(19) **DANMARK**

(10) **DK/EP 2826860 T3**

Patent- og Varemærkestyrelsen

(12) # Oversættelse af europæisk patentskrift

(51) Int.Cl.: *C 12 N 15/00 (2006.01)* *A 61 K 48/00 (2006.01)* *C 12 N 15/86 (2006.01)*

(45) Oversættelsen bekendtgjort den: **2018-12-03**

(80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2018-08-22**

(86) Europæisk ansøgning nr.: **14181861.7**

(86) Europæisk indleveringsdag: **2011-04-22**

(87) Den europæiske ansøgnings publiceringsdag: **2015-01-21**

(30) Prioritet: **2010-04-23 US 327627 P**

(62) Stamansøgningsnr: **11772784.2**

(84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

(73) Patenthaver: **University of Massachusetts, 225 Franklin Street, Boston, MA 02110, USA**

(72) Opfinder: **Gao, Guangping, 4 Edward Dunn Way, Westborough, MA 01581, USA**
ZHANG, Hongwei, 55 Lake Avenue North, Worcester, MA 01655, USA
WANG, Hongyan, 55 Lake Avenue North, Worcester, MA 01655, USA
XU, Zuoshang, 70 Manor Avenue, Wellesley, MA 02482, USA

(74) Fuldmægtig i Danmark: **Zacco Denmark A/S, Arne Jacobsens Allé 15, 2300 København S, Danmark**

(54) Benævnelse: **CNS-targeting AAV-vektorer og fremgangsmåder til anvendelse deraf**

(56) Fremdragne publikationer:

WO-A1-2010/129021
WO-A2-2007/127264
MARCO A. PASSINI ET AL: "CNS-targeted gene therapy improves survival and motor function in a mouse model of spinal muscular atrophy", JOURNAL OF CLINICAL INVESTIGATION, vol. 120, no. 4, 1 April 2010 (2010-04-01) , pages 1253-1264, XP055040159, ISSN: 0021-9738, DOI: 10.1172/JCI41615
DOLAN SONDHI ET AL: "Enhanced Survival of the LINCL Mouse Following CLN2 Gene Transfer Using the rh.10 Rhesus Macaque-derived Adeno-associated Virus Vector", MOLECULAR THERAPY, vol. 15, no. 3, 19 December 2006 (2006-12-19), pages 481-491, XP055084809, ISSN: 1525-0016, DOI: 10.1038/sj.mt.6300049
A S BEUTLER ET AL: "AAV for pain: steps towards clinical translation", GENE THERAPY, vol. 16, no. 4, 1 April 2009 (2009-04-01), pages 461-469, XP055070356, ISSN: 0969-7128, DOI: 10.1038/gt.2009.23
LUCY VULCHANOVA ET AL: "Differential adeno-associated virus mediated gene transfer to sensory neurons following intrathecal delivery by direct lumbar puncture", MOLECULAR PAIN, vol. 6, no. 1, 1 January 2010 (2010-01-01) , pages 31-31, XP055099564, ISSN: 1744-8069, DOI: 10.1186/1744-8069-6-31
CEARLEY ET AL: "Transduction characteristics of adeno-associated virus vectors expressing cap serotypes 7, 8, 9, and Rh10 in the mouse brain", MOLECULAR THERAPY, NATURE PUBLISHING GROUP, GB, vol. 13, no. 3, 1 March 2006 (2006-03-01), pages 528-537, XP005326765, ISSN: 1525-0016, DOI: 10.1016/J.YMTHE.2005.11.015
CASSIA N CEARLEY ET AL: "Expanded Repertoire of AAV Vector Serotypes Mediate Unique Patterns of Transduction in Mouse Brain", MOLECULAR THERAPY, vol. 16, no. 10, 19 August 2008 (2008-08-19), pages

Fortsættes ...

1710-1718, XP055022703, ISSN: 1525-0016, DOI: 10.1038/mt.2008.166
IWAMOTO NAOTAKA ET AL: "Global diffuse distribution in the brain and efficient gene delivery to the dorsal root ganglia by intrathecal injection of adeno-associated viral vector serotype 1", JOURNAL OF GENE MEDICINE, vol. 11, no. 6, June 2009 (2009-06), pages 498-505, XP002733270, ISSN: 1099-498X
D H MEIJER ET AL: "Controlling brain tumor growth by intraventricular administration of an AAV vector encoding IFN-[beta]", CANCER GENE THERAPY, vol. 16, no. 8, 6 February 2009 (2009-02-06), pages 664-671, XP055149461, GB ISSN: 0929-1903, DOI: 10.1038/cgt.2009.8
TENENBAUM L ET AL: "Recombinant AAV-mediated gene delivery to the central nervous system.", THE JOURNAL OF GENE MEDICINE FEB 2004, vol. 6 Suppl 1, February 2004 (2004-02), pages S212-S222, XP002715192, ISSN: 1099-498X
RALPH G SCOTT ET AL: "(S)ilencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model", NATURE MEDICINE, vol. 11, no. 4, 1 April 2005 (2005-04-01), pages 429-433, XP002400648,
BROOKE R. SNYDER ET AL: "Comparison of Adeno-Associated Viral Vector Serotypes for Spinal Cord and Motor Neuron Gene Delivery", HUMAN GENE THERAPY, vol. 22, no. 9, 1 September 2011 (2011-09-01), pages 1129-1135, XP055099540, ISSN: 1043-0342, DOI: 10.1089/hum.2011.008
H. WANG ET AL: "Widespread spinal cord transduction by intrathecal injection of rAAV delivers efficacious RNAi therapy for amyotrophic lateral sclerosis", HUMAN MOLECULAR GENETICS, vol. 23, no. 3, 1 February 2014 (2014-02-01), pages 668-681, XP055137556, ISSN: 0964-6906, DOI: 10.1093/hmg/ddt454

DESCRIPTION

[0001] The invention in some aspects relates to recombinant adeno-associated viruses useful for targeting transgenes to CNS tissue, and compositions comprising the same, and methods of use thereof.

BACKGROUND OF THE INVENTION

[0002] Gene therapy has been investigated for delivery of therapeutic genes to the CNS cells for treatment of various CNS disease, *e.g.*, Canavan disease, ALS, Parkinson disease (PD), *etc.* In some limited cases, therapeutic benefits have been observed using certain viruses, *e.g.*, recombinant adenovirus (rAd), lentivirus (LV) and adeno-associated virus (AAV) to express a variety of therapeutic genes. AAV2 has been used in clinical trials for treatment of PD and Leber congenital amaurosis (an eye disease) and preliminary findings suggest symptomatic improvements without noticeable toxicity [2-4].

[0003] However, AAV-based gene therapy to treat CNS disease has still faced major obstacle. Many CNS diseases including, for example, ALS affect both cortical and spinal motor neurons that are distributed in a very broad area in the CNS. It has frequently been the case that viral vectors injected into CNS tissue transduce cells only in the vicinity of the injection site, have a very limited spread and generally have not impacted the lifespan in CNS disease animal models [See, *e.g.*, Ref. 5]. Still, a variety of other viral administration methods have been tested. One example, involves injecting the viral particles into skeletal muscle and allowing the nerve terminals to internalize the viral genome, which is then retrogradely transported back to the spinal motor neurons. This approach has shown some positive results in certain mouse models [68]. However, to apply this method in larger mammals, like adult humans, would be impractical. Overall, the transduction efficiency observed with muscle injection is relatively low. Some investigators have tried to improve this efficiency by modifying viral capsid proteins with the nerve binding domains of tetanus toxin or botulinum toxin. These efforts have not been fruitful due to various technical difficulties. Another problem with muscle injection in larger mammals, is a need for large doses, which is technically challenging, expensive, and carries a high risk for adverse effects, ranging from immune reaction to transduction of unintended cells (*e.g.*, germ cells).

[0004] Another method that has been evaluated for delivering transgenes into motor neurons is to inject the virus into large nerves, which maximizes that exposure of the virus to motor axons, allowing the motor neurons to internalize the viral genome and retrogradely transport them back to the cell body. This method has been demonstrated to be more efficient in transducing motor neurons than muscle injection [9]. Still, to implement a method such as this in larger mammals would be challenging.

[0005] Passini et al. (2010) J. Clinical Investigation 120(4) 1253-1264 describes CNS-targeted gene therapy improving survival and motor function in a mouse model of spinal muscular atrophy; WO 2010/129021 describes gene therapy for neurodegenerative disorders; Sondhi (2006) Molec Ther. 15 pp 481-491 utilised a rh.10 Macaque derived AAV to deliver genes to a transgenic mouse exhibiting late infantile neuronal ceroid lipofuscinosis (LINCL). Beutler (2009) Gene Ther. 16 pp 461-469 reviewed the use of AAV for treatment of pain and in particular chronic neuropathic pain. Vulchanova et al. (2010) Molecular Pain 6(1) 31 describes differential adeno-associated virus mediated gene transfer to sensory neurons following intrathecal delivery by direct lumbar puncture; Cearley and Wolfe (2006) Molecular Therapy (13)3 528-537 describes transduction characteristics of adeno-associated virus vectors expressing cap serotypes 7,8,9, and Rh10 in the mouse brain; Cearley et al. (2008) Molecular Therapy 16(10) 1710-1718 describes an expanded repertoire of AAV serotypes mediating unique patterns of transduction in mouse brain; Iwamoto et al. J. Gene Medicine 11 498-505 describes global diffuse distribution in the brain and efficient gene delivery to the dorsal root ganglia by intrathecal injection of adeno-associated viral vector serotype 1; Meijer et al. Cancer Gene Therapy (2009) 16 664-671 describes controlling brain tumor growth by intraventricular administration of an AAV vector encoding IFN-β; Tenenbaum (2004) J. Gene Medicine 6 ppS212-S222 reviews the use of AAV mediated gene delivery to the CNS; Ralph et al. (2005) Nature Medicine 11(4) 429-433 describes silencing mutant SOD1 using RNAi protecting against neurodegeneration and extending survival in an ALS model; WO 2007/127264 describes the production and harvesting of AAV without the need for cell lysis.

SUMMARY

[0006] The present invention relates to the subject matter as defined in claim 1 and encompasses the embodiments as defined in claims 2-12.

[0007] Aspects of the disclosure provided herein, are based on the discovery of recombinant AAVs that achieve wide-spread distribution throughout CNS tissue of a subject. In some cases, the rAAVs spread throughout CNS tissue following direct administration into the cerebrospinal fluid (CSF), *e.g.*, via intrathecal and/or intracerebral injection. In other cases, the rAAVs cross the blood-brain-barrier and achieve wide-spread distribution throughout CNS tissue of a subject following intravenous administration. In some aspects the disclosure relates to rAAVs having distinct central nervous system tissue targeting capabilities (*e.g.*, CNS tissue tropisms), which achieve stable and nontoxic gene transfer at high efficiencies. Methods involving co-administration via intrathecal and intracerebral (e.g., intraventricular) injection of rAAVs are provided in some aspects. For example, it has been discovered that rAAVs having a capsid protein comprising a sequence as set forth in SEQ ID NO: 9 achieves wide-spread distribution following intrathecal injection throughout the CNS, and thus, are particularly useful for treating CNS-associated disorders such as, for example, ALS. In still further aspects of the disclosure methods are provided for treating Canavan disease.

[0008] According to some aspects of the disclosure herein, methods for delivering a transgene to CNS tissue in a subject are provided. In some cases, the methods comprise administering an effective amount of a rAAV by intrathecal administration, wherein the rAAV comprises (i) a capsid protein comprising a sequence as set forth in SEQ ID NO: 9 and (ii) a nucleic acid comprising a promoter operably linked with a transgene. In some cases, the methods further comprise administering an effective amount of the rAAV by intracerebral administration. In some cases, the methods comprise administering an effective amount of a rAAV by intrathecal administration and by intracerebral administration, wherein the rAAV infects cells of CNS tissue in the subject and comprises a nucleic acid comprising a promoter operably linked with a transgene. In certain cases, the intracerebral administration is an intraventricular administration. In one case, the intraventricular administration is an administration into a ventricular region of the forebrain of the subject. In certain cases, the intrathecal administration is in the lumbar region of the subject. In some cases, the dose of the rAAV for intrathecal administration is in a range of 10^{10} genome copies/subject to 10^{11} genome copies/subject. In some cases, the dose of the rAAV for intrathecal administration is in a range of 10^{11} genome copies/subject to 10^{12} genome copies/subject. In some cases, the dose of the rAAV for intrathecal administration is in a range of 10^{12} genome copies/subject to 10^{13} genome copies/subject. In some cases, the dose of the rAAV for intrathecal administration is in a range of 10^{13} genome copies/subject to 10^{14} genome copies/subject. In some cases, the dose of the rAAV for intracerebral administration is in a range of 10^{10} genome copies/subject to 10^{11} genome copies/subject. In some cases, the dose of the rAAV for intracerebral administration is in a range of 10^{11} genome copies/subject to 10^{12} genome copies/subject. In some cases, the dose of the rAAV for intracerebral administration is in a range of 10^{12} genome copies/subject to 10^{13} genome copies/subject. In some cases, the dose of the rAAV for intracerebral administration is in a range of 10^{13} genome copies/subject to 10^{14} genome copies/subject. In some cases, the dose of the rAAV for intracerebral or intrathecal administration is formulated for injection of a volume in a range of 1 μl to 10 μl. In some cases, the dose of the rAAV for intracerebral or intrathecal administration is formulated for injection of a volume in a range of 10 μl to 100 μl. In some cases, the rAAV for the intracerebral or intrathecal administration is formulated for injection of a volume in a range of 100 μl to 1 ml. In some cases, the rAAV for the intracerebral or intrathecal administration is formulated for injection of a volume of 1 ml or more. In some cases, the transgene encodes a reporter protein. In certain cases, the reporter protein is a fluorescent protein, an enzyme that catalyzes a reaction yielding a detectable product, or a cell surface antigen. In certain cases, the enzyme is a luciferase, a beta-glucuronidase, a chloramphenicol acetyltransferase, an aminoglycoside phosphotransferase, an aminocyclitol phosphotransferase, or a Puromycin N-acetyl-tranferase. In some cases, the transgene is a CNS-associated gene. In some cases, the CNS-associated gene is neuronal apoptosis inhibitory protein (NAIP), nerve growth factor (NGF), glial-derived growth factor (GDNF), brain-derived growth factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxlase (TH), GTP-cyclohydrolase (GTPCH), amino acid decorboxylase (AADC) or aspartoacylase (ASPA). In some cases, the transgene encodes an inhibitory RNA that binds specifically to SOD1 mRNA and inhibits expression of SOD1 in the subject. In some cases, the inhibitory RNA is an antisense RNA, a shRNA or a miRNA. In some cases, the inhibitory RNA has a sequence as set forth in SEQ ID NO: 26. Thus, according to some aspects of the disclosure a nucleic acid comprising a sequence as set forth in SEQ ID NO: 26 is provided. In some cases, a nucleic acid comprising a promoter operably linked with a region having a sequence as set forth in SEQ ID NO: 26 is provided.

[0009] In further aspects of the disclosure a recombinant AAV comprising a nucleic acid comprising a sequence as set forth in SEQ ID NO: 26 is provided. In some aspects of the disclosure a recombinant AAV comprising a nucleic acid comprising a promoter operably linked with a region having a sequence as set forth in SEQ ID NO: 26 is provided. In some cases the recombinant AAV further comprises a capsid protein comprising a sequence as set forth in SEQ ID NO: 9.

[0010] According to some aspects of the disclosure, methods for treating amyotrophic lateral sclerosis (ALS) in a subject in need thereof are provided. In some cases, the methods comprise administering an effective amount of a rAAV to CNS tissue of the subject, wherein the rAAV comprises (i) a capsid protein comprising a sequence as set forth in SEQ ID NO: 9 and (ii) a nucleic acid comprising a promoter operably linked with a region encoding an inhibitory RNA that binds specifically to SOD1 mRNA and inhibits expression of SOD1 in the subject. In some cases, the inhibitory RNA is an antisense RNA, a shRNA or a miRNA. In some cases, the inhibitory RNA has a sequence as set forth in SEQ ID NO: 26. In some cases, the methods comprise administering an effective amount of a rAAV to the subject, wherein the rAAV comprises a nucleic acid comprising a promoter operably linked with a region encoding a sequence as set forth in SEQ ID NO: 26 and wherein the rAAV infects cells of CNS tissue in the subject.

[0011] According to some aspects of the disclosure herein, methods for delivering a transgene to a CNS tissue in a subject are provided that comprise administering an effective amount of a rAAV by intravenous administration, wherein the rAAV infects cells of CNS tissue in the subject and comprises a nucleic acid comprising a promoter operably linked with a transgene. In some cases, the dose of the rAAV for intravenous administration is in a range of 10^{10} genome copies/subject to 10^{11} genome copies/subject. In some cases, the dose of the rAAV for intravenous administration is in a range of 10^{11} genome copies/subject to 10^{12} genome copies/subject. In some cases, the dose of the rAAV for intravenous administration is in a range of 10^{12} genome copies/subject to 10^{13} genome copies/subject. In some cases, the dose of the rAAV for intravenous administration is in a range of 10^{13} genome copies/subject to 10^{14} genome copies/subject. In some cases, the dose of the rAAV for intravenous administration is in a range of 10^{14} genome copies/subject to 10^{15} genome copies/subject. In some cases, the dose of the rAAV for intravenous administration is in a range of 10^{10} genome copies/kg to 10^{11} genome copies/kg. In some cases, the dose of the rAAV for intravenous administration is in a range of 10^{11} genome copies/kg to 10^{12} genome copies/kg. In some cases, the dose of the rAAV for intravenous administration is in a range of 10^{12} copies/kg to 10^{13} genome copies/kg. In some cases, the dose of the rAAV for intravenous administration is in a range of 10^{13}

genome copies/kg to 10^{14} genome copies/kg.

[0012] According to some aspects of the disclosure herein, methods for delivering a transgene to a CNS tissue in a subject are provided that comprise administering to the subject an effective amount of a rAAV that comprises (i) a capsid protein having a sequence as set forth in any one of SEQ ID NO: 10 to 12 and (ii) a nucleic acid comprising a promoter operably linked with a transgene. In some cases, the methods comprise administering to the subject an effective amount of a rAAV comprising a transgene to a subject, wherein the rAAV comprises a capsid protein of a AAV serotype, or serotype variant, selected from the group consisting of: AAV1, AAV2, AAV5, AAV6, AV6.2, AAV7, AAV8, AAV9, rh.10, rh.39, rh.43 and CSp3, and wherein: (a) if the AAV serotype is AAV1, the administration route is not intracerebral, intramuscular, intranerve, or intraventricular and/or the subject is not a mouse, rat or feline; (b) if the AAV serotype is AAV2, the administration route is not intracerebral or intraventricular administration and/or the subject is not a rat, mouse, feline, marmoset, or macaque; (c) if the AAV serotype is AAV5, the administration route is not intracerebral or intraventricular administration and/or the subject is not a rat, mouse, or marmoset; (d) if the AAV serotype is AAV6, the subject is not a mouse; (e) if the AAV serotype is AAV7, the administration route is not intracerebral administration and/or the subject is not a mouse or macaque; (f) if the AAV serotype is AAV8, the administration route is not intracerebral, intraperitoneal, or intravascular administration and/or the subject is not a mouse or macaque; (g) if the AAV serotype is AAV9, the administration route is not intracerebral or intravascular administration and/or the subject is not a rat or mouse; and (h) if the AAV serotype is AAVrh.10, the administration route is not intracerebral or intravascular administration and/or the subject is not a rat or mouse. In some cases, the AAV serotype, or serotype variant, is selected from AAV1, AAV6, AAV7, rh.39, rh.43, and CSp3, and the administration route is intravascular administration. In some cases, the AAV serotype is AAV7 and the administration route is intravascular administration. In some cases, the CNS tissue is selected from cortex, hippocampus, thalamus, hypothalamus, cerebellum, brain stem, cervical spinal cord, thoracic spinal cord, and lumbar spinal cord. In some cases, the transgene encodes a reporter protein. In certain cases, the reporter protein is a fluorescent protein, an enzyme that catalyzes a reaction yielding a detectable product, or a cell surface antigen. In certain cases, the enzyme is a luciferase, a beta-glucuronidase, a chloramphenicol acetyltransferase, an aminoglycoside phosphotransferase, an aminocyclitol phosphotransferase, or a Puromycin N-acetyl-tranferase. In some cases, the transgene is a CNS-associated gene. In certain cases, the CNS-associated gene is neuronal apoptosis inhibitory protein (NAIP), nerve growth factor (NGF), glial-derived growth factor (GDNF), brain-derived growth factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxlase (TH), GTP-cyclohydrolase (GTPCH), amino acid decorboxylase (AADC) or aspartoacylase (ASPA). In some cases, the rAAV is administered by intravenous injection.

[0013] According to some aspects of the disclosure herein a rAAV that comprises (i) a capsid protein having a sequence as set forth in any one of SEQ ID NO: 10 to 12 and (ii) a nucleic acid comprising a promoter operably linked with a CNS-associated gene is provided. In certain cases, the CNS-associated gene is neuronal apoptosis inhibitory protein (NAIP), nerve growth factor (NGF), glial-derived growth factor (GDNF), brain-derived growth factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxlase (TH), GTP-cyclohydrolase (GTPCH), amino acid decorboxylase (AADC) or aspartoacylase (ASPA). In some cases, mRNA expressed from the CNS-associated gene comprises a miRNA binding site of a miRNA that is preferentially expressed in non-CNS tissue. In certain cases, the miRNA binding site is a binding site for miR-122. In certain cases, the miRNA binding site is a binding site for miR-1. In some cases, mRNA expressed from the CNS-associated gene does not comprise a miRNA binding site of a miRNA that is preferentially expressed in CNS tissue. In some cases, the promoter is a CNS tissue specific promoter. In certain cases, the promoter is a promoter of a gene selected from: neuronal nuclei (NeuN), glial fibrillary acidic protein (GFAP), adenomatous polyposis coli (APC), and ionized calcium-binding adapter molecule 1 (Iba-1).

[0014] According to some aspects of the disclosure herein, a composition comprising a rAAV that comprises (i) a capsid protein having a sequence as set forth in SEQ ID NO: 10 to 12 and (ii) a nucleic acid comprising a promoter operably linked with a CNS-associated gene is provided. In certain cases the composition further comprises a pharmaceutically acceptable carrier. According to some aspects of the disclosure, a kit comprising a container housing the composition is provided. In some cases, the container is a sealed vial or ampule. In some cases, the container is a syringe.

[0015] According to some aspects of the disclosure herein, an isolated mammalian cell is provided that comprises a nucleic acid encoding a capsid protein having a sequence as set forth in any one of SEQ ID NO: 10 to 12 and a rAAV vector comprising a nucleic acid encoding a CNS-disease associated gene. In some cases, the isolated mammalian cell further comprises an AAV helper function vector. In some cases, isolated mammalian cell further comprises an accessory function vector. In certain cases, the CNS-associated gene is neuronal apoptosis inhibitory protein (NAIP), nerve growth factor (NGF), glial-derived growth factor (GDNF), brain-derived growth factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxlase (TH), GTP-cyclohydrolase (GTPCH), amino acid decorboxylase (AADC) or aspartoacylase (ASPA).

[0016] According to further aspects of the disclosure, a method for treating Canavan disease in a subject in need thereof is provided. In some cases, the methods comprise administering an effective amount of a rAAV to CNS tissue of the subject, wherein the rAAV comprises (i) a capsid protein other than a capsid protein of AAV serotype 2 and (ii) a nucleic acid comprising a promoter operably linked with a region encoding aspartoacylase (ASPA). Any of the rAAV serotypes disclosed herein may be used in the methods for treating Canavan disease. In some cases, the rAAV has a capsid protein having an amino acid sequence as set forth in SEQ ID NO: 8 or 9 or a variant thereof. In some cases, administering is performed intrathecally or intracerebrally. In some cases, administering is performed intravascularly.

[0017] In some cases, the methods comprise administering an effective amount of a rAAV to CNS tissue of the subject by a route other than intracerebral administration, wherein the rAAV comprises a nucleic acid comprising a promoter operably linked with a region encoding aspartoacylase (ASPA). In some cases, the methods comprise administering an effective amount of a rAAV to CNS tissue of the subject, wherein the rAAV comprises a nucleic acid comprising a promoter operably linked with a region encoding aspartoacylase (ASPA); and evaluating kidney function in the subject at least once after the administration. Any suitable method known in the art may be used to evaluate a subject's kidney function. The evaluation may involve, for example, an examination of blood or urine urea nitrogen levels, an examination of blood or urine creatinine levels, a creatinine clearance rate examination, a glomerular filtration rate examination, a filtration fraction examination, a renal plasma flow examination, an ultrasound examination, a microscopic examination of a kidney tissue biopsy or any other suitable kidney function test. It should be appreciated that in some cases an improvement in a subject's kidney function following treatment with an rAAV-mediated gene therapy is indicative of efficacy of the gene therapy for treating Canavan disease.

[0018] In some cases, the methods comprise administering an effective amount of a rAAV to CNS tissue of the subject, wherein the rAAV comprises a nucleic acid comprising a promoter operably linked with a region encoding aspartoacylase (ASPA); and evaluating vision of the subject at least once after the administration. Any suitable method known in the art may be used to evaluate a subject's vision. The evaluation may involve, for example, an external examination of the eye, a visual acuity examination, an examination of pupil function, a retinal examination, an ocular motility examination, an intraocular pressure test, or an ophthalmoscopic examination. The evaluation may involve a determination regarding a subject's ability to discriminate colors, objects or shapes or the ability of a subject to discern colors, objects or shapes from a particular distance. It should be appreciated that in some cases an improvement in a subject's vision following treatment with an rAAV-mediated gene therapy is indicative of efficacy of the gene therapy for treating Canavan disease.

[0019] In some cases, the nucleic acid expresses an aspartoacylase (ASPA) mRNA comprising one or more miRNA binds sites for one or more miRNAs that are more abundant in one or more non-CNS tissues in comparison to CNS tissue. Accordingly, in some cases, the mRNA is targeted for degradation by an miRNA in one or more non-CNS tissues. In some cases, the one or more non-CNS tissue is not kidney tissue or retinal tissue. In some cases, the one or more miRNAs that are more abundant in non-CNS tissues in comparison to CNS tissue are at least two-fold, at least three-fold, at least four-fold, at least five-fold, or at least ten-fold more abundant. MiRNAs that are more abundant in non-CNS tissue versus CNS tissue are known in the art. For example, one study discloses the expression levels of more than three-hundred different human miRNAs in 40 different tissues, including CNS tissue, kidney tissue. (*See* Liang Y, et al., Characterization of microRNA expression profiles in normal human tissues. BMC Genomics. 2007 Jun 12;8:166). Thus, in some cases, the skilled artisan could readily select (*e.g.*, based on data such as are disclosed in Liang *et al.*) a suitable miRNA that is more abundant in non-CNS tissue and incorporate a binding site for that miRNA into the encoded mRNA.

[0020] Each of the limitations described above can encompass various embodiments described above. It is, therefore, anticipated that each of the limitations involving any one element or combinations of elements can be included in each aspect of the disclosure. This disclosure is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The disclosure is capable of other embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021]

Figure 1 depicts quantitative results of EGFP intensities from fluorescence microscopic images of a panel of CNS tissue sections from neonatal mice infected with various rAAVs harboring EGFP expression vectors. Neonatal mice were administered the rAAVs by intravenous administration (superfacial temporal vein injection).

Figure 2 depicts quantitative results of EGFP intensities from fluorescence microscopic images of a panel of CNS tissue sections from adult mice infected with various rAAVs harboring EGFP expression vectors. Adult mice were administered the rAAVs by intravenous administration (tail vein injection).

Figure 3 depicts quantitation of EGFP expression in neonatal mice spinal cord (cervical, thoracic and lumber regions) 21 days post IV injection (5 mice per group). Neonatal mice were administered the rAAVs by intravenous administration (superfacial temporal vein injection).

Figure 4A depicts results showing that direct CSF injection of AAVrh.10 harboring a EGFP gene leads to EGFP expression in broad areas of the CNS. Tissue sections, prepared 60 days post virus injection, from brainstem, cervical spinal cord, thoracic spinal cord and lumbar spinal cord are shown. Gray/black pixels correspond with EGFP expression.

Figure 4B depicts results showing that direct CSF injection of AAVrh.10 harboring a EGFP gene leads to EGFP expression in astrocytes. Gray/black pixels correspond with EGFP expression.

Figure 5A depicts a rAAVrh.10 vector that expresses a microRNA targeting SOD1. The construct employs CAG (chicken β-actin promoter with a CMV enhancer) to drive the expression of EGFP and miR-SOD1 that is located in an intron in the 3'-UTR. pA stands for poly A signal. ITRs mark the inverted repeats of the AAV.

Figure 5B depicts results of experiments that test the silencing potency of 9 different miRNA constructs, miR-SOD1#5 was found to silence SOD1 expression most potently.

Figure 5C depicts results of experiments in which miR-SOD1#5 was packaged into AAVrh.10 and used to infect HEK293 cells. Total cellular protein was extracted 43 hours after the infection and blotted to detect SOD1. Scr stands for scrambled miRNA; Sod stands for miR-SOD1#5; and C stands for a control that expresses EGFP only.

Figure 5D depicts a plasmid map of pAAVscCB6 EGFPmir SOD5 (5243bp) (SEQ ID NO: 21).

Figure 6A depicts results of gene transfer studies in SOD1 (G93A) mutant mice showing that rAAV rh.10-SOD1 miRNA knockdowns levels of mutant SOD1 in astrocytes. Staining in motor neurons was also observed.

Figure 6B depicts results of gene transfer studies in SOD1 (G93A) mutant mice showing that rAAV rh.10-SOD1 shRNA increases live span, compared with a rAAV rh.10-scrambled miRNA.

Figure 7A depicts quantitation of EGFP expression in cervical, thoracic, and lumber spinal cord tissue compared with life spans individual mice infected with rAAV rh.10-SOD1 miRNA; rAAV rh.10-SOD1 was administered directly to the CSF.

Figure 7B depicts quantitation of EGFP expression in cervical, thoracic, and lumber spinal cord tissue compared with life spans of individual mice infected with rAAV rh.10-scrambled miRNA; rAAV rh. 10-scrambled miRNA was administered directly to the CSF.

Figure 8 depicts fluorescence microscopy analysis of mice that have been administered intrathecal injections of various AAVs. In this experiment, both AAV9 and AAVrh10 transduce cells along the full length of the spinal cord after a single injection into the CSF in lumbar subarachnoid space.

Figure 9 depicts the effects of AAV10-miR-SOD1 treatment. AAV10-miR-SOD1 treatment slows disease progression as indicated by the slower loss of body weight in treated compared with the control G93A mice.

Figure 10 depicts fluorescence microscopy analysis of mice that have been administered intrathecal injections of various AAVs. In this experiment, AAV9 and AAVrh10 can transduce cells in the broad forebrain areas after a single injection into the CSF in the third ventricle.

Figure 11 depicts fluorescence microscopy analysis of tissue sections from AAV9-injected mice. A single injection of AAV9 and AAVrh10 into the third ventricle can transduce cells in the broad forebrain areas, including cortex, hippocampus, striatum, thalamus, cerebellum and some scattered cells in the spinal cord. The same general pattern is also observed in AAV10-injected mice.

Figure 12 depicts an *in vitro* validation of artificial miRNA-binding sites for reporter silencing. Plasmids harboring the rAAVCBnLacZ genome with or without miR-1 or miR-122-binding sites were transfected into human hepatoma (HuH7) cells (a) which express miR-122 or cotransfected into 293 cells, together with a plasmid expressing either pri-miR-122 (b) or pri-miR-1 (c) at molar ratios of 1:3 (low) or 1:10 (high). 0X: no miRNA-binding site; 1X: one miRNA-binding site; 3X: three miRNA-binding sites. The cells were fixed and stained histochemically with X-gal 48 hours after transfection and blue cells counted. The percentage of nLacZ-positive cells in each transfection were compared to transfection of the control plasmid (prAAVCB*nLacZ*). CB, chicken β-actin; miR, microRNA; *nLacZ,* β-galactosidase reporter transgene; rAAV, recombinant adeno-associated viruses.

Figure 13 depicts an *in vivo* evaluation of endogenous miRNA-mediated transgene silencing in an rAAV9 transduction. (a-c) Adult male C588L/6 mice were injected intravenously with 5 x

10^{13} genome copies per kg (GC/kg) each of rAAV9CB*nLacZ* (no binding site), (a) rAAVCB9*nLacZ*miR-122BS (one miR-122-binding site) and rAAV9C8*nlacZ*-(miR-122BS)$_3$ (three miR-122-binding sites), (b) rAAV9CB*nLacZ*-miR-1 BS (one miR-1 binding site) and rAAV9CB*nLacZ*-(miR-1BS)$_3$ (three miR-1-binding sites, (c) rAAV9CB*nLacZ*-miR-1BS-miR-122BS (1X each binding site) and rAAV9CBnLacZ-(miR-1BS)$_3$-(miR-122BS)$_3$ (three miR-1 and three miR-122-binding sites). The animals were necropsied 4 weeks after vector administration, and appropriate tissues were harvested for cryosectioning and X-gal histochemical staining. miR, microRNA; *nLacZ*, β-galactosidase reporter transgene; rAAV, recombinant adeno-associated viruses, and (d) quantification of β-galactosidase activities in liver tissue from animals that received rAAV*nLacZ* vectors with and without miRNA-binding sites.

Figure 14 depicts an analysis of expression levels of cognate miRNA, mRNA, and protein of endogenous miRNA target genes in mice transduced with rAAV9CB*nLacZ* with or without miRNA-binding sites. Total cellular RNA or protein was prepared from (a-c) liver or (d) heart. (a) Northern blot detection of miRNAs. U6 small nuclear RNA provided a loading control. (b) Quantitative reverse-transcription PCR measuring cyclin Gl mRNA. The data are presented as relative *cyclin Gl* mRNA levels normalized to β-actin. (c,d) Western blot analyses of protein levels of endogenous targets of miR-122 and miR-1. Total cellular protein prepared from (c) liver or (d) heart was analyzed for cyclin G1 and calmodulin. (e) Serum cholesterol levels. Serum samples from mice that received rAAV9 with or without miRNA-binding sites were collected after 4 weeks and measured for total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL). miR, microRNA; *nLacZ*, β-galactosidase reporter transgene; rAAV, recombinant adeno-associated viruses.

Figure 15 depicts a molecular characterization of transgene mRNAs with or without miRNA binding sites. (a) Locations of the probes and primers, the sequences of mature miR-122 and its perfectly complementary binding site in the transgene mRNA are presented. (b) Total cellular RNA from liver was analyzed either by conventional reverse-transcription PCR (RT-PCR) by using primers that span a region between the 3' end of *nLacZ* and the 5' end of poly(A) signal (c) or by quantitative RT-PCR; data are presented as relative nLacZ mRNA levels normalized to β-actin. (d) For the northern blot analysis of *nLacZ* mRNA, 18S RNA served as a loading control, and the blots were hybridized with either a transgene DNA (e) or RNA probe. (f) In addition, poly(A) bearing mRNA from the liver of an animal received rAAV containing three miR-1- and three miR-122-binding sites was analyzed by 5' RACE; the PCR product was resolved on an ethidium bromide-stained agarose gel. miR, microRNA; *nLacZ,* β-galactosidase reporter transgene; rAAV, recombinant adeno-associated viruses.

Figure 16 depicts an alignment of sequences spanning the miRNA-binding sites and poly(A) signal regions recovered by 5 RACE. Poly(A)-containing mRNA was isolated from the (a) liver and (b) heart of an animal injected with rAAV9C*BnLacZ*-(*miR-1BS*)$_3$-(*miR-122BS*)$_3$. Twenty-one liver-derived and twenty-two heart-derived clones were sequenced. The putative cleavage sites in each clone are identified by arrows; the frequencies of miRNA-directed, site-specific cleavage for each miRNA-binding site are reported; triangles point to the positions of the expected miRNA-directed cleavage sites (a,b). miRNA, microRNA, *nLacZ,* β-galactosidase reporter transgene; rAAV, recombinant adeno-associated viruses.

Figure 17 depicts an endogenous miRNA-repressed, CNS-directed EGFP gene transfer by systemically delivered rAAV9. Ten-week-old male C57BL/6 mice were injected intravenously with scAAV9CBEGFP or scAAV9CB*nLacZ*(miR-1BS)$_3$-(miR-122BS)$_3$ at a dose of 2 x 10^{14} genome copies per kg (GC/ kg) body weight. The animals were necropsied 3 weeks later for whole body fixation by transcardiac perfusion. (a) Brain, spinal cord, liver, heart, and muscle were harvested for cryosectioning, immunofluorescent staining for EGFP (brain and cervical spinal cord), and fluorescence microscopy to detect EGFP. Total cellular DNA and RNA were extracted from brain, liver, heart and muscle to measure the amount of persistent vector genome by qPCR and EGFP mRNA by qRT-PCR. (b) For each tissue, the relative abundance of the EGFP mRNA containing miRNA-binding sites was compared to that of the EGFP mRNA lacking miRNA-binding sites. For each sample, mRNA abundance was normalized to the amount of vector genome detected in the tissue. EGFP, enhanced green fluorescent protein; miRNA, microRNA; *nLacZ,* β-galactosidase reporter transgene; qRT-PCR, quantitative reverse-transcription PCR; rAAV, recombinant adeno-associated viruses.

Figure 18 depicts a molecular model for endogenous miRNA-regulated rAAV expression. miRNA, microRNA; rAAV, recombinant adeno-associated viruses.

Figure 19 depicts a quantification of GFP intensity levels in the brain and spinal cord of neonatal mice transduced with various AAV vectors. 4×10^{11} genome copies (GCs) of ten different AAV vectors were injected into neonatal PI pups through superfacial vein. The mice were sacrificed 21 days after injection. The brain tissues were extracted and 40 μm thick cryosections were prepared. The sections were stained against anti-EGFP antibody. The images were analyzed and the intensity/pixel values of all AAV serotypes in various regions in brain and spinal cord (A) were calculated by using Nikon NIS elements AR software version 3.2. Average intensities of the brain and spinal cord regions for different rAAVs were also presented (B). Region of interest (ROI) of each anatomical structure was fixed for all vectors to ensure the parallel comparison.

Figure 20 depicts a strong and widespread EGFP expression in neonatal mouse brain after intravenous injection of rAAVs. 4×10^{11} genome copies (GCs) of rAAVs 7, 9, rh.10, rh.39 and rh.43 were injected into neonatal PI pups through superfacial vein. The mice were sacrificed 21 days after injection. The brain tissues were extracted and 40 μm thick cryosections were prepared. The sections were stained against anti-EGFP antibody. Bars represent 100 μm. The regions shown are: olfactory bulb, striatum, hippocampus, cortex, hypothalamus, cerebellum and medulla.

Figure 21 depicts EGFP expression in neonatal mouse spinal cord after intravenous injection of rAAVs. 4×10^{11} GCs of rAAVs 7, 9, rh.10, rh.39 and rh.43 were injected into neonatal PI pups through superfacial vein. The mice were sacrificed 21 days after injection. The spinal cord tissues were extracted and 40 μm thick cryosections were prepared. The sections from cervical, thoracic and lumbar regions were stained against anti-EGFP antibody. Bars represent

100 µm.

Figure 22 depicts EGFP expression in dorsal root ganglia transduced by intravascularly delivered rAAVs1, 2, 6, 6.2, 7, 9, rh.10 and rh.39. Neonatal pups received 4×10^{11} GCs of rAAVs at PI and were necropsied 21 days after injection. Forty µm thick cryosections were processed for double immunohistochemical staining for EGFP (green) and Neurons (NeuN, red). Bars represent 75 µm.

Figure 23 depicts confocal microscopic analysis of the transduced cell types in mouse CNS after systemic delivery of rAAVs to PI neonates. The 40 µm thick brain and spinal cord sections of the animals treated with different rAAVs were co-strained against anti-EGFP antibody and cell-type specific markers. Anti-NeuN was used to stain neuronal cells; anti-GFAP was used to stain astrocytes; anti-Calbindin was used to stain Purkinje cells; anti-ChAT was used to stain motor neurons; anti-DARPP was used to stain dopaminergic neurons in the substantia nigra. All rAAVs were examined, but for each cell type, only one representative picture was shown here.

Figure 24 depicts a transduction of the brain ventricular structures by intravascularly delivered rAAVs. Neonatal pups received 4×10^{11} GCs of rAAVs at PI and were necropsied 21 days after injection. The choroid plexuses in different ventricles were well preserved during tissue process. Forty µm thick cryosections were stained against anti-EGFP antibody. Bars represent 100 µm.

Figure 25 depicts an analysis of purity and morphological integrity of rAAV vectors. A. Silver stained SDS-Page analysis of CsCl gradient purified rAAVCBEGFP vectors used in this study. Approximately 1.5 x 1010 virus particles each of rAAVs 1, 2, 5, 6, 6.2, 7, 9, rh10, rh39 and rh43 were loaded in the corresponding lane. B. Transmission electron microscopy of negative stained recombinant AAV virions. rAAV virions were spread on a freshly prepared carbon coated- Formvar support film and stained with 1% uranyl acetate for transmission microscopy. The images of virus particles from representative vector lots were taken at 92,000X and presented.

Figure 26 depicts a transduction of neonatal mouse dorsal root ganglia by systemically delivered rAAVs 1, 6, 6.2 and rh43. Neonatal pups received 4×10^{11} GCs of rAAVs at P1 were necropsied 21 days after injection. Forty µm thick cryosections were stained against anti-EGFP antibody. Bars represent 75 µm.

Figure 27 depicts a transduction of the brain capillary vessels by intravascularly delivered rAAVs. Neonatal pups received 4×10^{11} GCs of rAAVs at P1 were necropsied 21 days after injection. Forty µm thick cryosections of the brains were stained against: (a) anti-EGFP antibody (AAV1, AAV6, AAV6.2, AAV7, AAV9, AAVrh.10, AAVrh.39 and AAVrh.43); (b) anti-EGFP and anti-CD34 antibodies (rh.10 only). Bars represent 100 µm.

Figure 28 depicts an evaluation of microgliosis in mice brain after systemic delivery of rAAVs to PI neonates. The 40 µm thick brain sections of the animals treated with different rAAVs were co-strained against anti-EGFP antibody and anti-IBa-1. Only the staining result of rAAVrh.10 was shown.

Figure 29 depicts native EGFP expression in mice CNS after systemic delivery of rAAVs to P1 neonates. Neonatal pups received 4×10^{11} GCs of rAAVs at PI were necropsied 21 days after injection. Forty µm thick cryosections were mounted and observed under microscope without immunostaining. The exposure times for each image were indicated.

Figure 30 depicts results showing the effects of rAAV based gene therapy in the treatment of Canavan disease. Figure 30A shows that treatment corrected gait and motor function of the CD mice. Figure 30B shows that treatment mitigated retinopathy and restored vision in CD mice. Figure 30C shows that NAA levels in the treated CD mice approach those in the normal mice. Figure 30D indicates that APSA activity is detected in the brains of CD mice. Figure 30E indicates APSA expression is detected in the brains of CD mice.

Figure 31A depicts that vacuolation in both brain and spinal cord of the treated mice is more patchy and variable with generally smaller-sized vacuoles and that some areas of the cerebral cortex show almost no vacuolation. Figure 31B shows ASPA expression in the cerebral cortex *in situ.*

Figure 32 depicts results of a quantitative analysis of vacuolation in various brain regions. Figure 32A shows that olfactory bulb had a dramatic mitigation in the white matter degeneration after gene therapy and that the large vacuoles were essentially eliminated in other tissues. Figure 32B shows results from a similar analysis on spinal cord sections.

Figure 33 depicts results from a histopathological evaluation of kidneys in the CD mice. Figure 33A shows that the renal tubular epithelium of the kidney was diffusely attenuated and exhibited enlargement of the tubular lumens in untreated CD mice. Figure 33B shows that treated CD mouse had normal glomeruli. Figure 33C and 33D depict results of an analysis of two lead candidate vectors, rAAV9 and rh.10, respectively, for efficiency of kidney transduction after IV delivery.

DETAILED DESCRIPTION

[0022] Adeno-associated virus (AAV) is a small (26 nm) replication-defective, nonenveloped virus, that depends on the presence of a second virus, such as adenovirus or herpes virus, for its growth in cells. AAV is not known to cause disease and induces a very mild immune response. AAV can infect both dividing and non-dividing cells and may incorporate its genome into that of the host cell. Aspects of the disclosure herein provide methods for delivering a transgene to a CNS tissue in a subject using recombinant AAV-based gene transfer. Accordingly, methods and compositions for treating CNS-related disorders are provided herein. Further aspects of the disclosure herein, are based on the discovery of rAAVs that achieve wide-spread distribution throughout CNS tissue. In some cases, the rAAVs spread throughout CNS tissue following direct administration into the cerebrospinal fluid (CSF), *e.g.*, via intrathecal and/or intracerebral injection. In other cases, the rAAVs cross the blood-brain-barrier and achieve wide-spread distribution throughout CNS tissue of a subject following intravenous administration. Such rAAVs are useful for the treatment of CNS-related disorders, including, for example, amyotrophic lateral sclerosis (ALS) and Canavan disease (CD).

Methods and Compositions for Targeting CNS tissue

[0023] Methods for delivering a transgene to central nervous system (CNS) tissue in a subject are provided herein. The methods typically involve administering to a subject an effective amount of a rAAV comprising a nucleic acid vector for expressing a transgene in the subject. An "effective amount" of a rAAV is an amount sufficient to infect a sufficient number of cells of a target tissue in a subject. An effective amount of a rAAV may be an amount sufficient to have a therapeutic benefit in a subject, *e.g.*, to extend the lifespan of a subject, to improve in the subject one or more symptoms of disease, *e.g.*, a symptom of ALS, a symptom of Canavan disease, *etc.* In some cases, an effective amount of a rAAV may be an amount sufficient to produce a stable somatic transgenic animal model. The effective amount will depend on a variety of factors such as, for example, the species, age, weight, health of the subject, and the CNS tissue to be targeted, and may thus vary among subject and tissue. An effective amount may also depend on the mode of administration. For example, targeting a CNS tissue by intravascular injection may require different (*e.g.*, higher) doses, in some cases, than targeting CNS tissue by intrathecal or intracerebral injection. In some cases, multiple doses of a rAAV are administered. An effective amount may also depend on the rAAV used. For example, dosages for targeting a CNS tissue may depend on the serotype (*e.g.*, the capsid protein) of the rAAV. For example, the rAAV may have a capsid protein of a AAV serotype selected from the group consisting of: AAV1, AAV2, AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, rh.10, rh.39, rh.43 and CSp3. In certain cases, the effective amount of rAAV is 10^{10}, 10^{11}, 10^{12}, 10^{13}, or 10^{14} genome copies per kg. In certain cases, the effective amount of rAAV is 10^{10}, 10^{11}, 10^{12}, 10^{13}, 10^{14}, or 10^{15} genome copies per subject.

[0024] A method for delivering a transgene to CNS tissue in a subject may comprise administering a rAAV by a single route or by multiple routes. For example, delivering a transgene to CNS tissue in a subject may comprise administering to the subject, by intravenous administration, an effective amount of a rAAV that crosses the blood-brain-barrier. Delivering a transgene to CNS tissue in a subject may comprise administering to the subject an effective amount of a rAAV by intrathecal administration or intracerebral administration, e.g., by intraventricular injection. A method for delivering a transgene to CNS tissue in a subject may comprise co-administering of an effective amount of a rAAV by two different administration routes, *e.g.*, by intrathecal administration and by intracerebral administration. Co-administration may be performed at approximately the same time, or different times.

[0025] The CNS tissue to be targeted may be selected from cortex, hippocampus, thalamus, hypothalamus, cerebellum, brain stem, cervical spinal cord, thoracic spinal cord, and lumbar spinal cord, for example. The administration route for targeting CNS tissue typically depends on the AAV serotype. For example, in certain instances where the AAV serotype is selected from AAV1, AAV6, AAV6.2, AAV7, AAV8, AAV9, rh.10, rh.39, rh.43 and CSp3, the administration route may be intravascular injection. In some instances, for example where the AAV serotype is selected from AAV1, AAV2, AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, rh.10, rh.39, rh.43 and CSp3, the administration route may be intrathecal and/or intracerebral injection.

Intravascular Administration

[0026] As used herein the term "intravascular administration" refers to the administration of an agent, *e.g.*, a composition comprising a rAAV, into the vasculature of a subject, including the venous and arterial circulatory systems of the subject. Typically, rAAVs that cross the blood-brain-barrier may be delivered by intravascular administration for targeting CNS tissue. In some cases, intravascular (*e.g.*, intravenous) administration facilitates the use of larger volumes than other forms of administration (*e.g.*, intrathecal, intracerebral). Thus, large doses of rAAVs (*e.g.*, up to 10^{15} GC/subject) can be delivered at one time by intravascular (*e.g.*, intravenous) administration. Methods for intravascular administration are well known in the art and include for example, use of a hypodermic needle, peripheral cannula, central venous line, *etc.*

Intrathecal and/or Intracerebral Administration

[0027] As used herein the term "intrathecal administration" refers to the administration of an agent, *e.g.*, a composition comprising a rAAV, into the spinal canal. For example, intrathecal administration may comprise injection in the cervical region of the spinal canal, in the thoracic region of the spinal canal, or in the lumbar region of the spinal canal. Typically, intrathecal administration is performed by injecting an agent, *e.g.*, a composition comprising a rAAV, into the subarachnoid cavity (subarachnoid space) of the spinal canal, which is the region between the arachnoid membrane and pia mater of the spinal canal. The subarchnoid space is occupied by spongy tissue consisting of trabeculæ (delicate connective tissue filaments that extend from the arachnoid mater and blend into the pia mater) and intercommunicating channels in which the cerebrospinal fluid is contained. In some cases, intrathecal administration is not administration into the spinal vasculature.

[0028] As used herein, the term "intracerebral administration" refers to administration of an agent into and/or around the brain. Intracerebral administration includes, but is not limited to, administration of an agent into the cerebrum, medulla, pons, cerebellum, intracranial cavity, and meninges surrounding the brain. Intracerebral administration may include administration into the dura mater, arachnoid mater, and pia mater of the brain. Intracerebral administration may include, in some cases, administration of an agent into the cerebrospinal fluid (CSF) of the subarachnoid space surrounding the brain. Intracerebral administration may include, in some cases, administration of an agent into ventricles of the brain, *e.g.*, the right lateral ventricle, the left lateral ventricle, the third ventricle, the fourth ventricle. In some cases, intracerebral administration is not administration into the brain vasculature.

[0029] Intracerebral administration may involve direct injection into and/or around the brain. In some cases, intracerebral administration involves injection using stereotaxic procedures. Stereotaxic procedures are well know in the art and typically involve the use of a computer and a 3-dimensional scanning device that are used together to guide injection to a particular intracerebral region, *e.g.*, a ventricular region. Micro-injection pumps (*e.g.*, from World Precision Instruments) may also be used. In some cases, a microinjection pump is used to deliver a composition comprising a rAAV. In some cases, the infusion rate of the composition is in a range of 1µl / minute to 100 µl / minute. As will be appreciated by the skilled artisan, infusion rates will depend on a variety of factors, including, for example, species of the subject, age of the subject, weight/size of the subject, serotype of the AAV, dosage required, intracerebral region targeted, *etc.* Thus, other infusion rates may be deemed by a skilled artisan to be appropriate in certain circumstances.

Methods and Compositions for Treating CNS-related Disorders

[0030] Methods and compositions for treating CNS-related disorders are also provided herein. As used herein, a "CNS-related disorder" is a disease or condition of the central nervous system. A CNS-related disorder may affect the spinal cord (*e.g.*, a myelopathy), brain (*e.g.*, a encephalopathy) or tissues surrounding the brain and spinal cord. A CNS-related disorder may be of a genetic origin, either inherited or acquired through a somatic mutation. A CNS-related disorder may be a psychological condition or disorder, *e.g.*, Attention Deficient Hyperactivity Disorder, Autism Spectrum Disorder, Mood Disorder, Schizophrenia, Depression, Rett Syndrome, *etc.* A CNS-related disorder may be an autoimmune disorder. A CNS-related disorder may also be a cancer of the CNS, *e.g.*, brain cancer. A CNS-related disorder that is a cancer may be a primary cancer of the CNS, *e.g.*, an astrocytoma, glioblastomas, etc., or may be a cancer that has metastasized to CNS tissue, e.g., a lung cancer that has metastasized to the brain. Further non-limiting examples of CNS-related disorders, include Parkinson's Disease, Lysosomal Storage Disease, Ischemia, Neuropathic Pain, Amyotrophic lateral sclerosis (ALS), Multiple Sclerosis (MS), and Canavan disease (CD).

[0031] Methods for treating amyotrophic lateral sclerosis (ALS) in a subject in need thereof are provided herein. A subject in need of a treatment for ALS is a subject having or suspected of having ALS. In some cases, ALS has been linked to a mutation in the gene coding for superoxide dismutase (SOD1). Elevated levels of SOD1 appear to be associated with ALS in some instances. It has been shown that transgenic expression of shRNA against SOD1 can knockdown mutant SOD1 expression, delay disease onset and extend survival (Xia et al. 2006, Neurobiol Dis 23: 578). Intrathecal infusion of siRNA against SOD1 at disease onset has also been found to knockdown mutant SOD1 expression and extend survival (Wang et al. 2008, JBC 283: 15845). Furthermore, nerve injection of adenovirus expressing shRNA against SOD1 at the disease onset can knockdown mutant SOD1 expression and extend survival (Wu et al. 2009, Antiox Redox Sig 11: 1523).

[0032] Aspects of the disclosure herein, are based on the discovery of AAV-based therapies that achieve, with low-toxicity, long-term inhibition of SOD1 expression that is wide-spread throughout CNS tissue of the subject. Methods for treating ALS that are provided herein, typically involve administering to CNS tissue of a subject an effective amount of a rAAV that harbors a nucleic acid comprising a promoter operably linked with a region encoding an inhibitory RNA that binds specifically to SOD1 mRNA (*e.g.*, that hybridizes specifically to a nucleic acid having a sequence as set forth in SEQ ID NO 17 or 19) and inhibits expression of SOD1 in the subject. It has been discovered that rAAVs having a capsid protein comprising a sequence as set forth in SEQ ID NO: 9 achieve wide-spread distribution throughout the CNS following intrathecal injection and/or intracerebral injection, and thus, are particularly useful for treating ALS. This result is surprising in light of certain other rAAVs that infect cells only within the immediate vicinity of the injection site, or the achieve only a limited distribution, following intrathecal injection. Thus, rAAVs that achieve wide-spread distribution throughout the CNS are particularly useful as gene transfer vectors for treating ALS.

[0033] In some cases, it has been discovered that co-administration by intrathecal injection and intracerebral injection, *e.g.*, intraventricular injection, of rAAVs having a capsid protein comprising a sequence as set forth in SEQ ID NO: 9 and a nucleic acid comprising a promoter operably linked with a region encoding an inhibitory RNA that binds specifically to SOD1 mRNA and inhibits expression of SOD1, achieves long-term inhibition of SOD1 and improves outcome (*e.g.*, lifespan) in an animal model of ALS (See, *e.g.*, Figure 6A). In some cases, the inhibitory RNA is an antisense RNA, a shRNA or a miRNA. The inhibitory RNA may have a sequence as set forth in SEQ ID NO: 26. The inhibitory RNA may have a sequence as set forth in any one of SEQ ID NO: 22 to 30. Thus, in some cases, a nucleic acid comprising a promoter operably linked with a nucleic acid having a sequence as set forth in any one of SEQ ID NO: 22 to 30 is provided. In some cases, a recombinant AAV that harbors a nucleic acid comprising a sequence as set forth in any one of SEQ ID NO: 22 to 30 is provided. The recombinant AAV may have a capsid protein comprising a sequence as set forth in SEQ ID NO: 9. The recombinant AAV may have a capsid protein comprising a sequence as set forth in any one of SEQ ID NO: 1 to 12.

[0034] Methods for treating Canavan disease (CD) in a subject in need thereof are provided herein. A subject in need of a treatment for CD is a subject having or suspected of having CD. Canavan disease is caused by a defective ASPA gene which is responsible for the production of the enzyme aspartoacylase. This enzyme normally breaks down the concentrated brain molecule N-acetyl aspartate. Decreased aspartoacylase activity in subjects with CD prevents the normal breakdown of N-acetyl aspartate, and the lack of breakdown appears to interfere with growth of the myelin sheath of the nerve fibers in the brain. Symptoms of Canavan disease, which may appear in early infancy and progress rapidly, may include mental retardation, loss of previously acquired motor skills, feeding difficulties, abnormal muscle tone (i.e., floppiness or stiffness), poor head control, and megalocephaly (abnormally enlarged head). Paralysis, blindness, or seizures may also occur. The disclosure herein may improve one or more symptoms of CD in a subject by administering to the subject a recombinant AAV harboring a nucleic acid that expresses aspartoacylase (ASPA). For example, a method for treating Canavan disease in a subject in need thereof may comprise administering an effective amount of a rAAV to CNS tissue of the subject by intravascular administration, wherein the rAAV comprises a nucleic acid comprising a promoter operably linked with a region encoding ASPA (*e.g.*, a region having a sequence as set forth in SEQ ID NO: 14 or 16). A method for treating Canavan disease in a subject in need thereof may comprise administering an effective amount of a rAAV to CNS tissue of the subject by intrathecal administration, wherein the rAAV comprises a nucleic acid comprising a promoter operably linked with a region encoding ASPA. In some cases, methods for treating CD involve administering, to CNS tissue of the subject, an effective amount of a rAAV that comprises a capsid protein other than a capsid protein of AAV serotype 2 (e.g., other than a protein having an amino acid sequence as set forth in SEQ ID NO: 2) and a nucleic acid comprising a promoter operably linked with a region encoding ASPA. In another example, a method for treating Canavan disease in a subject in need thereof comprises administering an effective amount of a rAAV to CNS tissue of the subject by a route other than intracerebral administration, wherein the rAAV comprises a nucleic acid comprising a promoter operably linked with a region encoding ASPA. In some cases, ASPA expressed in CNS tissue following administration of the rAAV results in a decrease in aspartoacylase activity and breakdown of N-acetyl aspartate in the CNS tissue. Thus, in some cases, a recombinant AAV vector is provided that comprises a nucleic acid encoding a sequence as set forth in SEQ ID NO: 14 or 16. In some cases, a recombinant AAV is provided that harbors a nucleic acid comprising a promoter operably linked with a region having a sequence as set forth in SEQ ID NO: 14 or 16. In some cases, a recombinant AAV is provided that harbors a nucleic acid comprising a promoter operably linked with a region encoding a protein having a sequence as set forth in SEQ ID NO: 13 or 15. The recombinant AAV may have a capsid protein comprising an amino acid sequence as set forth in any one of SEQ ID NO: 1 to 12. The recombinant AAV may have a capsid protein comprising a sequence as set forth in any one of SEQ ID NO: 1 and 3 to 12.

Recombinant AAVs

[0035] There is disclosed isolated AAVs. As used herein with respect to AAVs, the term "isolated" refers to an AAV that has been isolated from its natural environment (*e.g.*, from a host cell, tissue, or subject) or artificially produced. Isolated AAVs may be produced using recombinant methods. Such AAVs are referred to herein as "recombinant AAVs". Recombinant AAVs (rAAVs) preferably have tissue-specific targeting capabilities, such that a transgene of the rAAV will be delivered specifically to one or more predetermined tissue(s). The AAV capsid is an important element in determining these tissue-specific targeting capabilities. Thus, a rAAV having a capsid appropriate for the tissue being targeted can be selected. In some cases, the rAAV comprises a capsid protein having an amino acid sequence as set forth in any one of

SEQ ID NOs 1 to 12, or a protein having substantial homology thereto.

[0036] Methods for obtaining recombinant AAVs having a desired capsid protein are well known in the art (See, for example, US 2003/0138772). AAVs capsid protein that may be used in the rAAVs disclosed herein a include, for example, those disclosed in G. Gao, et al., J. Virol, 78(12):6381-6388 (June 2004); G. Gao, et al, Proc Natl Acad Sci USA, 100(10):6081-6086 (May 13, 2003); US 2003-0138772, US 2007/0036760, US 2009/0197338, and U.S. provisional application serial number 61/182,084, filed May 28, 2009. Typically the methods involve culturing a host cell which contains a nucleic acid sequence encoding an AAV capsid protein (*e.g.*, a nucleic acid encoding a protein having a sequence as set forth in any one of SEQ ID NOs 1-12) or fragment thereof; a functional *rep* gene; a recombinant AAV vector composed of, AAV inverted terminal repeats (ITRs) and a transgene; and sufficient helper functions to permit packaging of the recombinant AAV vector into the AAV capsid proteins.

[0037] The components to be cultured in the host cell to package a rAAV vector in an AAV capsid may be provided to the host cell in *trans.* Alternatively, any one or more of the required components (*e.g.*, recombinant AAV vector, rep sequences, cap sequences, and/or helper functions) may be provided by a stable host cell which has been engineered to contain one or more of the required components using methods known to those of skill in the art. Most suitably, such a stable host cell will contain the required component(s) under the control of an inducible promoter. However, the required component(s) may be under the control of a constitutive promoter. Examples of suitable inducible and constitutive promoters are provided herein, in the discussion of regulatory elements suitable for use with the transgene. In still another alternative, a selected stable host cell may contain selected component(s) under the control of a constitutive promoter and other selected component(s) under the control of one or more inducible promoters. For example, a stable host cell may be generated which is derived from 293 cells (which contain E1 helper functions under the control of a constitutive promoter), but which contain the rep and/or cap proteins under the control of inducible promoters. Still other stable host cells may be generated by one of skill in the art.

[0038] The recombinant AAV vector, rep sequences, cap sequences, and helper functions required for producing the rAAV disclosed herein may be delivered to the packaging host cell using any appropriate genetic element (vector). The selected genetic element may be delivered by any suitable method, including those described herein. The methods used to construct any example disclosed herein are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. See, *e.g.*, Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. Similarly, methods of generating rAAV virions are well known and the selection of a suitable method is not a limitation on the present disclosure. See, *e.g.*, K. Fisher et al, J. Virol., 70:520-532 (1993) and U.S. Pat. No. 5,478,745.

[0039] In some cases, recombinant AAVs may be produced using the triple transfection method (e.g., as described in detail in U.S. Pat. No. 6,001,650). Typically, the recombinant AAVs are produced by transfecting a host cell with a recombinant AAV vector (comprising a transgene) to be packaged into AAV particles, an AAV helper function vector, and an accessory function vector. An AAV helper function vector encodes the "AAV helper function" sequences (*i.e.*, rep and cap), which function in *trans* for productive AAV replication and encapsidation. Preferably, the AAV helper function vector supports efficient AAV vector production without generating any detectable wild-type AAV virions (*i.e.*, AAV virions containing functional rep and cap genes). Non-limiting examples of vectors suitable for use with the present disclosure include pHLP19, described in U.S. Pat. No. 6,001,650 and pRep6cap6 vector, described in U.S. Pat. No. 6,156,303. The accessory function vector encodes nucleotide sequences for non-AAV derived viral and/or cellular functions upon which AAV is dependent for replication (*i.e.*, "accessory functions"). The accessory functions include those functions required for AAV replication, including, without limitation, those moieties involved in activation of AAV gene transcription, stage specific AAV mRNA splicing, AAV DNA replication, synthesis of cap expression products, and AAV capsid assembly. Viral-based accessory functions can be derived from any of the known helper viruses such as adenovirus, herpesvirus (other than herpes simplex virus type-1), and vaccinia virus.

[0040] In some aspects, the disclosure provides transfected host cells. The term "transfection" is used to refer to the uptake of foreign DNA by a cell, and a cell has been "transfected" when exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are generally known in the art. See, *e.g.*, Graham et al. (1973) Virology, 52:456, Sambrook et al. (1989) Molecular Cloning, a laboratory manual, Cold Spring Harbor Laboratories, New York, Davis et al. (1986) Basic Methods in Molecular Biology, Elsevier, and Chu et al. (1981) Gene 13:197. Such techniques can be used to introduce one or more exogenous nucleic acids, such as a nucleotide integration vector and other nucleic acid molecules, into suitable host cells.

[0041] A "host cell" refers to any cell that harbors, or is capable of harboring, a substance of interest. Often a host cell is a mammalian cell. A host cell may be used as a recipient of an AAV helper construct, an AAV minigene plasmid, an accessory function vector, or other transfer DNA associated with the production of recombinant AAVs. The term includes the progeny of the original cell which has been transfected. Thus, a "host cell" as used herein may refer to a cell which has been transfected with an exogenous DNA sequence. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

[0042] In some aspects, the disclosure provides isolated cells. As used herein with respect to cell, the term "isolated" refers to a cell that has been isolated from its natural environment (*e.g.*, from a tissue or subject). As used herein, the term "cell line" refers to a population of cells capable of continuous or prolonged growth and division *in vitro*. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants. As used herein, the terms "recombinant cell" refers to a cell into which an exogenous DNA segment, such as DNA segment that leads to the transcription of a biologically-active polypeptide or production of a biologically active nucleic acid such as an RNA, has been introduced.

[0043] As used herein, the term "vector" includes any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, artificial chromosome, virus, virion, *etc*., which is capable of replication when associated with the proper control elements and which can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors. In some cases, useful vectors are contemplated to be those vectors in which the nucleic acid segment to be transcribed is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned," "under control" or "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid in which part or all of the nucleic acid encoding sequence is capable of being transcribed. In some cases, expression includes transcription of the nucleic acid, for example, to generate a biologically-active polypeptide product or inhibitory RNA (*e.g.*, shRNA, miRNA) from a transcribed gene.

[0044] The foregoing methods for packaging recombinant vectors in desired AAV capsids to produce the rAAVs of the disclosure are not meant to be limiting and other suitable methods will be apparent to the skilled artisan.

Recombinant AAV vectors

[0045] "Recombinant AAV (rAAV) vectors" are typically composed of, at a minimum, a transgene and its regulatory sequences, and 5' and 3' AAV inverted terminal repeats (ITRs). It is this recombinant AAV vector which is packaged into a capsid protein and delivered to a selected target cell. In some cases, the transgene is a nucleic acid sequence, heterologous to the vector sequences, which encodes a polypeptide, protein, functional RNA molecule (*e.g.*, miRNA, miRNA inhibitor) or other gene product, of interest. The nucleic acid coding sequence is operatively linked to regulatory components in a manner which permits transgene transcription, translation, and/or expression in a cell of a target tissue.

[0046] The AAV sequences of the vector typically comprise the cis-acting 5' and 3' inverted terminal repeat sequences (See, *e.g.*, B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp. 155 168 (1990)). The ITR sequences are about 145 bp in length. Preferably, substantially the entire sequences encoding the ITRs are used in the molecule, although some degree of minor modification of these sequences is permissible. The ability to modify these ITR sequences is within the skill of the art. (See, *e.g.*, texts such as Sambrook et al, "Molecular Cloning. A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory, New York (1989); and K. Fisher et al., J Virol., 70:520 532 (1996)). An example of such a molecule employed herein is a "cis-acting" plasmid containing the transgene, in which the selected transgene sequence and associated regulatory elements are flanked by the 5' and 3' AAV ITR sequences. The AAV ITR sequences may be obtained from any known AAV, including presently identified mammalian AAV types.

[0047] In addition to the major elements identified above for the recombinant AAV vector, the vector also includes conventional control elements which are operably linked to the transgene in a manner which permits its transcription, translation and/or expression in a cell transfected with the plasmid vector or infected with the virus produced herein. As used herein, "operably linked" sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in trans or at a distance to control the gene of interest. Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*i.e.*, Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. A great number of expression control sequences, including promoters which are native, constitutive, inducible and/or tissue-specific, are known in the art and may be utilized.

[0048] As used herein, a nucleic acid sequence (e.g., coding sequence) and regulatory sequences are said to be operably linked when they are covalently linked in such a way as to place the expression or transcription of the nucleic acid sequence under the influence or control of the regulatory sequences. If it is desired that the nucleic acid sequences be translated into a functional protein, two DNA sequences are said to be operably linked if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably linked to a nucleic acid sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide. Similarly two or more coding regions are operably linked when they are linked in such a way that their transcription from a common promoter results in the expression of two or more proteins having been translated in frame. In some cases, operably linked coding sequences yield a fusion protein. In some cases, operably linked coding sequences yield a functional RNA (*e.g.*, shRNA, miRNA).

[0049] For nucleic acids encoding proteins, a polyadenylation sequence generally is inserted following the transgene sequences and before the 3' AAV ITR sequence. A rAAV construct useful herein may also contain an intron, desirably located between the promoter/enhancer sequence and the transgene. One possible intron sequence is derived from SV-40, and is referred to as the SV-40 T intron sequence. Another vector element that may be used is an internal ribosome entry site (IRES). An IRES sequence is used to produce more than one polypeptide from a single gene transcript. An IRES sequence would be used to produce a protein that contain more than one polypeptide chains. Selection of these and other common vector elements are conventional and many such sequences are available [see, *e.g.*, Sambrook et al, and references cited therein at, for example, pages 3.18 3.26 and 16.17 16.27 and Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1989]. In some cases, a Foot and Mouth Disease Virus 2A sequence is included in polyprotein; this is a small peptide (approximately 18 amino acids in length) that has been shown to mediate the cleavage of polyproteins (Ryan, M D et al., EMBO, 1994; 4: 928-933; Mattion, N M et al., J Virology, November 1996; p. 8124-8127; Furler, S et al., Gene Therapy, 2001; 8: 864-873; and Halpin, C et al., The Plant Journal, 1999; 4: 453-459). The cleavage activity of the 2A sequence has previously been demonstrated in artificial systems including plasmids and gene therapy vectors (AAV and retroviruses) (Ryan, M D et al., EMBO, 1994; 4: 928-933; Mattion, N M et al., J Virology, November 1996; p. 8124-8127; Furler, S et al., Gene Therapy, 2001; 8: 864-873; and Halpin, C et al., The Plant Journal, 1999; 4: 453-459; de Felipe, P et al., Gene Therapy, 1999; 6: 198-208; de Felipe, P et al., Human Gene Therapy, 2000; 11: 1921-1931.; and Klump, H et al., Gene Therapy, 2001; 8: 811-817).

[0050] The precise nature of the regulatory sequences needed for gene expression in host cells may vary between species, tissues or cell types, but shall in general include, as necessary, 5' non-transcribed and 5' non-translated sequences involved with the initiation of transcription and translation respectively, such as a TATA box, capping sequence, CAAT sequence, enhancer elements, and the like. Especially, such 5' non-transcribed regulatory sequences will include a promoter region that includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory sequences may also include enhancer sequences or upstream activator sequences as desired. The vectors hereinmay optionally include 5' leader or signal sequences. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art.

[0051] Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, *e.g.*, Boshart et al, Cell, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the β-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1α promoter [Invitrogen].

[0052] Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, *e.g.*, acute phase, a particular differentiation state of the cell, or in replicating cells only. Inducible promoters and inducible systems are available from a variety of commercial sources, including, without limitation, Invitrogen, Clontech and Ariad. Many other systems have been described and can be readily selected by one of skill in the art. Examples of inducible promoters regulated by exogenously supplied promoters include the zinc-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No et al, Proc. Natl. Acad. Sci. USA, 93:3346-3351

(1996)), the tetracycline-repressible system (Gossen et al, Proc. Natl. Acad. Sci. USA, 89:5547-5551 (1992)), the tetracycline-inducible system (Gossen et al, Science, 268:1766-1769 (1995), see also Harvey et al, Curr. Opin. Chem. Biol., 2:512-518 (1998)), the RU486-inducible system (Wang et al, Nat. Biotech., 15:239-243 (1997) and Wang et al, Gene Ther., 4:432-441 (1997)) and the rapamycin-inducible system (Magari et al, J. Clin. Invest., 100:2865-2872 (1997)). Still other types of inducible promoters which may be useful in this context are those which are regulated by a specific physiological state, *e.g.*, temperature, acute phase, a particular differentiation state of the cell, or in replicating cells only.

[0053] In another case, the native promoter, or fragment thereof, for the transgene will be used. The native promoter may be preferred when it is desired that expression of the transgene should mimic the native expression. The native promoter may be used when expression of the transgene must be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further case, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression.

[0054] In some cases, the regulatory sequences impart tissue-specific gene expression capabilities. In some cases, the tissue-specific regulatory sequences bind tissue-specific transcription factors that induce transcription in a tissue specific manner. Such tissue-specific regulatory sequences (*e.g.*, promoters, enhancers, *etc.*.) are well known in the art. Exemplary tissue-specific regulatory sequences include, but are not limited to the following tissue specific promoters: neuronal such as neuron-specific enolase (NSE) promoter (Andersen et al., Cell. Mol. Neurobiol., 13:503-15 (1993)), neurofilament light-chain gene promoter (Piccioli et al., Proc. Natl. Acad. Sci. USA, 88:5611-5 (1991)), and the neuron-specific vgf gene promoter (Piccioli et al., Neuron, 15:373-84 (1995)). In some cases, the tissue-specific promoter is a promoter of a gene selected from: neuronal nuclei (NeuN), glial fibrillary acidic protein (GFAP), adenomatous polyposis coli (APC), and ionized calcium-binding adapter molecule 1 (Iba-1). Other appropriate tissue specific promoters will be apparent to the skilled artisan. In some cases, the promoter is a chicken Beta-actin promoter.

[0055] In some cases, one or more bindings sites for one or more of miRNAs are incorporated in a transgene of a rAAV vector, to inhibit the expression of the transgene in one or more tissues of a subject harboring the transgenes, e.g., non-CNS tissues. The skilled artisan will appreciate that binding sites may be selected to control the expression of a transgene in a tissue specific manner. For example, expression of a transgene in the liver may be inhibited by incorporating a binding site for miR-122 such that mRNA expressed from the transgene binds to and is inhibited by miR-122 in the liver. Expression of a transgene in the heart may be inhibited by incorporating a binding site for miR-133a or miR-1, such that mRNA expressed from the transgene binds to and is inhibited by miR-133a or miR-1 in the heart. The miRNA target sites in the mRNA may be in the 5' UTR, the 3' UTR or in the coding region. Typically, the target site is in the 3' UTR of the mRNA. Furthermore, the transgene may be designed such that multiple miRNAs regulate the mRNA by recognizing the same or multiple sites. The presence of multiple miRNA binding sites may result in the cooperative action of multiple RISCs and provide highly efficient inhibition of expression. The target site sequence may comprise a total of 5-100, 10-60, or more nucleotides. The target site sequence may comprise at least 5 nucleotides of the sequence of a target gene binding site.

Transgene Coding Sequences: CNS-Related Genes

[0056] The composition of the transgene sequence of a rAAV vector will depend upon the use to which the resulting vector will be put. For example, one type of transgene sequence includes a reporter sequence, which upon expression produces a detectable signal. In another example, the transgene encodes a therapeutic protein or therapeutic functional RNA. In another example, the transgene encodes a protein or functional RNA that is intended to be used for research purposes, *e.g.*, to create a somatic transgenic animal model harboring the transgene, *e.g.*, to study the function of the transgene product. In another example, the transgene encodes a protein or functional RNA that is intended to be used to create an animal model of disease. Appropriate transgene coding sequences will be apparent to the skilled artisan.

[0057] There is disclosed rAAV vectors for use in methods of preventing or treating one or more gene defects (*e.g.*, heritable gene defects, somatic gene alterations) in a mammal, such as for example, a gene defect that results in a polypeptide deficiency or polypeptide excess in a subject, and particularly for treating or reducing the severity or extent of deficiency in a subject manifesting a CNS-associated disorder linked to a deficiency in such polypeptides in cells and tissues. In some cases, methods involve administration of a rAAV vector that encodes one or more therapeutic peptides, polypeptides, shRNAs, microRNAs, antisense nucleotides, *etc.* in a pharmaceutically-acceptable carrier to the subject in an amount and for a period of time sufficient to treat the CNS-associated disorder in the subject having or suspected of having such a disorder.

[0058] A rAAV vector may comprise as a transgene, a nucleic acid encoding a protein or functional RNA that modulates or treats a CNS-associated disorder. The following is a non-limiting list of genes associated with CNS-associated disorders: neuronal apoptosis inhibitory protein (NAIP), nerve growth factor (NGF), glial-derived growth factor (GDNF), brain-derived growth factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxlase (TH), GTP-cyclohydrolase (GTPCH), aspartoacylase (ASPA), superoxide dismutase (SOD1) and amino acid decorboxylase (AADC). For example, a useful transgene in the treatment of Parkinson's disease encodes TH, which is a rate limiting enzyme in the synthesis of dopamine. A transgene encoding GTPCH, which generates the TH cofactor tetrahydrobiopterin, may also be used in the treatment of Parkinson's disease. A transgene encoding GDNF or BDNF, or AADC, which facilitates conversion of L-Dopa to DA, may also be used for the treatment of Parkinson's disease. For the treatment of ALS, a useful transgene may encode: GDNF, BDNF or CNTF. Also for the treatment of ALS, a useful transgene may encode a functional RNA, *e.g.*, shRNA, miRNA, that inhibits the expression of SOD1 . For the treatment of ischemia a useful transgene may encode NAIP or NGF. A transgene encoding Beta-glucuronidase (GUS) may be useful for the treatment of certain lysosomal storage diseases (*e.g.*, Mucopolysacharidosis type VII (MPS VII)). A transgene encoding a prodrug activation gene, *e.g.*, HSV-Thymidine kinase which converts ganciclovir to a toxic nucleotide which disrupts DNA synthesis and leads to cell death, may be useful for treating certain cancers, *e.g.*, when administered in combination with the prodrug. A transgene encoding an endogenous opioid, such a β-endorphin may be useful for treating pain. Other examples of transgenes that may be used in the rAAV will be apparent to the skilled artisan (See, e.g., Costantini LC, et al., Gene Therapy (2000) 7, 93-109).

[0059] In some cases, the cloning capacity of the recombinant RNA vector may be limited and a desired coding sequence may involve the complete replacement of the virus's 4.8 kilobase genome. Large genes may, therefore, not be suitable for use in a standard recombinant AAV vector, in some cases. The skilled artisan will appreciate that options are available in the art for overcoming a limited coding capacity. For example, the AAV ITRs of two genomes can anneal to form head to tail concatamers, almost doubling the capacity of the vector. Insertion of splice sites allows for the removal of the ITRs from the transcript. Other options for overcoming a limited cloning capacity will be apparent to the skilled artisan.

Recombinant AAV Administration

[0060] rAAVS are administered in sufficient amounts to transfect the cells of a desired tissue and to provide sufficient levels of gene transfer and expression without undue adverse effects. Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to the selected tissue (*e.g.*, intracerebral administration, intrathecal administration), intravenous, oral, inhalation (including intranasal and intratracheal delivery), intraocular, intravenous, intramuscular, subcutaneous, intradermal, intratumoral, and other parental routes of administration. Routes of administration may be combined, if desired.

[0061] Delivery of certain rAAVs to a subject may be, for example, by administration into the bloodstream of the subject. Administration into the bloodstream may be by injection into a vein, an artery, or any other vascular conduit. Moreover, in certain instances, it may be desirable to deliver the rAAVs to brain tissue, meninges, neuronal cells, glial cells, astrocytes, oligodendrocytes, cereobrospinal fluid (CSF), interstitial spaces and the like. In some cases, recombinant AAVs may be delivered directly to the spinal cord or brain by injection into the ventricular region, as well as to the striatum (*e.g.*, the caudate nucleus or putamen of the striatum), and neuromuscular junction, or cerebellar lobule, with a needle, catheter or related device, using neurosurgical techniques known in the art, such as by stereotactic injection (see, *e.g.*, Stein et al., J Virol 73:3424-3429, 1999; Davidson et al., PNAS 97:3428-3432, 2000; Davidson et al., Nat. Genet. 3:219-223, 1993; and Alisky and Davidson, Hum. Gene Ther. 11:2315-2329, 2000). In certain circumstances it will be desirable to deliver the rAAV-based therapeutic constructs in suitably formulated pharmaceutical compositions disclosed herein either subcutaneously, intrapancreatically, intranasally, parenterally, intravenously, intramuscularly, intracerebrally, intrathecally, intracerebrally, orally, intraperitoneally, or by inhalation. In some cases, the administration modalities as described in U.S. Pat. Nos.

5,543,158; 5,641,515 and 5,399,363 may be used to deliver rAAVs.

Recombinant AAV Compositions

[0062] The rAAVs may be delivered to a subject in compositions according to any appropriate methods known in the art. The rAAV, preferably suspended in a physiologically compatible carrier (e.g., in a composition), may be administered to a subject, *e.g.*, a human, mouse, rat, cat, dog, sheep, rabbit, horse, cow, goat, pig, guinea pig, hamster, chicken, turkey, or a non-human primate (e.g, Macaque). The compositions of the disclosure may comprise a rAAV alone, or in combination with one or more other viruses (*e.g.*, a second rAAV encoding having one or more different transgenes). In some cases, a compositions comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more different rAAVs each having one or more different transgenes.

[0063] Suitable carriers may be readily selected by one of skill in the art in view of the indication for which the rAAV is directed. For example, one suitable carrier includes saline, which may be formulated with a variety of buffering solutions (*e.g.*, phosphate buffered saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, and water. The selection of the carrier is not a limitation of the present disclosure.

[0064] Optionally, the compositions may contain, in addition to the rAAV and carrier(s), other conventional pharmaceutical ingredients, such as preservatives, or chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

[0065] The dose of rAAV virions required to achieve a desired effect or "therapeutic effect," *e.g.*, the units of dose in vector genomes/per kilogram of body weight (vg/kg), will vary based on several factors including, but not limited to: the route of rAAV administration, the level of gene or RNA expression required to achieve a therapeutic effect, the specific disease or disorder being treated, and the stability of the gene or RNA product. One of skill in the art can readily determine a rAAV virion dose range to treat a subject having a particular disease or disorder based on the aforementioned factors, as well as other factors that are well known in the art. An effective amount of the rAAV is generally in the range of from about 10 μl to about 100 ml of solution containing from about 10^9 to 10^{16} genome copies per subject. Other volumes of solution may be used. The volume used will typically depend, among other things, on the size of the subject, the dose of the rAAV, and the route of administration. For example, for intrathecal or intracerebral administration a volume in range of 1 μl to 10 μl or 10 μl to 100 μl may be used. For intravenous administration a volume in range of 10 μl to 100 μ1, 100 μl to 1 ml, 1 ml to 10 ml, or more may be used. In some cases, a dosage between about 10^{10} to 10^{12} rAAV genome copies per subject is appropriate. In certain cases, 10^{12} rAAV genome copies per subject is effective to target CNS tissues. In some cases the rAAV is administered at a dose of 10^{10}, 10^{11}, 10^{12}, 10^{13}, 10^{14}, or 10^{15} genome copies per subject. In some cases the rAAV is administered at a dose of 10^{10}, 10^{11}, 10^{12}, 10^{13}, or 10^{14} genome copies per kg.

[0066] In some cases, rAAV compositions are formulated to reduce aggregation of AAV particles in the composition, particularly where high rAAV concentrations are present (*e.g.*, ~10^{13} GC/ml or more). Methods for reducing aggregation of rAAVs are well known in the art and, include, for example, addition of surfactants, pH adjustment, salt concentration adjustment, *etc.* (See, *e.g.*, Wright FR, et al., Molecular Therapy (2005) 12, 171-178.)

[0067] Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens. Typically, these formulations may contain at least about 0.1% of the active ingredient or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 70% or 80% or more of the weight or volume of the total formulation. Naturally, the amount of active ingredient in each therapeutically-useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[0068] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. In many cases the form is sterile and fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0069] For administration of an injectable aqueous solution, for example, the solution may be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the host. The person responsible for administration will, in any event, determine the appropriate dose for the individual host.

[0070] Sterile injectable solutions are prepared by incorporating the active rAAV in the required amount in the appropriate solvent with various of the other ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0071] The rAAV compositions disclosed herein may also be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug-release capsules, and the like.

[0072] As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Supplementary active ingredients can also be incorporated into the compositions. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a host.

[0073] Delivery vehicles such as liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, may be used for the introduction of the compositions into suitable host cells. In particular, the rAAV vector delivered *transgenes* may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like.

[0074] Such formulations may be preferred for the introduction of pharmaceutically acceptable formulations of the nucleic acids or the rAAV constructs disclosed herein. The formation and use of liposomes is generally known to those of skill in the art. Recently, liposomes were developed with improved serum stability and circulation half-times (U.S. Pat. No. 5,741,516). Further, various methods of liposome and liposome like preparations as potential drug carriers have been described (; U.S. Pat. Nos. 5,567,434; 5,552,157; 5,565,213; 5,738,868 and 5,795,587).

[0075] Liposomes have been used successfully with a number of cell types that are normally resistant to transfection by other procedures. In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, drugs, radiotherapeutic agents, viruses, transcription factors and allosteric effectors into a variety of cultured cell lines and animals. In addition, several successful clinical trails examining the effectiveness of liposome-mediated drug delivery have been completed.

[0076] Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs). MLVs generally have diameters of from 25 nm to 4 μm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 .ANG., containing an aqueous solution in the core.

[0077] Alternatively, nanocapsule formulations of the rAAV may be used. Nanocapsules can generally entrap substances in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use.

[0078] In addition to the methods of delivery described above, the following techniques are also contemplated as alternative methods of delivering the rAAV compositions to a host. Sonophoresis (ie., ultrasound) has been used and described in U.S. Pat. No. 5,656,016 as a device for enhancing the rate and efficacy of drug permeation into and through the circulatory system. Other drug delivery alternatives contemplated are intraosseous injection (U.S. Pat. No. 5,779,708), microchip devices (U.S. Pat. No. 5,797,898), ophthalmic formulations (Bourlais et al., 1998), transdermal matrices (U.S. Pat. Nos. 5,770,219 and 5,783,208) and feedback-controlled delivery (U.S. Pat. No. 5,697,899).

Kits and Related Compositions

[0079] The agents described herein may, in some cases, be assembled into pharmaceutical or diagnostic or research kits to facilitate their use in therapeutic, diagnostic or research applications. A kit may include one or more containers housing the components disclosed above and instructions for use. Specifically, such kits may include one or more agents described herein, along with instructions describing the intended application and the proper use of these agents. In certain cases agents in a kit may be in a pharmaceutical formulation and dosage suitable for a particular application and for a method of administration of the agents. Kits for research purposes may contain the components in appropriate concentrations or quantities for running various experiments.

[0080] The kit may be designed to facilitate use of the methods described herein by researchers and can take many forms. Each of the compositions of the kit, where applicable, may be provided in liquid form (*e.g.*, in solution), or in solid form, (*e.g.*, a dry powder). In certain cases, some of the compositions may be constitutable or otherwise processable (*e.g.*, to an active form), for example, by the addition of a suitable solvent or other species (for example, water or a cell culture medium), which may or may not be provided with the kit. As used herein, "instructions" can define a component of instruction and/or promotion, and typically involve written instructions on or associated with packaging disclosed herein. Instructions also can include any oral or electronic instructions provided in any manner such that a user will clearly recognize that the instructions are to be associated with the kit, for example, audiovisual (*e.g.*, videotape, DVD, *etc.*), Internet, and/or web-based communications, *etc.* The written instructions may be in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which instructions can also reflects approval by the agency of manufacture, use or sale for animal administration.

[0081] The kit may contain any one or more of the components described herein in one or more containers. As an example, in one case, the kit may include instructions for mixing one or more components of the kit and/or isolating and mixing a sample and applying to a subject. The kit may include a container housing agents described herein. The agents may be in the form of a liquid, gel or solid (powder). The agents may be prepared sterilely, packaged in syringe and shipped refrigerated. Alternatively it may be housed in a vial or other container for storage. A second container may have other agents prepared sterilely. Alternatively the kit may include the active agents premixed and shipped in a syringe, vial, tube, or other container. The kit may have one or more or all of the components required to administer the agents to a subject, such as a syringe, topical application devices, or IV needle tubing and bag.

EXAMPLES

EXAMPLE 1: Characterization of 12 AAV vectors for intravascular delivery to target CNS and detarget non-CNS tissues by miRNA regulation

[0082] The CNS gene transfer properties of 12 scAAVEGFP vectors of different serotypes, or natural variants were evaluated. RAAVs that cross the blood-brain-barrier (BBB) and target oligodendrocytes were discovered. Experiments were performed in neonatal mice (1 day old) and in adult mice (10 week old) (C57BL/6). The following AAV serotypes were tested: AAV1, AAV2, AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, rh.10 (also referred to herein as AAVrh.10), rh.39, rh.43, CSp3.

[0083] The recombinant AAV vectors expressed an enhanced GFP reporter gene under the CMV-enhanced chicken β-actin hybrid promoter and were produced by transient transfection in 293 cells. The neonatal day 1 pups were anesthetized with isoflurane. Then 100 μL of rAAV vectors (4 x 10^{11} GC per mouse) was injected to the pups via superfacial temporal vein under a dissection microscope. In adult mice, rAAV was administered by tail vein injection (two different doses were evaluated 4 x 10^{11} GC per mouse or 4 x 10^{12} GC per mouse). Twenty-one days post injection, the treated animals were anesthetized and transcardially perfused with cold PBS and 4% (v/v) paraformaldehyde. Brains were extracted, immersed in 20% sucrose, and embedded in Tissue-Tek OCT. 40 μm thick sections were cut and stained in 12-well plate with primary antibodies, *e.g.*, anti-NeuN, anti-EGFP and anti-GFAP, overnight at 4°C, then with secondary antibodies for 2 h at room temperature. Control mice received PBS injections.

[0084] In the neonatal study, the distribution of EGFP (+) cells throughout the brain at 3 wks post-infusion was observed. Large numbers of EGFP (+) cells with variable intensities were visible in different regions of the brains from the animals treated with 10 out of 12 vectors. In many instances the choroid plexus showed very strong EGFP expression, and transduced brain parenchyma cells appeared predominantly in periventricular regions. This indicates that a fraction of IV delivered vectors may enter the CNS via the choroid plexus-blood interface. In adults, substantial staining of brain vasculature was observed. Overall targeting efficiencies by AAVs to different regions of the brain was ranked as hypothalamus > medulla > cortex > hippocampus > cerebellum > thalamus. EGFP expression was not detected at high levels in neonatal mice that were administered rAAV2 or rAAV5 harboring the EGFP reporter gene by injection of 4 x 10^{11} GC per mouse in the superfacial temporal vein. (See Table 1 and Figures 1 and 2 for summary data).

[0085] Tissue sections were also immunofluorescently stained with anti-EGFP and -cell type specific marker antibodies to classify EGFP (+) cell types in the CNS. Detection sensitivity for EGFP (+) cells, particularly neurons and oligodendrocytes, was improved dramatically. Although different vectors transduced neurons at variable efficiencies, all 10 vectors (including AAV9) exhibited stronger tropisms to non-neuronal cells, especially astrocytes. One vector (AAV7) targeted oligodendrocytes more efficiently than the other 9 vectors. Several rAAVs transduced both neurons and/or astrocytes at higher efficiencies as compared to rAAV9 (AAVrh.10, rh.34, and rh.43). Extensive astrocyte transduction was observed in hypothalamus and medulla. Injection of certain vectors resulted in substantial neuron transduction in different regions of the brain, including neocortex, hippocampus, and hypothalamus. Some vectors appeared to transduce Purkinje cells in cerebella cortex (*e.g.*, CSp3), while others effectively transduced blood vessel in neocortex, thalamus and hypothalamus. In addition, choroid plexuses in 3rd ventricle, lateral ventricle and 4th ventricle showed strong EGFP expression. EGFP expression was also evaluated in different spinal cord regions of neonatal and adult mice (results for neonatal studies are shown in Figure 3).

[0086] Transduction of non-CNS tissues such as heart and skeletal muscle was observed (*e.g.*, for AAV9, AAV8, and CSp3). In some cases, this may lead to some undesirable side effects. To address this issue, miRNA binding sites were incorporated into the 3' UTR of the transgene cassette and achieved highly specific and effective detargeting of AAV transduction from non-CNS tissues. To inhibit expression in liver, miRNA binding(s) for mR-122 were used. To inhibit expression in skeletal muscle and heart, miRNA binding(s) for mR-1 were used.

Table 1: AAV CNS TROPISMS

		AAV1	AAV2	AAV5	AAV6	AAV6.2	AAV7	AAV8	AAV9	rh.10	rh.39	rh.43	CSp3
[illegible]	Cortex	+			+	+	++	++	+++	++	+	-	+
	Hippocampus	+			+	+	++	++	+++	++	+	-	+
	Thalamus	+			+	+	++	++	++++	+++	++	+	+
	Hypothalamus	+			++	+	+++	++	++	+++	+++	+	++
	Cerebellum	+			++	+	++	+++	+++	++++	+	+	+
	Brain Stem	+			++	+	++	++	++++	+++	++	-	+
	Cervical	+++			+	+	+++	+++	++++	+++	+++	-	+
	Thoracic	+++			+	+	+++	+++	++++	+++	++	-	+
	Lumbar	+++			+	+	+++	+++	++++++	+++	++	-	+
Neo-Natal	Cortex	++	+	-	++	+	+	+++	++	++	++	++	++
	Hippocampus	+	+	-	-	-	+++	++	+	+	++	++	+
	Thalamus	+	+	-	-	+	++	+	+	+	++	+	+
	Hypothalamus	++	-	-	+	+	++++++	++++++	+	+	++++++	+++	-
	Cerebellum	++	-	-	+	-	+	+	+	+	++	+	+
	Brain Stem	++	-	-	+	-	++	+	+	+	+++++	+++	+++
	Cervical	-	-	-	+	++	++	+++	++	+++++	++++	+++	++
	Thoracic	+	-	-	+	++	+++	++	++	++	++++	+++	+
	Lumbar	+	-	-	+	++	++	+	+	++	+++	+++	+

Extent of Tissue Tropsim (- no tropism; ++++++ high tropism) Based on Data in Figures 1 and 2.

Example 2: Construction and evaluation of a recombinant AAVrh.10 vector to treat CD

[0087] Canavan disease (CD) is an inherited neurodegenerative disorder caused by mutations in the aspartoacylase gene (ASPA), leading to accumulation of N-acetyl-aspartic acid (NAA) in oligodendrocytes with resultant spongy degeneration of white matter in the brain. An initial clinical study on rAAV2-based ASPA gene therapy for CD achieved very limited success. It is believed, without wishing to be bound by theory, that an effective CD gene therapy will transduce oligodendrocytes throughout the CNS.

[0088] A rAAV vector is constructed that comprises a promoter operably linked with a region encoding ASPA protein (SEQ ID NO: 13 or 15) as a gene therapy vector for CD. The construct employs CAG (chicken β-actin promoter with CMV enhancer) to drive the expression of ASPA having a coding sequence as set forth in SEQ ID NO: 14 or 16. The rAAV vector is package into rAAV particles using the triple transfection method. To evaluate its effectiveness, rAAV-ASPA is examined in an ASAP knock-out mouse model of CD for its ability to eliminate or attenuate the CD-like phenotypic of homozygous ASPA knock-out mice (Matalon R et al. The Journal of Gene Medicine, Volume 2 Issue 3, Pages 165 - 175). Homozygous ASPA knock-out mice exhibit neurological impairment, macrocephaly, generalized white matter disease, deficient ASPA activity and high levels of NAA in urine. Magnetic resonance imaging (MRI) and spectroscopy (MRS) of the brain of the homozygous mice show white matter changes characteristic of Canavan disease and elevated NAA levels. Heterozygous ASPA knock-out mice, which have no overt phenotype at birth, serve as controls.

Example 3: Therapeutic Efficacy and Safety Evaluation of an AAV vector to treat CD

[0089] The mouse model of CD is a C57BL/6 derived ASPA gene KO strain. The homozygous KO animals present biochemical and neurological defects similar to those observed in CD patients. CD mice provide an animal model for evaluating gene therapy and other therapeutics for the treatment of CD. CD mice are used to study the efficacy and safety of the novel gene therapy strategies for the treatment of CD.

Experiment design

[0090] To examine therapeutic efficacy and safety, scAAV vectors (e.g., AAV7, AAV8, CSp3 and AAV9) carrying an optimized ASPA expression cassette are investigated in a preclinical gene therapy trial of CD. The vectors include miRNA binding site(s) to inhibit ASPA expression in non-CNS tissues. Both postnatal day-1 and 3-month-old adult animals are treated with each vector at two doses, 1 and 3 x 10^{14} GC/kg by intravenous administration. For the neonatal CD mice, two litters of animals receive each vector at each dose via temporal vein injections for necropsy of one litter each at 1- and 3-month time points. For the 3-month-old adult CD mice, 12 male animals are treated with each vector at each dose via tail vein injections. Six each of the treated animals are necropsied 1 and 3 months later. In further experiments, both postnatal day-1 and 3-month-old adult animals are treated with vectors at a dose in a range of 10^{11} to 10^{12} GC/subject by direct intraventricular administration.

Functional and neurological measurements during the live phase of the study

[0091]

1. 1). NAA metabolism. Urine samples are collected from the treated, untreated control, and wild type animals at days 14, 30, 45, 60, 75, and 90. The samples are analyzed by HPLC to determine the NAA levels.
2. 2). NAA accumulation and NAA-induced water retention in brain. MRI/MRS-based neuroimaging studies are performed on the live animals in all study groups at 1, 2, and 3 months after the vector treatment to measure spectral peak integrals for creatine/phosphocreatine and NAA as well as abnormal hyperintense areas in the brain.
3. 3). Liver function tests. Serum samples are collected from the animals in all study groups

at days 14, 30, 60, and 90 to measure the levels of alanine transaminase (ALT) and aspartate aminotransferase (AST) as indicators of vector-related liver toxicity.

4. 4). Neurological tests. Tremors, walking with splayed legs at a slow and shaky pace, and ataxia are among the prominent neurological features of the CD mice. At 1, 2, and 3 months after the gene therapy treatment, the animals in all study groups are subjected to a walking-pattern analysis by staining their feet with color ink and then recording their walking patterns as footprints on white paper. The animals also are tested and scored on a rotarod test for their ability to maintain balance.

Enzymatic and histopathological analyses at the endpoints of the study

[0092]

1. 1). ASPA activities in the brain and non-CNS tissues. On-target and off-target expression of ASPA are analyzed by collecting brain, liver, heart and pancreatic tissues at necropsy to measure ASAP activities in the respective tissue homogenates.
2. 2). Brain white matter and liver pathologies. To examine potential improvement in brain white-matter pathology and vector-related liver toxicity resulting from the gene therapy, brain and liver tissues are harvested and fixed, paraffin-embedded and sectioned, and stained with hematoxylin and eosin. Histopathological examination is performed by a pathologist.

Example 4: Delivery of therapeutic genes to the CNS cells by AAVrh.10

[0093] A screen of different AAV serotypes, was developed to identify candidates for a therapeutic gene transfer to the CNS. A recombinant AAV vector was constructed that expresses EGFP. The rAAV vector was packaged into four different AAVs: AAV1, 8, 9 and 10. Adult mice were injected with the AAVs into the CSF in the lumbar position. AAV1, 8 and 9 transduced cells only in the vicinity of the injection site at the lumber region of the spinal cord following administration of ~4.8 x 10^{10} particles. Surprisingly, AAVrh.10 transduced cells in the gray matter along the entire spinal cord and brainstem following the same injection protocol and dosage as AAV1, AAV8 and AAV9 (Fig. 4A). Recently, AAV9 has been shown to cross the blood brain barrier (BBB) and transduce spinal cord cells after intravenous injection. A weak signal was observed in the cerebellum and strong signals in the brainstem and spinal cord. A weak signal (similar to the cerebellum) in the forebrain was also observed. Without wishing to be bound by theory, it is believed that CSF flow and diffusion allows the virus spread along the entire spinal cord, but that the ability of a virus to flow and diffuse depends on the structure of the viral capsid. The transduced cell types include neurons and oligodendrocytes. But the majority appears to be astrocytes (Fig. 4B), as indicated by overlap of EGFP with GFAP-positive cells. Substantial overlap with the microglia marker, Iba-1 was not observed. A number of motor neurons were transduced as indicated by overlap of EGFP expression and NeuN staining. It was surprising that among the astrocytes, only those situated in the gray matter were transduced and those that were situated in the white matter and beneath the pia matter were not transduced. This was striking because the virus is likely to be exposed to astrocytes in these areas since it was administered in the subarachnoid space.

Example 5: Construction of a recombinant AAVrh.10 vector to treat ALS

[0094] An recombinant AAV system was developed as a treatment for ALS. A rAAVrh.10 vector was constructed that expresses a microRNA targeting SOD1 (Figure 5A). This microRNA was identified as miR-SOD1. The construct employed CAG (chicken β-actin promoter with CMV enhancer) to drive the expression of EGFP and miR-SOD1 that was located in an intron in the 3'-UTR.

[0095] The silencing potency of 9 miRNA constructs was evaluated. The constructs were transfected into HEK293 cells. After 48 hours, RNA was isolated and Northern blot was carried out to detect SOD1 mRNA (Figure 5B). MiR-SOD1#5 (SEQ ID NO: 26) silenced SOD1 expression most potently. Next, miR-SOD1#5 was packaged into AAVrh.10 (Figure 5D), which was used to infect HEK293 cells. Total cellular protein was extracted 43 hours after the infection and blotted to detect SOD1 (Figure 5C). Inhibition of expression of SOD1 at the protein level was observed.

Example 6: Delivery of therapeutic genes to the CNS cells to treat ALS

[0096] Large batches of AAVrh.10-miR-SOD1 and AAVrh.10-miR-Scr (scrambled miRNA) were produced using standard techniques. Self-complementary AAV (scAAV) was made because it mediates transduction with higher efficiency than conventional single stranded AAV[14]. A scAAVrh.10 was tested and found to express EGFP more rapidly (within 1 week) and stronger than a single stranded AAV.

[0097] AAVrh.10-miR-SOD1 was administered to one group of G93A mice (high SOD1 expressers) and AAVrh.10-miR-Scr to another group of G93A mice (n=15). The AAVrh.10 was injected intrathecally into the CSF in the lumbar area and injected intraventricularly into the forebrain in mice of 60 days of age (~4.8 x 10^{10} particles in 8ul).

[0098] The animals were allowed to live their natural lifespan before succumbing to ALS. The lifespan was compared between the two groups. It was found that mice receiving the AAVrh.10-miR-SOD1 virus, which expresses a SOD1miR5 (SEQ ID NO: 26), lived on average 135 days (±14 days), whereas mice receiving the AAVrh.10-miR-Scr, which expresses a scrambled miRNA (SEQ ID NO: 31), lived on average 122 days (±6 days) (Figure 6B). Moreover, by examining the extent of EGFP expression in cervical, thoracic, and lumber spinal cord tissue, a correlation in the levels of expression in these tissues, particularly with cervical tissue, and lifespan was observed in AAVrh.10-miR-SOD1 treated mice (Figure 7A), but not AAVrh.10-miR-Scr treated mice (Figure 7B). These results suggest that silencing mutant SOD1 expression in the cervical spinal cord is particularly beneficial in extending survival. A subset of the animals from each group were perfused with fixative, sectioned and stained for SOD1 in the spinal cord. SOD1 was detected using standard techniques [9]. SOD1 staining intensity in EGFP expressing cells was reduced compared with the non-EGFP cells that are transduced with AAVrh.10-miR-SOD1 (Figure 6A, showing knockdown of SOD1 expression in astrocytes). Reduction of expression of SOD1 was not observed in cells transduced with AAVrh.10-miR-Scr.

[0099] Tissues from another subset of animals in both groups were dissected to estimate transduction levels. The levels of transduction were estimated by determining the viral genome content using PCR on DNA samples obtained from different CNS and non-CNS regions. Measurements in non-CNS tissues (*e.g.* liver) provided an indication of whether virus had leaked to the periphery. Northern and Western analysis was performed to measure the SOD1 levels in the spinal cord. The antibody used for SOD1 detection was polyclonal, sheep anti-human SOD1, by Biodesign International, catalog#K90077C.

Example 7: Combined Intrathecal/Intraventricular Administration Protocol

[0100] AAV viruses were injected into mouse CSF by lumbar intrathecal injection and/or brain third ventricle injection. Injection into mice lumbar subarachnoid space was carried out using a method modified from Wu et al. [22]. A thin catheter (about 5cm) was made by stretching PE10 tube to the inner diameter 0.12 mm. The stretched section was cut to 1.7 to 1.9 mm, and two beads (1 mm apart) were made between the thin and the thick sections by heating and pressing the tube. To implant the catheter, the mouse was anesthetized by injection of Avertin (1.2% 2,2,2-tribromoethanol in 2% tert-amyl alcohol and PBS) intraperitoneally at 0.23 ml/10 g of body weight [23]. The catheter was then implanted between the L5 and L6 vertebra. The catheter was stitched to the surface muscle at the beaded area. Viruses of dose from 4.80E+10 Genome Copy (for virus screening, in 6ul) to 2.40E+10 Genome copy (for therapy, in 8ul) were injected via the catheter by a Hamilton syringe at a speed of 2 ul/minute. The catheter was sealed at the end by heat and left in place for one day. Wound was closed by clips. Injection into brain third ventricle was carried out using a Stoelting Stereotaxic Instrument and micro-injection pumps from World Precision Instruments following standard stereotaxic procedure. Same doses of virus were injected into the third ventricle at a rate of 1 ul/minutes.

[0101] Estimated doses for human and monkeys and comparison with IV injection are shown below. The two types of monkey are similar in size.

Table 2 - Estimated Doses for Human and Monkeys

Species	Avg CSF ml	Estimated CSF production rate ml/hour	Estimate dose(GC)	particles/g of body weight
mouse	0.035	0.018	2.40E+10	1.2E+09
human	140	21	9.6E+13	1.3E+09
Macaca mulatta (rhesus monkeys)	14	2.5	9.6E+12	1.7E+09
Macaca fascicularis (cynomolgus macaque)	See, Foust KD, et al., Nature Biotechnology, Volume 28, Number 3, March 2010, 271-274		1.00E+14	2.20E+11

References for Background and Examples 1-7

[0102]

1. 1. Daya S, Berns KI: Gene Therapy Using Adeno-Associated Virus Vectors. Clin Microbiol Rev 2008, 21:583-593.
2. 2. Eberling JL, Jagust WJ, Christine CW, Starr P, Larson P, Bankiewicz KS, Aminoff MJ: Results from a phase I safety trial of hAADC gene therapy for Parkinson disease. Neurology 2008, 70:1980-1983.
3. 3. Feigin A, Kaplitt MG, Tang C, Lin T, Mattis P, Dhawan V, During MJ, Eidelberg D: Modulation of metabolic brain networks after subthalamic gene therapy for Parkinson's disease. Proceedings of the National Academy of Sciences 2007, 104:19559-19564.
4. 4. Cideciyan AV, Hauswirth WW, Aleman TS, Kaushal S, Schwartz SB, Boye SL, Windsor EAM, Conlon TJ, Sumaroka A, Pang J-j, et al: Human RPE65 Gene Therapy for Leber Congenital Amaurosis: Persistence of Early Visual Improvements and Safety at 1 Year. Hum Gen Ther, 0.
5. 5. Raoul C, Abbas-Terki T, Bensadoun J-C, Guillot S, Haase G, Szulc J, Henderson CE, Aebischer P: Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. Nat Med 2005, 11:423-428.
6. 6. Kaspar BK, Llado J, Sherkat N, Rothstein JD, Gage FH: Retrograde Viral Delivery of IGF-1 Prolongs Survival in a Mouse ALS Model. Science 2003, 301:839-842.
7. 7. Azzouz M, Ralph GS, Storkebaum E, Walmsley LE, Mitrophanous KA, Kingsman SM, Carmeliet P, Mazarakis ND: VEGF delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model. Nature 2004, 429:413-417.
8. 8. Ralph GS, Radcliffe PA, Day DM, Carthy JM, Leroux MA, Lee DCP, Wong L-F, Bilsland LG, Greensmith L, Kingsman SM, et al: Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. Nat Med 2005, 11:429-433.
9. 9. Wu R, Wang H, Xia X, Zhou H, Liu C, Castro M, Xu Z: Nerve Injection of Viral Vectors

Efficiently Transfers Transgenes into Motor Neurons and Delivers RNAi Therapy Against ALS. Antioxidants & Redox Signaling 2009, 11:1523-1534.

10. 10. Foust KD, Nurre E, Montgomery CL, Hernandez A, Chan CM, Kaspar BK: Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. Nat Biotechnol 2009, 27:59-65.
11. 11. Duque S, Joussemet B, Riviere C, Marais T, Dubreil L, Douar AM, Fyfe J, Moullier P, Colle MA, Barkats M: Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. Mol Ther 2009, 17:1187-1196.
12. 12. Vandenberghe LH, Wilson JM, Gao G: Tailoring the AAV vector capsid for gene therapy. Gene Ther 2009, 16:311-319.
13. 13. McBride JL, Boudreau RL, Harper SQ, Staber PD, Monteys AM, Martins I, Gilmore BL, Burstein H, Peluso RW, Polisky B, et al: Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: Implications for the therapeutic development of RNAi. Proceedings of the National Academy of Sciences 2008, 105:5868-5873.
14. 14. McCarty DM: Self-complementary AAV Vectors; Advances and Applications. Mol Ther 2008, 16:1648-1656.
15. 15. Boillee S, Yamanaka K, Lobsiger CS, Copeland NG, Jenkins NA, Kassiotis G, Kollias G, Cleveland DW: Onset and progression in inherited ALS determined by motor neurons and microglia. Science 2006, 312:1389-1392.
16. 16. Xia X, Zhou H, Huang Y, Xu Z: Allele-specific RNAi selectively silences mutant SOD1 and achieves significant therapeutic benefit in vivo. Neurobiol Dis 2006, 23:578-586.
17. 17. Yamanaka K, Chun SJ, Boillee S, Fujimori-Tonou N, Yamashita H, Gutmann DH, Takahashi R, Misawa H, Cleveland DW: Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. Nat Neurosci 2008, 11:251253.
18. 18. Di Giorgio FP, Carrasco MA, Siao MC, Maniatis T, Eggan K: Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. Nat Neurosci 2007, 10:608-614.
19. 19. Nagai M, Re DB, Nagata T, Chalazonitis A, Jessell TM, Wichterle H, Przedborski S: Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. Nat Neurosci 2007, 10:615-622.
20. 20. Wang Y, Ou Mao X, Xie L, Banwait S, Marti HH, Greenberg DA, Jin K: Vascular Endothelial Growth Factor Overexpression Delays Neurodegeneration and Prolongs Survival in Amyotrophic Lateral Sclerosis Mice. J Neurosci 2007, 27:304-307.
21. 21. Storkebaum E, Lambrechts D, Dewerchin M, Moreno-Murciano M-P, Appelmans S, Oh H, Van Damme P, Rutten B, Man WY, De Mol M, et al: Treatment of motoneuron degeneration by intracerebroventricular delivery of VEGF in a rat model of ALS. Nat Neurosci 2005, 8:85-92.
22. 22. Wu, W. P., Xu, X. J., and Hao, J. X. (2004) J. Neurosci. Methods 133, 65-69
23. 23. Papaioannou, V. E., and Fox, J. G. (1993) Lab. Anim. Sci. 43, 189-192

Example 8. MicroRNA-regulated, Systemically Delivered rAAV9 Introduction to the Example

[0103] This example involves the use of tissue-specific, endogenous microRNAs (miRNAs) to repress rAAV expression outside the CNS, by engineering perfectly complementary miRNA-binding sites into the rAAV9 genome. The example describes recombinant adeno-associated viruses (rAAVs) that can cross the blood-brain-barrier and achieve efficient and stable transvascular gene transfer to the central nervous system (CNS), while de-targeting certain other tissues (e.g., liver, heart, skeletal muscle and other tissues) The approaches described in this example allowed simultaneous multi-tissue regulation and CNS-directed stable transgene expression without detectably perturbing the endogenous miRNA pathway. Regulation of rAAV expression by miRNA was primarily via site-specific cleavage of the transgene mRNA, generating specific 5' and 3' mRNA fragments.

[0104] Gene transfer mediated by recombinant adeno-associated virus (rAAV), as disclosed herein, is useful for treatment of a large number of neurological disorders. It has been found that rAAV vectors disclosed herein cross the blood-brain barrier and are specifically expressed in the CNS. Thus, the vectors may be used for intravascular delivery of rAAV for gene therapy of CNS diseases, including those that affect large areas of the brain and spinal cord.

[0105] This example describes the use of endogenous microRNAs (miRNAs) to suppress transgene expression outside the CNS. miRNAs are small, noncoding RNAs that regulate gene expression by post-transcriptional silencing. In general, miRNAs may silence genes by two mechanisms. When partially complementary to mRNA sequences, they typically reduce target mRNA stability and protein expression (e.g., by two- to fourfold or less), a mode of regulation thought to tune mRNA expression. In contrast, when miRNAs are nearly perfectly complementary to their mRNA targets, they typically bring about cleavage of the mRNA, triggering its wholesale destruction.

[0106] In particular, this example describes the use of miRNAs to detarget rAAV9 expression both separately and concurrently in the liver, heart, and skeletal muscle, the three tissues that are most efficiently targeted by intravenously delivered rAAV9. Silencing of transgene expression in liver, heart, and muscle exploited the natural expression of the abundant (≥60,000 copies/cell) miRNAs, miR-122, which is expressed in hepatocytes, and miR-1, a miRNA found in the heart and skeletal muscle of virtually all animals. miR-122-binding sites have been successfully used to prevent hepatotoxicity of a transgene from an adenovirus vector. Perfectly complementary sites for miR-1, miR-122, or both were engineered into the 3' untranslated region (UTR) of a nuclear-targeted, β-galactosidase (*nLacZ*) reporter transgene whose expression was driven by a cytomegalovirus-enhancer, chicken β-actin (CB) promoter. This example presents multiple independent results indicating that the miRNAs repress *nLacZ* expression by cleaving the transgene mRNA at exactly the same site as by all Argonaute-bound small RNAs in eukaryotic cells. When delivered systemically *in vivo*, the miRNA-detargeted rAAV9 vector successfully expressed the reporter transgene in the CNS, but not the liver or heart or skeletal muscle.

Results

miRNAs efficiently repress reporter gene expression in cultured cells

[0107] To evaluate a strategy for rAAV-mediated transduction, one or three tandem copies of a perfectly complementary binding site for miR-1 or miR-122 were introduced into the 3' UTR of *nLacZ* in a rAAV plasmid vector. The constructs were transfected into HuH7 cells, a human hepatoma cell line expressing -16,000 copies of miR-122 per cell, and measured the number of nLacZ-positive cells. The number of nLacZ-expressing HuH7 cells for the one-site plasmid was about half that of the no site control; three sites reduced the number of nLacZ-expressing cells more than sevenfold (Figure 12a).

[0108] Next, expression of the *nLacZ* constructs was analyzed in human embryonic kidney 293 cells, which naturally express low levels of both miR-122 and miR-1, when miR-1 or miR122 was introduced as a pri-miRNA from a second plasmid. 293 cells were transfected with the *nLacZ* reporter plasmids carrying 0, 1, or 3 miR-122 or miR-1-binding sites, together with a plasmid expressing either pri-miR-122 (Figure 12b) or pri-miR-1 (Figure 12c). To vary the concentration of the miRNA, either a low (1:3) or a high (1:10) molar ratio of the *nLacZ*-binding site plasmid to the miRNA expression plasmid was used. When miR-122 or miR-1 was introduced into the cells, *nLacZ* expression was repressed only when the *nLacZ* reporter mRNA contained the corresponding miRNA-binding sites; there was no reduction of nLacZ-positive cells when miR-1 was coexpressed with *nLacZ* containing miR-122-binding sites or when miR-122 was coexpressed with *nLacZ* containing miR-1-binding sites (Figure 12b,c).

Tissue-specific endogenous miRNAs regulate expression of rAAV9 delivered systemically in adult mice

[0109] To evaluate miRNA regulation of systemically delivered AAV9CB*nLacZ* vectors *in vivo*, AAV9CB*nLacZ* vectors carrying 0, 1, or 3 miRNA-binding sites perfectly complementary to either miR-122 or miR-1 were produced. The vectors were administered by tail vein injection to adult male C56BL/6 mice at a dose of 5 x 10^{13} genome copies per kg (GC/kg) body weight. Four weeks later, the liver and heart of the transduced animals were examined. LacZ staining revealed that the *nLacZ* transgene was silenced by the endogenous miRNAs in the cell type and organ in which they are predominantly expressed: the transgene was specifically silenced by miR-122 in the liver and by miR-1 in the heart (Figure 13a,b). While nLacZ positive cells were reduced in the livers of the animals treated with rAAV9CB*nLacZ* bearing one or three miR-122-binding sites, nLacZ expression levels in the hearts of the same animals were similar to those in the animals treated with AAV9CB*nLacZ* bearing no sites (Figure 13a). Similarly, nLacZ expression was not detected in the hearts of the animals that received AAV9CB*nLacZ* containing one or three miR-1-binding sites, but nLacZ expression in the livers of the same animals was not affected as compared to that in the control animal (Figure 13b). These data suggest that the greater the number of sites for a miRNA in rAAV, the lower the nLacZ expression in the tissue where the corresponding miRNA was expressed (Figure 13a,b).

[0110] Next, to evaluate whether transgene silencing could be achieved simultaneously in multiple tissues, different numbers of both miR-122- and miR-1-binding sites were inserted in the 3' UTR of the rAAV9CB*nLacZ* genome and examined for their expression in rAAV9 transduced mice. Histochemical staining of tissue sections showed that nLacZ expression was suppressed in both heart and liver for rAAV9CB*nLac* containing one or three copies each of the miR-1- and miR-122-binding sites, but nLacZ was readily detectable in pancreas, where expression of both miR-122 and miR-1 was low (Figure 13c). Quantitative, β-galactosidase assays of homogenized liver tissue similarly showed that nLacZ expression was significantly lower when the transgene contained the miRNA-binding sites (one miR-122-binding site: 7.8 ± 7.4%, *P* value = 0.005; three miR-122-binding sites: 1.6 ± 1.0%, *P* value = 0.005; one miR-1- plus one miR-122-binding site: 8.6 ± 5.7%, *P* value = 0.005; three miR-1-plus three miR-122-binding sites: 3.1 ± 1.2%, *P* value = 0.005; three miR-1-binding sites: 105.7 ± 11.6%) (Figure 13d).

miRNA repression of rAAV expression does not perturb endogenous miRNA pathways

[0111] Highly expressed transgenes bearing miRNA-complementary sites have been reported to promote degradation of the corresponding miRNA. The levels of miR- 122, miR-22, miR-26a, and *let-7* were determined in rAAV transduced liver. No difference in abundance of the four miRNAs was detected among the three study groups (Figure 14a). Moreover, data from high throughput sequencing analyses of small RNA from the livers of one animal each from the three study groups show no change in miRNA levels.

[0112] In order to determine whether the miRNA-binding sites in the transgene transcripts would deregulate the expression of the known endogenous target mRNAs of miR-122 or miR-1, the expression of cyclin G1, a miR-122 target in liver (Figure 14b,c) and calmodulin, a miR-1 target in heart (Figure 14d) were analyzed. No significant alteration in cyclin G1 or calmodulin expression was detected. miR-122 regulates cholesterol biosynthesis in the liver, and agents that block miR-122 function may produce readily detectable changes in serum cholesterol levels. No change in total cholesterol, high-density lipoprotein, or low-density lipoprotein levels was detected in mice 4 weeks after transduction with either control rAAV9 or rAAV9 expressing a transgene bearing miR-122-binding sites (Figure 14e). It was concluded that in this example miRNA-mediated detargeting of rAAV expression had no detectable effect on endogenous miRNA expression or function.

Endogenous miRNAs silence rAAV transduction by site-specific cleavage of transgene mRNA

[0113] To determine how miRNAs suppress expression of transgenes delivered by rAAV *in vivo*, the transgene mRNA in liver was characterized by conventional PCR (Figure 15b), quantitative reverse transcription PCR (qRT-PCR) (Figure 15c), Northern hybridization (Figure 15d,e), and rapid amplification of 5' complimentary DNA (cDNA) ends (5' RACE; Figure 15f). When primers were used that amplify the region between the 3' end of *nLacZ* (A^+F primer) and the 5' end of the poly(A) signal (A^+R primer), an amplicon that spans the miRNA-biding sites, a 145 basepair (bp) product was detected after 26 cycles of amplification for the samples that received control rAAV. An additional six cycles of amplification were required to detect a weak 220bp band for the samples transduced by rAAV containing three miR-122-binding sites. These data are consistent with low levels of intact *nLacZ* mRNA (Figure 15a,b).

[0114] To quantitatively assess the extent of the miRNA-directed repression of the transgene transcripts, qRT-PCR was performed using either oligo(dT) or random hexamer primers for reverse-transcription and PCR primer pairs that span either a 5' (nLacZ5'F/5'R), or 3' (nLacZ 3'F13'R) region of the *nLacZ* coding sequence (Figure 15a). The levels of *nLacZ* mRNA were examined with intact 5' and 3' ends in total liver RNA extracted from four animals that received the control rAAV9CB*nLacZ* and four that received rAAV9CB*nLacZ* containing three miR-122-binding sites in the 3' UTR. Reductions ranging from 3 ± 1 (random hexamer) to 7 ± 1 (oligo[dT])-fold in *nLacZ* mRNA with an intact 3' end were observed in the animals that had received rAAV9 containing miR-122-binding sites, relative to the control. In contrast, little or no decrease in *nLacZ* mRNA with an intact 5' end were detected for the same samples using the 5'F/5'R primer pair (Figure 15c). These results indicate that the primary mode of turnover of the mRNA that has been cleaved by a miRNA was 3'-to-5' exonucleolytic degradation.

[0115] To further characterize the fate of the transgene mRNA targeted by miR-1 or miR-122, Northern blot analyses was performed. A transgene probe binding to the 5' end of *nLacZ* mRNA detected a ~3.4 kb RNA in an animal injected with control rAAV9Cb*nLacZ*, the expected size of the of the full-length *nLacZ* transcript; a slightly larger band was detected in the liver sample from a mouse treated with rAAV9CB*nLacZ* bearing three miR1-binding sites (Figure 15a,d). In contrast to the single transcript detected for the rAAV9 expressing *nLacZ* bearing three miR-1- binding sites, two RNAs of different sizes were detected for the rAAV expressing *nLacZ* bearing three miR-122 sites (Figure 15d).

[0116] The lengths of these transcripts indicate that the longer transcript likely represents the full-length mRNA, whereas the shorter, more abundant transcript corresponds to 5' fragments of *nLacZ* RNA cleaved by miR-122 at the corresponding miR-122-binding sites in the 3' UTR (Figure 15d).

[0117] To confirm this observation, the Northern analysis was repeated using an RNA probe spanning a portion of 3' UTR of the transgene mRNA. In addition to detecting full-length *nLacZ* transcripts in the samples transduced by rAAV9 lacking miRNA-binding sites, two closely migrating species smaller than the 281 nucleotide RNA marker were detected. The size of these fragments was consistent with miRNA-directed 3' cleavage products of the *nLacZ* mRNA

(Figure 15e). These two 3' cleavage products were also detected by gel electrophoresis of the product from the 5' RACE experiment described below (Figure 15f).

[0118] To determine whether such target cleavage occurs *in vivo* when the *nLacZ* transcript contained miR-1 or miR-122-binding sites, rapid amplification of 5' cDNA ends (5' RACE) was performed. Figure 16 presents the sequences of 21 clones recovered using 5' RACE from liver RNA (Figure 16a) and 22 clones isolated from heart RNA (Figure 16b) from the animals injected with rAAV9 in which the *nLacZ* 3' UTR contained three miR-1 and three miR-122-binding sites. In liver, the sequence signatures for miR-122- directed cleavage of the transgene mRNA were detected at each miR- 122-binding site: 5% for the first binding site, 48% for the second binding site, and 43% for the third binding site. All 5' ends mapped to the phosphate that lies between the target nucleotides that pair with positions 10 and 11 of the sequence perfectly complementary to miR-122, the precise site cleaved by small RNAs bound to Argonaute proteins in all eukaryotes (Figure 17a). Similar results were obtained in the heart for the rniR-1 sites (Figure 17b).

[0119] Table 3 presents an expanded 5' RACE analysis for additional vector groups. It was noted that none of the 5' RACE products sequenced corresponded to miR-1-directed site-specific cleavage in liver or miR-122-directed site-specific cleavage in heart (Table 3). Although no cleavage was detected within miR-1-binding sites in the liver, some clones from heart were cleaved within the miR-122-binding sites, but not at the hallmark position for miRNA-directed cleavage.

Intravascularly delivered rAA V9 can be efficiently controlled by endogenous miRNAs

[0120] MiRNA-1 and miRNA-122-binding sites were added into the scAAV9CB enhanced GFP (EGFP) vector genome and injected 10-week-old C57BL/6 male mice with 2 x 10^{14} GC/kg. After 3 weeks, 40 μm sections of brain and spinal cord and 8 μm sections of liver, heart, and skeletal muscle were prepared and examined for EGFP protein expression. It was found that intravenously delivered scAAV9*CBEGFP* efficiently transduced the CNS; EGFP was readily detectable in the thalamus region of the brain and the cervical region of the spinal cord, but also in non-CNS tissues such as liver, heart, and muscle (Figure 17a). In contrast, transgene expression in those non-CNS tissues was reduced when miR-1 and miR122-binding sites were included in the transgene; EGFP expression was unaltered in the CNS, where miR-1 and miR-122 were not present (Figure 17a). Quantitative RT-PCR was used to measure the differential expression of the miRNA-repressed EGFP transgene in brain (41.2 ± 7.7%), liver (3.0 ± 0.5%), heart (0.4 ± 0.1%), and muscle (1.3 ± 0.4%), relative to the EGFP transgene lacking miRNA-binding sites (Figure 17b). To eliminate changes associated with transduction efficiency between experiments, the data were normalized to the number of vector genomes detected in the experimental and control samples. Similar to the microscopic analyses of native EGFP expression, the qRT-PCR data show that the presence of miR-122-or miR-1-binding sites reduced transgene expression in liver (20-fold), heart (100-fold), and muscle (50-fold), but did not detectably alter transgene expression in brain.

Discussion Of Results

[0121] This example shows that rAAV9 can be engineered so that endogenous miRNAs repress transgene expression outside the CNS. The results indicate that such engineered rAAV9s may be used in therapies for the degenerating neurons associated with Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis, by expressing neurotrophic growth factors such as insulin-like growth factor, brain-derived neurotrophic factor or glial-derived neurotrophic factor in the transduced astrocytes. This approaches eliminates or lessens non-CNS expression derived from the peripheral tissues transduced by systemically delivered rAAV9.

[0122] Achieving transgene expression in primarily only the target tissues is a consideration for the clinical development of safe CNS gene delivery. The results in this example indicate that endogenous miRNAs can be harnessed to restrict the tissue- and cell-type specificity of rAAV expression, as was initially shown for lentiviral vectors. The data demonstrate that endogenous miRNAs can effectively repress transgene expression from rAAV. In both heart and liver, the miRNAs repressed transgene expression by directing endonucleolytic cleavage of the transgene mRNA (Figure 18). MiRNA regulation of rAAV expression did not perturb the expression or function of the corresponding endogenous miRNA, allowing transgene expression to be restricted to the CNS in mice. The example indicates that a strategy that combines multiple binding sites for miRNAs expressed in the periphery but not the CNS is useful for the development of safer, CNS-specific gene therapy vectors.

Materials And Methods

[0123] *Vector design, construction, and production.* Perfectly complementary miRNA-binding sites were designed based on the annotated miR-1 and miR-122 sequences in miRBase and inserted into the *BstBI* restriction site in the 3' UTR of the nLacZ expression cassette of the ubiquitously expressed pAAVCB nuclear-targeted β-galactosidase (nLacZ) plasmid using synthetic oligonucleotides (Figure 15a and Table 3). This vector uses a hybrid cytomegalovirus enhancer/CB promoter cassette that is active in most cells and tissues. To express miR-122 and miR-1, pri-miR-122 and pri-miR-1 fragments were amplified by PCR from C57/B6 mouse genomic DNA (Table 4) and inserted into the XbaI restriction site 3' to a firefly luciferase cDNA in the pAAVCBELuc plasmid. The identity of each pri-miRNA was verified by sequencing. AAV9 vectors used in this study were generated, purified, and tittered.

[0124] *Cell culture and transfection.* HEK-293 and HuH7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 mg/l of penicillin-streptomycin (Hyclone, South Logan, UT). Cells were maintained in a humidified incubator at

37°C and 5% CO_2. Plasmids were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

[0125] *Mouse studies.* Male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were obtained and maintained. To monitor lipid profiles of the study animals, serum samples were collected 4 weeks after rAAV9 injection and analyzed for total cholesterol, high-density lipoprotein and low-density lipoprotein on a COBAS C 111 analyzer (Roche Diagnostics, Lewes, UK). To evaluate endogenous miRNA-mediated, CNS-restricted EGFP gene transfer, 10-week-old male C57BL/6 mice were injected intravenously (tail vein) with AAV9CBnLacZ-[miR-122-binding site $(BS)_1$]. AAV9CBnLacZ-$(miR\text{-}122BS)_3$. AAV9CBnLacZ-$(miR\text{-}1BS)_1$. AAV9CBnLacZ-$(miR\text{-}1BS)_3$. AAV9CBnLacZ-$(miR\text{-}1BS)_1$-$(miR\text{-}122BS)_1$, and AAV9CBnLacZ-$(miR\text{-}1BS)_3$-$(miR\text{-}122BS)_3$, respectively, at 5 x 10^{13} GC/kg body weight) or scAAV9CBEGFP at 2 x 10^{14} GC/kg body weight). Animals receiving nLacZ vectors were necropsied 4 weeks later; 8 µm cryosections of liver, heart, and pancreas tissues were prepared for X-gal-histochemical staining. Animals that received EGFP vectors were necropsied 3 weeks later and fixed by transcardial perfusion with 4% (wt/vol) paraformaldehyde. Brain, spinal cord, liver, heart, and muscle were harvested for cryosectioning. Brain and cervical spinal cord tissue were stained as floating sections in a 12-well plate using rabbit anti-EGFP antibody (Invitrogen) diluted 1:500, followed by goat anti-rabbit secondary antibody (Invitrogen) diluted 1:400. Outside the CNS, EGFP expression was detected directly by fluorescence. EGFP and antibody fluorescence was recorded using a Nikon TE-2000S inverted microscope at x 10 magnification and an exposure time of 3 seconds for liver, heart, and muscle, and 5 seconds for thalamus (brain) and cervical spinal cord.

[0126] *Vector genome quantification by qPCR.* Genome DNA was extracted from the selected tissues using QIAamp DNA Mini Kit (Qiagen, West Sussex, UK), according to the manufacturer's instructions. Quantitative PCR were carried out in triplicate using Ring DNA and 0.3 µmol/l EGFP-specific primers (EGFP-F and EGFP-R) using GoTaq qPCR master mix (Promega, Madison, WI) in a StepOne Plus real-time PCR instrument (Applied Biosystems, Foster City, CA).

[0127] *qRT-PCR analysis.* RNA was extracted using Trizol (Invitrogen), according to the manufacturer's instructions. Total RNA (0.5-1.0 µg) was primed with random hexamers or oligo(dT) and reverse-transcribed with MultiScribe Reverse Transcriptase (Applied Biosystems). Quantitative PCR were performed in triplicate with 0.3 µmol/l gene-specific primer pairs (nLacZ5'F/5'R, nLacZ 3'F/3'R, cyclinG1F/R and EGFP-F/EGFP-R) using the GoTaq qPCR master mix in a StepOne Plus Real-time PCR device. The specificity of qRT-PCR products derived from the 5' and 3' ends of *nlacZ* mRNA was confirmed by gel electrophoresis.

[0128] *Northern blot analysis.* Total RNA was extracted from mouse liver and analyzed by Northern hybridization. To detect *nLacZ* mRNA, a 618 bp fragment of *nLacZ* cDNA was isolated by NcoI and PciI digestion of pAAVCB*nLacZ* and labeled with α-^{32}P dCTP by random priming (Takara, Shiga, Japan). To detect 3' fragments of the cleaved nLacZ mRNA, an 111 bp fragment of the poly(A) sequence in the vector genome was cloned into pCR4-TOPO (Invitrogen) for preparation of antisense RNA probe labeled with α-^{32}P CTP during *in vitro* transcription using the Riboprobe System T7 kit (Promega). To detect miR-122, miR-26a, miR-22, and let-7 or U6 in total liver RNA, small RNAs were resolved by denaturing 15% polyacrylamide gels, transferred to Hybond N+ membrane (Amersham BioSciences, Pittsburgh, PA), and crosslinked with 254 nm light (Stratagene, La Jolla, CA). Synthetic oligonucleotides, 5' end-labeled with γ-^{32}P ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA), were used as DNA probes (Table 4) and hybridized in Church buffer (0.5 mol/l $NaHPO_4$, pH 7.2, 1 mmol/l EDTA, 7% (w/v) sodium dodecyl sulphate) at 37°C. Membranes were washed using 1 x SSC (150 mM sodium chloride, 15 mM sodium citrate), 0.1% sodium dodecyl sulphate buffer, and then visualized using an FLA-5100 Imager (Fujifilm, Tokyo, Japan).

[0129] *Western blot analysis.* Proteins were extracted with radioimmunoprecipitation assay buffer [25 mmol/l Tris-HCl, pH 7.6, 150 mmol/l NaCl, 1% (vol/vol) NP-40, 1% (wt/vol) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate] containing a protease inhibitor mixture (Boston BP, Boston, MA). Protein concentration was determined using the Bradford method (Bio-Rad, Melville, NY). Protein samples, 50 ug each, were loaded onto 12% polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membrane (Amersham BioSciences). Briefly, membranes were blocked with blocking buffer (LI-COR Biosciences, Lincoln, NE) at room temperature for 2 hours, followed by incubation with either anti-GAPDH (Millipore, Billerica, MA), anti-cyclin GI (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-calmodulin (Millipore) for 2 hours at room temperature. After three washes with PBS containing 0.1% (vol/vol) Tween-20, membranes were incubated with secondary antibodies conjugated to LI-COR IRDye for 1 hour at room temperature, and then antibodies detected using the Odyssey Imager (LI-COR).

[0130] *β-Galactosidase assay.* Proteins were extracted with radioimmunoprecipitation assay buffer and quantified as described above. Fifty micrograms of protein was used for each β-galactosidase assay using the Galacto-Star System (Applied Biosystems), according to the manufacturer's instructions.

[0131] *5' RACE.* 5' RACE was performed as described. The 5' RACE Outer Primer and the *nLacZ* gene-specific primer bGHpolyAR (Table 4) were used for the first round of nested PCR. The 5' RACE Inner Primer and the nLacZ gene-specific primer nLacZpolyR, which is located near the stop codon of nLacZ cDNA, were used for the second round of nested PCR (Table 4). PCR products were TOPO-cloned into pCR-4.0 (Invitrogen) and sequenced.

[0132] *Statistical analysis.* All results are reported as mean ± SD and compared between groups using the two-tailed Student's t-test.

Table 3 Summary of microRNA-guided transgene mRNA cleavage in mouse liver and heart

		Cleavage site						
miR BS cleavage	Position	Between 10 and 11 nt		Between 17 and 18 nt		Between 18 and 19 nt		Random site
Liver 1 Copy of miR-122 BS (21 clones)	1	17/21	81%	ND		ND		19%
3 Copies of miR-122 BS (11 clones)	1	ND	100%	ND		ND		0%
	2	4/11						
	3	7/11						
3 Copies each of miR-1 and miR 122 miR 1 3x BS	1	ND	ND	ND		ND		0%
BS in a single vector (21 clones)	2	ND						
	3	ND						
miR-122 3x BS	1	1/21	95%	ND		ND		5%
	2	10/21						
	3	9/21						
Heart 1 Copy of miR-1BS (12 clones)	1	12/12	100%	ND		ND		0%
3 Copies of miR 1BS (21 clones)	1	ND	80%	4/21	20%	ND		0%
	2	16/21		ND				
	3	1/21		ND				
3 Copies each of miR 1 and miR 122 miR-122 3x BS	1	ND	ND	ND		1/22	14%	4%
BS in a single vector (22 clones)	2	ND				1/22		
	3	ND				ND		
miR 1 3x BS	1	1/22	73%	ND	9%	ND		0%
	2	7/22		1/22				
	3	8/22		1/22				

Table 4. Oligonucleotide primers and probes used in Example 8.

Oligo nucleotides	Sequence	SEQ ID NO
$(miR\text{-}1)_1$ sense	[PHOS]CGAAATACATACTTCTTTACATTCC ATT	SEQ ID NO: 32
$(miR\text{-}1)_1$ anti-sense	[PHOS]CGAATGGAATGTAAAGAAGTATGT ATTT	SEQ ID NO: 33
$(miR\text{-}122)_1$ sense	[PHOS]CGAAACAAACACCATTGTCACACT CCATT	SEQ ID NO: 34
$(miR\text{-}122)_1$ anti-sense	[PHOS]CGAATGGAGTGTGACAATGGTGTT TGTTT	SEQ ID NO: 35
$(miR\text{-}1)_3$ sense	[PHOS]CGAAATACATACTTCTTTACATTCC AATACATACTTCTTTACATTCCAATACATA CTTCTTTACATTCCATT	SEQ ID NO: 36
$(miR\text{-}1)_3$ anti-sense	[PHOS]CGAATGGAATGTAAAGAAGTATGT ATTGGAATGTAAAGAAGTATGTATTGGAA TGTAAAGAAGTATGTATTT	SEQ ID NO: 37
$(miR\text{-}122)_3$ sense	[PHOS]CGAAACAAACACCATTGTCACACT CCAACAAACACCATTGTCACACTCCAACA AACACCATTGTCACACTCCATT	SEQ ID NO: 38
$(miR\text{-}122)_3$ anti-sense	[PHOS]CGAATGGAGTGTGACAATGGTGTT TGTTGGAGTGTGACAATGGTGTTTGTTGG AGTGTGACAATGGTGTTTGTTT	SEQ ID NO: 39
$(miR\text{-}1)_1$ -$(miR\text{-}122)_1$ sense	[PHOS]CGAAATACATACTTCTTTACATTCC AACAAACACCATTGTCACACTCCATT	SEQ ID NO: 40
$(miR\text{-}1)_1$ -$(miR\text{-}122)_1$ anti-sense	[PHOS]CGAATGGAGTGTGACAATGGTGTT TGTTGGAATGTAAAGAAGTATGTATTT	SEQ ID NO: 41
Synthesized $(miR\text{-}1)_3$ -$(miR\text{-}122)_3$ fragment	TTCGAACTCGAGATACATACTTCTTTACAT TCCAATACATACTTCTTTACATTCCAATAC ATACTTCTTTACATTCCACCATGGACTAGT ACAAACACCATTGTCACACTCCAACAAAC ACCATTGTCACACTCCAACAAACACCATT GTCACACTCCAGCGGCCGCTTCGAA	SEQ ID NO: 42

Oligo nucleotides	Sequence	SEQ ID NO
Pri-miR-122F	ATCGGGCCCGACTGCAGTTTCAGCGTTTG	SEQ ID NO: 43
Pri-miR-122R	CGCGGGCCCGACTTTACATTACACACAAT	SEQ ID NO: 44
Pri-miR-1F	CGCGGGCCCGACTGATGTGTGAGAGAGAC	SEQ ID NO: 45
Pri-miR-1R	CGCGGGCCCGACTTTCGGCCTCCCGAGGC	SEQ ID NO: 46
nLacZ5¢F(5¢F)	TGAAGCTGAAGCCTGTGATG	SEQ ID NO: 47
nLacZ 5¢R(5¢R)	GAGCACCTGACAGCATTGAA	SEQ ID NO: 48
nLacZ3¢F(3¢F)	CTCAGCAACAGCTCATGGAA	SEQ ID NO: 49
nLacZ3¢R(3¢R)	TTACTTCTGGCACCACACCA	SEQ ID NO: 50
nLacZpolyF(A^{+}F)	TGGTGTGGTGCCAGAAGTAA	SEQ ID NO: 51
nLacZpolyR(A^{+}R)	CAACAGATGGCTGGCAACTA	SEQ ID NO: 52
bGHpolyAR(bGH^{+}AR)	TGGGAGTGGCACCTTCCA	SEQ ID NO: 53
EGFP-F	CGACCACTACCAGCAGAACA	SEQ ID NO: 54
EGFP-R	CTTGTACAGCTCGTCCATGC	SEQ ID NO: 55
CyclinG1F	AATGGCCTCAGAATGACTGC	SEQ ID NO: 56
		SEQ

Oligo nucleotides	Sequence	SEQ ID NO
CyclinG1R	AGTCGCTTTCACAGCCAAAT	ID NO: 57
MM-ActinF	ATGCCA ACACAGTGCTGTCTGG	SEQ ID NO: 58
MM-ActinR	TGCTTGCTGATCCACATCTGCT	SEQ ID NO: 59
miR-122 probe	TGGAGTGTGACAATGGTGTTTG	SEQ ID NO: 60
Let-7 probe	AACTATACAACCTACTACCTCA	SEQ ID NO: 61
miR-26a probe	AGCCTATCCTGGATTACTTGAA	SEQ ID NO: 62
miR-22 Probe	ACA GTT CTT CAA CTG GCA GCT T	SEQ ID NO: 63
U6 probe	CTCTGTATCGTTCCAATTTTAGTATA	SEQ ID NO: 64

Example 9: Intravenous injection of rAA Vs mediated widespread transduction in neonatal mouse CNS

Introduction to the Example

[0133] This example describes an analysis of nine scAAV vectors for CNS gene transfer properties after systemic administration. This study involved identifying more effective vectors for the CNS gene transfer, In some aspects the study examined serotypes or natural variants of rAAVs for enhanced-permeation of the BBB. In some cases, the study sought to identify rAAV vectors with improved delivery of enhanced green fluorescent protein (EGFP) to the CNS following facial vein injection on postnatal day 1 (P1). AAV9 was included in the study. Except for rAAV2 and rAAV5, all other 7 vectors crossed the BBB with varied transduction efficiency, among which rAAVrh. 10, rAAVrh.39, rAAVrh.43, rAAV9 and rhAAV7 rank in the top 5, mediating robust EGFP expression in both neuronal and glial cells throughout the CNS in this study. The performance of rAAVrh.10 was comparable to that of rAAV9 and in some case better. Several rAAVs efficiently transduce neurons, motor neurons, astrocytes and Purkinje cells; among them, rAAVrh. 10 is at least as efficient as rAAV9 in many of the regions examined. Intravenously delivered rAAVs did not cause abnormal microgliosis in the CNS. The rAAVs that achieve stable widespread gene transfer in the CNS are useful as therapeutic vectors for neurological disorders affecting large regions of the CNS as well as convenient biological tools for neuroscience research.

Results

[0134] Twenty one days after vector administration in P1 mice, the CNS transduction profiles of the following recombinant AAV vectors encoding EGFP: rAAV1, rAAV2, rAAV5, rAAV6, rAAV6.2, rAAV7, rAAV9, rAAVrh.10, rAAVrh.39 and rhAAVrh.43 were compared. The vectors used in this study were comparable in purity and morphological integrity (Figure 19). As assessed by the scoring system described in the methods, rAAV9 was among the top performers; most other rAAVs tested (rAAV1, rAAV6, rAAV6.2, rAAV7, rAAVrh.10, rAAVrh.39 and rAAVrh.43) also gave rise to EGFP expression throughout the CNS (Table 2). The number of apparent EGFP positive cells (Table 5) among sub-anatomical structures was influenced by the particular vector used. For these seven rAAVs, and rAAV9 (total of eight rAAVs), that permeated the BBB and accomplished CNS transduction after i.v. delivery, EGFP positive cells were found in hypothalamus followed by medulla, striatum, hippocampus, cortex and cerebellum. In contrast, the transduction efficiency in olfactory bulb and thalamus was relatively low (Table 5). A quantitative assessment of EGFP gene transfer efficiency was made of each rAAV. 12 sub-anatomically and functionally important regions in the brain were selected for quantitative analysis of the mean EGFP intensity/pixel in each region for each rAAV by using Nikon NIS elements AR software V. 32 (Figure 19a) (see Methods). For the eight vectors that achieved CNS transduction after i.v. injection, the mean EGFP intensity/pixel was relatively low in cortex, habenular nucleus, cornu ammonis, dentate gyrus, thalamus, cerebellum and olfactory bulb, moderate in choroid plexus and caudate-putamen, but high in hypothalamus, medulla and amygdale (Figure 19a). The average EGFP intensities of all 12 regions for different rAAVs were compared in Figure 19b. AAVrh.10, AAVrh.39 and AAVrh.43 were noted for gene transduction efficiency in brain, followed by AAV7, AAV9, and AAV1 (Figures 19a and 19b). Those eight effective serotypes also mediated EGFP expression throughout the spinal cord, to different degrees. The same quantitative analysis was performed for each rAAV in the cervical, thoracic and lumbar sections of the spinal cord (Figure 19a); the average EGFP intensities of the three sections for different rAAVs were also compared (Figure 19b). AAV1, AAV9, AAVrh10, AAV.rh39 and AAV.rh43 displayed strong transduction in the spinal cord with the high EGFP intensity observed in the cervix, followed by thoracic and lumbar sections of the spinal cord (Figures 19a and 19b). For rAAV2 there were few EGFP-positive cells in hippocampus, cortex and hypothalamus. EGFP-positive cells were observed in the hypothalamus in AAV5-injected mice. A description of the observations made in different CNS structures is provided below. The subanatomic CNS structures may serve as a target for CNS gene therapy. In some cases, the subanatomic CNS structures are associated with pathological changes in one or more neurological disorders. In some cases, the subanatomic CNS structure have distinct transduction profiles for one or more rAAVs.

[0135] *Striatum.* Pathology of the striatum is associated with Huntington's disease, choreas, choreoathetosis, and dyskinesias. Addiction may involve plasticity at striatal synapses. Systemic injection of rAAV9 in neonatal mice tranduces striatal tissue. In this study, a large number of cells with neuronal morphology in this region were also transduced by rAAVrh.10 (Figure 20), which was confirmed by co-staining with a neuronal marker as described below. Other vectors, including rAAVrh.39 and rAAV7, also mediated moderate transduction in striatum (Figure 20). In contrast, rAAV6, rAAV6.2, and rAAV1 resulted in relatively lower EGFP expression in this structure (Figure 20).

[0136] *Hippocampus.* The hippocampus is a region associated with long-term memory and spatial navigation, which is usually damaged by stress and pathogenesis of diseases such as epilepsy and Schizophrenia. Large numbers of EGFP-positive neurons were observed bilaterally in all regions of the hippocampus, namely dentate gyrus, hilus, CA1, CA2 and CA3 for the mice received intravenous rAAVrh.10, rAAV9, rAAV7, rAAVrh.39, and rAAVrh.43 (ranked by transduction efficiency in this structure, Table 5 and Figures 19 and 20). In addition to the neuronal transduction pattern, EGFP-positive cells had morphologic appearance of astrocytes (Figure 20). This was further confirmed by double staining with antibodies against EGFP and astrocytic marker as described below. For intravenously delivered rAAV1, rAAV6 and rAAV6.2 vectors there were small numbers of EGFP-positive cells in the hippocampus (Figure 20).

[0137] *Cortex.* Pathological changes in the cortex have been implicated in Alzheimer's and Parkinson's diseases. AAV7, AAV9, AAVrh.10, AAVrh.39 and AAVrh.43 vectors achieved moderate EGFP transduction in cortex (Table 5 and Figures 19 and 20). The morphology of transduced cells was consistent with both neurons and astrocytes as further confirmed by cellular marker staining and confocal microscopic analysis described below. Prominent EGFP-positive cells were typically observed in the ventrolateral regions of the cortex, including posterior agranular insular cortex, piriform cortex, lateral entorhinal cortex, posterolateral cortical amygdaloid nucleus and posteromedial cortical amygdaloid nucleus (Figure 20). Strong EGFP signals spread from +1.5 to -3.3 mm in relation to the Bregma (0.0 mm). The cortical transduction efficiency of rAAVrh.10, rAAV9, rAAVrh.39 and rAAVrh.43 was comparable (Table 5 and Figures 19 and 20). AAV1, AAV6 and AAV6.2 vectors also transduced cells in the cortex (Figure 20).

[0138] *Hypothalamus.* A role for the hypothalamus is to secret neurohormones to control certain metabolic processes. The hypothalamus is also indicated in the etiology of diabetes. EGFP signal was observed in the hypothalamus for eight vectors. Intravenous administration of rAAVrh.10 resulted in the highest EGFP expression in the entire hypothalamus, followed by rAAVrh.39, rAAV7, rAAV6.2, rAAVrh.43, rAAV9, rAAV1 and rAAV6 (Figures 19 and 20 and Table 5). Interestingly most EGFP-positive cells in this structure have an astrocytic morphology which was ascertained by immunostaining for an astrocytic cell type specific marker as described below. The astrocytic EGFP signal tended to obscure direct examination of morphological details of other transduced cells. However, this was clarified by double immunofluorescent staining of tissue sections with antibodies for EGFP and neuronal cell markers as described below.

[0139] *Cerebellum.* The pathological lesions in cerebellum are often found in diseases such as cerebellar-cognitive affective syndrome, developmental coordination disorder, posterior fossa syndrome, linguistic deficits, aging, attention deficit hyperreactivity disorder, autism, dementia and schizophrenia. EGFP-positive cells and fibers were detected in cerebellum for most rAAV vectors (Table 5 and Figures 19 and 20). A large number of EGFP-expressing cells were found in the Purkinje and granule cell layers for rAAV7, rAAV9, rAAVrh.10, rAAVrh.39 and rAAVrh.43 (Figure 20). The transduction profile of rAAV1 vector indicated expression in cells in the granule cell layer, while rAAV6 and rAAV6.2 were localized in cells in the Purkinje cell layer (Figure 20).

[0140] *Medulla.* The medulla is a potential gene therapy target for treating chronic pain. Most rAAVs mediated moderate to robust EGFP expression in medulla with most green cells being present in the outer rim (Figure 20). Transduction efficiencies of these rAAV in this region are ranked in the following order: rAAVrh.39 = rAAVrh.43 > rAAV.rh10 > rAAV1 > rAAV9 > rAAV7 > rAAV6.2 > rAAV6 (Table 5 and Figure 19a). The morphology of most EGFP-transduced cells was consistent with the cells being astrocytes.

[0141] *Spinal cord.* The spinal cord is involved with motor neurons diseases. rAAVrh.10, rAAV9, rAAVrh.39 and rAAVrh.43 gave rise to very robust EGFP expression in cervical gray and white matter, while rAAV1, rAAV6.2 and rAAV7 showed moderate EGFP intensity (Table 5 and Figures 19 and 21). For rAAV1 the EGFP signal was observed in white matter. The transduction ability of all effective rAAVs decreased from cervical to lumbar spinal cord. EGFP-positive cells were visible in the latter region. Large populations of EGFP-positive cells with astrocytic morphology were observed throughout the spinal cord (Figure 21). In addition, rAAVrh.10, rAAV9, rAAVrh.39, rAAVrh.43 and rAAV7 also transduced cells with motor neuron morphology in the ventral regions of spinal cord (Figure 21). Ascending dorsal column fibers showed clear EGFP signal. In addition, dorsal root ganglia (DRG) displayed remarkable transduction with strong EGFP expression in DRG neurons (Figure 22 and Figure 26). The identities of rAAV transduced cell types in the spinal cord were characterized by co-immunofluorescence staining with antibodies against EGFP and cell type specific markers as described below.

IV administration of AAV vectors leads to transduction of different cell types in the CNS

[0142] To confirm the identity of transduced cells in different regions of the CNS, double immunofluorescent staining was performed with antibodies for EGFP and NeuN (generic neuronal marker), glial fibrillary acid protein (GFAP; astrocyte marker), calbindin-D28K (Purkinje cell marker), and choline acetyl transferase (ChAT; motor neuron marker) (Figure 23). The immunostaining results showed that a large number of NeuN positive cells expressed

EGFP throughout the mouse brain, which indicated widespread neuronal transduction. The regions with high density of transduced neurons included striatum, hippocampus, cortex and hypothalamus. rAAVrh.10, rAAV9, rAAV7 and rAAVrh.39 vectors were efficient in mediating neuronal transduction, followed by AAV6.2, AAV1 and AAV6 (Figures 19 and 23). In addition, dopaminergic neurons in substantia nigra were transduced by AAV.rh10 (Figure 23). Transduced cells in the CNS included GFAP-positive astrocytes with small cell bodies and highly ramified processes (Figure 23). The calbindin-D28K immunostaining confirmed the identity of a number of transduced cells in the cerebellum as Purkinje cells, with EGFP expression in both cell body and their tree-like processes (Figure 23). The rAAVs proficient in transducing Purkinje cells include: rAAVrh.10, rAAV9, rAAVrh.39, rAAV7, rAAV6.2 and rAAVrh.43. rAAV1 and rAAV6 transduced a portion of Purkinje cells with relatively low EGFP intensity (Figure 19). Transduction of motor neurons was confirmed by the presence of large EGFP+/ChAT+ cells in the ventral spinal cord for several rAAV vectors (Figure 23). rAAVrh.10, rAAV9, rAAV7, rAAVrh.39 showed comparable efficiency transduction of motor neurons (Figure 21).

IV administration of AAV vectors mediated robust transduction in ventricles and brain blood vessels

[0143] EGFP expression was observed in the choroid plexus cells in lateral, 3rd and 4th ventricles of the animals infused with rAAVrh.39, rAAVrh.10, rAAVrh.43, rAAV7 and rAAV9 (ranked by transduction efficiency, Table 5 and Figures 19 and 24). EGFP expression in different ventricles of the same mouse brain was similar (Figure 24). Ependymal cells lining the ventricles were also transduced. An observation regarding the distribution of EGFP-positive cells was the apparent gradient with the highest number of transduced cells in peri-ventricular regions and progressively lower numbers with increasing distance to the ventricles. This was apparent in areas around the 3rd and 4th ventricles than the lateral ventricles (Figure 24). Extensive EGFP signal was also found with blood vessels throughout mouse brain and spinal cord. This was verified by dual immunofluorescent staining with antibodies directed to EGFP and a blood vessel endothelium specific marker, CD34 (Figures 27a and 27b). Unlike the rAAV transduction profiles in different regions of the brain parenchyma, the EGFP transduction of the blood vessels throughout the CNS was relatively uniform for any given vector. However, transduction of blood vessels was influenced by the particular rAAV used. A majority of rAAVs mediated moderate (e.g., rAAV6) to highly efficient (e.g. rAAVrh.10) blood vessel transduction in the CNS.

IV injection of AAV vectors did not cause microgliosis

[0144] Brain sections were also stained with antibody against Iba-1 to label microgial cells. The Iba-1-positive cells in the sections from mice received rAAVrh.10 was no more than those in naive or PBS-injected mice (Figure 28). This result indicated that intravascularly delivered rAAVs do not cause sustained inflammation in the CNS of mice 3 weeks after the injection of P1 neonates.

Discussion of Results

[0145] In this study, the CNS transduction profile was evaluated for 10 different rAAV vectors delivered by intravascular infusion in neonatal mice. Most of the rAAVs can cross the BBB and mediate gene transfer to the neonatal mouse CNS with varying degrees of efficiency (Figures 19-21 and Table 5). After systemic administration, rAAVrh.10, rAAVrh.39, rAAVrh.43, and rAAV9 are the effective rAAVs with similar transduction capabilities and cellular tropism, as assessed by overall EGFP expression in the CNS. Specifically, a number of regions in the mouse CNS, including striatum, hippocampus, cortex, hypothalamus, cerebellum, medulla, and cervical spinal cord, revealed substantial EGFP expression. In addition, rAAV6.2 and rAAV7 were also effective. AAV1 and AAV6, achieved CNS transduction (Table 5). Native EGFP expression was detectable in brain and spinal cord sections for most of the rAAVs without immunostaining (Figure 29).

[0146] This example has clinical significance for gene therapy of CNS-related disorders, including for young patients. For a variety of neurological diseases, early treatment during infancy may be necessary to prevent irreversible CNS injury. The capacity of rAAVs to transduce large numbers of neuronal cells in different regions is relevant for treating neurological diseases such as spinal muscular atrophies, neuronal ceroid lipofuscinoses, and spinocerebellar degenerations. The efficiency of some rAAV vectors in transducing Purkinje and granule layer cells indicates that the vectors may be used for treating spinocerebellar ataxias. Transduction of astrocytes by rAAVs expressing secreted neurotrophic factors may be also beneficial for a number of neurodegenerative diseases such as Canavan's disease and amyotrophic lateral sclerosis. The vascular transduction in the CNS may be relevant for treating brain ischemia and stroke. The clinical application of intravascular rAAV-mediated gene delivery may also extend to the peripheral nervous system (PNS). Efficient transduction of DRG provides new therapeutic strategies for patients suffering from chronic pain.

[0147] Systemic gene delivery to the CNS is also useful as a method to manipulate gene expression in research. Effective and stable transgene expression in the CNS by intravenous administration of rAAVs may be applied to establish somatic transgenic animal models, which is a potentially cheaper, faster and simpler method than conventional transgenesis. Somatic CNS gene knock-down animal models may also be created using the method described herein.

[0148] Some rAAVs indeed demonstrated unique transduction profiles in the CNS. For instance, rAAV1 displayed transduced granule cells in the cerebellum, while rAAV6 and rAAV6.2 transduced Purkinje cells, and others transduced both types of cells (Figure 9). This indicates that once across the BBB, the rAAVs have distinct tropisms, which can be attributed to the capsid because that the vector genome used in all vectors was the same.

[0149] AAV serotypes disclosed herein can efficiently transduce brain capillary endothelial cells, neurons and astrocytes. This indicates that these vectors may extravasate from the circulation and reach the CNS parenchyma, possibly by crossing the BBB. AAV may cross the endothelial barrier by a transcytosis pathway. In this study, choroid plexuses and their surrounding parenchymal tissue were efficiently transduced. In addition, there was an apparent gradient of EGFP intensity from peri-ventricular (higher) to deep parenchymal (lower) tissue. These observations indicate that AAV may enter the neonatal mouse CNS through the choroid plexus, followed by widespread distribution via CSF and/or interstitial fluid flow to transduce neuronal and glial cells.

[0150] Neuronal- or glial-specific promoters, such as synapsin-1, and GFAP promoters may be used to restrict gene expression to a specific cell type. A further method to achieve targeted CNS gene delivery is to utilize RNA interference to detarget the peripheral tissues by post-transcriptional regulatory mechanisms. By adding microRNA binding sites into the 3' end of the transgene cassettes, transgene expression after systemic administration of AAV vectors may be reduced or eliminated in tissues such as liver, heart and skeletal muscle, while maintaining CNS transduction.

Materials And Methods

AAV production

[0151] ScAAV vectors were produced by trans-encapsidation of rAAV vector genome flanking by inverted terminal repeats (ITRs) from AAV2 with the capsids of different AAVs using the method transient transfection of 293 cells and CsCl gradient sedimentation as previously described. Vector preparations were titered by quantitative PCR. Purity of vectors was assessed by 4-12% SDS-acrylamide gel electrophoresis and silver staining (Invitrogen, Carlsbad, CA). Morphological integrity of each vector used in the study was examined by transmission electron microscopy of negative stained recombinant AAV virions. The expression of EGFP in the scAAV vector genome is directed by a hybrid CMV enhancer/chicken β-actin promoter.

Neonatal mouse injections

[0152] Wild-type C57BL/6 mice littermates were used. Mice breeding were conducted using programmatic timing method. Pregnant mice were monitored daily from embryonic day 17 to 21 to ensure the newborn pups could be dosed with vectors on P1. The mother (singly housed) of each litter to be injected was removed from the cage. Vectors were diluted to concentration of 4×10^{12} GCs/mL in PBS and 100 µl of solution was subsequently drawn into 31G insulin syringes (BD Ultra-Fine II U-100 Insulin Syringes). P1 pups of C57BL/6 mice were anesthetized using isoflurane and rested on ice. For intravenous injections, a dissection microscope was used to visualize the temporal vein (located just anterior to the ear). The needle was inserted into the vein and the plunger was manually depressed. Correct injection was verified by noting blanching of the vein. Each pup received 4×10^{11} GCs of different scrAAVCBEGFP vectors (rAAV1, rAAV2, rAAV5, rAAV6, rAAV6.2, rAAV7, rAAV9, rAAVrh.10, rAAVrh.39, rAAVrh.43; n=6-8 mice per group) via the superficial temporal vein. After the injection pups were carefully cleaned, rubbed with their original bedding, and then returned to their original cage. The mother was then reintroduced to the cage after brief nose numbing using ethanol pads.

Histological processing

[0153] The study animals were anesthetized 21 days post-injection, then transcardially perfused with 15 mL of cold PBS followed by 15 mL of fixation solution containing 4% paraformaldehyde (v/v) with 0.2% of glutaraldehyde (v/v) in PBS. Then the whole carcasses were post-fixed in fixation solution for 5 days. Spinal cords and brains were extracted under a bright-field dissecting microscope, rinsed in PBS, and then cryoprotected in 30% sucrose (w/v) in PBS at 4 °C. Once the tissues sank to the bottom of the sucrose solution, they were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and frozen in a dry ice/ethanol bath. The tissue blocks were stored at -80 °C until sectioning. Serial 40 µm floating sections of the entire brain were cut in a Cryostat (Thermo Microm HM 550). For the spinal cord, 3 mm length sections were taken from cervical, thoracic and lumbar regions, and then serial 40 µm transverse sections prepared as above.

Immunostaining and microscopy imaging analysis

[0154] Brain and spinal cord sections were stained as floating sections in 12-well plates. Sections were washed 3 times in PBS for 5 min each time, and then incubated in blocking solution containing 1% Triton-X100 (v/v) (Fisher, Pittsburg, PA), 5% dry-milk (w/v) and 10% goat serum (v/v) (Invitrogen) for 2h at room temperature. Then the sections were incubated with primary antibodies diluted in blocking solution at 4°C overnight. The following day tissue sections were washed twice in 0.05% Tween-20 (v/v) in PBS (PBST) and once with PBS, with each washing step lasting 10 min. Afterwards sections were incubated with appropriate secondary antibodies in blocking solution at room temperature for 2 h. Sections were washed again as above before mounting on glass slides. Vectashield with DAPI (Vector Laboratories, Burlingame, CA) was used to coverslip all slides, and then they were analyzed using a fluorescent inverted microscope (Nikon Eclipse Ti) or a Leica TSC-SP2 AOBS confocal microscope equipped with a 63× oil lens and a DM-IRE2 inverted microscope. The primary antibodies used in this study were as follows: rabbit anti-GFP (Invitrogen), goat anti-ChAT and mouse anti-NeuN (both from Millipore, Billerica, MA), mouse anti-GFAP (Cell signaling, Danvers, MA), rat anti-CD34 (Abcam, Cambridge, MA), mouse anti-Calbindin D-28k (Sigma, St

Louis, MO) and rabbit anti-DARPP (Abcam, Cambridge, MA). The secondary antibodies used in the study included: DyLight 488 AffiniPure Donkey Anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA); DyLight 549 AffiniPure Donkey Anti-Goat IgG (Jackson ImmunoResearch); DyLight 549 Affinipure Goat Anti-Rat IgG (Jackson ImmunoResearch); DyLight 594 AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch); goat anti-rabbit IgG-Alexa fluro 488 (Invitrogen) and goat anti-mouse IgG-Alexa fluro 568 (Invitrogen).

Semi-quantitative and quantitative comparison of EGFP transduction by different vectors

[0155] To generate a quantifiable and comparable data format, a semi-quantitative scoring system was develop to estimate transduction efficiency of different rAAV vectors in different regions of the mouse CNS. Briefly, regions with no EGFP positive cells were marked as (-). Regions with very few EGFP positive cells were scored (+), regions with some EGFP positive cells were ranked as (++), regions with many EGFP positive cells were marked as (+++). Finally, regions filled with EGFP positive cells were marked as (++++).

[0156] Next, 12 sub-anatomically and functionally important regions in the brain as well as cervical, thoracic and lumbar sections of the spinal cord were selected for quantitative analysis of images that were taken on a Nikon Eclipse Ti inverted microscope equipped with a Retiga 2000-RV CCD cooled camera. Nikon NIS elements AR software v. 3.2 was used for intensity quantification. Prior to quantification, optimal light source intensity and exposure times were obtained by plotting an intensity/exposure time curve using fluorescence reference slides (Ted Pella, prod. 2273). It was found that the intensity and exposure times had linear correlation. In addition, overexposure and extreme underexposure distorts the linear correlation. The maximum intensity (ND1) and a 20ms exposure were used for all sections to avoid overexposure. For quantification, fixed region of interest (ROI) was used to quantify the brightest area of any given brain region. A mean intensity (total intensity/size of ROI) was obtained for each region of all serotypes.

Table 5. Transduction characteristics of AAV serotypes following intravascular injections into neonatal mouse brain

	Olfactory Bulb		Striatum		Hippocampus		Cortex		Thalamus		Hypothalamus		Cerebellum		Medulla		Cervical		Thoracic		Lumber		Choroid Plexus	
	score	n	score	n	score	n	score	n	score	n	score	n	score	n	score	n	score	n	score	n	score	n	score	n
AAV1	+	3	++	3	++	3	+-	3	-	3	+++	3	+++	3	+++	3	+++	3	++-	3	+	3	+++	3
AAV2	-	3	-	3	+	3	+	3	-	3	+	3	+	3	+	3	+	3	-	3	-	3	++	3
AAV5	-	3	-	3	-	3	+	3	-	3	+	3	-	3	-	3	-	3	-	3	-	3	-	3
AAV6	+	3	+	3	++	3	+--	3	-	3	+++	2	-+	3	-+	3	++	3	+	3	+	3	+++	3
										++	1													
AAV6.2	-	3	+++	2	++	3	+-	3	-	3	-+++	3	-+	3	-+	3	++	3	++	3	+	3	++++	3
			++	1																				
AAV7	++-	1	+++	3	++-	2	+-	3	-	3	-+++	3	+++	1	-+	3	++	3	+	3	+	3	++++	3
	[illegible]	2			[illegible]	1							[illegible]	2										
AAV9	[illegible]	2	[illegible]	3	[illegible]	3	[illegible]	1		3	[illegible]	1	[illegible]	1	[illegible]	3	[illegible]	1	[illegible]	3	[illegible]	3	[illegible]	3
	++	1					+-	2			+++	2	-+	2			+++	2						
rh10	[illegible]	1	[illegible]	1	[illegible]	3	[illegible]	2	[illegible]	2	[illegible]	3	[illegible]	1	[illegible]	3	[illegible]	1	[illegible]	3	[illegible]	3	[illegible]	3
	++	2	++	2			+-	1	-	1			-+	2			+++	2						

rh39	++	1	+++	2	++	3	+++	1	-	3	+++	3	+++	1	++	1	+++	1	++	3	+	3	++++	3
	++	2	+++	1			++	2					++	2	+++	2	++	2						
rh43	++	3	+++	3	++	3	+++	3	-	3	+++	1	++	3	++	1	+++	2	++	3	+	3	++++	3
											+++	2			+++	2	++	1						

Scoring: (−) no transduction, (+) very few positive cells, (++) some positive cells, (+++) many positive cells, and (++++) region is almost saturated with EGFP-positive cells. The number of animals (n) with the particular score is given to the right of the score.

Example 10: Evaluation of an rAAV Based Treatment in a Canavan Disease Model
Introduction to the Example

[0157] CD is a rare and fatal childhood leukodystrophy caused by autosomal recessive mutations in the aspartoacylase gene (*ASPA*) [as established by G.G.'s graduate work (12)]. ASPA deficiency in CD patients leads to elevated N-Acetyl-Aspartic Acid (NAA) in urine (a hallmark of CD) and spongy degeneration of white matter throughout the CNS, producing severe psychomotor retardation and early death. An $ASPA^{-/-}$ mouse model mimics the neuropathology and clinical manifestations seen in CD patients, i.e., spongy degeneration of white matter, motor deficits, developmental delays, and early death (within 3 weeks after birth).

[0158] In this study, i.v. deliverable rAAVs were used to target the CNS globally to treat diffused WM degeneration in CD mice. Single i.v. injections of *ASPA* vector to the neonatal CD mice corrected metabolic defect, psychomotor malfunction and other disease phenotypes, and prolonged survival. While untreated CD mice started showing growth retardation, psychomotor malfunction in the 2^{nd} wk after birth and uniformly died soon after weaning, the treated mice began to gain weight 2 wks after vector injection and nearly caught up with their heterozygous littermates within 7 -8 weeks. Unlike CD mice, the mobility of the treated animals was similar to Wt littermates. Data from rotarod test on the treated mice showed no significant differences in the latency time among the treated CD mice and their age-matched Wt littermates, indicating that gene therapy corrected the ataxia, a typical neuromuscular symptom of CD. Biochemical characterization indicated reduction of NAA levels in the urine samples and restoration of ASPA activity in their brain and kidney tissues. Mitigation of the biochemical and clinical phenotypes was well correlated with globally ameliorated histopathology in not only the brain, spinal cord but also in the peripheral tissues such as kidney, indicating that CD is not just a CNS disorder.

Results

[0159] In CD mice were dosed at P1 (facial vein, $4x10^{11}$ GCs) with *AAV9ASPA*. The mice were monitored for growth, gait, motor function on rotarod, NAA levels in urine and ASPA activities in brain. The results showed that i) Untreated CD mice started losing weight at the 2^{nd} week and died in the 3^{rd} week after birth; ii) The treated animals recovered their capacity to grow in the 5^{th} week and caught up with $ASPA^{+/-}$ animal by the 10^{th} week; iii). Gene therapy completely corrected gait of CD mice as well as motor function of the CD mice treated at P1 (Figure 30A) as measured by rotarod test; iv). Gene therapy restored the vision of CD mice. The electroretinography (ERG) tests on the eyes of the CD mice showed non-recordable responses to light, while well-defined ERG responses were readily detectable in the treated CD mice (Figure 30B). These data indicate a severer retinopathy and loss of vision in CD mice and gene therapy can mitigate the retinopathy and restore the vision of CD mice; v). Gene therapy clearly improved metabolic defects of NAA as the NAA levels in the treated CD mice approach those in the control mice (Figure 30C); and vi) correction of NAA metabolism is well correlated with restoration of ASPA expression (Figure 30E) and activities (Figure 30D) in the brain of the treated CD mice.

[0160] To determine if the phenotypic corrections are correlated with alleviated neuropathology as well as in situ expression of ASPA in the brain sections of the treated CD mice, brain sections were analyzed at 3 months after gene therapy for neuropathology and ASPA immunohistochemistry. While the untreated mouse brain shows marked vacuolation that diffusely involves all regions of the brain and spinal cord, the vacuolation in both brain and spinal cord of the treated animal appears more patchy and variable with generally smaller-sized vacuoles. Some areas of the cerebral cortex show almost no vacuolation (Figure 31A). In addition, avidin-biotin complex (ABC) system was used to stain brain sections to detect ASPA expression in the cerebral cortex in situ (Figure 31B). To generate quantitative measurements on the improvement of neuropathology in the treated CD mice, the "vacuoles" in the brain and spinal cord sections caused by the white matter degeneration in the CD mice were quantified before and after gene therapy treatment. For this quantitative analysis, a Nikon Eclipse Ti inverted microscope and Nikon NIS elements AR software V.3.2 were used. Vacuoles that were > 3,000 pixels, 1,000 - 3,000 pixels and 100- 1,000 pixels were defined as large, medium and small vacuoles respectively. Among the 5 brain regions evaluated in this experiment, the olfactory bulb had the most dramatic mitigation in the white matter degeneration after gene therapy (Figure 32A). For the other 3 regions, while the large vacuoles were completely eliminated and the numbers of medium vacuoles were remarkably reduced, the reduction in the numbers of small vacuoles (<100 um) was not as significant in this experiment (Figure 32A). The same analysis on the spinal cord sections revealed a similar trend (Figure 32B).

[0161] Histopathology of the kidneys in the CD mice were evaluated. The glomeruli showed normal structure but were associated with dilation of Bowman's spaces. The renal tubular epithelium was diffusely attenuated (or atrophic) in association with enlargement of the tubular lumens (Figure 33A). In contrast, the treated CD mouse had normal glomeruli. Renal tubular epithelial cells were well-stained and normal in volume (Figure 33B). These results indicate the involvement of kidney in the pathophysiology of CD and kidney as a peripheral target for CD gene therapy. This result also indicates renal tropism of AAV vectors as a consideration for selection of a vector for CD gene therapy. Two vectors, rAAV9 and rh.10 were evaluated for efficiency of kidney transduction after IV delivery to 10 week old C57BL/6 mice. The results indicate the use of rAAVrh.10 (Figure 33D) as a useful vector for CD gene therapy because it transduces kidney efficiently in addition to its efficient CNS transduction (Figure 33C).

NUCLEIC ACID AND AMINO ACID SEQUENCES

[0162]

>gi|9632548|ref|NP_049542.1| capsid protein [Adeno-associated virus - 1] (SEQ ID NO: 1)

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLG
PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRVLEPLGLVEEGAKTAPGKKRPVEQSPQEPDSSSGIGKTGQQP
AKKRLNFGQTGDSESVPDPQPLGEPPATPAAVGPTTMASGGGAPMADNNEGADGV
GNASGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISSASTGASNDNHYFGYS
TPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTTNDGVTTIA
NNLTSTVQVFSDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRS
SFYCLEYFPSQMLRTGNNFTFSYTFEEVPFHSSYAHSQSLDRLMNPLIDQYLYYLNRT
QNQSGSAQNKDLLFSRGSPAGMSVQPKNWLPGPCYRQQRVSKTKTDNNNSNFTWT
GASKYNLNGRESIINPGTAMASHKDDEDKFFPMSGVMIFGKESAGASNTALDNVMIT
DEEEIKATNPVATERFGTVAVNFQSSSTDPATGDVHAMGALPGMVWQDRDVYLQG
PIWAKIPHTDGHFHPSPLMGGFGLKNPPPQILIKNTPVPANPPAEFSATKFASFITQYST
GQVSVEIEWELQKENSKRWNPEVQYTSNYAKSANVDFTVDNNGLYTEPRPIGTRYL
TRPL

>gi|10645923|ref|YP_680426.1| major coat protein VP1 [Adeno-associated virus - 2] (SEQ ID NO: 2)

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYKYLGPF
NGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEFQERLKEDTSFG
GNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSPVEPDSSSGTGKAGQQPA
RKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGTNTMATGSGAPMADNNEGADGVG
NSSGNWHCDSTWMGDRVITTSTRTWALPTYNNHLYKQISSQSGASNDNHYFGYSTP
WGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTIAN
NLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRS
SFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRT
NTPSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEYSWTG
ATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKTNVDIEKVMITD
EEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGVLPGMVWQDRDVYLQGPI
WAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANPSTTFSAAKFASFITQYSTG
QVSVEIEWELQKENSKRWNPEIQYTSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTR
NL

>gi|51593838|ref|YP_068409.1| capsid protein [Adeno-associated virus - 5] (SEQ ID NO: 3)

MSFVDHPPDWLEEVGEGLREFLGLEAGPPKPKPNQQHQDQARGLVLPGYNYLGPGN
GLDRGEPVNRADEVAREHDISYNEQLEAGDNPYLKYNHADAEFQEKLADDTSFGGN
LGKAVFQAKKRVLEPFGLVEEGAKTAPTGKRIDDHFPKRKKARTEEDSKPSTSSDAE
AGPSGSQQLQIPAQPASSLGADTMSAGGGGPLGDNNQGADGVGNASGDWHCDSTW
MGDRVVTKSTRTWVLPSYNNHQYREIKSGSVDGSNANAYFGYSTPWGYFDFNRFHS
HWSPRDWQRLINNYWGFRPRSLRVKIFNIQVKEVTVQDSTTTIANNLTSTVQVFTDD
DYQLPYVVGNGTEGCLPAFPPQVFTLPQYGYATLNRDNTENPTERSSFFCLEYFPSK
MLRTGNNFEFTYNFEEVPFHSSFAPSQNLFKLANPLVDQYLYRFVSTNNTGGVQFNK
NLAGRYANTYKNWFPGPMGRTQGWNLGSGVNRASVSAFATTNRMELEGASYQVPP
QPNGMTNNLQGSNTYALENTMIFNSQPANPGTTATYLEGNMLITSESETQPVNRVAY
NVGGQMATNNQSSTTAPATGTYNLQEIVPGSVWMERDVYLQGPIWAKIPETGAHFH
PSPAMGGFGLKHPPPMMLIKNTPVPGNITSFSDVPVSSFITQYSTGQVTVEMEWELKK
ENSKRWNPEIQYTNNYNDPQFVDFAPDSTGEYRTTRPIGTRYLTRPL

>gi|2766607|gb|AAB95450.1| capsid protein VP1 [Adeno-associated virus - 6] (SEQ ID NO: 4)

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLG

PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRVLEPFGLVEEGAKTAPGKKRPVEQSPQEPDSSSGIGKTGQQP
AKKRLNFGQTGDSESVPDPQPLGEPPATPAAVGPTTMASGGGAPMADNNEGADGV
GNASGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISSASTGASNDNHYFGYS
TPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTTNDGVTTIA
NNLTSTVQVFSDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRS
SFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLNRT
QNQSGSAQNKDLLFSRGSPAGMSVQPKNWLPGPCYRQQRVSKTKTDNNNSNFTWT
GASKYNLNGRESIINPGTAMASHKDDKDKFFPMSGVMIFGKESAGASNTALDNVMIT
DEEEIKATNPVATERFGTVAVNLQSSSTDPATGDVHVMGALPGMVWQDRDVYLQG
PIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANPPAEFSATKFASFITQYST
GQVSVEIEWELQKENSKRWNPEVQYTSNYAKSANVDFTVDNNGLYTEPRPIGTRYL
TRPL

>gi|171850125|gb|ACB55302.1| capsid protein VP1 [Adeno-associated virus - 6.2] (SEQ ID NO: 5)

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLG
PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRVLEPLGLVEEGAKTAPGKKRPVEQSPQEPDSSSGIGKTGQQP
AKKRLNFGQTGDSESVPDPQPLGEPPATPAAVGPTTMASGGGAPMADNNEGADGV
GNASGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISSASTGASNDNHYFGYS
TPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTTNDGVTTIA
NNLTSTVQVFSDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRS
SFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLNRT
QNQSGSAQNKDLLFSRGSPAGMSVQPKNWLPGPCYRQQRVSKTKTDNNNSNFTWT
GASKYNLNGRESIINPGTAMASHKDDKDKFFPMSGVMIFGKESAGASNTALDNVMIT
DEEEIKATNPVATERFGTVAVNLQSSSTDPATGDVHVMGALPGMVWQDRDVYLQG
PIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANPPAEFSATKFASFITQYST

GQVSVEIEWELQKENSKRWNPEVQYTSNYAKSANVDFTVDNNGLYTEPRPIGTRYL
TRPL

>gi|22652861|gb|AAN03855.1|AF513851_2 capsid protein [Adeno-associated virus - 7] (SEQ ID NO: 6)

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDNGRGLVLPGYKYLG
PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRVLEPLGLVEEGAKTAPAKKRPVEPSPQRSPDSSTGIGKKGQQ
PARKRLNFGQTGDSESVPDPQPLGEPPAAPSSVGSGTVAAGGGAPMADNNEGADGV
GNASGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISSETAGSTNDNTYFGYS
TPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKKLRFKLFNIQVKEVTTNDGVTTIA
NNLTSTIQVFSDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQSVGRSS
FYCLEYFPSQMLRTGNNFEFSYSFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLARTQ
SNPGGTAGNRELQFYQGGPSTMAEQAKNWLPGPCFRQQRVSKTLDQNNNSNFAWT
GATKYHLNGRNSLVNPGVAMATHKDDEDRFFPSSGVLIFGKTGATNKTTLENVLMT
NEEEIRPTNPVATEEYGIVSSNLQAANTAAQTQVVNNQGALPGMVWQNRDVYLQGP
IWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPANPPEVFTPAKFASFITQYSTG
QVSVEIEWELQKENSKRWNPEIQYTSNFEKQTGVDFAVDSQGVYSEPRPIGTRYLTR
NL

>gi|22652864|gb|AAN03857.1|AF513852_2 capsid protein [Adeno-associated virus - 8] (SEQ ID NO: 7)

MAADGYLPDWLEDNLSEGIREWWALKPGAPKPKANQQKQDDGRGLVLPGYKYLG
PFNGLDKGEPVNAADAAALEHDKAYDQQLQAGDNPYLRYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRVLEPLGLVEEGAKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQ
PARKRLNFGQTGDSESVPDPQPLGEPPAAPSGVGPNTMAAGGGAPMADNNEGADG
VGSSSGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISNGTSGGATNDNTYFG
YSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLSFKLFNIQVKEVTQNEGTKT

IANNLTSTIQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGR
SSFYCLEYFPSQMLRTGNNFQFTYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSR
TQTTGGTANTQTLGFSQGGPNTMANQAKNWLPGPCYRQQRVSTTTGQNNNSNFAW
TAGTKYHLNGRNSLANPGIAMATHKDDEERFFPSNGILIFGKQNAARDNADYSDVM
LTSEEEIKTTNPVATEEYGIVADNLQQQNTAPQIGTVNSQGALPGMVWQNRDVYLQ
GPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPTTFNQSKLNSFITQYS
TGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTSVDFAVNTEGVYSEPRPIGTRYL
TRNL

>gi|46487805|gb|AAS99264.1| capsid protein VP1 [Adeno-associated virus 9] (SEQ ID NO: 8)
MAADGYLPDWLEDNLSEGIREWWALKPGAPQPKANQQHQDNARGLVLPGYKYLG
PGNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLKYNHADAEFQERLKEDTS
FGGNLGRAVFQAKKRLLEPLGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQP
AKKRLNFGQTGDTESVPDPQPIGEPPAAPSGVGSLTMASGGGAPVADNNEGADGVG
SSSGNWHCDSQWLGDRVITTSTRTWALPTYNNHLYKQISNSTSGGSSNDNAYFGYST
PWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTDNNGVKTIA
NNLTSTVQVFTDSDYQLPYVLGSAHEGCLPPFPADVFMIPQYGYLTLNDGSQAVGRS
SFYCLEYFPSQMLRTGNNFQFSYEFENVPFHSSYAHSQSLDRLMNPLIDQYLYYLSKT
INGSGQNQQTLKFSVAGPSNMAVQGRNYIPGPSYRQQRVSTTVTQNNNSEFAWPGA

SSWALNGRNSLMNPGPAMASHKEGEDRFFPLSGSLIFGKQGTGRDNVDADKVMITN
EEEIKTTNPVATESYGQVATNHQSAQAQAQTGWVQNQGILPGMVWQDRDVYLQGP
IWAKIPHTDGNFHPSPLMGGFGMKHPPPQILIKNTPVPADPPTAFNKDKLNSFITQYST
GQVSVEIEWELQKENSKRWNPEIQYTSNYYKSNNVEFAVNTEGVYSEPRPIGTRYLT
RNL

>gi|29650526|gb|AAO88201.1| capsid protein [Non-human primate Adeno-associated virus] (SEQ ID NO: 9) rh-10
MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLG
PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRVLEPLGLVEEGAKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQ
PAKKRLNFGQTGDSESVPDPQPIGEPPAGPSGLGSGTMAAGGGAPMADNNEGADGV
GSSSGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISNGTSGGSTNDNTYFGY
STPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTQNEGTKTI
ANNLTSTIQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGR
SSFYCLEYFPSQMLRTGNNFEFSYQFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSR
TQSTGGTAGTQQLLFSQAGPNNMSAQAKNWLPGPCYRQQRVSTTLSQNNNSNFAW
TGATKYHLNGRDSLVNPGVAMATHKDDEERFFPSSGVLMFGKQGAGKDNVDYSSV
MLTSEEEIKTTNPVATEQYGVVADNLQQQNAAPIVGAVNSQGALPGMVWQNRDVY
LQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPTTFSQAKLASFIT
QYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTNVDFAVNTDGTYSEPRPIGT
RYLTRNL

>gi|171850147|gb|ACB55313.1| capsid protein VP1 [Adeno-associated virus - rh.39] (SEQ ID NO: 10)
MAADGYLPDWLEDNLSEGIREWWALKPGAPKPKANQQKQDDGRGLVLPGYKYLG
PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRVLEPLGLVEEAAKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQ
PAKKRLNFGQTGDSESVPDPQPIGEPPAGPSGLGSGTMAAGGGAPMADNNEGADGV
GSSSGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISNGTSGGSTNDNTYFGY
STPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLSFKLFNIQVKEVTQNEGTKTI
ANNLTSTIQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGR
SSFYCLEYFPSQMLRTGNNFEFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSR
TQSTGGTQGTQQLLFSQAGPANMSAQAKNWLPGPCYRQQRVSTTLSQNNNSNFAW
TGATKYHLNGRDSLVNPGVAMATHKDDEERFFPSSGVLMFGKQGAGRDNVDYSSV
MLTSEEEIKTTNPVATEQYGVVADNLQQTNTGPIVGNVNSQGALPGMVWQNRDVY
LQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPTTFSQAKLASFIT

QYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTNVDFAVNTEGTYSEPRPIGT
RYLTRNL

>gi|46487767|gb|AAS99245.1| capsid protein VP1 [Adeno-associated virus rh.43] (SEQ ID NO: 11)

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLG
PFNGLDKGEPVNAADAAALEHDKAYDQQLEAGDNPYLRYNHADAEFQERLQEDTS
FGGNLGRAVFQAKKRVLEPLGLVEEGAKTAPGKKRPVEQSPQEPDSSSGIGKKGQQP
ARKRLNFGQTGDSESVPDPQPLGEPPAAPSGVGPNTMAAGGGAPMADNNEGADGV
GSSSGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISNGTSGGATNDNTYFGY
STPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLSFKLFNIQVKEVTQNEGTKTI

ANNLTSTIQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGR
SSFYCLEYFPSQMLRTGNNFQFTYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSR
TQTTGGTANTQTLGFSQGGPNTMANQAKNWLPGPCYRQQRVSTTTGQNNNSNFAW
TAGTKYHLNGRNSLANPGIAMATHKDDEERFFPVTGSCFWQQNAARDNADYSDVM
LTSEEEIKTTNPVATEEYGIVADNLQQQNTAPQIGTVNSQGALPGMVWQNRDVYLQ
GPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPTTFNQSKLNSFITQYS
TGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTSVDFAVNTEGVYSEPRPIGTRYL
TRNL

>capsid protein VP1 [Adeno-associated virus] CSp3

(SEQ ID NO: 12)

MAADGYLPDWLEDNLSEGIREWWALKPGAPQPKANQQHQDNARGLVLPGYKYLG
PGNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLKYNHADAEFQERLKEDTS
FGGNLGRAVFQAKKRLLEPLGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQP
AKKRLNFGQTGDTESVPDPQPIGEPPAAPSGVGSLTIASGGGAPVADNNEGADGVGS
SSGNWHCDSQWLGDRVITTSTRTWALPTYNNHLYKRISNSTSGGSSNDNAYFGYSTP
WGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIRVKEVTDNNGVKTITN
NLTSTVQVFTDSDYQLPYVLGSAHEGCLPPFPADVFMIPQYGYLTLNDGSQAVGRSS
FYCLEYFPSQMLRTGNNFQFSYEFENVPFHSSYAHSQSLDRLMNPLIDQYLYYLSKTI
NGSGQNQQTLKFSVAGPSNMAVQGRNYIPGPSYRQQRVSTTVTRNNNSEFAWPGAS
SWALNGRNSLMNPGPAMASHKEGEDRFFPLSGSLIFGKQGTGRDNVDADKVMITNE
EEIKTTNPVATESYGQVATNHQSAQAQAQTGWVQNQGILPGMVWQDRDVYLQGPI
WAKIPHTDGNFHPSPLMGGFGVKHPPPQILIKNTPVPADPPTAFNKDKLNSFITQYST
GQVSVEIEWELQKENSKRWNPEIQYTSNYYKSNNVEFAVNTEGVYSEPRPIGTRYLT
RNL

>gi|189339202|ref|NP_001121557.1| aspartoacylase [Homo sapiens]

(SEQ ID NO: 13)

MTSCHIAEEHIQKVAIFGGTHGNELTGVFLVKHWLENGAEIQRTGLEVKPFITNPRAV
KKCTRYIDCDLNRIFDLENLGKKMSEDLPYEVRRAQEINHLFGPKDSEDSYDIIFDLH
NTTSNMGCTLILEDSRNNFLIQMFHYIKTSLAPLPCYVYLIEHPSLKYATTRSIAKYPV
GIEVGPQPQGVLRADILDQMRKMIKHALDFIHHFNEGKEFPPCAIEVYKIIEKVDYPR
DENGEIAAIIHPNLQDQDWKPLHPGDPMFLTLDGKTIPLGGDCTVYPVFVNEAAYYE
KKEAFAKTTKLTLNAKSIRCCLH

>gi|189339201:92-1033 Homo sapiens aspartoacylase (Canavan disease) (ASPA), transcript variant 2, mRNA

(SEQ ID NO: 14)

ATGACTTCTTGTCACATTGCTGAAGAACATATACAAAAGGTTGCTATCTTTGGAG
GAACCCATGGGAATGAGCTAACCGGAGTATTTCTGGTTAAGCATTGGCTAGAGA
ATGGCGCTGAGATTCAGAGAACAGGGCTGGAGGTAAAACCATTTATTACTAACC
CCAGAGCAGTGAAGAAGTGTACCAGATATATTGACTGTGACCTGAATCGCATTTT
TGACCTTGAAAATCTTGGCAAAAAAATGTCAGAAGATTTGCCATATGAAGTGAG
AAGGGCTCAAGAAATAAATCATTTATTTGGTCCAAAAGACAGTGAAGATTCCTAT

GACATTATTTTTGACCTTCACAACACCACCTCTAACATGGGGTGCACTCTTATTCT
TGAGGATTCCAGGAATAACTTTTTAATTCAGATGTTTCATTACATTAAGACTTCTC
TGGCTCCACTACCCTGCTACGTTTATCTGATTGAGCATCCTTCCCTCAAATATGCG
ACCACTCGTTCCATAGCCAAGTATCCTGTGGGTATAGAAGTTGGTCCTCAGCCTC

AAGGGGTTCTGAGAGCTGATATCTTGGATCAAATGAGAAAAATGATTAAACATG
CTCTTGATTTTATACATCATTTCAATGAAGGAAAAGAATTTCCTCCCTGCGCCATT
GAGGTCTATAAAATTATAGAGAAAGTTGATTACCCCCGGGATGAAAATGGAGAA
ATTGCTGCTATCATCCATCCTAATCTGCAGGATCAAGACTGGAAACCACTGCATC
CTGGGGATCCCATGTTTTTAACTCTTGATGGGAAGACGATCCCACTGGGCGGAGA
CTGTACCGTGTACCCCGTGTTTGTGAATGAGGCCGCATATTACGAAAAGAAAGA
AGCTTTTGCAAAGACAACTAAACTAACGCTCAATGCAAAAAGTATTCGCTGCTGT
TTACATTAG

>gi|31560279|ref|NP_075602.2| aspartoacylase [Mus Musculus]

(SEQ ID NO: 15)

MTSCVAKEPIKKIAIFGGTHGNELTGVFLVTHWLRNGTEVHRAGLDVKPFITNPRAV
EKCTRYIDCDLNRVFDLENLSKEMSEDLPYEVRRAQEINHLFGPKNSDDAYDLVFDL
HNTTSNMGCTLILEDSRNDFLIQMFHYIKTCMAPLPCSVYLIEHPSLKYATTRSIAKYP
VGIEVGPQPHGVLRADILDQMRKMIKHALDFIQHFNEGKEFPPCSIDVYKIMEKVDYP
RNESGDMAAVIHPNLQDQDWKPLHPGDPVFVSLDGKVIPLGGDCTVYPVFVNEAAY
YEKKEAFAKTTKLTLSAKSIRSTLH

>gi|142354273:148-1086 Mus musculus aspartoacylase (Aspa), mRNA

(SEQ ID NO: 16)

ATGACCTCTTGTGTTGCTAAAGAACCTATTAAGAAGATTGCCATCTTTGGAGGGA
CTCATGGAAATGAACTGACCGGAGTGTTTCTAGTTACTCACTGGCTAAGGAATGG
CACTGAAGTTCACAGAGCAGGGCTGGACGTGAAGCCATTCATTACCAATCCAAG
GGCGGTGGAGAAGTGCACCAGATACATTGACTGTGACCTGAATCGTGTTTTTGAC
CTTGAAAATCTTAGCAAAGAGATGTCTGAAGACTTGCCATATGAAGTGAGAAGG
GCTCAAGAAATAAATCATTTATTTGGTCCAAAAAATAGTGATGATGCCTATGACC
TTGTTTTTGACCTTCACAACACCACTTCTAACATGGGTTGCACTCTTATTCTTGAG
GATTCCAGGAATGACTTTTTAATTCAGATGTTTCACTATATTAAGACTTGCATGGC
TCCATTACCCTGCTCTGTTTATCTCATTGAGCATCCTTCACTCAAATATGCAACCA
CTCGTTCCATTGCCAAGTATCCTGTTGGTATAGAAGTTGGTCCTCAGCCTCACGGT
GTCCTTAGAGCTGATATTTTAGACCAAATGAGAAAAATGATAAAACATGCTCTTG
ATTTTATACAGCATTTCAATGAAGGAAAAGAATTTCCTCCCTGTTCTATTGACGTC
TATAAAATAATGGAGAAAGTTGATTATCCAAGGAATGAAAGTGGAGACATGGCT
GCTGTTATTCATCCTAATCTGCAGGATCAAGACTGGAAACCATTGCACCCTGGAG
ATCCTGTGTTTGTGTCTCTTGATGGAAAAGTTATTCCACTGGGTGGAGACTGTAC
CGTGTACCCAGTGTTTGTGAATGAAGCTGCATATTATGAAAAAAAAGAAGCATTT
GCAAAGACAACAAAACTAACACTCAGCGCAAAAAGCATCCGCTCCACTTTGCAC
TAA

>gi|48762945:149-613 Homo sapiens superoxide dismutase 1, soluble (SOD1), mRNA

(SEQ ID NO: 17)

ATGGCGACGAAGGCCGTGTGCGTGCTGAAGGGCGACGGCCCAGTGCAGGGCATC
ATCAATTTCGAGCAGAAGGAAAGTAATGGACCAGTGAAGGTGTGGGGAAGCATT
AAAGGACTGACTGAAGGCCTGCATGGATTCCATGTTCATGAGTTTGGAGATAATA
CAGCAGGCTGTACCAGTGCAGGTCCTCACTTTAATCCTCTATCCAGAAAACACGG
TGGGCCAAAGGATGAAGAGAGGCATGTTGGAGACTTGGGCAATGTGACTGCTGA
CAAAGATGGTGTGGCCGATGTGTCTATTGAAGATTCTGTGATCTCACTCTCAGGA
GACCATTGCATCATTGGCCGCACACTGGTGGTCCATGAAAAAGCAGATGACTTG

GGCAAAGGTGGAAATGAAGAAAGTACAAAGACAGGAAACGCTGGAAGTCGTTT
GGCTTGTGGTGTAATTGGGATCGCCCAATAA

>gi|4507149|ref|NP_000445.1| superoxide dismutase [Homo sapiens]
(SEQ ID NO: 18)
MATKAVCVLKGDGPVQGIINFEQKESNGPVKVWGSIKGLTEGLHGFHVHEFGDNTA
GCTSAGPHFNPLSRKHGGPKDEERHVGDLGNVTADKDGVADVSIEDSVISLSGDHCII
GRTLVVHEKADDLGKGGNEESTKTGNAGSRLACGVIGIAQ

>gi|45597446:117-581 Mus musculus superoxide dismutase 1, soluble (Sod1), mRNA
(SEQ ID NO: 19)
ATGGCGATGAAAGCGGTGTGCGTGCTGAAGGGCGACGGTCCGGTGCAGGGAACC
ATCCACTTCGAGCAGAAGGCAAGCGGTGAACCAGTTGTGTTGTCAGGACAAATT
ACAGGATTAACTGAAGGCCAGCATGGGTTCCACGTCCATCAGTATGGGGACAAT
ACACAAGGCTGTACCAGTGCAGGACCTCATTTTAATCCTCACTCTAAGAAACATG
GTGGCCCGGCGGATGAAGAGAGGCATGTTGGAGACCTGGGCAATGTGACTGCTG
GAAAGGACGGTGTGGCCAATGTGTCCATTGAAGATCGTGTGATCTCACTCTCAGG
AGAGCATTCCATCATTGGCCGTACAATGGTGGTCCATGAGAAACAAGATGACTT
GGGCAAAGGTGGAAATGAAGAAAGTACAAAGACTGGAAATGCTGGGAGCCGCT
TGGCCTGTGGAGTGATTGGGATTGCGCAGTAA

>gi|45597447|ref|NP_035564.1| superoxide dismutase [Mus musculus]
(SEQ ID NO: 20)
MAMKAVCVLKGDGPVQGTIHFEQKASGEPVVLSGQITGLTEGQHGFHVHQYGDNT
QGCTSAGPHFNPHSKKHGGPADEERHVGDLGNVTAGKDGVANVSIEDRVISLSGEH
SIIGRTMVVHEKQDDLGKGGNEESTKTGNAGSRLACGVIGIAQ

>pAAVscCB6 EGFPmir SOD5 (direct) 5243bp (SEQ ID NO: 21)
CTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCG
ACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGTAG
CCATGCTCTAGGAAGATCAATTCAATTCACGCGTCGACATTGATTATTGACTAGT
TATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCC
GCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCG
CCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTC
CATTGACGTCAATGGGTGGATATTTACGGTAAACTGCCCACTTGGCAGTACATCA
AGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCC
GCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACA
TCTACGTATTAGTCATCGCTATTACCATGTCGAGGCCACGTTCTGCTTCACTCTCC
CCATCTCCCCCCCCTCCCCACCCCCAATTTTGTATTTATTTATTTTTTAATTATTTT
GTGCAGCGATGGGGGCGGGGGGGGGGGGCGCGCGCCAGGCGGGGCGGGGCGGG
GCGAGGGGCGGGGCGGGGCGAGGCGGAGAGGTGCGGCGGCAGCCAATCAGAGC
GGCGCGCTCCGAAAGTTTCCTTTTATGGCGAGGCGGCGGCGGCGGCGGCCCTATA
AAAAGCGAAGCGCGCGGCGGGCGGGAGCAAGCTCTAGCCTCGAGAATTCACGCG
TGGTACCTCTAGAGCAGAGCTCGTTTAGTGAACCGTCAGTTCGAAATCGCCACCA
TGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGC
TGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCG
ATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCC
CGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGC

CGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAG
GCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCC
GCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGG
GCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACT
ACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGG
TGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACC
ACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACC
ACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATC
ACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGA
GCTGTACAAGTAAGTAACAGGTAAGTGCGATCGCTAATGCGGGAAAGCTCTTAT

TCGGGTGAGATGGGCTGGGGCACCATCTGGGGACCCTGACGTGAAGTTTGTCACT
GACTGGAGAACTCGGTTTGTCGTCTGTTGCGGGGGCGGCAGTTATGGCGGTGCCG
TTGGGCAGTGCACCCGTACCTTTGGGAGCGCGCGCCCTCGTCGTGTCGTGACGTC
ACCCGTTCTGTTGGTACCTGCTGTTGACAGTGAGCGACGCAATGTGACTTCGCTG
ACAAAGCTGTGAAGCCACAGATGGGCTTTGTCAGCAGTCACATTGCGCTGCCTAC
TGCCTCGGACTTCAAGGGCTCGAGAATTCAGGGTGGGGCCACCTGCCGGTAGGT
GTGCGGTAGGCTTTTCTCCGTCGCAGGACGCAGGGTTCGGGCCTAGGGTAGGCTC
TCCTGAATCGACAGGCGCCGGACCTCTGGCGGCCGCAACAACGCGTTCCTGACC
ATTCATCCTCTTTCTTTTTCCTGCAGGCTTGTGGAAGAAATGGGATCCGATCTTTT
TCCCTCTGCCAAAAATTATGGGGACATCATGAAGCCCCTTGAGCATCTGACTTCT
GGCTAATAAAGGAAATTTATTTTCATTGCAATAGTGTGTTGGAATTTTTTGTGTCT
CTCACTCGGCCTAGGTAGATAAGTAGCATGGCGGGTTAATCATTAACTACAAGG
AACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGA
GGCCGGGCGACCAAAGGTCGCCCGACGCCCGGGCTTTGCCCGGGCGGCCTCAGT
GAGCGAGCGAGCGCGCAGCCTTAATTAACCTAATTCACTGGCCGTCGTTTTACAA
CGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATC
CCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCA
ACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAG
CGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTA
GCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCC
CGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGC
ACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCC
CTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGAC
TCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTA
TAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAA
AATTTAACGCGAATTTTAACAAAATATTAACGCTTACAATTTAGGTGGCACTTTT
CGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATA
TGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAG
GAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCAT
TTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGA
AGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAA
GATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAA
GTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCG
GTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGA
AAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAAC
CATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAA
GGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGT
TGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATG

CCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTC
TAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGAC
CACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCC
GGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCC
TCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGA
AATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAG
ACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAA
AGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTG
AGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTG
AGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTA
CCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAA
CTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTT
AGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATC
CTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACT
CAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGT
GCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGC
GTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATC
CGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGA
AACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCG

ATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGC
GGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGC
GTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACC
GCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGA
AGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGC
AGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATT
AATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGG
CTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTA
TGACCATGATTACGCCAGATTTAATTAAGGCCTTAATTAGG

>sod1mir1 (direct) 108bp

(SEQ ID NO: 22)

TGCTGTTGACAGTGAGCGACATCATCAATTTTCCGAGCAGAACTGTGAAGCCACA
GATGGGTTCTGCTCGAAATTGATGATGCTGCCTACTGCCTCGGACTTCAAGGG

>sod1mir2 (direct) 106bp

(SEQ ID NO: 23)

TGCTGTTGACAGTGAGCGACGCATTAAAGGATCCTGACTGACTGTGAAGCCACA
GATGGGTCAGTCAGTCCTTTAATGCGCTGCCTACTGCCTCGGACTTCAAGGG

>sod1mir3 (direct) 108bp

(SEQ ID NO: 24)

TGCTGTTGACAGTGAGCGACTGCATGGATTCTCCATGTTCATCTGTGAAGCCACA
GATGGGATGAACATGGAATCCATGCAGCTGCCTACTGCCTCGGACTTCAAGGG

>sod1mir4 (direct) 106bp

(SEQ ID NO: 25)

TGCTGTTGACAGTGAGCGACAAGGATGAAGATCGAGGCATGCTGTGAAGCCACA
GATGGGCATGCCTCTCTTCATCCTTGCTGCCTACTGCCTCGGACTTCAAGGG

>sod1mir5 (direct) 110bp

(SEQ ID NO: 26)

TGCTGTTGACAGTGAGCGACGCAATGTGACTTCGCTGACAAAGCTGTGAAGCCA
CAGATGGGCTTTGTCAGCAGTCACATTGCGCTGCCTACTGCCTCGGACTTCAAGG
G

>sod1mir6 (direct) 108bp

(SEQ ID NO: 27)

TGCTGTTGACAGTGAGCGACCGATGTGTCTATCTTGAAGATTCTGTGAAGCCACA
GATGGGAATCTTCAATAGACACATCGGCTGCCTACTGCCTCGGACTTCAAGGG

>sod1mir7 (direct) 106bp

(SEQ ID NO: 28)

TGCTGTTGACAGTGAGCGACGGTGGAAATGATCAGAAAGTACTGTGAAGCCACA
GATGGGTACTTTCTTCATTTCCACCGCTGCCTACTGCCTCGGACTTCAAGGG

>sod1mir8 (direct) 110bp

(SEQ ID NO: 29)

TGCTGTTGACAGTGAGCGACGCTGTAGAAATTCGTATCCTGATCTGTGAAGCCAC
AGATGGGATCAGGATACATTTCTACAGCGCTGCCTACTGCCTCGGACTTCAAGGG

>sod1mir9 (direct) 106bp

(SEQ ID NO: 30)

TGCTGTTGACAGTGAGCGAGGTATTAAACTTGTCAGAATTTAGTGAAGCCACAGA
TGTAAATTCTGACAAGTTTAATACCCTGCCTACTGCCTCGGACTTCAAGGG

TGTAAATTCTGACAAGTTTAATACCCTGCCTACTGCCTCGGACTTCAAGGG

>pAAVscCB6 EGFPmir scr (1820bp - 1925bp, direct) 106bp
(SEQ ID NO: 31)
TGCTGTTGACAGTGAGCGACGATGCTCTAATCGGTTCTATCAAGTGAAGCCACAG
ATGTTGATAGAACCTTAGAGCATCGCTGCCTACTGCCTCGGACTTCAAGGG

REFERENCES CITED IN THE DESCRIPTION

Patent documents cited in the description

- WO2010129021A **[0005]**
- WO2007127264A **[0005]**
- US20030138772A **[0036]** **[0036]**
- US20070036760A **[0036]**
- US20090197338A **[0036]**
- US61182084A **[0036]**
- US5478745A **[0038]**
- US6001650A **[0039]** **[0039]**
- US6156303A **[0039]**
- WO9810088A **[0052]**
- US5543158A **[0061]**
- US5641515A **[0061]**
- US5399363A **[0061]**
- US5741516A **[0074]**
- US5567434A **[0074]**
- US5552157A **[0074]**
- US5565213A **[0074]**
- US5738868A **[0074]**
- US5795587A **[0074]**
- US5656016A **[0078]**
- US5779708A **[0078]**
- US5797898A **[0078]**
- US5770219A **[0078]**
- US5783208A **[0078]**

- US5697899A [0078]

Non-patent literature cited in the description

- **PASSINI et al.**J. Clinical Investigation, 2010, vol. 120, 41253-1264 [0005]
- **SONDHI**Molec Ther., 2006, vol. 15, 481-491 [0005]
- **BEUTLER**Gene Ther., 2009, vol. 16, 461-469 [0005]
- **VULCHANOVA et al.**Molecular Pain, 2010, vol. 6, 131- [0005]
- **CEARLEYWOLFE**Molecular Therapy, 2006, vol. 3, 13528-537 [0005]
- **CEARLEY et al.**Molecular Therapy, 2008, vol. 16, 101710-1718 [0005]
- **IWAMOTO et al.**J. Gene Medicine, vol. 11, 498-505 [0005]
- **MEIJER et al.**Cancer Gene Therapy, 2009, vol. 16, 664-671 [0005]
- **TENENBAUM**J. Gene Medicine, 2004, vol. 6, S212-S222 [0005]
- **RALPH et al.**Nature Medicine, 2005, vol. 11, 4429-433 [0005]
- **LIANG Y et al.**Characterization of microRNA expression profiles in normal human tissuesBMC Genomics, 2007, vol. 8, 166- [0019]
- **XIA et al.**Neurobiol Dis, 2006, vol. 23, 578- [0031]
- **WANG et al.**JBC, 2008, vol. 283, 15845- [0031]
- **WU et al.**Antiox Redox Sig, 2009, vol. 11, 1523- [0031]
- **G. GAO et al.**J. Virol, 2004, vol. 78, 126381-6388 [0036]
- **G. GAO et al.**Proc Natl Acad Sci USA, 2003, vol. 100, 106081-6086 [0036]
- **SAMBROOK et al.**Molecular Cloning: A Laboratory ManualCold Spring Harbor Press [0038]
- **K. FISHER et al.**J. Virol., 1993, vol. 70, 520-532 [0038]
- **GRAHAM et al.**Virology, 1973, vol. 52, 456- [0040]
- **SAMBROOK et al.**Molecular Cloning, a laboratory manualCold Spring Harbor Laboratories19890000 [0040]
- **DAVIS et al.**Basic Methods in Molecular BiologyElsevier19860000 [0040]
- **CHU et al.**Gene, 1981, vol. 13, 197- [0040]
- **B. J. CARTER**Handbook of ParvovirusesCRC Press19900000155-168 [0046]
- **SAMBROOK et al.**Molecular Cloning. A Laboratory ManualCold Spring Harbor Laboratory19890000 [0046]
- **K. FISHER et al.**J Virol.19960000vol. 70, 520-532 [0046]
- **AUSUBEL et al.**Current Protocols in Molecular BiologyJohn Wiley & Sons19890000 [0049]
- **RYAN, M D et al.**EMBO, 1994, vol. 4, 928-933 [0049] [0049]
- **MATTION, N M et al.**J Virology, 1996, 8124-8127 [0049] [0049]
- **FURLER, S et al.**Gene Therapy, 2001, vol. 8, 864-873 [0049] [0049]
- **HALPIN, C et al.**The Plant Journal, 1999, vol. 4, 453-459 [0049] [0049]
- **DE FELIPE, P et al.**Gene Therapy, 1999, vol. 6, 198-208 [0049]

- **DE FELIPE, P et al.**Human Gene Therapy, 2000, vol. 11, 1921-1931 [0049]
- **KLUMP, H et al.**Gene Therapy, 2001, vol. 8, 811-817 [0049]
- **BOSHART et al.**Cell, 1985, vol. 41, 521-530 [0051]
- **NO et al.**Proc. Natl. Acad. Sci. USA, 1996, vol. 93, 3346-3351 [0052]
- **GOSSEN et al.**Proc. Natl. Acad. Sci. USA, 1992, vol. 89, 5547-5551 [0052]
- **GOSSEN et al.**Science, 1995, vol. 268, 1766-1769 [0052]
- **HARVEY et al.**Curr. Opin. Chem. Biol., 1998, vol. 2, 512-518 [0052]
- **WANG et al.**Nat. Biotech., 1997, vol. 15, 239-243 [0052]
- **WANG et al.**Gene Ther., 1997, vol. 4, 432-441 [0052]
- **MAGARI et al.**J. Clin. Invest., 1997, vol. 100, 2865-2872 [0052]
- **ANDERSEN et al.**Cell. Mol. Neurobiol., 1993, vol. 13, 503-15 [0054]
- **PICCIOLI et al.**Proc. Natl. Acad. Sci. USA, 1991, vol. 88, 5611-5 [0054]
- **PICCIOLI et al.**Neuron, 1995, vol. 15, 373-84 [0054]
- **COSTANTINI LC et al.**Gene Therapy, 2000, vol. 7, 93-109 [0058]
- **STEIN et al.**J Virol, 1999, vol. 73, 3424-3429 [0061]
- **DAVIDSON et al.**PNAS, 2000, vol. 97, 3428-3432 [0061]
- **DAVIDSON et al.**Nat. Genet., 1993, vol. 3, 219-223 [0061]
- **ALISKYDAVIDSON**Hum. Gene Ther., 2000, vol. 11, 2315-2329 [0061]
- **WRIGHT FR et al.**Molecular Therapy, 2005, vol. 12, 171-178 [0066]
- Remington's Pharmaceutical Sciences1035-10381570-1580 [0069]
- **MATALON R et al.**The Journal of Gene Medicine, vol. 2, 3165-175 [0088]
- **FOUST KD et al.**Nature Biotechnology, 2010, vol. 28, 3271-274 [0101]
- **DAYA SBERNS KI**Gene Therapy Using Adeno-Associated Virus VectorsClin Microbiol Rev, 2008, vol. 21, 583-593 [0102]
- **EBERLING JLJAGUST WJCHRISTINE CWSTARR PLARSON PBANKIEWICZ KSAMINOFF MJ**Results from a phase I safety trial of hAADC gene therapy for Parkinson diseaseNeurology, 2008, vol. 70, 1980-1983 [0102]
- **FEIGIN AKAPLITT MGTANG CLIN TMATTIS PDHAWAN VDURING MJEIDELBERG D**Modulation of metabolic brain networks after subthalamic gene therapy for Parkinson's diseaseProceedings of the National Academy of Sciences, 2007, vol. 104, 19559-19564 [0102]
- **CIDECIYAN AVHAUSWIRTH WWALEMAN TSKAUSHAL SSCHWARTZ SBBOYE SLWINDSOR EAMCONLON TJSUMAROKA APANG J-J et al.**Human RPE65 Gene Therapy for Leber Congenital Amaurosis: Persistence of Early Visual Improvements and Safety at 1 YearHum Gen Ther, [0102]
- **RAOUL CABBAS-TERKI TBENSADOUN J-CGUILLOT SHAASE GSZULC JHENDERSON CEAEBISCHER P**Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALSNat Med, 2005, vol. 11, 423-428 [0102]
- **KASPAR BKLLADO JSHERKAT NROTHSTEIN JDGAGE FH**Retrograde Viral Delivery of IGF-1 Prolongs Survival in a Mouse ALS ModelScience, 2003, vol. 301, 839-842 [0102]
- **AZZOUZ MRALPH GSSTORKEBAUM EWALMSLEY LEMITROPHANOUS KAKINGSMAN SMCARMELIET PMAZARAKIS ND**VEGF delivery with retrogradely

transported lentivector prolongs survival in a mouse ALS modelNature, 2004, vol. 429, 413-417 [0102]

- **RALPH GSRADCLIFFE PADAY DMCARTHY JMLEROUX MALEE DCPWONG L-FBILSLAND LGGREENSMITH LKINGSMAN SM et al.**Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS modelNat Med, 2005, vol. 11, 429-433 [0102]
- **WU RWANG HXIA XZHOU HLIU CCASTRO MXU Z**Nerve Injection of Viral Vectors Efficiently Transfers Transgenes into Motor Neurons and Delivers RNAi Therapy Against ALSAntioxidants & Redox Signaling, 2009, vol. 11, 1523-1534 [0102]
- **FOUST KDNURRE EMONTGOMERY CLHERNANDEZ ACHAN CMKASPAR BK**Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytesNat Biotechnol, 2009, vol. 27, 59-65 [0102]
- **DUQUE SJOUSSEMET BRIVIERE CMARAIS TDUBREIL LDOUAR AMFYFE JMOULLIER PCOLLE MABARKATS M**Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neuronsMol Ther, 2009, vol. 17, 1187-1196 [0102]
- **VANDENBERGHE LHWILSON JMGAO G**Tailoring the AAV vector capsid for gene therapyGene Ther, 2009, vol. 16, 311-319 [0102]
- **MCBRIDE JLBOUDREAU RLHARPER SQSTABER PDMONTEYS AMMARTINS IGILMORE BLBURSTEIN HPELUSO RWPOLISKY B et al.**Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: Implications for the therapeutic development of RNAiProceedings of the National Academy of Sciences, 2008, vol. 105, 5868-5873 [0102]
- **MCCARTY DM**Self-complementary AAV Vectors; Advances and ApplicationsMol Ther, 2008, vol. 16, 1648-1656 [0102]
- **BOILLEE SYAMANAKA KLOBSIGER CSCOPELAND NGJENKINS NAKASSIOTIS GKOLLIAS GCLEVELAND DW**Onset and progression in inherited ALS determined by motor neurons and microgliaScience, 2006, vol. 312, 1389-1392 [0102]
- **XIA XZHOU HHUANG YXU Z**Allele-specific RNAi selectively silences mutant SOD1 and achieves significant therapeutic benefit in vivoNeurobiol Dis, 2006, vol. 23, 578-586 [0102]
- **YAMANAKA KCHUN SJBOILLEE SFUJIMORI-TONOU NYAMASHITA HGUTMANN DHTAKAHASHI RMISAWA HCLEVELAND DW**Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosisNat Neurosci, 2008, vol. 11, 251253- [0102]
- **DI GIORGIO FPCARRASCO MASIAO MCMANIATIS TEGGAN K**Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS modelNat Neurosci, 2007, vol. 10, 608-614 [0102]
- **NAGAI MRE DBNAGATA TCHALAZONITIS AJESSELL TMWICHTERLE HPRZEDBORSKI S**Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neuronsNat Neurosci, 2007, vol. 10, 615-622 [0102]
- **WANG YOU MAO XXIE LBANWAIT SMARTI HHGREENBERG DAJIN K**Vascular Endothelial Growth Factor Overexpression Delays Neurodegeneration and Prolongs Survival in Amyotrophic Lateral Sclerosis MiceJ Neurosci, 2007, vol. 27, 304-307 [0102]

- **STORKEBAUM ELAMBRECHTS DDEWERCHIN MMORENO-MURCIANO M-PAPPELMANS SOH HVAN DAMME PRUTTEN BMAN WYDE MOL M et al.**Treatment of motoneuron degeneration by intracerebroventricular delivery of VEGF in a rat model of ALSNat Neurosci, 2005, vol. 8, 85-92 [0102]
- **WU, W. P.XU, X. J.HAO, J. X.**J. Neurosci. Methods, 2004, vol. 133, 65-69 [0102]
- **PAPAIOANNOU, V. E.FOX, J. G.**Lab. Anim. Sci., 1993, vol. 43, 189-192 [0102]

Patentkrav

1. Rekombinant adeno-associeret virus (rAAV) omfattende (i) et capsidprotein af AAV9 eller AAVrh.10 og (ii) en nukleinsyre omfattende en promoter, der er operativt forbundet med et transgen til anvendelse som et medikament; hvor transgenet skal afgives til centralnervesystem (CNS)-væv hos et individ ved intratekal indgivelse og ved intracerebral indgivelse, og hvor rAAV'et inficerer celler i hjernen og i individets rygmarv.

2. rAAV til anvendelse ifølge krav 1, hvor den intracerebrale indgivelse er en intraventrikulær indgivelse.

3. rAAV til anvendelse ifølge krav 1 eller 2, hvor den intratekale indgivelse sker i individets cervikale region.

4. rAAV til anvendelse ifølge krav 1 eller 2, hvor den intratekale indgivelse sker i individets thorakale region.

5. rAAV til anvendelse ifølge krav 1 eller 2, hvor den intratekale indgivelse sker i individets lumbare region.

6. rAAV til anvendelse ifølge et hvilket som helst af kravene 1 til 5, hvor dosen af rAAV'en til intratekal eller intracerebral indgivelse er i et området på 10^{10} genomkopier til 10^{14} genomkopier.

7. rAAV til anvendelse ifølge et hvilket som helst af kravene 1 til 6, hvor transgenet er et CNS-associeret gen.

8. rAAV til anvendelse ifølge krav 7, hvor det CNS-associerede gen er udvalgt blandt NAIP (neuronal apoptosis inhibitory protein), nervevækstfaktor (NGF), GDNF (glial-derived growth factor), BDNF (brain-derived growth factor), ciliær neutrofisk faktor (CNTF), tyrosin-hydroxlase (TH), GTP-cyclohydrolase (GTPCH), aminosyre-decorboxylase (AADC) og aspartoacylase (ASPA).

9. rAAV til anvendelse ifølge et hvilket som helst af kravene 1 til 8, hvor rAAV'en er AAV rh.10, og transgenet koder for en inhibitorisk RNA, der binder specifikt til SOD1-mRNA og inhiberer ekspression af SOD1 i individet, og hvo rAAV'en er til anvendelse ved amyotrofisk lateral sklerose (ALS), hvor det inhibitoriske RNA eventuelt har en sekvens som angivet i SEQ ID NO: 26.

10. rAAV til anvendelse ifølge et hvilket som helst af kravene 1 til 9, hvor rAAV'et er formuleret i en sammensætning omfattende en farmaceutisk acceptabel bærer.

11. rAAV til anvendelse ifølge et hvilket som helst af kravene 1 til 10, hvor rAAv'en er AAV rh.10, og den intratekale indgivelse opnår fordeling gennem det intratekale rum, hvorved celler i den grå masse transduceres langs rygmarven og hjernestammen.

12. rAAV til anvendelse ifølge et hvilket som helst af kravene 1 til 11, hvor den intracerebrale indgivelse omfatter indgivelse i mindst en af følgende: cerebrum, medulla, pons, cerebellum, intrakranialt hulrum, meninges, der omgiver hjernen, dura mater, araknoid mater, pia mater, og/eller cerebrospinalvæske (CSF) af subaraknoidalrummet, der omgiver hjernen.

DRAWINGS

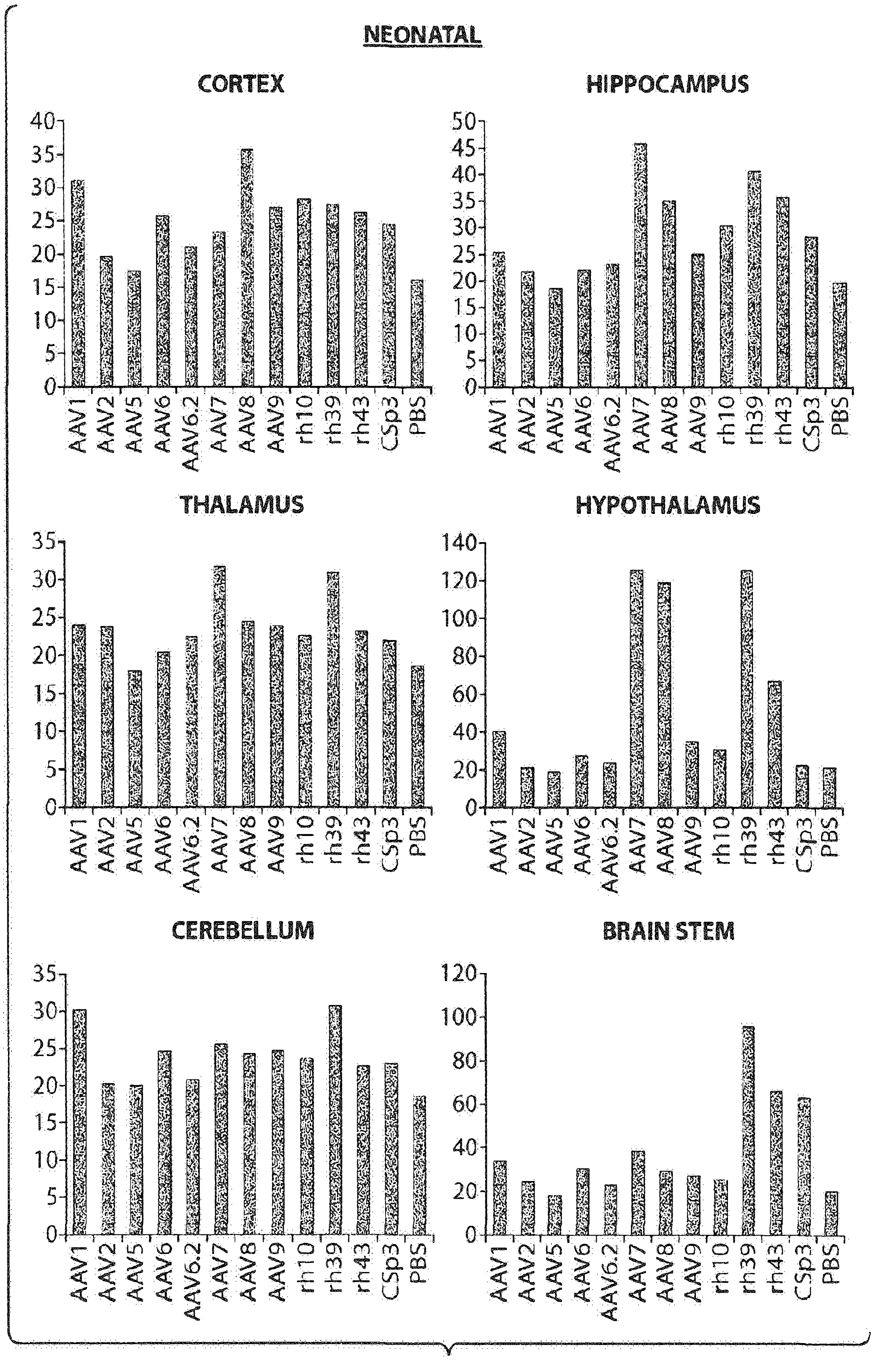


Fig. 1-1

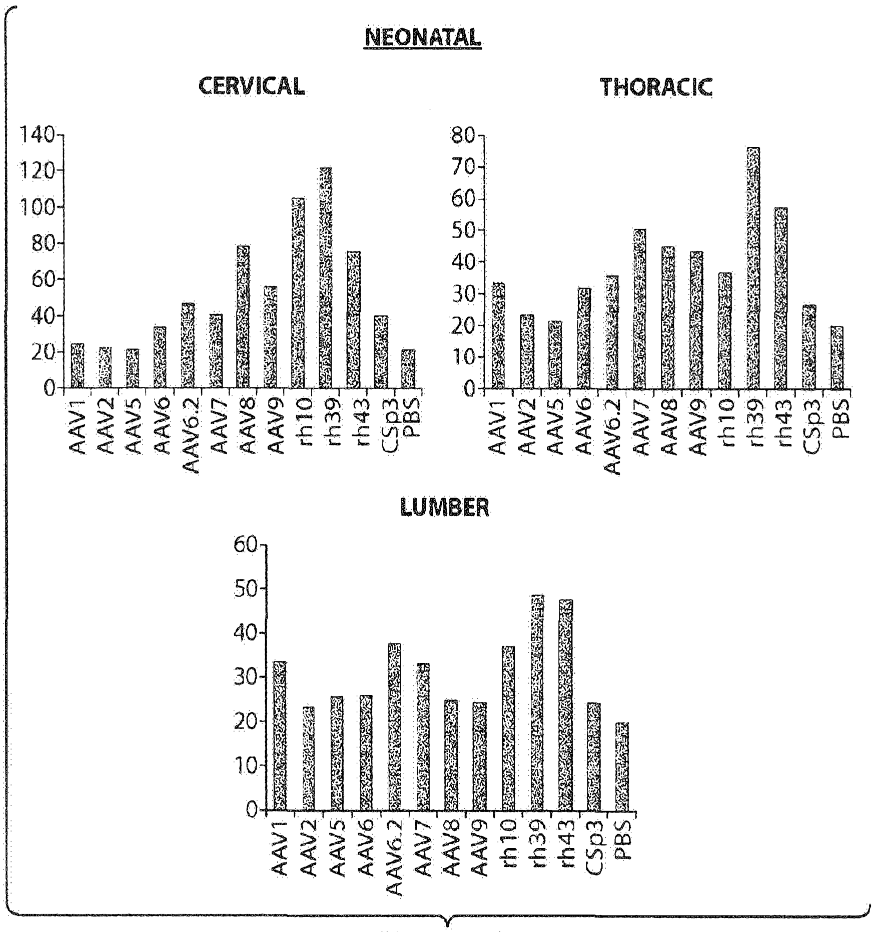

NEONATAL
CERVICAL
THORACIC
LUMBER
AAV1
AAV2
AAV5
AAV6
AAV6.2
AAV7
AAV8
AAV9
rh10
rh39
rh43
CSp3
PBS

Fig. 1-2

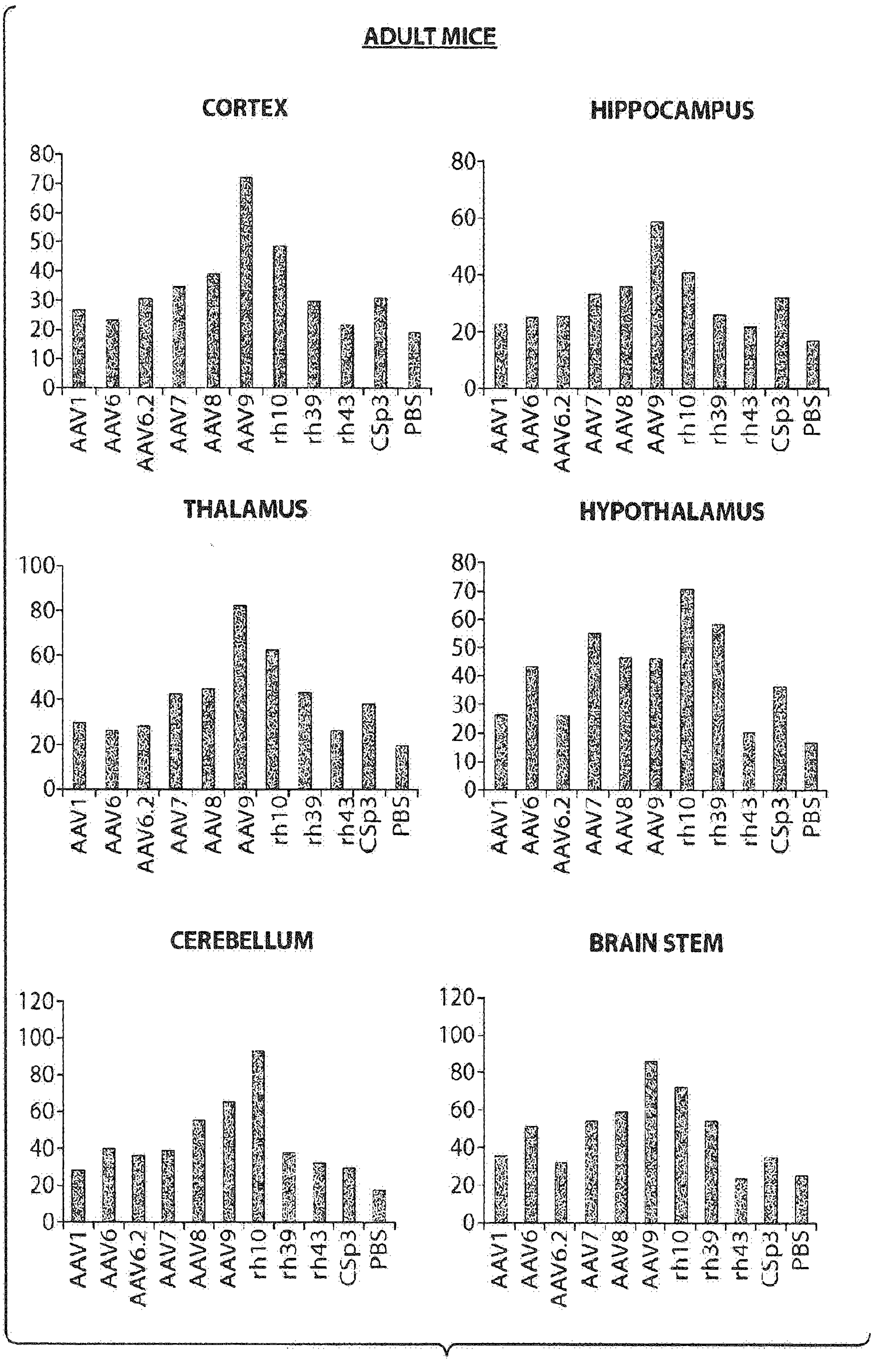

ADULT MICE
CORTEX
HIPPOCAMPUS
THALAMUS
HYPOTHALAMUS
CEREBELLUM
BRAIN STEM
AAV1
AAV6
AAV6.2
AAV7
AAV8
AAV9
rh10
rh39
rh43
CSp3
PBS

Fig. 2-1

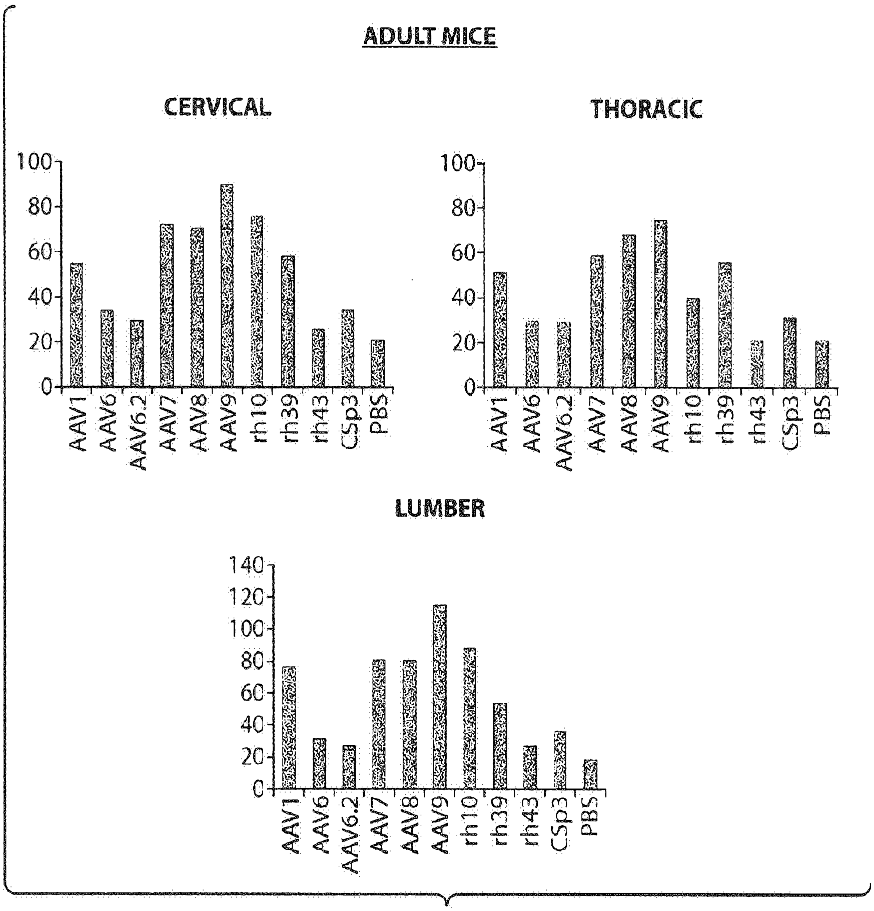

ADULT MICE
CERVICAL
THORACIC
LUMBER
100
80
60
40
20
0
140
120
AAV1
AAV6
AAV6.2
AAV7
AAV8
AAV9
rh10
rh39
rh43
CSp3
PBS

Fig. 2-2

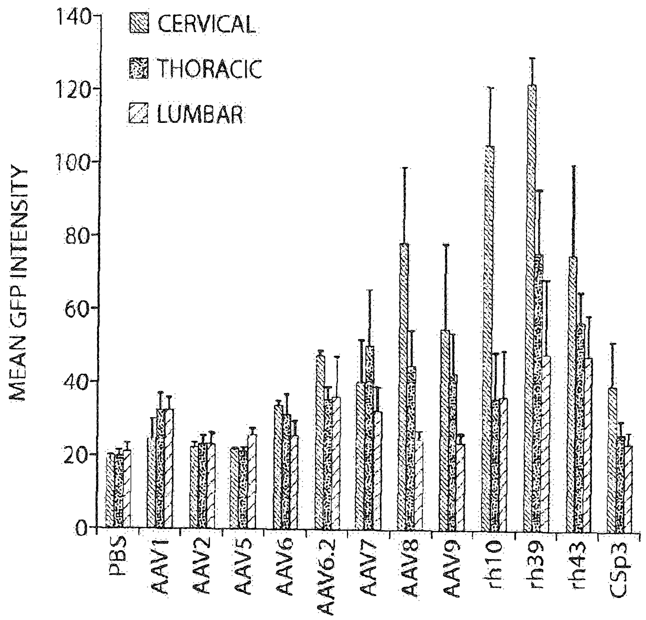

CERVICAL
THORACIC
LUMBAR
MEAN GFP INTENSITY
140
120
100
80
60
40
20
0
PBS
AAV1
AAV2
AAV5
AAV6
AAV6.2
AAV7
AAV8
AAV9
rh10
rh39
rh43
CSp3

Fig. 3

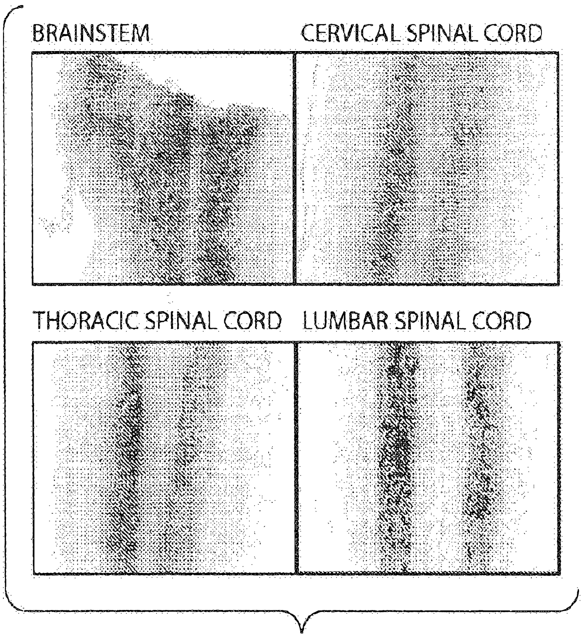

BRAINSTEM
CERVICAL SPINAL CORD
THORACIC SPINAL CORD
LUMBAR SPINAL CORD

Fig. 4A

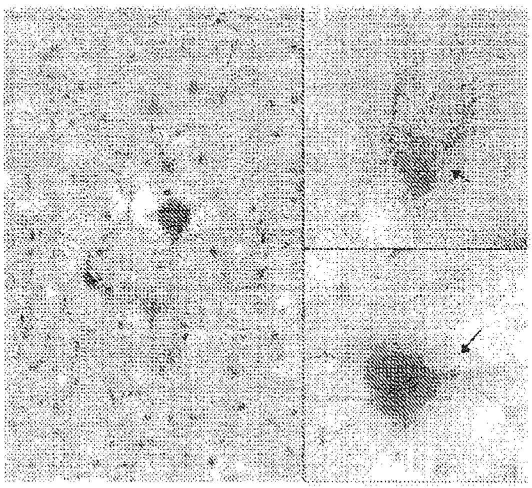


Fig. 4B

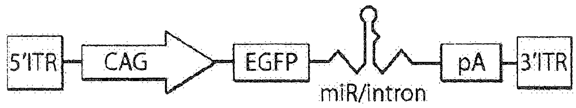


Fig. 5A

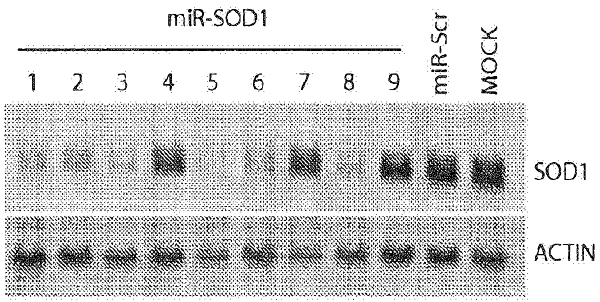


Fig. 5B

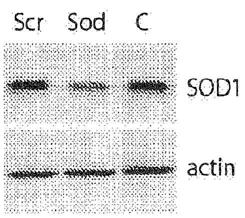


Fig. 5C

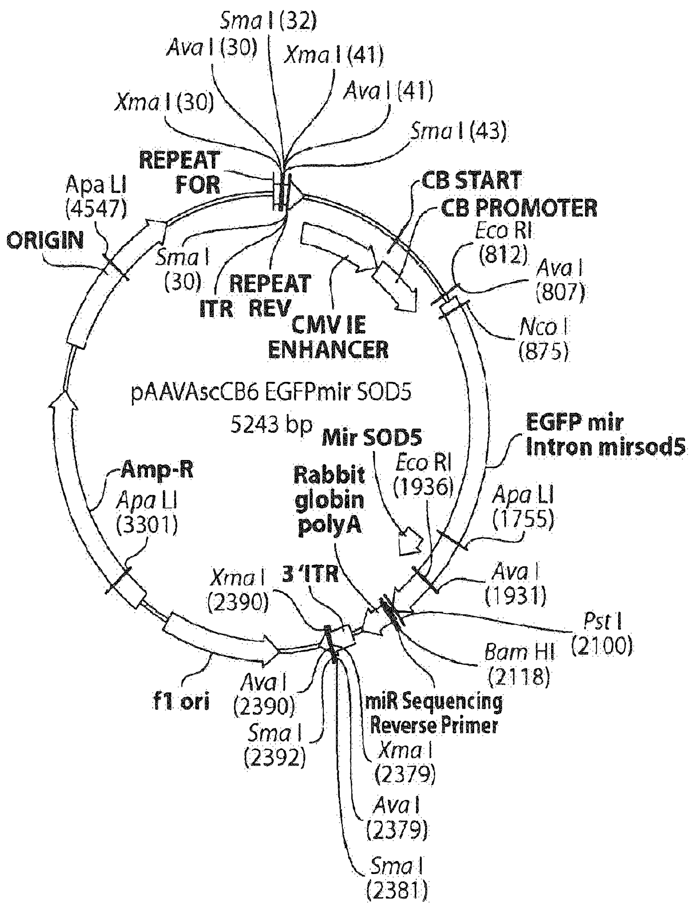

Sma I (32)
Ava I (30)
Xma I (41)
Ava I (41)
Xma I (30)
Sma I (43)
REPEAT FOR
Apa LI (4547)
CB START
CB PROMOTER
ORIGIN
Sma I (30)
ITR
REPEAT REV
Eco RI (812)
Ava I (807)
CMV IE ENHANCER
Nco I (875)
pAAVAscCB6 EGFPmir SOD5
5243 bp
Mir SOD5
EGFP mir Intron mirsod5
Amp-R
Apa LI (3301)
Rabbit globin polyA
Eco RI (1936)
Apa LI (1755)
Ava I (1931)
Xma I (2390)
3 'ITR
Pst I (2100)
Bam HI (2118)
f1 ori
Ava I (2390)
Sma I (2392)
miR Sequencing Reverse Primer
Xma I (2379)
Ava I (2379)
Sma I (2381)

Fig. 5D

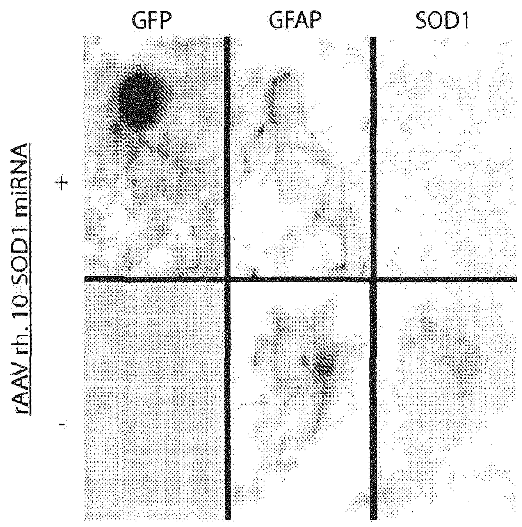

GFP
GFAP
SOD1
rAAV rh. 10-SOD1 miRNA
+
-

Fig. 6A

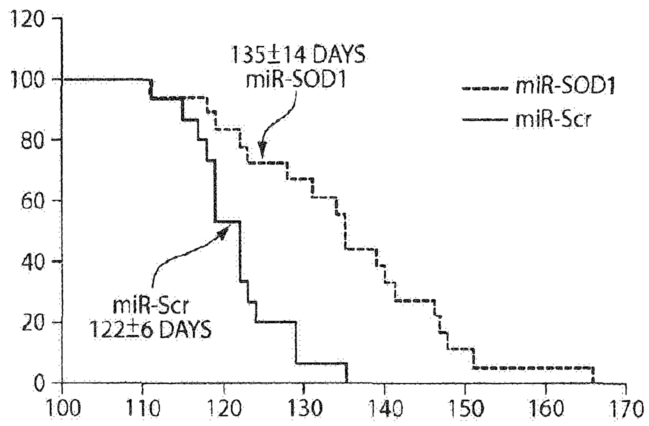

120
100
80
60
40
20
0
100
110
120
130
140
150
160
170
135±14 DAYS
miR-SOD1
miR-SOD1
miR-Scr
miR-Scr
122±6 DAYS

Fig. 6B

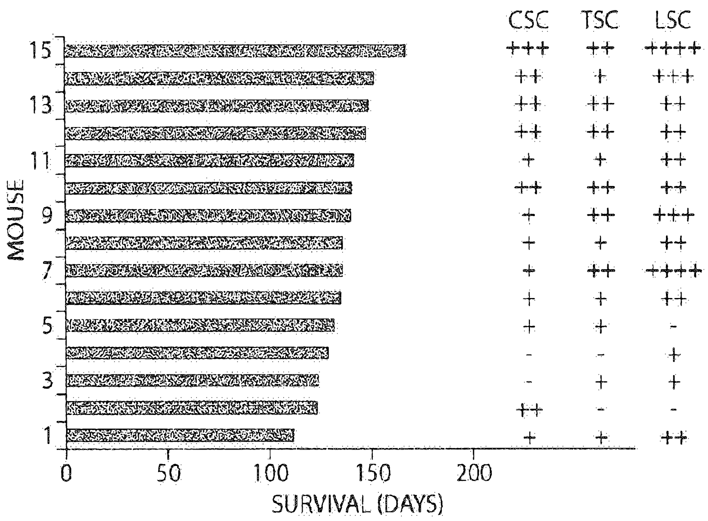

CSC
TSC
LSC
MOUSE
15
13
11
9
7
5
3
1
0
50
100
150
200
SURVIVAL (DAYS)

Fig. 7A

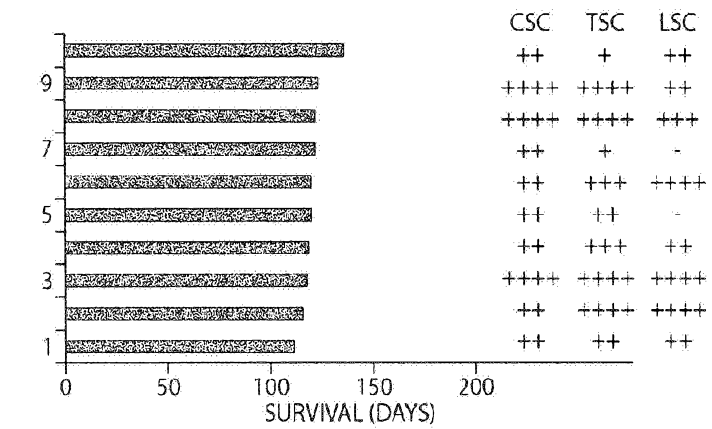

CSC
TSC
LSC
9
7
5
3
1
0
50
100
150
200
SURVIVAL (DAYS)

Fig. 7B

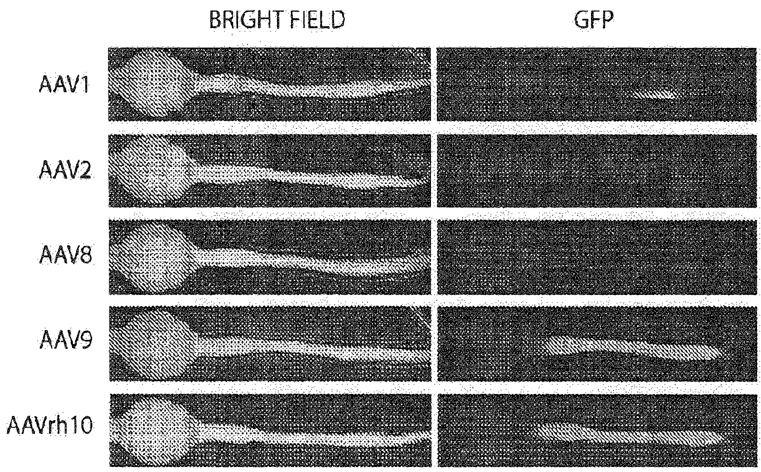

BRIGHT FIELD
GFP
AAV1
AAV2
AAV8
AAV9
AAVrh10

Fig. 8

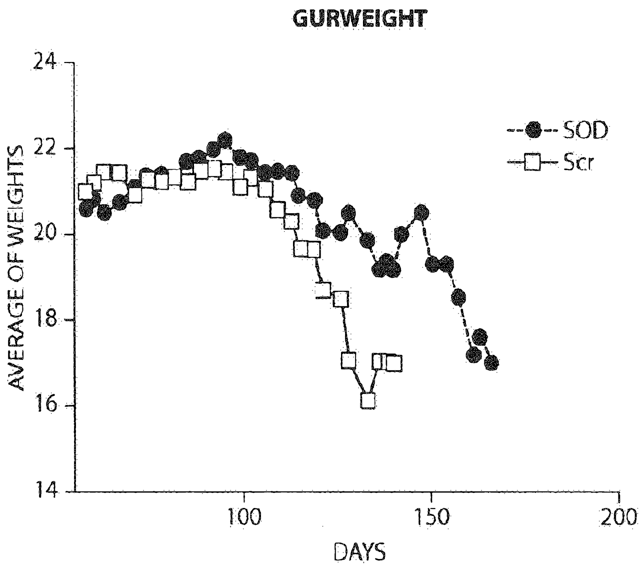

GURWEIGHT
24
22
20
18
16
14
AVERAGE OF WEIGHTS
100
150
200
DAYS
SOD
Scr

Fig. 9

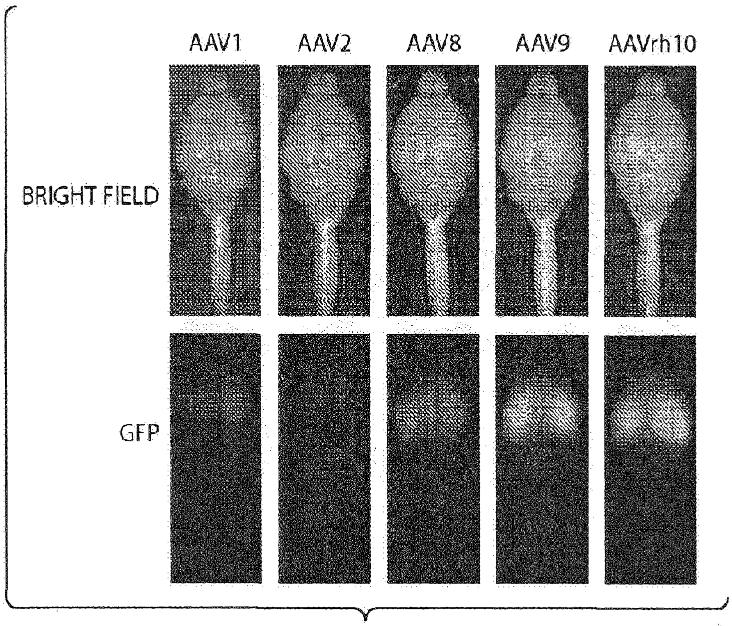


Fig. 10

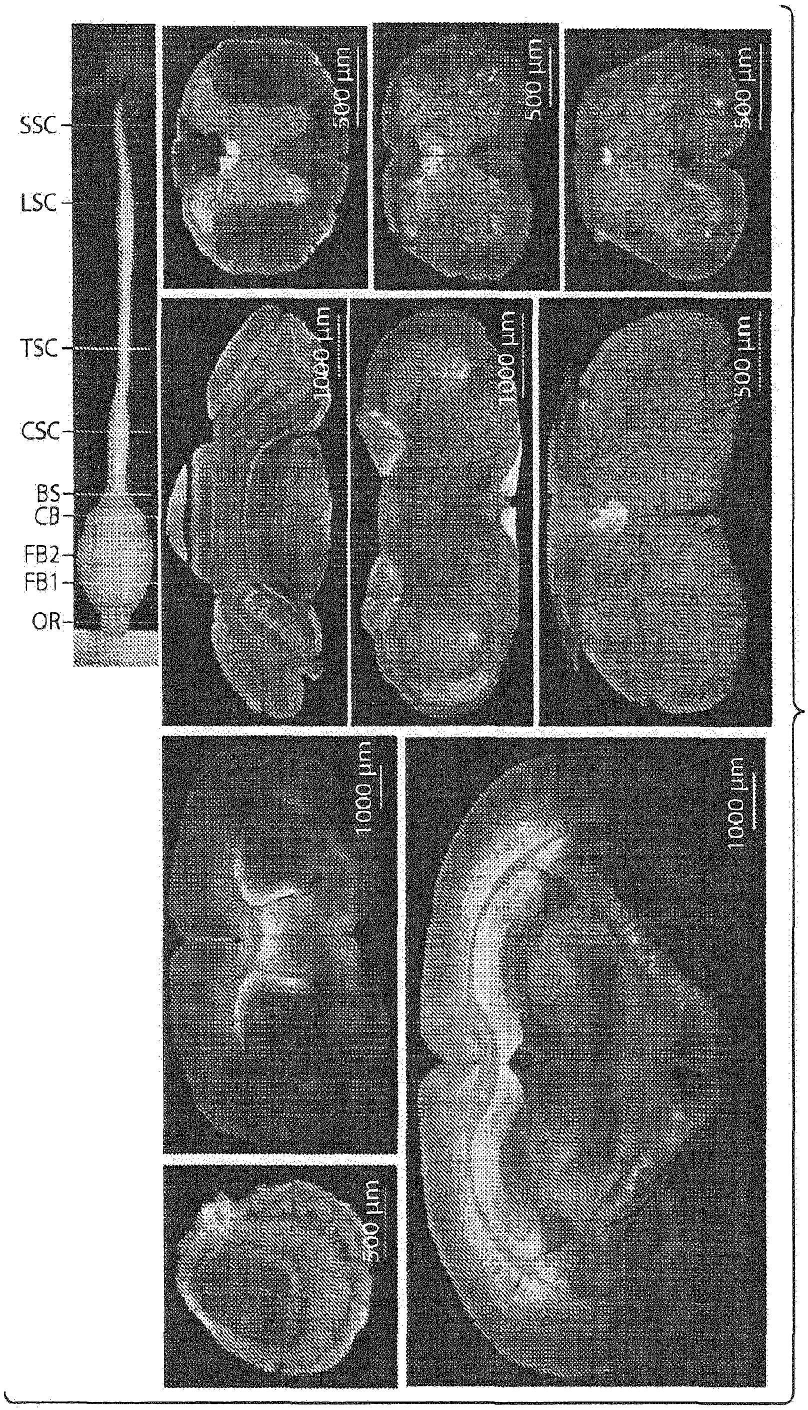

SSC
LSC
TSC
CSC
BS
CB
FB2
FB1
OR
500 µm
500 µm
500 µm
1000 µm
1000 µm
500 µm
1000 µm
1000 µm
500 µm

Fig. 11

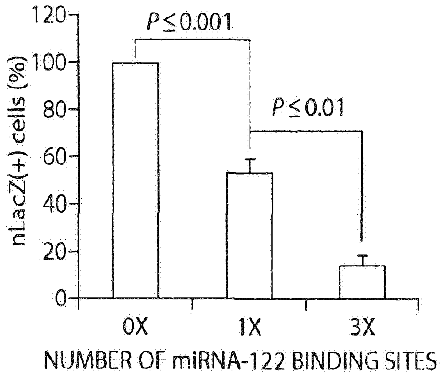


Fig. 12A

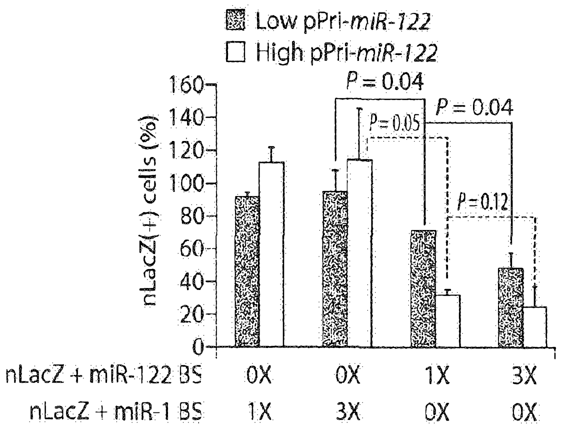


Fig. 12B

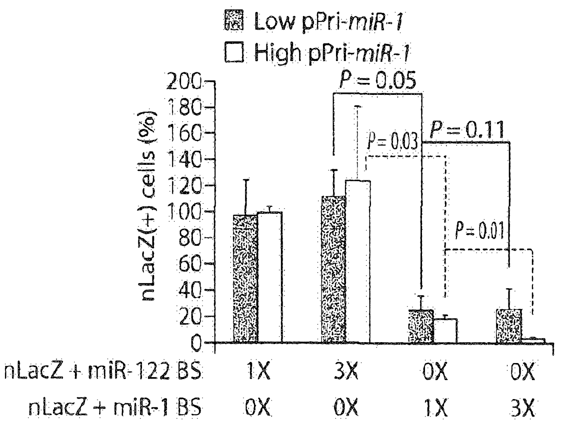


Fig. 12C

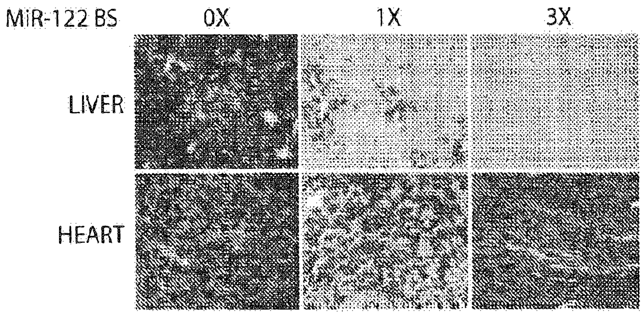


Fig. 13A

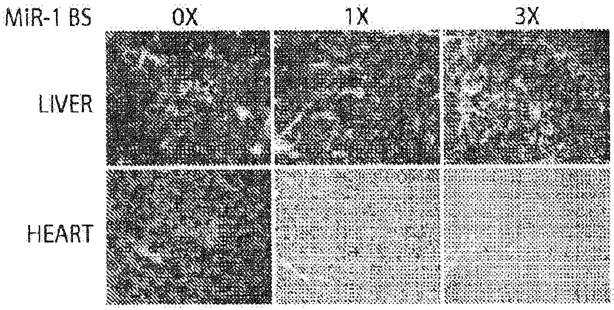


Fig. 13B

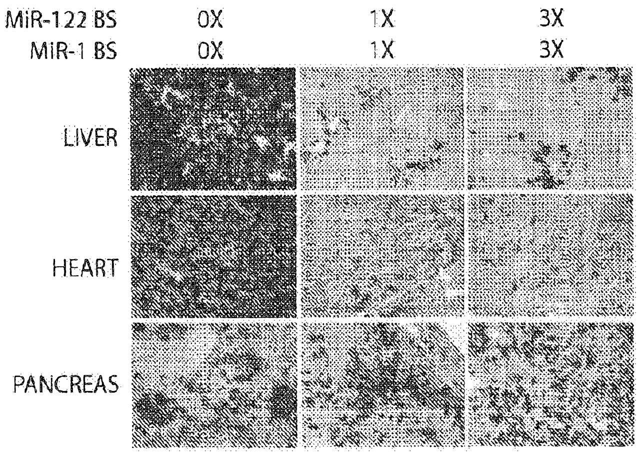


Fig. 13C

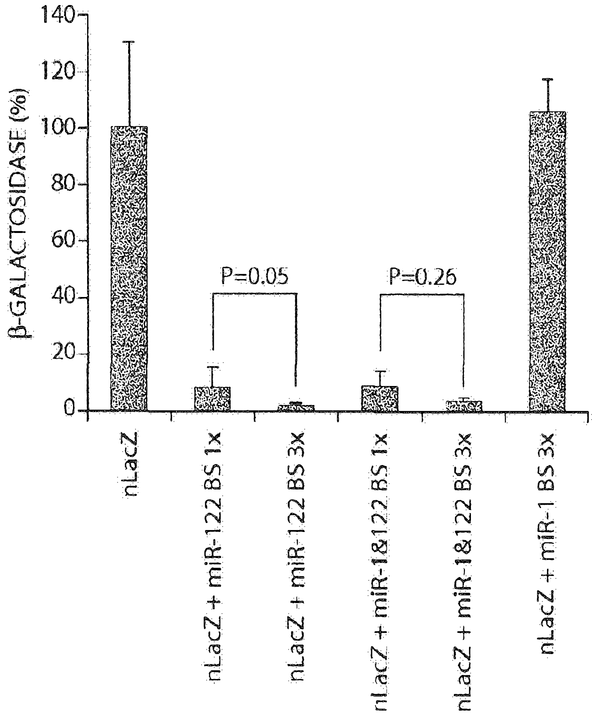

β-GALACTOSIDASE (%)
140
120
100
80
60
40
20
0
P=0.05
P=0.26
nLacZ
nLacZ + miR-122 BS 1x
nLacZ + miR-122 BS 3x
nLacZ + miR-1&122 BS 1x
nLacZ + miR-1&122 BS 3x
nLacZ + miR-1 BS 3x

Fig. 13D

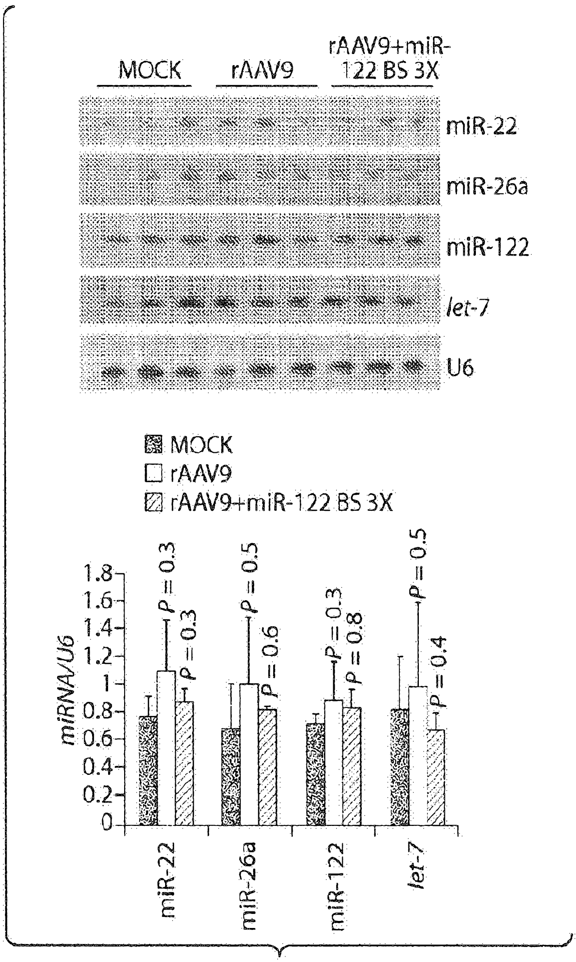

MOCK
rAAV9
rAAV9+miR-122 BS 3X
miR-22
miR-26a
miR-122
let-7
U6
MOCK
rAAV9
rAAV9+miR-122 BS 3X
miRNA/U6
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
P = 0.3
P = 0.3
P = 0.5
P = 0.6
P = 0.3
P = 0.8
P = 0.5
P = 0.4
miR-22
miR-26a
miR-122
let-7

Fig. 14A

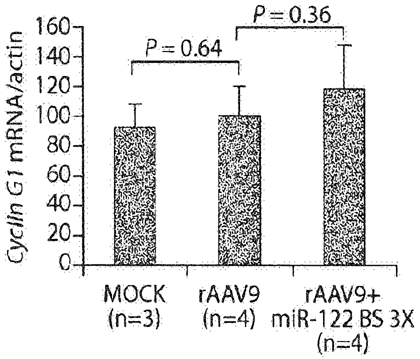


Fig. 14B

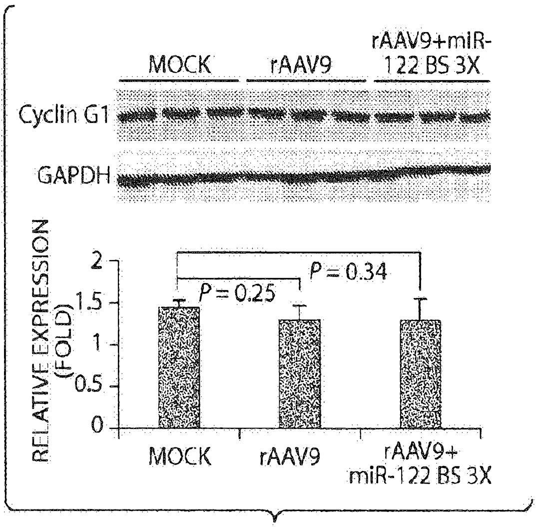


Fig. 14C

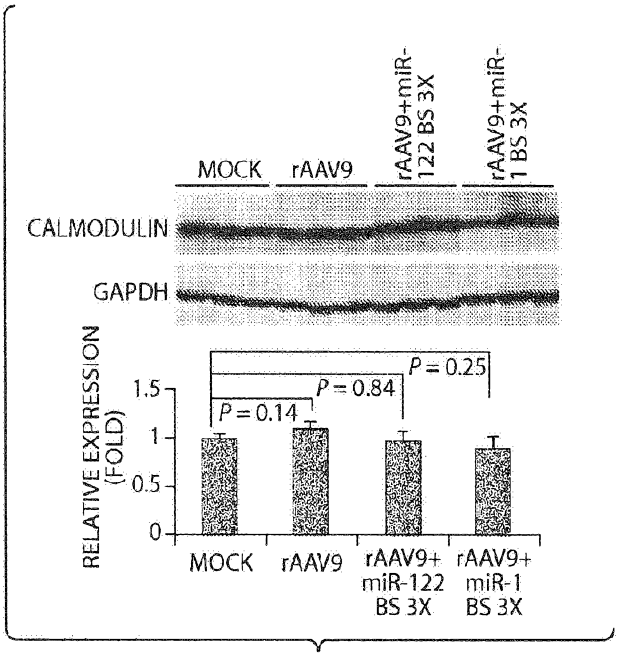


Fig. 14D

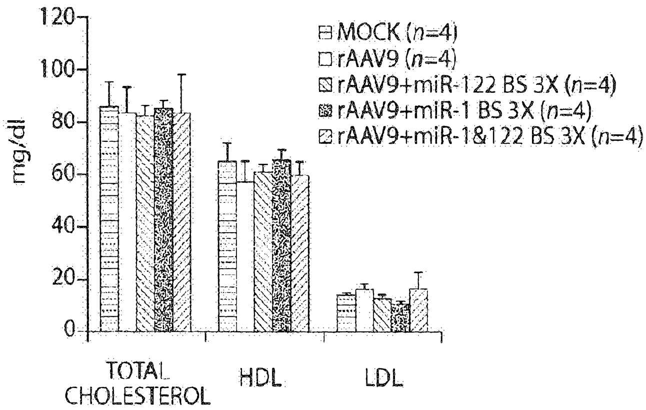


Fig. 14E

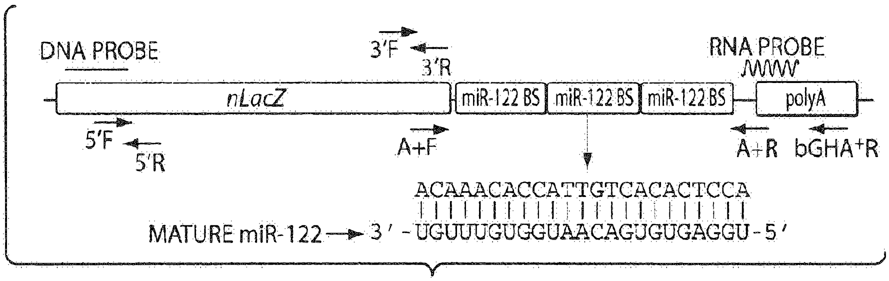


Fig. 15A

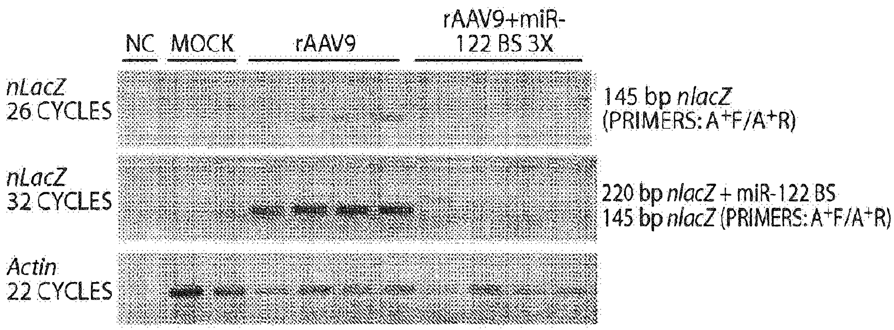


Fig. 15B

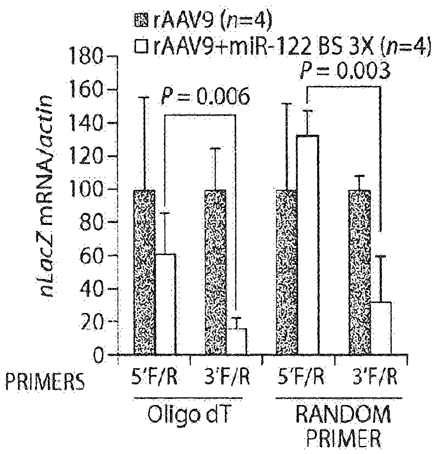
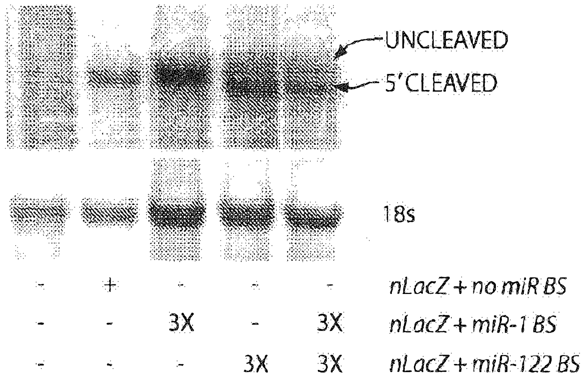
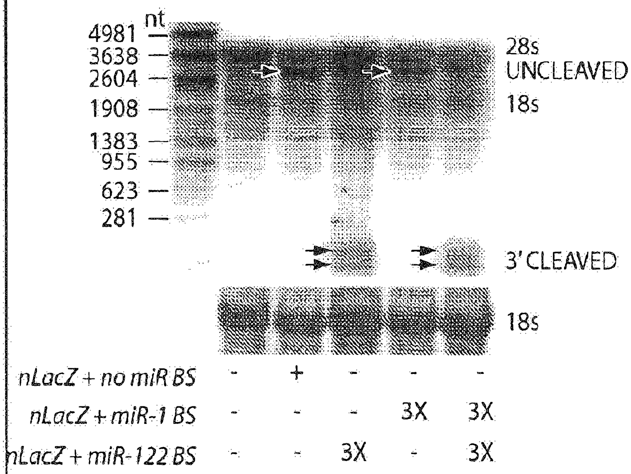


Fig. 15C



UC(nt)	3378	3447	3450	3550
5'PC-1			3171	3303
5'PC-2			3194	3280
5'PC-3			3217	3257

Fig. 15D



UC(nt)	3378	3450	3447	3550
3'PC-1		181		181
3'PC-2		158		158
3'PC-3		135		135

Fig. 15E

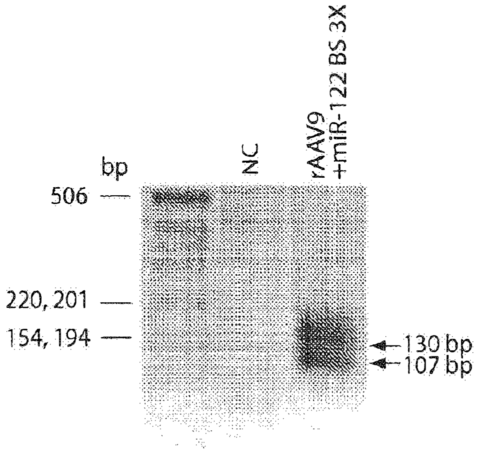


Fig. 15F

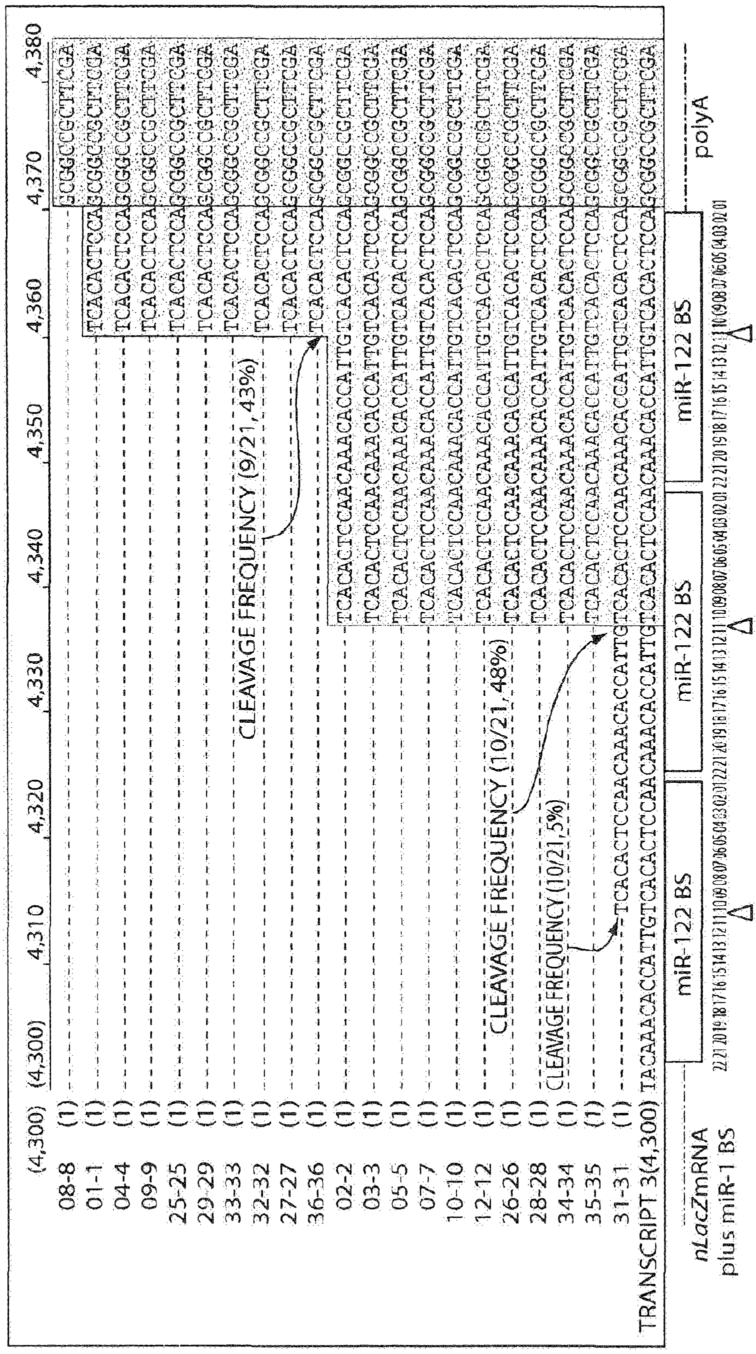

CLEAVAGE FREQUENCY (9/21, 43%)
CLEAVAGE FREQUENCY (10/21, 48%)
CLEAVAGE FREQUENCY (10/21, 5%)
TRANSCRIPT 3
miR-122 BS
miR-122 BS
miR-122 BS
polyA
nLacZmRNA
plus miR-1 BS

Fig. 16A

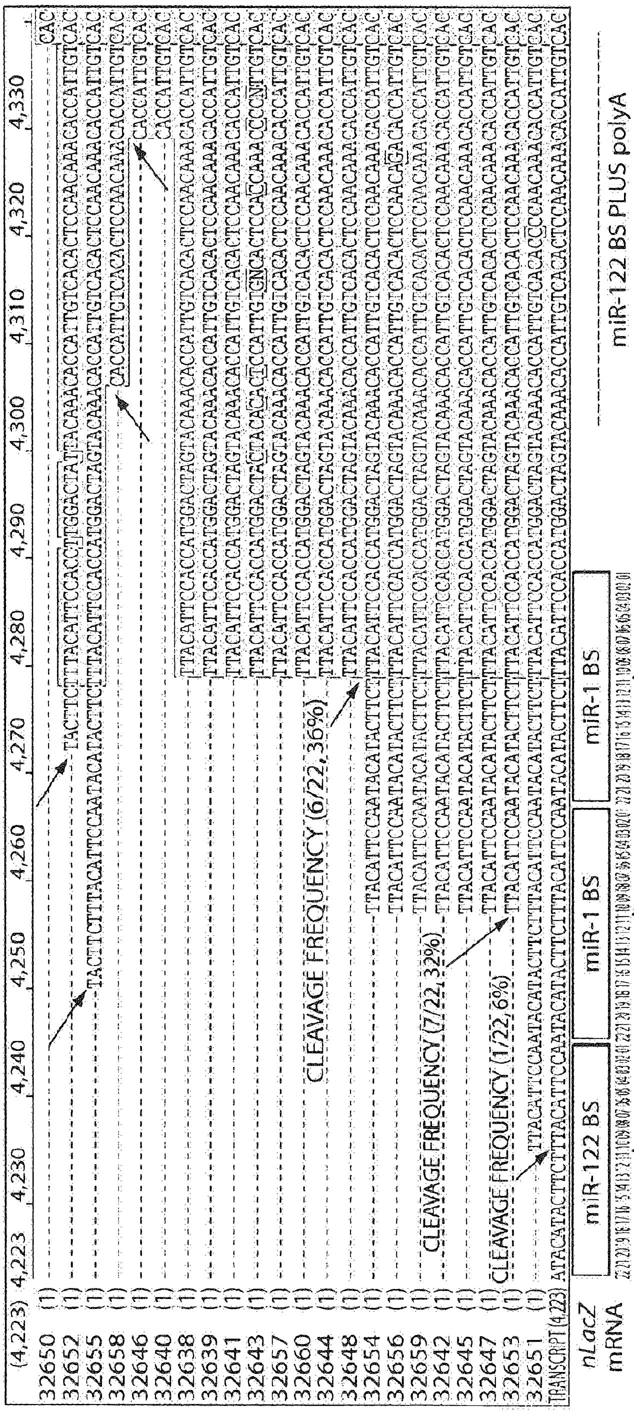

CLEAVAGE FREQUENCY (6/22, 36%)
CLEAVAGE FREQUENCY (7/22, 32%)
CLEAVAGE FREQUENCY (1/22, 6%)
nLacZ mRNA
miR-122 BS
miR-1 BS
miR-1 BS
miR-122 BS PLUS polyA

Fig. 16B

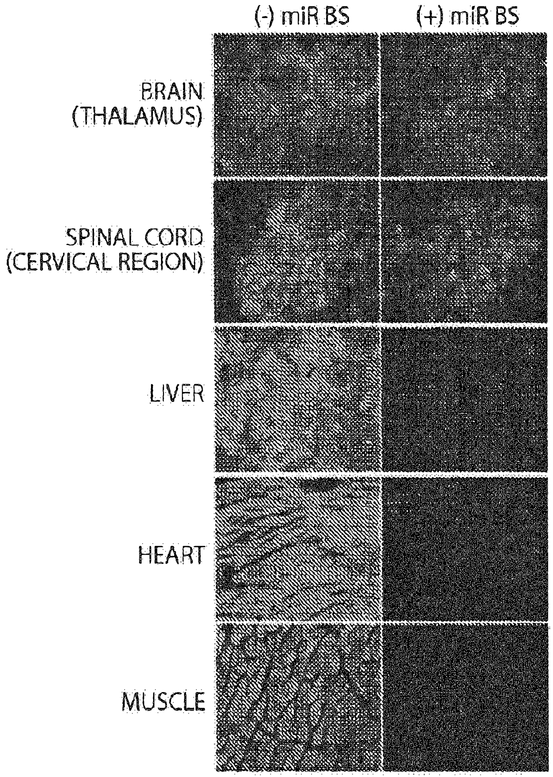


Fig. 17A

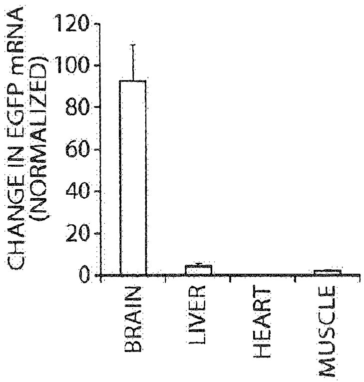


Fig. 17B

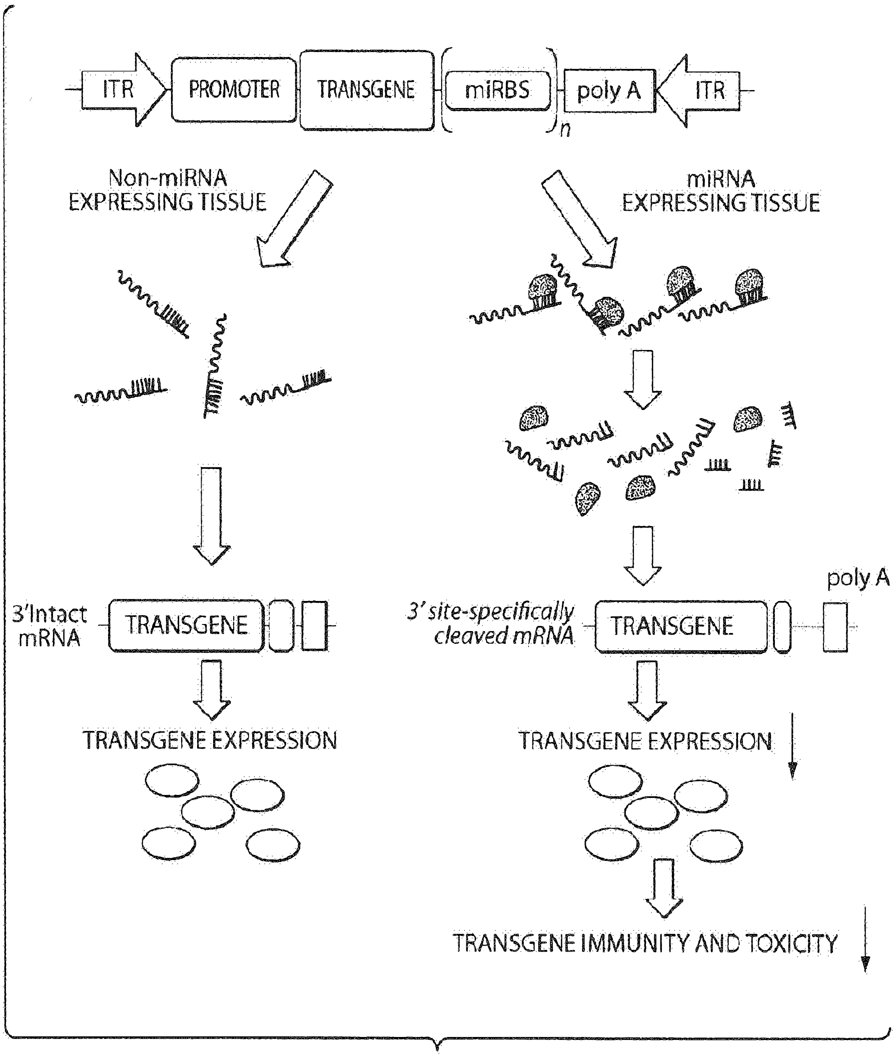

ITR
PROMOTER
TRANSGENE
miRBS
n
poly A
ITR
Non-miRNA
EXPRESSING TISSUE
miRNA
EXPRESSING TISSUE
3'Intact
mRNA
TRANSGENE
3' site-specifically
cleaved mRNA
TRANSGENE
poly A
TRANSGENE EXPRESSION
TRANSGENE EXPRESSION
TRANSGENE IMMUNITY AND TOXICITY

Fig. 18

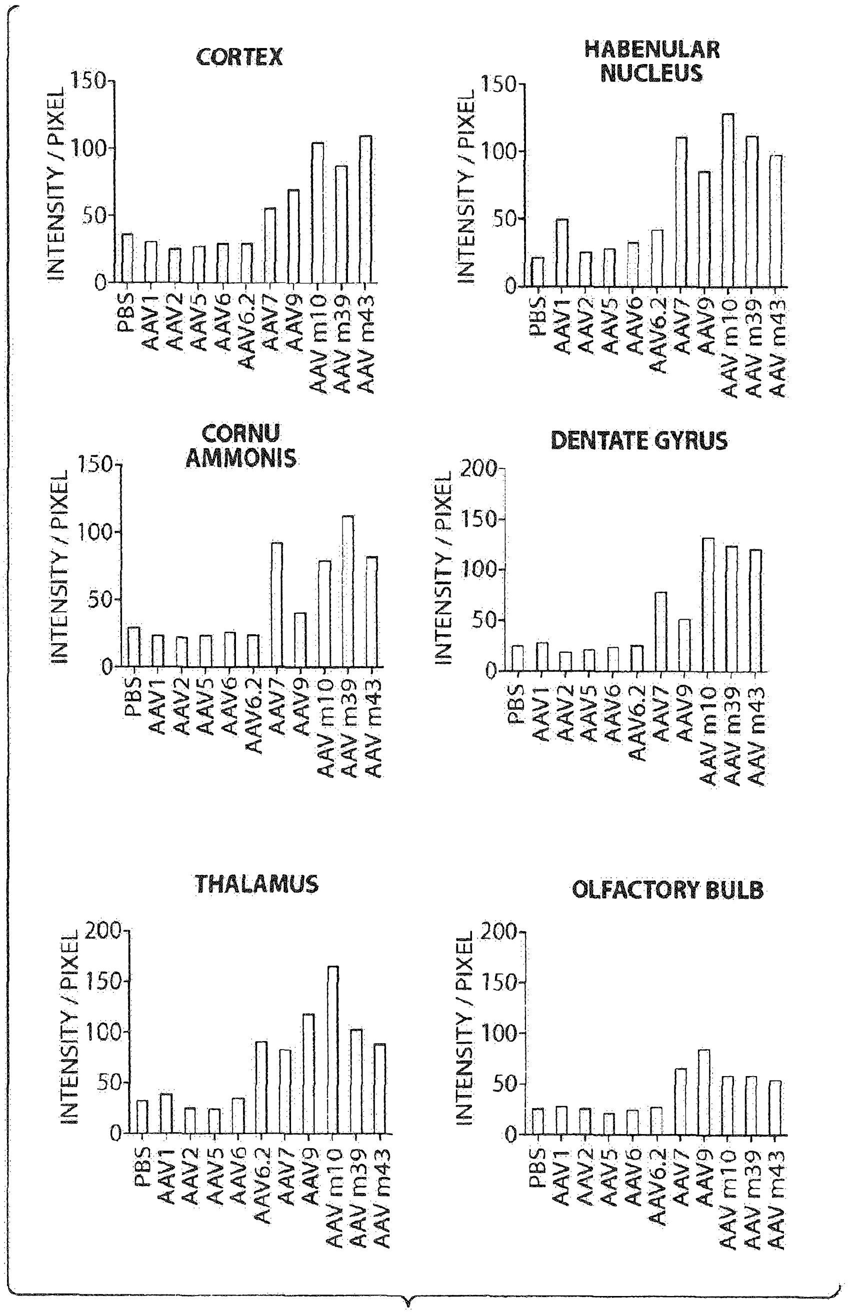

CORTEX
HABENULAR NUCLEUS
CORNU AMMONIS
DENTATE GYRUS
THALAMUS
OLFACTORY BULB
INTENSITY / PIXEL
0
50
100
150
200
PBS
AAV1
AAV2
AAV5
AAV6
AAV6.2
AAV7
AAV9
AAV m10
AAV m39
AAV m43

Fig. 19A-1

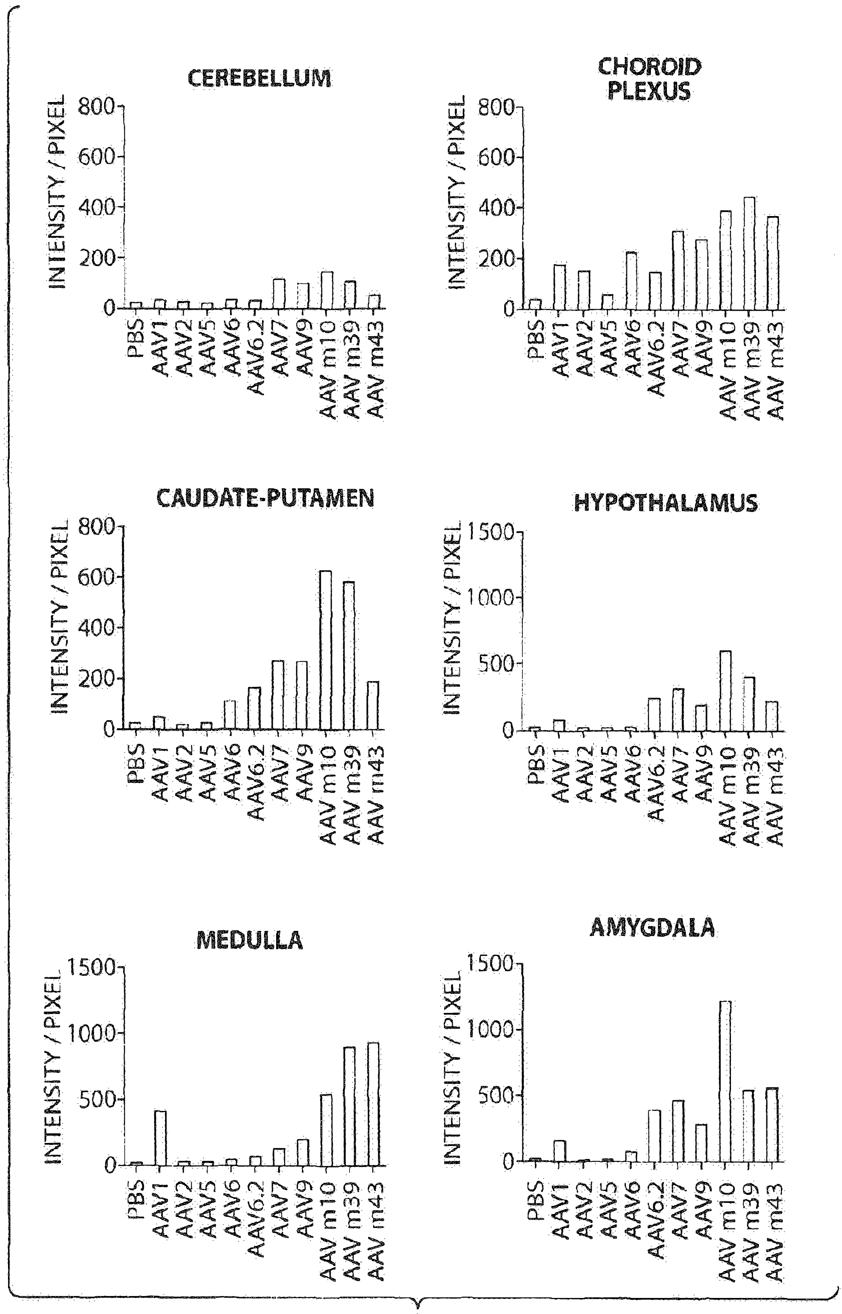

CEREBELLUM
CHOROID PLEXUS
CAUDATE-PUTAMEN
HYPOTHALAMUS
MEDULLA
AMYGDALA
INTENSITY / PIXEL
0
200
400
600
800
500
1000
1500
PBS
AAV1
AAV2
AAV5
AAV6
AAV6.2
AAV7
AAV9
AAV m10
AAV m39
AAV m43

Fig. 19A-2

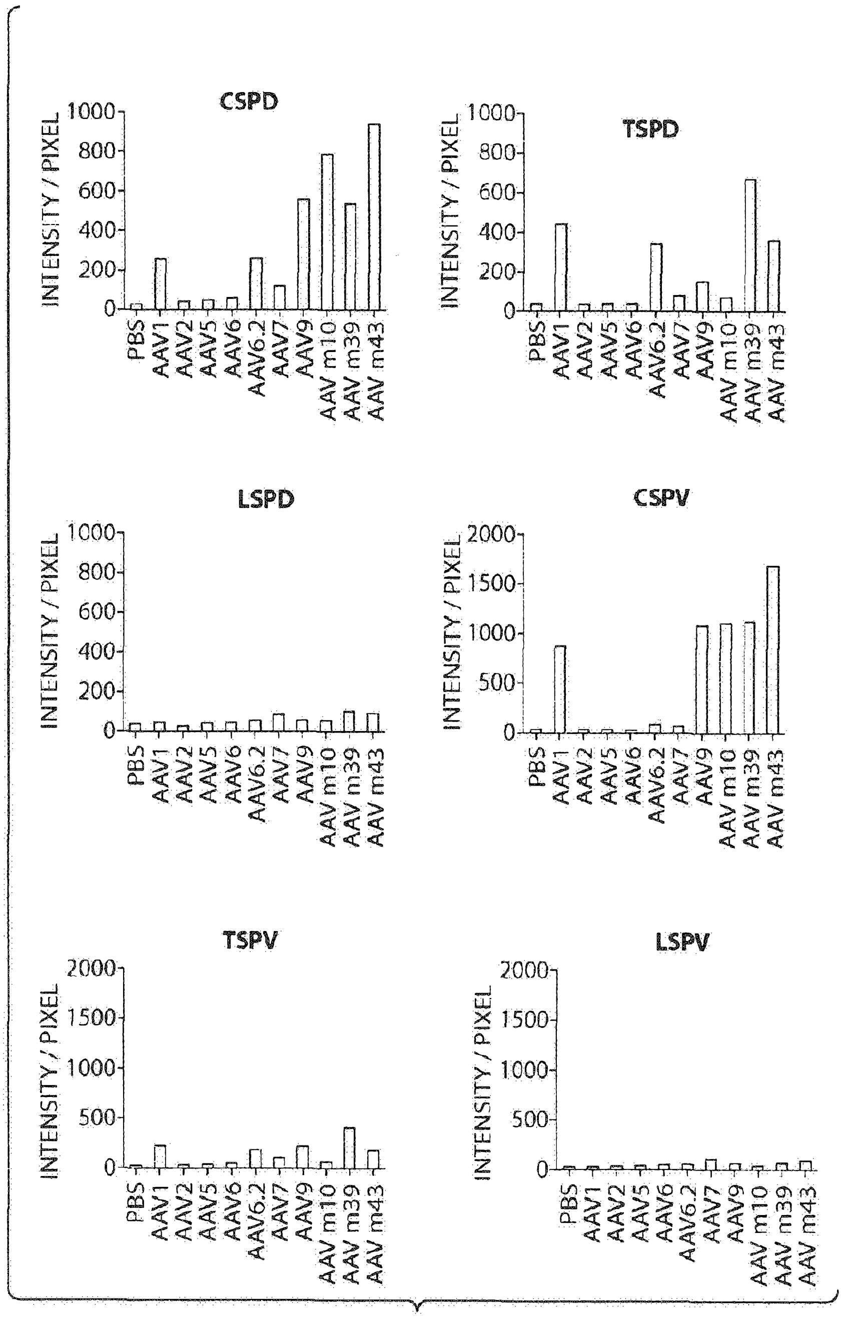

CSPD
TSPD
LSPD
CSPV
TSPV
LSPV
INTENSITY / PIXEL
0
200
400
600
800
1000
500
1500
2000
PBS
AAV1
AAV2
AAV5
AAV6
AAV6.2
AAV7
AAV9
AAV m10
AAV m39
AAV m43

Fig. 19A-3

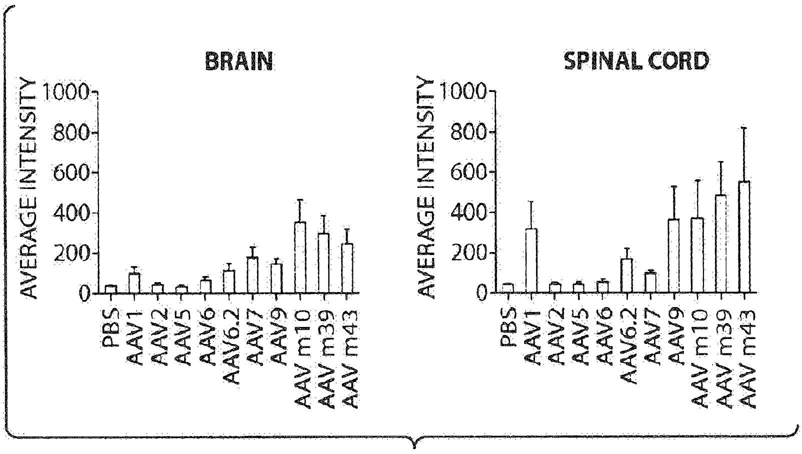

BRAIN
SPINAL CORD
AVERAGE INTENSITY
1000
800
600
400
200
0
PBS
AAV1
AAV2
AAV5
AAV6
AAV6.2
AAV7
AAV9
AAV m10
AAV m39
AAV m43

Fig. 19B

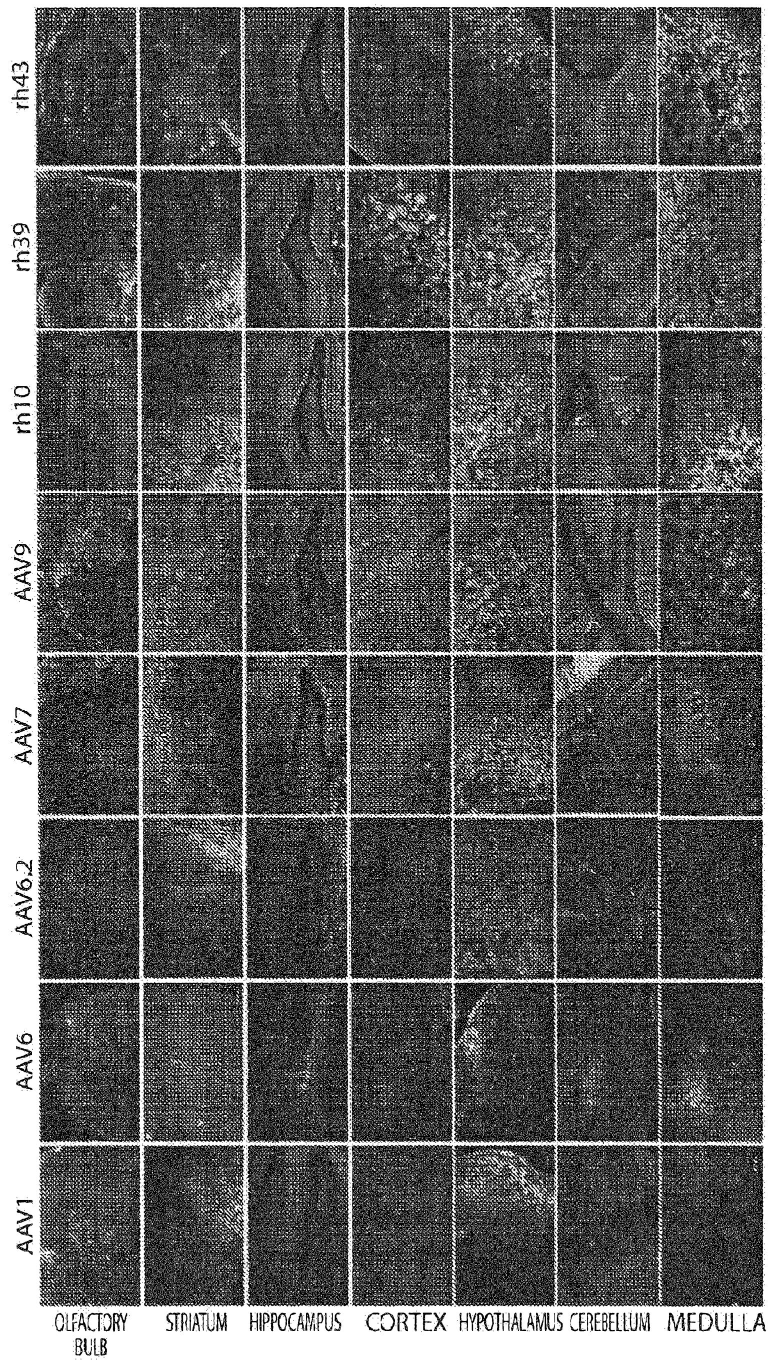

rh43
rh39
rh10
AAV9
AAV7
AAV6.2
AAV6
AAV1
OLFACTORY BULB
STRIATUM
HIPPOCAMPUS
CORTEX
HYPOTHALAMUS
CEREBELLUM
MEDULLA

Fig. 20

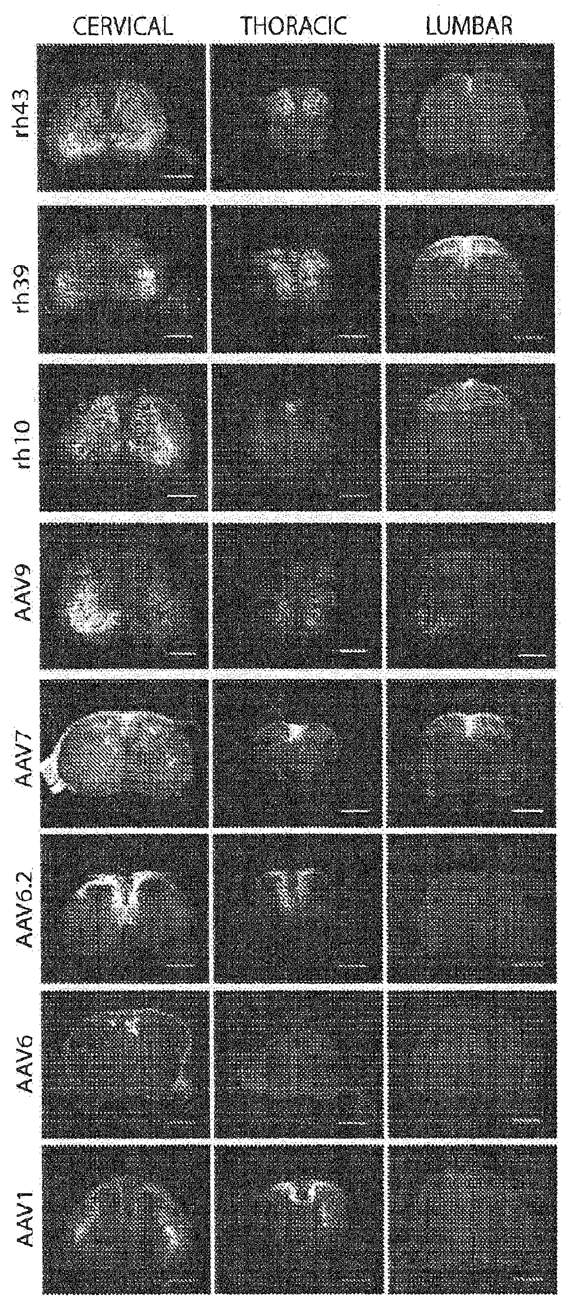

CERVICAL
THORACIC
LUMBAR
rh43
rh39
rh10
AAV9
AAV7
AAV6.2
AAV6
AAV1

Fig. 21

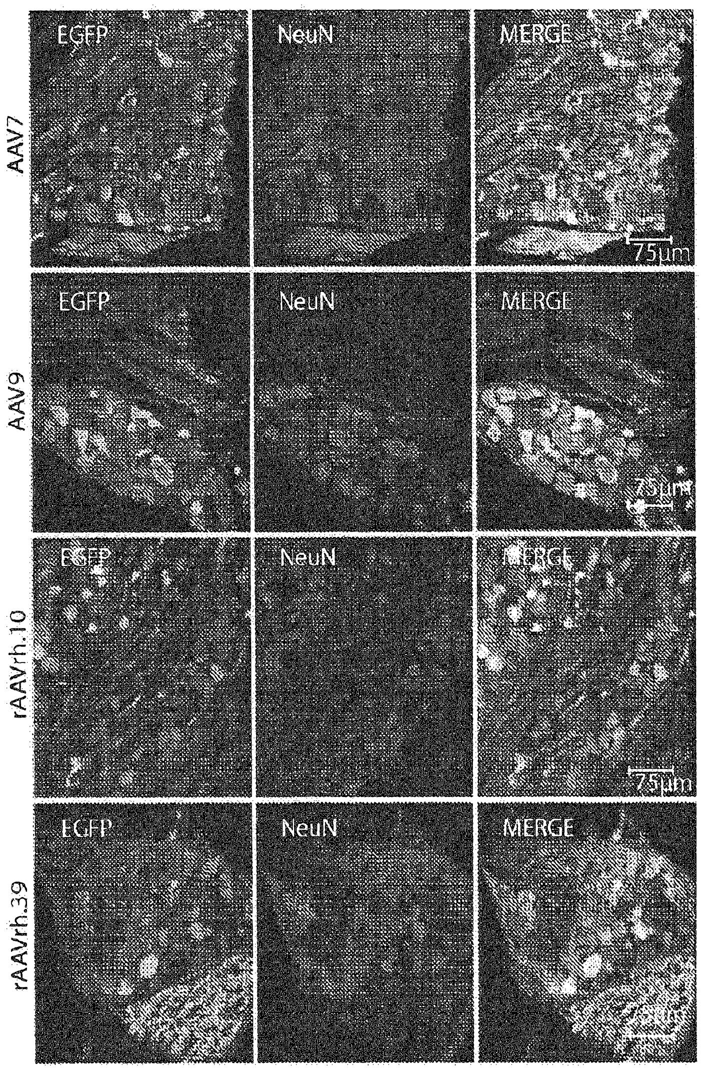

EGFP
NeuN
MERGE
AAV7
75μm
EGFP
NeuN
MERGE
AAV9
75μm
EGFP
NeuN
MERGE
rAAVrh.10
75μm
EGFP
NeuN
MERGE
rAAVrh.39

Fig. 22

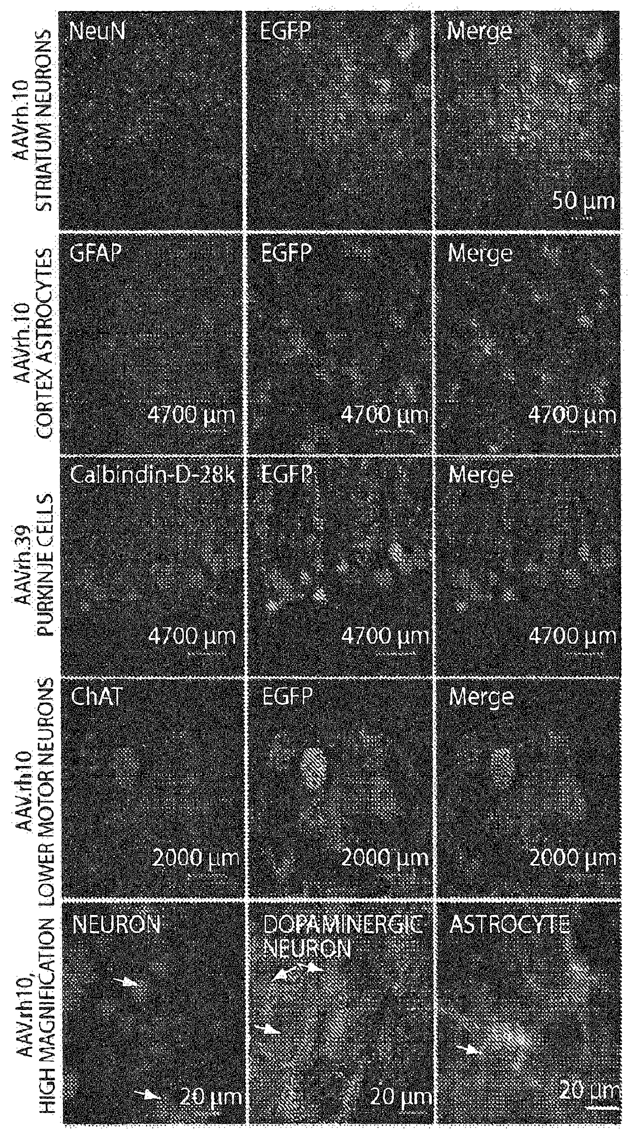

AAVrh.10
STRIATUM NEURONS
NeuN
EGFP
Merge
50 µm
AAVrh.10
CORTEX ASTROCYTES
GFAP
4700 µm
EGFP
4700 µm
Merge
4700 µm
AAVrh.39
PURKINJE CELLS
Calbindin-D-28k
4700 µm
EGFP
4700 µm
Merge
4700 µm
AAV.rh10
LOWER MOTOR NEURONS
ChAT
2000 µm
EGFP
2000 µm
Merge
2000 µm
AAV.rh10,
HIGH MAGNIFICATION
NEURON
20 µm
DOPAMINERGIC
NEURON
20 µm
ASTROCYTE
20 µm

Fig. 23

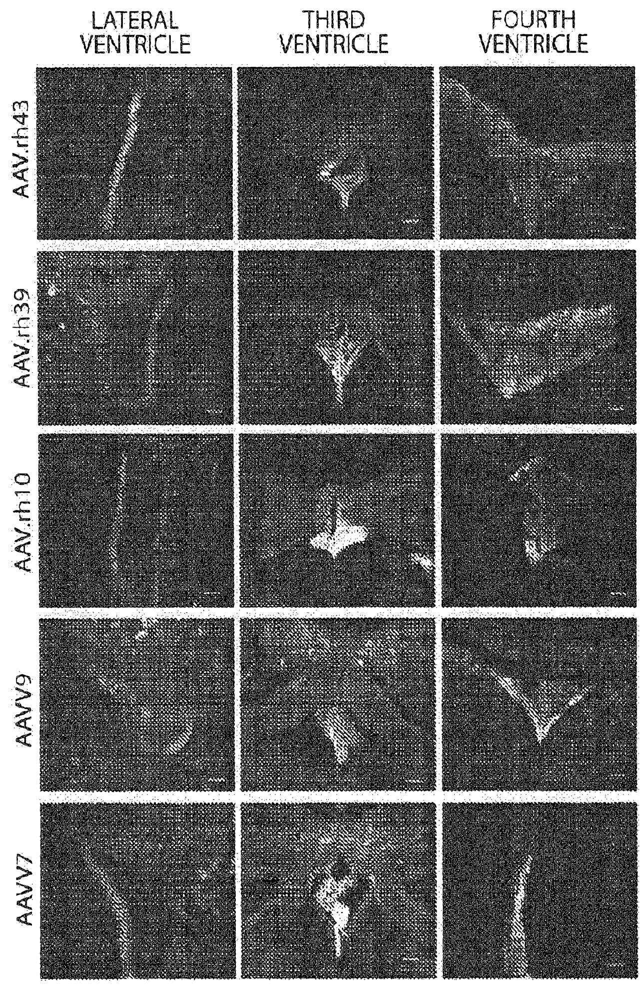

LATERAL VENTRICLE
THIRD VENTRICLE
FOURTH VENTRICLE
AAV.rh43
AAV.rh39
AAV.rh10
AAVV9
AAVV7

Fig. 24

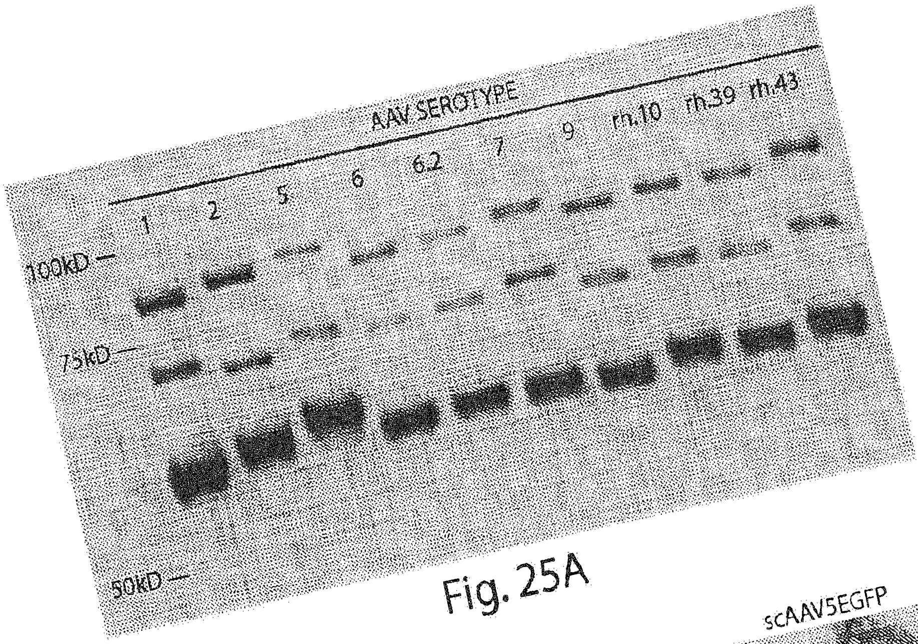


Fig. 25A

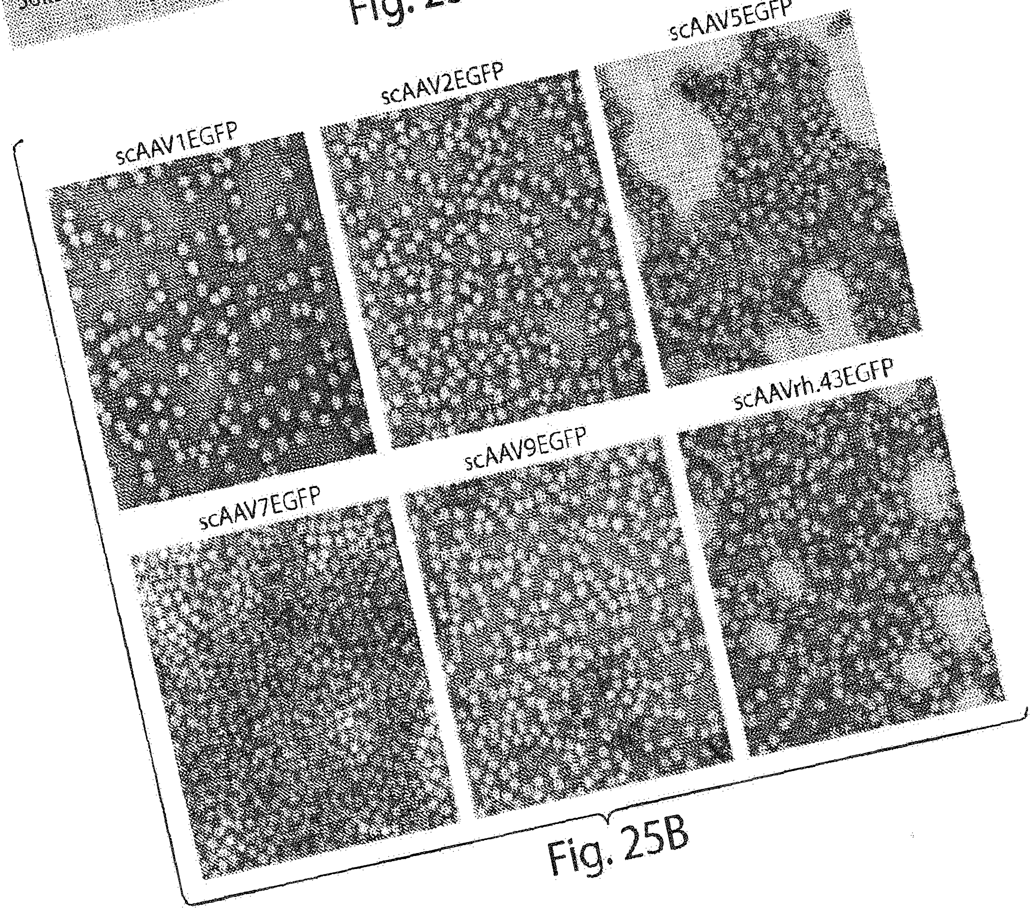


Fig. 25B

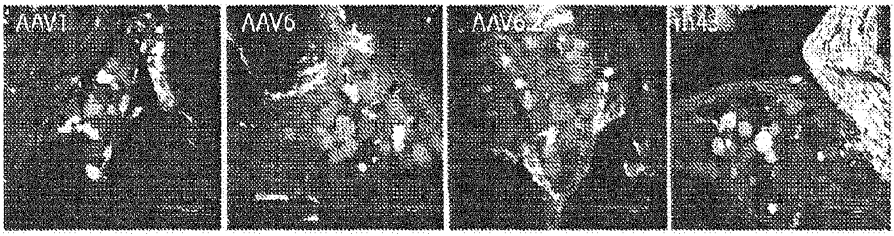


Fig. 26

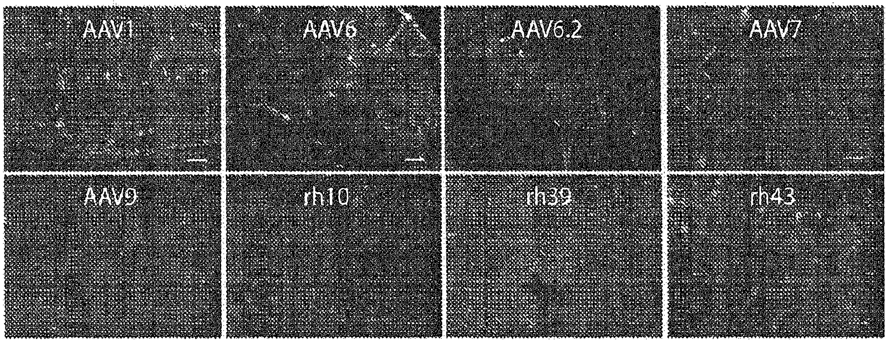


Fig. 27A

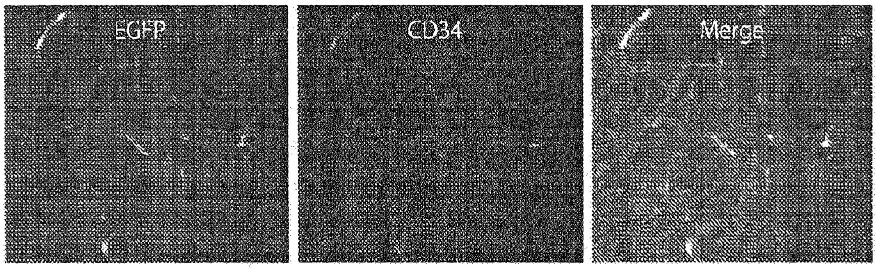


Fig. 27B

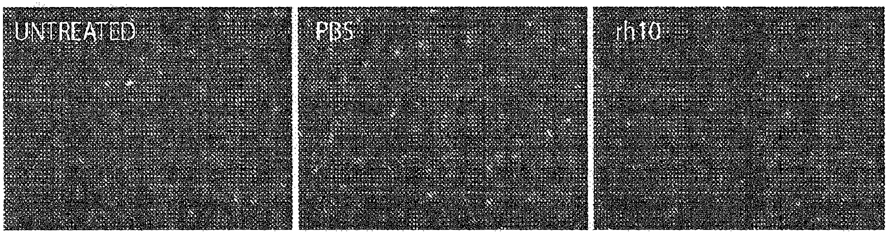


Fig. 28

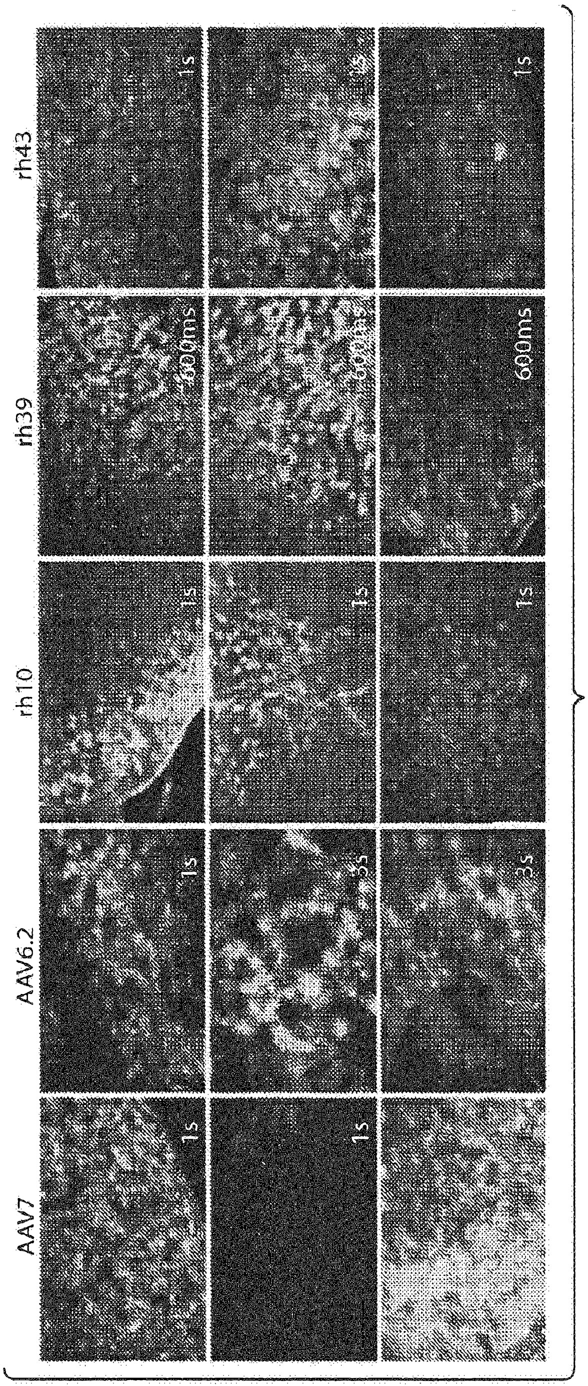


Fig. 29

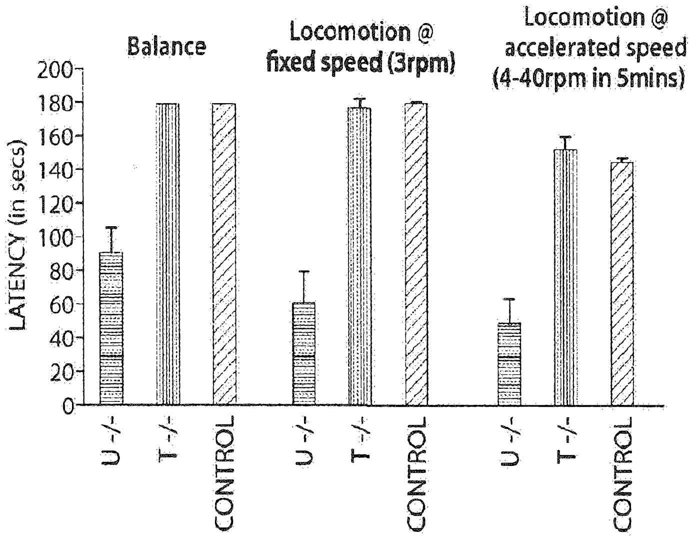


Fig. 30A

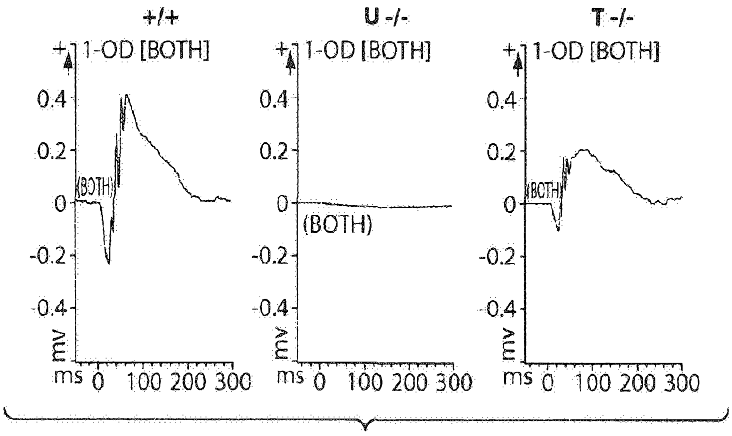


Fig. 30B

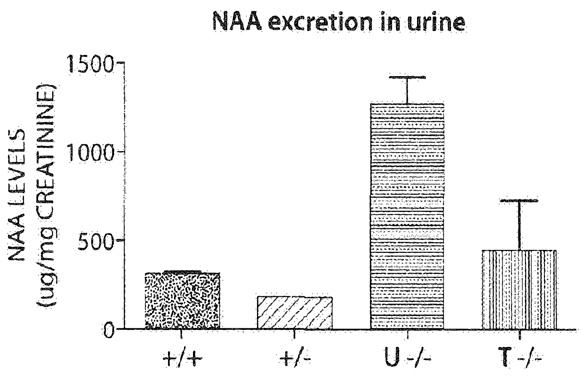


Fig. 30C

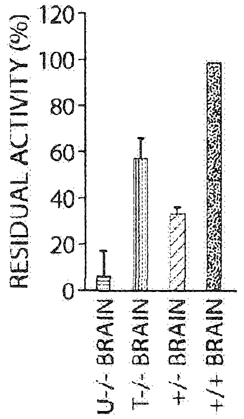


Fig. 30D

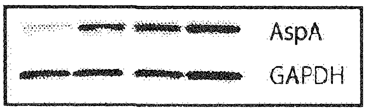


Fig. 30E

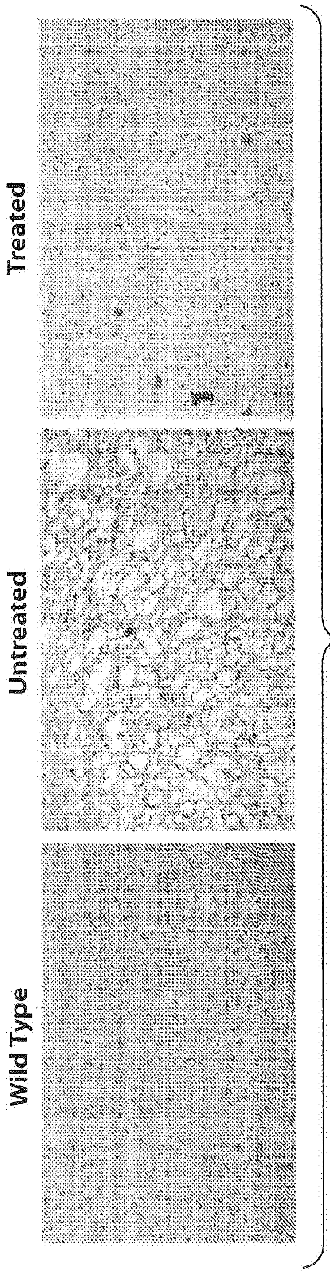


Fig. 31A

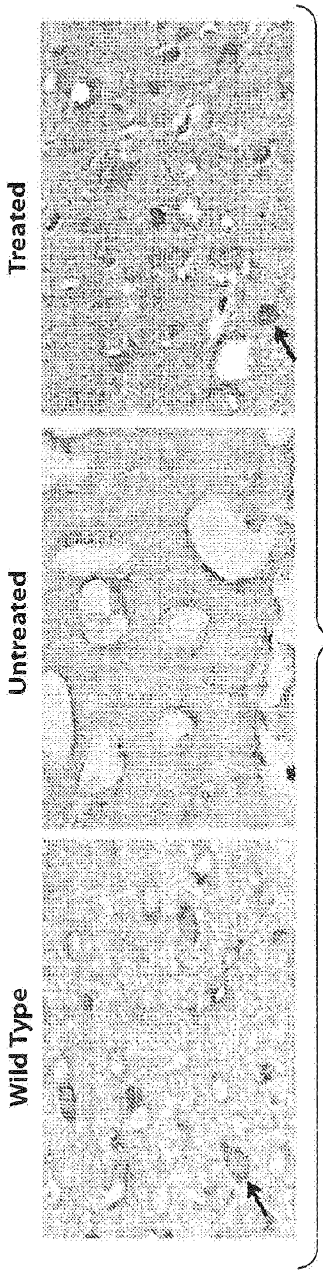


Fig. 31B

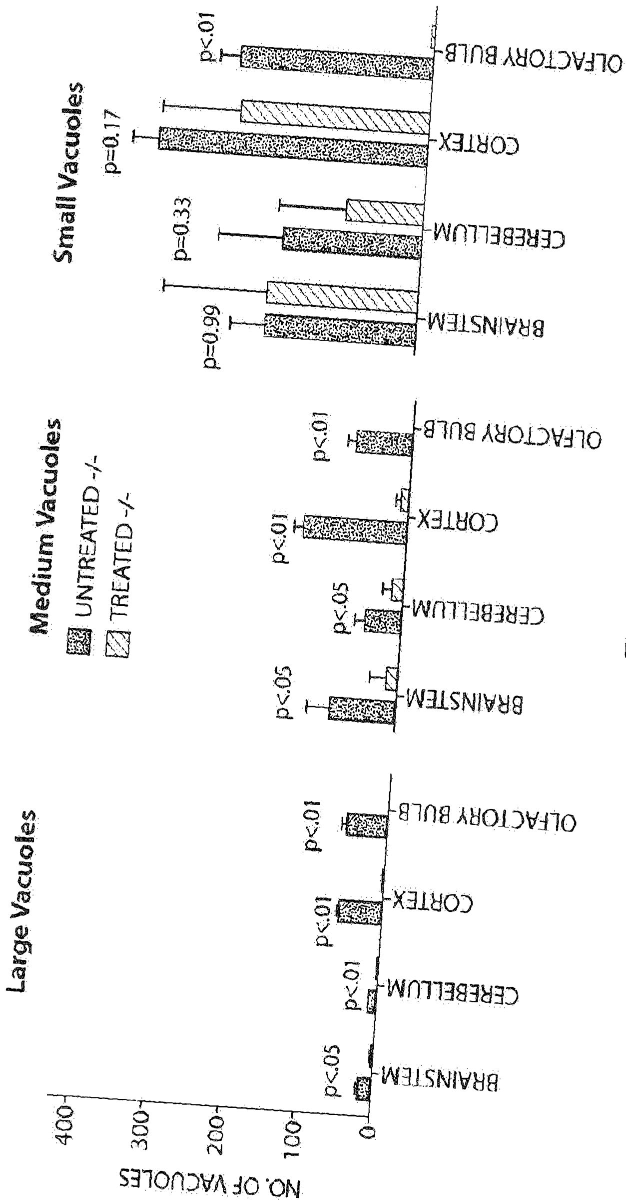

Large Vacuoles
Medium Vacuoles
Small Vacuoles
UNTREATED -/-
TREATED -/-
NO. OF VACUOLES
400
300
200
100
0
BRAINSTEM
CEREBELLUM
CORTEX
OLFACTORY BULB
p<.05
p<.01
p<.01
p<.01
p<.05
p<.05
p<.01
p<.01
p=0.99
p=0.33
p=0.17
p<.01

Fig. 32A


NO. OF VACUOLES
200
150
100
50
0
CERVICAL
THORACIC
LUMBAR
p<.01
p=0.101
p<.01
CERVICAL
THORACIC
LUMBAR
p<.01
p=0.109
p<.05
CERVICAL
THORACIC
LUMBAR
p<.01
p=0.265
p<0.87

Fig. 32B

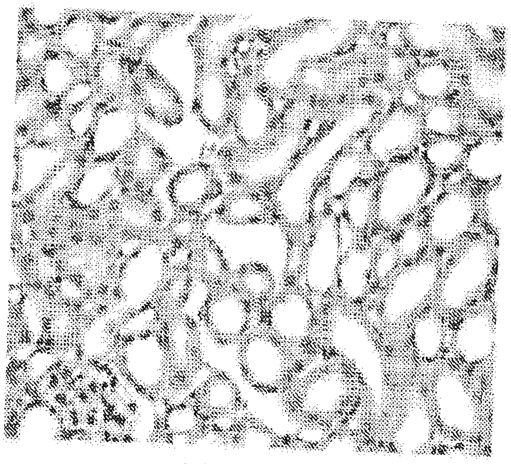


Fig. 33A

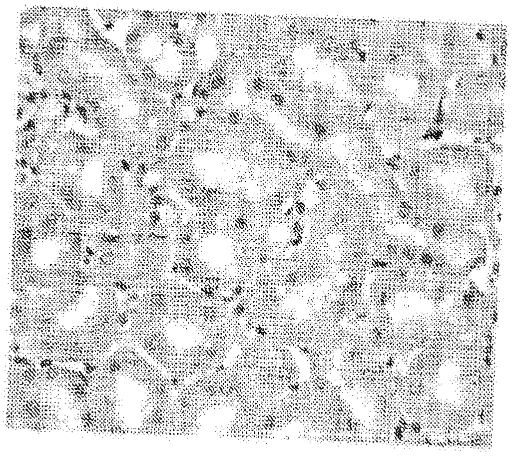


Fig. 33B

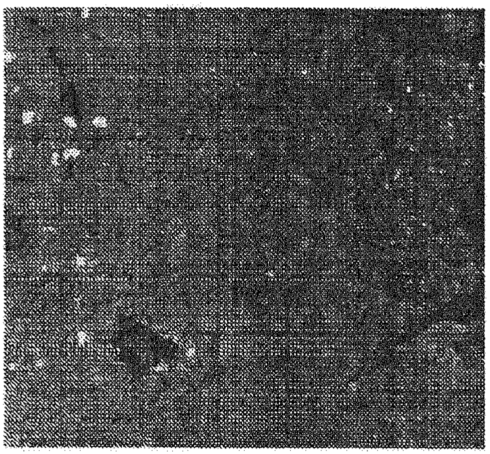


Fig. 33C

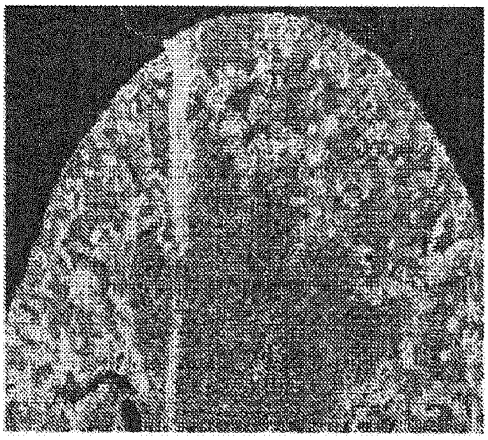


Fig. 33D