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(54) Benævnelse: **PRODUKTER OG FREMGANGSMÅDER TIL BEHANDLING AF AMYOTROFISK LATERAL SCLEROSE**

(56) Fremdragne publikationer:
AKIRA MACHIDA ET AL: "Intraperitoneal administration of AAV9-shRNA inhibits target gene expression in the dorsal root ganglia of neonatal mice", MOLECULAR PAIN, BIOMED CENTRAL, LONDON, GB, vol. 9, no. 1, 18 July 2013 (2013-07-18), page 36, XP021158065, ISSN: 1744-8069, DOI: 10.1186/1744-8069-9-36
KEVIN D FOUST ET AL: "Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes", NATURE BIOTECHNOLOGY, vol. 27, no. 1, 21 December 2008 (2008-12-21), pages 59-65, XP055023143, ISSN: 1087-0156, DOI: 10.1038/nbt.1515
GRIMM D ET AL: "ADENO-ASSOCIATED VIRUS VECTORS FOR SHORT HAIRPIN RNA EXPRESSION", METHODS IN ENZYMOLOGY, ACADEMIC PRESS, US, vol. 392, 1 January 2005 (2005-01-01), pages 381-405, XP008052578, ISSN: 0076-6879, DOI: 10.1016/S0076-6879(04)92023-X
DING H ET AL: "Selective silencing by RNAi of a dominant allele that causes amyotrophic lateral sclerosis", AGING CELL, BLACKWELL PUBLISHING, GB, vol. 2, 1 January 2003 (2003-01-01), pages 209-217, XP002389534, ISSN: 1474-9718, DOI: 10.1046/J.1474-9728.2003.00054.X
S. MUSATOV ET AL: "RNAi-mediated silencing of estrogen receptor in the ventromedial nucleus of hypothalamus abolishes female sexual behaviors", PROCEEDINGS OF THE NATIONAL ACADEMY OF

Fortsættes ...

SCIENCES, vol. 103, no. 27, 5 July 2006 (2006-07-05) , pages 10456-10460, XP055153583, ISSN: 0027-8424, DOI: 10.1073/pnas.0603045103
T. NAYAK ET AL: "Multimodality Imaging of RNA Interference", CURRENT MEDICINAL CHEMISTRY, vol. 20, no. 29, 1 August 2013 (2013-08-01), pages 3664-3675, XP055697267, NL ISSN: 0929-8673, DOI: 10.2174/09298673113202900012

DESCRIPTION

Statement of Government Interest

[0001] This invention was made with government support under U.S. National Institutes of Health R21-NS067238, NS027036, ROI NS064492 and RC2 NS69476-01. The Government has certain rights in the invention.

Incorporation By Reference Of Material Submitted Electronically

[0002] Incorporated by reference in its entirety is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: 14,350 byte ACII (Text) file named "47886PCT_SeqListing.txt," created on August 26, 2014.

Field of the Invention

[0003] The present invention relates to RNA-based methods for inhibiting the expression of the superoxide dismutase 1 (SOD-1) gene. Recombinant adeno-associated viruses of the invention deliver DNAs encoding RNAs that knock down the expression of SOD-1. The methods have application in the treatment of amyotrophic lateral sclerosis (ALS).

Background

[0004] ALS is an adult-onset, rapidly progressive and fatal neurodegenerative disease, characterized by selective degeneration of both upper and lower motor neurons. First characterized by Charcot in 1869, ALS is responsible for one in every 2000 deaths, affecting nearly 5 out of 100,000 individuals. ALS occurs when specific nerve cells in the brain and spinal cord that control voluntary movement degenerate. Within two to five years after clinical onset, the loss of these motor neurons leads to progressive atrophy of skeletal muscles, which results in loss of muscular function resulting in paralysis, speech deficits, and death due to respiratory failure.

[0005] Most ALS cases have no clear genetic linkage and are referred to as sporadic, but in 10% of instances disease is familial with dominant inheritance. Twenty percent of familial cases are caused by mutations in the enzyme superoxide dismutase 1 (SOD1), with over 140 distinct mutations identified to date[1, 2]. Many efforts to identify how mutations alter the function of SOD1 have produced a consensus view that SOD1 mutants acquire one or more toxicities, whose nature still remains controversial[3], but there is clear evidence that a proportion of

mutant SOD1 is misfolded and subsequently aggregates[4, 5]. SOD1 aggregates are, in fact, one of the histological hallmarks of SOD1-related ALS cases[4].

[0006] In the past 20 years, multiple animal models expressing mutant forms of human SOD1 have been generated. These models recapitulate the hallmarks of ALS, developing age-dependent motor axon degeneration and accompanying muscle denervation, glial inflammation and subsequent motor neuron loss. Selective gene excision experiments have determined that mutant SOD1 expression within motor neurons themselves contributes to disease onset and early disease progression[6], as does mutant synthesis in $NG2^+$ cells[7] that are precursors to oligodendrocytes. However, mutant SOD1 protein expression in microglia and astrocytes significantly drives rapid disease progressionf[6, 8], findings which have lead to the conclusion that ALS pathophysiology is non-cell autonomous[3].

[0007] Further, astrocytes have been found to be toxic to motor neurons in multiple *in vitro* models where mutant forms of human SOD1 were overexpressed[9-11]. A recent study derived astrocytes from post-mortem spinal cords of ALS patients with or without SOD1 mutations. In all cases, astrocytes from sporadic ALS patients were as toxic to motor neurons as astrocytes carrying genetic mutations in SOD1[12]. Even more strikingly, reduction of SOD1 in astrocytes derived from both sporadic and familial ALS patients decreased astrocyte-derived toxicity that is selective for motor, but not GABA, neurons. This remarkable finding, along with reports that misfolded SOD1 inclusions are found in the spinal cords of familial as well as some sporadic ALS patients[13,14, 15], has provided strong evidence for a pathogenic role of wild-type SOD1 in sporadic ALS.

[0008] Despite the insights that SOD1 mutant-expressing animal models have provided for understanding mechanisms involved in motor neuron degeneration, their utility for the development of therapeutic approaches has been questioned[16], as no drug with a reported survival benefit in mutant $SOD1^{G93A}$ mice has been effective in clinical trials with sporadic ALS patients. In all but one case the drugs taken to human trial had been reported only to extend mutant SOD 1 mouse survival when applied presymptomatically, and even then to provide a survival benefit solely by delaying disease onset with no benefit in slowing disease progression. The one exception to this was riluzole, which like the human situation, modestly extended survival of mutant $SOD1^{G93A}$ mice and did so by slowing disease progression[17]. Recognizing that success at human trial will require slowing of disease progression, the SOD1 mutant mice have perfectly predicted the success of riluzole and the failure of efficacy of each other drug attempted in human trial. What has been missing are additional therapies that affect disease progression in these mice.

[0009] Thus, riluzole is the only drug currently approved by the FDA as a therapy for ALS, providing a modest survival benefit[21]. For the 20% of familial cases caused by mutation in SOD1, attempts at improving therapy by reducing synthesis of SOD1 have been the focus of

multiple therapeutic development approaches. Antisense oligonucleotides and viral delivered RNA interference (RNAi) were tested in rat[22] and mouse models[23-25] that develop fatal paralysis from overexpressing human $SOD1^{G93A}$. Antisense oligonucleotides infused at disease onset produced SOD1 reduction and a modest slowing of disease progression[22]. Direct CSF infusion of antisense oligonucleotides has been tested clinically[26], leading to encouraging results in terms of tolerability and safety, but without significant reduction in SOD1 levels at the low dosages utilized. In each of the prior viral studies[23-25], SOD1 knockdown was achieved before disease onset by direct injection into the nervous system or taking advantage of axonal retrograde transport when a virus was injected intramuscularly[23, 24]. These studies led to varying degrees of success in extending survival or improving motor performance, depending on the time of treatment as well as level of SOD1 knockdown achieved in the spinal cord. Although these studies provided important proof of principle, the approaches were far from being readily translated into clinical strategies. Indeed, there have been controversial reports surrounding these initial viral mediated SOD1 suppression studies[23, 24, 27-29].

[0010] Adeno-associated virus (AAV) vectors have been used in a number of recent clinical trials for treatment of neurological disorders [Kaplitt et al., Lancet 369: 2097-2105 (2007); Marks et al., Lancet Neurol 7: 400-408 (2008); Worgall et al., Hum Gene Ther (2008)].

[0011] AAV is a replication-deficient parvovirus, the single-stranded DNA genome of which is about 4.7 kb in length including 145 nucleotide inverted terminal repeat (ITRs). The nucleotide sequence of the AAV serotype 2 (AAV2) genome is presented in Srivastava et al., J Virol, 45: 555-564 (1983) as corrected by Ruffing et al., J Gen Virol, 75: 3385-3392 (1994). *Cis*-acting sequences directing viral DNA replication (rep), encapsidation/packaging and host cell chromosome integration are contained within the ITRs. Three AAV promoters (named p5, p19, and p40 for their relative map locations) drive the expression of the two AAV internal open reading frames encoding rep and cap genes. The two rep promoters (p5 and p19), coupled with the differential splicing of the single AAV intron (at nucleotides 2107 and 2227), result in the production of four rep proteins (rep 78, rep 68, rep 52, and rep 40) from the rep gene. Rep proteins possess multiple enzymatic properties that are ultimately responsible for replicating the viral genome. The cap gene is expressed from the p40 promoter and it encodes the three capsid proteins VP1, VP2, and VP3. Alternative splicing and non-consensus translational start sites are responsible for the production of the three related capsid proteins. A single consensus polyadenylation site is located at map position 95 of the AAV genome. The life cycle and genetics of AAV are reviewed in Muzyczka, Current Topics in Microbiology and Immunology, 158: 97-129 (1992).

[0012] AAV possesses unique features that make it attractive as a vector for delivering foreign DNA to cells, for example, in gene therapy. AAV infection of cells in culture is noncytopathic, and natural infection of humans and other animals is silent and asymptomatic. Moreover, AAV infects many mammalian cells allowing the possibility of targeting many different tissues *in vivo.* Moreover, AAV transduces slowly dividing and non-dividing cells, and can persist essentially for

the lifetime of those cells as a transcriptionally active nuclear episome (extrachromosomal element). The AAV proviral genome is infectious as cloned DNA in plasmids which makes construction of recombinant genomes feasible. Furthermore, because the signals directing AAV replication, genome encapsidation and integration are contained within the ITRs of the AAV genome, some or all of the internal approximately 4.3 kb of the genome (encoding replication and structural capsid proteins, rep-cap) may be replaced with foreign DNA such as a gene cassette containing a promoter, a DNA of interest and a polyadenylation signal. The rep and cap proteins may be provided *in trans.* Another significant feature of AAV is that it is an extremely stable and hearty virus. It easily withstands the conditions used to inactivate adenovirus (56° to 65°C for several hours), making cold preservation of AAV less critical. AAV may even be lyophilized. Finally, AAV-infected cells are not resistant to superinfection.

[0013] Multiple serotypes of AAV exist and offer varied tissue tropism. Known serotypes include, for example, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV 10, AAV11 and AAVrh74. Advances in the delivery of AAV6 and AAV8 have made possible the transduction by these serotypes of skeletal and cardiac muscle following simple systemic intravenous or intraperitoneal injections. See Pacak et ah, Circ. Res., 99(4): 3-9 (1006) and Wang et ah, Nature Biotech., 23(3): 321-8 (2005). The use of AAV to target cell types within the central nervous system has involved surgical intraparenchymal injection. See, Kaplitt et ah, supra; Marks et ah, supra and Worgall et ah, supra. Regarding the use of AAV to target cell types within the nervous system, see International Publication No. WO 2010/071832. International Publication Nos. WO 2009/043936 and WO 2009/013290 state they relate to delivering genes to the central nervous system. International Publication No. WO 2011/133890 states it relates to recombinant adeno-associated viruses useful for targeting transgenes to central nervous system tissue.

[0014] Ding H et al: "Selective silencing by RNAi of dominant allele that causes amyotrophic lateral sclerosis", AGING CELL, BLACKWELL PUBLISHING, GB, vol. 2, 1 January 2003 (2003-01-01), pages 209-217 identified an siRNA and shRNA sequence that can selectively down-regulate the expression of mutant but not the wild-type SOD1.

[0015] There thus remains a need in the art for methods and materials for treatment of ALS

Summary

[0016] In a first aspect of the present invention there is provided a recombinant adeno-associated virus as set forth in the claims.

[0017] In a further aspect of the present invention there is also provided the recombinant adeno-associated virus of the present invention for use in therapy.

[0018] In another aspect of the present invention there is provided the recombinant adeno-associated virus of the present invention for use in treating amyotrophic lateral sclerosis (ALS).

[0019] In a further aspect of the present invention there is provided a composition comprising the recombinant adeno-associated virus of the present invention

[0020] The present invention provides products and their use for reducing mutant SODI protein levels in subjects in need thereof. The invention provides AAV-mediated delivery of RNAs including, but not limited to short hairpin RNAs, to reduce synthesis of ALS-causing human SODI mutants in subjects in need thereof. Recombinant AAV (rAAV) contemplated by the invention include, but are not limited to, rAAV9, rAAV2 and rAAVrh74. Delivery routes contemplated by the invention include, but are not limited to, systemic delivery and intrathecal delivery. Use of the methods and products of the invention is indicated, for example, in treating ALS.

[0021] The reference to methods of treatment in the subsequent paragraphs of the description are interpreted as reference to the compounds, pharmaceutical compositions and medicaments of the present invention for use in a method for treating of the human or animal body by therapy.

Detailed Description

[0022] In one aspect, the invention provides rAAV genomes comprising one or more AAV ITRs flanking a polynucleotide encoding one or more RNAs (including, but not limited to, small hairpin RNAs, antisense RNAs and/or microRNAs) that target mutant SOD1 polynucleotides. The examples describe the use of exemplary rAAV encoding small hairpin RNAs (shRNAs). In the rAAV genomes, the shRNA-encoding polynucleotide is operatively linked to transcriptional control DNA, specifically promoter DNA that is functional in target cells. Commercial providers such as Ambion Inc. (Austin, TX), Darmacon Inc. (Lafayette, CO), InvivoGen (San Diego, CA), and Molecular Research Laboratories, LLC (Herndon, VA) generate custom inhibitory RNA molecules. In addition, commercially kits are available to produce custom siRNA molecules, such as SILENCER™ siRNA Construction Kit (Ambion Inc., Austin, TX) or psiRNA System (InvivoGen, San Diego, CA). In some embodiments, the rAAV genome comprises a DNA encoding a SOD1 shRNA such as:

GCATCATCAATTTCGAGCAGAAGGAA (SEQ ID NO:1),

GAAGCATTAAAGGACTGACTGAA (SEQ ID NO:2),

CTGACTGAAGGCCTGCATGGATT (SEQ ID NO:3),

CATGGATTCCATGTTCATGA (SEQ ID NO:4) ("shRNA 130" or "SOD1 shRNA" herein),

GCATGGATTCCATGTTCATGA (SEQ ID NO:5),

GGTCTGGCCTATAAAGTAGTC (SEQ ID NO:6),

GGGCATCATCAATTTCGAGCA (SEQ ID NO:7),

GCATCATCAATTTCGAGCAGA (SEQ ID NO:8),

GCCTGCATGGATTCCATGTTC (SEQ ID NO:9),

GGAGGTCTGGCCTATAAAGTA (SEQ ID NO:10),

GATTCCATGTTCATGAGTTTG (SEQ ID NO:11),

GGAGATAATACAGCAGGCTGT (SEQ ID NO:12),

GCTTTAAAGTACCTGTAGTGA (SEQ ID NO:13),

GCATTAAAGGACTGACTGAAG (SEQ ID NO:14),

GCATCATCAATTTCGAGCAGAAGGAA (SEQ ID NO:1),

GAAGCATTAAAGGACTGACTGAA (SEQ ID NO:2),

CTGACTGAAGGCCTGCATGGATT (SEQ ID NO:3),

CATGGATTCCATGTTCATGA (SEQ ID NO:4),

GCATGGATTCCATGTTCATGA (SEQ ID NO:5),

GGTCTGGCCTATAAAGTAGTC (SEQ ID NO:6),

GGGCATCATCAATTTCGAGCA (SEQ ID NO:7),

GCATCATCAATTTCGAGCAGA (SEQ ID NO:8),

GCCTGCATGGATTCCATGTTC (SEQ ID NO:9),

GGAGGTCTGGCCTATAAAGTA (SEQ ID NO:10),

GATTCCATGTTCATGAGTTTG (SEQ ID NO:11),

GGAGATAATACAGCAGGCTGT (SEQ ID NO:12),

GCTTTAAAGTACCTGTAGTGA (SEQ ID NO:13),

GCATTAAAGGACTGACTGAAG (SEQ ID NO:14),

TCATCAATTTCGAGCAGAA (SEQ ID NO:15),

TCGAGCAGAAGGAAAGTAA (SEQ ID NO:16),

GCCTGCATGGATTCCATGT (SEQ ID NO:17),

TCACTCTCAGGAGACCATT (SEQ ID NO:18), or

GCTTTAAAGTACCTGTAGT (SEQ ID NO:19).

[0023] The rAAV genomes of the invention lack AAV rep and cap DNA. AAV DNA in the rAAV genomes (e.g., ITRs) may be from any AAV serotype for which a recombinant virus can be derived including, but not limited to, AAV serotypes AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10 and AAV-11. The nucleotide sequences of the genomes of the AAV serotypes are known in the art. For example, the complete genome of AAV-1 is provided in GenBank Accession No. NC_002077; the complete genome of AAV-2 is provided in GenBank Accession No. NC_001401 and Srivastava et al., J. Virol., 45: 555-564 {1983); the complete genome of AAV-3 is provided in GenBank Accession No. NC_1829; the complete genome of AAV-4 is provided in GenBank Accession No. NC_001829; the AAV-5 genome is provided in GenBank Accession No. AF085716; the complete genome of AAV-6 is provided in GenBank Accession No. NC_00 1862; at least portions of AAV-7 and AAV-8 genomes are provided in GenBank Accession Nos. AX753246 and AX753249, respectively; the AAV -9 genome is provided in Gao et al., J. Virol., 78: 6381-6388 (2004); the AAV-10 genome is provided in Mol. Ther., 13(1): 67-76 (2006); and the AAV-11 genome is provided in Virology, 330(2): 375-383 (2004). The AAVrh74 genome is provided in International Publication No. WO 2013/078316.

[0024] In another aspect, the invention provides DNA plasmids comprising rAAV genomes of the invention. The DNA plasmids are transferred to cells permissible for infection with a helper virus of AAV (e.g., adenovirus, E1-deleted adenovirus or herpesvirus) for assembly of the rAAV genome into infectious viral particles. Techniques to produce rAAV particles, in which an AAV genome to be packaged, rep and cap genes, and helper virus functions are provided to a cell are standard in the art. Production of rAAV requires that the following components are present within a single cell (denoted herein as a packaging cell): a rAAV genome, AAV rep and cap genes separate from (*i.e.*, not in) the rAAV genome, and helper virus functions. The AAV rep and cap genes may be from any AAV serotype for which recombinant virus can be derived and may be from a different AAV serotype than the rAAV genome ITRs, including, but not limited to, AAV serotypes AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10 and AAV-11. Production of pseudotyped rAAV is disclosed in, for example, WO 01/83692 which is incorporated by reference herein in its entirety. In various embodiments, AAV capsid proteins may be modified to enhance delivery of the recombinant vector. Modifications to capsid proteins are generally known in the art. See, for example, US 20050053922 and US 20090202490, the disclosures of which are incorporated by reference herein in their entirety.

[0025] A method of generating a packaging cell is to create a cell line that stably expresses all the necessary components for AAV particle production. For example, a plasmid (or multiple plasmids) comprising a rAAV genome lacking AAV rep and cap genes, AAV rep and cap genes separate from the rAAV genome, and a selectable marker, such as a neomycin resistance gene, are integrated into the genome of a cell. AAV genomes have been introduced into bacterial plasmids by procedures such as GC tailing (Samulski et al., 1982, Proc. Natl. Acad. S6. USA, 79:2077-2081), addition of synthetic linkers containing restriction endonuclease

cleavage sites (Laughlin et al., 1983, Gene, 23:65-73) or by direct, blunt-end ligation (Senapathy & Carter, 1984, J. Biol. Chem., 259:4661-4666). The packaging cell line is then infected with a helper virus such as adenovirus. The advantages of this method are that the cells are selectable and are suitable for large-scale production of rAAV. Other examples of suitable methods employ adenovirus or baculovirus rather than plasmids to introduce rAAV genomes and/or rep and cap genes into packaging cells.

[0026] General principles of rAAV production are reviewed in, for example, Carter, 1992, Current Opinions in Biotechnology, 1533-539; and Muzyczka, 1992, Curr. Topics in Microbial, and Immunol., 158:97-129). Various approaches are described in Ratschin et al., Mol. Cell. Biol. 4:2072 (1984); Hermonat et al., Proc. Natl. Acad. Sci. USA, 81:6466 (1984); Tratschin et al., Mol. Cell. Biol. 5:3251 (1985); McLaughlin et al., J. Virol., 62: 1963 (1988); and Lebkowski et al., 1988 Mol. Cell. Biol., 7:349 (1988). Samulski et al. (1989, J. Virol., 63:3822-3828); U.S. Patent No. 5,173,414; WO 95/13365 and corresponding U.S. Patent No. 5,658.776 ; WO 95/13392; WO 96/17947; PCT/US98/18600; WO 97/09441 (PCT/US96/14423); WO 97/08298 (PCT/US96/13872); WO 97/21825 (PCT/US96/20777); WO 97/06243 (PCT/FR96/01064); WO 99/11764; Perrin et al. (1995) Vaccine 13: 1244-1250; Paul et al. (1993) Human Gene Therapy 4:609-615; Clark et al. (1996) Gene Therapy 3: 1124-1132; U.S. Patent. No. 5,786,211; U.S. Patent No. 5,871,982; and U.S. Patent. No. 6,258,595. Single-stranded rAAV are specifically contemplated. The foregoing documents are hereby incorporated by reference in their entirety herein, with particular emphasis on those sections of the documents relating to rAAV production.

[0027] The embodiment thus provides packaging cells that produce infectious rAAV. In one embodiment packaging cells may be stably transformed cancer cells such as HeLa cells, 293 cells and PerC.6 cells (a cognate 293 line). In another embodiment, packaging cells are cells that are not transformed cancer cells such as low passage 293 cells (human fetal kidney cells transformed with El of adenovirus), MRC-5 cells (human fetal fibroblasts), WI-38 cells (human fetal fibroblasts), Vero cells (monkey kidney cells) and FRhL-2 cells (rhesus fetal lung cells).

[0028] In still another aspect, the invention provides rAAV (i.e., infectious encapsidated rAAV particles) comprising a rAAV genome of the invention. In some embodiments, the rAAV genome is a self-complementary genome. The genomes of the rAAV lack AAV rep and cap DNA, that is, there is no AAV rep or cap DNA between the ITRs of the genomes. Embodiments include, but are not limited to, the exemplary rAAV including a genome encoding the SOD1 shRNA named "AAV-SOD1-shRNA." A sequence including the AAV-SOD1-shRNA genome is set out below as an inverted sequence from a plasmid used in production.

```
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   CDS             complement(901..965)
                   /gene="SOD shRNA"
                   /SECDrawAs="Gene"
                   /SECStyleId=1
   misc_feature    complement(966..1064)
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                /SECDrawAs="Region"
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     misc_feature    1510..1779
                /gene="B-Actin promoter"
                /product="Chicken"
                /SECDrawAs="Region"
                /SECStyleId=1
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                /gene="SV40_late_19s_int"
                /SECDrawAs="Region"
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                /gene="modSV40_late_16s_int"
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     misc_feature    3009..3149

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               /SECDrawAs="Region"
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(SEQ ID NO:20)

[0029]

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 121 cgcattaagc gcggcgggtg tggtggttac gcgcagcgtg accgctacac ttgccagcgc
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 301 cgaccccaaa aaacttgatt agggtgatgg ttcacgtagt gggccatcgc cctgatagac
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 601 ctgattatca accggggtac atatgattga catgctagtt ttacgattac cgttcatcgc
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 781 atctgaattc aattcacgcg tggtacctac actttatgct tccggctcgt atgttgtgtg
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1021 tatggtgatt tcccagaaca catagcgaca tgcaaatatg aattcactgg ccgtcgtttt
1081 acaacgtcgt gactgggaaa accctggcgt tacccaactt aatcgccttg cagcacatcc
1141 cccttcgcc agctggcgta atagcgaaga ggcccgcacc gatcgccctt cccaacagtt
1201 gcgcagcctg tggtacctct ggtcgttaca taacttacgg taaatggccc gcctggctga
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1321 atagggactt tccattgacg tcaatgggtg gagtatttac ggtaaactgc ccacttggca
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1501 tactcgaggc cacgttctgc ttcactctcc ccatctcccc ccccttccca ccccccaattt
1561 tgtatttatt tatttttaa ttattttgtg cagcgatggg ggcgggggggg gggggggggc
1621 gcgcgccagg cggggcgggg cggggcgagg ggcggggcgg ggcgaggcgg agaggtgcgg
1681 cggcagccaa tcagagcggc gcgctccgaa agtttccttt tatggcgagg cggcggcggc
1741 ggcggccctа taaaaagcga agcgcgcggc gggcgggagc gggatcagcc accgcggtgg
1801 cggcctagag tcgacgagga actgaaaaac cagaaagtta actggtaagt ttagtctttt
1861 tgtcttttat ttcaggtccc ggatccggtg gtggtgcaaa tcaaagaact gctcctcagt
1921 ggatgttgcc tttacttcta ggcctgtacg gaagtgttac ttctgctcta aaagctgcgg
1981 aattgtaccc gcggccgatc caccggtcgc caccatggtg agcaagggcg aggagctgtt
2041 caccggggtg gtgcccatcc tggtcgagct ggacggcgac gtaaacggcc acaagttcag
2101 cgtgtccggc gagggcgagg gcgatgccac ctacggcaag ctgaccctga agttcatctg
2161 caccaccggc aagctgcccg tgccctggcc caccctcgtg accaccctga cctacggcgt
2221 gcagtgcttc agccgctacc ccgaccacat gaagcagcac gacttcttca agtccgccat
2281 gcccgaaggc tacgtccagg agcgcaccat cttcttcaag gacgacggca actacaagac
2341 ccgcgccgag gtgaagttcg agggcgacac cctggtgaac cgcatcgagc tgaagggcat
2401 cgacttcaag gaggacggca acatcctggg gcacaagctg gagtacaact acaacagcca

2461 caacgtctat atcatggccg acaagcagaa gaacggcatc aaggtgaact tcaagatccg
2521 ccacaacatc gaggacggca gcgtgcagct cgccgaccac taccagcaga acaccccccat
2581 cggcgacggc cccgtgctgc tgcccgacaa ccactacctg agcacccagt ccgccctgag
2641 caaagacccc aacgagaagc gcgatcacat ggtcctgctg gagttcgtga ccgccgccgg
2701 gatcactctc ggcatggacg agctgtacaa gtaaagcggc catcaagctt atcgataccg
2761 tcgactagag ctcgctgatc agcctcgact gtgccttcta gttgccagcc atctgttgtt
2821 tgcccctccc ccgtgccttc cttgaccctg gaaggtgcca ctcccactgt cctttcctaa
2881 taaaatgagg aaattgcatc gcattgtctg agtaggtgtc attctattct gggggggtggg
2941 gtggggcagg acagcaaggg ggaggattgg gaagacaata gcaggcatgc tggggagaga
3001 tcgatctgag gaacccctag tgatggagtt ggccactccc tctctgcgcg ctcgctcgct
3061 cactgaggcc gggcgaccaa aggtcgcccg acgcccgggc tttgcccggg cggcctcagt
3121 gagcgagcga gcgcgcagag agggagtggc ccccccccc ccccccccgg cgattctctt
3181 gtttgctcca gactctcagg caatgacctg atagcctttg tagagacctc tcaaaaatag
3241 ctaccctctc cggcatgaat ttatcagcta gaacggttga atatcatatt gatggtgatt
3301 tgactgtctc cggcctttct cacccgtttg aatctttacc tacacattac tcaggcattg
3361 catttaaaat atatgagggt tctaaaaatt tttatccttg cgttgaaata aaggcttctc
3421 ccgcaaaagt attacagggt cataatgttt ttggtacaac cgatttagct ttatgctctg
3481 aggctttatt gcttaatttt gctaattctt tgccttgcct gtatgattta ttggatgttg
3541 gaatcgcctg atgcggtatt ttctccttac gcatctgtgc ggtatttcac accgcatatg
3601 gtgcactctc agtacaatct gctctgatgc cgcatagtta gccagcccc gacacccgcc
3661 aacacccgct gacgcgccct gacgggcttg tctgctcccg gcatccgctt acagacaagc
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3781 gagacgaaag ggcctcgtga tacgcctatt tttataggtt aatgtcatga taataatggt
3841 ttcttagacg tcaggtggca cttttcgggg aaatgtgcgc ggaacccctа tttgtttatt
3901 tttctaaata cattcaaata tgtatccgct catgagacaa taaccctgat aaatgcttca
3961 ataatattga aaaaggaaga gtatgagtat tcaacatttc cgtgtcgccc ttattcccct
4021 ttttgcggca ttttgccttc ctgtttttgc tcacccagaa acgctggtga aagtaaaaga
4081 tgctgaagat cagttgggtg cacgagtggg ttacatcgaa ctggatctca acagcggtaa
4141 gatccttgag agttttcgcc ccgaagaacg ttttccaatg atgagcactt ttaaagttct
4201 gctatgtggc gcggtattat cccgtattga cgccgggcaa gagcaactcg gtcgccgcat
4261 acactattct cagaatgact tggttgagta ctcaccagtc acagaaaagc atcttacgga
4321 tggcatgaca gtaagagaat tatgcagtgc tgccataacc atgagtgata acactgcggc
4381 caacttactt ctgacaacga tcggaggacc gaaggagcta accgcttttt tgcacaacat
4441 gggggatcat gtaactcgcc ttgatcgttg ggaaccggag ctgaatgaag ccataccaaa
4501 cgacgagcgt gacaccacga tgcctgtagc aatggcaaca acgttgcgca aactattaac
4561 tggcgaacta cttactctag cttcccggca acaattaata gactggatgg aggcggataa
4621 agttgcagga ccacttctgc gctcggccct tccggctggc tggtttattg ctgataaatc
4681 tggagccggt gagcgtgggt ctcgcggtat cattgcagca ctggggccag atggtaagcc
4741 ctcccgtatc gtagttatct acacgacggg gagtcaggca actatggatg aacgaaatag
4801 acagatcgct gagataggtg cctcactgat taagcattgg taactgtcag accaagttta
4861 ctcatatata ctttagattg atttaaaact tcatttttaa tttaaaagga tctaggtgaa
4921 gatccttttt gataatctca tgaccaaaat cccttaacgt gagttttcgt tccactgagc
4981 gtcagacccc gtagaaaaga tcaaaggatc ttcttgagat cctttttttc tgcgcgtaat
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5101 gctaccaact ctttttccga aggtaactgg cttcagcaga gcgcagatac caaatactgt
5161 ccttctagtg tagccgtagt taggccacca cttcaagaac tctgtagcac cgcctacata
5221 cctcgctctg ctaatcctgt taccagtggc tgctgccagt ggcgataagt cgtgtcttac
5281 cgggttggac tcaagacgat agttaccgga taaggcgcag cggtcgggct gaacgggggg
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5341 ttcgtgcaca cagcccagct tggagcgaac gacctacacc gaactgagat acctacagcg
5401 tgagctatga gaaagcgcca cgcttcccga agggagaaag gcggacaggt atccggtaag
5461 cggcagggtc ggaacaggag agcgcacgag ggagcttcca gggggaaacg cctggtatct
5521 ttatagtcct gtcgggtttc gccacctctg acttgagcgt cgatttttgt gatgctcgtc
5581 aggggggcgg agcctatgga aaaacgccag caacgcggcc tttttacggt tcctggcctt
5641 ttgctggcct tttgctcaca tgttctttcc tgcgttatcc cctgattctg tggataaccg
5701 tattaccgcc tttgagtgag ctgataccgc tcgccgcagc cgaacgaccg agcgcagcga
5761 gtcagtgagc gaggaagcgg aagagc
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[0030] The SOD shRNA nucleotides 901-965 comprise the entire hairpin sequence including the sense and antisense arms, stem loop and termination sequence. The sequence in a forward orientation (with target sequences against SOD1 underlined) is:

5'AATTCATATTTGCATGTCGCTATGTGTTCTGGGAAATCACCATAAACGTGAAATGTCTTT
GGATTTGGGAATCTTATAAGTTCTGTATGAGACCACTCGGATCCATGGATTCCATGTTCA
TGATTCAAGAGATCATGAACATGGAATCCATGCTTTTTTGGAAA 3'(SEQ ID NO:21)

[0031] The rAAV of the invention may be purified by methods standard in the art such as by column chromatography or cesium chloride gradients. Methods for purifying rAAV vectors from helper virus are known in the art and include methods disclosed in, for example, Clark et al., Hum. Gene Ther., 10(6): 1031-1039 (1999); Schenpp and Clark, Methods Mol. Med., 69: 427-443 (2002); U.S. Patent No. 6,566,118 and WO 98/09657.

[0032] In another aspect, the invention contemplates compositions comprising rAAV of the present invention. Compositions of the invention comprise rAAV in a pharmaceutically acceptable carrier. The compositions may also comprise other ingredients such as diluents and adjuvants. Acceptable carriers, diluents and adjuvants are nontoxic to recipients and are preferably inert at the dosages and concentrations employed, and include buffers such as phosphate, citrate, or other organic acids; antioxidants such as ascorbic acid; low molecular weight polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, pluronics or polyethylene glycol (PEG).

[0033] Titers of rAAV to be administered in methods of the invention will vary depending, for example, on the particular rAAV, the mode of administration, the treatment goal, the individual, and the cell type(s) being targeted, and may be determined by methods standard in the art. Titers of rAAV may range from about about 1×10^2, about 1×10^3, about 1×10^4, about 1×10^5, about 1×10^6, about 1×10^7, about 1×10^8, about 1×10^9, about 1×10^{10}, about 1×10^{11}, about 1×10^{12}, about 1×10^{13} to about 1×10^{14} or more DNase resistant particles (DRP) per ml. Dosages may also be expressed in units of viral genomes (vg). Dosages may also vary based on the timing of the administration to a human. These dosages of rAAV may range from about 1×10^4, about 1×10^5, about 1×10^6, about 1×10^7, about 1×10^8, about 1×10^9, about 1×10^{10}, about 1×10^{11}, about 1×10^{12}, about 1×10^{13}, about 1×10^{14}, about 1×10^{15}, about 1×10^{16} or more

viral genomes per kilogram body weight in an adult. For a neonate, the dosages of rAAV may range from about about 1×10^{4}, about 3×10^{4}, about 1×10^{5}, about 3×10^{5}, about 1×10^{6}, about 3×10^{6}, about 1×10^{7}, about 3×10^{7}, about 1×10^{8}, about 3×10^{8}, about 1×10^{9}, about 3×10^{9}, about 1×10^{10}, about 3×10^{10}, about 1×10^{11}, about 3×10^{11}, about 1×10^{12}, about 3×10^{12}, about 1×10^{13}, about 3×10^{13}, about 1×10^{14}, about 3×10^{14}, about 1×10^{15}, about 3×10^{15}, about 1×10^{16}, about 3×10^{16} or more viral genomes per kilogram body weight.

[0034] In another aspect, the invention contemplates compositions comprising rAAV of the present invention. Compositions of the invention comprise rAAV in a pharmaceutically acceptable carrier. The compositions may also comprise other ingredients such as diluents and adjuvants. Acceptable carriers, diluents and adjuvants are nontoxic to recipients and are preferably inert at the dosages and concentrations employed, and include buffers such as phosphate, citrate, or other organic acids; antioxidants such as ascorbic acid; low molecular weight polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-formig counterions such as sodium; and/or nonionic surfactants such as Tween, pluronics or polyethylene glycol (PEG).

[0035] In still another aspect, the invention provides methods of transducing a target cell with a rAAV of the invention, *in vivo* or *in vitro.* The *in vivo* methods comprise the step of administering an effective dose, or effective multiple doses, of a composition comprising a rAAV of the invention to a subject, a subject (including a human being), in need thereof. If the dose is administered prior to onset/development of a disorder/disease, the administration is prophylactic. If the dose is administered after the onset/development of a disorder/disease, the administration is therapeutic. In embodiments of the invention, an effective dose is a dose that alleviates (eliminates or reduces) at least one symptom associated with the disorder/disease state being treated, that slows or prevents progression to a disorder/disease state, that slows or prevents progression of a disorder/disease state, that diminishes the extent of disease, that results in remission (partial or total) of disease, and/or that prolongs survival. An example of a disease contemplated for treatment with methods of the invention is ALS. "Treatment" according to the invention thus alleviates (eliminates or reduces) at least one symptom associated with the disorder/disease state being treated (for example, weight loss is eliminated or reduced by at least 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100% or greater), that slows or prevents progression to (onset/development) of a disorder/disease state, that slows or prevents progression of a disorder/disease state, that diminishes the extent of disease, that results in remission (partial or total) of disease, and/or that prolongs survival. In some embodiments, survival is prolonged by at least 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100% or greater.

[0036] Combination therapies are also contemplated by the invention. Combination as used herein includes both simultaneous treatment or sequential treatments. Combinations of methods of the invention with standard medical treatments (e.g., riluzole) are specifically contemplated, as are combinations with novel therapies.

[0037] Administration of an effective dose of the compositions may be by routes standard in the art including, but not limited to, systemic intramuscular, parenteral, intravenous, oral, buccal, nasal, pulmonary, intracranial, intrathecal, intraosseous, intraocular, rectal, or vaginal. Route(s) of administration and serotype(s) of AAV components of the rAAV (in particular, the AAV ITRs and capsid protein) of the invention may be chosen and/or matched by those skilled in the art taking into account the infection and/or disease state being treated and the target cells/tissue(s) that are to express the SOD1 shRNAs. In some embodiments, the route of administration is systemic. In some, embodiments the route of administration is intrathecal. In some, embodiments the route of administration is introcerebroventricular. In some, embodiments the route of administration is cisterna magna. In some, embodiments the route of administration is by lumbar puncture.

[0038] Transduction of cells with rAAV of the invention results in sustained expression of SOD1 shRNAs. In another aspect, the present invention thus provides methods of administering/delivering rAAV which express SOD1 shRNA to a subject, preferably a human being. The term "transduction" is used to refer to the administration/delivery of SOD1 shRNAs to a recipient cell either *in vivo* or *in vitro,* via a replication-deficient rAAV of the invention resulting in expression of a SOD1 shRNA by the recipient cell.

[0039] Thus, the invention provides methods of administering an effective dose (or doses, administered essentially simultaneously or doses given at intervals) of rAAV that encode SOD1 shRNAs to a subject in need thereof.

[0040] In one aspect, the invention provides methods of delivering a polynucleotide encoding an shRNA of the invention across the BBB comprising systemically administering a rAAV with a genome including the polynucleotide to a subject. In some embodiments, the rAAV genome is a self complementary genome. In other embodiments, the rAAV genome is a single-stranded genome. In some embodiments, the rAAV is a rAAV9. In some embodiments, the rAAV is a rAAV2. In some embodiments, the rAAV is a rAAVrh74.

[0041] In some embodiments, the methods systemically deliver polynucleotides across the BBB to the central and/or peripheral nervous system. Accordingly, a method is provided of delivering a polynucleotide to the central nervous system comprising systemically administering a rAAV with a self-complementary genome including the genome to a subject. In some embodiments, the polynucleotide is delivered to brain. In some embodiments, the polynucleotide is delivered to the spinal cord. Also provided is a method of delivering a polynucleotide to the peripheral nervous system comprising systemically administering a rAAV with a self-complementary genome including the polynucleotide to a subject is provided. In some embodiments, the polynucleotide is delivered to a lower motor neuron. In some

embodiments, the rAAV genome is a self complementary genome. In other embodiments, the rAAV genome is a single-stranded genome. In some embodiments, the rAAV is a rAAV9. In some embodiments, the rAAV is a rAAV2. In some embodiments, the rAAV is a rAAVrh74.

[0042] In another aspect, the invention provides methods of delivering a polynucleotide to the central nervous system of a subject in need thereof comprising intrathecal delivery of rAAV with a genome including the polynucleotide. In some embodiments, the rAAV genome is a self complementary genome. In other embodiments, the rAAV genome is a single-stranded genome. In some embodiments, the rAAV is a rAAV9. In some embodiments, the rAAV is a rAAV2. In some embodiments, the rAAV is a rAAVrh74. In some embodiments, a non-ionic, low-osmolar contrast agent is also delivered to the subject, for example, iobitridol, iohexol, iomeprol, iopamidol, iopentol, iopromide, ioversol or ioxilan.

[0043] Embodiments of the invention employ rAAV to deliver polynucleotides to nerve, glial cells and endothelial cells. In some embodiments, the nerve cell is a lower motor neuron and/or an upper motor neuron. In some embodiments, the glial cell is a microglial cell, an oligodendrocyte and/or an astrocyte. In other aspects the rAAV is used to deliver a polynucleotide to a Schwann cell.

Brief Description of the Drawings

[0044]

Figure 1. AAV9 transduction pattern and persistence in SOD1^{G93A} mice. SOD1^{G93A} mice were injected intravenously with AAV9-CB-GFP at P1, P21 and euthanized 21 days post injection (n=3 per time point). Spinal cords were examined for GFP, ChAT (motor neuron marker) and GFAP (astrocyte marker) expression. Temporal vein injection of AAV9-CB-GFP at P1 resulted in efficient transduction of motor neurons and glia in SOD1^{G93A} mice (**a,f,k,p**). Tail vein injection at P21 (**b**,**g**,**l**,**q**) predominantly targeted astrocytes with few GFP positive motor neurons. To test the persistence of transduced cells, AAV9-CB-GFP was intravenously injected at P1 and P21 in SOD1^{G93A} animals that were sacrificed at end stage (~P130). Immunofluorescence analysis of lumbar ventral horn (**c,d,h,i,m,n,r,s**) demonstrated that GFP expression was maintained in astrocytes throughout the disease course. To determine whether SOD1 mediated inflammation and damage would affect AAV9 transduction, we intravenously injected SOD1^{G93A} mice at P85 and harvested their spinal cords at endstage. There was no difference observed in the transduction pattern of SOD1^{G93A} mice treated at P21 or P85. Insets in (**r-t**) show co-localization between GFP and GFAP signal, (**u**) Quantification of transduced cells in ALS spinal cords (for each group tissues were analyzed from 3 animals). GFP and ChAT columns show numbers of cells counted. Bars = 100μm. AAV, adeno-associated virus; P1, postnatal day 1; P21, postnatal day 21; P85, postnatal day 85; GFP, green fluorescent protein; ChAT, choline acetyltransferase; GFAP, glial fibrillary acidic protein.

Figure 2. shRNA constructs show efficient reduction of human SOD1 protein *in vitro* and *in vivo*. (**a**) Sequence alignments between human and mouse SOD1 for the regions targeted by the 4 different shRNA constructs tested. (**b**) shRNA sequences were cloned into an H1 expression construct and transiently transfected into 293 cells. Lysates were collected 72 hours post transfection and analyzed by western blot. (**c**) Quantification of *in vitro* suppression of human SOD1 from three separate transient transfections showed >50% reduction in SOD1. (**d**) shRNA 130 was packaged into AAV9 and injected into SOD1^{G93A} mice at either P1 or P21. Spinal cords (*n*=3 per time point) were harvested three weeks post injection and analyzed by western blot for human SOD1 protein levels. (**e**) Quantification of *in vivo* suppression of human SOD1 within the spinal cord of ALS mice. P1 and P21 injected spinal cords showed 60% and 45% reductions in mutant SOD1 protein, respectively. hSOD1, human superoxide dismutase 1; mSOD1, mouse superoxide dismutase 1; GAPDH, glyceraldehyde 3 phosphate dehydrogenase.

Figure 3. Intravenous delivery of AAV9-SOD1-shRNA improves survival and motor performance in SOD1^{G93A} mice. SOD1^{G93A} mice received a single intravenous injection of AAV9-SODl-shRNA at P1 (n=6, green), P21 (n=9, red) or P85 (n=5, blue). Treated mice were monitored up to end stage and compared with non-injected control SOD1^{G93A} mice (n=15, gray). (**a,c**) AAV9-SOD1-shRNA injection into P1 SOD1^{G93A} mice significantly delayed median disease onset 39.5 days compared to control animals (**uninjected**, 103d; **P1**, 142.5d; $p<0.05$). Injection in P21 (red) or P85 (blue) ALS animals had no effect on disease onset (**P21**, 110d; P**85**, 105d). However, AAV9-SOD1-shRNA administered at P1, P21 or P85 all significantly extended median survival (**b**,**e**) (**uninjected,** 132d; **P1**, 183.5d **P21**, 171d; **P85**, 162d; all comparisons to control $p<0.001$). The P21 group had a significant extension in median disease duration (**d**) indicating a slowing of disease (**uninjected,** 29.5d; **P1,** 41d; **P21,** 49d; **P85,** 40d; Wilcoxon Signed Rank Test p= 0.06, 0.01 and 0.12, respectively). (**f-h**) P1 and P21 treated animals maintained their weights, had better hind limb grip strength and rotarod performance when compared to age-matched controls, indicating treated animals retained muscle tone and motor function during their prolonged survival. Lines between bars in (**c-e**) indicate statistically significant differences. * $p<0.05$. P1, postnatal day 1; P21, postnatal day 21; P85, postnatal day 85.

Figure 4. Intravenous injection of AAV9-SOD1-shRNA reduces mutant protein in spinal cords of SOD1^{G93A} mice. (**a-d**) Images of lumbar spinal cord sections from uninjected (**a**), P1 injected (**b**), P21 injected (**c**) and P85 injected (**d**) mice were captured with identical microscope settings to qualitatively show SOD1 levels at end stage. SOD1 levels inversely correlate with survival. (**e-t**) Co-labeling for GFP, ChAT and SOD1 shows that AAV9 transduced motor neurons had reduced SOD1 expression (arrows) while cells that lacked GFP maintained high levels of mutant protein (arrowheads). As described in **Figure 1u**, higher MN transduction and corresponding SOD1 reduction was observed in P1 injected mice (**i-l**) as compared to P21 injected (**m-p**) and P85 injected (**q-t**) mice. Bar = 100μm. P1, postnatal day 1; P21, postnatal day 21; P85, postnatal day 85; SOD1, superoxide dismutase 1; GFP, green fluorescent protein; ChAT, choline acetyltransferase.

Figure 5. AAV9-SOD1-shRNA improves survival and motor performance in SOD1^{G37R} mice treated after disease onset. (**a**) There was no difference in median disease onset between AAV9-SOD1-shRNA and control treated mice. (average age at treatment = 215d versus median onset of 194d control and 197d treated; Log Rank Test p= 0.46). (**b,f**) Median survival of AAV9-SOD1-shRNA treated SOD1^{G37R} mice (n=25) was significantly extended versus control mice (n=21). (**control**, n=21, 392d; **SOD1 shRNA**, *n*=25, 478.5d; Log Rank Test p<0.0001) (**c-e**) The early phase of disease was significantly slowed by 73 days in treated mice as compared to control mice (**control**, 89d; **SOD1 shRNA**, 162d; p<0.0001 Wilcoxon Signed Rank Test) while the late phase of disease showed a non-significant slowing (**control**, 63d; **SOD1 shRNA**, 81d; p=0.14 Wilcoxon Signed Rank Test). Together this amounted to a 66 day increase in median disease duration (**control**, 173d; **SOD1 shRNA**, 239d; p<0.0001 Wilcoxon Signed Rank Test). (**g**) A trend to improved hind limb grip strength appeared in AAV9-SOD1-shRNA treated mice compared to control mice.

Figure 6. Intravenous injection of AAV9 in adult SOD1^{G37R} mice targets astrocytes and motor neurons within the spinal cord. (**a-h**) Immunofluorescence analysis revealed neuronal as well as glial transduction in both AAV9-CB-GFP (**a-d**) and AAV9-SOD1-shRNA treated (**eh**) mice. (**i-p**) Human SOD1 levels appeared reduced in AAV9-SOD1-shRNA treated mice (**o**) compared with AAV9-GFP treated mice (**k**). Bar = 100 µm. GFP, green fluorescent protein; ChAT, choline acetyltransferase; GFAP, glial fibrillary acidic protein; SOD1, superoxide dismutase 1.

Figure 7. Intrathecal infusion of AAV9-SOD1-shRNA in non-human primates leads to efficient reduction in SOD1 levels. (**a**) A myelogram shortly after intrathecal infusion of AAV9-SOD1-shRNA mixed with contrast shows proper delivery into the subarachnoid space of a *cynomolgus macaque.* Arrows show diffusion of the contrast agent along the entire spinal cord. (**b**) Lumbar spinal cord sections from treated monkeys (*n*=3) were harvested two weeks post injection and stained for GFP using DAB staining. Sections had widespread GFP expression throughout the grey and white matter, (**c-e**) Immunofluorescence analysis of the lumbar spinal cord sections showed robust GFP (**c**) expression within ChAT (**d**) positive cells indicating motor neuron transduction (**e**, merge). (**f**) Western blot analysis of the lumbar spinal cords showed significant reduction in SOD1 levels in AAV9-SOD1-shRNA injected animals as compared to controls. (**g**) *In vivo* quantification of SOD1 knockdown in monkey lumbar spinal cord homogenate (*n*=3) showed an 87% reduction in animals that received AAV9-SOD1-shRNA compared to uninjected controls. (**h**) Laser capture microdissection was used to collect motor neurons or surrounding neuropil from injected and control lumbar monkey sections. Collected cells were analyzed for SOD1 levels by qRT-PCR. Motor neurons collected from AAV9-SOD1-shRNA animals (*n*=3) had a 95 ± 3% reduction in SOD1 RNA. Non-neurons had a 66 ± 9% reduction in SOD1 RNA in AAV9-SOD1-shRNA treated animals. Scale Bars: b = 100µm; e = 50 µm. SOD1: Superoxide dismutase 1.

Figure 8. Lumbar intrathecal infusion of AAV9-SOD1-shRNA leads to efficient transduction of motor neurons and non-neuronal cells in the cervical, thoracic and lumbar cord resulting in reduction of SOD1. (**a-c**) Immunofluorescence analysis of the three segments of the spinal cord; cervical **(a)**, thoracic **(b)** and lumbar **(c)**, showed robust GFP (green) expression within

Chat (red) positive cells indicating motor neuron transduction. (**d**) GFP+/Chat+ cell counts show a caudal to rostral gradient of motor neuron transduction ranging from 85% of transduced cells in the lumbar region to more than 50% in the cervical region. (**e**) SOD1 mRNA levels in cervical, thoracic and lumbar cord section homogenates analyzed by qRT-PCR show significant reduction in SOD1 transcript, consistently with motor neuron transduction. SOD1 levels were normalized to β-actin and AAV9-SOD1-shRNA injected animals were compared to an AAV9-CB-GFP injected control. Scale bars: (**a-c**) = 50μm; Error bars: (**d-e**) = SD.

Figure 9. Design of a clinical SOD1 shRNA construct. (a) Original AAV SOD1 shRNA construct contains shRNA sequence against human SOD1 under H1 promoter followed by the expression cassette for GFP which includes CMV enhancer, CBA promoter, modified SV40 intron, and GFP transgene sequence followed by bGH PolyA terminator. SOD1 shRNA expression cassette and GFP expression cassette are flanked by AAV2 ITRs which ensures the packaging of the complete flanked sequence in AAV9 capsid. (b) In clinical SOD1 shRNA construct, the GFP expression cassette is replaced by a stuffer element that contains tandem, noncoding sequences from FDA approved DNA vaccines. ITR: inverted terminal repeats; shRNA, small hairpin RNA; SOD1, superoxide dismutase 1; CMV, cytomegalo virus enhancer; CBA, Chicken β-actin promoter; GFP, green fluorescent protein; bGH pA, bovine growth hormone poly A terminator.

Figure 10. Schematic of clinical SOD1 shRNA construct. Different restriction sites are placed in the clinical SOD1 shRNA construct that allow the cloning of multiple shRNA expression cassettes while maintaining the total distance between the two ITRs.

Figure 11. *In vitro* transfection of clinical SOD1 shRNA construct efficiently reduces human SOD1 protein in HEK293 cells. Representative microscopic fields showing bright-field images of non-transfected control (a), AAV SOD1 shRNA transfected (b) and shuttle vector pJet SOD1 shRNA transfected (c,d) HEK 293 cells, 72 hrs post transfection. Corresponding fluorescence images reveal the lack of GFP fluorescence from pJet SOD1 shRNA transfected HEK 293 cells (g,h) as compared to AAV SOD1 shRNA transfected cells (f). (i) Western blot analysis of the cell lysates confirms the efficient knockdown of human SOD1 protein in pJet SOD1 shRNA transfected cells as compared to the non-transfected control cells. Immunoblot analysis also confirms removal of GFP transgene from pJet SOD1 shRNA construct. (j) Quantification of the in vitro downregulation of SOD1 by pJet SOD1 shRNA. pJet SOD1 shRNA reduces the protein levels of human SOD1 by almost 50% in HEK293 cells as compared to control. This reduction is similar to that achieved with AAV SOD1 shRNA construct.

Figure 12. Schematic of cloning strategy for clinical AAV SOD1 shRNA vector. Clinical SOD1 shRNA construct was cloned into AAV CB MCS vector using Kpn1/SPh1 sites. Kpn1/SPh1 double digest of AAV CB MCS plasmid results in the release of the complete transgene expression cassette from this vector which is further replaced with clinical SOD1 shRNA construct carrying SOD1 shRNA expression cassette and stuffer sequence.

Figure 13. Clinical AAV SOD1 shRNA efficiently reduces human SOD1 levels *in vitro.* HEK293 cells were transfected with clinical AAV SOD1 shRNA plasmid by Calcium phosphate method. Representative microscopic fields showing brightfield images of non-transfected control, AAV

SOD1 shRNA and Clinical AAV SOD1 shRNA transfected cells respectively, 72hrs post-transfection (a-c). Successful removal of GFP from clinical AAV SOD1 shRNA was confirmed by lack of GFP expression in Clinical AAV SOD1 shRNA transfected cells (f,g). (g) Western blot analysis of cell lysates, harvested 72hrs post-tranfection confirmed efficient downregulation of SOD1 in clinical AAV SOD1 shRNA transfected cells as compared to control. AAV SOD1 shRNA was used as a positive control. (h) Quantification of the in vitro knockdown of SOD1 by clinical AAV SOD1 shRNA.

Figure S1. AAV9-shRNA-SOD1 administration is well tolerated in WT mice. Female and male WT animals were injected with AAV9-SOD1-shRNA at P1 or P21 and monitored up to 6 months of age. (**a**,**b**) Both male and female treated mice showed steady increase in body mass as compared to control animals. (**c,d**) Rotarod performance and (**e**,**f**) hind limb grip strength were not affected by P1 or P21 treatment in both groups as compared to respective controls. n = 5 per group. WT, wild type; P1, postnatal day 1; P21, postnatal day 21.

Figure S2. Hematology and Serum Chemistry of AAV9-SODI-shRNA treated WT animals. (**a-m**) Blood was collected from P1 (green) or P21 (red) treated and control (gray) WT animals at 150 days of age for hematology studies. No significant differences were observed between treated and control animals, (**n-w**) Serum samples collected at 180 days of age from the same mice showed no significant differences in serum chemistry profile. Mean ± SEM. n = 5 per group. P1, postnatal day 1; P21, postnatal day 21.

Figure S3. AAV9-SODI-shRNA treatment in $SOD1^{G93A}$ mice reduces astrogliosis. End stage sections from control and AAV9-SOD1-shRNA treated animals were harvested and stained for GFAP, an astrocyte activation marker. P1 (**b**) and P85 (**d**) injected mice showed reduced levels of astrogliosis as compared to control (**a**) mice while P21(**c**) injected mice showed the maximum reduction. This was consistent with the percent astrocyte transduction achieved in these mice (**Figure 1u**). However, no effect was observed on microglia reactivity (**e-h**). Bar = 100 µm. P1, postnatal day 1; P21, postnatal day 21; P85, postnatal day 85.

Figure S4. Intravenous injection of AAV9-SOD1-shRNA efficiently reduces levels of mutant SOD1 protein in spinal cords of $SOD1^{G37R}$ mice. (**a**) Following disease onset, AAV9-CB-GFP or AAV9-SOD1-shRNA was injected in $SOD1^{G37R}$ mice and spinal cords were harvested at end stage and analyzed by western blot for human SOD1 protein levels. (**b**) Quantification of a) shows suppression of human SOD1 within the spinal cord of $SOD1^{G37R}$ mice (*n*=4 per group). hSOD1, human superoxide dismutase 1; GAPDH, glyceraldehyde 3 phosphate dehydrogenase.

Figure S5. shRNA 130 efficiently reduces the levels of monkey SOD1 *in vitro.* (**a**) Sequence alignment of the region targeted by SOD1 shRNA 130 and a single mismatch with the monkey sequence. Monkey sequence corresponds to SOD1 sequence from Rhesus monkey (NM 001032804.1), Cynomolgus monkey (sequenced in-house) and African green monkey. (**b**) The shRNA 130 expression cassette was cloned into lentiviral vector and used to infect Cos-7 cells. Lysates were analyzed 72 hours post infection by qRT PCR for SOD1. shRNA 130 reduced SOD1 transcript levels by 75% in Cos-7 cells.

Examples

[0045] The present invention is illustrated by the following examples. While the present invention has been described in terms of various embodiments and examples, it is understood that variations and improvements will occur to those skilled in the art. Therefore, only such limitations as appear in the claims should be placed on the invention.

Example 1

AAV9 transduction pattern and persistence in SOD1^{G93A} mice

[0046] We first evaluated the efficiency of AAV9 transduction in the SOD1^{G93A} mouse model that develops fatal paralytic disease. High copy SOD1^{G93A} mice were obtained from Jackson Laboratories (Bar Harbor, ME) and bred within the Kaspar lab. Animals were genotyped before the treatment to obtain SOD1^{G93A} expressing mice and their wild type littermates. Only female mice were included in the SOD1^{G93A} experiments. Animals were injected intravenously at postnatal day 1 or day 21 (to be referred to as P1 and P21, respectively) with self-complementary AAV9 expressing GFP from the CMV enhancer/beta-actin (CB) promoter (AAV9-CB-GFP) (*n*=3 per group). Three weeks post-injection, animals were sacrificed, and spinal cords examined for GFP expression (Figs 1a-u).

[0047] All procedures with animals described herein were performed in accordance with the NIH Guidelines and approved by the Research Institute at Nationwide Children's Hospital (Columbus, OH), University of California (San Diego, CA) or Mannheimer Foundation (Homestead, FL) Institutional Animal Care and Use Committees.

[0048] Transduction efficiency was high in SOD1^{G93A} astrocytes with GFP expressed in 34 ± 2% and 54 ± 3%, respectively, of P1 and P21 injected spinal grey matter astrocytes (defined by immunoreactivity for GFAP). This efficiency was similar to our previous report of 64 ± 1% in P21 injected wild type animals[18]. Motor neurons were a prominent cell type transduced at all levels of the spinal cords of P1 injected SOD1^{G93A} animals (62 ± 1%), compared with significantly lower targeting to motor neurons in P21 injected animals (8 ± 1%).

[0049] Although we have previously reported that transduced astrocytes in wild type spinal cords persist with continued GFP accumulation for at least 7 weeks post injection[18], longevity

of mutant SOD1 astrocytes (and their continued synthesis of genes encoded by the AAV9 episome) during active ALS-like disease was untested. Therefore, $SOD1^{G93A}$ mice were injected at P1 and P21 with AAV9-CB-GFP and followed to end-stage (~P130, *n*=3 per group) (Figs 1c,d,h,i,m,n,r,s). Immunofluorescent examination of the end-stage $SOD1^{G93A}$ spinal cords from animals injected at P1 and P21 showed a comparable number of GFP-expressing astrocytes as were found 21 days after AAV9 injection (P1: 42 ± 2%, P21: 61 ± 2%). These data are consistent with survival of transduced astrocytes for the duration of disease (~110 days post injection at P21) in $SOD1^{G93A}$ mice and that AAV9-encoded gene expression is maintained.

[0050] Further, recognizing that SOD1 mutant mediated damage, including astrocytic and microglial activation and early changes in the blood brain barrier develop during disease in mice in SOD1 mutant mice[20], we tested if this damage affected AAV9 transduction. $SOD1^{G93A}$ mice were injected at P85 with AAV9-CB-GFP and sacrificed at endstage (*n*=3) (Figs 1e,j,o,t). Analysis of the spinal cords revealed that the transduction pattern seen in P85 animals was similar to P21 treated animals with astrocytes as the predominant cell type transduced at all levels (51 ± 6% GFP+/GFAP+ cells in lumbar grey matter).

Example 2

Development of an shRNA sequence specific for human SOD1

[0051] To specifically target the human SOD1 mRNA, four shRNA constructs targeting human SOD1 were generated and obtained from the Life Technologies design tool. The constructs that had a minimum of four base mismatches compared to the mouse mRNA sequence (Fig. 2a). The base numbers for the human sequences shown correspond to record number CCDS33536.1 in the NCBI CCDS database. These constructs were cloned in pSilencer 3.1 (Genscript) under the human H1 promoter and tested *in vitro.* shRNA 130 along with H1 promoter was further cloned into an AAV vector along with a reporter GFP under Chicken Beta-Actin promoter to identify the transduced cells. Human 293 cells were transfected with each cassette. The HEK-293 cells were maintained in IMDM medium containing 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin. Upon reaching ~60% confluence, cells were transfected with pSilencer 3.1 containing the shRNAs being tested. Protein lysates were prepared 72 hours post transfection and analyzed for SOD1 levels by western blot. All four sequences reduced SOD1 protein levels by >50% (Figs 2b,c).
shRNA130 was selected for further experiments because it produced the most consistent knockdown across three separate transfection experiments. It was cloned into a self-complementary AAV9 vector that also contained a GFP gene whose expression would identify transduced cells (referred to as AAV9-SOD1-shRNA). Self-complementary AAV9-SOD1-shRNA was produced by transient transfection procedures using a double-stranded AAV2-ITR-based

CB-GFP vector, with a plasmid encoding Rep2Cap9 sequence as previously described along with an adenoviral helper plasmid pHelper (Stratagene, Santa Clara, CA) in 293 cells[18].

[0052] To confirm that the shRNA could suppress accumulation of human SOD1, SOD1^{G93A} mice (*n*=3) were injected intravenously with AAV9-SOD1-shRNA at either P1 or P21. For neonatal mouse injections, postnatal day 1-2 SOD1^{G93A} pups were utilized. Total volume of 50µl containing 5×10^{11} DNAse resistant viral particles of AAV9-SOD1-shRNA (Virapur LLC, San Diego, CA) was injected via temporal vein as previously described[18]. A correct injection was verified by noting blanching of the vein. After the injection, pups were returned to their cage. Animals were euthanized three weeks post injection and the spinal cords were harvested and analyzed by immunoblotting for both human (mutant) and murine (wild-type) SOD1 protein. P1 and P21 injected spinal cords showed 60% and 45% reductions in mutant SOD1 protein, respectively (Figs 2d,e). Murine SOD1 levels remained unchanged in response to human SOD1 knockdown.

Example 3

AAV9-SOD1-shRNA is safe and well tolerated in wild type mice

[0053] To determine whether high dose AAV9-SOD1-shRNA would be safe, normal mice of both sexes were intravenously injected at P1 or P21 (P1 = 5 males, 5 females at 5×10^{11}vg; P21 = 5 males, 5 females at 2×10^{12} vector genomes (vg)) and then monitored up to 6 months of age. Both P1 and P21 injected mice showed a steady increase in body mass similar to untreated mice (Figure S1), Weekly behavioral tests observed no significant differences between injected and control groups in motor skills (measured by rotarod) as well as in hind limb grip strength. At 150 and 180 days of age, blood samples were collected. Complete and differential blood counts of both treated and untreated groups showed similar blood chemistry parameters (Figure S2). Serum samples from both groups showed no significant differences in the levels of alkaline phosphatase, creatinine, blood urea nitrogen, potassium, sodium and chloride. Finally, all the animals were sacrificed at the age of 180 days. Histopathological analyses by a pathologist blinded to treatment group revealed no significant alterations in the AAV9-SOD1-shRNA treated animals compared to uninjected controls (data not shown). We conclude that both administration of AAV9 and sustained shRNA expression were apparently safe and well tolerated.

Example 4

Extended survival of SOD1 ^{G93A} mice from

AAV9 mediated reduction in mutant SOD1 even when initiated mid-disease

[0054] To test the efficacy of AAV9-mediated SOD1 reduction, we treated cohorts of $SOD1^{G93A}$ mice with a single intravenous injection of AAV9-SOD1-shRNA before (P1, 5×10^{11}vg, *n*=6 and P21, 2×10^{12}vg, *n*=9) or after (P85, 3×10^{12}vg, *n*=5) onset, recognizing that many astrocytes, but few motor neurons, would be transduced at the two later time points. For adult tail vein injections, animals were placed in a restraint that positioned the mouse tail in a lighted, heated groove. The tail was swabbed with alcohol then injected intravenously with AAV9-SOD1-shRNA.

[0055] Onset of disease (measured by weight loss from denervation-induced muscle atrophy) was significantly delayed by a median of 39.5 days (Fig. 3a,c; uninjected, 103d; P1, 142.5d; *p<0.05*, Wilcoxon Signed Rank Test) in the P1 injected cohort, but was not affected by either of the later injections (P21, 110d; P85, 105d). P1 and P21 treated animals maintained their weights, had better rotarod performance and hind limb grip strength when compared to age-matched controls, indicating treated animals maintained muscle tone and motor function during their prolonged survival (Figs 3f-h). Survival was significantly extended by AAV9 injection at all three ages, yielding survival times 30-51.5 days beyond that of uninjected $SOD1^{G93A}$ mice (uninjected, 132d; P1, 183.5d; P21, 171d; P85, 162d; Log-Rank Test p= <0.0001, 0.0003 and 0.001, respectively) (Figs 3b,e). Defining disease duration as the time from onset to end-stage revealed that the P21 treatment group had significantly increased duration, indicative of slowed disease progression, compared to uninjected controls (uninjected, 29.5d; P21, 49d; Wilcoxon Signed Rank Test p = 0.01), with trends toward slowed progression in animals injected at the other two ages (P1, 41d; P85, 40d; p = 0.06 and 0.12, respectively) (Fig. 3d). The lower percentage of targeted non-neuronal cells at P1 versus those targeted at P21 (Figure lu) suggests that a minimum number of non-neuronal cells must be targeted to slow disease progression in the fast progressive $SOD1^{G93A}$ model (Fig. 1u).

Example 5

Reduction of mutant SOD1 in AAV9 infected cells in treated $SOD1^{G93A}$ mice

[0056] Indirect immunofluorescence with an antibody that recognizes human, but not mouse SOD1, was used to determine accumulated mutant SOD1 levels in end-stage spinal cords of treated and control mice. Human SOD1 levels in end-stage spinal cord sections inversely correlated with increased survival (Figs. 4a-d). At end-stage, P1 (Fig. 4b), P21 (Fig. 4c) and P85 (Fig. 4d) AAV9-SOD1-shRNA injected animals had lower levels of mutant SOD1 when

compared with uninjected SOD1^{G93A} animals (Fig. 4a). SOD1 expression within transduced motor neurons (identified by GFP and ChAT expressing cells) was reduced compared to surrounding neurons that had not been transduced to express viral encoded GFP (Figs 4h,l,p,t; arrows versus arrowheads). Moreover, immunofluorescence imaging of end-stage spinal cords revealed corresponding reduction in astrogliosis, but no difference in microgliosis in AAV9-SOD1-shRNA treated animals versus controls (Figure S3).

Example 6

Therapeutic slowing of disease progression with peripheral injection of AAV9 after onset

[0057] To determine if AAV9-mediated mutant SOD1 reduction would slow disease progression, a cohort of SOD1^{G37R} mice[6] were injected intravenously with AAV9-SOD1-shRNA after disease onset (average age at treatment = 215d versus median onset of 197d in treated animals; Log Rank Test p= 0.46; Fig. 5a). *lox*SOD1^{G37R} ALS mice, carrying a human mutant SOD1^{G37R} transgene flanked by lox p sites under its endogenous promoter, were maintained in as previously described[37]. A combination of AAV9-CB-GFP (n=9) and uninjected (n=12) littermates were used as controls.

[0058] Post hoc analysis showed no differences between GFP and uninjected animals, therefore the groups were compiled as "control" in Fig. 5. Animals were evaluated weekly for body weight and hind limb grip strength and monitored until end-stage. AAV9-SOD1-shRNA treatment after disease onset significantly extended median survival by 86.5 days over control animals (control, n=21, 392d; SOD1 shRNA, *n*=25, 478.5d; Log Rank Test p<0.0001). Early disease duration, defined by the time from peak weight to 10% weight loss, was significantly slowed (control, 89d; SOD1 shRNA treated mice, 162 days; Wilcoxon Signed Rank Test p<0.01; Fig. 5c). A continuing trend toward slowing of later disease (10% weight loss to end stage) was also seen (control, 63d; SOD1 shRNA treated mice, 81d; Wilcoxon Signed Rank Test p=0.1389; Fig. 5d). Overall disease duration following AAV9-SOD1-shRNA therapy rose to 239d after disease onset versus 173d in control mice (Wilcoxon Signed Rank Test p<0.0001; Fig. 5e). Consistent with the slowed progression, AAV9 therapy maintained grip strength relative to control SOD1 mutant animals (Fig. 5g). The 86.5 day extension in survival surpassed the 62 day extension seen in transgenic studies that used astrocyte-specific Cre expression to inactivate the mutant SOD1^{G37R} transgene[8], presumably reflecting efficient AAV9 transduction of astrocytes after peripheral delivery and the possible transduction of other cell types (especially microglia[6]) whose synthesis of mutant SOD1 accelerates disease progression.

[0059] Histological examination of end-stage $SOD1^{G37R}$ treated animals revealed similar levels of intraspinal cell transduction in animals treated with AAV9-SOD1-shRNA or AAV9-GFP (Figure 6). GFP expression was predominantly observed within motor neurons and astrocytes of both groups, and SOD1 expression was detectably decreased only in animals that received AAV9-SOD1-shRNA (Figs 6k,o). Immunoblotting of whole spinal cord extracts from end stage $SOD1^{G37R}$ mice revealed an 80% reduction in hSOD1 protein levels in AAV9-SOD1-shRNA treated animals compared to controls (Figure S4).

Example 7

AAV9 mediated suppression of SOD1 in non-human primates

[0060] To test whether SOD1 levels could be efficiently lowered using AAV9 in the non-human primate spinal cord, AAV9 was injected intrathecally via lumbar puncture. This method was chosen over systemic delivery to decrease the amount of virus required and to minimize any effects from reduction of SOD1 in peripheral tissues. One year old cynomolgus macaques (*Macaca fascicularis*) with average body weight of 2 kg were used for this study at the Mannheimer Foundation. Regular monitoring of overall health and body weight was performed prior and after the injections to assess the welfare of the animals.

[0061] Sequencing of cDNA copied from mRNA isolated from African Green Monkey (COS cells) and the *Cynomolgus macaque* verified that the 130 shRNA had a single base mismatch to either sequence (Figure S5). The 130 shRNA expression cassette was inserted into a lentiviral vector which was then used to transduce COS cells. Cos-7 cells were maintained in DMEM with 10% FBS and 1% penicillin/streptomycin. Cells were infected with a lentiviral vector expressing SOD1 shRNA 130 under the H1 promoter and RFP under CMV promoter. RNA was extracted from infected and non-infected cells 72 hours post infection using an RNAeasy Kit (Qiagen). cDNA was prepared using RT^2 First strand synthesis kit (SABiosciences). SOD1 transcript levels were analyzed by qRT-PCR which revealed that the monkey SOD1 mRNA was reduced by ~75% in 130 shRNA transduced cells compared to mock transduced control cells (Figure S5).

[0062] The AAV9-SOD1-shRNA virus (1×10^{13} vg/kg) was infused along with contrast agent via lumbar puncture into the subarachnoid space of three male cynomolgus macaques and one control subject was injected with AAV9-CB-GFP (1×10^{13} vg/kg) (Fig. 7a). Each intrathecal injection was performed by lumbar puncture into the subarachnoid space of the lumbar thecal sac. AAV9 was resuspended with omnipaque (iohexol), an iodinated compound routinely used in the clinical setting. Iohexol is used to validate successful subarachnoid space cannulation and was administered at a dose of 100 mg/Kg. The subject was placed in the lateral decubitus position and the posterior midline injection site at ~L4/5 level identified (below the conus of the

spinal cord). Under sterile conditions, a spinal needle with stylet was inserted and subarachnoid cannulation was confirmed with the flow of clear CSF from the needle. In order to decrease the pressure in the subarachnoid space, 0.8ml of CSF was drained, immediately followed by injection with a mixture containing 0.7 mL iohexol (300 mg/ml formulation) mixed with 2.1mL of virus (2.8ml total).

[0063] No side effects from the treatments were identified. Two weeks post injection, the spinal cords were harvested for analysis of GFP expression and SOD1 RNA levels. GFP expression was seen broadly in neuronal and astrocytic cells throughout the grey and white matter of the lumbar spinal cord, the area closest to the site of injection (Figs 7b-e). Immunoblotting of extracts of lumbar spinal cord revealed 87% reduction in monkey SOD1 protein levels (Figs 7f,g). Laser capture microdissection was then used to isolate total RNA from motor neurons as well as from glia in the nearby neuropil. Analysis by quantitative RT-PCR using primers specific for monkey SOD1 (and normalized to actin) confirmed a 95 ± 3% knockdown in the motor neuron pool and a 66 ± 9% knockdown in the neuropil pool when compared to samples from a control animal (Fig 7h).

[0064] Next we examined the level of cell transduction throughout the spinal cord including cervical, thoracic and lumbar segments. GFP was found to be expressed broadly within all sections analyzed (Figs 8a-c). Motor neuron counts revealed a caudal to rostral gradient in cell transduction, with the cervical region showing more than 50% of GFP/Chat+ motor neurons, increasing to 65% in the thoracic region and reaching a remarkable 80% in the lumbar region (Fig 8d). In order to determine the overall level of SOD1 knockdown achieved with this transduction pattern, qRT-PCR for SOD1 was performed on whole section homogenates from cervical, thoracic and lumbar cord segments. The results confirmed robust SOD1 reduction at all three spinal cord levels, ranging from a 60% decrease in the cervical segment, a 70% decrease in the thoracic region and an 88% decrease in the lumbar region (Fig 8e), consistent with the proportion of cells transduced in each region.

Discussion

[0065] The examples above show that intravenous administration of AAV9-SOD1-shRNA is safe and well tolerated in wild type mice, with the absence of adverse effects after long-term assessment. Thiss approach have achieved one of the longest extensions in survival ever reported in the rapidly progressive $SOD1^{G93A}$ mouse model of ALS (increasing survival by 39% when treatment is initiated at birth). Even more encouraging, markedly slowed disease progression is seen even when AAV9 therapy to reduce mutant SOD1 synthesis is applied after disease onset in $SOD1^{G37R}$ mice, thereby significantly extending survival. Thus, the vascular delivery paradigm in mice represents a proof of concept that mutant SOD1 knockdown after disease onset can be beneficial in both rapid and more slowly progressive models of ALS at clinically relevant points in disease. Together, these data show that robust targeting and suppression of SOD1 levels via AAV9-mediated delivery of shRNA is effective in slowing

disease progression in mouse models of ALS, critically even when treatment is initiated after onset.

[0066] Multiple recent studies have brought forward the hypothesis that wild-type SOD1 may contribute through misfolding to the pathogenic mechanism(s) that underlie sporadic ALS through a pathway similar to that triggered by mutant SOD1[14, 30-32]. Included in this body of evidence is our own demonstration that astrocytes produced from sporadic ALS patients are toxic to co-cultured motor neurons and that toxicity is alleviated by siRNA-mediated reduction in wild type SOD1[30]. This evidence creates the potential that a proportion of sporadic ALS patients could also benefit from an AAV9-mediated SOD1 reduction approach that we have demonstrated to be effective in slowing disease progression in mice that develop fatal, ALS-like disease from expressing ALS-causing mutations in SOD1.

[0067] Finally, for translation of an AAV9-mediated suppression of SOD1 synthesis to the human setting, we have determined that infusion directly into the CSF at the lumbar level in a non-human primate produce substantial SOD1 reduction by targeting both motor neurons and non-neuronal cells. This outcome provides strong support for extending these efforts to an adult human by direct injection into CSF, as previously proposed[33, 34], so as to 1) limit the cost of viral production, 2) reduce the possibility that chronic suppression of SOD1 in the periphery may have deleterious consequences, and 3) reduce viral exposure to the peripheral immune system[33]. These data strongly indicate AAV9-SOD1-shRNA as a treatment for ALS.

Techiques/Methods Used in Examples 1-7

[0068] ***Perfusion and Tissue Processing.*** Control and treated SOD1^{G93A} mice were sacrificed at either 21 days post injection or at endstage for immunohistochemical analysis. Animals were anesthetized with xylazene/ketamine cocktail, transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde. Spinal cords were harvested, cut into blocks of tissue 5-6mm in length, and then cut into 40µm thick transverse sections on a vibratome (Leica, Bannockburn, IL). Serial sections were kept in a 96-well plate that contained 4% paraformaldehyde and were stored at 4°C. End stage *lox*SOD1^{G37R} mice were anesthetized using isoflurane and perfused with 4% paraformaldehyde. Spinal cord segments, including cervical, thoracic and lumbar segments were dissected. Following cryoprotection with 20% sucrose/4% paraformaldehyde overnight, spinal cords were frozen in isopentane at -65°C, and serial 30µm coronal sections were collected free floating using sliding microtome.

[0069] For safety studies, P1, P21 treated and control wild type mice were sacrificed at 180 days of age. Animals were anesthetized using xylazene/ketamine cocktail and perfused with 0.9% saline. Different tissues were removed and stored in 10% buffered formalin. These tissues were further processed, blocked and mounted for hematoxilin & eosin staining by the Nationwide Children's Hospital Morphology Core.

[0070] Cynomolgus monkeys injected with virus were euthanized 2 weeks post injection. Animals were anesthetized with sodium pentobarbital at the dose of 80 - 100 mg/kg intravenously and perfused with saline solution. Brain and spinal cord dissection were performed immediately and tissues were processed either for nucleic acid isolation (snap frozen) or post-fixed in 4% paraformaldehyde and subsequently cryoprotected with 30% sucrose and frozen in isopentane at -65°C. 12µm coronal sections were collected from lumbar cord using a cryostat for free floating immunostaining.

[0071] ***Immunohistochemistry.*** Mouse spinal cords were stained as floating sections. Tissues were washed three-times for 10 minutes each in TBS, then blocked in a solution containing 10% donkey serum, 1% Triton X-100 and 1% penicillin/streptomycin for two hours at room temperature. All the antibodies were diluted with the blocking solution. Primary antibodies used were as follows: rabbit anti-GFP (1:400, Invitrogen, Carlsbad, CA), rabbit anti-SOD1 (1:200, Cell signaling, Danvers, MA), goat anti-ChAT (1:50 Millipore, Billerica, MA), mouse anti-GFAP (1:200, Millipore, Billerica, MA), chicken anti GFAP (1:400, Abeam, Cambridge, MA), and rabbit anti-Iba1 (1:400, Wako, Richmond VA). Tissues were incubated in primary antibody at 4°C for 48-72 hours then washed three times with TBS. After washing, tissues were incubated for 2 hours at room temperature in the appropriate FITC-, Cy3-, or Cy5-conjugated secondary antibodies (1:200 Jackson Immunoresearch, Westgrove, PA) and DAPI (1:1000, Invitrogen, Carlsbad, CA). Tissues were then washed three times with TBS, mounted onto slides then coverslipped with PVA-DABCO. All images were captured on a Zeiss-laser-scanning confocal microscope.

[0072] For DAB staining, monkey spinal cord sections were washed three times in TBS, blocked for 2 h at RT in 10% donkey serum and 1% Triton X-100. Sections were then incubated overnight at 4°C with rabbit anti-GFP primary antibody (1:1000 Invitrogen, Carlsbad, CA) diluted in blocking buffer. The following day, tissues were washed with TBS 3 times, incubated with biotinylated secondary antibody anti-rabbit (1:200 Jackson Immunoresearch, Westgrove, PA) in blocking buffer for 30 min at RT, washed 3 times in TBS and incubated for 30 min at RT with ABC (Vector, Burlingame, CA). Sections were then washed for 3 times in TBS and incubated for 2 min with DAB solution at RT and washed with distilled water. These were then mounted onto slides and covered with coverslips in mounting medium. All images were captured with the Zeiss Axioscope.

[0073] ***Motor neuron and astrocyte quantification.*** For MN quantification, serial 40 µm thick lumbar spinal cord sections, each separated by 480 µm, were labeled as described for GFP and ChAT expression. Stained sections were serially mounted on slides from rostral to caudal, then coverslipped. Sections were evaluated using confocal microscopy (Zeiss) with a 40× objective and simultaneous FITC and Cy3 filters. The total number of ChAT positive cells found in the ventral horns with defined soma was tallied by careful examination through the entire z-extent of the section. GFP labeled cells were quantified in the same manner, while checking for co-localization with ChAT. For astrocyte quantification, as with MNs, serial sections were stained for GFP, GFAP and then mounted. Using confocal microscopy with a 63× objective and simultaneous FITC and Cy5 filters, random fields in the ventral horns of lumbar spinal cord

sections from tail vein injected animals were selected. The total numbers of GFP and GFAP positive cells were counted from a minimum of at least 24-fields per animal while focusing through the entire z extent of the section. Spinal cord sections of 3 animals per group were examined for MN and astrocyte quantification.

[0074] ***Immunoblot*** *analysis.* Spinal cords were harvested from P1, P21 injected and control $SOD1^{G93A}$ mice 21 days post injection and from treated and control monkeys 2 weeks post injection of AAV9-SOD1-shRNA. Spinal cords were homogenized and protein lysates were prepared using T-Per (Pierce) with protease inhibitor cocktail. Samples were resolved on SDS-PAGE according to manufacturer's instructions. Primary antibodies used were rabbit anti-SOD1 (1:750, Cell signaling, Danvers, MA) mouse anti-SOD1 (1:750, Millipore, Billerica, MA), rabbit anti-SOD1 (1:1000, Abcam, Cambridge, MA), rabbit anti-Actin (1:1000, Abcam, Cambridge, MA) and mouse anti-GAPDH (1:1000, Millipore, Billerica, MA). Secondary antibodies used were anti- rabbit HRP (1:10000-1:50000) and anti- mouse HRP (1:10000). Densitometric analysis was performed using Image J software.

[0075] ***Laser Capture Microdissection.*** 12 □ m lumbar spinal cord frozen sections were collected onto PEN membrane slides (Zeiss, Munich, Germany) and stained with 1% Cresyl violet (Sigma, St. Louis, MO) in methanol. Sections were air dried and stored at -80°C. After thawing, motor neurons were collected within 30 min from staining using the laser capture microdissector PALM Robo3 Zeiss) using the following settings: Cut energy: 48, LPC energy: 20, Cut focus: 80/81, LPC focus: 1, Position speed: 100, Cut speed: 50. About 500 MNs were collected per animal. Non-neuronal cells from the ventral horn were collected from the same sections after collecting the motor neurons.

[0076] ***qRT-PCR.*** RNA from laser captured cells or whole spinal cord sections from the cervical, thoracic and lumbar segments was isolated using the RNaqueous Micro Kit (Ambion, Grand Island, NY) according to manufacturer's instructions. RNA was then reverse-transcribed into cDNA using the RT^2 HT First Strand Kit (SABiosciences, Valencia, CA). 12.5 ng RNA were used in each Q-PCR reaction using SyBR Green (Invitrogen, Carlsbad, CA) to establish the relative quantity of endogenous monkey *SOD1* transcript in animals who had received the AAV9-SOD1-shRNA compared to animals who had received only AAV9-GFP. Each sample was run in triplicate and relative concentration calculated using the ddCt values normalized to endogenous actin transcript.

[0077] ***Behavior and Survival Analysis.*** Treated and control $SOD1^{G93A}$ mice were monitored for changes in body mass twice a week. *lox*$SOD1^{G37R}$ mice were weighed on a weekly basis. Motor coordination was recorded using a rotarod instrument (Columbus Instruments, Columbus, OH). Each weekly session consisted of three trials on the accelerating rotarod beginning at 5 rpm/min. The time each mouse remained on the rod was registered. Both $SOD1^{G93A}$ and *lox*$SOD1^{G37R}$ mice were subjected to weekly assessment of hindlimb grip strength using a grip strength meter (Columbus Instruments, Columbus, OH). Each weekly

session consisted of 3 ($SOD1^{G93A}$ mice) or 5 ($loxSOD1^{G37R}$ mice) tests per animal. Survival analysis was performed using Kaplan-Meier survival analysis. End stage was defined as an artificial death point when animals could no longer "right" themselves within 30 sec after being placed on its back. Onset and disease progression were determined from retrospective analysis of the data. Disease onset is defined as the age at which the animal reached its peak weight. Disease duration is defined as the time period between disease onset and end stage. Early disease duration is the period between peak weight and loss of 10% of body weight while late disease duration is defined as the period between 10% loss of body weight until disease end stage. Due to shorter life span of $SOD1^{G93A}$ animals, we did not assess the distinction between the early and late progression.

[0078] For toxicity analysis following injection at P1 or P21, treated and control WT mice were subjected to behavioral analysis starting at -30 days of age and monitored up to 6 months. Body mass was recorded weekly while rotarod performance and hindlimb grip strength were recorded biweekly.

[0079] ***Hematology and Serum Studies.*** Blood samples were collected in (K2) EDTA microtainer tubes (BD) from treated and control WT mice at 150 days of age by mandibular vein puncture. The same animals were bled at 180 days of age and blood was collected in serum separator microtainer tubes. The blood was allowed to clot for an hour and was then centrifuged at 10,000 rpm for 5 minutes. The clear upper phase (serum) was collected and frozen at -80°C. Hematological and serum analysis were conducted by Ani Lytics Inc, Gaithersburg, MD.

[0080] ***Statistical analysis.*** All statistical tests were performed using the GraphPad Prism (San Diego, CA) software package. Kaplan Meier survival analyses were analyzed by the Log Rank Test. Comparisons of median disease durations and survival times were analyzed by the Wilcoxon Signed Rank Test.

Example 8

Development of a clinical SOD1 shRNA construct

[0081] The AAV SOD1 shRNA vector described in Example 2 carries shRNA against human SOD1 sequence under the H1 promoter (Figure 9a). The same vector also contains a GFP expression cassette which expresses GFP under a CBA promoter. The other regulatory elements present in this cassette include CMV enhancer, SV40 intron and bGH PolyA terminator sequence. We show herein that AAV9 SOD1 shRNA administration results in efficient SOD1 downregulation along with robust expression of GFP *in vitro* as well as *in vivo.* No significant alterations were observed after the long term assessment of wild-type mice administered with AAV9 SOD1 shRNA. These results suggested that there are no evident off-

target effects due to the long-term expression of SOD1 shRNA as well as overexpression of GFP. Although we did not find GFP toxicity in our mice, several reports have shown the adverse effects of GFP overexpression *in vitro* and *in vivo.* Therefore, to eliminate the possibility of GFP toxicity altogether, the SOD1 shRNA construct of Example 2 was modified by replacing the GFP expression cassette with a non-coding stuffer sequence while maintaining the size of the total DNA construct flanked by the ITRs (Figure 9b). This is important as the distance between the two ITR sequences greatly affects the packaging capacity of the flanked construct into AAV9 capsids[321-324].

[0082] To date, none of the FDA approved stuffer sequences are readily available. There are, however, several plasmid backbones that are approved by FDA for the human administration. Small DNA fragments were picked from these plasmids which do not correspond to any essential DNA sequences necessary for selection and replication of the plasmid or the elements of the transcriptional units. The plasmid backbones are listed in Table 1. The DNA elements from different plasmids were arranged in tandem to generate a complete, 1607 bp stuffer sequence (SEQ ID NO: 22). Finally, a DNA construct containing the SOD1 shRNA expression cassette, followed by the stuffer sequence was synthesized from Genscript.

Table 1

Plasmid Backbone	**Condition**	**Intervention**	**Phase**	**ClinicalTrials.gov Identifier**
pVax1	Early Stage Non-	Recombinant DNA-pVAX/L523S	1	NCT00062907
	Small			
	Cell Lung Cancer			
pCDNA3	Chronic Hepatitis B	DNA vaccine pCMVS2.S	1,2	NCT00536627
pUCMV3	Stage III Ovarian	pUMVC3-hIGFBP-2 multi-epitope plasmid DNA vaccine	1	NCT01322802
	Epithelial Cancer			
	Stage III Ovarian			
	Germ Cell Tumor			
	Stage IV Ovarian			
	Epithelial Cancer			
	Stage IV Ovarian			
	Germ Cell Tumor			

Plasmid Backbone	Condition	Intervention	Phase	ClinicalTrials.gov Identifier
pBK-	Prostate Cancer	NY-ESO-1 plasmid DNA Cancer Vaccine	1	NCT00199849
CMV	Bladder Cancer			
	Non-Small Cell Lung			
	Cancer			
	Esophageal Cancer			
	Sarcoma			
pGA2	HIV Infections	pGA2/JS2 Plasmid DNA Vaccine	1	NCT00043511

[0083] Clinical SOD1 shRNA construct has shRNA against human SOD1 under H1 promoter which is followed by the non-coding stuffer sequence. This construct is designed in such a way that multiple shRNA expression cassettes can be added to the final vector by simultaneous removal of the stuffer sequence. Restriction endonuclease sites have been added to the stuffer sequence so that a part of the stuffer can be removed when another shRNA expression cassette is added (Figure 10). This simultaneous removal and addition of DNA sequences would help maintaining the optimal size of the whole construct between the ITRs (~2.0kb) to achieve efficient packaging.

[0084] Clinical SOD1 shRNA construct from Genscript was cloned into pJetI.2 shuttle vector via EcoRV. This parental clone was screened using various restriction endonucleases designed within the construct to confirm the correct clone. Kpn1/Sph1 double digestion of pJet SOD1 shRNA confirmed the presence of the complete construct (2023 bp) while Xbal digestion confirmed the presence of SOD1 shRNA expression cassette (414 bp) and the stuffer element, along with pJet backbone (~3000 bp). EcoRV/Pme1 double digestion also revealed the presence of stuffer element.

Example 9

Clinical SOD1 shRNA efficiently reduces human SOD1 protein levels *in vitro*

[0085] To determine the efficacy of the *de novo* synthesized SOD1 shRNA construct to downregulate SOD1 levels, HEK293 cells were transfected with pJet SOD1 shRNA plasmid using Calcium Phosphate method. AAV SOD1 shRNA plasmid was used as a positive control.

Immunofluorescence analysis of HEK293 cells, 72 hrs post transfection revealed the lack of native GFP fluorescence from pJet SOD1 shRNA transfected cells as compared to AAV9 SOD1 shRNA transfected cells. Immunoblot analysis of cell lysates from these cells further confirmed the successful replacement of GFP from pJet SOD1 shRNA plasmid. Importantly, pJet SOD1 shRNA resulted in efficient downregulation of SOD1 protein levels (>50%), similar to AAV SOD1 shRNA plasmid. See Figure 11.

Example 10

Generation of clinical AAV SOD1 shRNA

[0086] Clinical SOD1 shRNA construct was further cloned into an AAV.CB.MCS vector using Kpn1/Sph1 sites to generate clinical AAV SOD1 shRNA plasmid (Figure 12). AAV.CB.MCS was generated from AAV.CB.GFP plasmid obtained from merion Scientific by replacing GFP with a multiple cloning site (MCS). Cloning of clinical SOD1 shRNA construct at Kpn1/Sph1 sites puts it between the two AAV2 ITRs which facilitates the packaging of the construct in AAV9 viral capsids. See Figure 12.

[0087] Clinical AAV SOD1 shRNA plasmid was screened with restriction endonucleases to confirm the presence of SOD1 shRNA expression cassette (Xbal digest), stuffer sequence (EcoRV/Pmel double digest) and also intact ITR sequences (Smal digest).

Example 11

Clinical AAV SOD1 shRNA efficiently reduces human SOD1 protein levels *in vitro*

[0088] Clinical AAV SOD1 shRNA plasmid was transfected in HEK293 cells to determine its knockdown efficiency. Similar to the pJet SOD1 shRNA plasmid, clinical AAV SOD1 shRNA transfected cells were devoid of any GFP expression as evident by immunofluorescence (Figure 13a-f) and immunoblot assay (Figure 13g). More importantly, clinical AAV SOD1 shRNA efficiently reduced human SOD1 protein levels in HEK293 cells by more than 50% (Figure 13g,h). Altogether, these results confirmed the successful generation of clinical AAV SOD1 shRNA vector with functional SOD1 shRNA expression cassette and complete removal of the transgene expression cassette.

Documents Referenced

[0089]

1. 1. Da Cruz, S. & Cleveland, D.W. Understanding the role of TDP-43 and FUS/TLS in ALS and beyond. Curr Opin Neurobiol 21, 904-919 (2011).
2. 2. Rosen, D.R. et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362, 59-62 (1993).
3. 3. Ilieva, H., Polymenidou, M. & Cleveland, D.W. Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. The Journal of cell biology 187, 761-772 (2009).
4. 4. Chattopadhyay, M. & Valentine, J.S. Aggregation of copper-zinc superoxide dismutase in familial and sporadic ALS. Antioxidants & redox signaling 11, 1603-1614 (2009).
5. 5. Prudencio, M., Hart, P.J., Borchelt, D.R. & Andersen, P.M. Variation in aggregation propensities among ALS-associated variants of SOD1: correlation to human disease. Human molecular genetics 18, 3217-3226 (2009).
6. 6. Boillee, S. et al. Onset and progression in inherited ALS determined by motor neurons and microglia. Science 312, 1389-1392 (2006).
7. 7. Kang, S.H. et al. Degeneration and impaired regeneration of gray matter oligodendrocytes in amyotrophic lateral sclerosis. Nature neuroscience 16, 571-579 (2013).
8. 8. Yamanaka, K. et al. Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. Nature neuroscience 11, 251-253 (2008).
9. 9. Di Giorgio, F.P., Boulting, G.L., Bobrowicz, S. & Eggan, K.C. Human embryonic stem cell-derived motor neurons are sensitive to the toxic effect of glial cells carrying an ALS-causing mutation. Cell Stem Cell 3, 637-648 (2008).
10. 10. Di Giorgio, F.P., Carrasco, M.A., Siao, M.C., Maniatis, T. & Eggan, K. Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. Nature neuroscience 10, 608-614 (2007).
11. 11. Marchetto, M.C. et al. Non-cell-autonomous effect of human SOD1 G37R astrocytes on motor neurons derived from human embryonic stem cells. Cell Stem Cell 3, 649-657 (2008).
12. 12. Haidet-Phillips, A.M. et al. Astrocytes from familial and sporadic ALS patients are toxic to motor neurons. Nat Biotechnol 29, 824-828 (2011).
13. 13. Bosco, D.A. et al. Wild-type and mutant SOD1 share an aberrant conformation and a common pathogenic pathway in ALS. Nature neuroscience 13, 1396-1403 (2010).
14. 14. Pokrishevsky, E. et al. Aberrant localization of FUS and TDP43 is associated with misfolding of SOD1 in amyotrophic lateral sclerosis. PloS one 7, e35050 (2012).
15. 15. Forsberg, K. et al. Novel antibodies reveal inclusions containing non-native SOD1 in sporadic ALS patients. PLoS One 5, e11552 (2010).
16. 16. Aggarwal, S. & Cudkowicz, M. ALS drug development: reflections from the past and a way forward. Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics 5, 516-527 (2008).
17. 17. Gurney, M.E. et al. Benefit of vitamin E, riluzole, and gabapentin in a transgenic model of familial amyotrophic lateral sclerosis. Ann Neurol 39, 147-157 (1996).
18. 18. Foust, K.D. et al. Intravascular AAV9 preferentially targets neonatal neurons and

adult astrocytes. Nature biotechnology 27, 59-65 (2009).
19. 19. Duque, S. et al. Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. Mol Ther 17, 1187-1196 (2009).
20. 20. Zhong, Z. et al. ALS-causing SOD1 mutants generate vascular changes prior to motor neuron degeneration. Nature neuroscience 11, 420-422 (2008).
21. 21. Miller, R.G., Mitchell, J.D. & Moore, D.H. Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). Cochrane Database Syst Rev 3, CD001447 (2012).
22. 22. Smith, R.A. et al. Antisense oligonucleotide therapy for neurodegenerative disease. The Journal of clinical investigation 116, 2290-2296 (2006).
23. 23. Raoul, C. et al. Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. Nat Med 11, 423-428 (2005).
24. 24. Ralph, G.S. et al. Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. Nat Med 11, 429-433 (2005).
25. 25. Miller, T.M. et al. Virus-delivered small RNA silencing sustains strength in amyotrophic lateral sclerosis. Annals of neurology 57, 773-776 (2005).
26. 26. Miller, T.M. et al. An antisense oligonucleotide against SOD1 delivered intrathecally for patients with SOD1 familial amyotrophic lateral sclerosis: a phase 1, randomised, first-in-man study. Lancet neurology 12, 435-442 (2013).
27. 27. Towne, C., Raoul, C., Schneider, B.L. & Aebischer, P. Systemic AAV6 delivery mediating RNA interference against SOD1: neuromuscular transduction does not alter disease progression in fALS mice. Mol Ther 16, 1018-1025 (2008).
28. 28. Towne, C., Setola, V., Schneider, B.L. & Aebischer, P. Neuroprotection by gene therapy targeting mutant SOD1 in individual pools of motor neurons does not translate into therapeutic benefit in fALS mice. Mol Ther 19, 274-283 (2011).
29. 29. Mandel, R.J., Lowenstein, P.R. & Byrne, B.J. AAV6-mediated gene silencing fALS short. Mol Ther 19, 231-233 (2011).
30. 30. Synofzik, M. et al. Mutant superoxide dismutase-1 indistinguishable from wild-type causes ALS. Human molecular genetics 21, 3568-3574 (2012).
31. 31. Guareschi, S. et al. An over-oxidized form of superoxide dismutase found in sporadic amyotrophic lateral sclerosis with bulbar onset shares a toxic mechanism with mutant SOD1. Proc Natl Acad Sci U S A 109, 5074-5079 (2012).
32. 32. Haidet-Phillips, A.M. et al. Astrocytes from familial and sporadic ALS patients are toxic to motor neurons. Nat Biotechnol 29, 824-828 (2011).
33. 33. Bevan, A.K. et al. Systemic gene delivery in large species for targeting spinal cord, brain, and peripheral tissues for pediatric disorders. Mol Ther 19, 1971-1980 (2011).
34. 34. Gray, S.J. et al. Preclinical differences of intravascular AAV9 delivery to neurons and glia: a comparative study of adult mice and nonhuman primates. Mol Ther 19, 1058-1069 (2011).
35. 35. Lioy, D.T. et al. A role for glia in the progression of Rett's syndrome. Nature 475, 497-500 (2011).
36. 36. Miranda, C.J. et al. Aging brain microenvironment decreases hippocampal neurogenesis through Wnt-mediated survivin signaling. Aging Cell 11, 542-552 (2012).
37. 37. Yamanaka, K. et al. Mutant SOD1 in cell types other than motor neurons and

oligodendrocytes accelerates onset of disease in ALS mice. Proc Natl Acad Sci U S A 105, 7594-7599 (2008).

REFERENCES CITED IN THE DESCRIPTION

Cited references

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Patent documents cited in the description

- WO2010071832A [0013]
- WO2009043936A [0013]
- WO2009013290A [0013]
- WO2011133890A [0013]
- WO2013078316A [0023]
- WO0183692A [0024]
- US20050053922A [0024]
- US20090202490A [0024]
- US5173414A [0026]
- WO9513365A [0026]
- US5658776A [0026]
- WO9513392A [0026]
- WO9617947A [0026]
- US9818600W [0026]
- WO9709441A [0026]
- US9614423W [0026]
- WO9708298A [0026]
- US9613872W [0026]
- WO9721825A [0026]
- US9620777W [0026]
- WO9706243A [0026]
- FR9601064W [0026]
- WO9911764A [0026]

- US5786211A [0026]
- US5871982A [0026]
- US6258595B [0026]
- US6566118B [0031]
- WO9809657A [0031]

Non-patent literature cited in the description

- **KAPLITT et al.**Lancet, 2007, vol. 369, 2097-2105 [0010]
- **MARKS et al.**Lancet Neurol, 2008, vol. 7, 400-408 [0010]
- **WORGALL et al.**Hum Gene Ther, 2008, [0010]
- **SRIVASTAVA et al.**J Virol, 1983, vol. 45, 555-564 [0011]
- **RUFFING et al.**J Gen Virol, 1994, vol. 75, 3385-3392 [0011]
- **MUZYCZKA**Current Topics in Microbiology and Immunology, 1992, vol. 158, 97-129 [0011]
- **PACAK**Circ. Res., vol. 99, 43-9 [0013]
- **WANG**Nature Biotech., 2005, vol. 23, 3321-8 [0013]
- Selective silencing by RNAi of dominant allele that causes amyotrophic lateral sclerosis**DING H et al.**AGING CELLBLACKWELL PUBLISHING20030101vol. 2, 209-217 [0014]
- **SRIVASTAVA et al.**J. Virol., 1983, vol. 45, 555-564 [0023]
- **GAO et al.**J. Virol., 2004, vol. 78, 6381-6388 [0023]
- Mol. Ther., 2006, vol. 13, 167-76 [0023]
- Virology, 2004, vol. 330, 2375-383 [0023]
- **SAMULSKI et al.**Proc. Natl. Acad. S6. USA, 1982, vol. 79, 2077-2081 [0025]
- **LAUGHLIN et al.**Gene, 1983, vol. 23, 65-73 [0025]
- **SENAPATHYCARTER**J. Biol. Chem., 1984, vol. 259, 4661-4666 [0025]
- **CARTER**Current Opinions in Biotechnology, 1992, 1533-539 [0026]
- **MUZYCZKA**Curr. Topics in Microbial, and Immunol., 1992, vol. 158, 97-129 [0026]
- **RATSCHIN et al.**Mol. Cell. Biol., 1984, vol. 4, 2072- [0026]
- **HERMONAT et al.**Proc. Natl. Acad. Sci. USA, 1984, vol. 81, 6466- [0026]
- **TRATSCHIN et al.**Mol. Cell. Biol., 1985, vol. 5, 3251- [0026]
- **MCLAUGHLIN et al.**J. Virol., 1988, vol. 62, 1963- [0026]
- **LEBKOWSKI et al.**Mol. Cell. Biol., 1988, vol. 7, 349- [0026]
- **SAMULSKI et al.**J. Virol., 1989, vol. 63, 3822-3828 [0026]
- **PERRIN et al.**Vaccine, 1995, vol. 13, 1244-1250 [0026]
- **PAUL et al.**Human Gene Therapy, 1993, vol. 4, 609-615 [0026]
- **CLARK et al.**Gene Therapy, 1996, vol. 3, 1124-1132 [0026]
- **CLARK et al.**Hum. Gene Ther., 1999, vol. 10, 61031-1039 [0031]
- **SCHENPPCLARK**Methods Mol. Med., 2002, vol. 69, 427-443 [0031]

- **DA CRUZ, S.CLEVELAND, D.W.**Understanding the role of TDP-43 and FUS/TLS in ALS and beyondCurr Opin Neurobiol, 2011, vol. 21, 904-919 [0089]
- **ROSEN, D.R. et al.**Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosisNature, 1993, vol. 362, 59-62 [0089]
- **ILIEVA, H.POLYMENIDOU, M.CLEVELAND, D.W.**Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyondThe Journal of cell biology, 2009, vol. 187, 761-772 [0089]
- **CHATTOPADHYAY, M.VALENTINE, J.S.**Aggregation of copper-zinc superoxide dismutase in familial and sporadic ALSAntioxidants & redox signaling, 2009, vol. 11, 1603-1614 [0089]
- **PRUDENCIO, M.HART, P.J.BORCHELT, D.R.ANDERSEN, P.M.**Variation in aggregation propensities among ALS-associated variants of SOD1: correlation to human diseaseHuman molecular genetics, 2009, vol. 18, 3217-3226 [0089]
- **BOILLEE, S. et al.**Onset and progression in inherited ALS determined by motor neurons and microgliaScience, 2006, vol. 312, 1389-1392 [0089]
- **KANG, S.H. et al.**Degeneration and impaired regeneration of gray matter oligodendrocytes in amyotrophic lateral sclerosisNature neuroscience, 2013, vol. 16, 571-579 [0089]
- **YAMANAKA, K. et al.**Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosisNature neuroscience, 2008, vol. 11, 251-253 [0089]
- **DI GIORGIO, F.P.BOULTING, G.L.BOBROWICZ, S.EGGAN, K.C.**Human embryonic stem cell-derived motor neurons are sensitive to the toxic effect of glial cells carrying an ALS-causing mutationCell Stem Cell, 2008, vol. 3, 637-648 [0089]
- **DI GIORGIO, F.P.CARRASCO, M.A.SIAO, M.C.MANIATIS, T.EGGAN, K.**Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS modelNature neuroscience, 2007, vol. 10, 608-614 [0089]
- **MARCHETTO, M.C. et al.**Non-cell-autonomous effect of human SOD1 G37R astrocytes on motor neurons derived from human embryonic stem cellsCell Stem Cell, 2008, vol. 3, 649-657 [0089]
- **HAIDET-PHILLIPS, A.M. et al.**Astrocytes from familial and sporadic ALS patients are toxic to motor neuronsNat Biotechnol, 2011, vol. 29, 824-828 [0089] [0089]
- **BOSCO, D.A. et al.**Wild-type and mutant SOD1 share an aberrant conformation and a common pathogenic pathway in ALSNature neuroscience, 2010, vol. 13, 1396-1403 [0089]
- **POKRISHEVSKY, E. et al.**Aberrant localization of FUS and TDP43 is associated with misfolding of SOD1 in amyotrophic lateral sclerosisPloS one, 2012, vol. 7, e35050- [0089]
- **FORSBERG, K. et al.**Novel antibodies reveal inclusions containing non-native SOD1 in sporadic ALS patientsPLoS One, 2010, vol. 5, e11552- [0089]
- **AGGARWAL, S.CUDKOWICZ, M.**ALS drug development: reflections from the past and a way forwardNeurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics, 2008, vol. 5, 516-527 [0089]
- **GURNEY, M.E. et al.**Benefit of vitamin E, riluzole, and gabapentin in a transgenic model of familial amyotrophic lateral sclerosisAnn Neurol, 1996, vol. 39, 147-157 [0089]

- **FOUST, K.D. et al.**Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytesNature biotechnology, 2009, vol. 27, 59-65 [0089]
- **DUQUE, S. et al.**Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neuronsMol Ther, 2009, vol. 17, 1187-1196 [0089]
- **ZHONG, Z. et al.**ALS-causing SOD1 mutants generate vascular changes prior to motor neuron degenerationNature neuroscience, 2008, vol. 11, 420-422 [0089]
- **MILLER, R.G.MITCHELL, J.D.MOORE, D.H.**Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND)Cochrane Database Syst Rev, 2012, vol. 3, [0089]
- **SMITH, R.A. et al.**Antisense oligonucleotide therapy for neurodegenerative diseaseThe Journal of clinical investigation, 2006, vol. 116, 2290-2296 [0089]
- **RAOUL, C. et al.**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 [0089]
- **RALPH, G.S. et al.**Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS modelNat Med, 2005, vol. 11, 429-433 [0089]
- **MILLER, T.M. et al.**Virus-delivered small RNA silencing sustains strength in amyotrophic lateral sclerosisAnnals of neurology, 2005, vol. 57, 773-776 [0089]
- **MILLER, T.M. et al.**An antisense oligonucleotide against SOD1 delivered intrathecally for patients with SOD1 familial amyotrophic lateral sclerosis: a phase 1, randomised, first-in-man studyLancet neurology, 2013, vol. 12, 435-442 [0089]
- **TOWNE, C.RAOUL, C.SCHNEIDER, B.L.AEBISCHER, P.**Systemic AAV6 delivery mediating RNA interference against SOD1: neuromuscular transduction does not alter disease progression in fALS miceMol Ther, 2008, vol. 16, 1018-1025 [0089]
- **TOWNE, C.SETOLA, V.SCHNEIDER, B.L.AEBISCHER, P.**Neuroprotection by gene therapy targeting mutant SOD1 in individual pools of motor neurons does not translate into therapeutic benefit in fALS miceMol Ther, 2011, vol. 19, 274-283 [0089]
- **MANDEL, R.J.LOWENSTEIN, P.R.BYRNE, B.J.**AAV6-mediated gene silencing fALS shortMol Ther, 2011, vol. 19, 231-233 [0089]
- **SYNOFZIK, M. et al.**Mutant superoxide dismutase-1 indistinguishable from wild-type causes ALSHuman molecular genetics, 2012, vol. 21, 3568-3574 [0089]
- **GUARESCHI, S. et al.**An over-oxidized form of superoxide dismutase found in sporadic amyotrophic lateral sclerosis with bulbar onset shares a toxic mechanism with mutant SOD1Proc Natl Acad Sci U S A, 2012, vol. 109, 5074-5079 [0089]
- **BEVAN, A.K. et al.**Systemic gene delivery in large species for targeting spinal cord, brain, and peripheral tissues for pediatric disordersMol Ther, 2011, vol. 19, 1971-1980 [0089]
- **GRAY, S.J. et al.**Preclinical differences of intravascular AAV9 delivery to neurons and glia: a comparative study of adult mice and nonhuman primatesMol Ther, 2011, vol. 19, 1058-1069 [0089]
- **LIOY, D.T. et al.**A role for glia in the progression of Rett's syndromeNature, 2011, vol. 475, 497-500 [0089]
- **MIRANDA, C.J. et al.**Aging brain microenvironment decreases hippocampal neurogenesis through Wnt-mediated survivin signalingAging Cell, 2012, vol. 11, 542-

552 [0089]

- **YAMANAKA, K. et al.**Mutant SOD1 in cell types other than motor neurons and oligodendrocytes accelerates onset of disease in ALS miceProc Natl Acad Sci U S A, 2008, vol. 105, 7594-7599 [0089]

Patentkrav

1. Rekombinant adeno-associeret virus der omfatter superoxiddismutase-1 (SOD1)-shRNA-kodende DNA med sekvensen SEQ ID NO: 4, en hårnålesekvens og en sekvens der er komplementær til SEQ ID NO: 4, hvor virusgenomet mangler et rep-gen og mangler et cap-gen.

2. Rekombinant adeno-associeret virus ifølge krav 1 der yderligere omfatter en promotor, som er operativt forbundet med det superoxiddismutase-1 (SOD1) -shRNA-kodende DNA.

3. Rekombinant adeno-associeret virus ifølge krav 2, hvor promotoren er en H1-promotor.

4. Rekombinant adeno-associeret virus ifølge krav 3, hvor H1-promotoren omfatter nucleotiderne 966 til 1064 af SEQ ID NO: 20.

5. Rekombinant adeno-associeret virus ifølge et hvilket som helst af kravene 1-4 der yderligere der omfatter en stuffer-sekvens.

6. Rekombinant adeno-associeret virus ifølge krav 5, hvor stuffer-sekvensen omfatter SEQ ID NO: 22.

7. Rekombinant adeno-associeret virus ifølge et hvilket som helst af kravene 1-6, hvor virusgenomet er et selvkomplementært genom.

8. Rekombinant adeno-associeret virus ifølge et hvilket som helst af kravene 1-7 der omfatter et capsid, som er afledt af: AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV- 8, AAV-9, AAV-10, AAV-11, AAV-rh74.

9. Rekombinant adeno-associeret virus ifølge krav 8, hvor capsidet er et AAV9-capsid.

10. Sammensætning der omfatter det rekombinante adeno-associerede virus ifølge et hvilket som helst af kravene 1-9 og en farmaceutisk acceptabel bærer.

11. Sammensætningen ifølge krav 10 til anvendelse i kombination med et kontrastmiddel.

12. Sammensætningen ifølge krav 11, hvor kontrastmidlet er iobitridol, iohexol, iomeprol, iopamidol, iopentol, iopromid, ioversol eller ioxilan.

13. Rekombinant adeno-associeret virus ifølge et hvilket som helst af kravene 1-9 eller en sammensætning ifølge et hvilket som helst af kravene 10-12 til anvendelse ved behandling af amyotrofisk lateral sklerose (ALS).

14. Rekombinant adeno-associeret virus eller sammensætningen til anvendelse ifølge krav 13, hvor ALS er familiært.

15. Rekombinant adeno-associeret virus eller sammensætningen til anvendelse ifølge krav 14, hvor ALS er forårsaget af en eller flere mutationer i superoxid-dismutase-1 (SOD1).

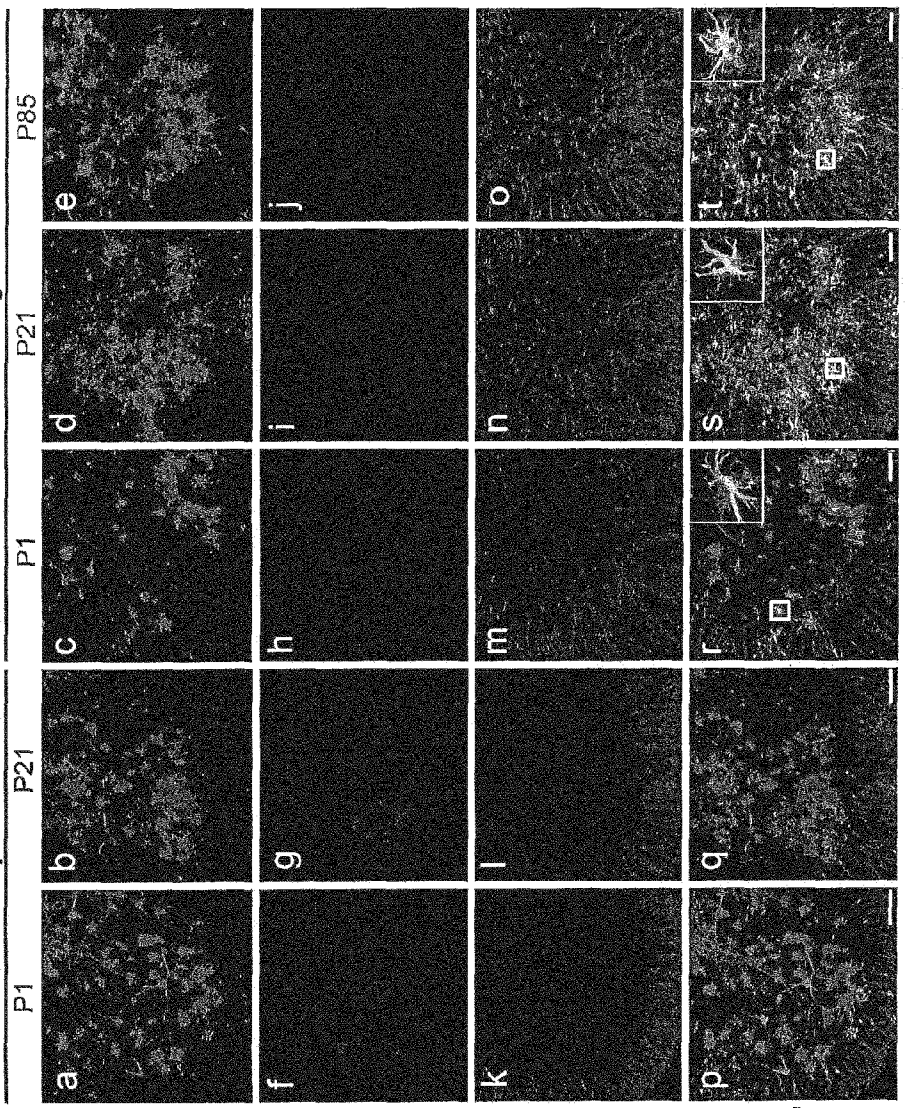
16. Rekombinant adeno-associeret virus eller sammensætningen til anvendelse ifølge et hvilket som helst af kravene 13-15, hvor det rekombinante adeno-associerede virus eller sammensætningen er til systemisk administration.

17. Rekombinant adeno-associeret virus eller sammensætningen til anvendelse ifølge et hvilket som helst af kravene 13-15, hvor den rekombinante adeno-associerede virus eller sammensætningen er til intratekal administration.

18. Rekombinant adeno-associeret virus ifølge et hvilket som helst af kravene 1 til 9 til anvendelse i terapi.

DRAWINGS

Figure 1



U

21d Post Injection	GFP	ChAT	% (Mean ± sem)	GFP	GFAP	% (Mean ± sem)
P1 Injected	186	300	62 ± 1	170	500	34 ± 2
P21 Injected	23	300	8 ± 1	272	500	54 ± 3

End Stage	GFP	ChAT	% (Mean ± sem)	GFP	GFAP	% (Mean ± sem)
P1 Injected	227	300	75 ± 4	211	500	42 ± 2
P21 Injected	29	300	10 ± 4	305	500	61 ± 2
P85 Injected	25	300	8 ± 2	257	500	51 ± 6

Figure 2

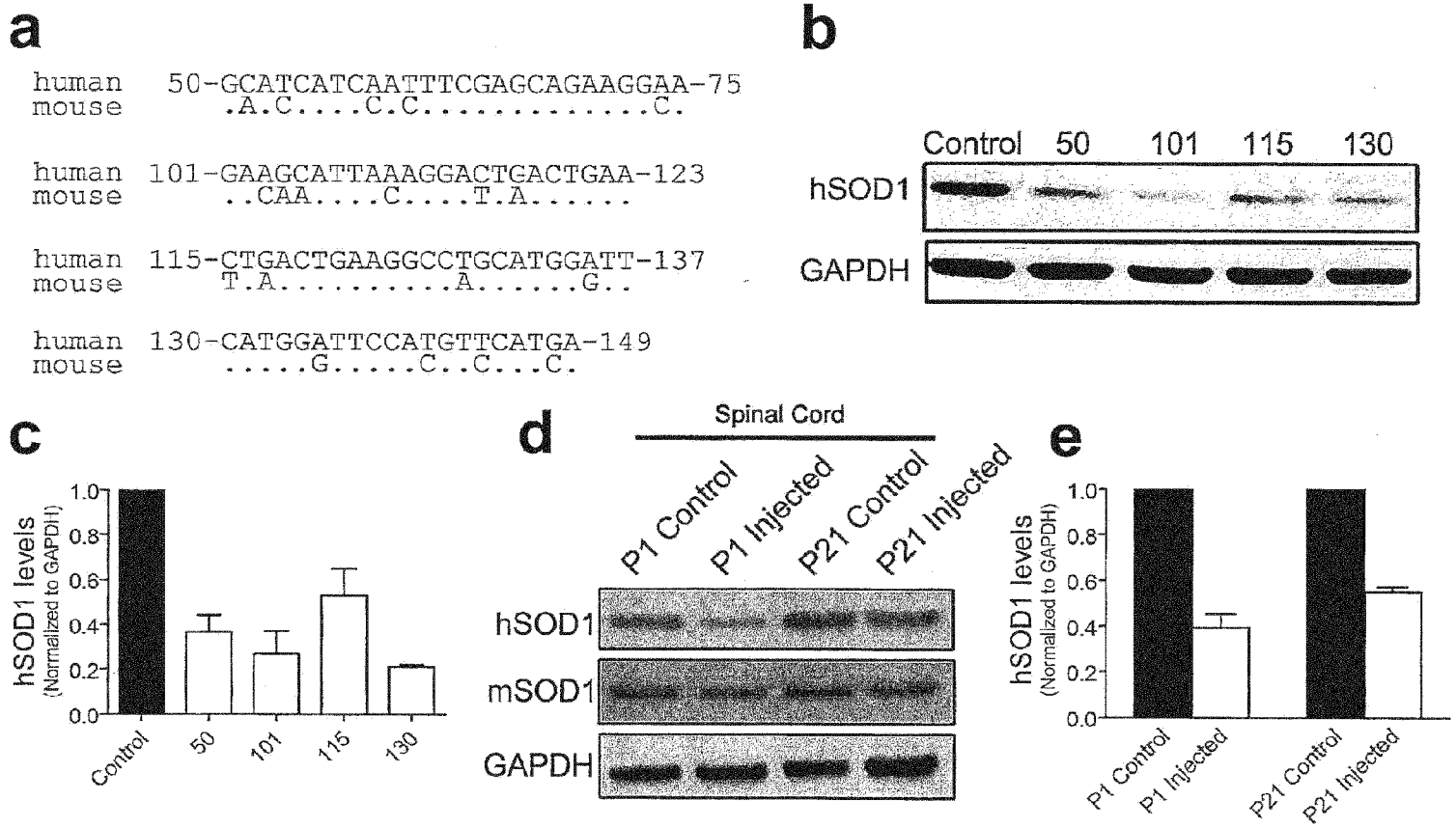


Figure 3

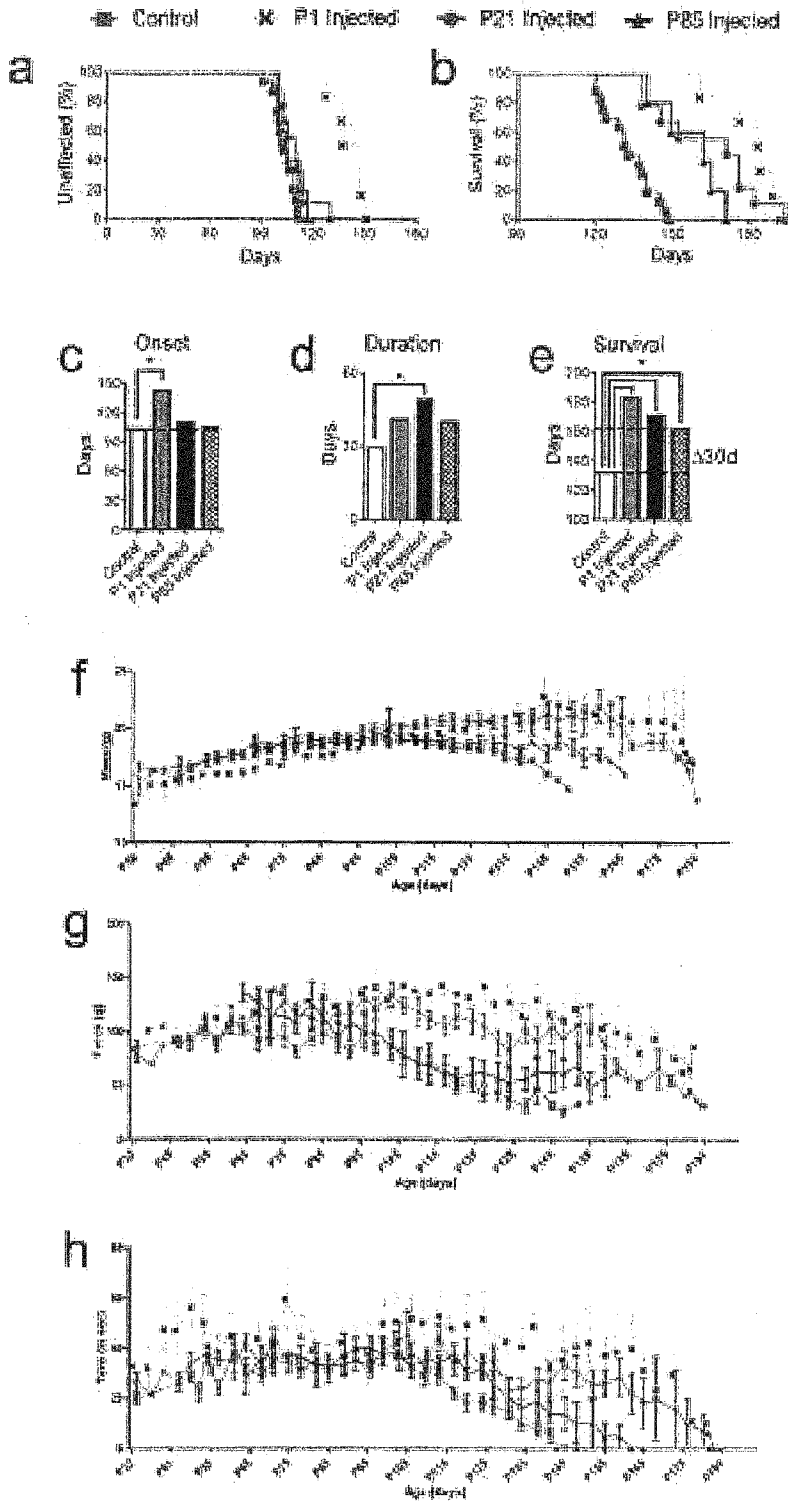
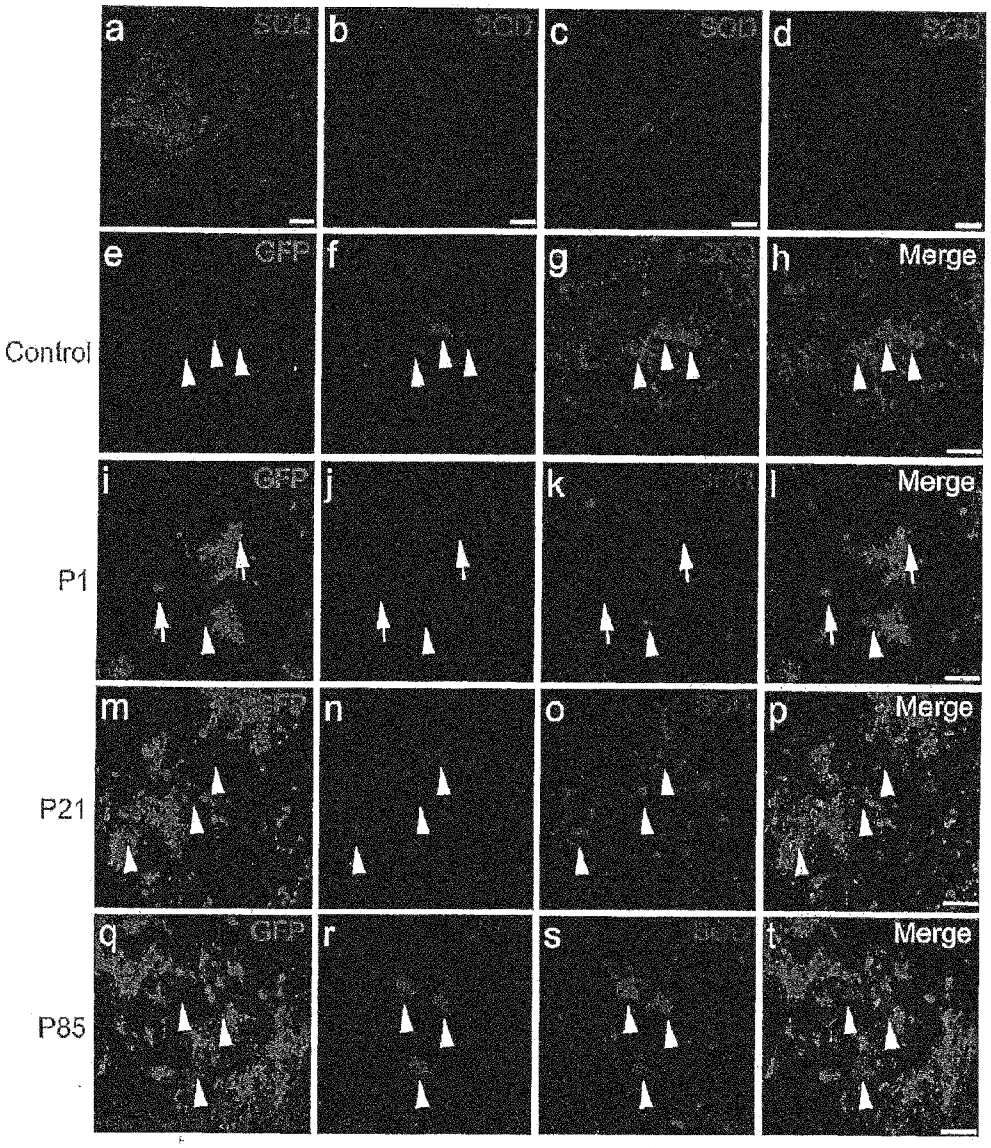

Control
P1 Injected
P21 Injected
PBS Injected
a
Unaffected (%)
Days
b
Survival (%)
Days
c
Onset
Days
d
Duration
Days
e
Survival
Days
Δ30d
f
g
h

Figure 4


a
b
c
d
e
GFP
f
g
h
Merge
Control
i
GFP
j
k
l
Merge
P1
m
n
o
p
Merge
P21
q
GFP
r
s
t
Merge
P85

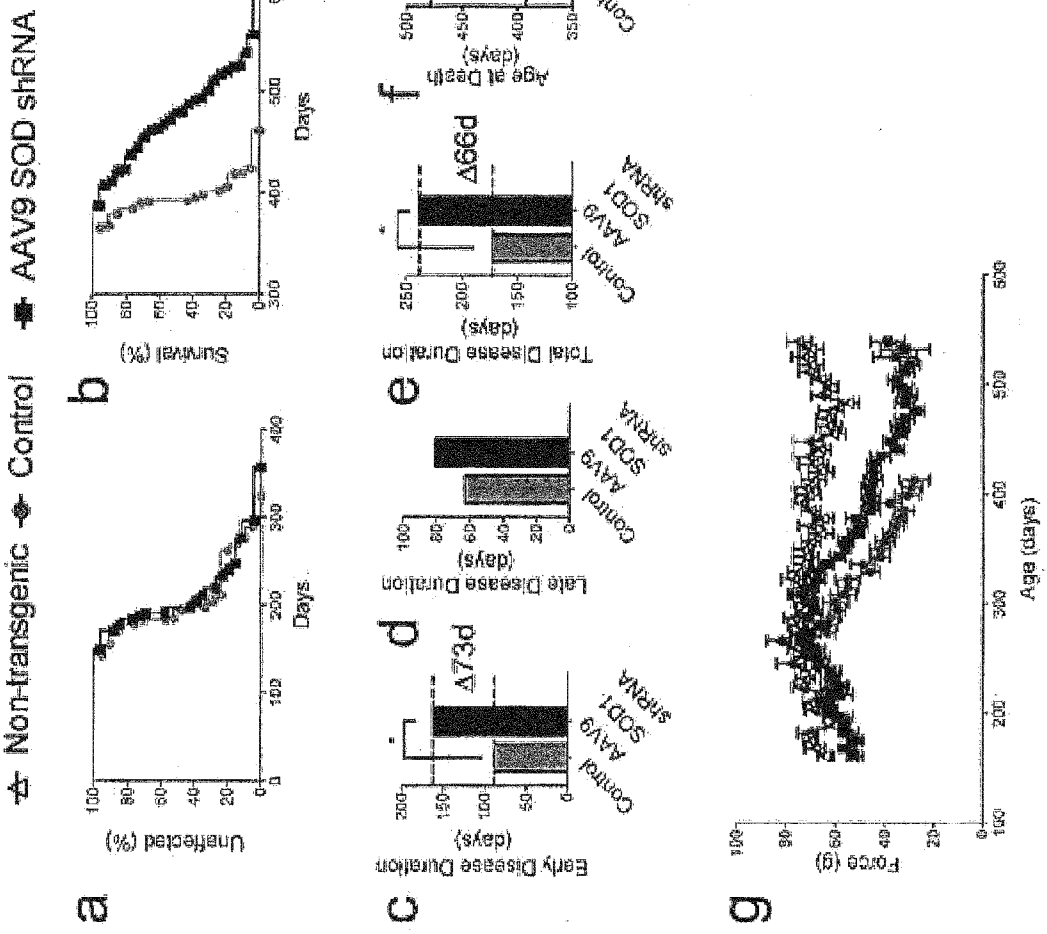

Non-transgenic
Control
AAV9 SOD shRNA
a
Unaffected (%)
Days
b
Survival (%)
Days
c
Early Disease Duration (days)
Δ73d
Control
AAV9 SOD1 shRNA
d
Late Disease Duration (days)
e
Total Disease Duration (days)
Δ66d
f
Age at Death (days)
Δ86.5d
g
Force (g)
Age (days)

Figure 5

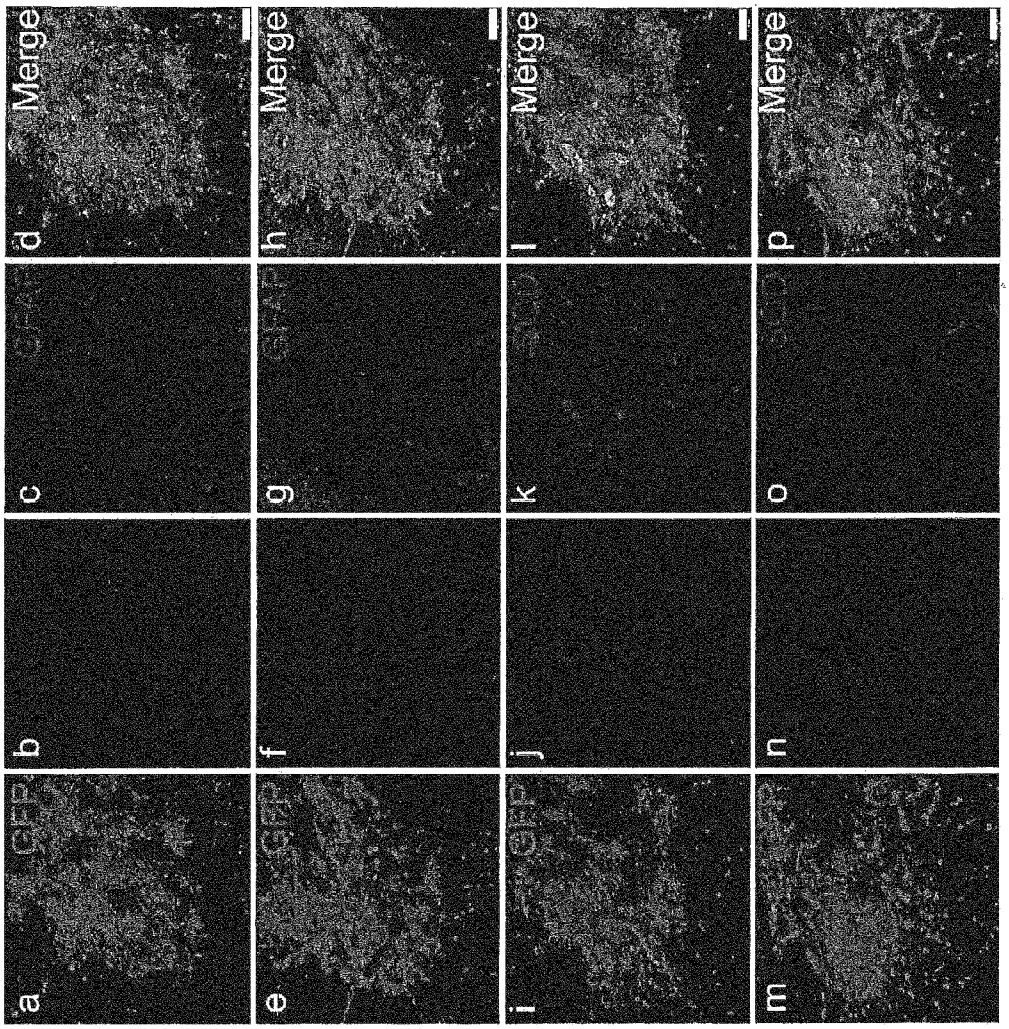

a
GFP
b
c
d
Merge
e
GFP
f
g
h
Merge
i
GFP
j
k
l
Merge
m
GFP
n
o
p
Merge
Control
AAV9
-SOD1
-shRNA
Control
AAV9
-SOD1
-shRNA

Figure 6

Figure 7

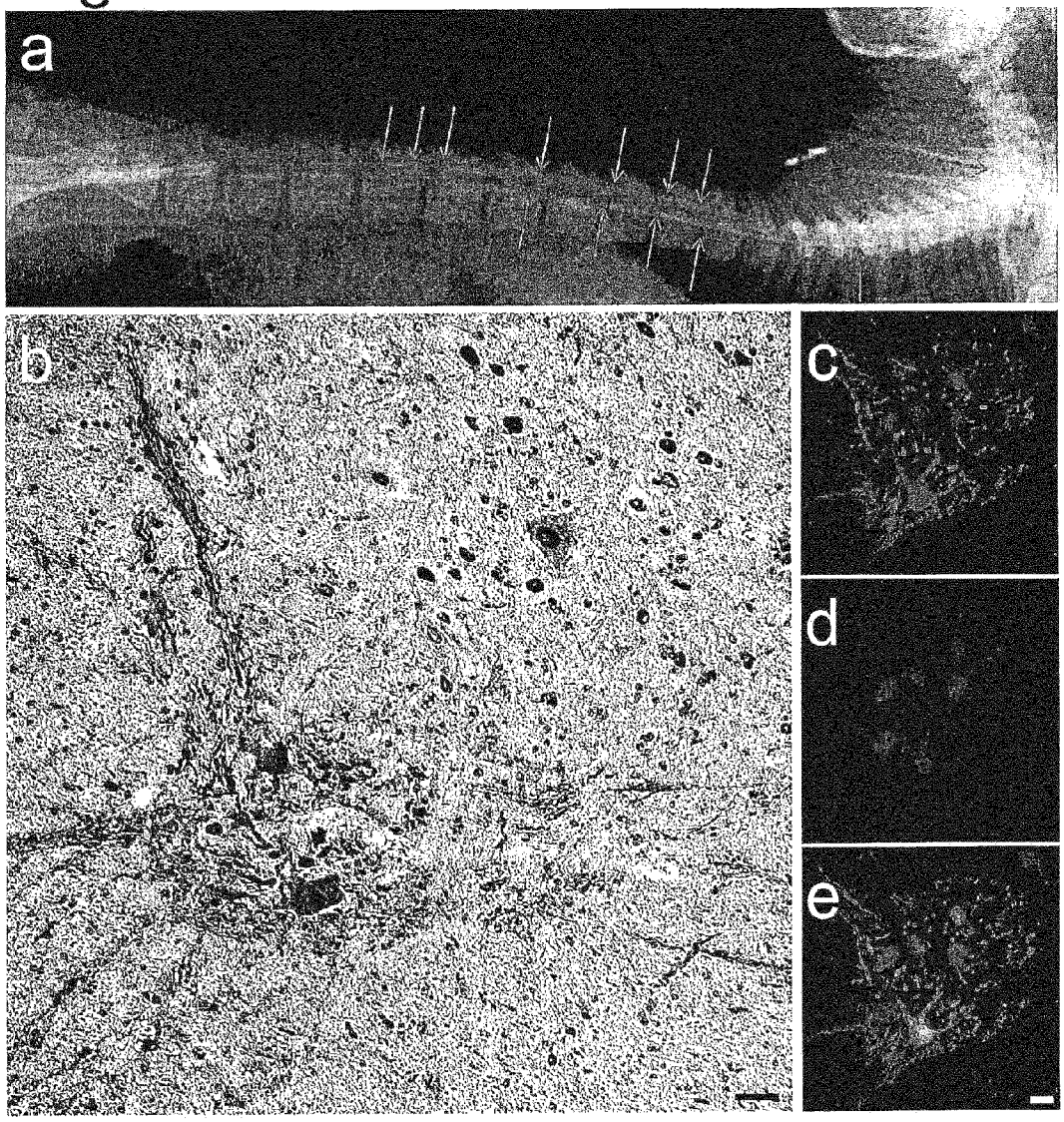

a
b
c
d
e
f
Control
Injected
SOD1
β-ACTIN
g
SOD1 Protein Levels
(Normalized to β-actin)
1.0
0.5
0.0
Control
Injected
h
SOD1 RNA Levels
1.5
1.0
0.5
0.0
Control Motor Neurons
Injected Motor Neurons
Control Non-Neuron
Injected Non-Neuron

Figure 8

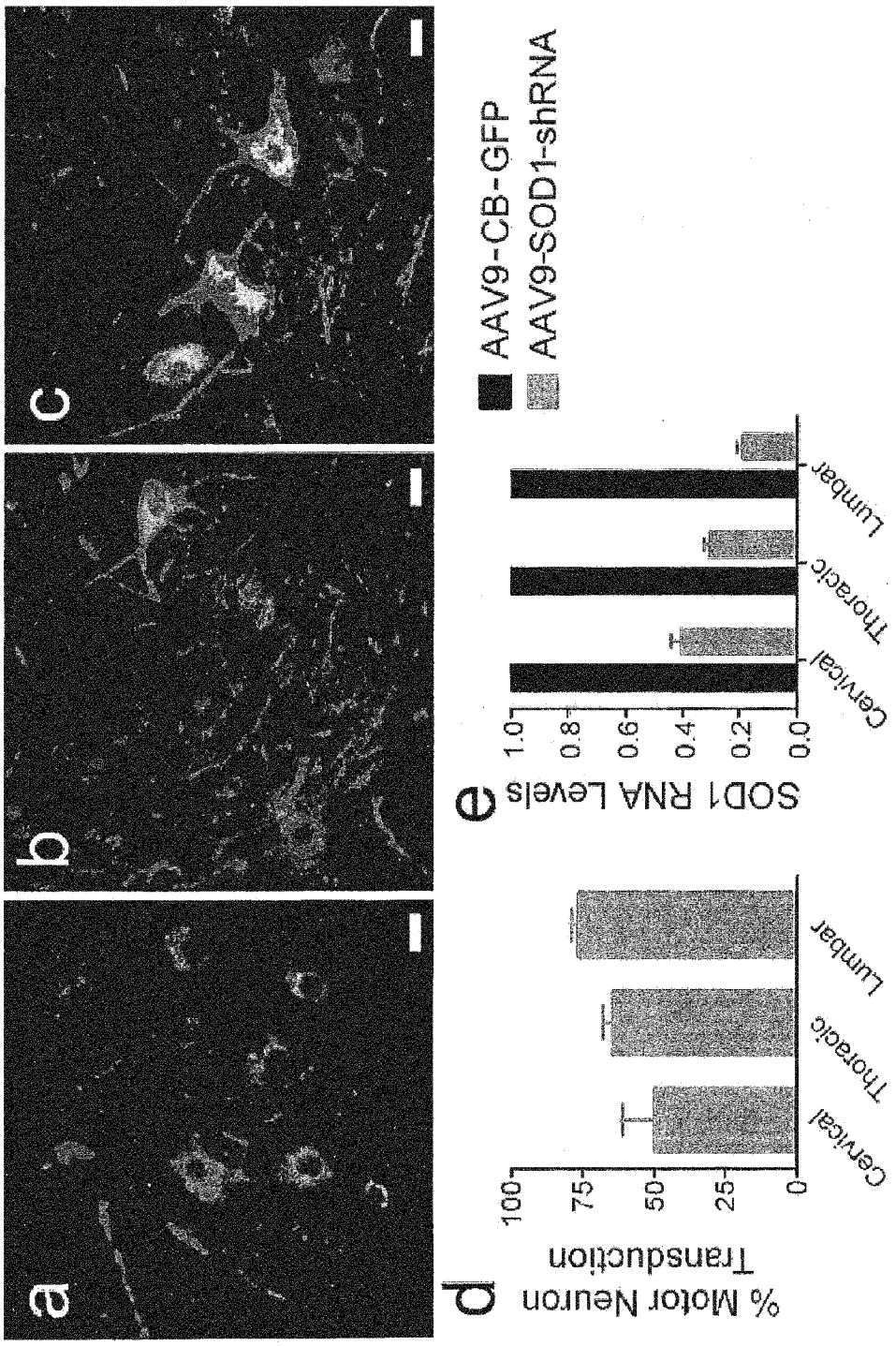

a
b
c
d
% Motor Neuron Transduction
100
75
50
25
0
Cervical
Thoracic
Lumbar
e
SOD1 RNA Levels
1.0
0.8
0.6
0.4
0.2
0.0
AAV9-CB-GFP
AAV9-SOD1-shRNA

Figure 9

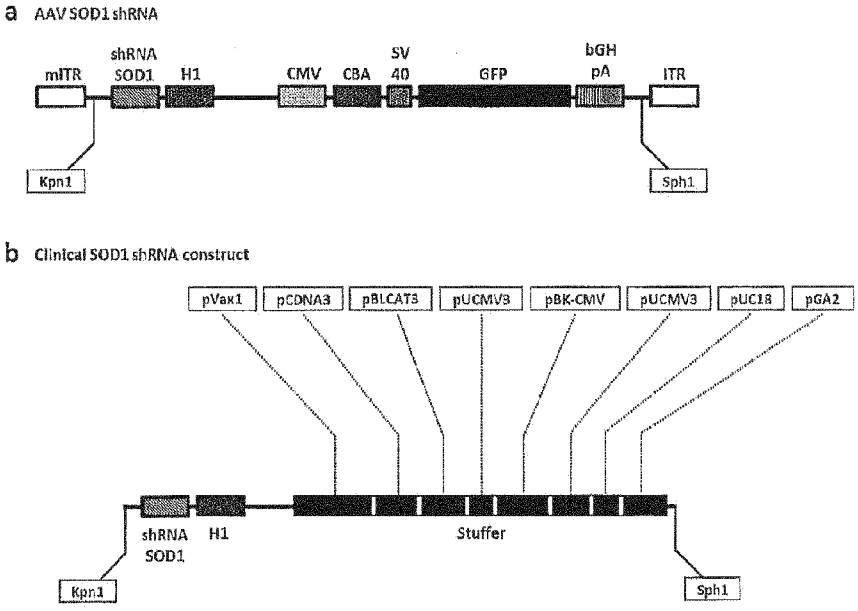

a AAV SOD1 shRNA
mITR
shRNA SOD1
H1
CMV
CBA
SV 40
GFP
bGH pA
ITR
Kpn1
Sph1
b Clinical SOD1 shRNA construct
pVax1
pCDNA3
pBLCAT3
pUCMV3
pBK-CMV
pUCMV3
pUC18
pGA2
shRNA SOD1
H1
Stuffer
Kpn1
Sph1

Figure 10

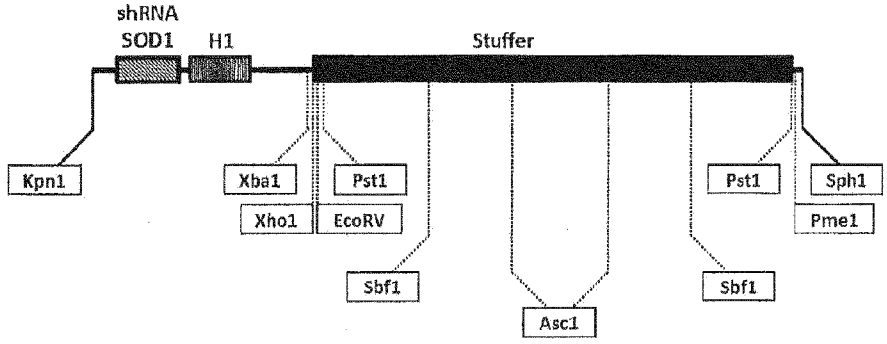

shRNA
SOD1
H1
Stuffer
Kpn1
Xba1
Xho1
Pst1
EcoRV
Sbf1
Asc1
Pst1
Sbf1
Sph1
Pme1

Figure 11

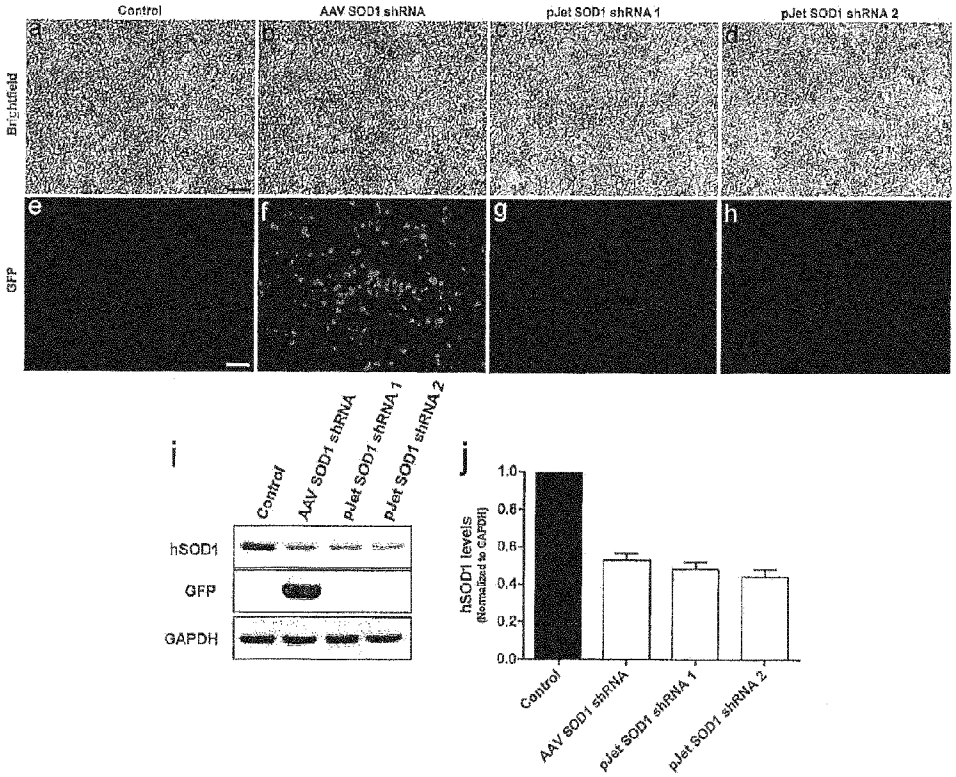

Control
AAV SOD1 shRNA
pJet SOD1 shRNA 1
pJet SOD1 shRNA 2
Brightfield
GFP
a
b
c
d
e
f
g
h
i
Control
AAV SOD1 shRNA
pJet SOD1 shRNA 1
pJet SOD1 shRNA 2
hSOD1
GFP
GAPDH
j
hSOD1 levels
(Normalized to GAPDH)
1.0
0.8
0.6
0.4
0.2
0.0
Control
AAV SOD1 shRNA
pJet SOD1 shRNA 1
pJet SOD1 shRNA 2

Figure 12

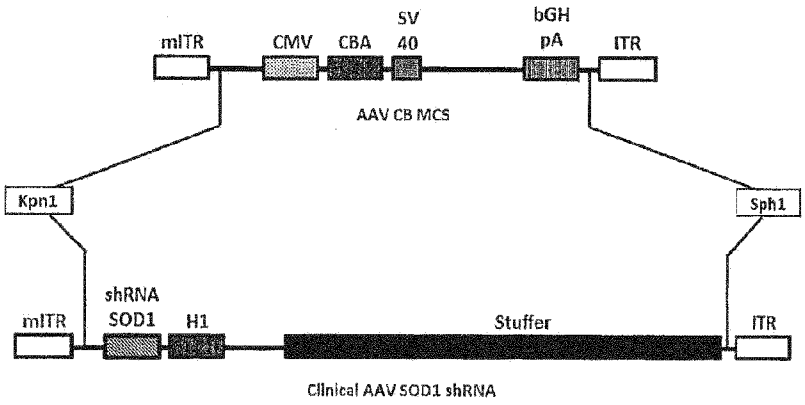
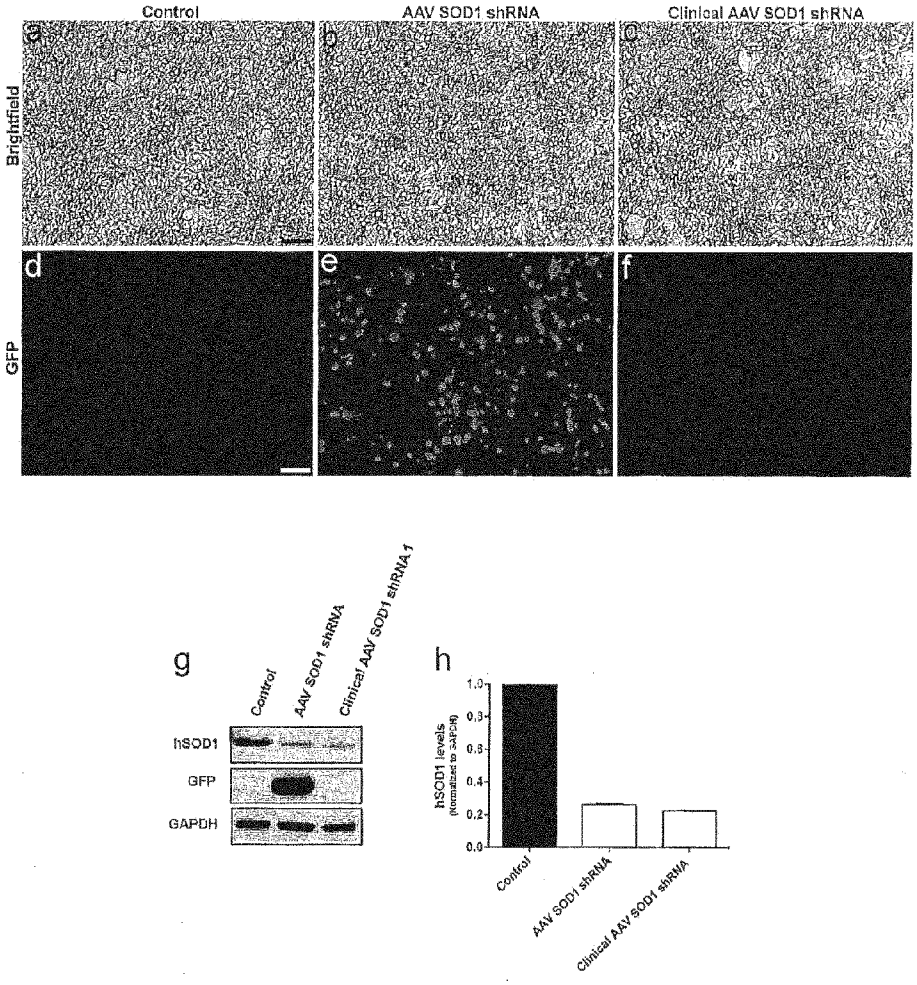
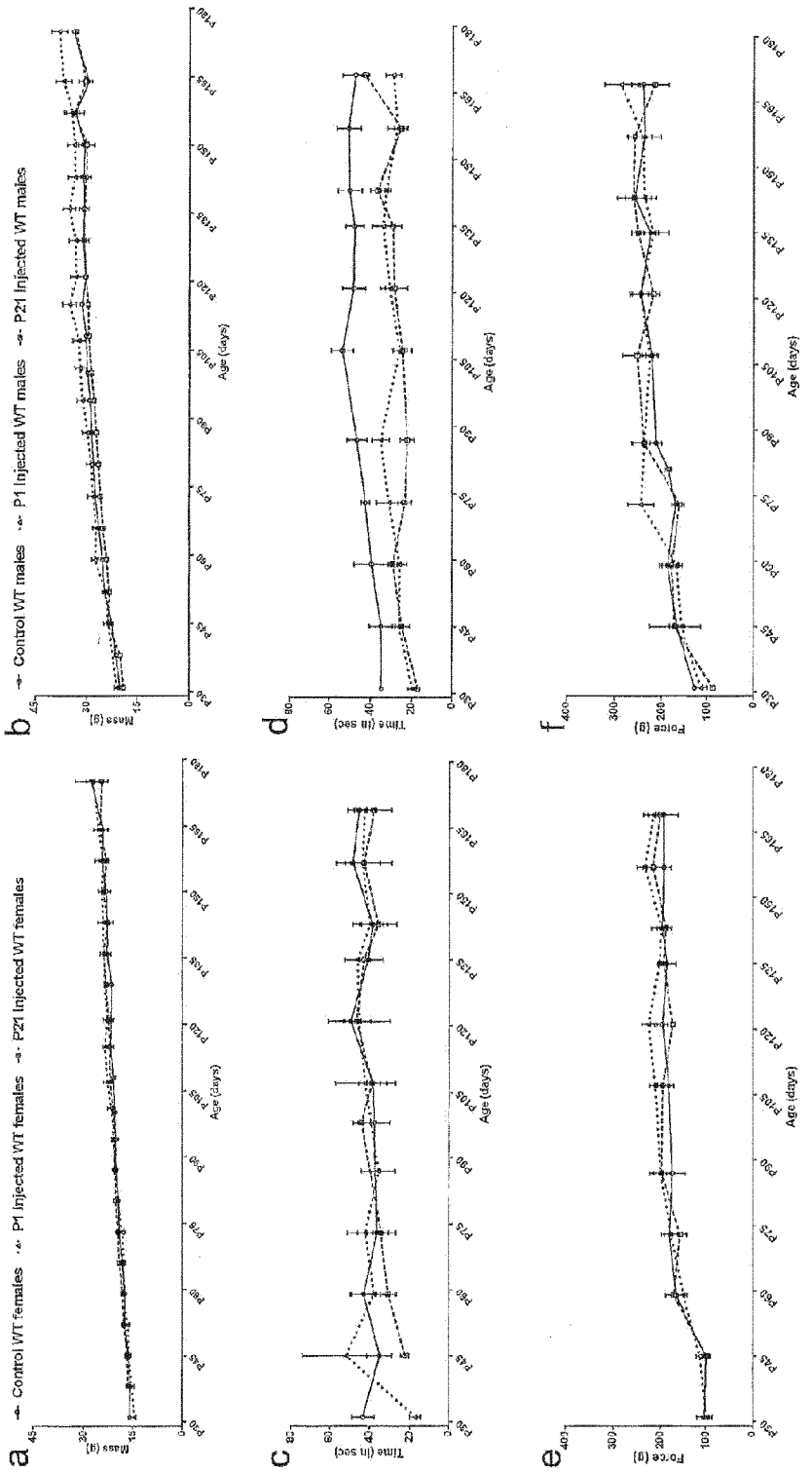

mITR
CMV
CBA
SV
40
bGH
pA
ITR
AAV CB MCS
Kpn1
Sph1
mITR
shRNA
SOD1
H1
Stuffer
ITR
Clinical AAV SOD1 shRNA

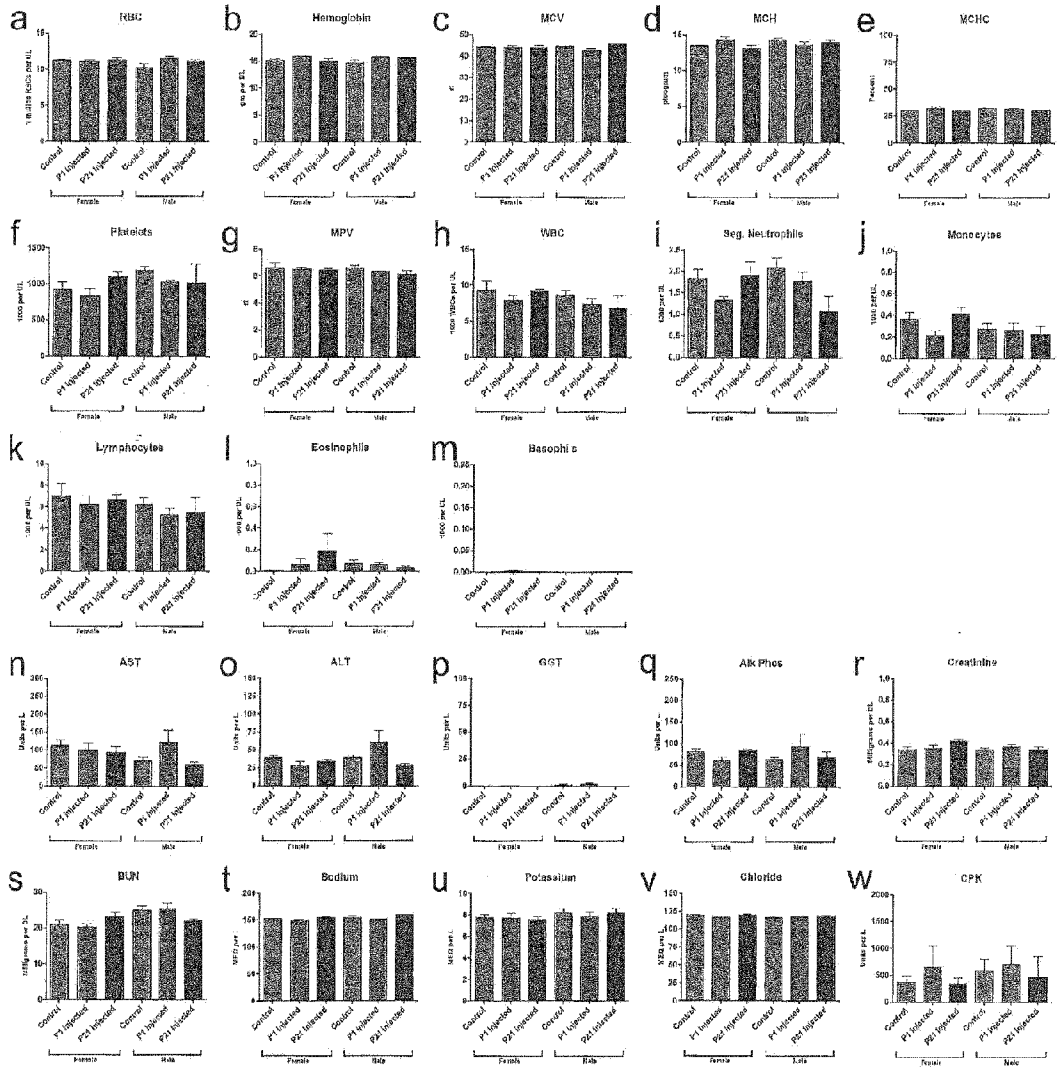
Figure 13


Control
AAV SOD1 shRNA
Clinical AAV SOD1 shRNA
Brightfield
GFP
a
b
c
d
e
f
g
Control
AAV SOD1 shRNA
Clinical AAV SOD1 shRNA 1
hSOD1
GFP
GAPDH
h
hSOD1 levels
(Normalized to GAPDH)
1.0
0.8
0.6
0.4
0.2
0.0
Control
AAV SOD1 shRNA
Clinical AAV SOD1 shRNA

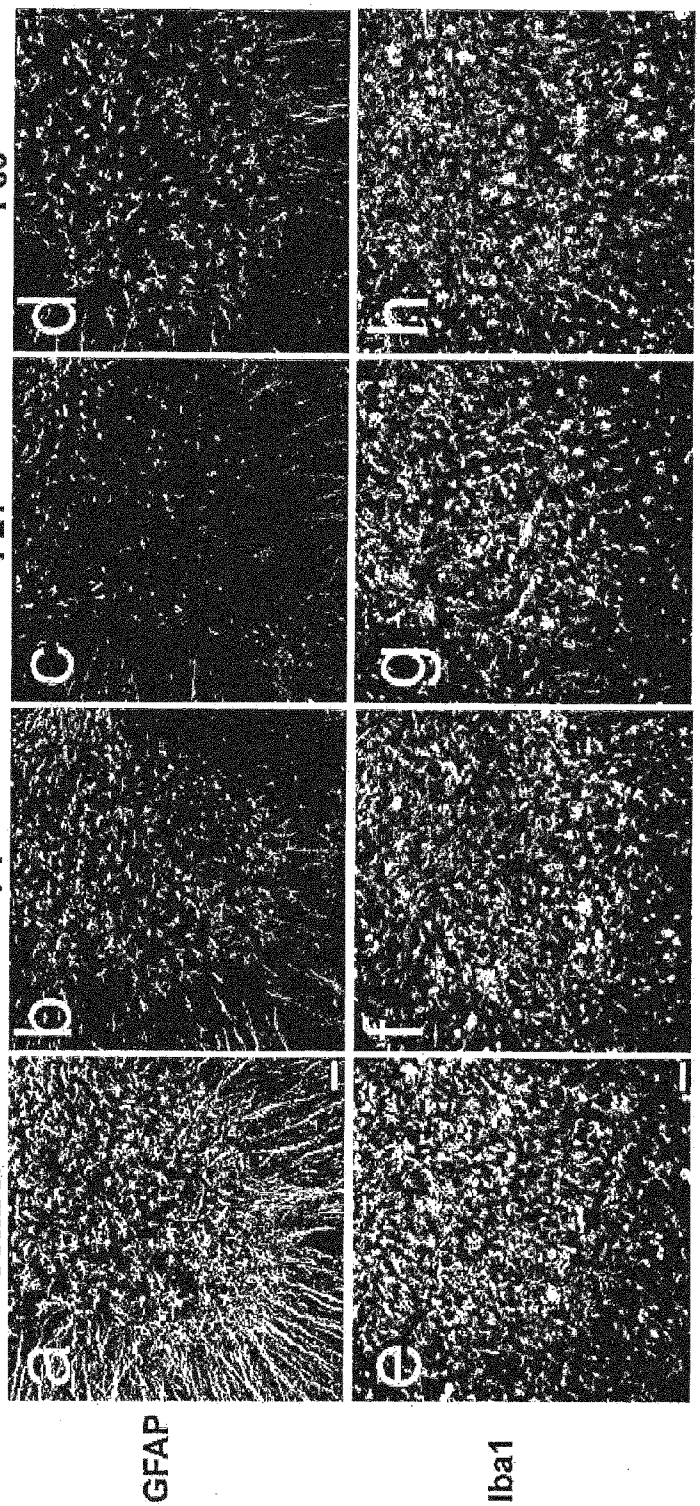
Supplementary Figure 1



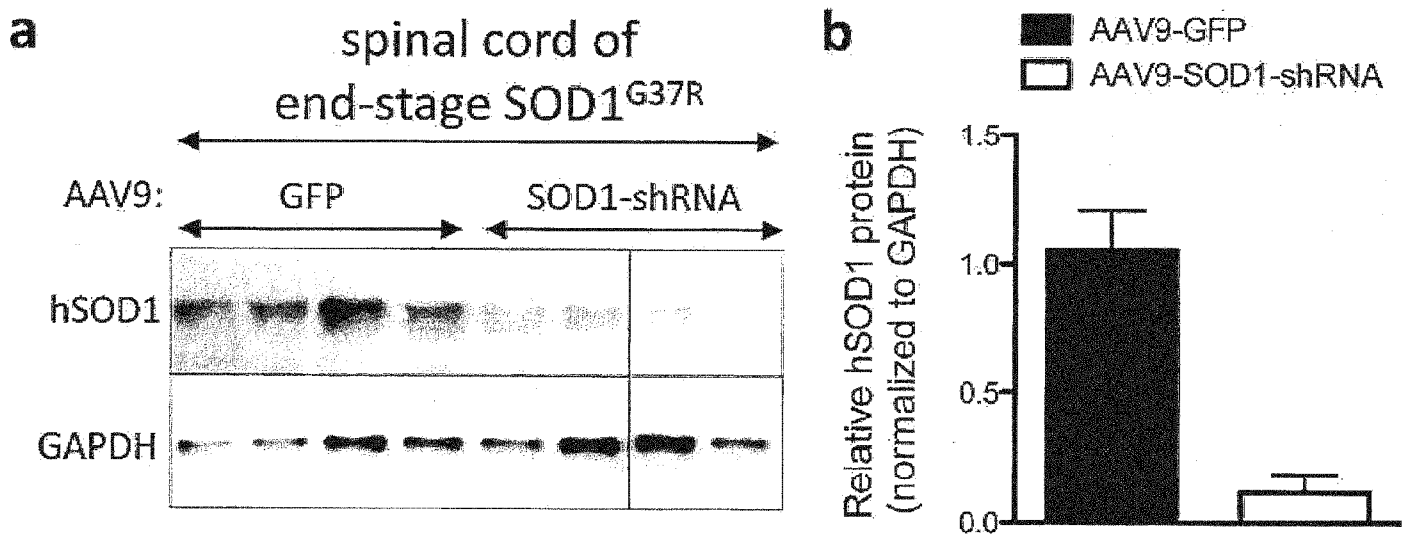
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4

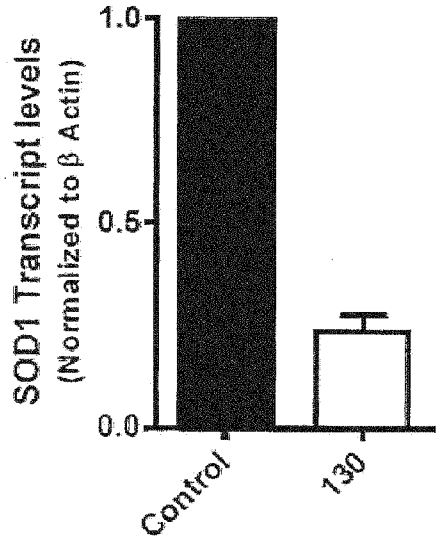


Supplementary Figure 5

a

Human 130-CATGGATTCCATGTTCATGA-149
Monkey .....C.

b



SEKVENSLISTE

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