effective” amount, i.e., an amount that is sufficient to alleviate and/or prevent at least one symptom associated with the disease, or provide improvement in the condition of the subject.

[0507] In some embodiments, the pharmaceutical compositions of the present disclosure may be administered by intraparenchymal injection or infusion. As used herein, “injection” and “infusion” may be used interchangeably and indicate the same. As a non-limiting example, the pharmaceutical compositions of the present disclosure may be administered to a subject by intraparenchymal injection. In certain embodiments, the intraparenchymal injection may be a spinal intraparenchymal injection, wherein the pharmaceutical compositions may be administered directly to the tissue of the spinal cord. In certain embodiments, the intraparenchymal injection may be a CNS (central nervous system) intraparenchymal injection wherein the pharmaceutical compositions may be administered directly to the tissue of the CNS.

[0508] In certain embodiments, the pharmaceutical compositions of the present disclosure may be administered to the cisterna magna in a therapeutically effective amount to transduce spinal cord motor neurons and/or astrocytes.

[0509] In certain embodiments, the pharmaceutical compositions of the present disclosure may be administered by intrastriatal infusion.

[0510] In some embodiments, the pharmaceutical compositions of the present disclosure may be administered by intraparenchymal injection as well as by another route of administration described herein.

[0511] In some embodiments, the pharmaceutical compositions of the present disclosure may be administered by intraparenchymal injection to the CNS, the brain and/or the spinal cord.

[0512] In some embodiments, the pharmaceutical compositions of the present disclosure may be administered by intraparenchymal injection and intrathecal injection. In certain embodiments, the pharmaceutical compositions of the present disclosure may be administered by intraparenchymal injection and intrastriatal injection.

[0513] In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at any level of the spinal cord, at a single or at multiple sites, at a volume of more than 1uL. In certain embodiments, a volume of 1uL-100uL is administered. In certain embodiments, a volume of 1uL-240uL is administered. In certain embodiments, a volume of 1uL-240uL is administered. In certain embodiments, a volume of 1uL-220uL is administered. In certain embodiments, a volume of between 1uL-200uL is administered. In certain embodiments, a volume of 1uL-180uL is administered. In certain embodiments, a volume of 1uL-160uL is administered. In certain embodiments, a volume of 1uL-150uL is administered. In certain embodiments, a volume of 1uL-140uL is administered. In certain embodiments, a volume of 1uL-130uL is administered. In certain embodiments, a volume of 1uL-120uL is administered. In certain embodiments, a volume of 1uL-110uL is administered. In certain embodiments, a volume of 1uL-90uL is administered. In certain embodiments, a volume of between 1uL-80uL is administered. In certain embodiments, a volume of 1uL-70uL is administered. In certain embodiments, a volume of 1uL-60uL is administered. In certain embodiments, a volume of 1uL-50uL is administered. In certain embodiments, a volume of 1uL-40uL is administered. In certain embodiments, a volume of 1uL-30uL is administered. In certain embodiments, a volume of 1uL-20uL is administered. In certain embodiments, a volume of 5uL-60uL is administered. In certain embodiments, a volume of 5uL-240uL is administered. In certain embodiments, a volume of 10uL-20uL is administered. In certain embodiments, a volume of 10uL-30uL is administered. In certain embodiments, a volume of 10uL-40uL is administered. In certain embodiments, a volume of 10uL-50uL is administered. In certain embodiments, a volume of 10uL-60uL is administered. In certain embodiments, a volume of 10uL-80uL is administered. In certain embodiments, a volume of 10uL-90uL is administered. In certain embodiments, a volume of 20uL-240 uL is administered. In certain embodiments, a volume of 20uL-200uL is administered. In certain embodiments, a volume of 20uL-180 uL is administered. In certain embodiments, a volume of 20uL-150uL is administered. In certain embodiments, a volume of 20uL-120uL is administered. In certain embodiments, a volume of 20uL-100uL is administered. In certain embodiments, a volume of 20uL-80uL is administered. In certain embodiments, a volume of 20uL-60uL is administered. In certain embodiments, a volume of 20uL-50uL is administered. In certain embodiments, a volume of 20uL-40uL is administered. In certain embodiments, a volume of 20uL-30uL is administered. In certain embodiments, a volume of 50uL-200 uL is administered. In certain embodiments, a volume of 50uL-180uL is administered. In certain

embodiments, a volume of 50uL-150uL is administered. In certain embodiments, a volume of 50uL-100uL is administered. In certain embodiments, a volume of 50uL-80uL is administered. In certain embodiments, a volume of 50uL-70uL is administered. In certain embodiments, a volume of 100uL-240uL is administered. In certain embodiments, a volume of 100uL-200 uL is administered. In certain embodiments, a volume of 100uL-180uL is administered. In certain embodiments, a volume of 100uL-150uL is administered.

[0514] The spinal cord is situated within the spine. The spine consists of a series of vertebral segments. There are 7 cervical (C1-C7), 12 thoracic (T1-T12), 5 lumbar (L1-L5), and 5 sacral (S1-S5) vertebral segments. Intraparenchymal injection or infusion into the spinal cord of AAV particles described herein may occur at one or multiple of these vertebral segments. For example, intraparenchymal injection or infusion into the spinal cord of AAV particles described herein may occur at 1, 2, 3, 4, 5, or more than 5 sites. The intraparenchymal injection or infusion sites may be at one or more regions independently selected from the cervical spinal cord, the thoracic spinal cord, the lumbar spinal cord, and the sacral spinal cord. In some embodiments, AAV particles described herein are administered via intraparenchymal (IPa) infusion at two sites into the spinal cord.

[0515] In some embodiments, the AAV particle described herein may be administered via intraparenchymal (IPa) infusion to one or more sites (e.g., 2, 3, 4 or 5 sites) selected from C1, C2, C3, C4, C5, C6, and C7. In some embodiments, the AAV particle described herein may be administered via intraparenchymal (IPa) infusion to two sites selected from C1, C2, C3, C4, C5, C6, and C7.

[0516] In some embodiments, the AAV particle described herein may be administered via intraparenchymal (IPa) infusion to one or more sites (e.g., 2, 3, 4 or 5 sites) selected from T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, and T12. In some embodiments, the AAV particle described herein may be administered via intraparenchymal (IPa) infusion to two sites selected from T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, and T12.

[0517] In some embodiments, the AAV particle described herein may be administered via intraparenchymal (IPa) infusion to one or more sites (e.g., 2, 3, 4 or 5 sites) selected from L1, L2, L3, L4, and L5. In some embodiments, the AAV particle described herein may be administered via intraparenchymal (IPa) infusion to two sites selected from L1, L2, L3, L4, and L5.

[0518] In some embodiments, the AAV particle described herein may be administered via intraparenchymal (IPa) infusion to one or more sites (e.g., 2, 3, 4 or 5 sites) selected from S1,

S2, S3, S4, and S5. In some embodiments, the AAV particle described herein may be administered via intraparenchymal (IPa) infusion to two sites selected from S1, S2, S3, S4, and S5.

[0519] In some embodiments, the AAV particle described herein may be administered via intraparenchymal (IPa) infusion at one or more sites (e.g., 2, 3, 4 or 5 sites) selected from C1, C2, C3, C4, C5, C6, C7, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, L1, L2, L3, L4, L5, S1, S2, S3, S4, and S5. In certain embodiments, the AAV particle described herein may be administered via intraparenchymal (IPa) infusion at two sites selected from C1, C2, C3, C4, C5, C6, C7, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, L1, L2, L3, L4, L5, S1, S2, S3, S4, and S5.

[0520] In some embodiments, the AAV particle described herein may be administered to one or more sites (e.g., 2, 3, 4 or 5 sites) selected from C1, C2, C3, C4, C5, C6, C7, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, L1, L2, L3, L4, and L5. In certain embodiments, the AAV particle described herein may be administered via intraparenchymal (IPa) infusion at two sites selected from C1, C2, C3, C4, C5, C6, C7, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, L1, L2, L3, L4, and L5.

[0521] In some embodiments, the AAV particle described herein may be administered to one or more levels (e.g., 2, 3, or 4 sites) selected from C1, C2, C3, C4, C5, C6, C7, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, and T12. In certain embodiments, the AAV particle described herein may be administered via intraparenchymal (IPa) infusion at two sites selected from C1, C2, C3, C4, C5, C6, C7, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, and T12. As a non-limiting example, the two sites may include one site from the cervical spinal cord region (e.g., C1-C7) and one site from the thoracic spinal cord region (e.g., T1-T12).

[0522] In some embodiments, the AAV particle described herein may be administered to one or more levels (e.g., 2, 3, or 4 sites) selected from C1, C2, C3, C4, C5, C6, C7, L1, L2, L3, L4, and L5. In certain embodiments, the AAV particle described herein may be administered via intraparenchymal (IPa) infusion at two sites selected from C1, C2, C3, C4, C5, C6, C7, L1, L2, L3, L4, and L5. As a non-limiting example, the two sites may include one site from the cervical spinal cord region (e.g., C1-C7) and one site from the lumbar spinal cord region (e.g., L1-L5).

[0523] In some embodiments, the AAV particle described herein may be administered to one or more levels (e.g., 2, 3, or 4 sites) selected from T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, L1, L2, L3, L4, and L5. In certain embodiments, the AAV particle described

herein may be administered via intraparenchymal (IPa) infusion at two sites selected from T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, L1, L2, L3, L4, and L5. As a non-limiting example, the two sites may include one site from the thoracic spinal cord region (e.g., T1-T12) and one site from the lumbar spinal cord region (e.g., L1-L5).

[0524] In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C1, C2, C3, C4, C5, C6, C7, and/or L1.

[0525] In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C1. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C2. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C3. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C4. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C5. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C6. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C7.

[0526] In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at two sites. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C1 and C2. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C1 and C3. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C1 and C4. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C1 and C5. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C1 and C6. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C1 and C7.

[0527] In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at two sites. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C2 and C3. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C2 and C4. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C2 and C5. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C2 and

C6. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C2 and C7.

[0528] In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at two sites. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C3 and C4. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C3 and C5. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C3 and C6. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C3 and C7.

[0529] In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at two sites. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C4 and C5. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C4 and C6. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C4 and C7.

[0530] In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at two sites. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C5 and C6. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C5 and C7.

[0531] In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at two sites. In certain embodiments, the AAV particle described herein is administered via intraparenchymal (IPa) infusion at C6 and C7 of the spinal cord.

[0532] In certain embodiments, the AAV particle described herein is administered via spinal cord infusion at two sites. In another embodiment, the AAV particle described herein comprises administration at level C3 or C5 of the spinal cord. In yet another embodiment, the AAV particle described herein are administered at levels C3 and C5 of the spinal cord.

[0533] The intraparenchymal (IPa) infusion may be for 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60 or more than 60 minutes. As a non-limiting example, the infusion is for 10 minutes. As a non-limiting

example, the infusion is for 11 minutes. As a non-limiting example, the infusion is for 12 minutes. As a non-limiting example, the infusion is for 13 minutes. As a non-limiting example, the infusion is for 14 minutes. As a non-limiting example, the infusion is for 15 minutes.

[0534] The intraparenchymal (IPa), *e.g.*, spinal cord, infusion may be, independently, a dose volume of about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 80, 120, 240 or more than 240 uL. As a non-limiting example, the dose volume is about 20 uL. As a non-limiting example, the dose volume is about 25 uL. As a non-limiting example, the dose volume is about 30 uL. As a non-limiting example, the dose volume is about 35 uL. As a non-limiting example, the dose volume is about 40 uL. As a non-limiting example, the dose volume is about 45 uL. As a non-limiting example, the dose volume is about 50 uL. As a non-limiting example, the dose volume is about 60 uL. As a non-limiting example, the dose volume is about 80 uL. As a non-limiting example, the dose volume is about 120 uL. As a non-limiting example, the dose volume is about 240 uL.

[0535] In certain embodiments, the dose volume is 5uL-60uL per site of administration. In another embodiment, the dose volume is 25uL-40uL per site of administration. In certain embodiments, the dose volume is 5uL-60uL for administration to level C3, C5, C6, or C7 of the spinal cord. In certain embodiments, the dose volume is 5uL-60uL for administration to level C3 of the spinal cord. In another embodiment, the dose volume is 5uL-60uL for administration to level C5 of the spinal cord. In yet another embodiment, the dose volume is 5uL-60uL for administration to level C3 of the spinal cord and the dose volume for administration to level C5 of the spinal cord is 5uL-60uL. In certain embodiments, the dose volume is 25uL-40uL for administration to level C3, C5, C6, or C7 of the spinal cord. In certain embodiments, the dose volume is 25uL-40uL for administration to level C3 of the spinal cord. In another embodiment, the dose volume is 25uL-40uL for administration to level C5 of the spinal cord. In yet another embodiment, the dose volume is 25uL-40uL for administration to level C3 of the spinal cord and the dose volume for administration to level C5 of the spinal cord is 25uL-40uL.

[0536] The intraparenchymal (IPa), *e.g.*, spinal cord, infusion may be at an injection rate of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more than 15 uL/min. As a non-limiting example, the injection rate is 5 uL/min.

[0537] The intraparenchymal (IPa), *e.g.*, spinal cord, infusion may be at a dose between about 1×10^6 VG and about 1×10^{16} VG. In some embodiments, delivery may comprise a

composition concentration of about 1×10^6 , 2×10^6 , 3×10^6 , 4×10^6 , 5×10^6 , 6×10^6 , 7×10^6 , 8×10^6 , 9×10^6 , 1×10^7 , 2×10^7 , 3×10^7 , 4×10^7 , 5×10^7 , 6×10^7 , 7×10^7 , 8×10^7 , 9×10^7 , 1×10^8 , 2×10^8 , 3×10^8 , 4×10^8 , 5×10^8 , 6×10^8 , 7×10^8 , 8×10^8 , 9×10^8 , 1×10^9 , 2×10^9 , 3×10^9 , 4×10^9 , 5×10^9 , 6×10^9 , 7×10^9 , 8×10^9 , 9×10^9 , 1×10^{10} , 2×10^{10} , 3×10^{10} , 4×10^{10} , 5×10^{10} , 6×10^{10} , 7×10^{10} , 8×10^{10} , 9×10^{10} , 1×10^{11} , 2×10^{11} , 2.1×10^{11} , 2.2×10^{11} , 2.3×10^{11} , 2.4×10^{11} , 2.5×10^{11} , 2.6×10^{11} , 2.7×10^{11} , 2.8×10^{11} , 2.9×10^{11} , 3×10^{11} , 4×10^{11} , 4.1×10^{11} , 4.2×10^{11} , 4.3×10^{11} , 4.4×10^{11} , 4.5×10^{11} , 4.6×10^{11} , 4.7×10^{11} , 4.8×10^{11} , 4.9×10^{11} , 5×10^{11} , 6×10^{11} , 6.1×10^{11} , 6.2×10^{11} , 6.3×10^{11} , 6.4×10^{11} , 6.5×10^{11} , 6.6×10^{11} , 6.7×10^{11} , 6.8×10^{11} , 6.9×10^{11} , 7×10^{11} , 7.1×10^{11} , 7.2×10^{11} , 7.3×10^{11} , 7.4×10^{11} , 7.5×10^{11} , 7.6×10^{11} , 7.7×10^{11} , 7.8×10^{11} , 7.9×10^{11} , 8×10^{11} , 9×10^{11} , 1×10^{12} , 1.1×10^{12} , 1.2×10^{12} , 1.3×10^{12} , 1.4×10^{12} , 1.5×10^{12} , 1.6×10^{12} , 1.7×10^{12} , 1.8×10^{12} , 1.9×10^{12} , 2×10^{12} , 3×10^{12} , 4×10^{12} , 4.1×10^{12} , 4.2×10^{12} , 4.3×10^{12} , 4.4×10^{12} , 4.5×10^{12} , 4.6×10^{12} , 4.7×10^{12} , 4.8×10^{12} , 4.9×10^{12} , 5×10^{12} , 6×10^{12} , 7×10^{12} , 8×10^{12} , 8.1×10^{12} , 8.2×10^{12} , 8.3×10^{12} , 8.4×10^{12} , 8.5×10^{12} , 8.6×10^{12} , 8.7×10^{12} , 8.8×10^{12} , 8.9×10^{12} , 9×10^{12} , 1×10^{13} , 2×10^{13} , 3×10^{13} , 4×10^{13} , 5×10^{13} , 6×10^{13} , 6.7×10^{13} , 7×10^{13} , 8×10^{13} , 9×10^{13} , 1×10^{14} , 2×10^{14} , 3×10^{14} , 4×10^{14} , 5×10^{14} , 6×10^{14} , 7×10^{14} , 8×10^{14} , 9×10^{14} , 1×10^{15} , 2×10^{15} , 3×10^{15} , 4×10^{15} , 5×10^{15} , 6×10^{15} , 7×10^{15} , 8×10^{15} , 9×10^{15} , or 1×10^{16} VG. As a non-limiting example, the dose is 4.4×10^{10} VG. As a non-limiting example, the dose is 1.4×10^{11} VG. As a non-limiting example, the dose is 4.1×10^{11} VG. As a non-limiting example, the dose is 4.4×10^{11} VG. As a non-limiting example, the dose is 5.0×10^{11} VG. As a non-limiting example, the dose is 5.1×10^{11} VG. As a non-limiting example, the dose is 6.6×10^{11} VG. As a non-limiting example, the dose is 7.2×10^{11} VG. As a non-limiting example, the dose is 8.0×10^{11} VG. As a non-limiting example, the dose is 8.1×10^{11} VG. As a non-limiting example, the dose is 1.0×10^{12} VG. As a non-limiting example, the dose is 1.1×10^{12} VG. As a non-limiting example, the dose is 1.2×10^{12} VG. As a non-limiting example, the dose is 1.3×10^{12} VG. As a non-limiting example, the dose is 1.0×10^{10} vg- 1.0×10^{12} VG. As a non-limiting example, the dose is 5.0×10^{11} vg- 8.0×10^{11} VG.

[0538] In certain embodiments, the intraparenchymal (IPa), *e.g.*, spinal cord, infusion may be between about 1.0×10^{13} VG/ml and about 3×10^{13} VG/ml. In another embodiment, the intraparenchymal (IPa), *e.g.*, spinal cord, infusion is 1.5×10^{13} VG/ml- 3.0×10^{13} VG/ml. In yet another embodiment, the intraparenchymal (IPa), *e.g.*, spinal cord, infusion is 1.8×10^{13} VG/ml - 2.5×10^{13} VG/ml. In certain embodiments, the intraparenchymal (IPa), *e.g.*, spinal cord, infusion is 1.8×10^{13} VG/ml, 1.85×10^{13} VG/ml, 1.9×10^{13} VG/ml, 1.95×10^{13} VG/ml, 2×10^{13} VG/ml, 2.01×10^{13} VG/ml, 2.02×10^{13} VG/ml, 2.03×10^{13} VG/ml, 2.04×10^{13} VG/ml,

2.05x10¹³ VG/ml, 2.06x10¹³ VG/ml, 2.07x10¹³ VG/ml, 2.08x10¹³ VG/ml, 2.09x10¹³ VG/ml, or 2.10x10¹³ VG/ml.

[0539] In certain embodiments, the dose volume is 5uL-60uL per site of administration and the dose is 1.0x10¹⁰ VG- 1.0x10¹² VG. In certain embodiments, the dose volume is 5uL-60uL per site of administration and the dose is 5.0x10¹¹ VG- 8.0x10¹¹ VG. In another embodiment, the dose volume is 25uL-40uL per site of administration and the dose is 1.0x10¹⁰ VG- 1.0x10¹² VG. In another embodiment, the dose volume is 25uL-40uL per site of administration and the dose is 5.0x10¹¹ VG- 8.0x10¹¹ VG. In certain embodiments, the dose volume is 5uL-60uL for administration to level C3, C5, C6, or C7 of the spinal cord and the dose is 1.0x10¹⁰ VG- 1.0x10¹² VG. In certain embodiments, the dose volume is 5uL-60uL for administration to level C3, C5, C6, or C7 of the spinal cord and the dose is 5.0x10¹¹ VG- 8.0x10¹¹ VG. In certain embodiments, the dose volume is 5uL-60uL for administration to level C3 of the spinal cord and the dose is 1.0x10¹⁰ VG- 1.0x10¹² VG. In certain embodiments, the dose volume is 5uL-60uL for administration to level C3 of the spinal cord and the dose is 5.0x10¹¹ VG- 8.0x10¹¹ VG. In another embodiment, the dose volume is 5uL-60uL for administration to level C5 of the spinal cord and the dose is 1.0x10¹⁰ VG- 1.0x10¹² VG. In another embodiment, the dose volume is 5uL-60uL for administration to level C5 of the spinal cord and the dose is 5.0x10¹¹ VG- 8.0x10¹¹ VG. In yet another embodiment: i) the dose volume is 5uL-60uL for administration to level C3 of the spinal cord and the dose is 1.0x10¹⁰ VG- 1.0x10¹² VG, for example, 5.0x10¹¹ VG- 8.0x10¹¹ VG, and ii) the dose volume for administration to level C5 of the spinal cord is 5uL-60uL and the dose is 1.0x10¹⁰ VG- 1.0x10¹² VG, for example, 5.0x10¹¹ VG- 8.0x10¹¹ VG. In certain embodiments, the dose volume is 25uL-40uL for administration to level C3, C5, C6, or C7 of the spinal cord and the dose is 1.0x10¹⁰ VG- 1.0x10¹² VG. In certain embodiments, the dose volume is 25uL-40uL for administration to level C3, C5, C6, or C7 of the spinal cord and the dose is 5.0x10¹¹ VG- 8.0x10¹¹ VG. In certain embodiments, the dose volume is 25uL-40uL for administration to level C3 of the spinal cord and the dose is 1.0x10¹⁰ VG- 1.0x10¹² VG. In certain embodiments, the dose volume is 25uL-40uL for administration to level C3 of the spinal cord and the dose is 5.0x10¹¹ VG- 8.0x10¹¹ VG. In another embodiment, the dose volume is 25uL-40uL for administration to level C5 of the spinal cord and the dose is 1.0x10¹⁰ VG- 1.0x10¹² VG. In another embodiment, the dose volume is 25uL-40uL for administration to level C5 of the spinal cord and the dose is 5.0x10¹¹ VG- 8.0x10¹¹ VG. In yet another embodiment, i) the dose volume is 25uL-40uL for administration to level C3 of the spinal cord, and the dose is

1.0x10¹⁰ VG- 1.0x10¹² VG, for example, 5.0x10¹¹ VG- 8.0x10¹¹ VG, and ii) the dose volume for administration to level C5 of the spinal cord is 25uL-40uL, and the dose is 1.0x10¹⁰ VG- 1.0x10¹² VG, for example, 5.0x10¹¹ VG- 8.0x10¹¹ VG.

[0540] In certain embodiments, the AAV particle described herein encoding siRNA molecules may be administered via intraparenchymal (IPa) infusion at two sites. The AAV particles may be delivered at the same or different volume for both sites. The AAV particles may be delivered at the same or different volumes for both sites. The AAV particles may be delivered at the same or different infusion rates for both sites.

[0541] In certain embodiments, the AAV particle described herein encoding siRNA molecules may be administered via intraparenchymal (IPa) infusion at two sites. The AAV particles may be delivered at the same volume for both sites. The AAV particles may be delivered at the same dose for both sites. The AAV particles may be delivered at the same infusion rates for both sites.

[0542] In certain embodiments, the AAV particle described herein encoding siRNA molecules may be administered via intraparenchymal (IPa) infusion at two sites. The AAV particles may be delivered at different volumes for both sites. The AAV particles may be delivered at different doses for both sites. The AAV particles may be delivered at different infusion rates for both sites.

[0543] In certain embodiments, the AAV particle described herein encoding siRNA molecules may be administered via intraparenchymal (IPa) infusion at two sites. The AAV particles may be delivered at the same volume for both sites. The AAV particles may be delivered at different dose for both sites. The AAV particles may be delivered at different infusion rates for both sites.

[0544] In certain embodiments, the AAV particle described herein encoding siRNA molecules may be administered via intraparenchymal (IPa) infusion at two sites. The AAV particles may be delivered at the same volume for both sites. The AAV particles may be delivered at different dose for both sites. The AAV particles may be delivered at the same infusion rates for both sites.

[0545] In certain embodiments, the AAV particle described herein encoding siRNA molecules may be administered via intraparenchymal (IPa) infusion at two sites. The AAV particles may be delivered at the same volume for both sites. The AAV particles may be delivered at the same dose for both sites. The AAV particles may be delivered at different infusion rates for both sites.

[0546] In certain embodiments, the AAV particle described herein encoding siRNA molecules may be administered via intraparenchymal (IPa) infusion at two sites. The AAV particles may be delivered at different volumes for both sites. The AAV particles may be delivered at the same dose for both sites. The AAV particles may be delivered at the same infusion rates for both sites.

[0547] In certain embodiments, the AAV particle described herein encoding siRNA molecules may be administered via intraparenchymal (IPa) infusion at two sites. The AAV particles may be delivered at different volume for both sites. The AAV particles may be delivered at different dose for both sites. The AAV particles may be delivered at the same infusion rates for both sites.

[0548] In certain embodiments, the AAV particle described herein encoding siRNA molecules may be administered via intraparenchymal (IPa) infusion at two sites. The AAV particles may be delivered at different volumes for both sites. The AAV particles may be delivered at the same dose for both sites. The AAV particles may be delivered at different infusion rates for both sites.

[0549] In certain embodiments, the AAV particle described herein encoding siRNA molecules may be administered via intraparenchymal (IPa) infusion at C3 and C5. For the infusion at C3, the volume may be 25 uL and the dose may be 4.1×10^{11} vg. For the infusion at C5, the volume may be 40 uL and the dose may be 6.6×10^{11} vg. The injection rate for both infusions may be 5 uL/min for about 13 minutes.

[0550] In some embodiments, IPa infusions (e.g., spinal cord) may result in delivery of the pharmaceutical compositions (i.e., AAV particles) along the extent of the rostral-caudal axis of the spinal cord. In some embodiments, IPa infusions (e.g., spinal cord) yield a rostrocaudal gradient of AAV particle transmission. In some embodiments, IPa infusions (e.g., spinal cord) result in delivery of the pharmaceutical compositions to regions distal to the injection site. While not wishing to be bound by theory, AAV particles of the disclosure may travel the length of the rostral caudal axis of the spinal cord subsequent to IPa infusion at a particular site. In other words, the AAV particles may not be confined to the immediate vicinity of the injection site. As a non-limiting example, the AAV particles may be transported by a trans-synaptic (across the synapse) mechanism. This trans-synaptic mechanism may follow a tract or channel present along the rostral-caudal axis of the spinal cord.

Devices

[0551] As used herein, the term “device” refers to any article constructed or modified to suit a particular purpose, such as facilitating the delivery of the pharmaceutical compositions to a subject or the detection of the administered pharmaceutical compositions in a subject.

[0552] In some embodiments, the devices may be utilized for intraparenchymal injection of the pharmaceutical compositions. Devices may also be used to administer the pharmaceutical compositions to the spinal cord.

[0553] In some embodiments, the device may be a custom floating cannula. In certain embodiments, the custom infusion cannula with a narrow diameter is used for the injections. The cannula may include a 30-gauge beveled needle of fixed length connected to a 30-gauge flexible silastic tubing of variable length. The distal end may be fitted with a Hamilton luer lock, which, in turn, may be attached to a microinjector pump. The proximal silastic tubing may be ensheathed within a 24-gauge rigid outer cannula that is seated on the proximal end of the injection needle flange. The flange seats the outer cannula and may serve as a depth stop for the injection needle

[0554] In certain embodiments, the device may be an intraspinal cannula. The intraspinal cannula may include proximal syringe connection and a distal tip. The proximal syringe connection comprises a female luer lock syringe connector which may be connected to a 3-20' cannula with protective sheathing. The cannula may include a single internal lumen from the distal tip to the syringe. The cannula may include a 4-6" flexible portion near the distal tip. The distal tip includes a flange/depth stop and a blunt rigid tip. The intraspinal cannula may also include a mechanism for attachment to the subject.

[0555] In certain embodiments, the device may be a complex stereotactic frame.

[0556] In certain embodiments, the device may be a simplified stereotactic frame.

[0557] In certain embodiments, the pharmaceutical compositions may be delivered without a frame.

[0558] In certain embodiments, the device may be magnetic resonance imager. Such imagers when used in conjunction with contrast agents such as Gadolinium can detect the administered pharmaceutical compositions in a subject.

[0559] In certain embodiments, any of the devices described herein may be combined to deliver and/detect the administered pharmaceutical compositions.

Dosing

[0560] The pharmaceutical compositions of the present disclosure may be administered to a subject using any amount effective for preventing and treating a SOD1 associated disorder (*e.g.*, ALS). The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the disease, the particular composition, its mode of administration, its mode of activity, and the like.

[0561] The compositions of the present disclosure are typically formulated in unit dosage form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of the compositions of the present disclosure may be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effectiveness for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, and route of administration; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts.

[0562] In some specific embodiments, the doses of AAV vectors for delivering siRNA duplexes of the present disclosure may be adapted dependent on the disease condition, the subject and the treatment strategy, etc. Typically, about 10^5 , 10^6 , 10^{12} , 10^{13} , 10^{14} , 10^{15} to 10^{16} viral genome (unit) may be administered per dose.

[0563] The desired dosage may be delivered three times a day, two times a day, once a day, every other day, every third day, every week, every two weeks, every three weeks, or every four weeks.

[0564] In certain embodiments, the desired dosage may be delivered using multiple administrations (*e.g.*, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or more administrations). When multiple administrations are employed, split dosing regimens such as those described herein may be used. As used herein, a "split dose" is the division of single unit dose or total daily dose into two or more doses, *e.g.*, two or more administrations of the single unit dose. As used herein, a "single unit dose" is a dose of any modulatory polynucleotide therapeutic administered in one dose/at one time/single route/single point of contact, *i.e.*, single administration event. As used herein, a "total daily dose" is an amount given or prescribed in 24 hr period. It may be administered as a single unit dose. In certain embodiments, the viral vectors comprising the SOD1 targeting

polynucleotides of the present disclosure are administered to a subject in split doses. They may be formulated in buffer only or in a formulation described herein.

Methods of treatment of disorders associated with the spinal cord, including ALS

[0565] Provided in the present disclosure are methods for introducing the SOD1 targeting polynucleotides described herein into cells, the method comprising introducing into said cells any of the polynucleotides in an amount sufficient for degradation of target SOD1 mRNA to occur. In some aspects, the cells may be stem cells, neurons such as motor neurons, muscle cells and glial cells such as astrocytes.

[0566] Described here are methods for delivering AAV particles to the spinal cord, for the treatment of disorders associated with the spinal cord, such as, but not limited to motor neuron disease (e.g., ALS). In certain embodiments, these methods result in trans-synaptic transmission.

[0567] Disclosed herein are also methods for treating ALS associated with abnormal SOD1 function in a subject in need of treatment. The method optionally comprises administering to the subject a therapeutically effective amount of a composition comprising or encoding at least one siRNA duplex targeting SOD1 gene. Said siRNA duplex will silence SOD1 gene expression and inhibit SOD1 protein production and reduce one or more symptoms of ALS in the subject such that ALS is therapeutically treated.

[0568] In some embodiments, the SOD1 targeting polynucleotide of the present disclosure or the composition comprising or encoding is administered to the central nervous system of the subject. In other embodiments, the siRNA duplex of the present disclosure or the composition comprising it is administered to the muscles of the subject

[0569] In particular, the SOD1 targeting polynucleotides may be delivered into specific types of targeted cells, including motor neurons; glial cells including oligodendrocyte, astrocyte and microglia; and/or other cells surrounding neurons such as T cells. Studies in human ALS patients and animal SOD1 ALS model implicated that glial cells play an early role in the dysfunction and death of ALS neurons. Normal SOD1 in the surrounding, protective glial cells can prevent the motor neurons from dying even though mutant SOD1 is present in motor neurons (e.g., reviewed by Philips and Rothstein, *Exp. Neurol.*, 2014, May 22. pii: S0014-4886(14)00157-5; the content of which is incorporated herein by reference in its entirety).

[0570] In some specific embodiments, at least one siRNA duplex targeting SOD1 gene used as a therapy for ALS is inserted in a viral vector, such as an AAV vector.

[0571] In some embodiments, the present composition is administered as a single therapeutic or combination therapeutics for the treatment of ALS.

[0572] The viral vectors comprising or encoding siRNA duplexes targeting SOD1 gene may be used in combination with one or more other therapeutic agents. By "in combination with," it is not intended to imply that the agents must be administered at the same time and/or formulated for delivery together, although these methods of delivery are within the scope of the present disclosure. Compositions can be administered concurrently with, prior to, or subsequent to, one or more other desired therapeutics or medical procedures. In general, each agent will be administered at a dose and/or on a time schedule determined for that agent.

[0573] Therapeutic agents that may be used in combination with the SOD1 targeting polynucleotides of the present disclosure can be small molecule compounds which are antioxidants, anti-inflammatory agents, anti-apoptosis agents, calcium regulators, antiglutamatergic agents, structural protein inhibitors, and compounds involved in metal ion regulation.

[0574] Compounds used in combination for treating ALS may include, but are not limited to, agents that reduce oxidative stress, such as free-radical scavengers, or Radicava (edaravone), antiglutamatergic agents: Riluzole, Topiramate, Talampanel, Lamotrigine, Dextromethorphan, Gabapentin and AMPA antagonist; Anti-apoptosis agents: Minocycline, Sodium phenylbutyrate and Arimoclomol; Anti-inflammatory agent: ganglioside, Celecoxib, Cyclosporine, Azathioprine, Cyclophosphamide, Plasmaphoresis, Glatiramer acetate and thalidomide; Ceftriaxone (Berry et al., *Plos One*, 2013, 8(4)); Beta-lactam antibiotics; Pramipexole (a dopamine agonist) (Wang et al., *Amyotrophic Lateral Scler.*, 2008, 9(1), 50-58); Nimesulide in U.S. Patent Publication No. 20060074991; Diazoxide disclosed in U.S. Patent Publication No. 20130143873); pyrazolone derivatives disclosed in US Patent Publication No. 20080161378; free radical scavengers that inhibit oxidative stress-induced cell death, such as bromocriptine (US. Patent Publication No. 20110105517); phenyl carbamate compounds discussed in PCT Patent Publication No. 2013100571; neuroprotective compounds disclosed in US Pat. Nos. 6,933,310 and 8,399,514 and US Patent Publication Nos. 20110237907 and 20140038927; and glycopeptides taught in U.S. Patent Publication No. 20070185012; the content of each of which is incorporated herein by reference in their entirety.

[0575] Therapeutic agents that may be used in combination therapy with the siRNA duplexes targeting SOD1 gene of the present disclosure may be hormones or variants that can protect neuron loss, such as adrenocorticotrophic hormone (ACTH) or fragments thereof (e.g., U.S. Patent Publication No. 20130259875); Estrogen (e.g., U.S. Pat. Nos. 6,334,998 and 6,592,845); the content of each of which is incorporated herein by reference in their entirety.

[0576] Neurotrophic factors may be used in combination therapy with the siRNA duplexes targeting SOD1 gene of the present disclosure for treating ALS. Generally, a neurotrophic factor is defined as a substance that promotes survival, growth, differentiation, proliferation and /or maturation of a neuron, or stimulates increased activity of a neuron. In some embodiments, the present methods further comprise delivery of one or more trophic factors into the subject in need of treatment. Trophic factors may include, but are not limited to, IGF-I, GDNF, BDNF, CTNF, VEGF, Colivelin, Xaliproden, Thyrotrophin-releasing hormone and ADNF, and variants thereof.

[0577] In one aspect, the AAV vector comprising at least one siRNA duplex targeting SOD1 gene may be co-administered with AAV vectors expressing neurotrophic factors such as AAV-IGF-I (Vincent et al., *Neuromolecular medicine*, 2004, 6, 79-85; the content of which is incorporated herein by reference in its entirety) and AAV-GDNF (Wang et al., *J Neurosci.*, 2002, 22, 6920-6928; the content of which is incorporated herein by reference in its entirety).

[0578] In some embodiments, the composition of the present disclosure for treating ALS is administered to the subject in need intravenously, intramuscularly, subcutaneously, intraperitoneally, intrathecally, intraparenchymally (CNS, brain, and/or spinal cord) and/or intraventricularly, allowing the siRNA duplexes or vectors comprising the siRNA duplexes to pass through one or both the blood-brain barrier and the blood spinal cord barrier. In some aspects, the method includes administering (e.g., intraparenchymally administering, intraventricularly administering and/or intrathecally administering) directly to the central nervous system (CNS) of a subject (using, e.g., an infusion pump and/or a delivery scaffold) a therapeutically effective amount of a composition comprising at least one siRNA duplex targeting SOD1 gene or AAV vectors comprising at least one siRNA duplex targeting SOD1 gene, silencing/suppressing SOD1 gene expression, and reducing one or more symptoms of ALS in the subject such that ALS is therapeutically treated.

[0579] In some embodiments, the composition of the present disclosure for treating ALS is administered to the subject in need intraparenchymally (CNS, brain, and/or spinal cord),

allowing the siRNA duplexes or vectors comprising the siRNA duplexes to pass through one or both the blood-brain barrier and the blood spinal cord barrier.

[0580] In certain aspects, the symptoms of ALS including motor neuron degeneration, muscle weakness, muscle atrophy, the stiffness of muscle, difficulty in breathing, slurred speech, fasciculation development, frontotemporal dementia and/or premature death are improved in the subject treated. In other aspects, the composition of the present disclosure is applied to one or both of the brain and the spinal cord. In other aspects, one or both of muscle coordination and muscle function are improved. In other aspects, the survival of the subject is prolonged.

Definitions

[0581] Unless stated otherwise, the following terms and phrases have the meanings described below. The definitions are not meant to be limiting in nature and serve to provide a clearer understanding of certain aspects of the present disclosure.

[0582] As used herein, the term “nucleic acid”, “polynucleotide” and “oligonucleotide” refer to any nucleic acid polymers composed of either polydeoxyribonucleotides (containing 2-deoxy-D-ribose), or polyribonucleotides (containing D-ribose), or any other type of polynucleotide which is an N glycoside of a purine or pyrimidine base, or modified purine or pyrimidine bases. There is no intended distinction in length between the term “nucleic acid”, “polynucleotide” and “oligonucleotide”, and these terms will be used interchangeably. These terms refer only to the primary structure of the molecule. Thus, these terms include double- and single-stranded DNA, as well as double- and single stranded RNA.

[0583] As used herein, the term “RNA” or “RNA molecule” or “ribonucleic acid molecule” refers to a polymer of ribonucleotides; the term “DNA” or “DNA molecule” or “deoxyribonucleic acid molecule” refers to a polymer of deoxyribonucleotides. DNA and RNA can be synthesized naturally, e.g., by DNA replication and transcription of DNA, respectively; or be chemically synthesized. DNA and RNA can be single-stranded (i.e., ssRNA or ssDNA, respectively) or multi-stranded (e.g., double stranded, i.e., dsRNA and dsDNA, respectively). The term “mRNA” or “messenger RNA”, as used herein, refers to a single stranded RNA that encodes the amino acid sequence of one or more polypeptide chains.

[0584] As used herein, the term “RNA interfering” or “RNAi” refers to a sequence specific regulatory mechanism mediated by RNA molecules which results in the inhibition or interfering or “silencing” of the expression of a corresponding protein-coding gene. RNAi

has been observed in many types of organisms, including plants, animals and fungi. RNAi occurs in cells naturally to remove foreign RNAs (e.g., viral RNAs). Natural RNAi proceeds via fragments cleaved from free dsRNA which direct the degradative mechanism to other similar RNA sequences. RNAi is controlled by the RNA-induced silencing complex (RISC) and is initiated by short/small dsRNA molecules in cell cytoplasm, where they interact with the catalytic RISC component argonaute. The dsRNA molecules can be introduced into cells exogenously. Exogenous dsRNA initiates RNAi by activating the ribonuclease protein Dicer, which binds and cleaves dsRNAs to produce double-stranded fragments of 21-25 base pairs with a few unpaired overhang bases on each end. These short double stranded fragments are called small interfering RNAs (siRNAs).

[0585] As used herein, the term “small/short interfering RNA” or “siRNA” refers to an RNA molecule (or RNA analog) comprising between about 5-60 nucleotides (or nucleotide analogs) which is capable of directing or mediating RNAi. Preferably, a siRNA molecule comprises between about 15-30 nucleotides or nucleotide analogs, more preferably between about 16-25 nucleotides (or nucleotide analogs), even more preferably between about 18-23 nucleotides (or nucleotide analogs), and even more preferably between about 19-22 nucleotides (or nucleotide analogs) (e.g., 19, 20, 21 or 22 nucleotides or nucleotide analogs). The term “short” siRNA refers to a siRNA comprising 5-23 nucleotides, preferably 21 nucleotides (or nucleotide analogs), for example, 19, 20, 21 or 22 nucleotides. The term “long” siRNA refers to a siRNA comprising 24-60 nucleotides, preferably about 24-25 nucleotides, for example, 23, 24, 25 or 26 nucleotides. Short siRNAs may, in some instances, include fewer than 19 nucleotides, e.g., 16, 17 or 18 nucleotides, or as few as 5 nucleotides, provided that the shorter siRNA retains the ability to mediate RNAi. Likewise, long siRNAs may, in some instances, include more than 26 nucleotides, e.g., 27, 28, 29, 30, 35, 40, 45, 50, 55, or even 60 nucleotides, provided that the longer siRNA retains the ability to mediate RNAi or translational repression absent further processing, e.g., enzymatic processing, to a short siRNA. siRNAs can be single stranded RNA molecules (ss-siRNAs) or double stranded RNA molecules (ds-siRNAs) comprising a sense strand and an antisense strand which hybridized to form a duplex structure called siRNA duplex. According to the present disclosure, recombinant AAV vectors may encode one or more RNAi molecules such as an siRNA, shRNA, microRNA or precursor thereof.

[0586] As used herein, the term “the antisense strand” or “the first strand” or “the guide strand” of a siRNA molecule refers to a strand that is substantially complementary to a

section of about 10-50 nucleotides, e.g., about 15-30, 16-25, 18-23 or 19-22 nucleotides of the mRNA of the gene targeted for silencing. The antisense strand or first strand has sequence sufficiently complementary to the desired target mRNA sequence to direct target-specific silencing, e.g., complementarity sufficient to trigger the destruction of the desired target mRNA by the RNAi machinery or process.

[0587] As used herein, the term “the sense strand” or “the second strand” or “the passenger strand” of a siRNA molecule refers to a strand that is complementary to the antisense strand or first strand. The antisense and sense strands of a siRNA molecule are hybridized to form a duplex structure. As used herein, a “siRNA duplex” includes a siRNA strand having sufficient complementarity to a section of about 10-50 nucleotides of the mRNA of the gene targeted for silencing and a siRNA strand having sufficient complementarity to form a duplex with the siRNA strand. According to the present disclosure, recombinant AAV vectors may encode a sense and/or antisense strand.

[0588] As used herein, the term “complementary” refers to the ability of polynucleotides to form base pairs with one another. Base pairs are typically formed by hydrogen bonds between nucleotide units in antiparallel polynucleotide strands. Complementary polynucleotide strands can form base pair in the Watson-Crick manner (e.g., A to T, A to U, C to G), or in any other manner that allows for the formation of duplexes. As persons skilled in the art are aware, when using RNA as opposed to DNA, uracil rather than thymine is the base that is considered to be complementary to adenosine. However, when a U is denoted in the context of the present disclosure, the ability to substitute a T is implied, unless otherwise stated. Perfect complementarity or 100% complementarity refers to the situation in which each nucleotide unit of one polynucleotide strand can form hydrogen bond with a nucleotide unit of a second polynucleotide strand. Less than perfect complementarity refers to the situation in which some, but not all, nucleotide units of two strands can form hydrogen bond with each other. For example, for two 20-mers, if only two base pairs on each strand can form hydrogen bond with each other, the polynucleotide strands exhibit 10% complementarity. In the same example, if 18 base pairs on each strand can form hydrogen bonds with each other, the polynucleotide strands exhibit 90% complementarity.

[0589] As used herein, “targeting” means the process of design and selection of nucleic acid sequence that will hybridize to a target nucleic acid and induce a desired effect.

[0590] The term “gene expression” refers to the process by which a nucleic acid sequence undergoes successful transcription and in most instances translation to produce a protein or

peptide. For clarity, when reference is made to measurement of “gene expression”, this should be understood to mean that measurements may be of the nucleic acid product of transcription, e.g., RNA or mRNA or of the amino acid product of translation, e.g., polypeptides or peptides. Methods of measuring the amount or levels of RNA, mRNA, polypeptides and peptides are well known in the art.

[0591] As used herein, the term “mutation” refers to any changing of the structure of a gene, resulting in a variant (also called “mutant”) form that may be transmitted to subsequent generations. Mutations in a gene may be caused by the alternation of single base in DNA, or the deletion, insertion, or rearrangement of larger sections of genes or chromosomes.

[0592] As used herein, the term “vector” means any molecule or moiety which transports, transduces or otherwise acts as a carrier of a heterologous molecule such as the SOD1 targeting polynucleotides of the disclosure. A “viral vector” is a vector which comprises one or more polynucleotide regions encoding or comprising a molecule of interest, e.g., a transgene, a polynucleotide encoding a polypeptide or multi-polypeptide or a modulatory nucleic acid such as small interfering RNA (siRNA). Viral vectors are commonly used to deliver genetic materials into cells. Viral vectors are often modified for specific applications. Types of viral vectors include retroviral vectors, lentiviral vectors, adenoviral vectors and adeno-associated viral vectors.

[0593] The term “adeno-associated virus” or “AAV” or “AAV vector” as used herein refers to any vector which comprises or derives from components of an adeno associated vector and is suitable to infect mammalian cells, preferably human cells. The term AAV vector typically designates an AAV type viral particle or virion comprising a nucleic acid molecule encoding a siRNA duplex. The AAV vector may be derived from various serotypes, including combinations of serotypes (i.e., “pseudotyped” AAV) or from various genomes (e.g., single stranded or self-complementary). In addition, the AAV vector may be replication defective and/or targeted.

[0594] As used herein, the phrase “inhibit expression of a gene” means to cause a reduction in the amount of an expression product of the gene. The expression product can be a RNA molecule transcribed from the gene (e.g., an mRNA) or a polypeptide translated from an mRNA transcribed from the gene. Typically, a reduction in the level of an mRNA results in a reduction in the level of a polypeptide translated therefrom. The level of expression may be determined using standard techniques for measuring mRNA or protein.

[0595] As used herein, the term “*in vitro*” refers to events that occur in an artificial environment, *e.g.*, in a test tube or reaction vessel, in cell culture, in a Petri dish, *etc.*, rather than within an organism (*e.g.*, animal, plant, or microbe).

[0596] As used herein, the term “*in vivo*” refers to events that occur within an organism (*e.g.*, animal, plant, or microbe or cell or tissue thereof).

[0597] As used herein, the term “modified” refers to a changed state or structure of a molecule of the disclosure. Molecules may be modified in many ways including chemically, structurally, and functionally.

[0598] As used herein, the term “synthetic” means produced, prepared, and/or manufactured by the hand of man. Synthesis of polynucleotides or polypeptides or other molecules of the present disclosure may be chemical or enzymatic.

[0599] As used herein, the term “transfection” refers to methods to introduce exogenous nucleic acids into a cell. Methods of transfection include, but are not limited to, chemical methods, physical treatments and cationic lipids or mixtures. The list of agents that can be transfected into a cell is large and includes, but is not limited to, siRNA, sense and/or anti-sense sequences, AAV vectors or particles, DNA encoding one or more genes and organized into an expression plasmid, proteins, protein fragments, and more.

[0600] As used herein, “off target” refers to any unintended effect on any one or more target, gene, or cellular transcript.

[0601] As used herein, the phrase “pharmaceutically acceptable” is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[0602] As used herein, the term “effective amount” of an agent is that amount sufficient to effect beneficial or desired results, for example, clinical results, and, as such, an “effective amount” depends upon the context in which it is being applied. For example, in the context of administering an agent that treats ALS, an effective amount of an agent is, for example, an amount sufficient to achieve treatment, as defined herein, of ALS, as compared to the response obtained without administration of the agent.

[0603] As used herein, the term “therapeutically effective amount” means an amount of an agent to be delivered (*e.g.*, nucleic acid, drug, therapeutic agent, diagnostic agent, prophylactic agent, *etc.*) that is sufficient, when administered to a subject suffering from or

susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition.

[0604] As used herein, the term "subject" or "patient" refers to any organism to which a composition in accordance with the disclosure may be administered, *e.g.*, for experimental, diagnostic, prophylactic, and/or therapeutic purposes. Typical subjects include animals (*e.g.*, mammals such as mice, rats, rabbits, non-human primates such as chimpanzees and other apes and monkey species, and humans) and/or plants.

[0605] As used herein, the term "preventing" or "prevention" refers to delaying or forestalling the onset, development or progression of a condition or disease for a period of time, including weeks, months, or years.

[0606] The term "treatment" or "treating", as used herein, refers to the application of one or more specific procedures used for the cure or amelioration of a disease. In certain embodiments, the specific procedure is the administration of one or more pharmaceutical agents. In the context of the present disclosure, the specific procedure is the administration of one or more siRNA duplexes or dsRNA targeting SOD1 gene.

[0607] As used herein, the term "amelioration" or "ameliorating" refers to a lessening of severity of at least one indicator of a condition or disease. For example, in the context of neurodegeneration disorder, amelioration includes the reduction of neuron loss.

[0608] As used herein, the term "administering" refers to providing a pharmaceutical agent or composition to a subject.

[0609] As used herein, the term "neurodegeneration" refers to a pathologic state which results in neural cell death. A large number of neurological disorders share neurodegeneration as a common pathological state. For example, Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS) all cause chronic neurodegeneration, which is characterized by a slow, progressive neural cell death over a period of several years, whereas acute neurodegeneration is characterized by a sudden onset of neural cell death as a result of ischemia, such as stroke, or trauma, such as traumatic brain injury, or as a result of axonal transection by demyelination or trauma caused, for example, by spinal cord injury or multiple sclerosis. In some neurological disorders, mainly one type of neuron cells is degenerative, for example, motor neuron degeneration in ALS.

EXAMPLES

Example 1. SOD1 Targeting Polynucleotide Design (siRNA)

[0610] siRNA design is carried out to identify siRNAs targeting human SOD1 gene. The design uses the SOD1 transcripts from human (GenBank access No. NM_000454.4 (SEQ ID NO: 10)), cynomolgus (GenBank access No. NM_001285406.1 (SEQ ID NO: 11)), rhesus SOD1 transcript (GenBank access No. NM_001032804.1 (SEQ ID NO: 11)), and *Sus scrofa* (GenBank access No. NM_001190422.1 (SEQ ID NO: 12)), respectively (Table 10). The siRNA duplexes are designed with 100% identity to the human SOD1 transcript for positions 2-18 of the antisense strand, and partial or 100% identity to the non-human SOD1 transcript for positions 2-18 of the antisense strand. In all siRNA duplexes, position 1 of the antisense strand is engineered to a U and position 19 of the sense strand is engineered to a C, in order to unpair the duplex at this position.

Table 10. SOD1 gene sequences

SOD1 transcripts	Access No.	SEQ ID NO.	Sequence
Human (Homo sapiens) SOD1 cDNA (981bp)	NM_000454.4	10	GTTTGGGGCCAGAGTGGGCGAGGCGCGGAGGTCTGGCC TATAAAGTAGTCGCGGAGACGGGGTGTGGTTTGCCTCG TAGTCTCCTGCAGCGTCTGGGGTTTCCGTTGCAGTCTCTG GAACCAGGACCTCGGCGTGGCCTAGCGAGTTATGGCGA CGAAGGCCGTGTGCGTGCTGAAGGGCGACGGCCAGTG CAGGGCATCATCAATTTTCGAGCAGAAGGAAAGTAATGG ACCAGTGAAGGTGTGGGGAAGCATTAAAGGACTGACTG AAGGCCATGCATGGATTCCATGTTTCATGAGTTTGGAGATA ATACAGCAGGCTGTACCAGTGCAGGTCCTCACTTAAATC CTCTATCCAGAAAACACGGTGGGCCAAAGGATGAAGAG AGGCATGTTGGAGACTTGGGCAATGTGACTGCTGACAA AGATGGTGTGGCCGATGTGTCTATTGAAGATTCTGTGAT CTCACTCTCAGGAGACCATTGCATCATTGGCCGCACACT GGTGGTCCATGAAAAAGCAGATGACTTGGGCAAAGGTG GAAATGAAGAAAGTACAAAGACAGGAAACGCTGGAAGT CGTTTGGCTTGTGGTGAATTGGGATCGCCCAATAAACA TTCCCTTGGATGTAGTCTGAGGGCCCCTTAACTCATCTGTT ATCCTGCTAGCTGTAGAAATGTATCCTGATAAACATTA ACACTGTAATCTTAAAAGTGAATTGTGTGACTTTTTCA GAGTTGCTTTAAAGTACCTGTAGTGAGAAACTGATTTAT GATCACTTGAAGATTTGTATAGTTTTATAAACTCAGT TAAAATGTCTGTTTCAATGACCTGTATTTTGGCCAGACTTA AATCACAGATGGGTATTAACCTTGTGAGAATTTCTTTGT CATTCAAGCCTGTGAATAAAAACCCTGTATGGCACTTAT TATGAGGCTATTAAGAATCCAAATTCAAACTAAAAA AAAAAAAAAAAAA
Cynomolgus (Macaca fascicularis) SOD1 cDNA (465bp)	NM_001285406.1	11	ATGGCGATGAAGGCCGTGTGCGTGTGAAGGGCGACAG CCCAGTGCAGGGCACCATCAATTTTCGAGCAGAAGGAAA GTAATGGACCAGTGAAGGTGTGGGGAAGCATTACAGGA TTGACTGAAGGCCTGCATGGATTCCATGTTTCATCAGTTT GGAGATAATACACAAGGCTGTACCAGTGCAGGTCCTCA CTTTAATCCTCTATCCAGACAACACGGTGGGCCAAAGGA TGAAGAGAGGCATGTTGGAGACCTGGGCAATGTGACTG CTGGCAAAGATGGTGTGGCCAAGGTGTCTTTTGAAGATT

			CTGTGATCTCGCTCTCAGGAGACCATTCCATCATTGGCC GCACATTGGTGGTCCATGAAAAAGCAGATGACTTGGGC AAAGGTGGAAATGAAGAAAGTAAAAAGACAGGAAACG CTGGAGGTCGTCTGGCTTGTGGTGTAAATGGGATCGCCC AATAA
rhesus (Macaca mulatta) SOD1 cDNA (465bp)	NM_001 032804.1	11	ATGGCGATGAAGGCCGTGTGCGTGTGAAGGGCGACAG CCCAGTGCAGGGCACCATCAATTTTCGAGCAGAAGGAAA GTAATGGACCAGTGAAGGTGTGGGGAAGCATTACAGGA TTGACTGAAGGCCTGCATGGATTCCATGTTTCATCAGTTT GGAGATAATACACAAGGCTGTACCAGTGCAGGTCCTCA CTTTAATCCTCTATCCAGACAACACGGTGGGCCAAAGGA TGAAGAGAGGCATGTTGGAGACCTGGGCAATGTGACTG CTGGCAAAGATGGTGTGGCCAAGGTGTCTTTCGAAGATT CTGTGATCTCGCTCTCAGGAGACCATTCCATCATTGGCC GCACATTGGTGGTCCATGAAAAAGCAGATGACTTGGGC AAAGGTGGAAATGAAGAAAGTAAAAAGACAGGAAACG CTGGAGGTCGTCTGGCTTGTGGTGTAAATGGGATCGCCC AATAA
Pig (Sus scrofa) SOD1 cDNA (658 bp)	NM_001 190422.1	12	CGTCGGCGTGTACTGCGGCCTCTCCCGCTGTTCTGGTA CCCTCCCAGCCCGGACCGGAGCGCGCCCCCGCAGTCAT GGCGACGAAGGCCGTGTGTGTGCTGAAGGGCGACGGCC CGGTGCAGGGCACCATCTACTTCGAGCTGAAGGGAGAG AAGACAGTGTAGTAACGGGAACCATTAAGGACTGGC TGAAGGTGATCATGGATTCCATGTCCATCAGTTTGGAGA TAATACACAAGGCTGTACCAGTGCAGGTCCTCACTCAA TCCTGAATCCAAAAACATGGTGGGCCAAAGGATCAAG AGAGGCACGTTGGAGACCTGGGCAATGTGACTGTGGC AAAGATGGTGTGGCCACTGTGTACATCGAAGATTCTGTG ATCGCCCTCTCGGGAGACCATTCCATCATTGGCCGCACA ATGGTGGTCCATGAAAAACCAGATGACTTGGGCAGAGG TGGAAATGAAGAAAGTACAAAGACGGGAAATGCTGGAA GTCGTTTGGCCTGTGGTGTAAATGGGATCACCCAGTAAA CATTCCCTCATGCCATGGTCTGAATGCCAGTAACTCATC TGTTATCTTGCTAGTTGTAGTTGTAGAAATTTAACTTGAT AAACATTAACACTGTAACCTTAAAAAAAAAAAAAAAAAA AA

Example 2. Intraparenchymal delivery of AAV to spinal cord

[0611] Traditional routes of AAV delivery, such as intrathecal or intravenous administration, have not yielded robust transduction of the cervical and thoracic spinal cord in large mammals so a new route of AAV delivery - intraparenchymal injection - was evaluated for improved cervical spinal cord transduction efficiency. Biodistribution of viral genomes and SOD1 mRNA knockdown were evaluated in the ventral horn at multiple levels of the spinal cord, including the cervical level.

[0612] In the first experiment, three Göttingen adult (6 months of age), female mini-pigs weighing 14-20 kg each were utilized for the study. Animals were not pre-screened for neutralizing antibodies to AAV. A 4-5 cm laminectomy was performed between C3 and C5, allowing for 3 cm between injections. Self-complementary (sc) AAV vectors (scAAV) with ITR to ITR sequence of SEQ ID NO: 9, including an H1 promoter and modulatory

polynucleotide (SEQ ID NO: 6) comprising siRNA targeting SOD1 were packaged in AAVrh10 (scAAV-miRSOD1).

[0613] Two injections of the scAAV-miRSOD1 (titer 2.03×10^{13} vg/mL) were administered, for a total dose/animal of 1.3×10^{12} vg. At the rostral end of the laminectomy, i.e. at the C3 level of the spinal cord, a single 25 μ L (5.1×10^{11} vg) volume was injected into the ventral horn of the spinal cord. At the caudal end of the laminectomy, i.e. at the C5 level of the spinal cord, a single 40 μ L (8.1×10^{11} vg) volume was injected into the ventral horn of the contralateral side. Both injections were administered at the rate of 5 μ L/min, yielding an approximately 13-minute total infusion time. Four weeks following the procedure, animals were sacrificed, and spinal cord tissue was collected for analyses.

[0614] To determine if intraparenchymal administration of the scAAV-miRSOD1 leads to transduction of the spinal cord and knockdown of SOD1 mRNA, ventral horn punches were analyzed by the branched DNA (bDNA) method to quantify levels of SOD1 mRNA, normalized to the geometric mean of beta-actin (ACTB), TATA-box binding protein (TBP) and peptidylprolyl isomerase A (PPIA) mRNA levels. These normalized SOD1 mRNA levels were then expressed relative to normalized SOD1 mRNA levels in ventral horn punches from the lumbar region of the spinal cord (L1-L3) from the same animals.

[0615] Significant SOD1 mRNA knockdown was evident in ventral horn punches from C1 to T7-10, relative to SOD1 mRNA levels in ventral horn punches from L1-L3, with similar SOD1 mRNA levels in ventral horn punches from both sides of the spinal cord. One-way ANOVA and Dunnett's test indicated significant SOD1 mRNA knockdown at each level of the spinal cord (C1-T5 $p < 0.0001$; T7-10 $p < 0.05$). As shown in Table 11, spinal cord segments closest to the injections exhibited the greatest SOD1 mRNA knockdown. Spinal segments C1 through C8 had robust and significant knockdown of SOD1 mRNA (approximately 50-75% knockdown). Even at spinal segment T5, distant from the site of vector injection, significant knockdown of SOD1 mRNA ($32.6 \pm 5.1\%$ knockdown) was observed.

Table 11. SOD1 mRNA levels relative to L1-L3

Spinal cord segment	SOD1 mRNA level normalized to geomean (ACTB, TBP and PPIA) (relative to L1-L3, %)						Mean \pm Standard Error
	Fig #301		Fig #302		Fig #303		
	Ventral Horn 1	Ventral Horn 2	Ventral Horn 1	Ventral Horn 2	Ventral Horn 1	Ventral Horn 2	
C1	47.2	49.9	43.2	48.7	48.3	44.8	47.0 \pm 1.0
C2	39.9	41.3	42.3	41.0	49.6	46.5	43.4 \pm 1.5
C3-rostral	25.1	28.2	18.1	15.0	22.6	32.5	23.6 \pm 2.6
C3-caudal	14.0	17.0	33.1	35.7	29.7	21.3	25.1 \pm 3.7

C5-rostral	21.4	19.0	14.6	21.0	36.1	35.3	24.6±3.7
C5-caudal	25.4	26.6	38.2	33.8	31.7	32.8	31.4±2.0
C7	31.9	15.6	53.6	48.9	44.1	39.2	38.9±5.6
C8	50.1	45.1	50.5	48.7	36.7	42.8	45.7±2.2
T1-T2	54.0	77.7	55.6	56.0	55.3	55.2	59.0±3.8
T5	65.3	53.1	63.0	58.0	84.9	80.2	67.4±5.1
T7-T10	84.6	81.5	83.0	68.0	94.3	93.1	84.1±3.9
L1-L3	98.2	93.8	102.0	96.8	105.2	103.9	100.0±1.8

[0616] Normalized SOD1 mRNA levels in ventral horn punches from AAV particle-treated pigs were also expressed relative to normalized SOD1 mRNA levels in ventral horn punches from the spinal cord of a single naïve pig. SOD1 mRNA levels were normalized to the geometric mean of beta-actin (ACTB), TATA-box binding protein (TBP) and peptidylprolyl isomerase A (PPIA) mRNA levels. SOD1 mRNA levels from each cervical segment of the treated pigs were then expressed relative to normalized SOD1 mRNA levels using C2 SOD1 mRNA levels from the naïve pig. Thoracic SOD1 mRNA levels (treated pigs) were normalized using T2 SOD1 mRNA levels (naïve pig), and lumbar SOD1 mRNA levels (treated pigs) were normalized using L2 SOD1 mRNA levels from the naïve pig. Ventral horn punches from the naïve pig spinal cord were collected from C2, T2 and L2 levels. As shown in Table 12, SOD1 mRNA levels in the ventral horn punches of the scAAV-miRSOD1 administered pigs showed significant knockdown relative to the naïve pig (one-way ANOVA and Dunnett’s test; $p < 0.0001$) at all spinal cord levels tested. Similar SOD1 mRNA levels were observed in ventral horn punches from both sides of the spinal cord. SOD1 mRNA knockdown was strongest near the C3 and C5 injection sites (79-84% knockdown). Even at spinal cord levels distant from the sites of AAV injection, ventral horn punches exhibited significant SOD1 mRNA knockdown. At the T5, T7-T10, and L1 spinal cord levels, ventral horn punches showed significant $55.1 \pm 3.4\%$, $44.0 \pm 2.6\%$ and $33.4 \pm 1.2\%$ knockdown of SOD1 mRNA, respectively.

Table 12. SOD1 mRNA levels relative to naïve control

Spinal cord segment	SOD1 mRNA level normalized to geomean (ACTB, TBP and PPIA) (relative to naïve control, %)						Mean ± Standard Error
	Fig #301		Fig #302		Fig #303		
	Ventral Horn Punch 1	Ventral Horn Punch 2	Ventral Horn Punch 1	Ventral Horn Punch 2	Ventral Horn Punch 1	Ventral Horn Punch 2	
C1	31.5	33.2	28.8	32.4	32.1	29.8	31.3±0.7
C2	26.6	27.5	28.2	27.3	33.0	30.9	28.9±1.0
C3-rostral	16.7	18.8	12.1	10.0	15.0	21.6	15.7±1.8
C3-caudal	9.3	11.3	22.0	23.8	19.8	14.2	16.7±2.4
C5-rostral	14.3	12.6	9.7	14.0	24.0	23.5	16.4±2.4

C5-caudal	16.9	17.7	25.5	22.5	21.1	21.8	20.9±1.3
C7	21.2	10.4	35.7	32.6	29.3	26.1	25.9±3.7
C8	33.3	30.0	33.6	32.4	24.5	28.5	30.4±1.4
T1-2	36.0	51.7	37.0	37.3	36.8	36.8	39.3±2.5
T5	43.5	35.3	41.9	38.6	56.5	53.4	44.9±3.4
T7-10	56.4	54.2	55.3	45.2	62.8	62.0	56.0±2.6
L1	65.4	62.5	67.9	64.5	70.1	69.2	66.6±1.2

[0617] As shown in Table 13, the analysis of vector genome biodistribution by digital droplet PCR showed high vector genome copy number per diploid cell in ventral horn punches of the cervical spinal cord nearest the injection sites. Vector genome copy numbers dropped steeply (>10-fold) from C3 to C2, and from C7 to C8 spinal cord levels. However, even at spinal cord levels distant from the C3 and C5 sites of AAV injection, ventral horn punches exhibited significant vector genome copies. At the T5, T7-T10, and L1-L3 spinal cord levels, ventral horn punches showed significant 1.7 ± 1.2 , 0.2 ± 0.0 , and 0.5 ± 0.2 vector genome copies per diploid cell, respectively.

Table 13. Vector Genome Quantification

Spinal cord segment	Vector Genome/Diploid Cell (vg/dc)						Mean ± Standard Error
	Fig #301		Fig #302		Fig #303		
	Ventral Horn Punch 1	Ventral Horn Punch 2	Ventral Horn Punch 1	Ventral Horn Punch 2	Ventral Horn Punch 1	Ventral Horn Punch 2	
C1	4.4	4.8	10.7	7.2	4.7	3.1	5.8 ± 1.1
C2	14.1	6.6	25.4	32.1	19.8	13.2	18.5 ± 3.8
C3-rostral	763.1	1305.8	618.1	62.2	798.4	286.2	638.9 ± 177.3
C3-caudal	147.3	837.6	1445.7	79.5	185.4	817.1	585.4 ± 221.0
C5-Rostral	677.7	448.0	1703.8	41.1	70.5	138.2	513.2 ± 278.7
C5-Caudal	644.3	60.7	564.6	70.2	78.0	174.7	265.4 ± 109.0
C7	29.4	1225.7	5.5	6.1	24.4	9.3	216.7 ± 201.8
C8	12.0	22.4	4.2	1.7	7.7	11.6	9.9 ± 3.0
T1-T2	6.7	0.4	1.3	1.6	3.3	1.9	2.5 ± 0.9
T5	0.6	7.7	0.6	0.5	0.4	0.2	1.7 ± 1.2
T7-T10	0.3	0.3	0.3	0.4	0.1	0.2	0.2 ± 0.0
L1-L3	0.5	0.4	0.3	0.1	1.4	0.2	0.5 ± 0.2

[0618] Vector genome distribution showed a linear correlation to levels of SOD1 mRNA knockdown in both analyses, i.e., when SOD1 knockdown was compared to L1-L3 ($r^2=0.26$, $p<0.0001$) and when compared to naïve control ($r^2=0.26$, $p<0.0001$). Low vector genome copy number per diploid cell (< 1 vg/dc) such as 0.2 or 0.5 vector genome copies per diploid cell on average, still yielded substantial SOD1 mRNA knockdown.

[0619] In a second experiment, six Göttingen adult (>9 months of age) female and male mini-pigs weighing 15-30 kg each were utilized. Animals were not pre-screened for neutralizing antibodies to AAV. A multi-level laminectomy was performed at the C3 to C5 levels to access the spinal cord, allowing for 3 cm between injections.

[0620] In the first group of three pigs, two injections of the scAAV-miRSOD1 (titer 2.03×10^{13} vg/mL) were administered, for a total dose/animal of 1.6×10^{12} vg. At the rostral end of the laminectomy, a single 40 μ L (8.1×10^{11} vg) volume was injected into the ventral horn at rostral C3 on the right side. At the caudal end of the laminectomy, a single 40 μ L (8.1×10^{11} vg) volume was injected into the ventral horn at caudal C5 on the left side. Both injections were administered at the rate of 5 μ L/min, yielding an approximately 16-minute total infusion time. In the second group of three pigs, vehicle was injected with the same dosing paradigm. Four weeks following the procedure, animals were sacrificed, and spinal cord tissue was collected for analyses.

[0621] Ventral horn punches were analyzed by the branched DNA (bDNA) method for knockdown of SOD1 mRNA, normalized to the geometric mean of beta-actin (ACTB), TATA-box binding protein (TBP) and peptidylprolyl isomerase A (PPIA) mRNA levels, and expressed relative to normalized SOD1 mRNA levels in ventral horn punches from the same spinal cord level of vehicle treated animals. Significant SOD1 mRNA knockdown was evident in punches taken from the left ventral horn from C1 to T12 and in punches taken from the right ventral horn from C1 to L1, with similar SOD1 mRNA levels in ventral horn punches from both sides of the spinal cord. Two-way ANOVA and Sidak's multiple comparisons test indicated significant SOD1 mRNA knockdown at each level of the spinal cord relative to the vehicle control group (left side: C1-T7 $p < 0.0001$; T10 $p < 0.001$, T12 $P < 0.01$; right side: C1-T10 $p < 0.0001$; T12 $p < 0.001$, L1 $P < 0.01$). As shown in Table 14, spinal cord segments closest to the injections exhibited the greatest SOD1 mRNA knockdown, with the maximal SOD1 mRNA knockdown at C5. Spinal segments C1 through T5 had robust and significant knockdown of SOD1 mRNA (50-82% knockdown). Even at spinal segment T12 on the left side and at spinal cord segment L1 at the right side, distant from the site of vector injection, significant knockdown of SOD1 mRNA ($35.22 \pm 2.76\%$; $29.14 \pm 10.36\%$ knockdown, respectively) was observed.

Table 14. SOD1 mRNA levels relative to vehicle group

Spinal cord Segments	SOD1 mRNA level normalized to geometric mean of housekeeping genes ACTB, TBP and PPIA (relative to vehicle control, %)							
	Left Ventral Horn							
	Vehicle				AAV			
	Pig #1001	Pig #1002	Pig #1003	Mean ± Standard Error	Pig #1005	Pig #1004	Pig #1006	Mean ± Standard Error
C1	101.66	91.48	106.86	100±4.52	38.45	32.18	37.13	35.92±1.91
C2	79.26	103.29	117.45	100±11.15	27.49	30.11	35.06	30.88±2.22
C3	110.32	107.20	82.48	100±8.81	35.71	41.30	34.96	37.32±2.00
C5-Rostral	98.87	122.26	78.86	100±12.54	11.33	14.71	28.73	18.26±5.33
C5-Caudal	99.80	91.21	108.99	100±5.13	21.56	20.63	15.97	19.39±1.73
C7	99.00	96.50	104.49	100±2.36	22.55	23.66	26.87	24.36±1.30
C8	106.34	92.34	101.31	100±4.09	23.27	32.11	29.59	28.32±2.63
T1	89.95	92.54	117.51	100±8.78	30.34	41.09	35.28	35.57±3.11
T4	84.33	100.11	115.56	100±9.02	40.53	45.11	41.78	42.47±1.37
T5	100.90	97.81	101.30	100±1.10	44.22	43.50	41.29	43.00±0.88
T7	91.75	102.39	105.85	100±4.24	63.00	49.46	49.49	53.98±4.51
T10	88.91	111.54	99.55	100±6.54	62.41	50.33	61.55	58.10±3.89
T12	87.58	107.32	105.09	100±6.24	60.37	64.13	69.85	64.78±2.76
L1	102.65	104.40	92.95	100±3.56	65.43	58.89	107.53	77.29±15.24
Spinal cord Segments	Right Ventral Horn							
	Vehicle				AAV			
	Pig #1001	Pig #1002	Pig #1003	Mean ± Standard Error	Pig #1005	Pig #1004	Pig #1006	Mean ± Standard Error
C1	93.18	91.40	115.42	100±7.73	56.06	33.77	41.78	43.87±6.52
C2	100.83	91.11	108.07	100±4.91	31.20	31.88	39.79	34.29±2.76
C3	115.93	96.42	87.65	100±8.36	27.03	18.23	33.33	26.20±4.38
C5-Rostral	81.86	99.31	118.83	100±10.68	23.50	13.53	30.29	22.44±4.87
C5-Caudal	106.72	92.42	100.86	100±4.15	32.02	18.18	19.09	23.10±4.47
C7	108.37	88.48	103.15	100±5.95	31.51	27.44	24.66	27.87±1.99
C8	107.49	86.23	106.29	100±6.90	23.05	26.39	33.22	27.55±2.99
T1	87.95	96.86	115.19	100±8.02	46.68	38.91	37.15	40.91±2.93
T4	93.02	99.40	107.57	100±4.21	41.22	41.82	47.36	43.47±1.96
T5	90.86	99.08	110.06	100±5.56	55.96	45.69	42.32	47.99±4.10
T7	89.52	99.61	110.87	100±6.17	52.97	51.09	53.84	52.63±0.81
T10	98.17	92.35	109.47	100±5.03	55.06	54.18	61.05	56.76±2.16
T12	86.00	102.54	111.46	100±7.46	57.66	55.25	70.35	61.09±4.69
L1	96.12	94.31	109.57	100±4.81	67.49	54.85	90.26	70.86±10.36

[0622] As shown in Table 15, the analysis of vector genome biodistribution by digital droplet PCR showed high vector genome copy number per diploid cell in ventral horn punches of the cervical spinal cord nearest the injection sites. Vector genome copy numbers dropped steeply (>10-fold on average) from C3 to C2, and from C5 to C7 spinal cord levels. However, even at spinal cord levels distant from the C3 and C5 sites of AAV injection, ventral horn punches exhibited vector genome copies well above background levels. At the T5, T7, T10, T12, and L1 spinal cord levels, ventral horn punches showed 0.73±0.18,

0.35±0.03, 0.27±0.04, 0.25±0.03, and 0.38±0.19 vector genome copies per diploid cell, respectively.

Table 15: Vector Genome Quantification

Spinal Cord Segments	Vector Genome/Diploid Cell (vg/dc)							
	Left Ventral Horn				Right Ventral Horn			
	Pig #1005	Pig #1004	Pig #1006	Mean ± Standard Error	Pig #1005	Pig #1004	Pig #1006	Mean ± Standard Error
C1	1.74	3.02	1.16	1.97±0.55	2.59	2.12	1.29	2.00±0.38
C2	9.57	9.73	4.52	7.94±1.71	10.69	14.29	4.42	9.80±2.88
C3	29.66	35.55	27.98	31.06±2.29	585.67	633.71	28.65	416.01±194.17
C5-Rostral	92.67	187.13	439.19	239.66±103.42	45.37	201.47	554.95	267.27±150.74
C5-Caudal	3029.44	760.00	332.53	1373.99±836.88	132.37	960.73	290.71	461.27±253.88
C7	18.39	28.38	56.81	34.53±11.51	9.52	27.43	41.11	26.02±9.14
C8	5.15	6.82	11.99	7.98±2.06	3.57	10.37	15.65	9.86±3.50
T1	2.03	4.03	7.06	4.37±1.46	2.66	4.40	5.83	4.30±0.92
T4	0.51	0.52	0.84	0.63±0.11	0.63	1.15	0.95	0.91±0.15
T5	0.43	1.54	0.58	0.85±0.35	0.35	0.92	0.55	0.60±0.17
T7	0.23	0.42	0.31	0.32±0.06	0.40	0.41	0.32	0.38±0.03
T10	0.13	0.27	0.36	0.25±0.07	0.26	0.36	0.24	0.29±0.04
T12	0.21	0.17	0.28	0.22±0.03	0.27	0.22	0.38	0.29±0.05
L1	1.32	0.16	0.12	0.53±0.39	0.25	0.30	0.13	0.22±0.05

[0623] Vector genome distribution showed linear correlation to levels of SOD1 mRNA knockdown, when compared to vehicle control ($r^2=0.15$, $p=0.0002$). 50% SOD1 knockdown was achieved with low vector genome copy number per diploid cell (< 1vg/dc) such as 0.2 or 0.5 vector genome copies per diploid cell on average, in ventral horn punches ~30cm caudal to the injection site.

Example 3: SOD1 reduction in tissues and cells

[0624] In situ hybridization studies of SOD1 mRNA were conducted using tissue sections derived from the ventral horn of the spinal cord of the animals used in the intraparenchymal delivery study (Example 2).

[0625] The ventral horn of the C6 and the T5 spinal cord segments of pig 302 injected with scAAV-miRSOD1 particles showed little to no SOD1 mRNA specific staining, indicating SOD1 knockdown. A substantial reduction in the endogenous SOD1 mRNA expression was observed in the large motor neurons in a rostrocaudal gradient, with strongest reduction in the cervical region. SOD1 mRNA specific staining was observed in the L1-L3 spinal cord segments of pig 302 injected with scAAV particles, which is consistent with the

bDNA method data for L1-L3- showing limited knockdown of SOD1 in the L1-L3 spinal cord segments. As expected, the ventral horn of the spinal cord segment L2 of naïve uninjected pigs showed strong staining for SOD1 mRNA.

[0626] SOD1 mRNA levels were measured in motor neuron pools isolated from the spinal cord segment T13 by laser capture, in depleted grey matter or a cross section of the whole spinal cord segment from the study described in Example 2. The levels of Choline Acetyl Transferase (ChAT), a motor neuron cytoplasmic marker were also measured to confirm the enrichment of motor neurons in the isolated motor neuron samples. The results are shown in Table 16a, where VH indicates ventral horn, MN indicates motor neuron, DGM indicates depleted grey matter and left/right indicate the side of cord from which the sample was obtained. The SOD1 fold change values in Table 16a are relative to vehicle group and the ChAT enrichment was measured relative to vehicle T13 cross section of the entire spinal cord. The values are represented as averages \pm standard error.

Table 16a. Relative SOD1 mRNA levels and ChAT enrichment in motor neurons

Sample and Side of cord	SOD1 mRNA		ChAT (fold)
	Vehicle	AAV	
VH MN Left	1.00 \pm 0.05	0.71 \pm 0.09	36.03 \pm 3.14
VH MN Right	1.00 \pm 0.10	0.72 \pm 0.05	31.14 \pm 2.73
DGM Left	1.00 \pm 0.04	0.92 \pm 0.02	17.39 \pm 3.59
DGM Right	1.00 \pm 0.06	0.83 \pm 0.05	18.24 \pm 4.54
T13 Cord Cross section	1.02 \pm 0.25	0.95 \pm 0.09	1.16 \pm 0.57

[0627] Isolated motor neurons obtained from both the left and the right ventral horn, showed a significant reduction of SOD1 mRNA levels ($p < 0.05$, 2-way ANOVA, Sidak's Test compared to matched vehicle control). These results are similar to the SOD1 mRNA levels (bDNA assay) in T12 and L1 segments from the same pigs. ChAT enrichment was observed in the isolated motor neurons but not in the T13 cord cross section samples.

[0628] SOD1 mRNA levels were measured in motor neurons isolated from the spinal cord segment C4 by laser capture and in depleted grey matter from the study described in Example 2. The results are shown in Table 16b, where VH indicates ventral horn, MN indicates motor neuron, DGM indicates depleted grey matter and left/right indicate the side of cord from which the sample was obtained. The SOD1 fold change values in Table 16b are relative to vehicle group was measured relative to vehicle C4 cross section. The values are represented as averages \pm standard error.

Table 16b. Relative SOD1 mRNA levels in motor neurons

Sample and Side of cord	SOD1 mRNA	
	Vehicle	AAV
VH MN Left	1.00±0.07	0.03±0.00
VH MN Right	1.00±0.04	0.03±0.00
DGM Left	1.04±0.19	0.32±0.05
DGM Right	1.01±0.08	0.28±0.02

[0629] Both isolated motor neurons and motor neuron depleted grey matter (DGM) at C4 show a significant reduction in SOD1 mRNA levels ($p < 0.05$, 2-way ANOVA, Sidak's Test compared to matched vehicle control). These data are consistent with the SOD1 mRNA levels at C3 and C5 (bdNA assay). These data also demonstrate a further specific increase in observed reduction of SOD1 mRNA in motor neurons compared to grey matter, resulting in almost complete suppression of SOD1 mRNA (knockdown of 97%) in cervical spinal cord motor neurons.

[0630] The hypoglossal nucleus and the nucleus ambiguus are regions of the brain stem nuclei that can be affected by ALS. The hypoglossal nucleus contains a prominent cluster of large motor neurons that supply the muscles of the tongue and the nucleus ambiguus contains large motor neurons which are strongly associated with speech and swallowing. In situ hybridization of SOD1 mRNA was conducted using tissue sections derived from the brain stem of pigs injected intraparenchymally to the spinal cord with scAAV-miRSOD1. SOD1 mRNA levels were found to be similar in the vehicle treated and the SOD1 AAV particle treated groups as measured by *in situ* hybridization. To determine if intraparenchymal spinal cord administration of the AAV particles led to transduction of the brain stem and knockdown of SOD1 mRNA, left and right caudal brain stem samples were also analyzed by the branched DNA (bdNA) method. The mRNA levels were normalized to the geometric mean of beta-actin (ACTB), TATA-box binding protein (TBP) and peptidylprolyl isomerase A (PPIA) mRNA levels. The normalized SOD1 mRNA levels are expressed relative to normalized SOD1 mRNA levels in the brain stem from animals treated with vehicle control (Table 17). Vector genome biodistribution was measured by digital droplet PCR for both doses of the scAAV-miRSOD1 and the number of vector genomes/diploid cell was measured (Table 17). In Table 17, BLLQ stands for "below the lower limit of quantification" and is approximately < 0.005 vg/dc for a 40ng template input.

Table 17. SOD1 mRNA and vector genome distribution in brainstem

Parameter	Caudal				Rostral			
	Left side		Right side		Left side		Right side	
	Vehicle	AAV	Vehicle	AAV	Vehicle	AAV	Vehicle	AAV
SOD1 mRNA	100±3.03	83.16±1.51	100±4.89	78.22±1.65	100±4.62	95.46±1.72	100±4.85	89.41±9.82
Vector genome/ diploid cell	BLLQ	0.46±0.17	BLLQ	0.63±0.22	BLLQ	0.29±0.05	BLLQ	0.25±0.06

[0631] Statistically significant SOD1 mRNA knock down was observed in left and right sides of caudal brainstem with a p value <0.01 and p value < 0.001 respectively (one way-ANOVA and Dunnett's multiple comparison test). Vector genomes were detected in brainstem regions at levels similar to those observed at spinal cord segments T5 through L1 after IPa dosing.

[0632] Serum neutralizing antibody levels in the plasma of pigs injected with scAAV-miRSOD1 or vehicle control were measured. No correlation between the neutralizing antibody status and the levels of SOD1 mRNA or viral genome were observed. These results suggest that the neutralizing antibodies do not impact the observed SOD1 mRNA levels.

Example 4: Effect of SOD1 siRNA *in vitro*

[0633] miR788.2 siRNA targeting SOD1 was assayed for inhibition of endogenous human SOD1 expression in HuH-7 cells. Transfection of HuH-7 cells with varying doses of siRNA was carried out with Lipofectamine 2000 (Invitrogen/Life Technologies) according to the manufacturer's instructions. Quantitation of human SOD1 and GAPDH (control) mRNA levels was performed using the bDNA (branched DNA) assay. The percent human SOD1 mRNA expression levels are shown in Figure 1. As seen in Figure 1, increasing the concentrations of the siRNA decreased the relative human SOD1 mRNA expression levels. The IC50 is the concentration of siRNA required to achieve 50% human SOD1 mRNA expression levels as indicated by the dotted line in Figure 1.

[0634] To test if miR788.2 is selective to human SOD1, bioinformatics analysis of the antisense strand was used to identify 9 potential human off-target genes. These genes included Slit Guidance Ligand 2 (SLIT2), Nuclear Receptor Coactivator 2 (NCOA2), Phospholipase C Eta 1 (PLCH1), BRD4 Interacting Chromatin Remodeling Complex Associated Protein Like (BICRAL), Bromodomain Containing 1 (BRD1), Scm Like With Four Mbt Domains 1 (SFMBT1), Dynein Axonemal Heavy Chain 7 (DNAH7), Zinc Finger Matrin-Type 3 (ZMAT3) and Malate Dehydrogenase 1B (MDH1B). Cell lines that

expressed both human SOD1 and one of these potential off-targets were selected, and SOD1 siRNA containing the guide strand of miR788.2 was transfected, and the levels of SOD1, the potential off-target and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression were evaluated. The activity of the SOD1 siRNA containing the guide strand of miR788.2 on any given on- or off-target was expressed as percent on- or off-target mRNA level (normalized to GAPDH mRNA) in treated cells, relative to the mean on- or off-target mRNA levels (normalized to GAPDH mRNA), respectively, across control wells. IC₅₀ values for SOD1 knockdown by the SOD1 siRNA containing the guide strand of miR788.2 were <0.02 nM in Huh-7 cells and <0.15 nM in C42 cells, indicating potent on-target knockdown. In contrast, no IC₅₀ value for any potential off-target could be calculated with a concentration range of 0.1 pM to 24 nM of the SOD1 siRNA containing the guide strand of miR788.2. These results show that there is an IC₅₀ separation of on-target (human SOD1) versus off-target mRNA suppression of at least 160-fold for the 9 potential off-targets. Thus, the guide strand of miR788.2 is selective for SOD1 over the nearest predicted potential off targets by at least 160-fold.

Example 5. In Vitro Activity of AAV-miRNA Vectors Targeting SOD1

[0635] The miRNA expression vectors were designed by engineering VOYSOD1miR104-788.2 targeting SOD1 (modulatory polynucleotide SEQ ID NO: 6), within an ITR to ITR sequence comprising one of two different filler sequences i.e. ITR to ITR with a lentivirus derived filler (SEQ ID NO. 9) or ITR to ITR with an albumin filler (SEQ ID NO. 25). The ITR to ITR sequences were packaged in AAVrh10 to generate scAAVrh10.H1.mir104-788.2 (lenti) or scAAVrh10.H1.mir104-788.2 (albumin) constructs respectively. As used herein the term, "lenti" indicated in parenthesis of the construct name indicates that the construct comprises a lentivirus derived filler sequence, whereas the term "albumin" indicated in parenthesis of the construct name means that the construct comprises an albumin gene derived filler sequence. AAV particles were produced using the HEK293T and triple transfection (TT) method using roller bottles. The particles were infected into HEK293T cells. A vector comprising AAVrh10 with a green fluorescent protein (GFP) transgene was used as a negative control. HEK293T cells were plated into 96-well plates (2.0 x 10⁴ cells/well in 100 μL cell culture medium) and infected with 9 different multiplicity of infections (MOIs) ranging from 1.52 x 10³ to 1 x 10⁷, with triplicate wells per condition. Forty-eight hours after infection, the cells were harvested for immediate cell lysis. Cell lysates were used for quantitative RT-PCR to quantify human SOD1 mRNA levels as well as

mRNA levels of housekeeping genes. Human SOD1 mRNA levels were normalized to the geometric mean of alanyl-tRNA synthetase (AARS) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels, and then further normalized to the GFP control group to obtain relative human SOD1 mRNA levels. The MOIs and relative human SOD1 mRNA levels normalized to geometric mean of AARS and GAPDH (relative to GFP control, %) are shown in Table 18 for both vectors tested.

Table 18. Human SOD1 mRNA levels with different doses of AAV-miRSOD1 vectors

MOI	Relative remaining human SOD1 mRNA (average ± standard error)	
	scAAVrh10.H1.mir104-788.2 (lenti)	scAAVrh10.H1.mir104-788.2 (albumin)
1.00E+07	5.7 ± 0.2	5.8 ± 0.3
3.33E+06	6.0 ± 0.4	6.6 ± 0.5
1.11E+06	7.9 ± 0.3	9.9 ± 0.4
3.70E+05	17.8 ± 0.8	24.9 ± 1.8
1.23E+05	37.7 ± 2.3	46.4 ± 1.3
4.12E+04	61.8 ± 6.4	71.3 ± 3.3
1.37E+04	79.6 ± 3.9	79.6 ± 2.9
4.57E+03	88.9 ± 5.6	92.2 ± 3.0
1.52E+03	91.5 ± 3.7	97.7 ± 3.3
0	95.3 ± 4.4	100.9 ± 4.3

[0636] Dose dependent knockdown of human SOD1 mRNA was observed for both vectors scAAVrh10.H1.mir104-788.2 (lenti) and scAAVrh10.H1.mir104-788.2 (albumin) in HEK293T cells. The relative mRNA values of human SOD1 were also fitted onto a curve and the values are shown in Table 19.

Table 19. Best fit values for AAV-miRSOD1

Best Fit Values	scAAVrh10.H1.mir104-788.2 (lenti)	scAAVrh10.H1.mir104-788.2 (albumin)
Bottom	4.08	1.99
Top	94.13	99.58
Hillslope	1.02	0.86
IC50	7.18E+04	9.62E+04
logIC50	4.86	4.98
p value	0.08	

[0637] As shown in Table 19, similar potency was observed with both vectors with a p value of 0.08. IC50 values were also similar and in the 10⁴ range for both vectors.

[0638] Viral genomes and capsid proteins were independently extracted from purified AAV preparations. Genome integrity was evaluated using denaturing gel which detected an

approximately 3 kb band. Capsid integrity was measured using silver staining of capsid proteins with polyacrylamide gel electrophoresis which showed 3 bands in the 75kDa range.

Example 6. *In vivo* human SOD1 knockdown in transgenic mouse model

[0639] Self-complementary (sc) AAV vectors (scAAV) with an siRNA construct (VOYSOD1miR104-788.2) targeting SOD1 and containing different filler sequences within the ITR to ITR as described in Example 4 were packaged in AAVrh10 and formulated in phosphate buffered saline (PBS) with 0.001% F-68. Female or male Tg(SOD1)3Cjc/J mice (Jackson Laboratory, Bar Harbor, ME), 14-30 weeks of age, which express human SOD1, received bilateral intrastriatal infusions (5 μ L at 0.5 μ L/min) of scAAVrh10.H1.mir104-788.2 (lenti), scAAVrh10.H1.mir104-788.2 (albumin), or vehicle (n of 3 to 5 per group). For scAAVrh10.H1.mir104-788.2 (lenti), vector concentrations were 1.5×10^{13} , 3.0×10^{12} , 5.6×10^{11} or 1.9×10^{11} vg/mL, corresponding to total doses of 7.5×10^{10} , 1.5×10^{10} , 2.8×10^9 or 9.4×10^8 vg. For scAAVrh10.H1.mir104-788.2 (albumin), vector concentrations were 1.5×10^{13} , 3.0×10^{12} , 5.7×10^{11} or 1.9×10^{11} vg/mL, corresponding to total doses of 7.6×10^{10} , 1.5×10^{10} , 2.9×10^9 or 9.5×10^8 vg. Four weeks after dosing, animals were euthanized, brains were removed, and left and right striatal regions were dissected and flash frozen. For each animal, the entire striatal sample was evaluated for human SOD1 mRNA suppression by qRT-PCR. Total RNA was extracted from striatal tissue samples using the RNeasy Mini Kit according to the manufacturer's protocol (QIAGEN). Complementary DNA synthesis was performed by reverse transcription using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). All TaqMan assays and master mixes were ordered from Life Technologies and used according to the manufacturer's recommendations. qRT-PCR was performed using the CFX384 real-time system (BIO-RAD) and data were analyzed with the $\Delta\Delta$ CT method. Human SOD1 mRNA levels were normalized to murine GAPDH (mGAPDH) mRNA levels, and then further normalized to the vehicle control group. These group averages were calculated to obtain a group (treatment) average. The qRT-PCR mRNA results are shown below in Table 20. The human SOD1 mRNA levels are represented as percent averages \pm standard deviation (SD).

Table 20. Human SOD1 mRNA suppression in wild-type human SOD1 transgenic mouse striatum

Groups	Dose (vg/5 μ L)	Normalized Human SOD1 mRNA
Vehicle	0	100 \pm 29.4
scAAVrh10.H1.mir104-788.2 (lenti)	9.4×10^8	39.65 \pm 12.63
	2.8×10^9	51.86 \pm 39.68
	1.5×10^{10}	36.36 \pm 18.11

scAAVrh10.H1.mir104-788.2 (albumin)	9.5×10^8	33.93±22.20
	2.9×10^9	40.10±8.90
	1.5×10^{10}	29.78±11.95
	7.6×10^{10}	21.27±5.46

[0640] In human SOD1 transgenic mouse striatum, scAAVrh10.H1.mir104-788.2 (lenti) caused about 48% to 64% silencing of human SOD1 mRNA at about 28 days after intrastriatal infusion of 9.4×10^8 vg to 1.5×10^{10} vg per striatum. scAAVrh10.H1.mir104-788.2 (albumin) caused about 60% to 79% silencing of human SOD1 mRNA at about 28 days after intrastriatal infusion of 1.0×10^9 vg to 8.0×10^{10} vg per striatum. Maximum knockdown of 79% was observed with scAAVrh10.H1.mir104-788.2 (albumin) 8.0×10^{10} dose of viral genome (vg)/5 μ L. A substantial knockdown was observed even with the lowest dose of either vector tested.

[0641] The tolerability of the AAV vectors administered by intrastriatal infusion was investigated in human SOD1 transgenic mice. Body weight was recorded before and after dosing with the vehicle, scAAVrh10.H1.mir104-788.2 (lenti), or scAAVrh10.H1.mir104-788.2 (albumin). The body weight change obtained with each group is shown as the percentage of body weight measured prior to dosing in Table 21.

Table 21. Body weight change in human SOD1 transgenic mouse striatum

Groups	Dose (vg/ 5 μ L)	Body weight change (% of pre-dosing)
Vehicle	0	-1.28±2.93
scAAVrh10.H1.mir104-788.2 (lenti)	9.4×10^8	2.23±3.34
	2.8×10^9	4.54±5.35
	1.5×10^{10}	-14.92±8.86
scAAVrh10.H1.mir104-788.2 (albumin)	9.5×10^8	3.24±3.93
	2.9×10^9	1.30±6.17
	1.5×10^{10}	-2.04±3.90
	7.6×10^{10}	-22.87±11.62

[0642] The p value was calculated using the one-way ANOVA, Dunnett's test. A p value of < 0.05 was obtained with the highest dose 1.60×10^{10} (vg/ 5 μ L) of the scAAVrh10.H1.mir104-788.2 (lenti) vector and a p value of <0.001 was obtained with the highest dose 8.00×10^{10} (vg/ 5 μ L) of the scAAVrh10.H1.mir104-788.2 (albumin) vector suggesting that a significant weight loss occurred at the highest doses of the vectors. Morbidity was also observed in the higher dose groups. At a dose of 1.60×10^{10} (vg/ 5 μ L), 2/6 mice in the scAAVrh10.H1.mir104-788.2 (albumin) group and 5/5 mice in the scAAVrh10.H1.mir104-788.2 (lenti) group were either found dead or euthanized by week 4 after the injection. 2/5 mice in the highest dose (8.00×10^{10}) of the scAAVrh10.H1.mir104-

788.2 (albumin) group were found dead at 2 days and 3.5 weeks respectively. Postmortem analysis revealed that the death may have been due to *Klebsiella oxytoca* or *Klebsiella pneumoniae* infection.

Example 7. Effect of SOD1 siRNA *in vitro*

[0643] VOYSOD1miR104-788.2, VOYSOD1miR127-860, VOYSOD1miR114-806 and VOYSOD1miR114-861 were engineered into scAAVDJ vectors with a CBA promoter. The porcine epithelial cell line, SK-RST was cultured *in vitro* and infected with the described vectors at 3 different MOIs, namely 4.00E+03, 2.00E+04, and 1.00E+05. A control scAAVDJ. EGFP vector was also evaluated at these MOIs. The expression of SOD1 mRNA was measured and normalized to porcine GAPDH mRNA. The relative SOD1 mRNA levels are shown as relative to % GFP expression in Figure 2. VOYSOD1miR104-788.2 showed the strongest dose dependent knockdown.

Example 8. Intraparenchymal delivery of SOD1 siRNA to spinal cord

[0644] Biodistribution of viral genomes and SOD1 mRNA knockdown were evaluated in the ventral horn at multiple levels of the spinal cord, including the cervical level in pigs.

[0645] Three Göttingen adult (6 months of age), female mini-pigs weighing 14-20 kg each were utilized for each of the groups in the study. Animals were not pre-screened for neutralizing antibodies to AAV. A 4-5 cm laminectomy was performed between C3 and C5, allowing for 3 cm between injections. Self-complementary (sc) AAV vector (scAAV) with modulatory polynucleotide (SEQ ID NO: 6) comprising siRNA targeting SOD1 and ITR to ITR sequence of (SEQ ID NO: 25) which includes an albumin derived filler sequence were packaged into AAVrh10 vector to generate scAAVrh10.H1.mir104-788.2 (albumin).

[0646] For a high dose, two injections of the scAAV (titer 1.73×10^{13} vg/mL) were administered. At the rostral end of the laminectomy, i.e. at the C3 level of the spinal cord, a single 40 μ L (6.9×10^{11} vg/injection) volume was injected into the ventral horn of the spinal cord. At the caudal end of the laminectomy, i.e. at the C5 level of the spinal cord, a single 40 μ L (6.9×10^{11} vg/injection) volume was injected into the ventral horn of the contralateral side, for a total dose of 1.38×10^{12} vg. For the lower of the two doses, two injections of the scAAV (titer 5.8×10^{11} vg/mL,) were administered (1/30th of high dose). At the rostral end of the laminectomy, i.e. at the C3 level of the spinal cord, a single 40 μ L (2.3×10^{10} vg/injection) volume was injected into the ventral horn of the spinal cord. At the caudal end of the laminectomy, i.e. at the C5 level of the spinal cord, a single 40 μ L (2.3×10^{10} vg/injection)

volume was injected into the ventral horn of the contralateral side, for a total dose of 4.6×10^{10} vg. All injections were administered at the rate of $5 \mu\text{L}/\text{min}$, yielding an approximately 13-minute total infusion time. Four weeks following the procedure, animals were sacrificed, and spinal cord tissue was collected for analyses.

[0647] To determine if intraparenchymal administration of the AAV particles led to transduction of the spinal cord and knockdown of SOD1 mRNA, ventral horn punches were analyzed by the branched DNA (bDNA) method. mRNA levels of SOD1 mRNA were normalized to the geometric mean of beta-actin (ACTB), TATA-box binding protein (TBP) and peptidylprolyl isomerase A (PPIA) mRNA levels. The normalized SOD1 mRNA levels are expressed relative to normalized SOD1 mRNA levels in ventral horn punches from animals treated with vehicle control.

[0648] Significant SOD1 mRNA knockdown was evident in ventral horn punches from C1 to L1 of the pigs treated with $6.9E+11$ vg/injection. The mRNA knockdown was assessed relative to SOD1 mRNA levels in ventral horn punches from vehicle control treated animals. The results are shown in Table 22a. Similar SOD1 mRNA levels were obtained from the ventral horn punches from both sides of the spinal cord. Two-way ANOVA and Sidak’s multiple comparison test indicated significant SOD1 mRNA knockdown at each level of the spinal cord (C1-T12 left side $p < 0.0001$; T12 right side $p < 0.001$, L1 $p < 0.01$). As shown in Table 22a, spinal cord segments closest to the injections exhibited the greatest SOD1 mRNA knockdown. Spinal segments C1 through T12 had robust and significant knockdown of SOD1 mRNA (approximately 50-75% knockdown). Even at spinal cord segment L1, distant from the site of vector injection, significant knockdown of SOD1 mRNA (approximately, 30% knockdown) was observed.

Table 22a. SOD1 mRNA levels (high dose group) relative to vehicle group (%)

Spinal Cord Segments	Left Ventral Horn							
	Vehicle				scAAVrh10.H1.miR104.788.2 (high dose)			
	Pig #1001	Pig #1002	Pig #1003	Mean \pm Standard Error	Pig #1007	Pig #1008	Pig #1009	Mean \pm Standard Error
C1	99.52	82.32	118.16	100 \pm 10.35	28.35	35.77	36.21	33.44 \pm 2.55
C2	80.14	104.77	115.09	100 \pm 10.37	29.17	30.85	29.35	29.79 \pm 0.53
C3	113.62	104.09	82.29	100 \pm 9.27	24.00	32.45	27.66	28.04 \pm 2.45
C5-Rostral	98.62	98.05	103.34	100 \pm 6.08	8.43	22.08	8.64	16.28 \pm 2.01
C5-Caudal	103.97	88.05	107.97	100 \pm 1.68	20.29	13.99	14.57	13.05 \pm 4.52
C7	105.90	90.03	104.07	100 \pm 5.02	22.13	23.29	25.95	23.79 \pm 1.13
C8	102.23	91.45	106.32	100 \pm 4.44	22.93	48.48	61.17	44.20 \pm 11.25
T1	90.02	93.74	116.24	100 \pm 8.19	34.67	37.90	42.68	38.42 \pm 2.33
T4	92.86	93.95	113.19	100 \pm 6.60	44.20	34.30	47.17	41.89 \pm 3.89
T5	90.21	92.92	116.87	100 \pm 8.47	44.77	35.12	46.37	42.09 \pm 3.52
T7	91.07	99.06	109.87	100 \pm 5.45	55.20	42.85	56.77	51.61 \pm 4.40

Spinal Cord Segments	Right Ventral Horn							
	Vehicle				scAAVrh10.H1.mir104.788.2 (high dose)			
	Pig #1001	Pig #1002	Pig #1003	Mean ± Standard Error	Pig #1007	Pig #1008	Pig #1009	Mean ± Standard Error
T10	94.64	102.90	102.46	100±2.69	58.37	43.06	54.12	51.85±4.56
T12	88.93	102.39	108.69	100±5.83	63.12	23.62	59.50	48.75±12.61
L1	104.69	110.00	85.31	100±7.50	63.08	58.74	72.94	64.92±4.20
L4	103.62	98.20	98.18	100±1.81	87.88	77.19	76.96	80.68±3.60
L5	92.66	102.98	104.36	100±3.69	81.76	81.98	79.15	80.96±0.91
C1	92.12	92.70	115.18	100±7.59	37.53	44.21	48.46	43.40±3.18
C2	98.71	89.22	112.07	100±6.63	39.02	38.39	43.18	40.20±1.50
C3	113.69	100.21	86.10	100±7.96	19.00	36.45	17.37	24.27±6.11
C5-Rostral	80.97	98.73	120.30	100±3.50	27.63	35.75	26.55	25.39±2.36
C5-Caudal	105.99	93.87	100.14	100±11.37	29.00	20.94	26.23	29.98±2.90
C7	107.82	94.14	98.04	100±4.07	30.91	29.34	34.60	31.62±1.56
C8	101.64	96.70	101.67	100±1.65	29.67	31.25	36.78	32.57±2.15
T1	92.80	90.42	116.78	100±8.42	44.93	42.69	49.11	45.58±1.88
T4	93.18	100.01	106.81	100±3.93	53.31	44.59	58.06	51.98±3.95
T5	90.78	97.88	111.34	100±6.03	49.25	40.02	61.22	50.16±6.14
T7	86.50	109.82	103.69	100±6.98	56.43	46.52	72.73	58.56±7.64
T10	101.21	97.75	101.04	100±1.13	54.48	52.29	68.33	58.37±5.02
T12	94.32	99.59	106.08	100±3.40	57.18	53.37	82.46	64.34±9.13
L1	96.60	94.26	109.14	100±4.62	64.35	60.99	86.46	70.60±7.99
L4	103.62	90.24	106.13	100±4.93	81.26	74.38	88.89	81.51±4.19
L5	104.84	95.90	99.27	100±2.61	91.68	87.28	90.66	89.87±1.33

[0649] Comparing the SOD1 mRNA levels obtained with scAAVrh10.H1.mir104-788.2 (lenti) (Table 11) to the levels obtained with scAAVrh10.H1.mir104-788.2 (albumin) (Table 22a) showed that similar SOD1 mRNA knockdown was achieved with both the AAV containing the lentivirus derived filler (1.6E+12 vg total) or containing the albumin derived filler (1.4E+12vg total).

[0650] The results for the pigs injected with the lower dose of the scAAVrh10.H1.mir104-788.2 (albumin) are shown in Table 22b.

Table 22b. SOD1 mRNA levels (lower dose group) relative to vehicle group (%)

Spinal Cord Segments	Left Ventral Horn							
	Vehicle				scAAVrh10.H1.mir104.788.2 (low dose)			
	Pig #1001	Pig #1002	Pig #1003	Mean ± Standard Error	Pig #1010	Pig #1011	Pig #1012	Mean ± Standard Error
C1	99.52	82.32	118.16	100±10.35	79.40	84.98	65.41	76.60±5.82
C2	80.14	104.77	115.09	100±10.37	70.49	79.44	56.18	68.70±6.77
C3	113.62	104.09	82.29	100±9.27	71.23	36.43	39.05	48.90±11.19
C5-Rostral	98.62	98.05	103.34	100±6.08	35.41	47.21	20.12	48.19±9.61
C5-Caudal	103.97	88.05	107.97	100±1.68	64.13	49.52	30.93	34.25±7.84
C7	105.90	90.03	104.07	100±5.02	77.51	77.03	59.26	71.27±6.00
C8	102.23	91.45	106.32	100±4.44	63.60	75.69	64.47	67.92±3.89
T1	90.02	93.74	116.24	100±8.19	98.42	110.11	90.03	99.52±5.82
T4	92.86	93.95	113.19	100±6.60	94.22	93.60	87.65	91.82±2.09
T5	90.21	92.92	116.87	100±8.47	92.11	90.63	76.65	86.47±4.92
T7	91.07	99.06	109.87	100±5.45	96.43	83.33	83.99	87.92±4.26

T10	94.64	102.90	102.46	100±2.69	85.88	84.50	74.36	81.58±3.63
T12	88.93	102.39	108.69	100±5.83	84.25	89.38	80.24	84.63±2.65
L1	104.69	110.00	85.31	100±7.50	88.86	111.16	87.87	95.97±7.60
Spinal Cord Segments	Right Ventral Horn							
	Vehicle				scAAVrh10.H1.mir104.788.2 (low dose)			
	Pig #1001	Pig #1002	Pig #1003	Mean ± Standard Error	Pig #1010	Pig #1011	Pig #1012	Mean ± Standard Error
C1	92.12	92.70	115.18	100±7.59	98.99	102.92	79.90	93.94±7.11
C2	98.71	89.22	112.07	100±6.63	84.94	94.50	78.86	86.10±4.55
C3	113.69	100.21	86.10	100±7.96	65.48	39.95	35.08	46.84±9.43
C5-Rostral	80.97	98.73	120.30	100±3.50	42.50	38.75	38.70	49.55±5.11
C5-Caudal	105.99	93.87	100.14	100±11.37	59.42	46.92	42.31	39.98±1.26
C7	107.82	94.14	98.04	100±4.07	76.81	89.17	71.36	79.11±5.27
C8	101.64	96.70	101.67	100±1.65	74.10	94.59	84.69	84.46±5.92
T1	92.80	90.42	116.78	100±8.42	96.90	110.21	87.72	98.28±6.53
T4	93.18	100.01	106.81	100±3.93	97.48	95.65	97.57	96.90±0.63
T5	90.78	97.88	111.34	100±6.03	90.17	82.25	85.27	85.90±2.31
T7	86.50	109.82	103.69	100±6.98	104.90	91.90	100.84	99.21±3.84
T10	101.21	97.75	101.04	100±1.13	99.26	98.84	96.51	98.20±0.85
T12	94.32	99.59	106.08	100±3.40	123.15	103.11	104.85	110.37±6.41
L1	96.60	94.26	109.14	100±4.62	93.11	104.16	96.39	97.89±3.27

[0651] Pigs injected with the lower of the two doses, (2.3E+10 vg/injection) showed SOD1 mRNA knockdown in the ventral horn punches from spinal cord segments, C2 to C8. Similar SOD1 mRNA levels were obtained from the ventral horn punches from both sides of the spinal cord. Two-way ANOVA and Sidak’s multiple comparison test indicated significant SOD1 mRNA knockdown at each level of the spinal cord (C3-C5 p< 0.0001 with 50 % knockdown).

[0652] The SOD1 mRNA levels obtained with 6.9E+11 vg/injection were compared to the SOD1 mRNA levels obtained with 2.3E+10 vg/injection. Two-way ANOVA and Sidak’s multiple comparison test indicated that the SOD1 mRNA knockdown is significantly lower in the lower dose groups at the following spinal cord segments: C1 right side, C2 right side, C7, C8 right side, T1-T4, T7 left side, T10 right side, T12 right side (p< 0.0001); C1 left side, T5 (p< 0.001); C2 left side, T7 right side, T12 left side (p< 0.01; C5 left side, and L1 (p< 0.05). No significant difference in the knockdown was observed at injection site C3-C5.

[0653] Vector genome biodistribution was measured by digital droplet PCR for both doses of the scAAVrh10.H1.mir104-788.2 (albumin). The results for both dose levels are shown in Table 23 as mean of vector genome (vg) per diploid cell (dc) ± standard error of the mean (SEM).

Table 23. Vector genome biodistribution

Spinal cord Segments	Vector Genome/Diploid Cell (vg/dc)							
	High dose (6.9E+11 vg/injection)							
	Left Ventral Horn				Right Ventral Horn			
	Pig #1007	Pig #1008	Pig #1009	Mean ± Standard Error	Pig #1007	Pig #1008	Pig #1009	Mean ± Standard Error
C1	1.21	0.60	1.57	1.13±0.28	1.04	0.63	0.95	0.87±0.12
C2	6.21	2.48	5.87	4.85±1.19	6.54	1.83	4.33	4.23±1.36
C3	62.07	8.35	31.38	33.93±15.46	265.36	18.57	349.19	211.04±99.23
C5-Rostral	517.66	14.36	293.54	275.19±145.38	23.92	13.25	73.08	36.75±18.42
C5-Caudal	29.57	210.86	163.63	134.69±54.30	13.47	86.29	18.31	39.36±23.51
C7	8.36	16.83	6.71	10.63±3.13	3.42	8.18	2.70	4.77±1.72
C8	2.14	5.80	0.12	2.69±1.66	1.74	3.56	2.39	2.56±0.53
T1	0.85	1.33	0.88	1.02±0.16	0.58	1.28	0.74	0.87±0.21
T4	0.20	0.28	0.20	0.23±0.03	0.17	0.29	0.19	0.22±0.04
T5	0.15	0.20	0.13	0.16±0.02	0.09	0.23	0.18	0.17±0.04
T7	0.13	0.15	0.14	0.14±0.01	0.10	0.16	0.11	0.12±0.02
T12	0.11	0.13	0.14	0.13±0.01	0.20	0.13	0.09	0.14±0.03
T10	0.13	0.09	0.09	0.10±0.01	0.07	0.13	0.08	0.09±0.02
L1	0.04	0.38	0.11	0.18±0.10	0.05	0.11	0.05	0.07±0.02
Spinal cord Segments	Low dose (2.3E+10 vg/injection)							
	Left Ventral Horn				Right Ventral Horn			
	Pig #1010	Pig #1011	Pig #1012	Mean ± Standard Error	Pig #1010	Pig #1011	Pig #1012	Mean ± Standard Error
	C1	0.20	0.14	0.23	0.19±0.03	0.04	0.07	0.12
C2	0.25	0.30	0.72	0.42±0.15	0.19	3.21	0.50	1.30±0.96
C3	0.71	2.30	2.88	1.96±0.65	0.61	5.90	10.33	5.61±2.81
C5-Rostral	5.63	0.70	21.65	9.33±6.32	2.11	3.15	5.95	3.73±1.15
C5-Caudal	0.72	0.65	4.58	1.98±1.30	0.45	0.85	1.11	0.80±0.19
C7	0.51	0.37	0.81	0.56±0.13	0.13	0.26	0.48	0.29±0.10
C8	0.15	0.13	0.35	0.21±0.07	0.13	0.16	0.30	0.20±0.05
T1	0.05	0.08	0.16	0.10±0.03	0.04	0.07	0.13	0.08±0.03
T4	0.03	0.04	0.10	0.06±0.02	0.02	0.02	0.05	0.03±0.01
T5	0.03	0.04	0.07	0.05±0.01	0.27	0.03	0.08	0.13±0.07
T7	0.05	0.02	0.07	0.05±0.01	0.04	0.03	0.06	0.04±0.01
T12	0.01	0.03	0.03	0.02±0.01	0.17	0.10	0.05	0.11±0.04
T10	0.02	0.11	0.02	0.05±0.03	0.04	0.06	0.31	0.14±0.09
L1	0.03	0.17	0.02	0.07±0.05	0.01	0.02	0.01	0.01±0.00

[0654] The high dose (6.9E+11 vg/injection) group showed high vector genome copy number per diploid cell in ventral horn punches of the cervical spinal cord nearest the infusion sites. Vector genome copy numbers then dropped steeply (>10-fold) from C3 to C1, and from C7 to T1 spinal cord levels, and then held constant from T4 through L1. The ratio of the mean for vector genome copy numbers of both dose groups was calculated and is shown in Table 24, where VH indicates ventral horn.

Table 24. Ratio of means for vg/dc of 6.9E+11 vg/injection:2.3E+10 vg/injection dose groups

Spinal Cord Level	Left VH	Right VH	Overall VH
C1	5.93	11.53	7.53
C2	11.46	3.26	5.28
C3	17.28	37.59	32.33
C5-R	29.51	9.84	23.88
C5-C	67.92	49.01	62.47
C7	18.87	16.43	18.04
C8	12.8	12.86	12.83
T1	10.55	10.81	10.67
T4	4	7.01	5.06
T5	3.43	1.31	1.88
T7	3	2.81	2.91
T10	5.43	1.31	2.05
T12	2.07	0.67	1.04
L1	2.41	5.9	2.9

[0655] Vector genome distribution levels were found to be similar on both sides of the spinal cord, except close to the injection sites. The ratio of the vector genome between high dose (6.9E+11 vg/injection) and low dose (2.3E+10 vg/injection) groups near the injection sites is similar to the 30-fold difference in dose, but this ratio gradually decreased to 1-3 fold in regions distal to the injection site (T5 through L1).

[0656] Similar vector genome distribution was observed with scAAVrh10.H1.mir104-788.2 (lenti) and scAAVrh10.H1.mir104-788.2 (albumin) as shown in Table 25, where VH indicates ventral horn.

Table 25. Comparison of vector genome copies in ventral horn punches of scAAVrh10.H1.mir104-788.2 (lenti) and scAAVrh10.H1.mir104-788.2 (albumin) groups

Spinal cord Segments	Vector Genome/Diploid Cell (vg/dc)							
	scAAVrh10.H1.mir104-788.2 (albumin) @ High dose (6.9E+11 vg/injection)							
	Left Ventral Horn				Right Ventral Horn			
	Pig #1007	Pig #1008	Pig #1009	Mean ± Standard Error	Pig #1007	Pig #1008	Pig #1009	Mean ± Standard Error
C1	1.21	0.60	1.57	1.13±0.28	1.04	0.63	0.95	0.87±0.12
C2	6.21	2.48	5.87	4.85±1.19	6.54	1.83	4.33	4.23±1.36
C3	62.07	8.35	31.38	33.93±15.46	265.36	18.57	349.19	211.04±99.23
C5-Rostral	517.66	14.36	293.54	275.19±145.38	23.92	13.25	73.08	36.75±18.42
C5-Caudal	29.57	210.86	163.63	134.69±54.30	13.47	86.29	18.31	39.36±23.51
C7	8.36	16.83	6.71	10.63±3.13	3.42	8.18	2.70	4.77±1.72
C8	2.14	5.80	0.12	2.69±1.66	1.74	3.56	2.39	2.56±0.53
T1	0.85	1.33	0.88	1.02±0.16	0.58	1.28	0.74	0.87±0.21
T4	0.20	0.28	0.20	0.23±0.03	0.17	0.29	0.19	0.22±0.04
T5	0.15	0.20	0.13	0.16±0.02	0.09	0.23	0.18	0.17±0.04
T7	0.13	0.15	0.14	0.14±0.01	0.10	0.16	0.11	0.12±0.02
T12	0.11	0.13	0.14	0.13±0.01	0.20	0.13	0.09	0.14±0.03
T10	0.13	0.09	0.09	0.10±0.01	0.07	0.13	0.08	0.09±0.02

L1	0.04	0.38	0.11	0.18±0.10	0.05	0.11	0.05	0.07±0.02
Spinal cord Segments	scAAVrh10.H1.mir104-788.2 (lenti) @ 8.1E+11 vg/injection							
	Left Ventral Horn				Right Ventral Horn			
	Pig #1005	Pig #1004	Pig #1006	Mean ± Standard Error	Pig #1005	Pig #1004	Pig #1006	Mean ± Standard Error
C1	1.74	3.02	1.16	1.97±0.55	2.59	2.12	1.29	2.00±0.38
C2	9.57	9.73	4.52	7.94±1.71	10.69	14.29	4.42	9.80±2.88
C3	29.66	35.55	27.98	31.06±2.29	585.67	633.71	28.65	416.01±194.17
C5-Rostral	92.67	187.13	439.19	239.66±103.42	45.37	201.47	554.95	267.27±150.74
C5-Caudal	3029.44	760	332.53	1373.99±836.88	132.37	960.73	290.71	461.27±253.88
C7	18.39	28.38	56.81	34.53±11.51	9.52	27.43	41.11	26.02±9.14
C8	5.15	6.82	11.99	7.98±2.06	3.57	10.37	15.65	9.86±3.50
T1	2.03	4.03	7.06	4.37±1.46	2.66	4.4	5.83	4.30±0.92
T4	0.51	0.52	0.84	0.63±0.11	0.63	1.15	0.95	0.91±0.15
T5	0.43	1.54	0.58	0.85±0.35	0.35	0.92	0.55	0.60±0.17
T7	0.23	0.42	0.31	0.32±0.06	0.4	0.41	0.32	0.38±0.03
T12	0.13	0.27	0.36	0.25±0.07	0.26	0.36	0.24	0.29±0.04
T10	0.21	0.17	0.28	0.22±0.03	0.27	0.22	0.38	0.29±0.05
L1	1.32	0.16	0.12	0.53±0.39	0.25	0.3	0.13	0.22±0.05

[0657] Similar vector genome distributions were observed, with a trend (albeit small), towards more vector genomes observed with scAAVrh10.H1.mir104-788.2 (lenti). The largest difference between the two groups was observed at C5 where the vector comprising the lentivirus derived filler sequence had a value that was 4-9-fold higher than the vector comprising the albumin derived filler sequence. Statistically significant difference in vector genome distribution between the two groups was observed at T4 right side, T7 right side and T12 right side (p<0.01).

[0658] Histopathological analysis was conducted using H&E staining of tissue sections from the C3 injection site in the spinal cord. Change in histopathology relative to vehicle control was assessed for all the constructs shown in Table 26. The samples were graded as one of the following: Grade 1: Minimal, Grade 2: Mild, Grade 3: Moderate, Grade 4: Marked, or Grade 5: Severe difference between the construct and vehicle control. In Table 26 the number in parenthesis adjacent to the grade indicates the number of specimens (pigs) that showed the indicated phenotype.

Table 26. Spinal cord histopathology

Parameters measured	Vehicle	scAAVrh10.H1.mir104-788.2 (lenti) 8.1E+11 vg/injection	scAAVrh10.H1.mir104-788.2 (albumin)	
			6.9E+11 vg/injection high dose	2.3E+10 vg/injection low dose
Axonal degeneration	Grade 1 (2), Grade 1-3 (1)	Grade 1 (2), Grade 1-2 (1)	Grade 1 (1), Grade 1-2 (2)	Grade 1 (2), Grade 1-2 (1)
Dystrophic axon	Grade 1 (2)	Grade 1 (2)	Grade 1 (1)	Grade 1 (2)

Gliosis	None	Grade 1 (2)	Grade 1 (1), Grade 2 (1)	None
Infiltrates	None	Grade 1 (1)	Grade 2 (2)	None
Necrosis	None	None	Grade 2 (1)	None
Neuronophagia	None	Grade 1 (1)	Grade 1 (1)	Grade 1 (1)
Chromatolysis	None	Grade 1 (2), Grade 2 (1)	Grade 1 (2)	Grade 1 (3)

[0659] Minimal to mild changes were observed in AAV-treated groups.

[0660] *In situ* hybridization (ISH) for pig SOD1 mRNA and vector genome was conducted on cross-sections of the cervical and thoracic spinal cord from animals treated with vehicle or scAAVrh10.H1.mir104-788.2 (albumin). Vector genome signal in the motor neurons of both sides of the ventral horn was observed in AAV treated animals. The vg signal was more abundant in the motor neurons on the side closest to the injection. A substantial reduction in the endogenous SOD1 mRNA signal was observed in the large motor neurons in a rostrocaudal gradient, with strongest reduction in the cervical region. Dramatic reduction of SOD1 mRNA signal was observed in the motor neurons in the ventral horn of both sides of the C5 spinal cord segments of animals injected with AAV. Reduction of SOD1 mRNA by ISH correlated with the SOD1 mRNA knockdown in ventral horn punches as assessed by bDNA.

Equivalents and Scope

[0661] Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments in accordance with the disclosure herein. The scope of the present disclosure is not intended to be limited to the above Description, but rather is as set forth in the appended claims.

[0662] In the claims, articles such as “a,” “an,” and “the” may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The disclosure includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The disclosure includes embodiments in which more than one, or the entire group members are present in, employed in, or otherwise relevant to a given product or process.

[0663] It is also noted that the term “comprising” is intended to be open and permits but does not require the inclusion of additional elements or steps. When the term “comprising” is used herein, the term “consisting of” is thus also encompassed and disclosed.

[0664] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the disclosure, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[0665] In addition, it is to be understood that any particular embodiment of the present disclosure that falls within the prior art may be explicitly excluded from any one or more of the claims. Since such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the compositions of the disclosure (e.g., any antibiotic, therapeutic or active ingredient; any method of production; any method of use; etc.) can be excluded from any one or more claims, for any reason, whether or not related to the existence of prior art.

[0666] It is to be understood that the words which have been used are words of description rather than limitation, and that changes may be made within the purview of the appended claims without departing from the true scope and spirit of the disclosure in its broader aspects.

[0667] While the present disclosure has been described at some length and with some particularity with respect to the several described embodiments, it is not intended that it should be limited to any such particulars or embodiments or any particular embodiment, but it is to be construed with references to the appended claims so as to provide the broadest possible interpretation of such claims in view of the prior art and, therefore, to effectively encompass the intended scope of the disclosure.

[0668] All cited sources, for example, references, publications, databases, database entries, and art cited herein, are incorporated into this application by reference, even if not expressly stated in the citation. In case of conflicting statements of a cited source and the instant application, the statement in the instant application shall control.

[0669] Section and table headings are not intended to be limiting.

CLAIMS

We claim:

1. A method for inhibiting the expression of the SOD1 gene in a cell comprising administering to a subject at one or more sites by intraparenchymal delivery a composition comprising an adeno-associated viral (AAV) vector, said AAV vector comprising a nucleic acid sequence positioned between two inverted terminal repeats (ITRs) for inhibiting or suppressing expression of SOD1 in a cell, wherein said nucleic acid sequence comprises a sense strand sequence and an antisense strand sequence, wherein the sense strand sequence comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO: 7 and the antisense strand sequence comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO: 8 and wherein said sense strand sequence and antisense strand sequence share a region of complementarity of at least four nucleotides in length.
2. The method of claim 1, wherein the expression of SOD1 is inhibited or suppressed.
3. The method of claim 2, wherein the SOD1 is wild type SOD1, mutated SOD1 with at least one mutation or both wild type SOD1 and mutated SOD1 with at least one mutation.
4. The method of claim 2, wherein the expression of SOD1 is inhibited or suppressed by about 20% to about 100%.
5. The method of any of claims 1-4, wherein the nucleic acid sequence comprises a sense strand sequence and an antisense strand sequence of an siRNA duplex.
6. The method of claims 1-5, wherein said nucleic acid sequence positioned between two inverted terminal repeats (ITRs) including said ITRs consists of SEQ ID NO: 25.
7. The method of any of claims 1-5, wherein the region of complementarity is at least 17 nucleotides in length.

8. The method of claim 7, wherein the region of complementarity is between 19 and 21 nucleotides in length.
9. The method of claim 8, wherein the region of complementarity is 19 nucleotides in length.
10. The method of any of claims 1-5, wherein the sense strand sequence and the antisense strand sequence are, independently, 30 nucleotides or less.
11. An adeno-associated viral (AAV) vector, said AAV vector comprising a nucleic acid sequence positioned between two inverted terminal repeats (ITRs) for inhibiting or suppressing expression of SOD1 in a cell, wherein said nucleic acid sequence comprises a sense strand sequence and an antisense strand sequence, wherein the sense strand sequence comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO: 7 and the antisense strand sequence comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO: 8 and wherein said sense strand sequence and antisense strand sequence share a region of complementarity of at least four nucleotides in length.
12. A polynucleotide sequence comprising SEQ ID NO: 9.
13. A polynucleotide sequence consisting of SEQ ID NO: 25.
14. An adeno-associated viral (AAV) vector, said AAV vector comprising a vector genome sequence comprising SEQ ID NO: 25 packaged in an AAV capsid.
15. The adeno-associated viral (AAV) vector of claim 14, wherein the AAV capsid serotype is selected from AAVrh10 and AAV9.
16. The adeno-associated viral (AAV) vector of claim 14 wherein the vector genome is self-complementary (scAAV).

17. A method for treating and/or ameliorating a neurodegenerative or spinal cord related disease in a subject in need of treatment, the method comprising administering to the subject at more than one site by intraparenchymal delivery a therapeutically effective amount of a composition comprising an adeno-associated viral (AAV) vector, said AAV vector comprising a nucleic acid sequence positioned between two inverted terminal repeats (ITRs) for inhibiting or suppressing expression of a gene associated with said neurodegenerative or spinal cord related disease in a cell, wherein said nucleic acid sequence comprises a sense strand sequence and an antisense strand sequence, wherein the sense strand sequence comprises at least 15 contiguous nucleotides and the antisense strand sequence comprises at least 15 contiguous nucleotides and wherein said sense strand sequence and antisense strand sequence share a region of complementarity of at least four nucleotides in length.

18. The method of any of claim 17, wherein the region of complementarity is at least 17 nucleotides in length.

19. The method of claim 18, wherein the region of complementarity is between 19 and 21 nucleotides in length.

20. The method of claim 19, wherein the region of complementarity is 19 nucleotides in length.

21. The method of claim 17, wherein the sense strand sequence and the antisense strand sequence are, independently, 30 nucleotides or less.

22. The method of claim 17, wherein the AAV vector comprises a capsid serotype selected from the group consisting of AAV1, AAV2, AAV2G9, AAV3, AAV3a, AAV3b, AAV3-3, AAV4, AAV4-4, AAV5, AAV6, AAV6.1, AAV6.2, AAV6.1.2, AAV7, AAV7.2, AAV8, AAV9, AAV9.11, AAV9.13, AAV9.16, AAV9.24, AAV9.45, AAV9.47, AAV9.61, AAV9.68, AAV9.84, AAV9.9, AAV10, AAV11, AAV12, AAV16.3, AAV24.1, AAV27.3, AAV42.12, AAV42-1b, AAV42-2, AAV42-3a, AAV42-3b, AAV42-4, AAV42-5a, AAV42-5b, AAV42-6b, AAV42-8, AAV42-10, AAV42-11, AAV42-12, AAV42-13, AAV42-15, AAV42-aa, AAV43-1, AAV43-12, AAV43-20,

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 AAVrh.61, AAVrh.64, AAVrh.64R1, AAVrh.64R2, AAVrh.67, AAVrh.73, AAVrh.74,
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AAV, bovine AAV, AAVhE1.1, AAVhEr1.5, AAVhER1.14, AAVhEr1.8, AAVhEr1.16, AAVhEr1.18, AAVhEr1.35, AAVhEr1.7, AAVhEr1.36, AAVhEr2.29, AAVhEr2.4, AAVhEr2.16, AAVhEr2.30, AAVhEr2.31, AAVhEr2.36, AAVhER1.23, AAVhEr3.1, AAV2.5T, AAV-PAEC, AAV-LK01, AAV-LK02, AAV-LK03, AAV-LK04, AAV-LK05, AAV-LK06, AAV-LK07, AAV-LK08, AAV-LK09, AAV-LK10, AAV-LK11, AAV-LK12, AAV-LK13, AAV-LK14, AAV-LK15, AAV-LK16, AAV-LK17, AAV-LK18, AAV-LK19, AAV-PAEC2, AAV-PAEC4, AAV-PAEC6, AAV-PAEC7, AAV-PAEC8, AAV-PAEC11, AAV-PAEC12, AAV-2-pre-miRNA-101, AAV-8h, AAV-8b, AAV-h, AAV-b, AAV SM 10-2, AAV Shuffle 100-1, AAV Shuffle 100-3, AAV Shuffle 100-7, AAV Shuffle 10-2, AAV Shuffle 10-6, AAV Shuffle 10-8, AAV Shuffle 100-2, AAV SM 10-1, AAV SM 10-8, AAV SM 100-3, AAV SM 100-10, BNP61 AAV, BNP62 AAV, BNP63 AAV, AAVrh.50, AAVrh.43, AAVrh.62, AAVrh.48, AAVhu.19, AAVhu.11, AAVhu.53, AAV4-8/rh.64, AAVLG-9/hu.39, AAV54.5/hu.23, AAV54.2/hu.22, AAV54.7/hu.24, AAV54.1/hu.21, AAV54.4R/hu.27, AAV46.2/hu.28, AAV46.6/hu.29, AAV128.1/hu.43, true type AAV (ttAAV), UPENN AAV 10, Japanese AAV 10 serotypes, AAV CBr-7.1, AAV CBr-7.10, AAV CBr-7.2, AAV CBr-7.3, AAV CBr-7.4, AAV CBr-7.5, AAV CBr-7.7, AAV CBr-7.8, AAV CBr-B7.3, AAV CBr-B7.4, AAV CBr-E1, AAV CBr-E2, AAV CBr-E3, AAV CBr-E4, AAV CBr-E5, AAV CBr-e5, AAV CBr-E6, AAV CBr-E7, AAV CBr-E8, AAV CHt-1, AAV CHt-2, AAV CHt-3, AAV CHt-6.1, AAV CHt-6.10, AAV CHt-6.5, AAV CHt-6.6, AAV CHt-6.7, AAV CHt-6.8, AAV CHt-P1, AAV CHt-P2, AAV CHt-P5, AAV CHt-P6, AAV CHt-P8, AAV CHt-P9, AAV CKd-1, AAV CKd-10, AAV CKd-2, AAV CKd-3, AAV CKd-4, AAV CKd-6, AAV CKd-7, AAV CKd-8, AAV CKd-B1, AAV CKd-B2, AAV CKd-B3, AAV CKd-B4, AAV CKd-B5, AAV CKd-B6, AAV CKd-B7, AAV CKd-B8, AAV CKd-H1, AAV CKd-H2, AAV CKd-H3, AAV CKd-H4, AAV CKd-H5, AAV CKd-H6, AAV CKd-N3, AAV CKd-N4, AAV CKd-N9, AAV CLg-F1, AAV CLg-F2, AAV CLg-F3, AAV CLg-F4, AAV CLg-F5, AAV CLg-F6, AAV CLg-F7, AAV CLg-F8, AAV CLv-1, AAV CLv1-1, AAV Clv1-10, AAV CLv1-2, AAV CLv-12, AAV CLv1-3, AAV CLv-13, AAV CLv1-4, AAV Clv1-7, AAV Clv1-8, AAV Clv1-9, AAV CLv-2, AAV CLv-3, AAV CLv-4, AAV CLv-6, AAV CLv-8, AAV CLv-D1, AAV CLv-D2, AAV CLv-D3, AAV CLv-D4, AAV CLv-D5, AAV CLv-D6, AAV CLv-D7, AAV CLv-D8, AAV CLv-E1, AAV CLv-K1, AAV CLv-K3, AAV CLv-K6, AAV CLv-L4, AAV CLv-L5, AAV CLv-L6, AAV CLv-M1, AAV CLv-M11, AAV CLv-M2, AAV CLv-M5, AAV CLv-M6,

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23. The method of claim 22, wherein the capsid serotype is AAVrh.10.

24. The method of claim 22, wherein the AAV vector comprises a promoter, and wherein the promoter is H1.

25. The method of claim 22, wherein the AAV comprises a filler sequence.

26. The method of claim 25, wherein the filler sequence is selected from a lentivirus derived filler sequence and an albumin gene derived filler sequence.

27. The method of claims 1 or 17, wherein the administering step by intraparenchymal delivery occurs at two sites within the spinal cord.

28. The method of claims 1 or 17, wherein the administration by intraparenchymal delivery occurs at two sites within the cervical spinal cord.
29. The method of claims 1 or 17, wherein the two sites of administration are at levels C3 and C5 of the spinal cord.
30. The method of claims 1 or 17, wherein the volume of administration is from about 5 μ L to about 240 μ L at level C3 of the spinal cord and from about 5 μ L to about 240 μ L at level C5 of the spinal cord.
31. The method of claims 1 or 17, wherein the volume of administration is from about 5 μ L to about 60 μ L at level C3 of the spinal cord and from about 5 μ L to about 60 μ L at level C5 of the spinal cord.
32. The method of claim 31, wherein the volume of administration is from about 25 to about 40 μ L at level C3 of the spinal cord and from about 25 to about 40 μ L at level C5 of the spinal cord.
33. The method of claims 1 or 17, wherein the dose is from about 1×10^{10} vg to about 1×10^{12} vg at level C3 of the spinal cord and from about 1×10^{10} vg to about 1×10^{12} vg at level C5 of the spinal cord.
34. The method of claim 33, wherein the dose is from about 5×10^{11} vg to about 8×10^{11} vg at level C3 of the spinal cord and from about 5×10^{11} vg to about 8×10^{11} vg at level C5 of the spinal cord.
35. The method of claim 33, wherein the dose is from about 2×10^{10} vg to about 7×10^{11} vg at level C3 of the spinal cord and from about 2×10^{10} vg to about 7×10^{12} vg at level C5 of the spinal cord.
36. The method of any of claims 30-35, wherein the injection rate is 5 μ L/min.

Figure 1

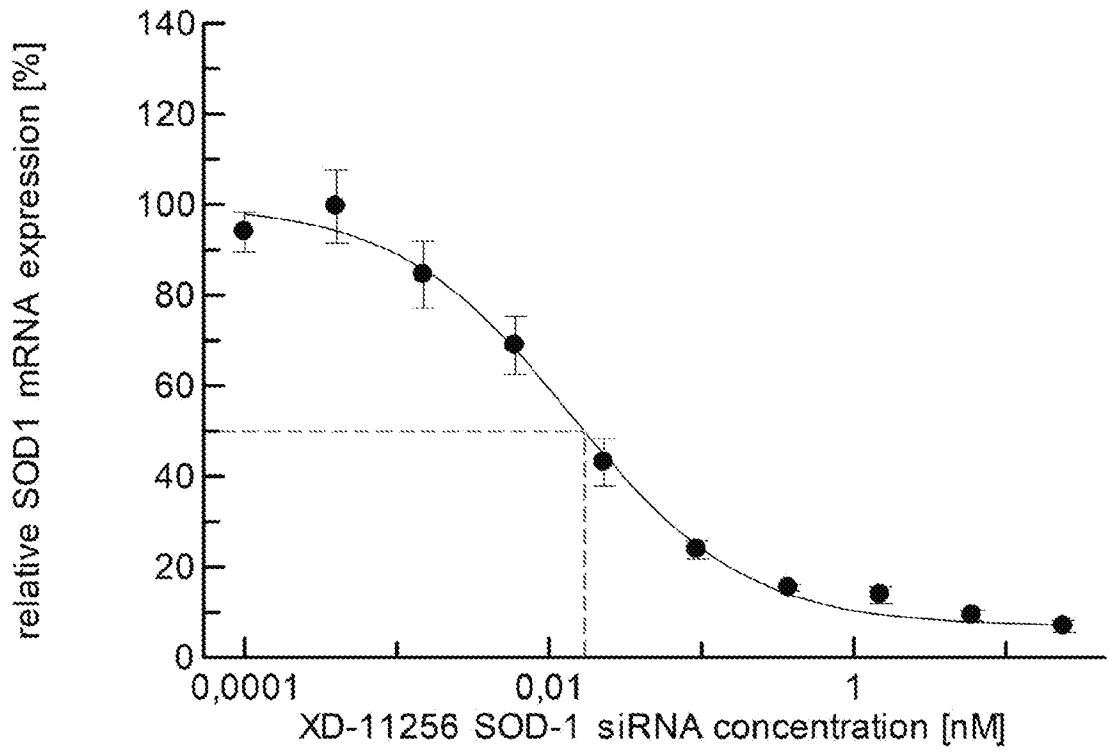
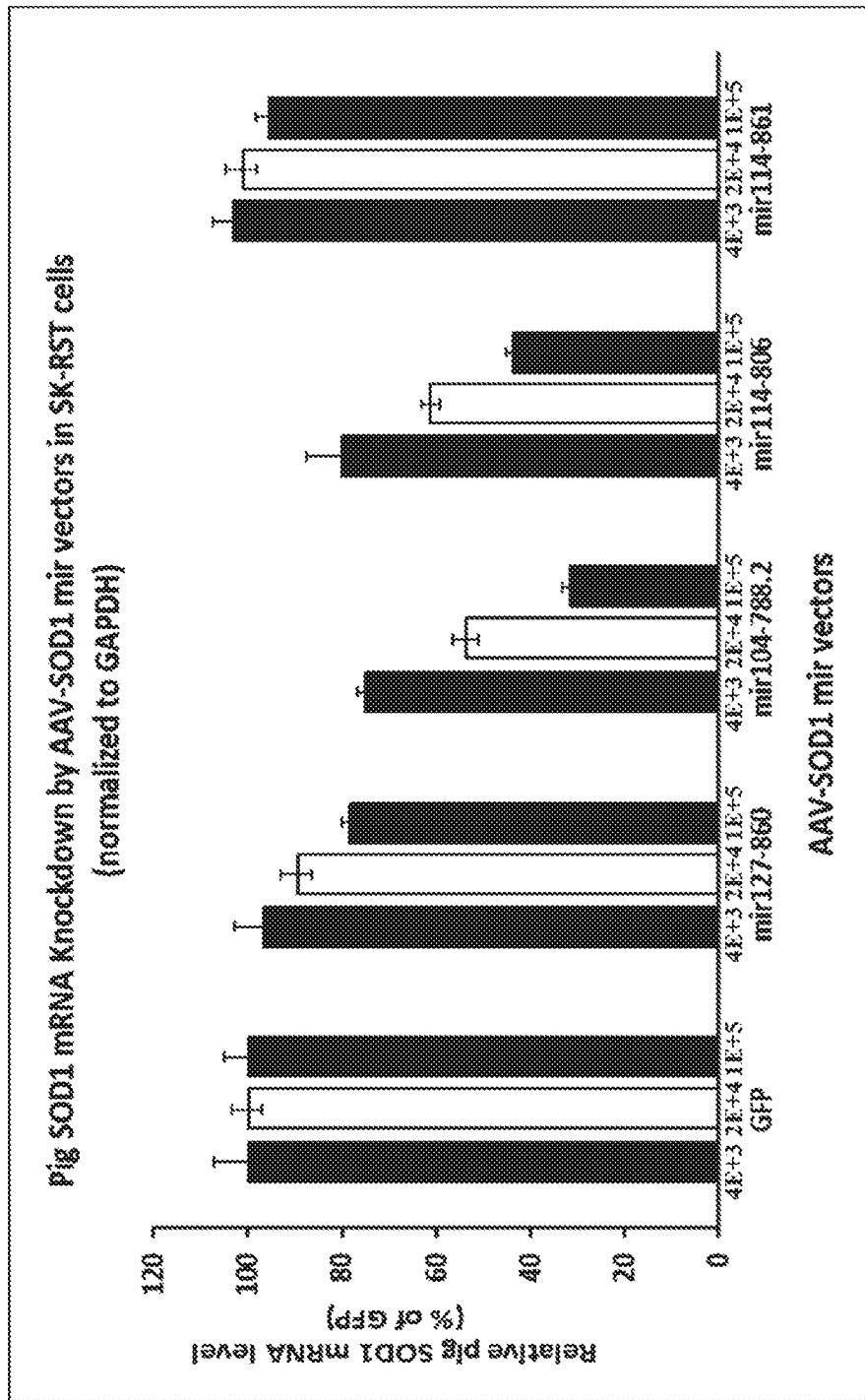


Figure 2



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<141> 2019-07-02

<150> 62/746,104

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 <213> Artificial Sequence

<220>
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<400> 26
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 <211> 570
 <212> DNA
 <213> Homo sapiens

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<210> 29
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<220>
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<400> 30

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gagcgcgag 130