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(57) Abstract: A gene therapy vector comprising an expression cassette coding for a mammalian apolipoprotein E that has a residue other than arginine at at least one of positions 112, 136, or 158, but is not a mammalian apolipoprotein E that has R112, R136 and R158 or a mammalian apolipoprotein E that has C112, R136 and C158, or coding for an antibody that binds to APOE4 or disrupts the binding of APOE to heparan sulfate proteoglycans, and methods of using the vector, are provided.

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APOE GENE THERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of the filing date of U.S. application No. 62/939999, filed on November 25, 2019, the disclosure of which is incorporated by reference herein.

BACKGROUND

Alzheimer's disease (AD) directly affects 5 million Americans and is rapidly increasing in prevalence and economic impact. Existing drugs have little impact on the underlying disease process and no preventive therapies are currently available. Inheritance of the variant APOE4 conveys a high risk for the development of AD, while inheritance of APOE2 gene is protective, reducing the risk of developing AD by ~50% and delaying the age of onset. APOE4 is associated with increased brain amyloid load and greater memory impairment in AD. Conversely, APOE2 attenuates these effects. Previous studies have shown that adeno-associated virus (AAV) gene delivery of the human APOE2 coding sequence to the CNS of a murine model expressing human APOE4 reduces the amount of amyloid-ß peptide and amyloid burden (Zhao et al. 2016). In humans, the odds ratio of developing AD with E4/E4 homozygous genotype is 14.9 and is reduced to 2.6 in E2/E4 heterozygotes. Recent reports have suggested that APOE4 is associated with abnormal brain function apart from its role in promoting amyloid production.

SUMMARY

The disclosure provides for a gene therapy vector comprising an expression cassette coding for a mammalian apolipoprotein E (APOE) that is protective or includes a substitution, e.g., relative to APOE4, in a region that binds a lipoprotein receptor, e.g., LDLR, or binds heparan sulfate proteoglycan (HSPG), e.g., a region such as that from position 135 to 151. In one embodiment, the mammalian APOE in the gene therapy vector has a residue other than arginine, histidine or lysine at at least one of positions 112, 136, or 158. In one embodiment, the mammalian APOE in the gene therapy vector has a residue other than arginine at positions 112 and 136. In one embodiment, the mammalian APOE in the gene therapy vector has a residue other than arginine at positions 136 and 158. In one embodiment, the mammalian APOE in the gene therapy vector has a residue other than arginine at positions 112, 136 and 158. In one embodiment, the apolipoprotein E is a human apolipoprotein E. In one embodiment, the residue other than arginine is serine, leucine, valine, glycine, isoleucine, alanine, threonine, asparagine, cysteine, or methionine. In one embodiment, the residue other than arginine is serine, threonine, asparagine, cysteine, or glutamine. In one embodiment, position 112 has a cysteine, methionine, valine, threonine, alanine, serine, arginine, glycine or isoleucine. In one embodiment, position 112 has a cysteine. In one embodiment, position 136 has a threonine, cysteine, methionine, arginine or a serine. In one embodiment, position 136 has a serine. In one embodiment, position 158 has a cysteine, methionine, valine, threonine, alanine, serine, arginine, glycine or isoleucine. In one embodiment, position 158 has an arginine or a cysteine. In one embodiment, two of positions 112, 136 or 158 have an arginine. In one embodiment, position 112 does not have an arginine. In one embodiment, position 136 does not have an arginine. In one embodiment, position 158 does not have an arginine. In one embodiment, the mammalian APOE in the gene therapy vector has a Cys at position 112, a Ser at position 136 and a Cys at position 158. In one embodiment, the mammalian APOE in the gene therapy vector has a Cys at position 112, a Ser at

position 136 and a Arg at position 158. In one embodiment, the gene therapy vector is a viral gene therapy vector. In one embodiment, the gene therapy vector is an adenovirus, adeno-associated virus (AAV), retrovirus or lentivirus vector. In one embodiment, the viral gene therapy vector is a rAAV vector. In one embodiment, the AAV vector is pseudotyped, e.g., with AAVrh.10, AAV8, AAV9, AAV5, AAVhu.37, AAVhu.20, AAVhu.43, AAVhu.8, AAVhu.2, or AAV7 capsid. In one embodiment, the AAV vector is pseudotyped with AAVrh.10, AAV8, or AAV5. In one embodiment, the AAV vector is AAV2, AAV5, AAV7, AAV8, AAV9 or AAVrh.10. In one embodiment, the gene therapy vector further comprises a nucleotide sequence having RNAi sequences corresponding to APOE4 for inhibition of APOE4 mRNA. In one embodiment, a second gene therapy vector comprises a nucleotide sequence having RNAi sequences corresponding to APOE4 for inhibition of APOE4 mRNA. In one embodiment, the nucleotide sequence is linked to a second promoter. In one embodiment, the second promoter is a PolIII promoter. In one embodiment, the RNAi comprises miRNA including a plurality of miRNA sequences. In one embodiment, the RNAi comprises siRNA including a plurality of siRNA sequences. In one embodiment, the vector is a plasmid. In one embodiment, the mammalian apolipoprotein gene in the vector has C112, S136 and R158 or a C112, S136 and C158. In one embodiment, a second gene therapy vector comprises a nucleotide sequence having nucleic acid sequences encoding an anti-APOE4 or anti-heparan antibody.

Also provided is a pharmaceutical composition comprising the gene therapy vector. In one embodiment, the vector is a viral vector. In one embodiment, the vector is a rAAV vector. In one embodiment, the amount of the vector in the composition is about 1×10^{11} to about 1×10^{16} genome copies. In one embodiment, the amount of the vector in the composition is about 1×10^{12} to about 1×10^{15} genome copies. In one embodiment, the amount of the vector in the composition is about 1×10^{11} to about 1×10^{13} genome copies. In one embodiment, the amount of the vector in the composition is about 1×10^{13} to about 1×10^{15} genome copies. In one embodiment, the pharmaceutical composition further comprises a pharmaceutically acceptable carrier.

Further provided is a method to prevent, inhibit or treat Alzheimer's disease including preventing or treating one or more symptoms thereof such as dementia, or other tauopathy or cognitive impairment in a mammal. In one embodiment, prior to administration, the mammal has or is at risk of increased amyloid plaque and/or increased tau in the brain, e.g., relative to an age-matched mammal or a mammal with a decreased risk of Alzheimer's disease or other tauopathy or cognitive impairment. In one embodiment, the mammal has cognitive impairment. The method includes administering to the mammal an effective amount of a composition comprising the gene therapy vector. In one embodiment, the composition comprises liposomes comprising the vector. In one embodiment, the composition comprises nanoparticles comprising the vector. In one embodiment, the mammal is a E2/E4 heterozygote. In one embodiment, the mammal is a E4/E4 homozygote. In one embodiment, the mammal is a E3/E4 heterozygote. In one embodiment, the mammal is a E2/E3 heterozygote. In one embodiment, the mammal is a human. In one embodiment, the human is an adult, e.g., over the age of 20 years old, e.g., at least 40, 50 or 60 years old. In one embodiment, the human is a juvenile, e.g., from 10 to 20 years old. In one embodiment, the human is a child, e.g., under the age of 10 years old. In one embodiment, the composition is administered to a human at birth or within 1, 2, 3, 4 or 5 years of birth. In one embodiment, the composition is systemically administered. In one embodiment, the composition is orally administered. In one embodiment, the composition is intravenously administered. In one embodiment, the composition is locally administered. In one embodiment, the composition is injected. In one embodiment, the composition

is administered to the central nervous system. In one embodiment, the composition is administered to the brain. In one embodiment, the composition is a sustained release composition. In one embodiment, administration of the gene therapy vector is effective to prevent or inhibit accumulation of amyloid plaques. In one embodiment, administration of the gene therapy vector is effective to prevent or inhibit tau pathology. In one embodiment, administration of the gene therapy vector is effective to prevent or inhibit accumulation of amyloid plaques and prevent or inhibit tau pathology.

In addition, a method to prevent, inhibit or treat a disease associated with APOE4 expression in a mammal is provided. The method includes administering to the mammal an effective amount of a composition comprising the gene therapy vector. In one embodiment, the composition comprises liposomes comprising the vector. In one embodiment, the composition comprises nanoparticles comprising the vector. In one embodiment, the mammal is a E2/E4 heterozygote. In one embodiment, the mammal is a E4/E4 homozygote. In one embodiment, the mammal is a E3/E4 heterozygote. In one embodiment, the mammal is a E4/E4 homozygote. In one embodiment, the mammal is a human. In one embodiment, the composition is systemically administered. In one embodiment, the composition is orally administered. In one embodiment, the composition is intravenously administered. In one embodiment, the composition is locally administered. In one embodiment, the composition is injected. In one embodiment, the composition is administered to the central nervous system. In one embodiment, the composition is administered to the brain. In one embodiment, the composition is a sustained release composition.

A method to reduce binding to heparin sulfate proteoglycans in a mammal is provided that includes administering to the mammal an effective amount of a composition comprising the gene therapy vector. In one embodiment, the composition comprises liposomes comprising the vector. In one embodiment, the composition comprises nanoparticles comprising the vector. In one embodiment, the mammal is a E2/E4 heterozygote. In one embodiment, the mammal is a E4/E4 homozygote. In one embodiment, the mammal is a E3/E4 heterozygote. In one embodiment, the mammal is a E4/E4 homozygote. In one embodiment, the mammal is a human. In one embodiment, the composition is systemically administered. In one embodiment, the composition is orally administered. In one embodiment, the composition is intravenously administered. In one embodiment, the composition is locally administered. In one embodiment, the composition is injected. In one embodiment, the composition is administered to the central nervous system. In one embodiment, the composition is administered to the brain. In one embodiment, the composition is a sustained release composition.

In one embodiment, the composition is administered via a catheter. In one embodiment, the composition is intraventricularly administered. In one embodiment, the composition is intracranially administered. In one embodiment, the composition is administered to the lumbar region. In one embodiment, the composition is administered to the cisterna magna. In one embodiment, the composition is administered via burr holes to the brain. In one embodiment, the composition is administered below C1-C2.

In one embodiment, the composition is administered so that expression of the encoded gene product, e.g., modified APOE or an antibody that binds heparan, is similar to expression levels of APOE4 in the mammal. In one embodiment, the ratio of secreted vector encoded gene product to secreted APOE4 in the mammal is 1:1. In one embodiment, the ratio of secreted vector encoded gene product to secreted APOE4 in the mammal is 2:1. In one embodiment, the ratio of secreted vector encoded gene product to secreted APOE4 in the mammal is 0.5:1. In one embodiment, the ratio of secreted vector

encoded gene product to secreted APOE4 in the mammal is 0.1:1. In one embodiment, the ratio of secreted vector encoded gene product to secreted APOE4 in the mammal is 3:1. In one embodiment, the levels of expression of the encoded gene product in the mammal prevent or inhibit cognitive decline (deterioration). In one embodiment, the levels of expression of the encoded gene product in the mammal reduce tau tangles and amyloid accumulation.

A method to prevent, inhibit or treat a lipid disorder in a mammal is provided that includes administering to the mammal an effective amount of a composition comprising the gene therapy vector. In one embodiment, the composition comprises liposomes comprising the vector. In one embodiment, the composition comprises nanoparticles comprising the vector. In one embodiment, the mammal is a E2/E4 heterozygote. In one embodiment, the mammal is a E4/E4 homozygote. In one embodiment, the mammal is a E3/E4 heterozygote. In one embodiment, the mammal is a E4/E4 homozygote. In one embodiment, the mammal is a human. In one embodiment, the composition is systemically administered. In one embodiment, the composition is orally administered. In one embodiment, the composition is intravenously administered. In one embodiment, the composition is locally administered. In one embodiment, the composition is injected. In one embodiment, the composition is administered to the central nervous system. In one embodiment, the composition is administered to the brain. In one embodiment, the composition is a sustained release composition.

In one embodiment, a gene therapy vector comprises an AAV expression vector encoding a human APOE gene modified as described herein and either in *cis* or in *trans* artificial miRNA(s) that target endogenous APOE4. This vector system silences the expression of detrimental endogenous APOE4 in combination with supplementation of the beneficial APOE gene from a gene therapy vector, e.g., an AAV vector. Exemplary microRNA sequences were incorporated in the CAG promoter intron or polyA tail of the vector transgene plasmid coding for the human APOE coding sequence. Alternatively, the microRNA(s) may be inserted between a PolIII promoter, e.g., a U6 promoter, and terminator following the polyA site of the APOE expression cassette. The vector-derived human APOE DNA sequence may include silent nucleotide changes to ensure that it is not suppressed by the microRNAs and can include a tag such as a HA tag for detection, e.g., for pre-clinical detection studies. In one embodiment, the expression construct is packaged into an AAV capsid of a serotype that targets astrocytes and glial cells (for example AAV9 but can be other vectors), the prominent sites of endogenous APOE expression in the CNS.

The vectors described herein may be employed to prevent, inhibit or treat cognitive impairment, dementia, Alzheimer's disease, e.g., autosomal dominant, late onset, or early onset. In one embodiment, the mammal has reduced fibrillar amyloid-beta plaque burden, reduced levels of paired helical filament tau, or decreased neurodegeneration, post-administration of the vector. In one embodiment, the vectors described herein may be employed to prevent, inhibit or treat cognitive impairment, e.g., associated with traumatic brain injury, stroke, transient ischemic attack, dementia, Creutzfeldt-Jakob disease, multiple sclerosis, prion disease, Pick's disease, corticobasal degeneration, Parkinson's disease, Lewy body dementia, Progressive supranuclear palsy; Dementia pugilistica (chronic traumatic encephalopathy); frontotemporal dementia and parkinsonism linked to chromosome 17; Lytico-Bodig disease; Tangle-predominant dementia; Ganglioglioma and gangliocytoma; Meningioangiomatosis; Subacute sclerosing panencephalitis; lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease, and lipofuscinosis; Argyrophilic grain disease; or Frontotemporal lobar degeneration.

The vectors described herein may also be employed to prevent, inhibit or treat one or more symptoms associated with hyperlipoproteinemia, e.g., Type I, Type 2, Type 3, Type 4 or Type 5. In one embodiment, the amount of vector administered decreases circulating cholesterol, e.g., decreases VLDL and/or LDL, decreases intermediate density lipoproteins, decreases plasma levels of cholesterol and/or triglycerides, decreases triglycerides containing VLDL, decreases lipid accumulation in the glomerular capillaries, decreases intraglomerular lipoprotein or decreases thrombi.

BRIEF DESCRIPTION OF FIGURES

Figure 1. APOE alleles, prevalence and associated risk of developing Alzheimer's disease.

Figure 2. Exemplary viral gene therapy.

Figure 3. Exemplary route of administration of gene therapy.

Figure 4. Selected amino acid residues for different APOE alleles, prevalence and associated risk of developing Alzheimer's disease.

Figure 5. Nucleotide residues at two positions in different APOE alleles.

Figure 6. APOE3ch mutation.

Figure 7. Impact of APOE3ch mutation.

Figure 8. Affinity of APOE3ch for heparin.

Figure 9. Possible mechanism for APOE or tau binding to HSPGs.

Figure 10. Exemplary gene therapy approaches to prevent, inhibit or treat diseases associated with expression of certain APOE alleles.

Figure 11. Association of APOE alleles with Dominant type III hyperlipoproteinemia and lipoprotein glomerulopathy.

Figure 12. Gene therapy with AAV-mediated delivery of APOE AD decreased risk variants. Based on the knowledge that APOE4 homozygotes have a 15-20-fold increased risk for AD (Reiman et al., 2020; Corder et al., 1993; Hefferman et al., 2016), a therapeutic strategy is employed using an AA V vector to deliver the coding sequence of APOE2 and APOE3 variants with the Christchurch variant to decrease the risk for AD.

Figure 13. APOE variants, the risk for AD, and proposed gene therapy to protect against APOE4. APOE2, 3 and 4 are common variants (Hefferman et al., 2016; ALXFORUM, 2010). APOE3ChC is the variant superimposed on PSEN 1-E290A in the case report of the Christchurch mutation on the APOE3 background protecting from the PSEN 1-E290A dominant variant causing early onset AD19. APOE3ChC and APOE2ChC variants may be therapies to mitigate the high risk of AD in APOE4 homozygotes. The APOE2ChC variant has not been observed in nature, but it may be more effective than APOE3ChC. *Low in the context of PSEN 1-E290A.

Figure 14. Studies supporting the relative effect of APOE3ChC in a female with the PSEN1-E280 mutation. Shown are quantitative data for mean cortical amyloid plaque burden (Pittsburgh Compound 8, PET), inferior temporal cortex PHF tau burden (Floratаucipir PET), hippocampal volume (MRI) and precuneus glucose metabolism (Fludeoxyglucose PET). Black dots- PSEN1 carriers with MCI onset at typical young age; grey dots- PSEN1 carriers that have not yet developed MCI; the subject with PSEN1 carrier + APOE3ChC variant (red dot with arrow). Figure modified from Arboleda-Velasquez et al. (2019).

Figure 15. The Christchurch mutation impairs heparin binding of APOE. ELISA was used to quantify differences in the NaCl elution pattern of different APOE isoforms from heparin columns. Figure taken from Arboleda-Velasquez et. al. (2019). Arrow designates the subject with the APOEChC variant.

Figure 16. AAVrh.10hAPOE2 vector. The AAVrh.10hAPOE2 vector expresses the human APOE2 transgene behind the constitutive CAG promoter, consisting of the cytomegalovirus (CMV) enhancer, chicken β actin promoter, splice donor and intron, and rabbit β-globin splice acceptor, and followed by the rabbit β-globin polyadenylation signal (A_{β}). The hAPOE2 expression cassette is flanked by the AAV2 inverted terminal repeats (ITR) and packaged into the rhesus adeno-associated viral vector serotype 10 (AAVrh.10) capsid. All APOE vectors used in this proposal are identical except for the APOE coding sequence. The AAVrh.10Null vector is identical but the expression cassette AAVrh.10hAPOE2 is replaced by a nontranslatable sequence.

Figure 17. Using AAVrh.10hAPOE2 as an example, shown is SDS polyacrylamide gel electrophoresis which shows the 3 bands associated with the 3 AAV vector capsid proteins that align with the expected molecular weights.

Figure 18. Assessment of the distribution of the APOE2 protein in the brain of nonhuman primates following intracisternal administration of AAVrh.10APOE2. NHPs administered AAVrh.10hAPOE2 ($5x10^{13}$ gc, APOE2 transgene) assessed 8 week post-therapy. The right hemispheres were subdivided into 1 cm^3 cubes and analyzed for protein APOE2 levels by ELISA. A. Human APOE2 protein in brain sections. B. CSF (10 µl/time point) was sampled from the 3 NHPs at 3 time points [pre-administration (day 0), and days 28 and 56 post-administration] and analyzed by SOS-PAGE followed by antibody detection of APOE2 by Western. See Rosenberg et al. (2018).

Figures 19A-19B. Resolution of amyloid pathology in the PDAPP mouse model. AAVrh.10hAPOE2 or the control AAVrh.10mCherry (10^{10} gc) were administered bilaterally into the hippocampus of 9-month old PDAPP mice. Eight week post-administration, the mice were sacrificed and the hippocampus of the left hemisphere was dissected, homogenized, and sequentially extracted with RIPA (representing soluble Aβ) and 5.5 M guanidine (representing insoluble Aβ). Aβ1-42, and Aβ1-40 levels were determined by ELISA. A) Tissue levels of Aβ1-42; and B) Tissue levels of Aβ1-40 in hippocampus of PDAPP mice injected with AAVrh.10hAPOE2 or AAVrh.10mCherry. n=10-12 animals per group. Data presented as mean± SD.* $p<0.05$, *** $p<0.001$. See Zhao et al. (2016).

Figures 20A-20B. Dose-dependent effects of intrahippocampal delivery of APOE2 on AP levels in the hippocampus of 2.5-month old APP.PS1/TRE4 mice. AAVrh.10hAPOE2 ($0.25x10^{10}$, $0.5x10^{10}$ or $1x10^{10}$ gc) or AAVrh.10mCherry ($1x10^{10}$ gc) were injected bilaterally into the hippocampus of 2.5-month old male APP.PS1/TRE4 mice. Eight-week post-administration, the mice were sacrificed. The hippocampus of the left hemisphere was dissected, homogenized, and sequentially extracted with RIPA (representing soluble Aβ) and 5.5 M guanidine (representing insoluble Aβ). Aβ1-42 levels in each extract were determined by ELISA. A) Insoluble Aβ1-42 levels (guanidine extractable) in hippocampus. B) Soluble Aβ1-42 levels (RIPA extractable) in hippocampus. n=3-5 animals per group. Data presented as mean± SD; *$p < 0.05$, *** $p<0.001$. Zhao et al. (2016).

Figures 21A-21B.Intrathalamic delivery of AAV9-hAPOE2 on APOE and Aβ levels in the brain in APP.PS1/TRE4 mice. Ten wk old mice were administered AAV9-hAPOE2 or AAV9-GFP by bilateral intrathalamic injection (10^{10} gc) and sacrificed after 8 wk. Thalamus, hippocampus, and cerebral cortex

from the left hemisphere were dissected and homogenized. A) APOE and B) Aβ1-42 levels were quantified by ELISA. Adapted from Zhao et al.

Figure 22. Knockdown of APOE *in vitro*. U87 human astroglioma cells were transfected with siRNAs targeting APOE or non-targeting (NT) control (5 pmol). Cells were collected 72 hr later and endogenous *APOE* mRNA levels were quantified by RT-qPCR. Four different siRNAs targeting the coding sequence of APOE were generated based on a comparison of multiple siRNA design algorithms. The identified sequences were as follows: GGUGGAGCAAGCGGUGGAGuu (SEQ ID NO:20); GGAGUUGAAGGCCUACAAAuu (SEQ ID NO:21); GGAAGACAUGCAGCGCCAGuu (SEQ ID NO:22); and GCGCGCGGAUGGAGGAGAUuu (SEQ ID NO:23).

Figures 23A-23B. hAPOE2-mirAPOE4 expression cassette. A) Schematic of mirAPOE4. The siRNA targeting sequence (guide strand) and antisense sequence with selected mismatches (passenger strand) to facility processing are incorporated into the enhanced mir155 backbone. B) Schematic of the AAV9-hAPOE2-mirAPOE4 expression construct. The construct contains the human *APOE2* coding sequence fused to a hemagglutinin tag (HA) behind the constitutive CAG promoter [cytomegalovirus (CMV) enhancer, chicken β actin promoter, splice donor and intron, and rabbit β-globin splice acceptor], and followed by the rabbit β-globin polyadenylation (polyA) signal. mirAPOE4 tandem repeats are inserted into either the CAG intron and/or between the transgene stop codon and the polyA sequence. The hAPOE2 expression cassette is flanked by the AAV2 inverted terminal repeats (ITR) and packaged into the AAV9 capsid. A miR from siRNA#2 may be employed, e.g.,
CTGGAGGCTTGCTGAAGGCTGTATGCTGATTTGTAGGCCTTCAACTCCT GTTTTGGCCACTGACTGACAGGAGTGAGGCCTACAAATCAGGACACAAGGCCTGTTACTAGCACTCA CATGGAACAAATGGCC (SEQ ID NO:24);
CTGGAGGCTTGCTTTGGGCTGTATGCTGATTTGTAGGCCTTCAACTCCTGTTTTGGCCACTGACTGA CAGGAGTTGAAGTCACAAATCAGGACACAAGGCCCTTTATCAGCACTCACATGGAACAAATGGCCAC CGTGGGAGGATGACAA (SEQ ID NO:25); or
CTGGAGGCTTGCTTTGGGCTGTATGCTGTTCCGATTTGTAGGCCTTCAAGTTTTGGCCACTGACTGA CTTGAAGTCACAAATCGGAACAGGACACAAGGCCCTTTATCAGCACTCACATGGAACAAATGGCCAC CGTGGGAGGATGACAA (SEQ ID NO:26).

Figure 24. Testing the knockdown efficiency of miRs located in the intron and polyA.

Figure 25. Luciferase results for knock-down efficiency.

Figure 26. Testing APOE Target Site Only Control

Figure 27. Identifying the miRNA target site in the vector-derived APOE2 with silent nucleotide changes.

Figure 28. Generating pAAV-miRNA-APOE-HA.

Figure 29. *In vivo* studies.

Figure 30. Luciferase expression in controls.

Detailed Description

In the following description, reference is made to the accompanying drawings that form a part hereof, and in which is shown by way of illustration specific embodiments which may be practiced. These embodiments are described in detail to enable those skilled in the art to practice the invention, and it is to be understood that other embodiments may be utilized and that logical changes may be made without

departing from the scope of the present invention. The following description of example embodiments is, therefore, not to be taken in a limited sense, and the scope of the present invention is defined by the appended claims.

DETAILED DESCRIPTION

Definitions

A "vector" refers to a macromolecule or association of macromolecules that comprises or associates with a polynucleotide, and which can be used to mediate delivery of the polynucleotide to a cell, either *in vitro* or *in vivo*. Illustrative vectors include, for example, plasmids, viral vectors, liposomes and other gene delivery vehicles. The polynucleotide to be delivered, sometimes referred to as a "target polynucleotide" or "transgene," may comprise a coding sequence of interest in gene therapy (such as a gene encoding a protein of therapeutic interest), a coding sequence of interest in vaccine development (such as a polynucleotide expressing a protein, polypeptide or peptide suitable for eliciting an immune response in a mammal), and/or a selectable or detectable marker.

"Transduction," "transfection," "transformation" or "transducing" as used herein, are terms referring to a process for the introduction of an exogenous polynucleotide into a host cell leading to expression of the polynucleotide, e.g., the transgene in the cell, and includes the use of recombinant virus to introduce the exogenous polynucleotide to the host cell. Transduction, transfection or transformation of a polynucleotide in a cell may be determined by methods well known to the art including, but not limited to, protein expression (including steady state levels), e.g., by ELISA, flow cytometry and Western blot, measurement of DNA and RNA by hybridization assays, e.g., Northern blots, Southern blots and gel shift mobility assays. Methods used for the introduction of the exogenous polynucleotide include well-known techniques such as viral infection or transfection, lipofection, transformation and electroporation, as well as other non-viral gene delivery techniques. The introduced polynucleotide may be stably or transiently maintained in the host cell.

"Gene delivery" refers to the introduction of an exogenous polynucleotide into a cell for gene transfer, and may encompass targeting, binding, uptake, transport, localization, replicon integration and expression.

"Gene transfer" refers to the introduction of an exogenous polynucleotide into a cell which may encompass targeting, binding, uptake, transport, localization and replicon integration, but is distinct from and does not imply subsequent expression of the gene.

"Gene expression" or "expression" refers to the process of gene transcription, translation, and post-translational modification.

An "infectious" virus or viral particle is one that comprises a polynucleotide component which it is capable of delivering into a cell for which the viral species is trophic. The term does not necessarily imply any replication capacity of the virus.

The term "polynucleotide" refers to a polymeric form of nucleotides of any length, including deoxyribonucleotides or ribonucleotides, or analogs thereof. A polynucleotide may comprise modified nucleotides, such as methylated or capped nucleotides and nucleotide analogs, and may be interrupted by non-nucleotide components. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The term polynucleotide, as used herein, refers interchangeably to double- and single-stranded molecules. Unless otherwise specified or required, any embodiment

described herein that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

An "isolated" polynucleotide, e.g., plasmid, virus, polypeptide or other substance refers to a preparation of the substance devoid of at least some of the other components that may also be present where the substance or a similar substance naturally occurs or is initially prepared from. Thus, for example, an isolated substance may be prepared by using a purification technique to enrich it from a source mixture. Isolated nucleic acid, peptide or polypeptide is present in a form or setting that is different from that in which it is found in nature. For example, a given DNA sequence (e.g., a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs that encode a multitude of proteins. The isolated nucleic acid molecule may be present in single-stranded or double-stranded form. When an isolated nucleic acid molecule is to be utilized to express a protein, the molecule will contain at a minimum the sense or coding strand (i.e., the molecule may single-stranded), but may contain both the sense and anti-sense strands (i.e., the molecule may be double-stranded). Enrichment can be measured on an absolute basis, such as weight per volume of solution, or it can be measured in relation to a second, potentially interfering substance present in the source mixture. Increasing enrichments of the embodiments of this invention are envisioned. Thus, for example, a 2-fold enrichment, 10-fold enrichment, 100-fold enrichment, or a 1000-fold enrichment.

A "transcriptional regulatory sequence" refers to a genomic region that controls the transcription of a gene or coding sequence to which it is operably linked. Transcriptional regulatory sequences of use in the present invention generally include at least one transcriptional promoter and may also include one or more enhancers and/or terminators of transcription.

"Operably linked" refers to an arrangement of two or more components, wherein the components so described are in a relationship permitting them to function in a coordinated manner. By way of illustration, a transcriptional regulatory sequence or a promoter is operably linked to a coding sequence if the TRS or promoter promotes transcription of the coding sequence. An operably linked TRS is generally joined in *cis* with the coding sequence, but it is not necessarily directly adjacent to it.

"Heterologous" means derived from a genotypically distinct entity from the entity to which it is compared. For example, a polynucleotide introduced by genetic engineering techniques into a different cell type is a heterologous polynucleotide (and, when expressed, can encode a heterologous polypeptide). Similarly, a transcriptional regulatory element such as a promoter that is removed from its native coding sequence and operably linked to a different coding sequence is a heterologous transcriptional regulatory element.

A "terminator" refers to a polynucleotide sequence that tends to diminish or prevent read-through transcription (i.e., it diminishes or prevent transcription originating on one side of the terminator from continuing through to the other side of the terminator). The degree to which transcription is disrupted is typically a function of the base sequence and/or the length of the terminator sequence. In particular, as is well known in numerous molecular biological systems, particular DNA sequences, generally referred to as "transcriptional termination sequences" are specific sequences that tend to disrupt read-through transcription by RNA polymerase, presumably by causing the RNA polymerase molecule to stop and/or disengage from the DNA being transcribed. Typical example of such sequence-specific terminators include polyadenylation ("polyA") sequences, e.g., SV40 polyA. In addition to or in place of such

sequence-specific terminators, insertions of relatively long DNA sequences between a promoter and a coding region also tend to disrupt transcription of the coding region, generally in proportion to the length of the intervening sequence. This effect presumably arises because there is always some tendency for an RNA polymerase molecule to become disengaged from the DNA being transcribed, and increasing the length of the sequence to be traversed before reaching the coding region would generally increase the likelihood that disengagement would occur before transcription of the coding region was completed or possibly even initiated. Terminators may thus prevent transcription from only one direction ("uni-directional" terminators) or from both directions ("bi-directional" terminators), and may be comprised of sequence-specific termination sequences or sequence-non-specific terminators or both. A variety of such terminator sequences are known in the art; and illustrative uses of such sequences within the context of the present invention are provided below.

"Host cells," "cell lines," "cell cultures," "packaging cell line" and other such terms denote higher eukaryotic cells, such as mammalian cells including human cells, useful in the present invention, e.g., to produce recombinant virus or recombinant fusion polypeptide. These cells include the progeny of the original cell that was transduced. It is understood that the progeny of a single cell may not necessarily be completely identical (in morphology or in genomic complement) to the original parent cell.

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

A "control element" or "control sequence" is a nucleotide sequence involved in an interaction of molecules that contributes to the functional regulation of a polynucleotide, including replication, duplication, transcription, splicing, translation, or degradation of the polynucleotide. The regulation may affect the frequency, speed, or specificity of the process, and may be enhancing or inhibitory in nature. Control elements known in the art include, for example, transcriptional regulatory sequences such as promoters and enhancers. A promoter is a DNA region capable under certain conditions of binding RNA polymerase and initiating transcription of a coding region usually located downstream (in the 3' direction) from the promoter. Promoters include AAV promoters, e.g., P5, P19, P40 and AAV ITR promoters, as well as heterologous promoters.

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

The terms "polypeptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, acetylation, phosphonylation, lipidation, or conjugation with a labeling component.

The term "exogenous," when used in relation to a protein, gene, nucleic acid, or polynucleotide in a cell or organism refers to a protein, gene, nucleic acid, or polynucleotide which has been introduced into the cell or organism by artificial or natural means. An exogenous nucleic acid may be from a different organism or cell, or it may be one or more additional copies of a nucleic acid which occurs naturally within the organism or cell. By way of a non-limiting example, an exogenous nucleic acid is in a chromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature, e.g., an expression cassette which links a promoter from one gene to an open reading frame for a gene product from a different gene.

"Transformed" or "transgenic" is used herein to include any host cell or cell line, which has been altered or augmented by the presence of at least one recombinant DNA sequence. The host cells of the present invention are typically produced by transfection with a DNA sequence in a plasmid expression vector, as an isolated linear DNA sequence, or infection with a recombinant viral vector.

The term "sequence homology" means the proportion of base matches between two nucleic acid sequences or the proportion amino acid matches between two amino acid sequences. When sequence homology is expressed as a percentage, e.g., 50%, the percentage denotes the proportion of matches over the length of a selected sequence that is compared to some other sequence. Gaps (in either of the two sequences) are permitted to maximize matching; gap lengths of 15 bases or less are usually used, 6 bases or less e.g., with 2 bases or less. When using oligonucleotides as probes or treatments, the sequence homology between the target nucleic acid and the oligonucleotide sequence is generally not less than 17 target base matches out of 20 possible oligonucleotide base pair matches (85%); not less than 9 matches out of 10 possible base pair matches (90%), or not less than 19 matches out of 20 possible base pair matches (95%).

Two amino acid sequences are homologous if there is a partial or complete identity between their sequences. For example, 85% homology means that 85% of the amino acids are identical when the two sequences are aligned for maximum matching. Gaps (in either of the two sequences being matched) are allowed in maximizing matching; gap lengths of 5 or less or with 2 or less. Alternatively, two protein sequences (or polypeptide sequences derived from them of at least 30 amino acids in length) are homologous, as this term is used herein, if they have an alignment score of at more than 5 (in standard deviation units) using the program ALIGN with the mutation data matrix and a gap penalty of 6 or greater. The two sequences or parts thereof are more homologous if their amino acids are greater than or equal to 50% identical when optimally aligned using the ALIGN program.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is structurally related to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is structurally related to all or a portion of a reference polypeptide sequence, e.g., they have at least 80%, 85%, 90%, 95% or more, e.g., 99% or 100%, sequence identity. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing

two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denote a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, e.g., at least 90 to 95 percent sequence identity, or at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 20-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison.

"Conservative" amino acid substitutions are, for example, aspartic-glutamic as polar acidic amino acids; lysine/arginine/histidine as polar basic amino acids; leucine/isoleucine/methionine/valine/alanine/glycine/proline as non-polar or hydrophobic amino acids; serine/ threonine as polar or uncharged hydrophilic amino acids. Conservative amino acid substitution also includes groupings based on side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. For example, it is reasonable to expect that replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the properties of the resulting polypeptide. Whether an amino acid change results in a functional polypeptide can readily be determined by assaying the specific activity of the polypeptide. Naturally occurring residues are divided into groups based on common side-chain properties: (1) hydrophobic: norleucine, met, ala, val, leu, ile; (2) neutral hydrophilic: cys, ser, thr; (3) acidic: asp, glu; (4) basic: asn, gln, his, lys, arg; (5) residues that influence chain orientation: gly, pro; and (6) aromatic; trp, tyr, phe.

The disclosure also envisions polypeptides with non-conservative substitutions. Non-conservative substitutions entail exchanging a member of one of the classes described above for another.

Rationale

Existing drugs have little effect on the underlying disease processes of AD and no preventive therapies are currently available. As there are no effective therapies for AD, an effective therapy for AD would be a major advance (Conrado et al., 2020). Worldwide, there are approximately 35 million cases of AD affecting 6% of individuals >65 years (Fann et al., 2018), and it is responsible for 1.9 million deaths/year (Collaborators GBDD, 2019; Hebert et al., 2013). In the US, there are over 122,000 deaths/year. The estimated financial burden of caring for AD patients in the US is $305 billion/year. The APOE4 allele represents 13.7% of worldwide populations and with a much higher frequency in patients with AD (Safieh et al., 2019). Inheritance of APOE4 is associated with early cognitive decline, earlier onset AD and more severe disease with earlier death (Safieh et al., 2019; Williams 2020; Raber et al.,

2004). There have been many attempts at therapy for AD directed at amyloid, tau and other pathologies. While inheritance of the *APOE4* allele represents the strongest genetic risk factor for the development of late-onset AD, the *APOE2* allele is protective. Gene therapy to permanently deliver *APOE2* while decreasing endogenous *APOE4* expression could effectively convert *APOE4* homozygous individuals to *APOE2/4* heterozygotes with lowered APOE4 expression, substantially reducing the risk of AD development. Further *APOE4* homozygous individuals could receive the AAV gene therapy according to their genotype years before the onset of symptoms. The present strategy represents the development of a strategy, capitalizing on the observation that the APOE3ChC variant protected an individual in the PSEN1-E290A kindred who develop early-onset AD (Arboleda-Velasquez et al., 2019). AAV-mediated gene transfer of APOE3ChC and APOE2ChC may be efficacious in amyloid and/or tau pathology, and more so than AAV delivery of APOE2 alone.

Based on the knowledge that APOE4 enhances the risk for amyloid and tau-based AD pathology, it was demonstrated (Zhao et al., 2016; Shi et al., 2017; Dodart et al., 2005; Hudry et al., 2013)that CNS gene therapy with APOE2 can prevent APOE4-based AD pathology in mouse models. The primary innovation of this proposal is converting a unique clinical observation (Arboleda-Velasquez et al., 2019) into a 2nd generation, more effective gene therapy. There is extensive epidemiologic data demonstrating that the APOE genotype plays an important role in the pathogenesis of AD, with APOE2 protecting and APOE4 increasing the risk for development of AD-related pathology (Corder et al., 1994; Raber 2004; Genin et al., 2011; Sando et al., 2008). The observation that an individual in the Colombian PSEN1-E290A kindred was prevented from developing early onset AD by homozygous co-inheritance of homozygous APOE3ChC, led to APOE3ChC gene therapy based genetic modification of the CNS of APOE4 homozygotes topre vent the high risk of APOE4-driven AD-related pathology. Further, based prior studies demonstrating that gene therapy with APOE2 suppresses E4-driven amyloid-related pathology, APOE2ChC gene therapy may prevent both APOE4-driven amyloid and tau pathology and do so more effectively than APOE2 or APOE3ChC.

Exemplary APOE Nucleic Acid and Amino Acid Sequences

Apolipoprotein E (APOE) is a 299 amino acid protein involved in the metabolism of fats in the body. It is a family of proteins that binds fats and interact with the low density lipoprotein receptor (LDLR) which is important for normal processing of triglyceride rich lipoproteins. In peripheral tissues, APOE is produced by the liver and macrophages, and mediates cholesterol metabolism. In the central nervous system, APOE is produced by astrocytes and transports cholesterol to neurons via APO receptors, which are members of the LDLR family. There are three major APOE alleles, APOE2 (Cys 112, Cys 158), APOE3 (Cys112, Arg158) and APOE4 (Arg112, Arg158).

Exemplary human APOE sequences include but are not limited to:

mkvlwaallv tflagcqakv eqavetepep elrqqtewqs gqrwelalgr fwdylrwvqt
lseqvqeell ssqvtqelra lmdetmkelk aykseleeql tpvaeetrar lskelqaaqa
rlgadmedvc grlvqyrgev qamlgqstee lrvrlashlr klrkrllrda ddlqkrlavy
qagaregaer glsairerlg plveqgrvra atvgslagqp lqeraqawge rlrarmeemg
srtrdrldev keqvaevrak leeqaqqirl qaeafqarlk swfeplvedm qrqwaglvek
vqaavgtsaa pvpsdnh (SEQ ID NO:1) as well as sequences with at least 80%, 85%, 90%, 95% or more, e.g., 99% or 100%, sequence identity thereto,

where in one embodiment, APOE4 may have 31K, 46P, 79T, 130R, 163C, 292H and/or 314R, and APOE2 may have 43C, 152Q, 154C/S, 163C/P, 164Q, 172A, 176C, 242Q, 246C, 254E.

Exemplary human APOE sequences, e.g., those for silent nucleotide substitutions if they encode APOE2, include but are not limited to:

ggaacttgat gctcagagag gacaagtcat ttgcccaagg tcacacagct ggcaactggc
agagccagga ttcacgccct ggcaatttga ctccagaatc ctaaccttaa cccagaagca
cggcttcaag cccctggaaa ccacaatacc tgtggcagcc agggggaggt gctggaatct
catttcacat gtggggaggg ggctcccctg tgctcaaggt cacaaccaaa gaggaagctg
tgattaaaac ccaggtccca tttgcaaagc ctcgactttt agcaggtgca tcatactgtt
cccacccctc ccatcccact tctgtccagc cgcctagccc cactttcttt ttttctttt
tttgagacag tctccctctt gctgaggctg gagtgcagtg gcgagatctc ggctcactgt
aacctccgcc tcccgggttc aagcgattct cctgcctcag cctcccaagt agctaggatt
acaggcgccc gccaccacgc ctggctaact tttgtatttt tagtagagat ggggtttcac
catgttggcc aggctggtct caaactcctg accttaagtg attcgcccac tgtggcctcc
caaagtgctg ggattacagg cgtgagctac cgcccccagc ccctcccatc ccacttctgt
ccagcccccc agccctactt tctttctggg atccaggagt ccagatcccc agcccccctct
ccagattaca ttcatccagg cacaggaaag gacagggtca ggaaaggagg actctgggcg
gcagcctcca cattcccctt ccacgcttgg cccccagaat ggaggagggt gtctggatta
ctgggcgagg tgtcctccct tcctggggac tgtggggggt ggtcaaaaga cctctatgcc
ccacctcctt cctccctctg ccctgctgtg cctggggcag ggggagaaca gcccacctcg
tgactgggggg ctggcccagc ccgccctatc cctgggggag ggggcgggac agggggagcc
ctataattgg acaagtctgg gatccttgag tcctactcag cccagcgga ggtgaaggac
gtccttcccc aggagccg (SEQ ID NO:2)

or

ccccagcgga ggtgaaggac gtccttcccc aggagccgac tggccaatca caggcaggaa
gatgaaggtt ctgtgggctg cgttgctggt cacattcctg gcaggatgcc aggccaaggt
ggagcaagcg gtggagacag agccggagcc cgagctgcgc cagcagaccg agtggcagag
cggccagcgc tgggaactgg cactgggtcg cttttgggat tacctgcgct gggtgcagac
actgtctgag caggtgcagg aggagctgct cagctcccaa gtcacccaag aactgagggc
gctgatggac gagaccatga aggagttgaa ggcctacaaa tcggaactgg aggaacaact
gaccccggta gcggaggaga cgcgggcacg gctgtccaag gagctgcaga cggcgcaggc
ccggctgggc gcggacatgg aggacgtgtg cggccgcctg gtgcagtacc gcggcgaggt
gcaggccatg ctcggccaga gcaccgagga gctgcgggtg cgcctcgcct cccacctgcg
caagctgcgt aagcggctcc tccgcgatcc cgatgacctg cagaagcgcc tggcagtgta
ccaggccggg gcccgcgagg gcgccgagcg cggcctcagc gccatccgcg agcgcctggg
gcccctggtg gaacagggcc gcgtgcgggc cgccactgtg ggctccctgg ccggccagcc
gctacaggag cgggcccagg cctggggcga gcggctgcgc gcgcggatgg aggagatggg
cagtcggacc cgcgaccgcc tggacgaggt gaaggagcag gtggcggagg tgcgcgccaa
gctggaggag caggcccagc agatacgcct gcaggccgag gccttccagg cccgcctcaa
gagctggttc gagccccctgg tggaagacat gcagcgccag tgggccgggc tggtggagaa
ggtgcaggct gccgtgggca ccagcgccgc ccctgtgccc agcgacaatc actgaacgcc

gaagcctgca gccatgcgac cccacgccac cccgtgcctc ctgcctccgc gcagcctgca gcgggagacc ctgtccccgc cccagccgtc ctcctggggt ggaccctagt ttaataaaga ttcaccaagt ttcacgc (SEQ ID NO:3)

or have

MKVLWAALLVTFLAGCQAKVEQAVETEPEPELRQQTEWQSGQRWELALGRFWDYLRWVQTLSEQVQE ELLSSQVTQELRALMDETMKELKAYKSELEEQLTPVAEETRARLSKELQTAQARLGADMEDVCGRLVQY RGEVQAMLGQSTEELRVRLASHLRKLRKRLLRDPDDLQKRLAVYQAGAREGAERGLSAIRERLGPLVEQ GRVRAATVGSLAGQPLQERAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEEQAQQIRLQA EAFQARLKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNH (SEQ ID NO:4)

as well as sequences with at least 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95% or more, e.g., 99% or 100%, sequence identity thereto that encode an APOE.

Other exemplary APOE sequence include

mkvlwaallv tflagcqakv eqavetepep elrqqtewqs gqrwelalgr fwdylrwvqt lseqvqeell ssqvtqelra lmdetmkelk aykseleeql tpvaeetrar lskelqtaqa rlgadmedvc grlvqyrgev qamlgqstee lrvrlashlr klrkrllrdp ddlqkrlavy qagaregaer glsairerlg plveqgrvra atvgslagqp lqeraqawge rlrarmeemg srtrdrldev keqvaevrak leeqaqqirl qaeafqarlk swfeplvedm qrqwaglvek vqaavgtsaa pvpsdnh (SEQ ID NO:5);

mkvlwaallv tflagcqakv eqavetepep elrqqtewqs gqrwelalgr fwdylrwvqt lseqvqeell ssqvtqelra lmdetmkelk aykseleeql tpvaeetrar lskelqtaqa rlgadmedvc grlvqyrgev qamlgqstee lrvrlashlr klrkrllrda ddlqkrlavy qagaregaer glsairerlg plveqgrvra atvgslagqp lqeraqawge rlrarmeemg srtrdrldev keqvaevrak leeqaqqirl qaeafqarlk swfeplvedm qrqwaglvek vqaavgtsaa pvpsdnh (SEQ ID NO:6);

mkvlwaallv tflagcqakv eqavetepep elrqqtewqs gqrwelalgr fwdylrwvqt lseqvqeell ssqvtqelra lmdetmkelk aykseleeql tpvaeetrar lskelqtaqa rlgadmedvc grlvqyrgev qamlgqstee lrvrlashlr klrkrllrda ddlqkrlavy qagaregaer glsairerlg plveqgrvra atvgslagqp lqeraqawge rlrarmeemg srtrdrldev keqvaevrak leeqaqqirl qaeafqarlk swfeplvedm qrqwaglvek vqaavgtsaa pvpsdnh (SEQ ID NO:7).

For positions 112 and 158, E2 contains two cysteine residues, E3 contains a cysteine and an arginine, and E4 contains arginine residues at both sites. In one embodiment, the AAV vectors are prepared to express the Christchurch mutant in an ApoE2 or ApoE3 background. The vectors may be introduced to wild-type and disease model mice, and expression and biological measures of efficacy determined.

Gene Delivery Vectors

Gene delivery vectors within the scope of the invention include, but are not limited to, isolated nucleic acid, e.g., plasmid-based vectors which may be extrachromosomally maintained, and viral vectors, e.g., recombinant adenovirus, retrovirus, lentivirus, herpesvirus, poxvirus, papilloma virus, or adeno-associated virus, including viral and non-viral vectors which are present in liposomes, e.g., neutral or cationic liposomes, such as DOSPA/DOPE, DOGS/DOPE or DMRIE/DOPE liposomes, and/or associated with other molecules such as DNA-anti-DNA antibody-cationic lipid (DOTMA/DOPE) complexes. The gene therapy vectors may encode an APOE, RNAi, and/or an antibody or fragment thereof, as disclosed herein. Exemplary viral gene delivery vectors are described below. Gene delivery vectors may be administered via any route including, but not limited to, intracranial, intrathecal, intramuscular, buccal, rectal, intravenous or intracoronary administration, and transfer to cells may be enhanced using

electroporation and/or iontophoresis, and/or scaffolding such as extracellular matrix or hydrogels, e.g., a hydrogel patch. In one embodiment, a permeation enhancer is not employed to enhance indirect delivery to the CNS.

Retroviral vectors

Retroviral vectors exhibit several distinctive features including their ability to stably and precisely integrate into the host genome providing long-term transgene expression. These vectors can be manipulated *ex vivo* to eliminate infectious gene particles to minimize the risk of systemic infection and patient-to-patient transmission. Pseudotyped retroviral vectors can alter host cell tropism.

Lentiviruses

Lentiviruses are derived from a family of retroviruses that include human immunodeficiency virus and feline immunodeficiency virus. However, unlike retroviruses that only infect dividing cells, lentiviruses can infect both dividing and nondividing cells. For instance, lentiviral vectors based on human immunodeficiency virus genome are capable of efficient transduction of cardiac myocytes *in vivo*. Although lentiviruses have specific tropisms, pseudotyping the viral envelope with vesicular stomatitis virus yields virus with a broader range (Schnepp et al., Meth. Mol. Med., 69:427 (2002)).

Adenoviral vectors

Adenoviral vectors may be rendered replication-incompetent by deleting the early (E1A and E1B) genes responsible for viral gene expression from the genome and are stably maintained into the host cells in an extrachromosomal form. These vectors have the ability to transfect both replicating and nonreplicating cells and, in particular, these vectors have been shown to efficiently infect cardiac myocytes *in vivo*, e.g., after direction injection or perfusion. Adenoviral vectors have been shown to result in transient expression of therapeutic genes *in vivo*, peaking at 7 days and lasting approximately 4 weeks. The duration of transgene expression may be improved in systems utilizing neural specific promoters. In addition, adenoviral vectors can be produced at very high titers, allowing efficient gene transfer with small volumes of virus.

Adeno-associated virus vectors

Recombinant adeno-associated viruses (rAAV) are derived from nonpathogenic parvoviruses, evoke essentially no cellular immune response, and produce transgene expression lasting months in most systems. Moreover, like adenovirus, adeno-associated virus vectors also have the capability to infect replicating and nonreplicating cells and are believed to be nonpathogenic to humans. Moreover, they appear promising for sustained cardiac gene transfer (Hoshijima et al., Nat. Med., 8:864 (2002); Lynch et al., Circ. Res., 80:197 (1997)).

AAV vectors include but are not limited to AAV1, AAV2, AAV5, AAV7, AAV8, AAV9 or AAVrh10, including chimeric viruses where the AAV genome is from a different source than the capsid.

Plasmid DNA vectors

Plasmid DNA is often referred to as "naked DNA" to indicate the absence of a more elaborate packaging system. Direct injection of plasmid DNA to myocardial cells *in vivo* has been accomplished. Plasmid-based vectors are relatively nonimmunogenic and nonpathogenic, with the potential to stably integrate in the cellular genome, resulting in long-term gene expression in postmitotic cells *in vivo*. For example, expression of secreted angiogenesis factors after muscle injection of plasmid DNA, despite relatively low levels of focal transgene expression, has demonstrated significant biologic effects in animal models and appears promising clinically (Isner, Nature, 415:234 (2002)). Furthermore, plasmid DNA is

rapidly degraded in the blood stream; therefore, the chance of transgene expression in distant organ systems is negligible. Plasmid DNA may be delivered to cells as part of a macromolecular complex, e.g., a liposome or DNA-protein complex, e.g., see below, and delivery may be enhanced using techniques including electroporation.

Exemplary Non-Viral Formulations

Biodegradable particles comprising, e.g., isolated nucleic acid for protective APOE expression, a vector for antibody expression, or a vector for RNAi expression, may include or may be formed from biodegradable polymeric molecules which may include, but are not limited to polylactic acid (PLA), polyglycolic acid (PGA), co-polymers of PLA and PGA (i.e., polyactic-co-glycolic acid (PLGA)), poly-ε-caprolactone (PCL), polyethylene glycol (PEG), poly(3-hydroxybutyrate), poly(p-dioxanone), polypropylene fumarate, poly(orthoesters), polyol/diketene acetals addition polymers, poly-alkyl-cyano-acrylates (PAC), poly(sebacic anhydride) (PSA), poly(carboxybiscarboxyphenoxyphenoxy hexone (PCPP) poly[bis (p-carboxypheonoxy)methane](PCPM), copolymers of PSA, PCPP and PCPM, poly(amino acids), poly(pseudo amino acids), polyphosphazenes, derivatives of poly[(dichloro)phosphazenes] and poly[(organo)phosphazenes], poly-hydroxybutyric acid, or S-caproic acid, elastin, or gelatin. (See, e.g., Kumari et al., Colloids and Surfaces B: Biointerfaces 75 (2010) 1-18; and U.S. Pat. Nos. 6,913,767; 6,884,435; 6,565,777; 6,534,092; 6,528,087; 6,379,704; 6,309,569; 6,264,987; 6,210,707; 6,090,925; 6,022,564; 5,981,719; 5,871,747; 5,723,269; 5,603,960; and 5,578,709; and U.S. Published Application No. 2007/0081972; and International Application Publication Nos. WO 2012/115806; and WO 2012/054425; the contents of which are incorporated herein by reference in their entireties).

The biodegradable nanoparticles may be prepared by methods known in the art. (See, e.g., Nagavarma et al., Asian J. of Pharma. And Clin. Res., Vol 5, Suppl 3, 2012, pages 16-23; Cismaru et al., Rev. Roum. Chim., 2010, 55(8), 433-442; and International Application Publication Nos. WO 2012/115806; and WO 2012/054425; the contents of which are incorporated herein by reference in their entireties). Suitable methods for preparing the nanoparticles may include methods that utilize a dispersion of a preformed polymer, which may include but are not limited to solvent evaporation, nanoprecipitation, emulsification/solvent diffusion, salting out, dialysis, and supercritical fluid technology. In some embodiments, the nanoparticles may be prepared by forming a double emulsion (e.g., water-in-oil-in-water) and subsequently performing solvent-evaporation. The nanoparticles obtained by the disclosed methods may be subjected to further processing steps such as washing and lyophilization, as desired. Optionally, the nanoparticles may be combined with a preservative (e.g., trehalose).

Typically, the nanoparticles have a mean effective diameter of less than 1 micron, e.g., the nanoparticles have a mean effective diameter of between about 25 nm and about 500 nm, e.g., between about 50 nm and about 250 nm, about 100 nm to about 150 nm, or about 450 nm to 650 nm. The size of the particles (e.g., mean effective diameter) may be assessed by known methods in the art, which may include but are not limited to transmission electron microscopy (TEM), scanning electron microscopy (SEM), Atomic Force Microscopy (AFM), Photon Correlation Spectroscopy (PCS), Nanoparticle Surface Area Monitor (NSAM), Condensation Particle Counter (CPC), Differential Mobility Analyzer (DMA), Scanning Mobility Particle Sizer (SMPS), Nanoparticle Tracking Analysis (NTA), X-Ray Diffraction (XRD), Aerosol Time of Flight Mass Spectroscopy (ATFMS), and Aerosol Particle Mass Analyzer (APM).

The biodegradable nanoparticles may have a zeta-potential that facilitates uptake by a target cell. Typically, the nanoparticles have a zeta-potential greater than 0. In some embodiments, the nanoparticles have a zeta-potential between about 5 mV to about 45 mV, between about 15 mV to about 35 mV, or between about 20 mV and about 40 mV. Zeta-potential may be determined via characteristics that include electrophoretic mobility or dynamic electrophoretic mobility. Electrokinetic phenomena and electroacoustic phenomena may be utilized to calculate zeta-potential.

In one embodiment, a non-viral delivery vehicle comprises polymers including but not limited to poly(lactic-co-glycolic acid) (PLGA), polylactic acid (PLA), linear and/or branched PEI with differing molecular weights (e.g., 2, 22 and 25 kDa), dendrimers such as polyamidoamine (PAMAM) and polymethoacrylates; lipids including but not limited to cationic liposomes, cationic emulsions, DOTAP, DOTMA, DMRIE, DOSPA, distearoylphosphatidylcholine (DSPC), DOPE, or DC-cholesterol; peptide based vectors including but not limited to Poly-L-lysine or protamine; or poly(β-amino ester), chitosan, PEI-polyethylene glycol, PEI-mannose-dextrose, DOTAP-cholesterol or RNAiMAX.

In one embodiment, the delivery vehicle is a glycopolymer-based delivery vehicle, poly(glycoamidoamine)s (PGAAs), that have the ability to complex with various polynucleotide types and form nanoparticles. These materials are created by polymerizing the methylester or lactone derivatives of various carbohydrates (D-glucarate (D), *meso*-galactarate (G), D-mannarate (M), and L-tartarate (T)) with a series of oligoethyleneamine monomers (containing between 1-4 ethylenamines (Liu and Reineke, 2006). A subset composed of these carbohydrates and four ethyleneamines in the polymer repeat units yielded exceptional delivery efficiency.

In one embodiment, the delivery vehicle comprises polyethyleneimine (PEI), Polyamidoamine (PAMAM), PEI-PEG, PEI-PEG-mannose, dextran-PEI, OVA conjugate, PLGA microparticles, or PLGA microparticles coated with PAMAM, or any combination thereof. The disclosed cationic polymer may include, but are not limited to, polyamidoamine (PAMAM) dendrimers. Polyamidoamine dendrimers suitable for preparing the presently disclosed nanoparticles may include 3rd-, 4th-, 5th-, or at least 6th-generation dendrimers.

In one embodiment, the delivery vehicle comprises a lipid, e.g., *N*-[1-(2,3-dioleoyloxy)propel]-*N*,*N*,*N*-trimethylammonium (DOTMA), 2,3-dioleyloxy-*N*-[2-spermine carboxamide] ethyl-*N*,*N*-dimethyl-1-propanammonium trifluoracetate (DOSPA, Lipofectamine); 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); *N*-[1-(2,3-dimyristloxy) propyl]; *N*,*N*-dimethyl-*N*-(2-hydroxyethyl) ammonium bromide (DMRIE), 3-β-[*N*-(*N*,*N*-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol); dioctadecyl amidoglyceryl spermine (DOGS, Transfectam); or imethyldioctadeclyammonium bromide (DDAB). The positively charged hydrophilic head group of cationic lipids usually consists of monoamine such as tertiary and quaternary amines, polyamine, amidinium, or guanidinium group. A series of pyridinium lipids have been developed (Zhu et al., 2008; van der Woude et al., 1997; Ilies et al., 2004). In addition to pyridinium cationic lipids, other types of heterocyclic head group include imidazole, piperizine and amino acid. The main function of cationic head groups is to condense negatively charged nucleic acids by means of electrostatic interaction to slightly positively charged nanoparticles, leading to enhanced cellular uptake and endosomal escape.

Lipids having two linear fatty acid chains, such as DOTMA, DOTAP and SAINT-2, or DODAC, may be employed as a delivery vehicle, as well as tetraalkyl lipid chain surfactant, the dimer of *N*,*N*-

dioleyl-*N*,*N*- dimethylammonium chloride (DODAC). All the *trans*-orientated lipids regardless of their hydrophobic chain lengths ($C_{18:1}$, $C_{18:1}$ and $C_{20:1}$) appear to enhance the transfection efficiency compared with their *cis*-orientated counterparts.

The structures of cationic polymers useful as a delivery vehicle include but are not limited to linear polymers such as chitosan and linear poly(ethyleneimine), branched polymers such as branch poly(ethyleneimine) (PEI), circle-like polymers such as cyclodextrin, network (crosslinked) type polymers such as crosslinked poly(amino acid) (PAA), and dendrimers. Dendrimers consist of a central core molecule, from which several highly branched arms 'grow' to form a tree-like structure with a manner of symmetry or asymmetry. Examples of dendrimers include polyamidoamine (PAMAM) and polypropylenimine (PPI) dendrimers.

DOPE and cholesterol are commonly used neutral co-lipids for preparing cationic liposomes. Branched PEI-cholesterol water-soluble lipopolymer conjugates self-assemble into cationic micelles. Pluronic (poloxamer), a non-ionic polymer and SP1017, which is the combination of Pluronics L61 and F127, may also be used.

In one embodiment, PLGA particles are employed to increase the encapsulation frequency although complex formation with PLL may also increase the encapsulation efficiency. Other cationic materials, for example, PEI, DOTMA, DC-Chol, or CTAB, may be used to make nanospheres.

In one embodiment, complexes are embedded in or applied to a material including but not limited to hydrogels of poloxamers, polyacrylamide, poly(2-hydroxyethyl methacrylate), carboxyvinyl-polymers (e.g., Carbopol 934, Goodrich Chemical Co.), cellulose derivatives, e.g., methylcellulose, cellulose acetate and hydroxypropyl cellulose, polyvinyl pyrrolidone or polyvinyl alcohols, or combinations thereof.

In some embodiments, a biocompatible polymeric material is derived from a biodegradable polymeric such as collagen, e.g., hydroxylated collagen, fibrin, polylactic-polyglycolic acid, or a polyanhydride. Other examples include, without limitation, any biocompatible polymer, whether hydrophilic, hydrophobic, or amphiphilic, such as ethylene vinyl acetate copolymer (EVA), polymethyl methacrylate, polyamides, polycarbonates, polyesters, polyethylene, polypropylenes, polystyrenes, polyvinyl chloride, polytetrafluoroethylene, N-isopropylacrylamide copolymers, poly(ethylene oxide)/poly(propylene oxide) block copolymers, poly(ethylene glycol)/poly(D,L-lactide-co-glycolide) block copolymers, polyglycolide, polylactides (PLLA or PDLA), poly(caprolactone) (PCL), or poly(dioxanone) (PPS).

In another embodiment, the biocompatible material includes polyethyleneterephalate, polytetrafluoroethylene, copolymer of polyethylene oxide and polypropylene oxide, a combination of polyglycolic acid and polyhydroxyalkanoate, gelatin, alginate, poly-3-hydroxybutyrate, poly-4-hydroxybutyrate, and polyhydroxyoctanoate, and polyacrylonitrilepolyvinylchlorides.

In one embodiment, the following polymers may be employed, e.g., natural polymers such as starch, chitin, glycosaminoglycans, e.g., hyaluronic acid, dermatan sulfate and chrondrotin sulfate, and microbial polyesters, e.g., hydroxyalkanoates such as hydroxyvalerate and hydroxybutyrate copolymers, and synthetic polymers, e.g., poly(orthoesters) and polyanhydrides, and including homo and copolymers of glycolide and lactides (e.g., poly(L-lactide, poly(L-lactide-co-D,L-lactide), poly(L-lactide-co-glycolide, polyglycolide and poly(D,L-lactide), pol(D,L-lactide-coglycolide), poly(lactic acid colysine) and polycaprolactone.

In one embodiment, the biocompatible material is derived from isolated extracellular matrix

(ECM). ECM may be isolated from endothelial layers of various cell populations, tissues and/or organs, e.g., any organ or tissue source including the dermis of the skin, liver, alimentary, respiratory, intestinal, urinary or genital tracks of a warm blooded vertebrate. ECM employed in the invention may be from a combination of sources. Isolated ECM may be prepared as a sheet, in particulate form, gel form and the like.

The biocompatible scaffold polymer may comprise silk, elastin, chitin, chitosan, poly(d-hydroxy acid), poly(anhydrides), or poly(orthoesters). More particularly, the biocompatible polymer may be formed polyethylene glycol, poly(lactic acid), poly(glycolic acid), copolymers of lactic and glycolic acid, copolymers of lactic and glycolic acid with polyethylene glycol, poly(E-caprolactone), poly(3-hydroxybutyrate), poly(p-dioxanone), polypropylene fumarate, poly(orthoesters), polyol/diketene acetals addition polymers, poly(sebacic anhydride) (PSA), poly(carboxybiscarboxyphenoxyphenoxy hexone (PCPP) poly[bis (p-carboxypheonoxy) methane] (PCPM), copolymers of SA, CPP and CPM, poly(amino acids), poly(pseudo amino acids), polyphosphazenes, derivatives of poly[(dichloro)phosphazenes] or poly[(organo) phosphazenes], poly-hydroxybutyric acid, or S-caproic acid, polylactide-co-glycolide, polylactic acid, polyethylene glycol, cellulose, oxidized cellulose, alginate, gelatin or derivatives thereof.

Thus, the polymer may be formed of any of a wide range of materials including polymers, including naturally occurring polymers, synthetic polymers, or a combination thereof. In one embodiment, the scaffold comprises biodegradable polymers. In one embodiment, a naturally occurring biodegradable polymer may be modified to provide for a synthetic biodegradable polymer derived from the naturally occurring polymer. In one embodiment, the polymer is a poly(lactic acid) ("PLA") or poly(lactic-co-glycolic acid) ("PLGA"). In one embodiment, the scaffold polymer includes but is not limited to alginate, chitosan, poly(2-hydroxyethylmethacrylate), xyloglucan, co-polymers of 2-methacryloyloxyethyl phosphorylcholine, poly(vinyl alcohol), silicone, hydrophobic polyesters and hydrophilic polyester, poly(lactide-co-glycolide), N-isoproylacrylamide copolymers, poly(ethylene oxide)/poly(propylene oxide), polylactic acid, poly(orthoesters), polyanhydrides, polyurethanes, copolymers of 2-hydroxyethylmethacrylate and sodium methacrylate, phosphorylcholine, cyclodextrins, polysulfone and polyvinylpyrrolidine, starch, poly-D,L-lactic acid-para-dioxanone-polyethylene glycol block copolymer, polypropylene, poly(ethylene terephthalate), poly(tetrafluoroethylene), poly-epsilon-caprolactone, or crosslinked chitosan hydrogels.

Alternatively, the nucleic acids or vectors, can be administered in dosages of at least about 0.0001 mg/kg to about 1 mg/kg, of at least about 0.001 mg/kg to about 0.5 mg/kg, at least about 0.01 mg/kg to about 0.25mg/kg or at least about 0.01 mg/kg to about 0.25 mg/kg of body weight, although other dosages may provide beneficial results.

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Pharmaceutical Compositions

The invention provides a composition comprising, consisting essentially of, or consisting of the above-described gene transfer vector(s) and a pharmaceutically acceptable (e.g., physiologically acceptable) carrier. When the composition consists essentially of the gene transfer vector and a pharmaceutically acceptable carrier, additional components can be included that do not materially affect the composition (e.g., adjuvants, buffers, stabilizers, anti-inflammatory agents, solubilizers, preservatives,

etc.). When the composition consists of the inventive gene transfer vector and the pharmaceutically acceptable carrier, the composition does not comprise any additional components. Any suitable carrier can be used within the context of the invention, and such carriers are well known in the art. The choice of carrier will be determined, in part, by the particular site to which the composition may be administered and the particular method used to administer the composition. The composition optionally can be sterile with the exception of the gene transfer vector described herein. The composition can be frozen or lyophilized for storage and reconstituted in a suitable sterile carrier prior to use. The compositions can be generated in accordance with conventional techniques described in, e.g., *Remington: The Science and Practice of Pharmacy, 21st Edition*, Lippincott Williams & Wilkins, Philadelphia, PA (2001).

Suitable formulations for the composition include aqueous and non-aqueous solutions, isotonic sterile solutions, which can contain anti-oxidants, buffers, and bacteriostats, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, immediately prior to use. Extemporaneous solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. In one embodiment, the carrier is a buffered saline solution. In one embodiment, the inventive gene transfer vector is administered in a composition formulated to protect the gene transfer vector from damage prior to administration. For example, the composition can be formulated to reduce loss of the gene transfer vector on devices used to prepare, store, or administer the gene transfer vector, such as glassware, syringes, or needles. The composition can be formulated to decrease the light sensitivity and/or temperature sensitivity of the gene transfer vector. To this end, the composition may comprise a pharmaceutically acceptable liquid carrier, such as, for example, those described above, and a stabilizing agent selected from the group consisting of polysorbate 80, L-arginine, polyvinylpyrrolidone, trehalose, and combinations thereof. Use of such a composition will extend the shelf life of the gene transfer vector, facilitate administration, and increase the efficiency of the inventive method. Formulations for gene transfer vector -containing compositions are further described in, for example, Wright et al., *Curr. Opin. Drug Discov. Devel.*, 6(2): 174-178 (2003) and Wright et al., *Molecular Therapy*, *12*: 171-178 (2005))

The composition also can be formulated to enhance transduction efficiency. In addition, one of ordinary skill in the art will appreciate that the inventive gene transfer vector can be present in a composition with other therapeutic or biologically-active agents. For example, factors that control inflammation, such as ibuprofen or steroids, can be part of the composition to reduce swelling and inflammation associated with *in vivo* administration of the gene transfer vector. Immune system stimulators or adjuvants, e.g., interleukins, lipopolysaccharide, and double-stranded RNA. Antibiotics, i.e., microbicides and fungicides, can be present to treat existing infection and/or reduce the risk of future infection, such as infection associated with gene transfer procedures.

Injectable depot forms are made by forming microencapsule matrices of the subject compounds in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable

formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

In certain embodiments, a formulation comprises a biocompatible polymer selected from the group consisting of polyamides, polycarbonates, polyalkylenes, polymers of acrylic and methacrylic esters, polyvinyl polymers, polyglycolides, polysiloxanes, polyurethanes and co-polymers thereof, celluloses, polypropylene, polyethylenes, polystyrene, polymers of lactic acid and glycolic acid, polyanhydrides, poly(ortho)esters, poly(butic acid), poly(valeric acid), poly(lactide-co-caprolactone), polysaccharides, proteins, polyhyaluronic acids, polycyanoacrylates, and blends, mixtures, or copolymers thereof.

The composition can be administered in or on a device that allows controlled or sustained release, such as a sponge, biocompatible meshwork, mechanical reservoir, or mechanical implant. Implants (see, e.g., U.S. Patent No. 5,443,505), devices (see, e.g., U.S. Patent No. 4,863,457), such as an implantable device, e.g., a mechanical reservoir or an implant or a device comprised of a polymeric composition, are particularly useful for administration of the inventive gene transfer vector. The composition also can be administered in the form of sustained-release formulations (see, e.g., U.S. Patent No. 5,378,475) comprising, for example, gel foam, hyaluronic acid, gelatin, chondroitin sulfate, a polyphosphoester, such as bis-2-hydroxyethyl-terephthalate (BHET), and/or a polylactic-glycolic acid.

The dose of the gene transfer vector in the composition administered to the mammal will depend on a number of factors, including the size (mass) of the mammal, the extent of any side-effects, the particular route of administration, and the like. In one embodiment, the inventive method comprises administering a "therapeutically effective amount" of the composition comprising the inventive gene transfer vector described herein. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time as necessary, to achieve a desired therapeutic result. The therapeutically effective amount may vary according to factors such as the extent of the disease or disorder, age, sex, and weight of the individual, and the ability of the gene transfer vector to elicit a desired response in the individual. The dose of gene transfer vector in the composition to achieve a particular therapeutic effect typically is administered in units of vector genome copies per cell (gc/cell) or vector genome copies/per kilogram of body weight (gc/kg). One of ordinary skill in the art can readily determine an appropriate gene transfer vector dose range to treat a patient having a particular disease or disorder, based on these and other factors that are well known in the art. The therapeutically effective amount may be between 1×10^{10} genome copies to 1×10^{13} genome copies. The therapeutically effective amount may be between 1×10^{11} genome copies to 1×10^{14} genome copies. The therapeutically effective amount may be between 1×10^{7} genome copies to 1×10^{10} genome copies. The therapeutically effective amount may be between 1×10^{14} genome copies to 1×10^{17} genome copies. Assuming a 70 kg human, the dose ranges may be from 1.4×10^{8} gc/kg to 1.4×10^{11} gc/kg, 1.4×10^{9} gc/kg to 1.4×10^{12} gc/kg, 1.4×10^{10} gc/kg to 1.4×10^{13} gc/kg, or 1.4×10^{11} gc/kg to 1.4×10^{14} gc/kg.

In one embodiment, the composition is administered once to the mammal. It is believed that a single administration of the composition may result in persistent expression in the mammal with minimal side effects. However, in certain cases, it may be appropriate to administer the composition multiple times during a therapeutic period to ensure sufficient exposure of cells to the composition. For example, the composition may be administered to the mammal two or more times (e.g., 2, 3, 4, 5, 6, 6, 8, 9, or 10 or more times) during a therapeutic period.

The present disclosure provides pharmaceutically acceptable compositions which comprise a therapeutically-effective amount of gene transfer vector comprising a nucleic acid sequence as described above.

Routes of Administration, Dosages and Dosage Forms

Administration of, for example, the gene delivery vectors in accordance with the present invention, may be continuous or intermittent, depending, for example, upon the recipient's physiological condition, and other factors known to skilled practitioners. The administration of the gene delivery vector(s) may be essentially continuous over a preselected period of time or may be in a series of spaced doses. Both local administration, e.g., intracranial, intranasal or intrathecal, and systemic administration, e.g., using viruses that cross the blood-brain barrier, are contemplated. Any route of administration may be employed, e.g., intravenous, intranasal or intrabronchial, direct administration to the lung and intrapleural. In one embodiment, compositions may be delivered to the pleura.

One or more suitable unit dosage forms comprising the gene delivery vector(s), which may optionally be formulated for sustained release, can be administered by a variety of routes including intracranial, intrathecal, or intranasal, or other means to deliver to the CNS, or oral, or parenteral, including by rectal, buccal, vaginal and sublingual, transdermal, subcutaneous, intravenous, intramuscular, intraperitoneal, intrathoracic, or intrapulmonary routes. The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods well known to pharmacy. Such methods may include the step of bringing into association the vector with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the product into the desired delivery system.

The amount of gene delivery vector(s) administered to achieve a particular outcome will vary depending on various factors including, but not limited to, the genes and promoters chosen, the condition, patient specific parameters, e.g., height, weight and age, and whether prevention or treatment, is to be achieved.

Vectors of the invention may conveniently be provided in the form of formulations suitable for administration, e.g., into the brain. A suitable administration format may best be determined by a medical practitioner for each patient individually, according to standard procedures. Suitable pharmaceutically acceptable carriers and their formulation are described in standard formulations treatises, e.g., Remington's Pharmaceuticals Sciences. By "pharmaceutically acceptable" it is meant a carrier, diluent, excipient, and/or salt that is compatible with the other ingredients of the formulation, and not deleterious to the recipient thereof.

Vectors of the invention may be formulated in solution at neutral pH, for example, about pH 6.5 to about pH 8.5, or from about pH 7 to 8, with an excipient to bring the solution to about isotonicity, for example, 4.5% mannitol or 0.9% sodium chloride, pH buffered with art-known buffer solutions, such as sodium phosphate, that are generally regarded as safe, together with an accepted preservative such as metacresol 0.1% to 0.75%, or from 0.15% to 0.4% metacresol. Obtaining a desired isotonicity can be accomplished using sodium chloride or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol, polyols (such as mannitol and sorbitol), or other inorganic or organic solutes. Sodium chloride is useful for buffers containing sodium ions. If desired, solutions of the above compositions can also be prepared to enhance shelf life and stability. Therapeutically useful compositions of the invention can be prepared by mixing the ingredients following generally accepted

procedures. For example, the selected components can be mixed to produce a concentrated mixture which may then be adjusted to the final concentration and viscosity by the addition of water and/or a buffer to control pH or an additional solute to control tonicity.

The vectors can be provided in a dosage form containing an amount of a vector effective in one or multiple doses. For viral vectors, the effective dose may be in the range of at least about 10^7 viral particles, e.g., about 10^9 viral particles, or about 10^{11} viral particles. The number of viral particles added may be up to 10^{14}. For example, when a viral expression vector is employed, about 10^8 to about 10^{60} gc of viral vector can be administered as nucleic acid or as a packaged virion. In some embodiments, about 10^9 to about 10^{15} copies of viral vector, e.g., per 0.5 to 10 mL, can be administered as nucleic acid or as a packaged virion. Alternatively, the nucleic acids or vectors, can be administered in dosages of at least about 0.0001 mg/kg to about 1 mg/kg, of at least about 0.001 mg/kg to about 0.5 mg/kg, at least about 0.01 mg/kg to about 0.25mg/kg or at least about 0.01 mg/kg to about 0.25 mg/kg of body weight, although other dosages may provide beneficial results. The amount administered will vary depending on various factors including, but not limited to, the nucleic acid or vector chosen for administration, the disease, the weight, the physical condition, the health, and/or the age of the mammal. Such factors can be readily determined by the clinician employing animal models or other test systems that are available in the art. As noted, the exact dose to be administered is determined by the attending clinician, but may be in 1 mL phosphate buffered saline. For delivery of plasmid DNA alone, or plasmid DNA in a complex with other macromolecules, the amount of DNA to be administered will be an amount which results in a beneficial effect to the recipient. For example, from 0.0001 to 1 mg or more, e.g., up to 1 g, in individual or divided doses, e.g., from 0.001 to 0.5 mg, or 0.01 to 0.1 mg, of DNA can be administered.

For example, when a viral expression vector is employed, about 10^8 to about 10^{60} gc of viral vector can be administered as nucleic acid or as a packaged virion. In some embodiments, about 10^9 to about 10^{15} copies of viral vector, e.g., per 0.5 to 10 mL, can be administered as nucleic acid or as a packaged virion. Alternatively, the nucleic acids or vectors, can be administered in dosages of at least about 0.0001 mg/kg to about 1 mg/kg, of at least about 0.001 mg/kg to about 0.5 mg/kg, at least about 0.01 mg/kg to about 0.25 mg/kg or at least about 0.01 mg/kg to about 0.25 mg/kg of body weight, although other dosages may provide beneficial results.

In one embodiment, administration may be by intracranial, intraventricular, intracisternal, lumbar, intrahepatic, intratracheal or intrabronchial injection or infusion using an appropriate catheter or needle. A variety of catheters may be used to achieve delivery, as is known in the art. For example, a variety of general purpose catheters, as well as modified catheters, suitable for use in the present invention are available from commercial suppliers. Also, where delivery is achieved by injection directly into a specific region of the brain or lung, a number of approaches can be used to introduce a catheter into that region, as is known in the art.

By way of illustration, liposomes and other lipid-containing gene delivery complexes can be used to deliver one or more transgenes. The principles of the preparation and use of such complexes for gene delivery have been described in the art (see, e.g., Ledley, (1995); Miller et al., (1995); Chonn et al., (1995); Schofield et al., (1995); Brigham et al., (1993)).

Pharmaceutical formulations containing the gene delivery vectors can be prepared by procedures known in the art using well known and readily available ingredients. For example, the agent can be formulated with common excipients, diluents, or carriers, and formed into tablets, capsules, suspensions,

powders, and the like. The vectors of the invention can also be formulated as elixirs or solutions appropriate for parenteral administration, for instance, by intramuscular, subcutaneous or intravenous routes.

The pharmaceutical formulations of the vectors can also take the form of an aqueous or anhydrous solution, e.g., a lyophilized formulation, or dispersion, or alternatively the form of an emulsion or suspension.

In one embodiment, the vectors may be formulated for administration, e.g., by injection, for example, bolus injection or continuous infusion via a catheter, and may be presented in unit dose form in ampules, pre-filled syringes, small volume infusion containers or in multi-dose containers with an added preservative. The active ingredients may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredients may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

These formulations can contain pharmaceutically acceptable vehicles and adjuvants which are well known in the prior art. It is possible, for example, to prepare solutions using one or more organic solvent(s) that is/are acceptable from the physiological standpoint.

For administration to the upper (nasal) or lower respiratory tract by inhalation, the vector is conveniently delivered from an insufflator, nebulizer or a pressurized pack or other convenient means of delivering an aerosol spray. Pressurized packs may comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount.

Alternatively, for administration by inhalation or insufflation, the composition may take the form of a dry powder, for example, a powder mix of the therapeutic agent and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules or cartridges, or, e.g., gelatine or blister packs from which the powder may be administered with the aid of an inhalator, insufflator or a metered-dose inhaler.

For intra-nasal administration, the vector may be administered via nose drops, a liquid spray, such as via a plastic bottle atomizer or metered-dose inhaler. Typical of atomizers are the Mistometer (Wintrop) and the Medihaler (Riker).

The local delivery of the vectors can also be by a variety of techniques which administer the vector at or near the site of disease, e.g., using a catheter or needle Examples of site-specific or targeted local delivery techniques are not intended to be limiting but to be illustrative of the techniques available. Examples include local delivery catheters, such as an infusion or indwelling catheter, e.g., a needle infusion catheter, shunts and stents or other implantable devices, site specific carriers, direct injection, or direct applications.

The formulations and compositions described herein may also contain other ingredients such as antimicrobial agents or preservatives.

Subjects

The subject may be any animal, including a human. human and non-human animals. Non-human animals includes all vertebrates, e.g., mammals and non-mammals, such as non-human primates, sheep,

dogs, cats, cows, horses, chickens, amphibians, and reptiles, although mammals, such as non-human primates, sheep, dogs, cats, cows and horses may be the subject. The subject may also be livestock such as, cattle, swine, sheep, poultry, and horses, or pets, such as dogs and cats.

In one embodiment, subjects include human subjects. The subject is generally diagnosed with the condition by skilled artisans, such as a medical practitioner.

The methods of the invention described herein can be employed for subjects of any species, gender, age, ethnic population, or genotype. Accordingly, the term subject includes males and females, and it includes elderly, elderly-to-adult transition age subjects adults, adult-to-pre-adult transition age subjects, and pre-adults, including adolescents, childrens, and infants.

Examples of human ethnic populations include Caucasians, Asians, Hispanics, Africans, African Americans, Native Americans, Semites, and Pacific Islanders. The methods of the invention may be more appropriate for some ethnic populations such as Caucasians, especially northern European populations, as well as Asian populations.

The term subject also includes subjects of any genotype or phenotype as long as they are in need of the invention, as described above. In addition, the subject can have the genotype or phenotype for any hair color, eye color, skin color or any combination thereof.

The term subject includes a subject of any body height, body weight, or any organ or body part size or shape.

Exemplary Embodiments

In one embodiment, a gene therapy vector is provided comprising an expression cassette coding for a mammalian apolipoprotein E that has a residue other than arginine at at least one of positions 112, 136, or 158, or coding for an antibody or antigen binding fragment thereof that specifically binds APOE4, such as an antibody that has the binding specificity or is derived from 9D11, 4E4 (binds to residues between 100 and 150), 5B5, E29, 1343A (Arboleda-Velasquez et al., 2019), or one disclosed in U.S. Patent No. 8,741,298, the disclosures of which are incorporated by reference herein, or one that binds heparan sulfate, e.g., heparan sulfate proteoglycans (HPSGs). In one embodiment, the mammalian apolipoprotein E is not APOE2, APOE3, or APOE4 but rather is a modified APOE that is protective, e.g., decreases or delays cognitive impairment or deterioration, when exogenously expressed in a mammal, or may have reduced binding to HSPGs. In one embodiment, the apolipoprotein E in the vector is a human apolipoprotein E, e.g., modified as described herein. In one embodiment, the residue in the APOE in the vector other than arginine is serine, threonine, asparagine, cysteine, or glutamine. In one embodiment, position 112 in the APOE in the vector is a cysteine. In one embodiment, position 136 in the APOE in the vector i a serine. In one embodiment, position 158 in the APOE in the vector is an arginine or a cysteine. In one embodiment, two of positions 112, 136 or 158 in APOE in the vector have an arginine. In one embodiment, position 112 in the APOE in the vectors does not have an arginine. In one embodiment, position 158 in APOE in the vector does not have an arginine. In one embodiment, the gene therapy is a viral gene therapy vector, e.g., an adenovirus, adeno-associated virus (AAV), retrovirus or lentivirus vector. In one embodiment, the viral gene therapy vector is a rAAV vector. In one embodiment, the AAV vector has an AAVrh.10, AAV8, AAV9, AAV5, AAVhu.37, AAVhu.20, AAVhu.43, AAVhu.8, AAVhu.2, or AAV7 capsid. In one embodiment, the AAV vector is AAV2, AAV5, AAV7, AAV8, AAV9 or AAVrh.10. Th vector may be present in a pharmaceutical composition. In one embodiment, the amount of the viral vector in the composition is about 1×10^{11} to about 1×10^{16} genome copies.

The vector may be introduced to cells *in vitro* or *in vivo*. In one embodiment, a method to prevent, inhibit or treat Alzheimer's disease in a mammal includes administering to the mammal an effective amount of the gene therapy vector. In one embodiment, a method to prevent, inhibit or treat a disease associated with APOE4 expression in a mammal includes administering to the mammal an effective amount of the gene therapy vector. In one embodiment, a method to prevent, inhibit or treat a lipid disorder in a mammal includes administering to the mammal an effective amount the gene therapy vector. In one embodiment, the mammal is a E2/E4 heterozygote. In one embodiment, the mammal is a E4/E4 homozygote. In one embodiment, the mammal is a E2/E2 homozygote. In one embodiment, the mammal is a E3/E3 homozygote. In one embodiment, the mammal is a E3/E4 heterozygote. In one embodiment, the mammal is a E2/E3 heterozygote. In one embodiment, the mammal is a human. In one embodiment, the vector is systemically administered. In one embodiment, the vector is injected. In one embodiment, the vector is administered to the central nervous system. In one embodiment, the vector is administered to the brain. In one embodiment, the vector encodes an apolipoprotein E having C112, S136 and R158 or having C112, S136 and C158.

In one embodiment, a gene therapy vector comprising a promoter operably linked to a nucleic acid sequence comprising an open reading frame encoding APOE that is not APOE2, APOE3, or APOE4 but is modified and is protective, and a 3' untranslated region (3' UTR), and a nucleotide sequence having RNAi sequences corresponding to APOE4 for inhibition of APOE4 mRNA. In one embodiment, the vector comprises the nucleotide sequence. In one embodiment, the nucleotide sequence is 5' or 3' to the open reading frame. In one embodiment, the nucleotide sequence is 5' and 3' to the open reading frame. In one embodiment, the nucleotide sequence is on a different vector. In one embodiment, the vector is a viral vector. In one embodiment, the viral vector is an AAV, adenovirus, lentivirus, herpesvirus or retrovirus vector. In one embodiment, the AAV is AAV5, AAV9 or AAVrh10. In one embodiment, the nucleotide sequence is linked to a second promoter. In one embodiment, the second promoter is a PolIII promoter. In one embodiment, the RNAi comprises miRNA including a plurality of miRNA sequences. In one embodiment, the RNAi comprises siRNA including a plurality of siRNA sequences. In one embodiment, the open reading frame comprises a plurality of silent nucleotide substitutions. In one embodiment, the vector has at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the codons have a silent nucleotide substitution. In one embodiment, the open reading frame further comprises a peptide tag, e.g., a HA, histidine tag, AviTag, maltose binding tag, Strep-tag, FLAG-tag, V5-tag, Myc-tag, Spot-tag, T7 tag, or NE-tag. In one embodiment, the vector is in a host cell, e.g., in a host organism such as a mammal, or a non-human primate or a human. The host mammal may be administered the vector in an amount effective to, for example, prevent, inhibit or treat Alzheimer's disease in a mammal, or prevent, inhibit or treat a disease associated with APOE4 expression in a mammal. In one embodiment, the vector and/or the nucleotide sequence is systemically administered. In one embodiment, the vector and/or the nucleotide sequence is orally administered. In one embodiment, the vector and/or the nucleotide sequence is intravenously administered.

The invention will be further described by the following non-limiting examples.

Example 1

Overview

The pathogenesis of Alzheimer's disease (AD) is complex, characterized by central nervous system (CNS) accumulation of amyloid-beta (Aβ), and amyloid plaques, abnormal phosphorylation of tau,

tau tangles, inflammation and progressive loss of neurons, resulting in progressive cognitive decline1. Genetics play a major role in the risk for these pathogenic processes (DeTure & Dickson, 2019; Holtzman et al., 2012; Safieh et al., 2019; Fernandez et al., 2019). Early-onset autosomal dominant AD is caused by mutations in amyloid protein precursor (APP) and presenilin (PSEN) 1 and 2 (Campion et al., 1999; Carmona et al., 2018), genes that affect APP processing, altering production of Aβ peptides, resulting in aggregation and plaque formation (Carmona et al., 2018; Dai et al., 2018). The major genetic factors for sporadic late-onset AD are variants of apolipoprotein E (APOE), a lipid transport protein (Tzioras et al., 2019; Wolters et al., 2019). APOE has 3 common isoforms, the common ε3 isoform is associated with an average risk for AD, ε4 increases risk 15-20-fold while ε2 decreases risk 66 to 99% lower than ε3 and ε4, respectively (Wolters et al., 2019; Corder et al., 1994; Naj et al.,. 2011; Reiman et al., 2020). At the biologic level, the APOE genotype (ε4>ε3>ε2) predicts timing and amount of brain amyloid, p-tau and tau tangles in humans and AD mouse models (Tzioras et al., 2019; Shi et al., 2017; Abner et al., 2018). The differences in E2 and E4 are dictated by amino acids 112 and 158; APOE2 (C112 C158) conveys low risk and APOE4 (R112 R158) high risk (Corder et al., 1994; Reiman et al., 2020; Corder et al., 1993). Within this 112-158 "Alzheimer's risk region" is coding for the LDL receptor and binding to heparan proteoglycans (Mahley et al., 1999).

Since APOE4 conveys high risk and APOE2 low risk, to develop a gene therapy to prevent/treat the development of AD-related pathology in APOE4 homozygotes with AAVrh.10hAPOE2, a serotype rh.10 adeno-associated virus (AAV) gene transfer vector coding for human APOE2 was prepared. CNS administration of AAVrh.10hAPOE2 prevented APOE4-associated Aβ and amyloid burden in mouse models (Zhao et al., 2016), supporting the concept that APOE4-associated risk for AD might be mitigated using APOE2 gene therapy to convert the APOE4 homozygote brain to an APOE2-APOE4 heterozygote state (Zhao et al., 2016).

The importance of the APOE 112-158 region in mediating AD risk was highlighted by the report of a female carrier of the PSEN1 E280 mutation but also homozygote for APOE3ChC, the Christchurch R136S APOE3 variant (Arboleda-Velasquez et al., 2019). The dominant PSEN1-E290A gene defines a Colombian family of >1,200 individuals all with early onset CNS Aβ and amyloid accumulation; heterozygotes develop dementia in their mid-forties (Lopera et al., 1997). Strikingly, the female who co-inherited APOE3ChC and PSEN1-E290A was in good cognitive condition at age 70, well past the age when the kindred typically develop cognitive decline (Arboleda-Velasquez et al., 2019). Clinical assessment revealed accumulation of amyloid plaques, but low levels of tau pathology, leading to the concept that the APOE3ChC variant blocked development of tau pathology. This was corroborated with *in vitro* experiments demonstrating that APOE3ChC behaves like the APOE2 allele; both bind poorly to heparin (APOE3ChC>APOE2) compared to APOE4 which binds tightly (Mahley et al., 1999; Zhao et al., 2016; Rosenberg et al., 2018; Arboleda-Velasquez et al., 2019). The observation that the inheritance of APOE3ChC prevents PSEN1-E280A-driven development of tau pathology but not amyloid pathology (Arboleda-Velasquez et al., 2019), together with gene therapy studies demonstrating that APOE4-driven amyloid pathology can be suppressed by AAV-mediated delivery of APOE2 to the CNS, led to testing, in mouse models of AD amyloid and tau pathology, whether AAVrh.10-mediated delivery to the CNS of APOE3ChC (APOE3 with Christchurch variant) suppresses tau-related pathology and whether delivery of APOE2ChC (APOE2 with Christchurch variant) suppresses both amyloid and tau pathology. 2 mouse,

human E4-based models are used: APP.PS1/TRE4 exhibiting APP and APOE4-associated amyloid pathology (Zhao et al., 2016; Rosenberg et al., 2018; Arboleda-Velasquez et al., 2019; Lopera et al., 1997; Kim et al, 2011) and P301S/E4 exhibiting tau and APOE4 associated tau pathology (Liu et al., 2016; Allen et al., 2002).

Strategy

Heredity plays a major role in the risk for Alzheimer's disease (DeTure & Dickson, 2019; Holtzman et al., 2012; Safieh et al., 2019; Fernandez et al., 2019). Early-onset autosomal dominant AD is caused by mutations in amyloid protein precursor (APP) and presenilin 1 (PSEN1) and 2 (PSEN2) (Campion et al., 1999; Carmona et al., 2018), genes that affect the processing of APP, altering the production of Aβ peptides, resulting in aggregation and increased plaque formation (Carmona et al., 2018; Dai et al., 2018). The major genetic factors for sporadic late-onset AD are variants of apolipoprotein E (APOE), a lipid transport protein (Tzioras et al., 2019; Wolters et al., 2019). APOE has three common isoforms, ε2, ε3, ε4; the common ε3 isoform is associated with an average risk for AD, ε4 increases the risk and reduces the age of onset of AD, while ε2 decreases risk and delays the age of onset (Wolters et al., 2019; Corder et al., 1994; Naj et al., 2011). At the biologic level, the APOE genotype ε4 > ε3 > ε2 predicts the timing and amount of brain amyloid, p-tau and tau tangles in humans and mouse models of AD (Tzioras et al., 2019; Shi et al., 2017; Abner et al., 2018). Based on the knowledge that inheritance of different variants of the APOE genes can dictate the AD risk, age of onset and severity (DeTure & Dickson, 2019; Shinohara et al., 2016), it was envisioned that a therapy to reduce the CNS-related Alzheimer's pathology in APOE4 homozygotes could be accomplished by delivering "APOE AD decreased risk" variants to the CNS. Prior studies demonstrated that APOE2 delivery to the CNS mediated by AAV serotype rh.10 (AAVrh.10), reduced Aβ and amyloid burden in mouse models of AD (Zhao et al., 2016). The ability of APOE2 to reduce brain Aβ burden in these mouse models was dependent on gene dose and the amount of pre-existing Aβ1-42 deposition, suggesting that, if sufficient brain APOE2 levels could be achieved early in the course of the disease, gene delivery of APOE2 using an AAV vector was a viable therapeutic approach for treating or preventing AD (Zhao et al., 2016). This data, along with studies demonstrating the ability to deliver and safely achieve high levels of APOE2 expression in the CNS of non-human primates using intracisternal route (Rosenberg et al., 2018), led to a first-in-human ongoing clinical study to treat APOE4 homozygotes with intracisternal administration of AAVrh.10hAPOE2.

An "APOE AD decreased risk" gene therapy strategy that is more effective than gene therapy with APOE2 in mitigating development of APOE4-driven AD-related pathology is envisioned. The approach is based on a recent report of the consequences of the APOE3-Christchurch (APOE3ChC) mutation superimposed on the Colombian PSEN1-E290A mutation, a PSEN1 variant in a family affecting >1,200 individuals (Lopera et al., 1997; Acosta-Baena et al., 2011). The PSEN1-E280A mutation is associated with an increased Aβ production, leading to amyloid accumulation; heterozygotes develop dementia in their mid-forties (Lopera et al., 1997). A female from this kindred with the autosomal dominant PSEN1 E280A mutation, but also homozygous for the APOE3ChC variant, is in good cognitive condition at age 70 and remains independent, well past the age of 40 yr, when those with the same PSEN1 mutation typically demonstrate cognitive decline (Arboleda-Velsquez et al., 2019). Clinical assessment revealed significant accumulation of amyloid plaques, but low tau/p-tau pathology suggesting that the APOE3ChC mutation may have blocked development of tau pathology (Arboleda-Velsquez et al., 2019). Of interest, the

APOE3ChC variant (R136S) is in the same region of the APOE protein as the common ε2, 3 and 4 alleles (112-158), consistent with the concept that this region of APOE is related to risk for AD (Mahley et al., 1999).

The ability of the APOE3ChC variant to suppress tau pathology was corroborated with *in vitro* experiments demonstrating that APOE3ChC behaves like the APOE2 allele; both bind poorly to heparin (APOE3ChC<APOE2) (Mahley et al., 1999; Arboleda-Velsquez et al., 2019). These findings suggest that the AD-related chain of pathogenic events is likely broken by APOE3ChC after amyloid formation, and independent of the mechanism, the Christchurch mutation is protective, can be used to treat APOE4 homozygotes, and likely will be more effective than gene therapy with the APOE2 allele (Figures 12-13). Using the AAVrh.10 capsid as a delivery system in relevant AD mouse models, it is assessed AAVrh.10-mediated delivery to the CNS of either APOE3 or APOE2 each harboring the Christchurch mutation, are superior to AA Vrh.10hAPOE2 as a treatment for Alzheimer's disease in APOE4 homozygotes. Based on the prior demonstration that AAVrh.10hAPOE2 suppresses amyloid accumulation in murine models (Zhao et al., 2016), and the clinical case report on the Christchurch variant, it is likely that AAVrh.10hAPOE3ChC suppresses tau, but not amyloid, pathology, and that AAVrh.10hAPOE2ChC may be more effective, suppressing both amyloid and tau pathology.

PSEN1-APOE3ChC. While the report of an individual who inherited the APOE3ChC variant along with Colombian PSEN1-E280 mutation only represents a single case, the report included additional studies that support the conclusion that APOE3ChC is an "APOE AD decreased risk" variant. Although amyloid PET scanning revealed a significant accumulation of CNS amyloid plaques, very little tau pathology was observed, and was mostly confined to the medial temporal lobe. In addition, brain glucose metabolism was almost normal (Figure 14). The concept that the APOE3ChC mutation blocks development of tau pathology is similar to the concept in the study by Reiman et al. (2020) that observed in 24 autopsy samples from APOE2 homozygotes that APOE2 was associated with decreased tau pathology. The ability of the APOE3ChC variant to suppress tau pathology was supported by *in vitro* experiments demonstrating that APOE3ChC behaves like the APOE2 allele; and that compared to E4, E2 and E3ChC both bind poorly to heparin (binding: E4>E3>E2>>E3ChC, Figure 15). HSPGs have been implicated in the buildup of amyloid plaques, as well as in promoting microglial response to amyloid (Hefferman et al., 2016; ALXFORUM, 2010; Snow et al., 1988; Liu et al., 2016; O'Callaghan et al., 2018). HSPG have also been linked to tau pathology; it has been suggested that HSPG allow tau fibrils to attach to neurons, facilitating uptake and propagation of toxic forms of tau (Rauch et al., 2018; Zhao et al., 2019). *In vitro* studies also demonstrated that APOE3 bound with an antibody to residues 130-143 (which includes the region of the Christchurch mutation) converted APOE3 functionally to APOE3ChC as measured by heparin binding. Together, these findings suggest that the Alzheimer's related chain of pathogenic events is likely broken by APOE3ChC after amyloid formation, and independent of the mechanism, since the Christchurch mutation is protective, it be used to treat APOE4 homozygotes.

APOE4-associated risk for AD. APOE, a lipid binding protein, is the major carrier of cholesterol in the CNS (Puglielli et al., 2003; Williams et al., 2020). In the brain it is mainly produced by astroglia and microglia (Flowers & Rebeck, 2020; Pitas et al., 1987; Holtzman et al., 2012). The APOE particles are formed in the extracellular milieu (Mahley et al., 1999; Flowers & Rebeck, 2020); the APOE particle transports cholesterol to neurons via APOE receptors, members of the low density lipoprotein receptor gene family (Liu et al., 2013; Mondadoori et al., 2007; Zhang et al., 2013). The APOE protein (299 amino

acids) has N-terminal (1-167) and C-terminal (206-299)domains connected by a hinge region (Safieh et al, 2019; Flowers & Rebeck, 2020). The C-terminal region contains the low density lipoprotein receptor binding site41 **(Figure 2).** In addition to its role in transporting cholesterol in the CNS, APOE has myriad other functions (Holtzman et al., 2012; Castellano et al., 2011; Deane et al., 2008; Hashimoto et al., 2012; Hatters et al., 2006a; Hatters et al., 2006b; Li et al., 2012; Manelli et al., 2005; Walker et al., 2000; Yu et al., 2014; Zhao et al., 2009). Human APOE is commonly expressed in 3 isoforms (APOE2, 3, and 4) that differ only by two residues (Mahley et al., 1996; Zannis et al., 1981) (Figure 13). Inheritance of APOE4 is the most important genetic risk factor for AD. APOE3 is neutral, the E4 allele increases risk and reduces age of onset and E2 decreases risk and delays age of onset (Reiman et al., 2020; Mahley, 2016; Conejero-Goldberg et al., 2014; Nagy et al., 1995; Raber et al., 2004). Epidemiologic data suggest that APOE4 and APOE2 are co-dominant, that is, E2/E4 heterozygotes, instead of having the 4-fold higher risk for AD of E3/E4 heterozygotes, have close to the normal risk of E3/E3 homozygotes (Corder et al., 1994; Liu et al., 2013; Genin et al., 2011), i.e., roughly equivalent expression of E2 cancels out the deleterious effect of the E4 allele (Corder et al., 1994; Farrer et al., 1997; Coon et al., 2007). The key conclusions from epidemiologic studies of the protective effects of E2 studies include: the E2 genotype is markedly underrepresented in AD (Corder et al., 1994; Reiman et al., 2020); the E2 allele is associated with a delayed age-of-onset of AD (Reiman et al., 2020; Benjamin et al., 1994); E2 carriers have reduced AD-related pathology (Reiman et al., 2020; Nagy et al., 1995); E2 is associated with slower cognitive decline (Small et al., 2004); E2 carriers harbor more robust white matter integrity that may be associated with decreased vulnerability to developing AD (Chiang et al., 2012); and in normal elderly, E2 carriers have lower levels of cerebrospinal fluid p-Tau and marginally lower tau (Chiang et al.,, 2010). Experimental studies demonstrate that E2 promotes metabolism of Aβ; neuronal repair and neurite outgrowth; and acts as an anti-oxidant and anti-inflammatory agent (Rebeck et al., 2002). Further, animal and clinical studies have shown that APOE genotype also predicts the timing and amount of brain amyloid β (Aβ) peptide deposition as well as amyloid burden (E4>E3>E2) (Castellano et al., 2011; Raber et al., 2004; Bales et al., 2009; Holtzman et al., 2000; Reiman et al., 2009; Schmechel et al., 1993), i.e, APOE4 impairs amyloid β clearance and increases amyloid formation 70,71 .(Liao et l., 2017; Hu et al., 2015). Murine studies in mice have shown that targeted deletion of the endogenous *ApoE* gene results in a dramatic decrease in fibrillary amyloid and total brain Aβ deposition in the PDAPP mouse model of AD (these mice express a mutant APP transgene resulting in high brain levels of human Aβ) (Bales et al., 1997). By crossing PDAPP mice to human APOE targeted replacement (TRE) mice, APOE isoform-dependent effects were observed in brain Aβ deposition and amyloid burden (E4>>E3>E2) in a manner that recapitulates what is observed in AD patients (Castellano et al., 2011; Bales et al., 2009; Holtzman et al., 2000; Fagan et al., 2002), suggesting that the APOE isoforms play an important role in determining brain amyloid burden. Further, studies in tau pathology-related P301S/E3, E2 and E4 mice demonstrate ApoE affects neurodegeneration in the context of tau pathology independently of amyloid-β. ApoE4 resulted in aggravated neurodegeneration whereas the absence of ApoE was neuroprotective (Shi et al., 2017).

APOE2 gene therapy for APOE4-related CNS pathology. Based on the epidemiologic data of APOE2 being protective, a gene therapy program was developed to assess the hypothesis that transfer of APOE2 to the CNS of APOE4 homozygotes would mitigate the E4 risk (Zhao et al., 2016; Rosenberg et al., 2018). Evidence in the literature of the feasibility of this approach came from studies of Dodart et al.

(2005) who demonstrated that administration of APOE4 and APOE2 to the CNS of murine AD models using lentivirus vectors increased and decreased, respectively, brain Aβ/amyloid burden. The finding that APOE2 decreased brain Al3/amyloid burden was replicated by Hudry and colleagues (2013) using an AAV4 vector to deliver APOE2 into the lateral ventricle of mutant APP mice. Using AAV8 vectors administered to the CSF, Hu et a1. (2015) found that increasing APOE2 in TRE4 mouse models, was an efficacious strategy to treat AD; whereas, increasing APOE4 in TRE4 mice was deleterious. As described in detail below, an AAVrh.10 serotype vector coding for human APOE2 reduces the amyloid burden in 2 murine models (Zhao et al., 2016). Irrespective of the molecular mechanisms by which these APOE alleles determine AD risk, given the fact that APOE2 is strongly protective and when delivered to the mouse brain via either gene therapy vector markedly reduces Aβ/amyloid burden, CNS gene delivery of APOE2 via viral vectors represents a therapeutic strategy for APOE4 homozygous AD.

AAVrh.10. The AAVrh.10 serotype is derived from a rhesus macaque. This serotype has been administered with various expression cassettes to the CNS of experimental animals (mice, nonhuman primates), to children with CLN2 disease and adults with Alzheimer's disease (clinicaltrials.gov; NCT01414985, NCT01161576, NCT03634007). The AAVrh.10 vectors are designed with a highly active constitutive promoter, intron coding sequence and the rabbit β-globin polyA flanked by AAV2 inverted terminal repeats (as an example see Figure 16 for details of the AAVrh.10hAPOE2 vector). The AAVrh.10 capsid is highly effective at mediating gene expression in the CNS, equivalent to AAV9, but with less potential toxicity (Rosenberg et al., 2018; Rosenberg et al., 2014; Sondhi et al., 2012; Tardieu et al., 2014; Zerah et al., 20150. All vectors are characterized for numerous measures of identity, potency and purity. Purity by polyacrylamide gel electrophoresis establish the absence of contaminant proteins from the cells used in vector production (Figure 17). As a demonstration of the effectiveness of the intracisternal administration of a vector coding human APOE2 to nonhuman primates (NHP) provide broad distribution of vector derived protein throughout the CNS including the cerebral spinal fluid (Figure 18). These observations support that CNS administration of an AAVrh.10 vector coding for a human APOE isoform can effectively distribute the coding APOE isoform throughout the CNS.

AA Vrh.10hAPOE2 ameliorates pathology in AD animal models. The PDAPP mouse is a well characterized mouse model of brain amyloidosis that overexpresses a human APP mutation and develops age-dependent Aβ and amyloid deposition in the hippocampus and cerebral cortex (Games et al., 1995; Schenk et al., 1999). At 9 months of age, there are abundant Aβ and amyloid plaques in the hippocampus80. Expression of the AAVrh.10hAPOE2 vector demonstrated significant resolution of the amyloid pathology observed in control animals treated with a similar viral vector expressing an irrelevant gene (Figure 19) (Zhao et al., 2016). Expression of APOE2 using the AAVrh.10hAPOE2 vector resulted in a marked decrease in insoluble Aβ1-42 (70.4% reduction. $p<0.001$ *vs* the mCherry control) and soluble Aβ1-42 (27.2% reduction, $p<0.05$ *vs* mCherry control) levels in the hippocampus of PDAPP mice. Insoluble Aβ1-40 was also dramatically decreased (61.2% reduction. $p<0.001$ *vs* control) although the relatively already low levels of soluble Aβ1-40 were not altered.

To further assess the role of APOE2 expression on brain Aβ burden, the impact of AAVrh.10hAPOE2 dose on Aβ/amyloid pathology in APP.PS1/TRE4 triple transgenic mice was also evaluated. APP.PS1/TRE4 mice carry the APP Swedish (APPswe) and PS1 mutations as well as the human APOE4 gene (Kim et al., 2011; Jankowsky et al., 2018). APP.PS1/TRE4 mice develop robust age-

dependent Aβ and amyloid pathology in multiple brain regions including the cerebral cortex, hippocampus, and thalamus. Amyloid plaques are observed in the cortex of these mice as early as 2 months of age and the brain AJ3/amyloid burden that develops is dependent on APOE4 expression (Kim et al., 2011). Similar to the observation in the PDAPP mice, dose-dependent suppression of amyloid-beta levels was observed in the hippocampus of APP.PS1/TRE4 mice treated with intra-hippocampal delivery of AAVrh.10hAPOE2 (Figure 20).

Mouse models of APOE4-related AD pathology. The clinical goal is to suppress both amyloid and tau pathology in the CNS of APOE4 homozygotes, and thus murine models were chosen with murine ApoE knockouts and human APOE4 knockins. One murine model for APOE4-related amyloid pathology and another one is for APOE4-related tau pathology. The APP.PS1/TRE4 mouse expresses the human β-amyloid (Aβ) precursor protein (APP) with the Swedish mutation (APPswe), mutant (L 166P) human presenilin 1 and the human APOE4 gene replacing the endogenous mouse ApoE gene. These mice express high levels of human Aβ protein and human AD-like amyloid pathology (Kim et al., 2011) and were used in our prior studies to demonstrate that AAVrh.10hAPOE2 will suppress AD amyloid-related pathology (Zhao et al., 2016) (Figure 20). The P301S/E4 mouse harbor the human P301S 1N4R tau, deletion of the murine ApoE gene and addition of the human APOE4 gene (Shi et al., 2017; Allen et al., 2002). These mice have APOE4-related tau pathology and will be used to evaluate the ability of the gene therapy vector to suppress tau pathology. These mice also have microglial and astrocyte activation, representing AD-related inflammation (Shi et al., 2017).

Approach. The Christchurch variant (APOE3-R 136S) is in the APOE 112-158 "Alzheimer's risk region" that includes residues 112 and 118 that define APOE2, 3 and 4, the LDL binding region (136-150) and the proteoglycan heparan sulfate binding region (Figure 13). Thedemonstration that the APOE4-associated Abeta and amyloid burden in an APOE4-related mouse model could be prevented with CNS administration of AAVrh.10APOE2 supports the concept that the APOE4-associated risk for AD might be mitigated by using gene therapy delivering the APOE2 coding sequence to convert the APOE4 homozygote brain to an APOE2-APOE4 heterozygote state (Zhao et al., 2016). The report of a woman in the PSEN1-E290A kindred with concomitant homozygous inheritance of APOE3ChC that negated the PSEN1-E290A driven AD-related tau pathology, led to the focus of this proposal: to develop a 2nd generation gene therapy for APOE4 homozygotes using an AAVrh.10 vector to deliver the Christchurch mutation on either the APOE3 or APOE2 background. We will test this in 2 human APOE4-related mouse models: APP.PS1ffRE4 demonstrating primarily amyloid-related pathology and P301S/E4 demonstrating primarily tau-related pathology (Shi et al., 2017; Kim et al., 2011). If the Christchurch variant on the APOE3 background is effective in the mouse models equivalent to the case report of dual inheritance of PSEN1-E290A and APOE3ChC, then gene therapy with AAVrh.10APOE3ChC should suppress tau pathology in the P301S/E4 mouse but not the amyloid pathology in the APP.PS1ffRE4 mouse. However, based on the effectiveness of AAVrh.10hAPOE2 in suppressing amyloid pathology in the APP.PS1ffRE4 mouse (Zhao et al., 2016), we hypothesize that AAVrh.10hAPOE2ChC will suppress both the amyloid pathology in the APP.PS1ffRE4 mouse and also the tau pathology in the PSEN1-E290A mouse. If the hypothesis is correct, AAVrh.10hAPOEChC will be an ideal 2nd generation candidate to move to the clinic to treat APOE4 homozygotes. Other than the different vectors (AAVrh.10hAPOE3ChC and AAVrh.10hAPOE3, or AAVrh.10hAPOE2ChC and AAVrh.10hAPOE2), everything is identical. The

statistical analysis includes comparison of the parameters within each experiment, and separately,, to compare the differences of AAVrh.10hAPOE3ChC and AAVrh.10hAPOE2ChC.

For each mouse model (APP.PS1ffRE4, P301S/E4) the vectors AAVrh.10hAPOE3ChC, AAVrh.10hAPOE3; AAVrh.10hAPOE2ChC, AAVrh.10hAPOE2; controls for both, AAVrh.10Null (no translatable transgene) or PBS] are administered at 2 months of age bilaterally to the hippocampus in 2 µl. The vectors are administered with 2 doses $0.4x10^{10}$ genome copies (gc) or $1.0x10^{10}$ gc. Assessments will be at 5 and 9 months. Ten males and 10 females will be used for all data points (mouse strain, vector or PBS, assessment time points; Table I). The assessments to be conducted related to the vectors, general pathology, amyloid, tau and inflammation are listed in Table II. Based on AAVrh.10hAPOE2 effectively suppressing amyloid-related pathology in APP.PS1ffRE4 mice (Zhao et al., 2016), and the case report of homozygous inheritance of APOE3ChC allowing for amyloid pathology but preventing the tau pathology in a female carrier in the PSEN1-E280A kindred (Arboleda-Velasquez et al., 2019), we both the hAPOE3ChC and hAPOE2ChC vectors are likely more effective than the hAPOE3 and hAPOE2 vectors without the ChC variant, and the hAPOE2ChC construct is likely more effective than the hAPOE3ChC construct in preventing both amyloid and tau pathology. Also tested are: (1) the effectiveness of the hAPOE3ChC and hAPOE2 vectors on more general pathology (quantitative volumes of AD-relevant CNS structures, transcriptome analysis of general cell functions); and (2) AD-related inflammation (microglia and astrocyte activation, transcriptome analysis of proinflammatory genes) because the E4 variant is associated with greater innate immune-related inflammation than the E2 and E3 variants (Vitek et al., 2009; Gale et al., 2014).

Table I. AAVrh.10-mediated Therapy with APOE Variants of APP.PS1/TRE4 and P301S/E4 Mouse Models of Human APOE4-related AD Pathology

Parameter	**Variable**
Mouse model	APP.PS1/TRE4, P301S/E4
Transgene (AAVrh.10 vectors)	APOE3ChC, APOE3; APOE2ChC, APOE2; -Null, PBS[1]
Route, dose	Bilateral hippocampus, $0.4x10^{10}$ gc, $1x10^{10}$ gc2
Age at vector administration	2 months[2]
Age of assessment	5, 9 months[3]
Number of mice/time-point, dose	10 M, 10F
Total number of mice	880

Each mouse model is treated with PBS or AAVrh.10 vectors coding for the listed transgenes; All administrations are at 2 months, bilateral to the hippocampus at the listed doses in 2 µl each; Assessment of the CNS is at 5 and 9 months, see Table II for list of parameters to be assessed

Table II. Assessment of the Efficacy of APOE Variants in the APP.PS1/TRE4 and P301S/E4 Mouse Models of Human APOE4-related AD Pathology

Parameter	**Assessment**
Vector-related	Transgene DNA, mRNA, protein (Western, immunohistochemistry)

General pathology	Quantitative volumes, transcriptome of general cell function (RNAseq)
Amyloid-related	Soluble, insoluble Aβ-1-42, Aβ-1-40 (ELISA, immunohistochemistry)
Tau-related	Total tau, p-tau (ELISA, immunohistochemistry)
Inflammation-related	Microglia, astrocyte activation (CD68 immunohistochemistry, RNAseq proinflammatory genes)

Each mouse model is administered AAVrh.10hAPOE3ChC, hAPOE3, hAPOE2ChC, hAPOE2, Null ($0.4x10^{10}$ gc or $1x10^{10}$ gc; bilateral, hippocampus) or PBS at 2 months of age and evaluated at 5 and 9 months of age. Ten males, 10 females for each vector, each dose, each time-point; PBS 10M, 10F each timepoint; DNA, mRNA by qPCR, APOE proteins are assessed by quantitative Western, quantitative immunohistochemistry; Quantitative volumes of piriform/entorhinal cortex, hippocampus, posterior lateral ventricle, and thickness of dentate gyrus are assessed; RNAseq of quantitative transcriptome of general cell function-related is assessed; Soluble (RIPA) and insoluble Aβ-1-42 and Aβ-1-40 are assessed by ELISA and immunohistochemistry of Aβ (positive % area of hippocampus, Aβ plaque burden); ELISA and immunohistochemistry of tau and p-tau (positive % area of hippocampus, tau tangle quantification are assessed); Quantification of microglia (CD68, Iba1) and astrocytes (GFAP) are assessed by cell count and% area of hippocampus, RNAseq quantitative transcriptome of proinflammatory genes.

Methods.

Vector-related. The MVrh.10 vectors are produced and purified as described previously ((Sondhi et al., 2012; Sondhi et al., 2007). Briefly, the vectors are produced by cotransfection of HEK293T cells with an expression cassette plasmid and adenoviral helper plasmids. The packaging cell line, HEK293T, is maintained in Dulbecco's modified Eagles medium, supplemented with 5% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and maintained at 37°C with 5% CO_2. The cells are grown in CellSTACKS (Corning) for 24 hours followed by transfection with plasmids using the PEIpro procedure. The cells are incubated 3 days before harvesting and lysed by 5 freeze/thaw cycles. The resulting cell lysate is treated with 50 U/ml of benzonase at 37°C, 30 minutes. The cell lysate is purified by iodixanol density gradient followed by Q-HP ion-exchange chromatography. The purified AAVrh.10 vectors are concentrated in phosphate buffered saline (PBS). Vector genome titer is determined by TaqMan quantitative polymerase chain reaction. The purified vectors are sterile filtered; tested 14 days for growth on medium supporting the growth of aerobic bacteria, anaerobic bacteria, or fungi; tested for endotoxin; and demonstrated to be mycoplasma free.

Mouse models. The APP.PS1/TRE4 mouse colony, derived from crossing APP.PS1-21 mice with human APOE4 knockin mice, are established (Zhao et al., 2016). The P301S/E4 mice are generated by crossing human tau mutant P301S mice with human APOE4 knockin mice. The genome of the mice is validated by PCR of the relevant knockin genes. Intracerebral injection of AAV vectors is carried out by stereotaxic surgery (Dodart et al., 2005). Briefly, under sterile conditions, mice are anesthetized with isoflurane and secured on a stereotaxic frame (David Kopf Instruments). An incision is made over the skull, and burr holes of the size of the injection needle are made using a high-speed drill. The AAV preparation (2 µL, gc at the indicated dose) is bilaterally injected into the targeted brain regions using a 33-gauge needle (Hamilton) and a syringe pump (KD Scientific) at a rate of 0.2 µL/min. Stereotactic coordinates used for injections are as follows: ±1.7 mm anteroposterior from bregma; ±1.2 mm mediolateral from bregma, and ±1. 7 mm dorsoventral below the dura. This is based on an atlas of the mouse brain95. After each injection, the needle is left in place for 5 minutes to minimize backflow and

then slowly withdrawn. Animals are individually housed and monitored until they regained full consciousness. At 5 months and 9 months of age, the mice are deeply anesthetized with ketamine/xylazine and transcardially perfused with 0.3% heparinized saline (2500 IU/L). Brains are rapidly collected, and each brain will be divided along the sagittal plane with 1 hemibrain processed for histologic and immunohistochemistry (IHC) analyses, and the other hemibrain microdissected into the hippocampus, thalamus, and entorhinal cortex then rapidly frozen on dry ice and stored at -80° for biochemical analyses.

Brains are post-fixed in 4% paraformaldehyde for ≥ 48 hours, transferred to 30% sucrose for ≥ 48 hours before freezing, and sectioned using a CM3050-S cryostat (Leica). Serial coronal sections aree prepared at 50 µm intervals. IHC analyses will be performed using standard methods (Bales et al., 2009; Holtzman et al., 2000; Bales et al., 1997). Free-floating sections are washed and incubated with primary antibodies overnight at 4°C. Immunoreactivity (IR) are visualized using Alexa Fluor 488 or 594-conjugated secondary antibodies (1:200; Invitrogen) or the Vectastain ABC Elite kit (Vector) and 3,3-diaminobenzidine solution. The specificity of the primary antibodies staining is verified by the lack of IR signal when the primary antibodies were omitted. Thioflavin-S staining is performed as to detect amyloid plaques (Bales et al., 2009; Holtzman et al., 2000; Bales et al., 1997). Fixed brain sections are briefly incubated with 0.1 % freshly made thioflavin-S solution (in 50% ethanol) for 8 minutes and then cleared with 80% ethanol for 1 minute followed by washing with H_2O 3x for 1 minute each before being cover slipped with mounting media. Images are taken using an epifluorescence microscope (Nikon H550L).

Vector-derived APOE. For tissue homogenates processed for vector DNA, the DNeasy Blood and Tissue Kit (cat #69506; Qiagen) will be used with about 50 mg of homogenized tissue sample. For tissue homogenates processed for vector transgene mRNA, the RNeasy Lipid Tissue Mini kit (Qiagen) will be used with ~50 mg of homogenized tissue sample. Quantitative analysis of vector DNA and transgene mRNA copies in the brain sections will be performed by PCR using a TaqMan-based analysis (Applied Biosystems "Universal Master Mix II, no UNG" reagent) and a human-specific primer/probe set (Applied Biosystems) to detect the 3' terminus of the human APOE2 or E3 or E4 cDNA from viral genomes, in the background of murine genomic DNA (forward primer: 5'GTGGAGAAGGTGCAGGCT-3' (SEQ ID NO:10); reverse primer: 5'-AAGCGTAATCTGGAACATCGT-3' (SEQ ID NO:11); probe, 5'CCCTGTGCCCAGCGACAATC-3' (SEQ ID NO:12). The PCR is performed using the QuantStudio6 Flex system (Applied Biosystems) (Rosenberg et al., 2018). APOE levels are measured in brains using a sandwich ELISA (Bales et al., 2009). Tissue homogenates are diluted in sample dilution buffer (PBST containing 0.4% glycine). Samples were loaded into 96-well plates coated with an anti-APOE antibody (1:2000; Millipore). After overnight incubation at 4°C, a biotinylated anti-APOE antibody (1:10,000; Meridian Life Science) is used for detection. The IR signal after incubation with HRP-conjugated streptavidin (Research Diagnostics) is developed with a TMB substrate (Thermo Scientific) and read on a Synergy H1 Hybrid plate reader (BioTek). Levels of APOE are calculated using a standard curve generated with recombinant human APOE (Meridian Life Science). Levels of APOE in brain homogenates are determined in triplicate, normalized to protein content, and expressed as the amount of APOE/ mg protein. Examination of APOE and other protein levels in mouse brain tissue homogenates is also carried out by Western analysis (Zhao et al., 2016; Sacramento et al., 2020). The total protein concentration in tissue homogenates was determined using a BCA protein assay kit (Thermo Scientific). For sodium dodecyl sulfate (SOS) polyacrylamide gel electrophoresis, equivalent amounts of protein (25 mg) samples are mixed with loading dye containing 2% SDS and 1 % β-mercaptoethanol, incubated for 10 minutes at

90°C, and resolved on 10% Tris-glycine polyacrylamide gels. For nondenaturing PAGE, equivalent amounts of protein (25 µg) samples are mixed with nondenaturing loading dye to a final concentration of 0.04% bromophenol blue, 4.0% glycerol, and 100 mM Tris (pH 6.8) and resolved on 4-12% nondenaturing Tris-glycine polyacrylamide gels (Invitrogen). Proteins are transferred onto polyvinylidene difluoride (Millipore) membranes at 100 V. After blocking with Superblock (Thermo Scientific) for 1 hour, 23°C, membranes are probed with primary antibodies overnight at 4°C followed by species-specific HRP-conjugated secondary antibodies (1:8000; Invitrogen) for 1 hour, 23°C. Enhanced chemiluminescence (GE Healthcare Biosciences) is used to identify the bands. Protein molecular weights on denaturing gels are determined by comparison with the kaleidoscope molecular weight marker standard (Bio-Rad). Particle diameters on native gels is determined by comparison with a native high molecular weight marker standard (GE Healthcare Biosciences). Signal quantification is done using densitometry analysis of the scanned, autoradiograms with Image Lab software (Bio-Rad). Loading is normalized by stripping blots and reprobing with a β-actin antibody (Sigma-Aldrich).

General pathology.

Volumetric analysis. Every 6th coronal brain section (300 µm between sections) starting rostrally at bregma +2.1 mm to the dorsal end of the hippocampus at bregma -3.9 mm are cryosectioned for each mouse. The mounted sections are stained with 0.1 % Sudan black in 70% ethanol at RT for 20 minutes, then washed in 70% ethanol for 1 minute, 3 times. The sections are washed in Milli-Q water for 3x and coverslipped with Fluoromount. The stained slices are imaged with the NanoZoomer and areas of interest traced and measured in each slice using the NOP viewer. The volume is calculated using the formula: volume = (sum of area) x 0.3 mm. For hippocampus and posterior lateral ventricle, quantification starts from bregma -1.1 and ends at bregma -3.9. For piriform/entorhinal cortex, quantification starts at bregma -2.3 and ends at bregma -3.9.

Neuronal layer thickness measurement. Three sections (bregma -1.4, -1.7, and -2.0 mm) from each mouse are mounted and stained in cresyl violet for 5 minutes at RT. The slices are then sequentially dehydrated in 50%, 70%, 95% (3 times) and 100% ethanol (twice) for 1 minute, then cleared in xylene for 4 minutes (twice), and cover slipped in cytoseal (Coon et al., 2007) (Thermo Fisher Scientific, 8310-16). The thickness of the CA1 pyramidal cell layer and dentate gyrus granular cell layer are measured by drawing a scale perpendicular to the cell layer at two spots in all 3 slices and taking the average value for each mouse.

RNASeq transcriptome analysis. Total RNA is extracted using the TRIzol method and subsequent clean-up was performed on RNeasy columns (Qiagen). RNA quantity was assessed by Nanodrop ND-1000 (Thermo Scientific) and RNA quality assessed by Bioanalyzer (Agilent Technologies) (Raman et al., 2009; Tumor Analysis, 2004). Total RNA is purified, amplified and loaded onto an Illumina flowcell for paired-end sequencing reactions using the Illumina HiSeq 2500 (Illumina) (Ryan et al., 2014). The library is prepared using (0.5 µg total RNA) TruSeq RNA Library Prep Kit v2. Illumina HiSeq paired-end reads are aligned to GRCh37/hg19 human reference genome and RefSeq gene definitions (2014-06-02) using STAR (2.3.1z13_r470). Cufflinks (2.2) is used to convert aligned reads into fragments per kilobase of exon per million fragments sequenced (FPKM) using RefSeq gene definitions. Genes with FPKM >0.125 are included in the analysis. The dysregulated genes are functionally annotated using Gene Ontology (GO) and the Human Protein Reference Data Base (www.hprd.org). A 2-tailed Student's t-test is used for comparison of numerical data and a chi-square test is used with Yates' correction is used for comparison

of categorical data. The files are processed in Partek Genomics Suite software version 6.6, 2012 (Partek). A 1-way ANOVA is used to compare the matched groups, and a 2-or 3-way ANOVA is used to compare groups with parameters identified as a source of variation. A p value <0.05 is considered significant (p value was corrected for multiple testing using with Benjamini-Hochberg correction). Relevant to "general pathology," normal cell function genes and autophagy-related genes are assessed as detailed in the Shi et al. (2017) study.

Amyloid-related. Quantification of amyloid/Al3 burden is performed using standard methods (Zhao et al., 2016; Bales et al., 2009; Holtzman et al., 2000; Bales et al., 1997). Briefly, 4 brain sections per mouse, each separated by 300 µm, are selected for quantification. These sections correspond roughly to sections at Bregma -1.4, -1.7, -2.0, and -2.3 mm in the mouse brain atlas (Franklin et al., 2019). Quantification of brain Aβ burden is completed after immunostaining with the biotinylated 3D6 antibody (Zhao et al., 2016). Images are thresholded to highlight plaques and then analyzed by the "Analyze Particles" function of the ImageJ software (National Institutes of Health). After thresholding, identified objects are individually inspected to confirm each object quantified is a plaque. The percentage of surface area covered by Aβ IR (Aβ burden) or thioflavin-S (amyloid burden) is determined for specific areas of the hippocampal formation comprising the stratum oriens, pyramidal layer, stratum radiatum, and the dentate gyrus. Aβ or thioflavin-S burden is then represented as a % of the total area quantified. An average of 4 tissue sections is used to quantify the plaque load for each mouse. All analyses are performed in a blinded manner, with the examiner unaware of the treatment condition of any animal.

Aβ levels are assayed in brain homogenates from mice using a sandwich enzyme-linked immunosorbent assay (ELISA) using standard methods (Bales et al., 2009; Holtzman et al., 2000; Bales et al., 1997). Briefly, brain tissue is homogenated serially with RIPA and a 5.5-M guanidine buffer containing a cocktail of protease inhibitors (1:1000; Roche). Aβ measured after the RIPA extraction represents the soluble pool of Aβ, whereas Aβ measured after guanidine extraction represents the insoluble pool. The homogenates are diluted with a cold sample dilution buffer (1% bovine serum albumin in phosphate buffered saline-0.05% tween 20 [PBST]) before measurement of Aβ1-40 or Aβ1-42. Samples are loaded onto plates coated with an antibody that specifically recognizes the C-terminal domain of Aβ1-42 (21F12), or Aβ1-40 (2G3) as the capture antibody, and biotinylated 3D6 is used for detection. The IR signal after incubation with horseradish peroxidase (HRP)-conjugated streptavidin (Research Diagnostics) is developed with a TMB substrate (Thermo Scientific) and read on a Synergy H1 Hybrid plate reader (BioTek). Levels of Aβ are calculated using a standard curve generated with recombinant human Aβ (American Peptide Company). Levels of Aβ in brain homogenates are determined in triplicate, normalized to protein content, and expressed as the amount of Aβ/mg protein. Aβ oligomers are quantified with the 3D6/biotin-3D6 sandwich ELISA (Immuno-Biological Laboratories), in which the same N-terminal (residues 1-16) antibodies are used for both capture and detection.

Tau-related. *ELISAs.* Human total tau, and p-tau are quantitated by pS202/T205-tau (ATS) and pT212/pS214-tau (AT100) antibodies as sandwich ELISAs as described by Chai et al. (2011). Briefly, 96-well plates are precoated with 5 µg/ml ATS, or 2 µg/ml AT100 (Thermo Fisher Scientific) overnight at 4°C followed by blocking with Starting Block blocking buffer (Thermo Fisher Scientific). Samples (S1 or P1) are diluted in Superblock buffer (Thermo Fisher Scientific) and loaded onto the plates together with biotinylated-HT7 antibody (1:300; Thermo Fisher Scientific). After incubation for 1 hour, 23°C, samples

are washed 9 times with TBS/0.5% Tween 20 wash buffer followed by incubation with streptavidin-HRP (Jackson Immunoresearch) for 30 minutes. The plates are then developed by incubating with one-step 3,3,5,5-tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific) for 30 minutes and stopped with 2N H_2SO_4 and then read using a BioTek Synergy H1 Hybrid Reader at 450 nm. The amount of ATS or AT100 immunoreactive tau is determined using a standard curve derived from human AD brain homogenates.

Inflammation-related. The extent and characteristics of gene therapy on the inflammation associated with the 2 murine models are assessed as described by Shi et al. (2017). Immunohistochemistry is assessed by CD68-positive and Iba-1 microglial straining and astrocyte straining (GFAP). In addition, the RNAseq data are assessed using the same proinflammatory genes used by Shi et al. (2017), with particular focus on the proinflammatory gene cluster 1 and astrocyte A1-specific (inflammation only) genes (Shi et al., 2017).

Statistical considerations. For each measurement within the categories vector-derived APOE, general pathology, Amyloid-related, Tau-related, inflammation-related (Table II) a multi-way ANOVA model is applied. The main ANOVA model includes five factors: mouse model (APP.PS1/TRE4, P301S/E4), vector (AAVrh.10-APOE3ChC, -APOE3, AAVrh.10APOE2ChC, -APOE2, AAVrh.10Null), dose ($0.5x10^{10}$ and $1x10^{10}$ gc), time (5 and 9 months), and sex (M/F), where additional separate ANOVAs comparing one vector at a time to no therapy-PBS (but otherwise the same factor) are used to explore impacts compared to the PBS control. The central hypotheses are assessed with the main ANOVA models with the following pre-hoc contrasts for the Amyloid-related and Tau-related measurements: AA Vrh.1 0-APOE3ChC vs AA Vrh.1 0Null (Aim 1) and AAVrh.10APOE2ChC vs AAVrh.10Null and AAVrh.10-APOE3ChC vs AAVrh.10APOE2ChC with each of these applied between mouse strains, between doses within each mouse strain, and between time points within each mouse strain. This comprises 45 (= vector comparisons x factor comparisons x measurements) planned tests and a Bonferroni correction is used to assess significance controlling an overall 0.05 type I error. As an empirical benchmark to approximate the lower bound on power of this design, differences in soluble Aβ1-42 of 27 .2% and the observed standard deviation of each group when comparing AAVrh.10hAPOE2 to control in the PDAPP mouse brain amyloidosis experiments (Figure 8) produces power > 0.85, where considering the empirical differences and standard deviations for insoluble Aβ1-42 the power would be even greater, indicating the overall design is well-powered to assess the central hypotheses. As well as these prehoc comparisons, an exploratory post-hoc testing approach (with appropriate corrections) is additionally applied to assess the amount of brain vector derived APOE for different mouse models, doses, and time of measurement, as well as to explore how the broader vector impacts (e.g., AAVrh.10-APOE3ChC and AAVrh.10APOE2ChC in comparison to each other and to AAVrh.10-APOE3 and AAVrh.10-APOE2) on general pathology and inflammation related measurements (volume and RNA-Seq) and to estimate contributions of mouse model, vector, dose, time of measurement and sex to these impacts.

Example 2

Overview

Alzheimer's disease (AD), a degenerative brain disease and the most common cause of dementia, currently affects 5.8 million Americans and 50 million people worldwide. AD symptoms include progressive decline of cognitive and functional abilities and brain pathology, including extracellular beta-

amyloid plaques, intracellular tau tangles, chronic inflammation, and brain atrophy. The strongest genetic risk factor for susceptibility to late-onset AD concerns polymorphisms in the apolipoprotein E (*APOE*) allele. *APOE4* is found at high frequency in AD patients and homozygous inheritance is associated with a 14.5-fold increased risk of developing AD. In contrast, *APOE2* decreases risk of AD development and delays onset of disease, e.g., reducing the risk of developing AD by ≥50% and delaying the age of onset. Based on the epidemiologic data, *APOE4* is associated with increased brain amyloid load and greater memory impairment in AD, while *APOE2* attenuates these effects. In humans, the odds ratio of developing AD with *APOE4/4* homozygous genotype is 14.5 and is reduced to 2.6 in *APOE2/4* heterozygotes. Thus, the presence of APOE4 still constitutes a risk even when supplemented by the protective APOE2. In addition, APOE4 is associated with increased innate immune activation, differential signaling in neurons, exacerbation of tau pathology, and abnormal brain function apart from its role in promoting β-amyloid production.

Delivery of *APOE2* directly to the CNS of AD murine models using an adeno-associated virus (AAV) vector could provide significant APOE2 expression and decrease the levels of soluble and insoluble amyloid-β peptide and amyloid burden. However, even with APOE2 supplementation, the presence of APOE4 still constitutes an increased risk as *APOE2/4* heterozygotes have a 2.6-fold increased risk of developing AD. In one embodiment, an AAV-based gene therapy to introduce expression of the protective APOE2, a modified APOE2 or APOE3, while simultaneously decreasing the levels of deleterious endogenous APOE4 in *APOE4* homozygotes is provided. In one embodiment, artificial microRNAs (miRNA) targeting the endogenous APOE4 are introduced into an AAV expression cassette along with the cDNA for the human *APOE2* gene (hAPOE2-mirAPOE4). In one embodiment, the AAV9 serotype capsid is employed to package the expression cassette because it mediates efficient transduction of astrocytes, the main producers of APOE, as well as microglia and neurons. The established P301S/E4 AD mouse model that expresses mutant human tau and human *APOE4* and has high phosphorylated tau burden, chronic inflammation, and extensive neurodegeneration is used to assess therapy efficacy. To evaluate the strategy with AAV9-hAPOE2-mirAPOE4, an AAV construct is tested for silencing of endogenous APOE4 expression and delivery of the APOE2 coding sequence *in vitro*. In addition, it is determined if augmentation of APOE2 with reduction of endogenous APOE4 protects against tau pathology, neurodegeneration, and neuroinflammation *in vivo*.

Adeno-associated virus (AAV) serotype rh.10 and serotype 9 delivery of the human *APOE2* coding sequence to the central nervous system (CNS) of two AD murine models by intrahippocampal or intrathalamic routes yielded significant levels of human APOE2 expression across the brain. Further, the amount of soluble and insoluble amyloid-β peptide and amyloid burden was reduced in portions of the brain. However, genetically modifying the brain from *APOE4/4* to *APOE2/4* by only providing APOE2 does not completely mitigate the risk of the presence of APOE4. Decreasing expression of endogenous APOE4 along with delivering the protective APOE2 using AAV gene therapy can diminish the risk of *APOE4* homozygotes than gene therapy with APOE2 alone.

The coding sequences for human *APOE2* and *APOE4* differ by two nucleotides. Artificial microRNA (miRNA) sequences were designed that target the endogenous *APOE4* mRNA for suppression. Artificial miRNAs are screened *in vitro* for their ability to silence APOE expression in a human astroglioma cell line. Selected miRNAs are incorporated into the AAV expression cassette along with a modified human *APOE2* cDNA that cannot be silenced by the miRNAs. The selected AAV

expression cassettes containing *APOE2* and the artificial miRNAs targeting *APOE4* are packaged into the AAV9 capsid in order to target expression to astrocytes, the main producers of APOE in the CNS.

P301S/E4 AD murine model mice express both human mutant tau and human APOE4 and develop high levels of phosphorylated tau, brain atrophy, and neuroinflammation from microglia activation. Because APOE in the CNS is expressed mainly from astrocytes, an AAV9 vector is used that efficiently transduces astrocytes as well as microglia and neurons in this study. AAV9-hAPOE2-mirAPOE4 and AAV9-hAPOE2 vectors are administered directly to the CNS of P301S/E4 mice by the intracisternal route. Mice are evaluated for changes in behavior and cognitive function over time. After sacrifice, mice will be assessed for expression of vector-derived hAPOE2 and endogenous hAPOE4 in the brain as well as β-amyloid, total and phosphorylated tau levels and pathology, microglial activation, and brain atrophy.

Strategy. Alzheimer's symptoms include progressive decline of cognitive and functional abilities, including memory loss, confusion, personality and mood changes, and loss of ability to perform basic activities1. Brain pathology associated with AD includes the accumulation of extracellular beta-amyloid (Aβ) plaques and intracellular neurofibrillary tau tangles. Chronic neuroinflammation from glial activation and cell loss leading to brain atrophy are present in the advanced AD brain (Association As., 2019).

The strongest genetic risk factor for susceptibility to late-onset AD concerns polymorphisms in the apolipoprotein E (*APOE*) allele. *APOE* has three isoforms: *APOE2, APOE3*, and *APOE4. APOE3* is the most common allele while the allelic frequency of *APOE4* is approximately 15% and *APOE2* 8% (Farrer et al., 1997). However, the frequency of AD patients with *APOE4* is 40-65% (Farrer et al., 1997; Corder et al., 1993; Ward et al., 2012). *APOE4* is associated with accelerated rate and severity of cognitive decline and an earlier age of onset of AD. In contrast, *APOE2* decreases the risk of AD development by 50% and delays onset by up to 10 years (Corder et al., 1993; Corder et al., 1997; Sando et al., 2008; Shinohara et al., 2016).

APOE is a secreted protein with a primary role in lipid transport in both the periphery and central nervous system (CNS). The APOE isoforms differ at only two amino acids positions 112 and 158 (APOE2: Cys/Cys, APOE3: Cys/Arg, APOE4: Arg/Arg). However, these differences profoundly affect the structure and function of APOE (Hatters & Peters-Libeu, 2006). Serum and brain concentration of APOE differ between isoforms (APOE2 > APOE3 > APOE4). There are differences in the lipidation status with APOE2 and APOE3 usually associating with high-density lipoproteins (HDL) and APOE4 with low- and very low-density lipoproteins (LDL, VLDL) (Hatters & Peters-Libeu, 2006; Dong et al., 1994). Additionally, APOE isoforms bind differentially to receptors including low-density lipoprotein receptor-related 1 (LRP1), heparan sulfate proteoglycans (HSPG), and LDL receptor (LDLR) with APOE2 having a lower affinity than APOE3 or APOE4 (Bu, 2009, Arboleda-Velasquez et al., 2019).

APOE plays numerous roles in the pathology and neurodegeneration found in AD. Extracellular Aβ plaques are a hallmark of AD pathology. Aβ deposition is more abundant in *APOE4* carriers (Dorey et al., 2014). APOE4 enhances Aβ production, reduces Aβ clearance by impairing lysosomal degradation and transport across the blood brain barrier, and promotes Aβ aggregation and oligomer stabilization, representing a toxic gain-of-function (Jiang et al., 2008; Du et al., 2009; Nielsen et al., 2010; Rodriguez et al., 2014; Zekonyte et al., 2016; Lin et al., 2018; Wang et al., 2018). Hyperphosphorylated tau is the primary constituent of neurofibrillary tangles found in later stages of AD disease (Hampel et al., 2010). APOE4 expression exacerbates tau pathology (Shi et al., 2017). APOE4 shows weaker binding to tau than other isoforms (Strittmatter et al., 1994; Fleming et al., 1996), and expression of APOE4 in neurons

can increase tau phosphorylation (Wang et al., 2018; Brecht et al., 2004; Harris et al., 2004). APOE also has immunomodulatory functions and greater immune response is associated with the *APOE4* allele (Shi & Holtzman, 2018). Recent studies showed that innate immune activation in the CNS plays a key role in neuroinflammation and subsequent neuronal loss and brain atrophy in AD, and microglial activation is promoted by the presence of APOE4 (Rodriguez et al., 2014; Shi et al., 2017; Shi et al., 2019). APOE4 binds with higher affinity than APOE2 to receptors on neurons and stimulates enhanced activation of multiple signaling pathways including synapse formation and increased amyloid precursor protein (APP) transcription (Ohkubo et al., 2001; Huang et al., 2017; Huang et al., 2019). APOE4 binding to these receptors on damaged neurons also may act as an opsonin to promote neuronal phagocytosis by microglia via the interaction with the microglial expressed APOE binding partner 'triggering receptor expressed on myeloid cells-2' (TREM2) leading to enhanced neuronal loss and brain atrophy (Atagi et al., 2015; Bailey et al., 2015; Yeh et al., 2016; Jendresen et al., 2017). Further, APOE4 disrupts normal astrocyte and microglia homeostatic function (Fernandez et al., 2019). Clinically, *APOE4* is associated with accelerated cognitive decline while *APOE2* is protective (Shinohara et al., 2016; Helkala et al., 1996; Staehelin et al., 1999; Wilson et al., 2002). Thus, APOE4 plays a central role in multiple processes connected to AD development and is a prime target for intervention.

The human genetic data indicates that the *APOE4* and *APOE2* alleles have semi-dominant inheritance (Corder et al., 1994; Genin et al., 2011). While *APOE4* homozygous individuals have a 14.5-fold increased risk of developing AD, *APOE2/4* individuals have only a 2.6-fold increased risk, indicating that the presence of the protective *APOE2* allele can partially mitigate the deleterious effects of the *APOE4* allele. Although the risk of AD development is substantially reduced in *APOE2/4* heterozygotes, it is still significantly higher than in individuals with *APOE2/3* or *APOE2/2* genotypes (1.8- and 7.7-fold decreased risk of AD development, respectively (Genin et al., 2011). This genetic data suggests that reduction in the level of *APOE4* along with increasing *APOE2* in the CNS would be beneficial for therapy for *APOE4* carriers at high risk for AD development.

Human induced pluripotent stem cell derived neurons expressing APOE4 had increased Aβ production, higher levels of tau phosphorylation, and more degeneration that was corrected by converting *APOE4* to *APOE3* through gene editing (Lin et al., 2018; Wang et al., 2018). Previous studies from our group and others demonstrated that delivery of *APOE2* to the CNS by viral vectors both increased the expression of APOE2 and decreased brain Aβ and amyloid burden in mouse models of AD41-43. The dose of APOE2 delivered and the amount of pre-existing Aβ1-42 deposition were important factors in determining the efficacy of the treatment (Zhao et al., 2016). However, in these previous studies, endogenous APOE4 was still present.

The disclosed therapies could be delivered to *APOE4* homozygotes long before symptom onset to prevent or halt disease processes before they lead to AD.

Studies. Previous studies tested AAV serotype rh.10 and serotype 9 delivery of the human *APOE2* coding sequence to the CNS of two AD murine models by intrahippocampal or intrathalmic routes. Human APOE2 was expressed to high levels across the brain using intrathalmic delivery of AAV9-APOE2 vector in the APP.PS1/TRE4 AD murine model of Aβ amyloidosis expressing human amyloid precursor protein (APP) and presenilin 1 (PS1) mutations and human *APOE4* instead of murine *APOE*43 (Figure 21A). Further, *APOE2* augmentation reduced Aβ and amyloid loads in this AD mouse (Zhao et al., 2016)

(Figure 21B). One strategy is to encode artificial microRNAs (miRNA) targeting the endogenous *APOE4* into the AAV expression cassette along with the cDNA for human *APOE2* gene.

Target sequence selection. The *APOE4* sequence was evaluated for the best targeting sequences for small interfering RNAs (siRNAs) using multiple algorithms and screened to minimize the potential for off-target interactions. Knockdown ability of the selected siRNAs was tested *in vitro* for endogenous APOE in the U87 human astroglioma cell line (Figure 22). The siRNA sequences are not specific for *APOE4* and will inhibit expression of all *APOE* isoforms. The best siRNA sequence was selected for incorporation into an artificial miRNA cassette for expression in the AAV vector.

The effect of the gene therapy focuses on tau pathology, neuroinflammation, and neurodegeneration. The AAV vector delivers persistent expression of human *APOE2* along with artificial miRNAs targeting the endogenous *APOE4* mRNA to downregulate expression (hAPOE2-mirAPOE4). AAV9 efficiently transduces astrocytes, which are the primary producers of APOE in the brain, as well as microglia and neurons and will be used for this study (Xu et al., 2006; Foust et al., 2009; Gray et al., 2011; Gong et al., 2015; Vagner et al., 2016; Zhang et al., 2014; Zhang et al., 2-2014). This AAV gene therapy provides enhanced APOE2 expression relative to APOE4 in *APOE4* homozygotes, reduce neurofibrillary tau tangles, decrease microglial activation, reduce hippocampus and cortex pathology, and improve behavior.

AAV construct to silence endogenous *APOE4* expression and deliver the *APOE2* coding sequence *in vitro*. siRNA sequences were identified that effectively knockdown the expression of APOE in U87 human astroglioma cells. These sequences target segments of *APOE* that are common to all allele sequences. The most effective siRNA sequences are selected and incorporated into an enhanced mir155 artificial miRNA backbone to yield mirAPOE4 (Fowler et al., 2016) (Figure 23A). As the placement of the mismatches in the hairpin stem and GC content can affect the processing of miRNAs in cells (Fowler et al., 2016; Fang & Bartel, 2015), several iterations of the mirAPOE4s are tested for ability to knockdown APOE *in vitro* to ensure the most effective design. Two to four tandem copies of the optimized mirAPOE4 are cloned into either the intron present in the AAV expression cassette promoter (hybrid CMV/Chicken β-actin; CAG) or into the intervening sequence between the transgene (mCherry or hAPOE2-HA) stop codon and the polyadenylation signal to determine the optimal number and positioning of mirAPOE4 in the expression cassette (Mueller et al., 2012) (Figure 23B). The AAV mirAPOE4 expression cassette plasmids are tested *in vitro* for their ability to reduce expression of APOE by transfecting U87 cells for 72 hours before harvesting the culture media and cells. Expression of *APOE* mRNA is evaluated by RT-qPCR and secreted APOE protein by western blot and ELISA.

In parallel, silent mutations are made in the coding sequence of the human *APOE2* cDNA at the recognition sites for the chosen mirAPOE4s to ensure that vector derived *APOE2* expression cannot be silenced. Vector-derived human *APOE2* has a C-terminal hemagglutinin (HA) tag to facilitate detection. Expression of vector-derived hAPOE2-HA in the presence of mirAPOE4 is confirmed by co-transfection and analysis of hAPOE2-HA expression by western blotting with an anti-HA antibody. The optimized hAPOE2-mirAPOE4 cassette is packaged into an AAV9 serotype capsid. AAV9 efficiently transduces astrocytes, which are the primary producers of APOE in the brain, as well as microglia and neurons. Functionality of the AAV9 vectors to express hAPOE2-HA and mirAPOE4 is confirmed *in vitro* before proceeding to *in vivo* experiments.

Augmentation of APOE2 may correlate with reduction of endogenous APOE4 protects against tau pathology, neurodegeneration, and neuroinflammation *in vivo*. Administration of AAV9-hAPOE2 to the CNS increased APOE levels across the brain and reduced Aβ1-42 levels in the APP.PS1/TRE4 AD mouse model of Aβ amyloidosis in the context of human APOE4 (Zhao et al., 2016; Kim et al, 2011) (Figure 21). Because of the importance of neurofibrillary tau tangles and innate immune response in AD brain atrophy and cognitive dysfunction independent of Aβ load (Arboleda-Velasquez et al., 2019; Shi et al., 2017; Shi et al., 2019; Josephs et al., 2008; Mattsson et al,. 2018), tau pathology, microglial activation, and neurodegeneration are determined. P301S/E4 mice overexpress 1N4R human tau containing the P301S mutation, linked to frontotemporal dementia, and human *APOE4* replacing the mouse *APOE* (Shi et al., 2017; Yoshiyama et al., 2007). The P301S/E4 mice have higher brain phosphorylated tau burdens and more extensive neurodegeneration and neuroinflammation than mice expressing human *APOE3* or *APOE2* (Shi et al., 2017; Shi et al., 2019), but do not show amyloid plaque formation (Shi et al., 2017; Yoshiyama et al., 2007; Haurigot et al., 2013). P301S 1N4R tau B6/C3 mice (Jackson Laboratories 008169) and human *APOE4* knockin mice (TRE4 - Taconic 1549: B6.129P2-Apoetm3 (APOE*4)Mae N8) can recreate the published model by crossing the P301S and TRE4 mice.. C57B1/6 mice are used as wild-type controls.

The impact of vector-mediated delivery of AAV9-hAPOE2-mirAPOE4 expressing human *APOE2* plus miRNA against endogenous *APOE4* is assessed after administration by the intracisternal (IC) route in the P301S/E4 mice. The IC route provides wide vector distribution throughout the brain with the least invasive administration (Haurigot et al., 2013; Hinderer et al., 2018; Rosenberg et al., 2018; Markmann et al., 2018). AAV9-hAPOE2, AAV9-mCherry-mirAPOE4, AAV9-null, and no therapy (PBS) are used as controls. Mice are evaluated for behavior and neurologic assessment monthly. After sacrifice, mice are evaluated for vector-derived hAPOE2 and endogenous APOE4 expression, Aβ peptide burden, total soluble and insoluble tau burden, hyperphosphorylated tau tangles, microglial activation, and brain neurodegeneration at 1 and 7 months post-administration (total age: 3 and 9 months, respectively) (Table 3).

Table 3. Mouse Study

Mouse model (genotype)	Vector	Administration dose and route	Parameters to be evaluated
P301S/E4	AAV9-hAPOE2		• Behavior and neurologic assessment (monthly) • Brain expression of hAPOE4 and hAPOE2 • Soluble and insoluble Aβ levels • Assessment of phosphorylated tau
	AAV9-hAPOE2-mirAPOE4		
	AAV9-mCherry-mirAPOE4		
	10^{11} gc, via intracisternal (IC) route		
	No therapy (PBS)	None (PBS, IC)	

C57Bl/6 (controls)	No therapy (PBS)	None (PBS, IC)	and total tau levels in brain homogenates • CNS assessment of neurofibrillary tangles • Microglial activation • Pathology of hippocampus and cortex[13,14]
2 strains	**5 vectors**	**1 route**	**Necropsy @ 3 and 9 months of age**

Eight wk old adult mice, n=16 males and n=16 females per treatment cohort (n=192 total). All mice are randomly assigned to cohort groups, to avoid potential study bias, with staggered dosing of animals across all groups. Cages are numbered without treatment qualifiers to keep researchers blinded to treatment status.

Treatment cohorts involve double mutant mouse lines (P301S/E4) with human tau and APOE4 gene replacements. Wild-type controls with the same genetic background, C57B1/6 strain, are used for 'No therapy' controls.

Vectors tested include: AAV9-hAPOE2, AAV9-hAPOE2-mirAPOE4, AAV9-mCherry-mirAPOE4, AAV9-null, or no therapy (PBS).

All vectors are administered at 10^{11} genome copies (gc) or PBS (10 µl) into the CSF via the IC route. This dose scales to a dose (10^{14} gc) that is safe to deliver in humans (Sharma & McNeill, 2009).

Health checks are performed 3 times/weeks for the first two weeks, and then weekly afterwards on all surviving mice, with observation for any unusual or altered behavior. As part of the weekly health checks, the mice are weighed and recorded by personnel blinded to the treatment cohorts.

Mice are sacrificed at 2 time points (3 and 9 months of age, 1 and 7 months post-vector administration), half of each cohort is sacrificed (n=8M/8F). During necropsy, the mouse CSF is sampled first from the cisterna magna, then the mice are perfused with cold PBS and the brain is collected and flash-frozen in liquid nitrogen and stored at -80°C. Half of the brain is used for vector DNA, mRNA, and protein assays. The remaining hemisphere is fixed and sectioned coronally at 50 µM slices for immunohistochemistry (IHC), immunofluorescence (IF), or pathology.

At monthly intervals, mice are subjected to a behavior and neurologic assessment. Mouse locomotor activity and ability to stand/rear are evaluated in the open field chamber equipped with infrared beam arrays to monitor and record mouse movements64. Second, the mice are tested for cognitive abilities by use of novel object recognition 24 hours after introduction of a new object. The test is performed inside of the open field chamber and the amount of time exploring the new object will be recorded (Webster et al., 2013). AD mice show decreased performance in this test with age. All testing is videotaped for examination of altered behavior by blinded personnel.

hAPOE2-HA and hAPOE4 protein are detected in brain tissue samples from multiple regions by western blotting/ELISA with antibodies specific for hAPOE4 and the hAPOE2 HA tag; mRNA are detected by RTqPCR using allele specific probes (Zhong et al., 2016).

Microdissected sections of the hippocampus, thalmus, and cerebral cortex are homogenized and sequentially extracted with RIPA (soluble Aβ) and 5.5 M guanidine (insoluble Aβ). Aβ1-42/1-40 levels are quantified by ELISA (Zhao et al., 2016).

Brain lysates are probed with antibodies against total tau (BT2) and phosphorylated-tau (CP13) by western blotting (Sacramento et al., 2020).

Brain slices are stained by anti-phospho-tau antibody AT8 for IHC/IF (Shi et al., 2017; Sacramento et al., 2020).

Microglia activation is assessed in brain sections by staining for CD68 positive cells by IHC (Shi et al., 2017).

Multiple brain slices, 300 µM apart, are stained with 0.1% Sudan black for brain volume analysis. The areas of interest, including hippocampus, posterior lateral ventricles, and piriform/entorhinal cortex, are traced and measured in the imaging software. Three sections are stained with cresyl violet for neuronal layer thickness measurements of the CA1 pyramidal cell layer and dentate gyrus granular cell layer for observational changes in the hippocampal region. Measurements are recorded using ImageJ software at all sites and averaged (Shi et al., 2017).

Neuronal toxicity of the AAV9 vector itself is evaluated in the cresyl violet stained brain sections by assessing the percentage of positively stained cells for each of the three sections.

Statistics. Studies are performed in triplicate for three independent experiments, and data will be presented as means ± standard deviation (SD) unless otherwise stated. Differences between groups are analyzed using a one-way analysis of variance (ANOVA) for multiple comparisons. Aim 2 studies will be performed with n=8 mice per cohort (8M/8F). If the coefficient of variation is 35% for both groups, then the use of n=8 per group allows for a 2.8-fold difference between groups ($p<0.05$, power=0.95). Differences between groups for biochemical measurements are analyzed by ANOVA for multiple comparisons. Behavioral assessments are analyzed by two-way repeated measures ANOVA with multiple comparisons.

Exemplary Modified APOE2 sequences:

ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGCCAAGGTGG
AGCAAGCGGTGGAGACAGAGCCGGAGCCCGAGCTGCGCCAGCAGACCGAGTGGCAGAGCGGCCA
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TGAAAGAACTCAAAGCTTATAAGAGCGAGCTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACG
CGGGCACGGCTGTCCAAGGAGCTGCAGGCGGCGCAGGCCCCGGCTGGGCGCGGACATGGAGGACG
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CGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGGGCCGCGTGCGGGCCGCCACTGTGGGC
TCCCTGGCCGGCCAGCCGCTACAGGAGCGGGCCCAGGCCTGGGGCGAGCGGCTGCGCGCGCGGA
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GGAGATGGGCAGCCGGACCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGCGC
GCCAAGCTGGAGGAGCAGGCCCAGCAGATACGCCTGCAGGCCGAGGCCTTCCAGGCCCGCCTCAA
GAGCTGGTTCGAGCCCCTGGTGGAAGACATGCAGCGCCAGTGGGCCGGGCTGGTGGAGAAGGTGC
AGGCTGCCGTGGGCACCAGCGCCGCCCCTGTGCCCAGCGACAATCAC (SEQ ID NO:33).

References

2020 Alzheimer's disease facts and figures. Alzheimers Dement. 2020.

Abner et al., J. Alzheimers Dis., 64:1307 (2018).

Acosta-Baena et al., Lancet. Neural., 10:213 (2011).

Allen et al., J. Neurosci., 22:9340 (2002).

Arboleda-Velasquez et al., Nat. Med., 25:1680 (2019).

Association As. 2019 Alzheimer's disease facts and figures. Alzheimer's & Dementia. 2019; 15:321-387.

Atagi et al., J. Biol. Chem., 290:26043 (2015).

Bailey et al., J. Biol. Chem., 290:26033 (2015).

Bales et al., J. Neurosci., 29:6771 (2009).

Bales et al., Nat. Genet., 17:263 (1997).

Benjamin et al., Psychol. Aging, 19:592 (2004).

Brecht et al., J. Neurosci., 24:2527 (2004).

Bu, Nat. Rev. Neurosci., 10:333 (2009).

Campion et al., Am. J. Hum. Genet., 65:664 (1999).

Carmona et al., Handb. Clin. Neural., 148:395 (2018).

Castellano et al., Sci. Transl. Med., 3:89ra57 (2011).

Chai et al., J. Biol. Chem., 286:34457 (2011).

Chiang et al., AJNR Am. J. Neuroradiol., 33:1392 (2012).

Chiang et al., Neurology, 75:1976 (2010).

Collaborators GBDD, Lancet Neural., 18:88 (2019).

Conejero-Goldberg et al., Mol. Psychiatry., 19:1243 (2014).

Conrado et al., Clin. Pharmacol. Ther., 107:796 (2020).

Coon et al., J. Clin. Psychiatry, 68:613 (2007).

Corder et al., Nat. Genet., 7:180 (1994).

Corder et al., Science, 261:921 (1993).

Dai et al., Oncotarget., 9:15132 (2018).

Deane et al., J. Clin. Invest., 118:4002 (2008).

DeTure & Dickson, Mol. Neurodegener., 14:32 (2019).

Dodart et al., Proc. Natl. Acad. Sci. U.S.A., 102:1211 (2005).

Dong et al., J. Biol. Chem., 269:22358 (1994).

Dorey et al., Neurosci. Bull., 30:317 (2014).

Du et al., Neurosci. Lett., 464:140 (2009).

Exceptionally low likelihood of alzheimer's dementia in APOE2 homozygotes [Internet]. 2019.

Fagan et al., Neurobiol. Dis., 9:305 (2002).

Fang and Bartel, Mol. Cell., 60:131 (2015).

Fann et al., Lancet. Psychiatry., 5:424 (2018).

Farrer et al., JAMA, 278:1349 (1997).

Fernandez et al., Front Aging Neurosci., 11:14 (2019).

Fernandez et al., Neurosci., 11:14 (2019).

Fleming et al., Exp. Neurol., 138:252 (1996).

Flowers & Rebeck, Neurobiol. Dis., 136:104724 (2020).

Foust et al., Nat. Biotechnol., 27:59 (2009).

Fowler et al., Nucleic Acids Res., 44:e48 (2016).

Franklin K, Paxinos G. Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates, Compact Academic Press; 2019.

Gale et al., J. Allergy Clin. Immunol., 134:127 (2014).

Games et al., Nature, 373:523 (1995).

Gatz et al., Nat. Rev. Neurosci., 19:583 (2018).

Genin et al., Mol. Psychiatry., 16:903 (2011).

Gong et al., Mol. Ther., 23:824 (2015).

Gray et al., Mol. Ther., 19:1058 (2011).

Hampel et al., Exp. Gerontol., 45:30 (2010).

Harris et al., J. Biol. Chem., 279:44795 (2004).

Hashimoto et al., J. Neurosci., 32:15181 (2012).

Hatters et al., J. Mol. Biol., 361:932 (2006b).

Hatters et al., Trends Biochem. Sci., 31:445 (2006a).

Haurigot et al., J. Clin. Invest., 123:3254 (2013).

Hebert et al., Neurology, 80:1778 (2013).

Heffernan et al., J. Mol. Neurosci., 60:316 (2016).

Helkala et al., Neurosci. Lett., 204:177 (1996).

Hinderer et al., Hum. Gene. Ther., 29:15 (2018).

Holtzman et al., Cold Spring Harb. Perspect Med., 2:a006312 (2012).

Holtzman et al., Proc. Natl. Acad. Sci. U.S.A., 97:2892 (2000).

Hu et al., Mol. Neurodegener., 10:6 (2015).

Huang et al., Cell., 168:427 (2017).

Huang et al., J. Neurosci., 39:7408 (2019).

Hudry et al., Sci. Transl. Med., 5:212ra161 (2013).

Jankowsky et al., Biomol. Eng., 17:157 (2001).

Jendresen et al., J. Neuroinflammation., 14:59 (2017).

Jiang et al., Neuron., 58:681 (2008).

Josephs et al., Ann. Neurol., 63:204 (2008).

Kim et al., J. Neurosci., 31:18007 (2011).

Li et al., J. Biol. Chem., 287:44593 (2012).

Liao et al., Curr. Opin. Lipidol., 28:60 (2017).

Lin et al., Neuron., 98:1141 (2018).

Liu et al., J. Neurosci., 36:12425 (2016).

Liu et al., Nat. Rev. Neural., 9:106 (2013).

Liu et al., Sci. Transl. Med., 8:332ra344 (2016).
Lopera et al., JAMA, 277:793 (1997).
Mahley et al., Ann. NY Acad. Sci., 777:139 (1996).
Mahley et al., J. Lipid. Res., 40:1933 (1999).
Mahley, Arterioscler. Thromb. Vase. Biol., 36:1305 (2016).
Manelli et al., J. Mol. Neurosci., 23:235 (2004).
Markmann et al., Exp. Neurol., 306:22 (2018).
Mattsson et al., Alzheimers Res. Ther., 10:77 (2018).
Mondadori et al., Cereb. Cortex., 17:1934 (2007).
Mueller et al., Mol. Ther., 20:590 (2012).
Nagy et al., Neuroscience, 69:757 (1995).
Naj et al., Nat. Genet., 43:436 (2011).
Nielsen et al., Glia., 58:1235 (2010).
O'Callaghan et al., J. Histochem. Cytochem., 66:305 (2018).
Ohkubo et al., J. Biol. Chem., 276:3046 (2001).
Phillips, IUBMB Life, 66:616 (2014).
Pitas et al., Biochim. Biophys. Acta., 917:148 (1987).
Prince et al., Alzheimers Dement., 9:63 (2013).
Puglielli et al., Nat. Neurosci., 6:345 (2003).
Raber et al., Neurobiol. Aging, 25:641 (2004).
Raman et al., BMC Genomics, 10:493 (2009).
Rauch et al., Sci. Rep., 8:6382 (2018).
Rebeck et al., J. Alzheimers Dis., 4:145 (2002).
Reiman et al., Nat. Commun., 11:667 (2020).
Reiman et al., Proc. Natl. Acad. Sci. U.S.A., 106:6820 (2009).
Rodriguez et al., J. Neuroinflammation., 11:111 (2014).
Rosenberg et al., Hum. Gene Ther. Clin. Dev., 25:164 (2014).
Rosenberg et al., Hum. Gene. Ther. Clin. Dev., 29:24 (2018).
Ryan et al., PLoS One, 9:e88051 (2014).
Sacramento et al., Hum. Gene. Ther., 31:57 (2020).
Safieh et al., BMC Med., 17:64 (2019).
Sando et al., BMC Neurol., 8:9 (2008).
Schenk et al., Nature, 400:173 (1999).
Schmechel et al., Proc. Natl. Acad. Sci. U.S.A., 90:9649 (1993).
Sharma & McNeill, Br. J. Pharmacol., 157:907 (2009).
Shi & Holtzman, Nat. Rev. Immunol., 18:759 (2018).
Shi et al., J. Exp. Med., 216:2546 (2019).
Shi et al., Nature., 549:523 (2017).
Shinohara et al., Ann. Neurol., 79:758 (2016).
Snow et al., Am. J. Pathol., 133:456 (1988).
Sondhi et al., Hum. Gene Ther. Methods, 23:324 (2012).
Sondhi et al., Mol. Ther., 15:481 (2007).

Staehelin et al., Acta. Neurol. Scand., 100:53 (1999).

Strittmatter et al., Proc. Natl. Acad. Sci. U.S.A., 91:11183 (1994).

Sullivan et al., J. Stroke Cerebrovasc. Dis., 17:303 (2008).

Tardieu et al., Hum. Gene Ther., 25:506 (2014).

Tatem et al., J. Visualized Exp., JoVE. ___:51785 (2014).

Tumor Analysis Best Practices Working G. Expression profiling--best practices for data generation and interpretation in clinical trials, Nat. Rev. Genet., 5:229 (2004).

Tzioras et al., Neuropathol. Appl. Neurobiol., 45:327 (2019).

Vagner et al., Mol. Cell. Neurosci., 77:76 (2016).

Vitek et al., Neurobiol. Aging, 30:1350 (2009).

Walker et al., Acta. Neuropathol., 100:36 (2000).

Wang et al., Nat. Med., 24:647 (2018).

Ward et al., Neuroepidemiology, 38:1 (2012).

Webster et al., Alzheimer's research & therapy, 5:28 (2013).

Wildsmith et al., Anal. Biochem., 395:116 (2009).

Williams et al., Mol. Neurodegener., 15:8 (2020).

Wilson et al., J. Neurol. Neurosurg. Psychiatry., 73:672 (2002).

Wolters et al., PLoS One., 14:e0219668 (2019).

World Health Organization. The Epidemiology and impact of dementia current state and future trends. 2015https://www.who.int/mental_health/neurology/dementia/dementia_thematicbrief _epidemiology . pdf [last accessed 6/3/20]

Xu et al., J. Neurosci., 26:4985 (2006).

Yeh et al., Neuron., 91:328 (2016).

Yoshiyama et al., Neuron., 53:337 (2007).

Yu et al., Annu. Rev. Neurosci., 37:79 (2014).

Zannis & Breslow, Biochemistry, 20:1033 (1981).

Zekonyte et al., Biochim. Biophys. Acta., 1862:1047 (2016).

Zerah et al., Hum. Gene Ther. Clin. Dev., 26:113 (2015).

Zhang et al., Genet. Test Mol. Biomarkers., 17:47 (2013).

Zhang et al., J. Neurosci., 34:11929 (2014).

Zhang et al., Neuron., 89:37 (2016).

Zhao et al., Angew. Chem. Int. Ed. Engl., (2019).

Zhao et al., J. Neurosci., 29:3603 (2009).

Zhao et al., Neurobiol. Aging, 44:159 (2016).

Zhong et al., Mol. Neurodegener., 11:2 (2016).

All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

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Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

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WHAT IS CLAIMED IS:

1. An adeno-associated virus (AAV) vector comprising in the 5' to 3' direction:
a first AAV2 ITR sequence;
a CMV enhancer sequence;
a chicken beta-actin promoter sequence;
a nucleic acid sequence encoding an apolipoprotein 2 (APOE2) polypeptide comprising a Christchurch mutation;
a polyA sequence; and
a second AAV2 ITR sequence.

2. The AAV vector of claim 1, wherein the Christchurch mutation is R136S in the mature form of APOE2.

3. The AAV vector of claim 1 or 2, wherein the APOE2 polypeptide comprises substitutions R136S and R158C in the mature form of the amino acid sequence set forth in SEQ ID NO: 1.

4. The AAV vector of any one of claims 1-3, wherein the AAV capsid protein is an AAV1 capsid protein, an AAV2 capsid protein, an AAV4 capsid protein, an AAV5 capsid protein, an AAV7 capsid protein, an AAV8 capsid protein, an AAV9 capsid protein, or an AAVrh10 capsid protein.

5. An AAV viral vector comprising an AAV capsid protein, wherein the AAV viral vector is produced by packaging the AAV vector of any one of claims 1-4.

6. A pharmaceutical composition comprising the AAV viral vector of claim 5.

7. Use of the AAV viral vector of claim 5 or the pharmaceutical composition of claim 6 in the preparation of a medicament comprising a therapeutically effective amount of the AAV viral vector for treating Alzheimer's disease in a human subject.

8. A method of treating Alzheimer's disease in a human subject, comprising administering to the subject a therapeutically effective amount of the AAV viral vector of claim 5 or the pharmaceutical composition of claim 6.

9. The use of claim 7 or the method of claim 8, wherein the therapeutically effective amount comprises about 1×10^{11} to about 1×10^{16} genome copies.

10. The use of claim 7 or 9 or the method of claim 8 or 9, wherein the subject is an APOE2/APOE4 heterozygote, an APOE4/APOE4 homozygote or an APOE3/APOE4 heterozygote.

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11. The use of any one of claims 7, 9 and 10 or the method of any one of claims 8-10, wherein the medicament is formulated for systemic administration or wherein the AAV viral vector or pharmaceutical composition is administered systematically.

12. The use of any one of claims 7, 9 and 10 or the method of any one of claims 8-10, wherein the medicament is formulated for intracisternal administration or wherein the AAV viral vector or pharmaceutical composition is administered intracisternally.

13. A method to prevent, inhibit or treat one or more symptoms of Alzheimer's disease in a human, comprising: administering to the human a composition comprising an effective amount of a recombinant adeno-associated virus (rAAV) vector comprising an open reading frame encoding an apolipoprotein 2 (APOE2) polypeptide comprising a Christchurch mutation, the expression of which in the human prevents, inhibits or treats the one or more symptoms.

14. The method of claim 13, wherein the therapeutically effective amount comprises wherein the amount of the vector is about 1×10^{11} to about 1×10^{16} genome copies.

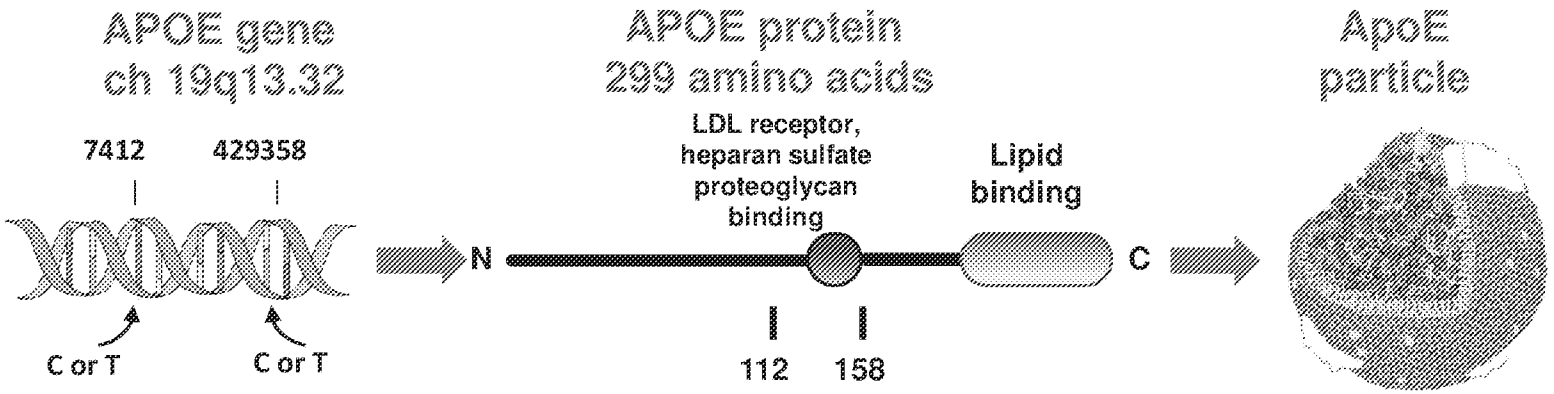
15. The method of claim 13 or 14, wherein the human t is an APOE2/APOE4 heterozygote, an APOE4/APOE4 homozygote or an APOE3/APOE4 heterozygote.

16. The method of any one of claims 13-15, wherein the composition is systemically administered.

17. The method of any one of claims 13-15, wherein the composition is administered intracisternally.

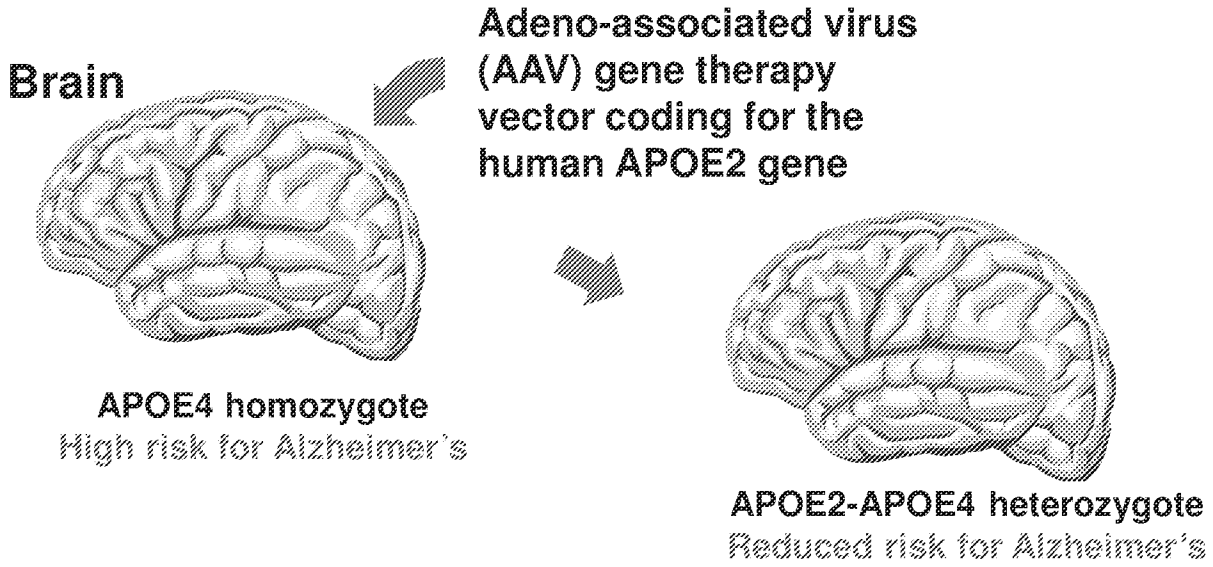
18. Use of a recombinant adeno-associated virus (rAAV) vector in the preparation of a medicament for treating one or more symptoms of Alzheimer's disease in a human, comprising, wherein the rAAV vector comprises an open reading frame encoding an apolipoprotein 2 (APOE2) polypeptide comprising a Christchurch mutation, the expression of which in the human prevents, inhibits or treats the one or more symptoms of Alzheimer's disease.

Figure 1. APOE4 Gene, Protein and Risk for Alzheimer's Disease



APOE genetic variant	Site in the genome 7412	429358	112	158	Prevalence	Risk for Alzheimer's
APOE2	T	T	Cys	Cys	7%	Low
APOE3	C	T	Cys	Arg	79%	Average
APOE4	C	C	Arg	Arg	13%	High

Figure 2. APOE2 Gene Therapy to Convert the APOE4 Brain to APOE2E4



- Adeno-associated virus delivery of the APOE2 gene to the CNS will genetically convert the E4 brain into an E2/E4 brain, thus reducing or preventing the pathology of Alzheimer's disease in APOE4 homozygotes

Figure 3. AAVrh.10APOE2 Is Administered by the Intracisternal Route Directly to Brain Cerebral Spinal Fluid

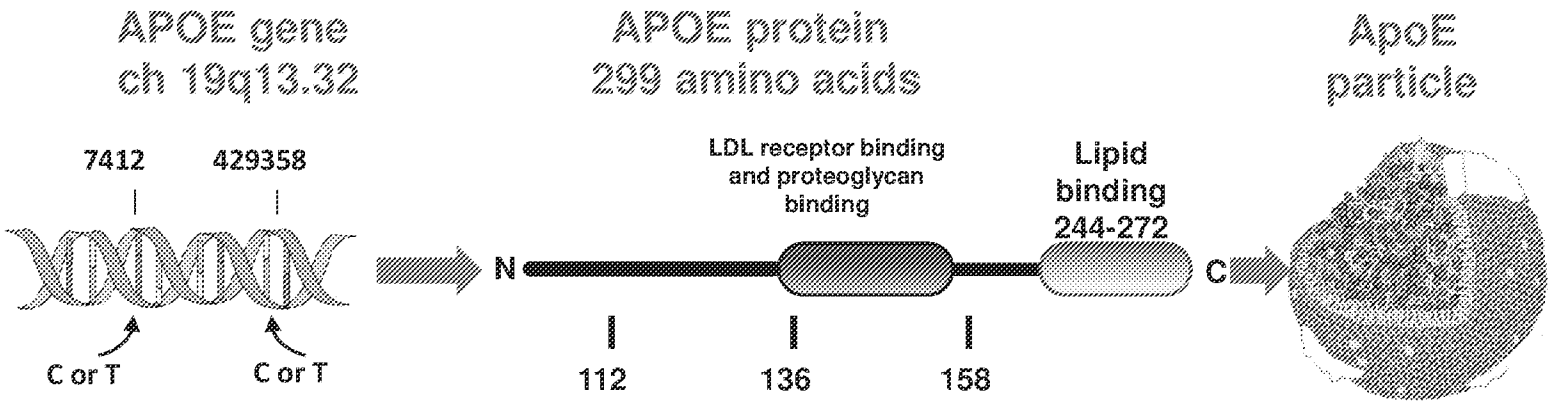
- Administration to the cisterna magna is an outpatient procedure

Figure 4. APOE3 ChristChurch Alzheimer's Disease-Protective APOE mutation

APOE genetic variant	Amino acid 112	136	158	Prevalence	Risk for Alzheimer's
APOE2	Cys	Arg	Cys	7%	Low
APOE3	Cys	Arg	Arg	79%	Average
APOE3Chc	*Cys*	*Ser*	*Arg*	*Very low*	*Low*
APOE4	Arg	Arg	Arg	13%	High

Arboleda-Velasquez et al 2019 Resistance to autosomal dominant Alzheimer's disease in an APOE3 Christchurch homozygote: a case report. Nature Medicine
Zalocusky et al 2019 An Alzheimer's-disease-protective APOE mutation, Nature Medicine
Reiman et al 2019 Exceptionally low likelihood of Alzheimer's dementia in APOE2 homozygotes. medRχiv

Figure 5. APOE3ChristChurch Gene and Protein



APOE genetic variant	Site in the genome 7412	429358	112	136	158	Prevalence	Risk for Alzheimer's
APOE2	T	T	**Cys**	Arg	**Cys**	7%	Low
APOE3	C	T	Cys	Arg	Arg	79%	Average
APOE3ch	C	T	Cys	Ser	Arg	Very low	Low
APOE4	C	C	**Arg**	Arg	**Arg**	13%	High

Figure 6. APOE3ch Mutation

- Case of Colombian woman with the autosomal-dominant E280A mutation in presenilin 1 (Paisa mutation)
- Cognitively well for decades (age 70) past this mutation's typical age of disease onset (40 yr)
- This mutation causes Aβ overproduction, associated with neurodegeneration and cognitive decline
- At age 70 she has short-term memory but remains independent
- Amyloid PET scanning revealed a massive buildup of amyloid plaques in her brain, far higher than those seen in young mutation carriers who are cognitively impaired
- Very little tau pathology, mostly confined to the medial temporal lobe, brain glucose metabolism is almost normal
- It's as if the chain of pathogenic events is broken after amyloid formation
- Independent of the mechanism, can this mutation be used to treat APOE4 homozygotes?

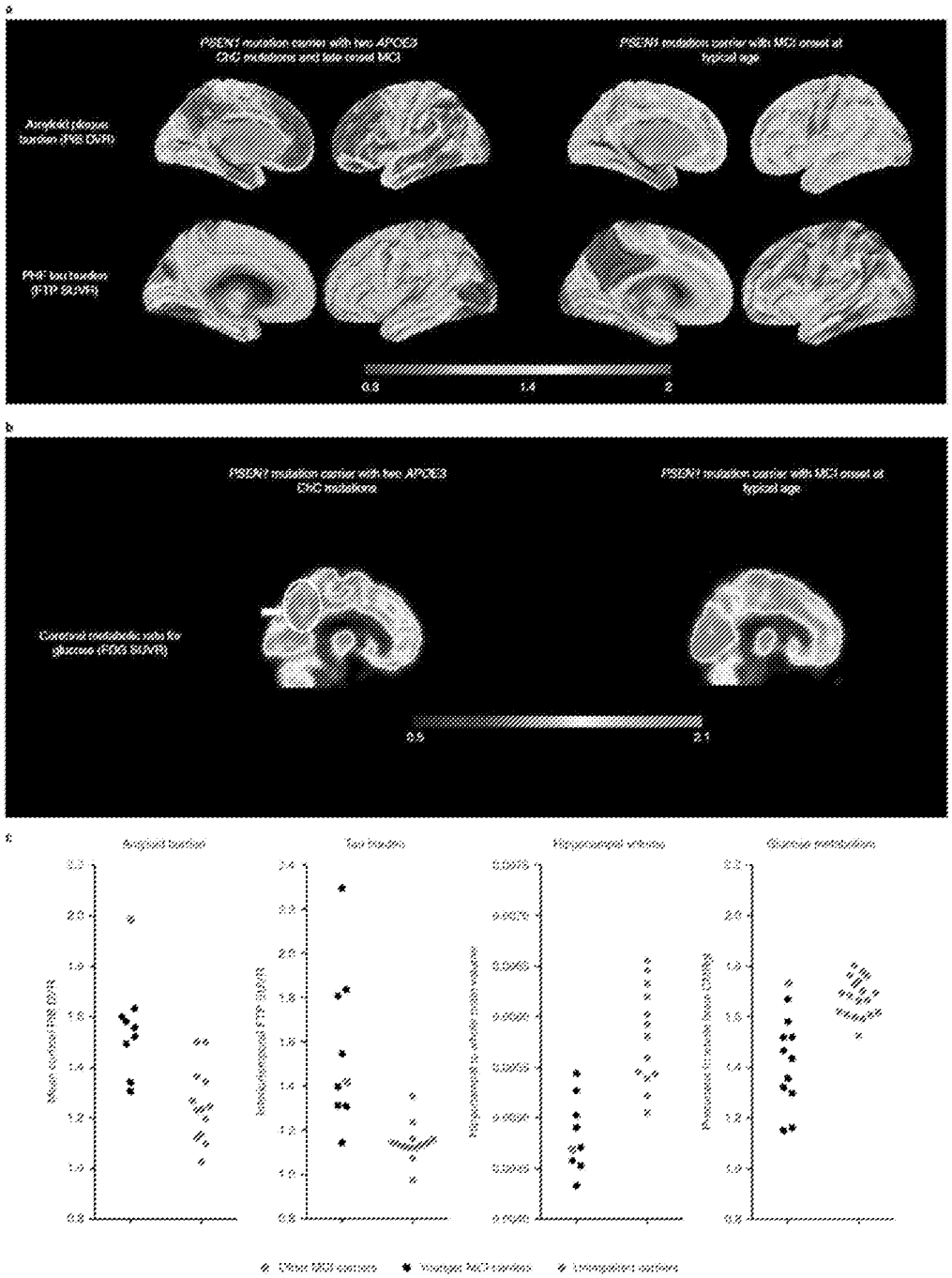
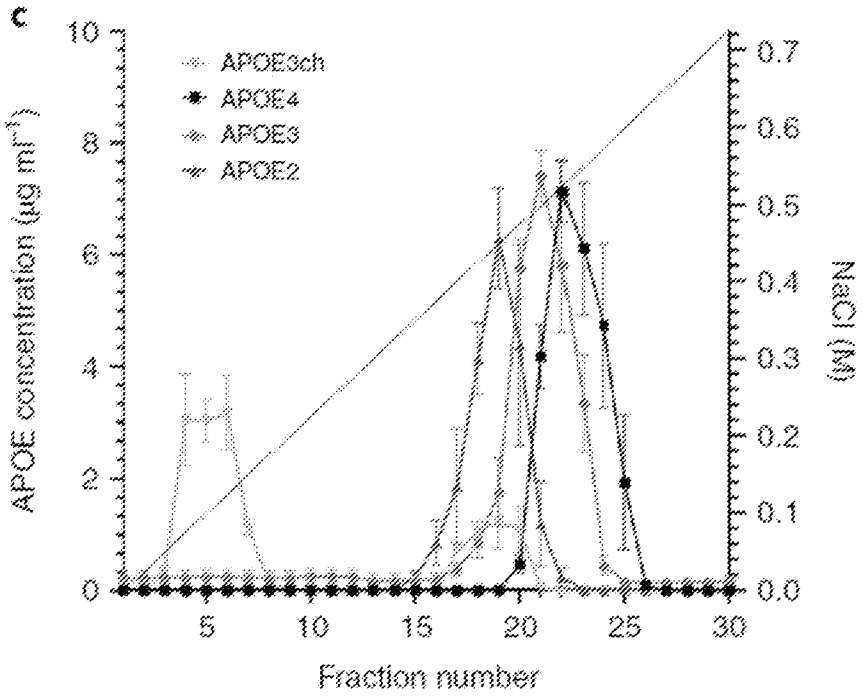


Figure 7. Impact of the APOE3ch Mutation

- The Colombian woman inherited two copies of this ApoE3 variant, known as the Christchurch mutation
- The Christchurch mutation appears to be recessive. Four other Paisa mutation carriers in the Colombian kindred inherited a single copy, and all developed cognitive impairment at the expected age
- The ApoEch mutation seems to block secondary tau pathology (latest Reiman study shows that this is also the case for ApoE2 homozygotes, as seen from autopsy samples from 24 ApoE2 homozygotes)
- This protective effect is similar to, but greater than, that of ApoE2
- *In vitro* experiments showed that ApoE3ch behaves like the ApoE2 allele; both isoforms poorly bind heparin, corresponding to the extracellular matrix molecules implicated in the propagation and uptake of toxic forms of tau
- Binding to heparin: E4>E3.E2>>E3ch
- Protection therefore may correlate with lost binding to HSPGs, believed to propagate tangles

Figure 8. Low Affinity of APOE3ch Binding to Heparin

- HSPGs have been implicated in the buildup of amyloid plaques, as well as in promoting the microglial response to amyloid
- HSPGs have also been linked to tau pathology and suggested that these sticky receptors allow tau fibrils to get attached to neurons, facilitating uptake and propagation
- ApoE3 bound with an Ab to the aa 130- 143 (which includes the region of the Christchurch mutation) converts ApoE3 to ApoEch as measured by heparin binding



ApoE3ch was eluted from the heparin column with the lowest NaCl concentration, revealing impaired heparin binding compared to other ApoE isoforms.

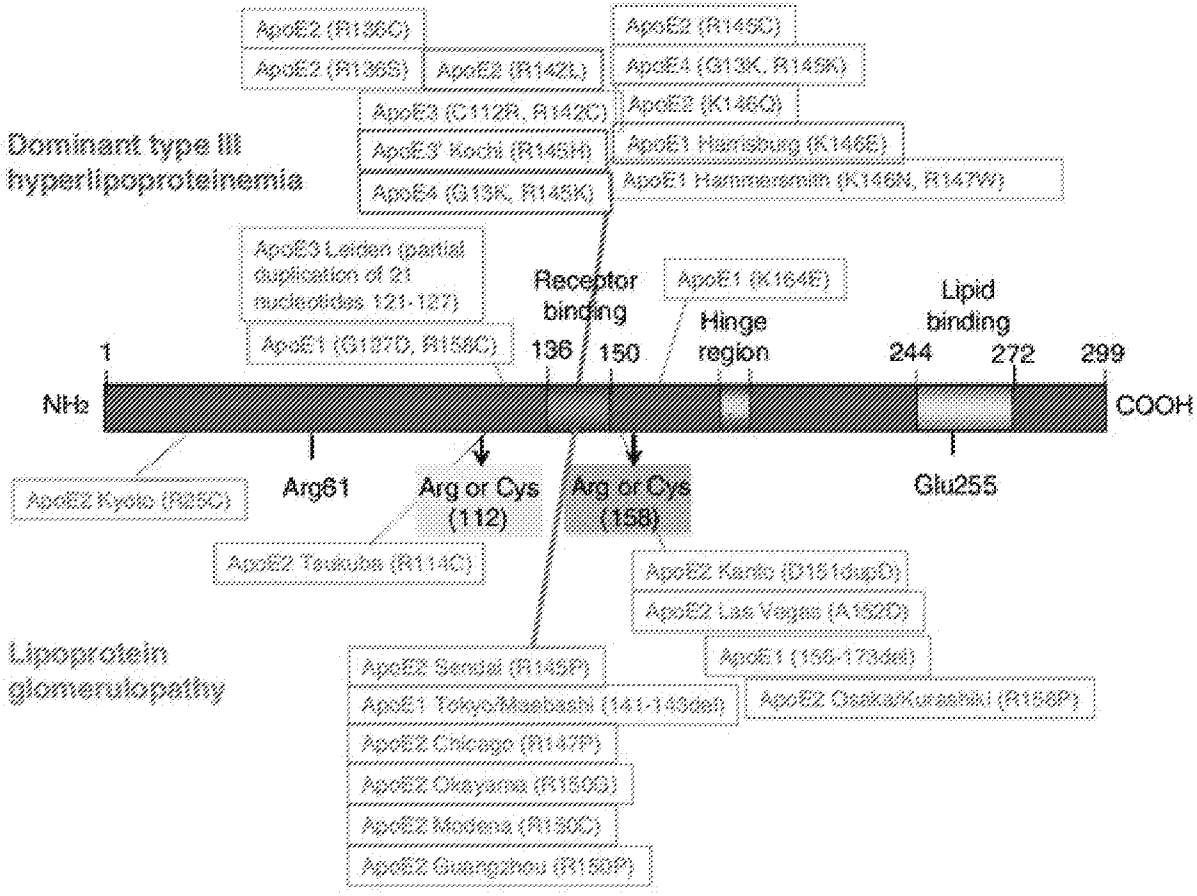
Figure 9

- ApoE could be facilitating tau binding to HSPGs which in turn may play a role in tau pathology
- Since the ChristChurch variant's weaker affinity for HSPG binding disrupts the facilitation of tau pathology it may represent a design for therapy
- Alteratively, any interventions that weakens ApoE-HSPG binding might have therapeutic potential

Figure 10

- Gene therapy with AAV-APOE3ch
- Gene therapy with AAV-APOE2ch
- Target ApoE-HSPG binding with an antibody delivered by AAV
- Use an SiRNA to silence ApoE4 in combo with AAV-APOE3ch delivery in same construct
- Dyslipidemia is unlikely because the gene therapy is mostly confined to the CNS; any dyslipidemia can be treated with current lipid therapies

Figure 11. ApoE Protein Mutations that Result in Lipid Disorders May Alter Alzheimer's Predilection



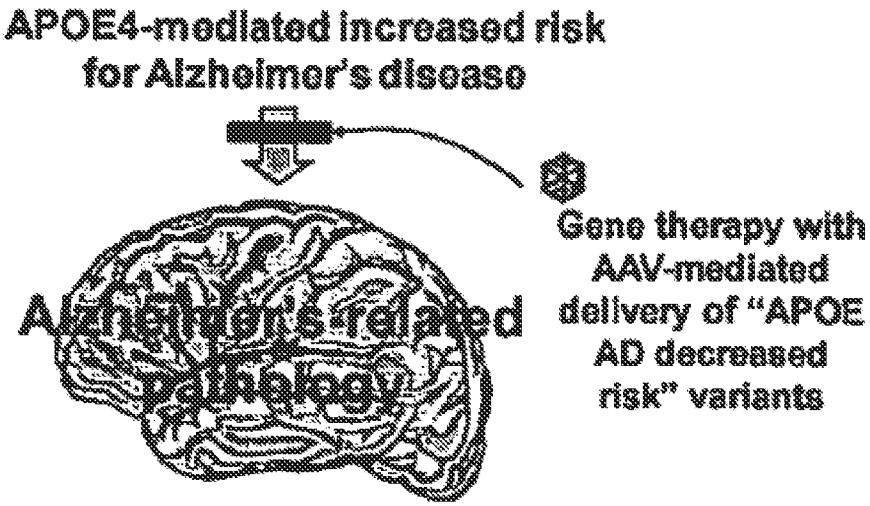
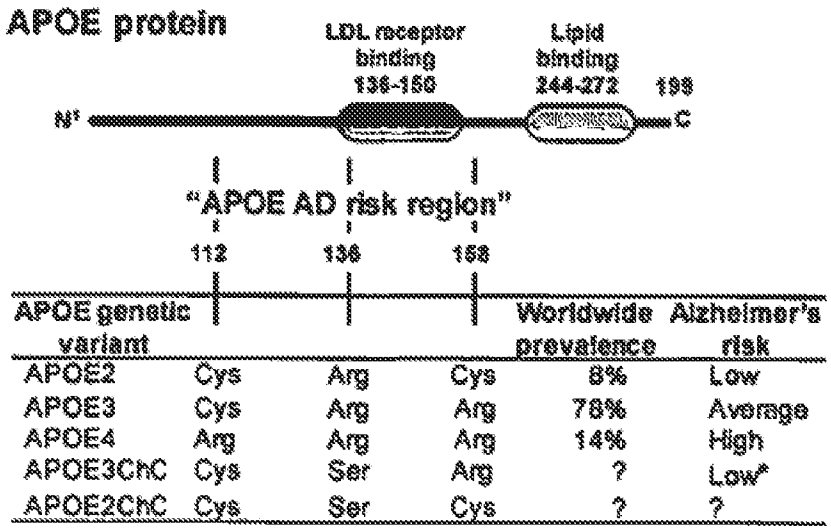


Figure 12



APOE genetic variant				Worldwide prevalence	Alzheimer's risk
APOE2	Cys	Arg	Cys	8%	Low
APOE3	Cys	Arg	Arg	78%	Average
APOE4	Arg	Arg	Arg	14%	High
APOE3ChC	Cys	Ser	Arg	?	Low*
APOE2ChC	Cys	Ser	Cys	?	?

Figure 13

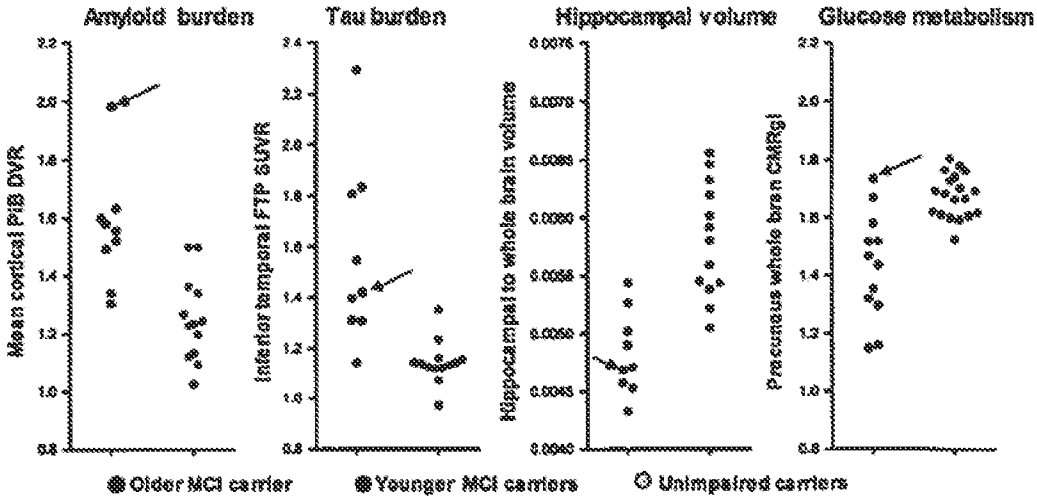


Figure 14

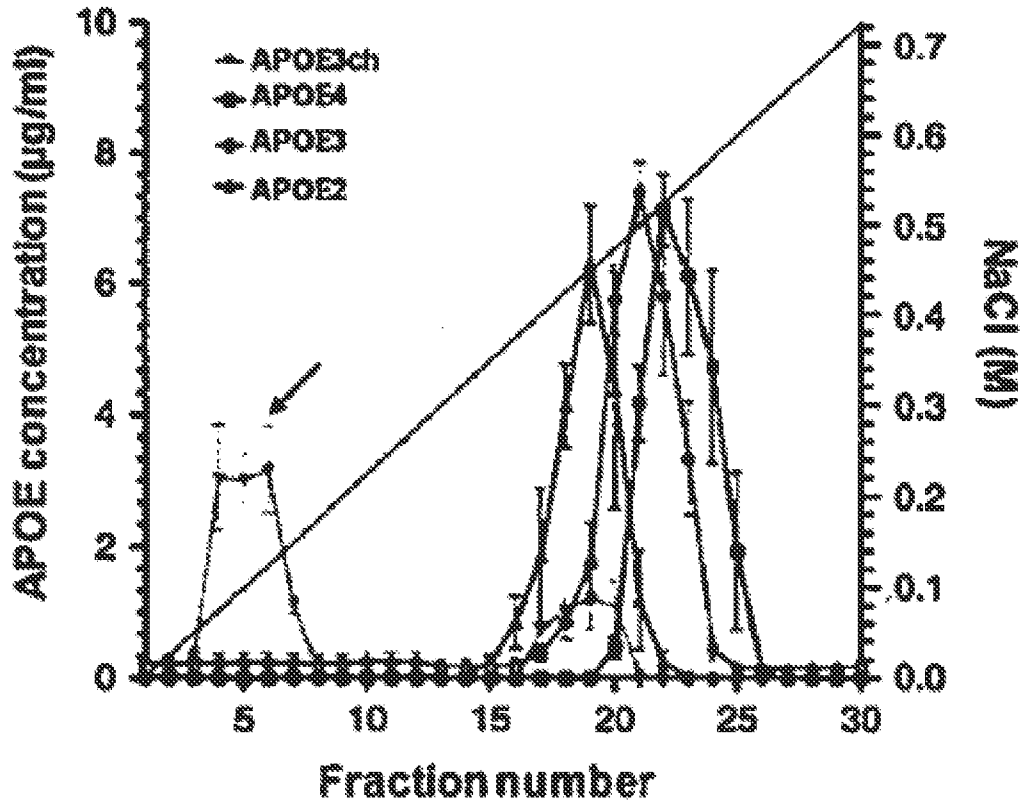


Figure 15

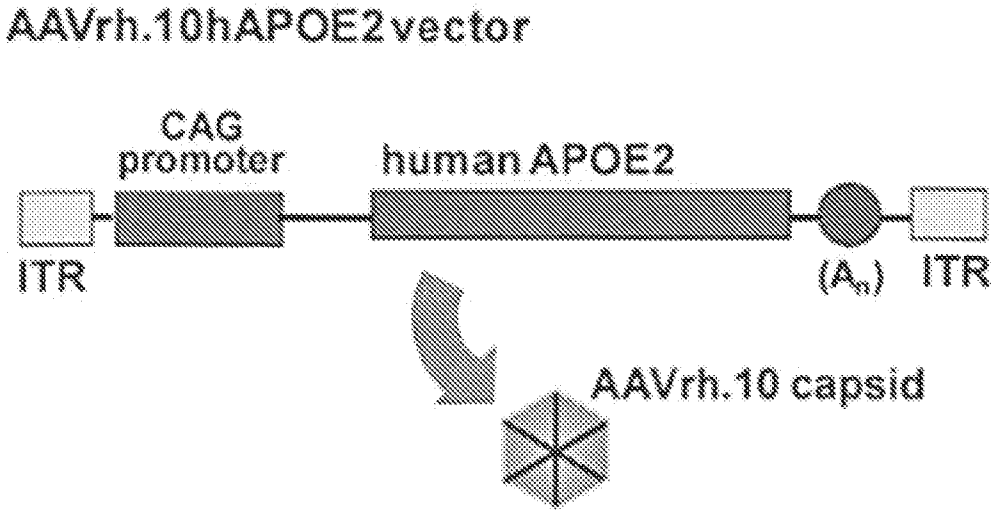


Figure 16

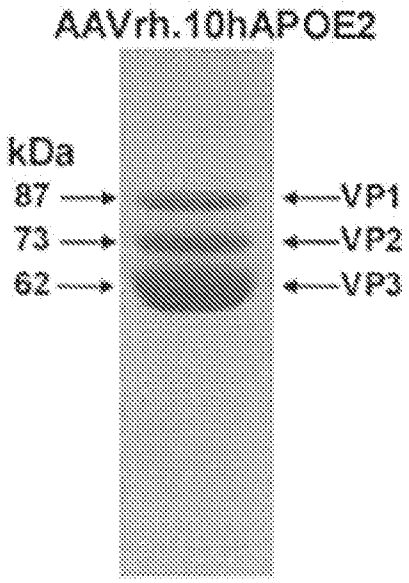


Figure 17

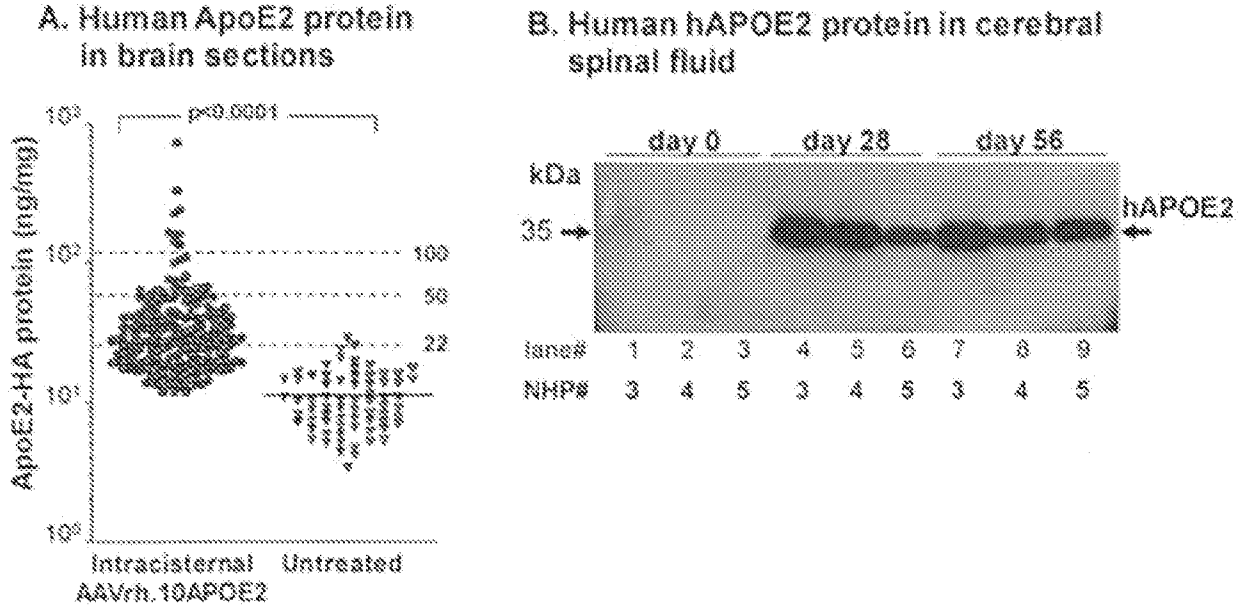


Figure 18

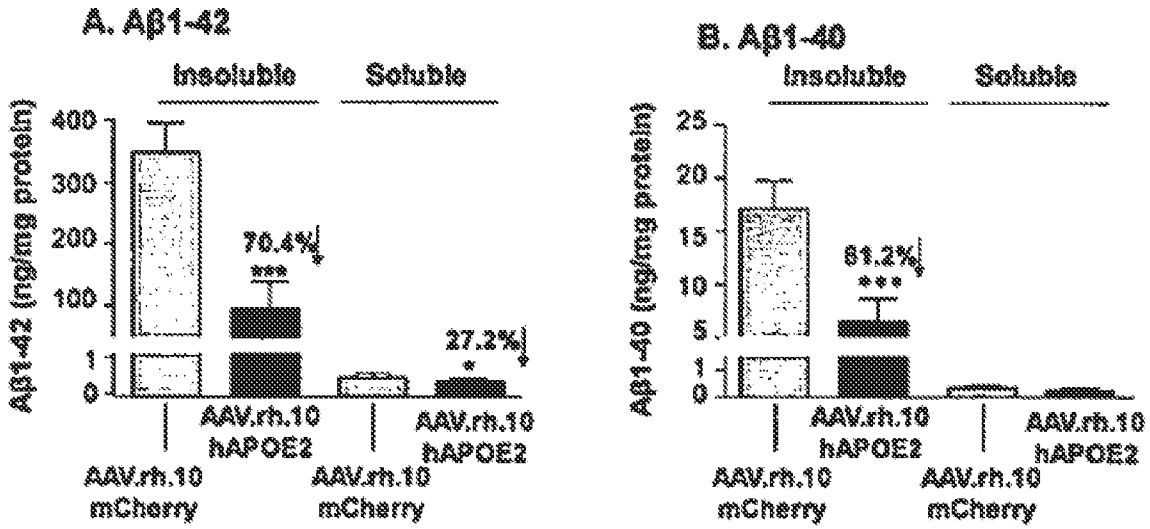


Figure 19

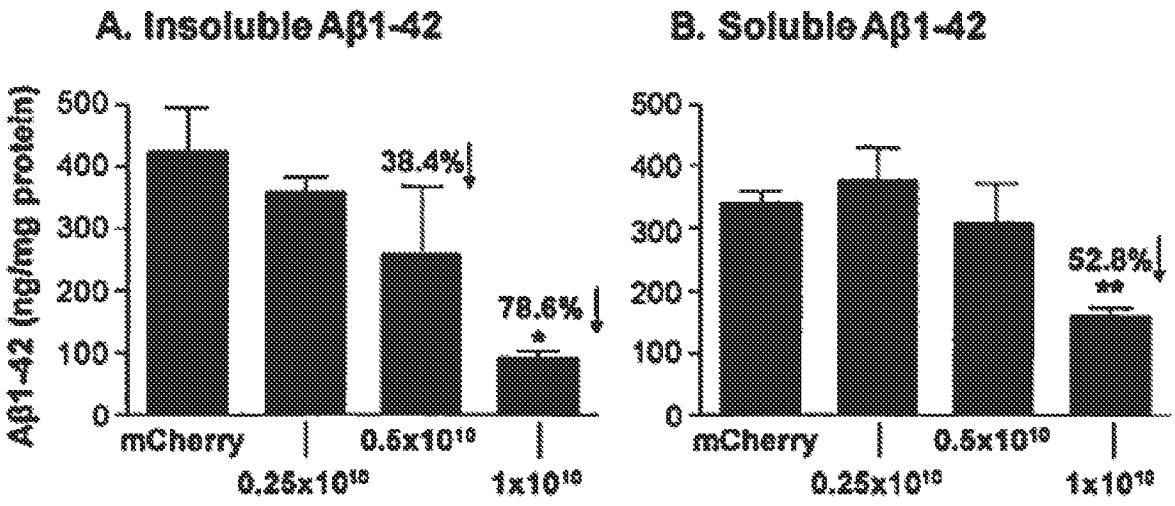

A. Insoluble Aβ1-42
B. Soluble Aβ1-42
Aβ1-42 (ng/mg protein)
500
400
300
200
100
0
38.4%
78.6%
*
52.8%
**
mCherry
0.25x10¹⁰
0.5x10¹⁰
1x10¹⁰

Figure 20

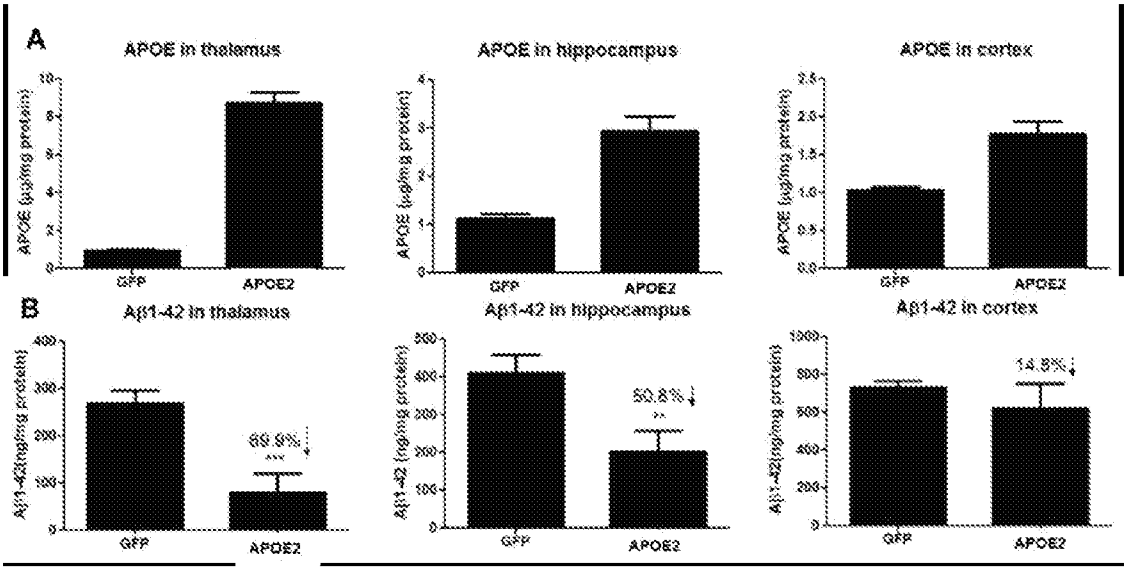


Figure 21

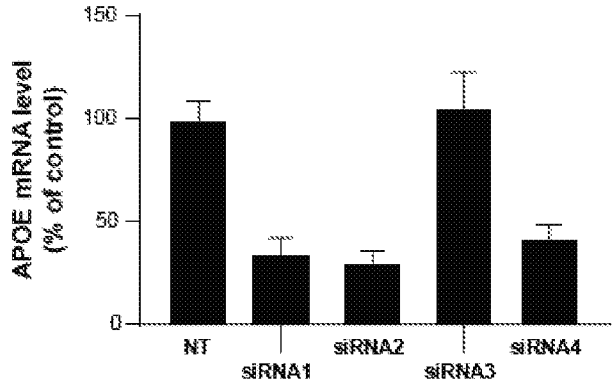


Figure 22

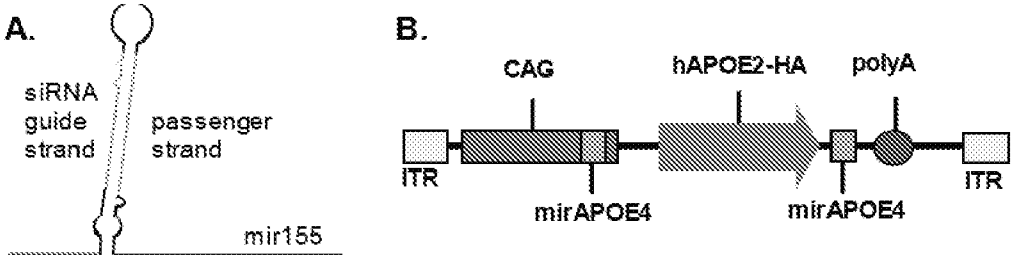
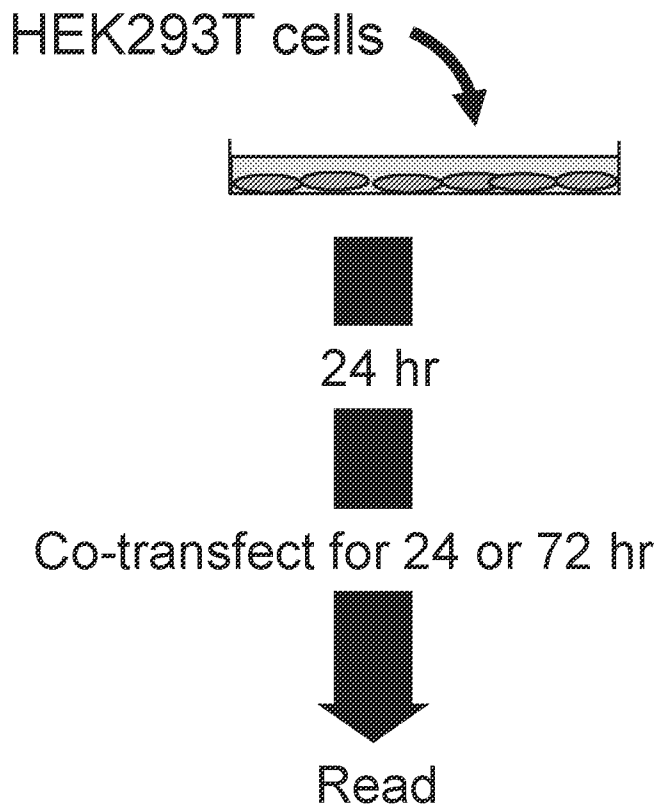


Figure 23

Figure 24. 293T Transfection

Transfection Scheme

- Co-transfect pAAVcag-miRAPOE4 with pmirGLO containing target sites or the vector alone



- Target 1
  - miRNA in the intron
  - miRNA in the polyA
  - New controls
- Null vector
  - miRNA in the intron
  - miRNA in the polyA
  - New controls

- This experiment tests the knockdown efficiency of miRs located in the intron and polyA

Figure 25. Luciferase Results

Conclusions

- miR2A in the intron or polyA appears to exhibit greater knockdown as compared to miR2B

Comments on Controls

- The empty vector controls were removed since the target site may decrease luciferase expression
- Another assay was set up to test luciferase expression with the pmirGLO containing target site, alone.

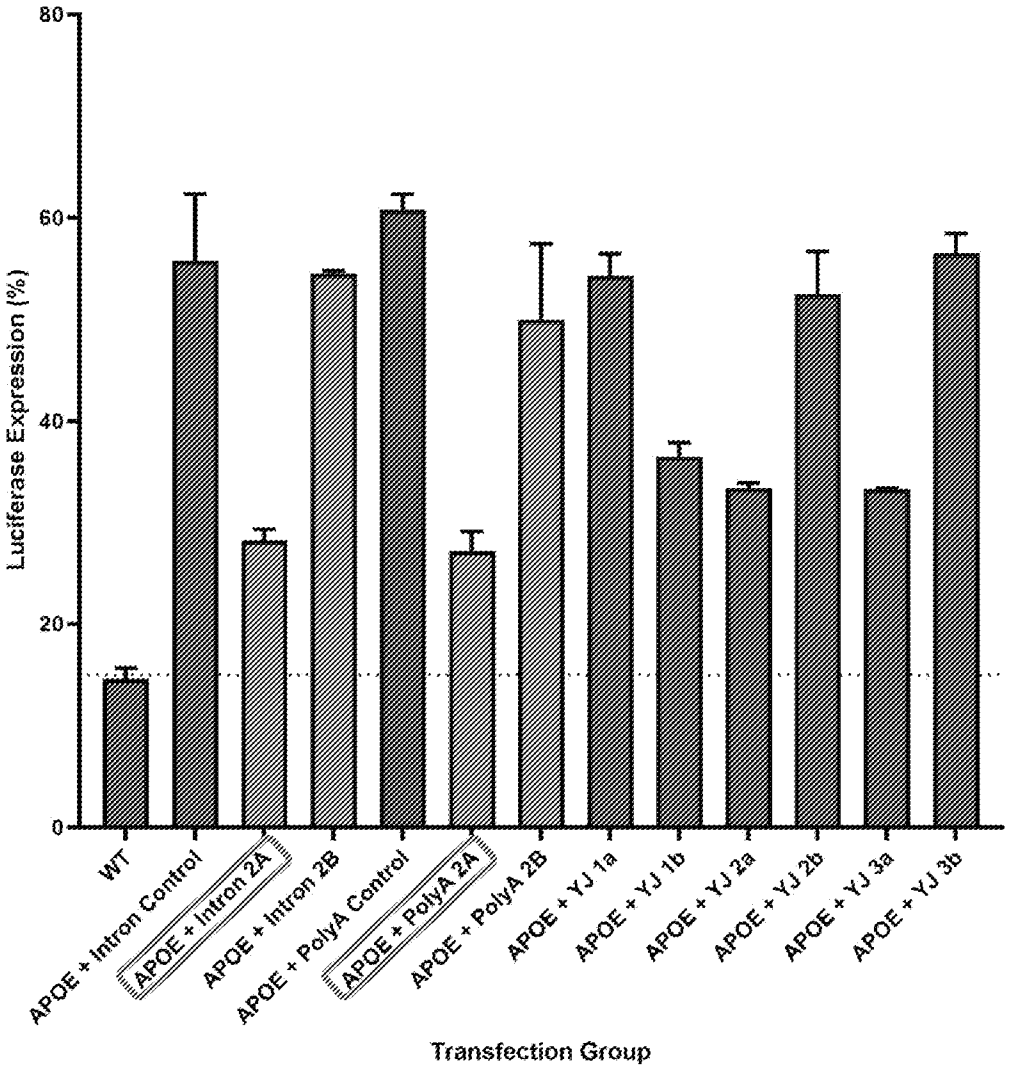


Figure 26. Testing APOE Target Site Only Control

Rationale

- If the control pmirGLO containing target site alone decreases luciferase expression, then pmirGLOluc (null) vector cannot be used as a negative control.
- The target site may affect the stability of the mRNA, may contain cryptic binding site or may interact with translation machinery.

Conclusions

- miR2A in the intron or polyA appears to exhibit greater knockdown as compared to miR2B

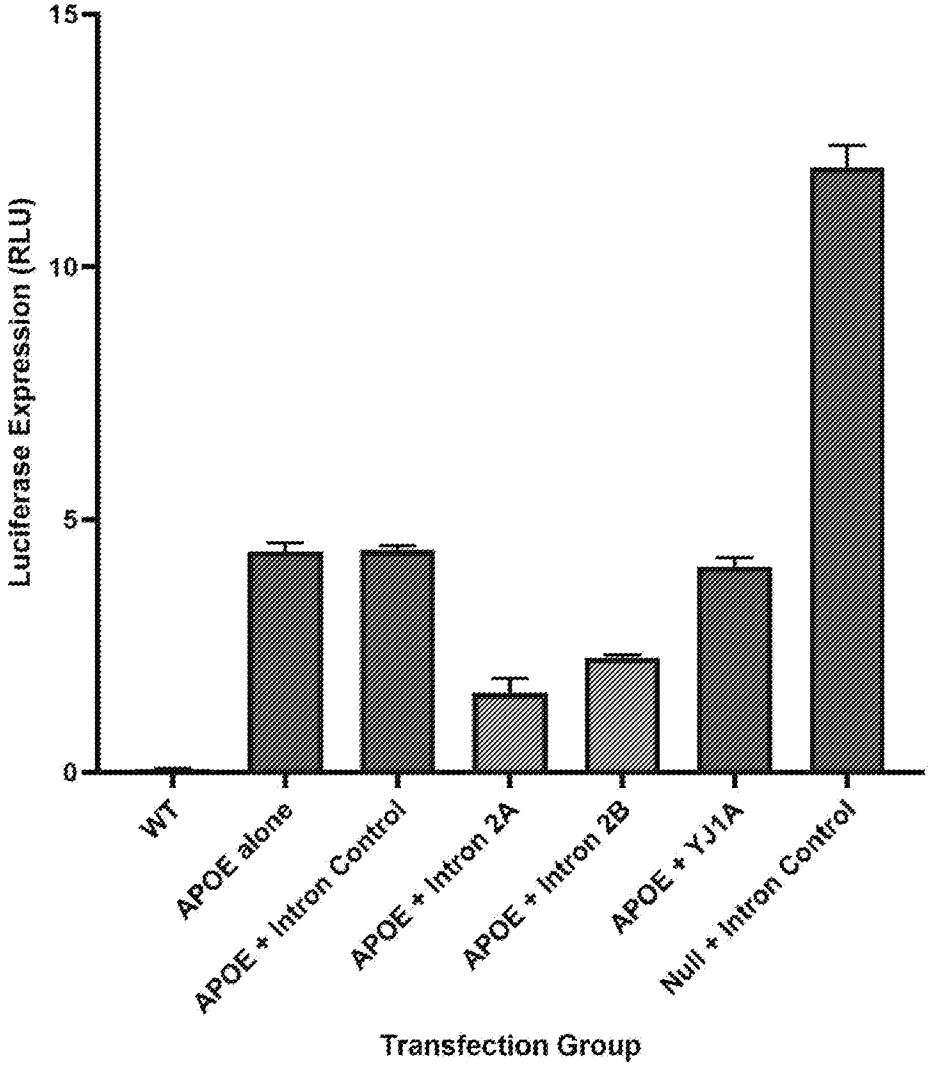


Figure 27.BsaI - BmgBI

Rationale

- Approximately 200bp piece in APOE2 overlapping with the miRNA site
- Replace the miRNA target site in the vector-derived APOE2 with silent nucleotide changes
- Therapeutic APOE2 being delivered will not also be silenced

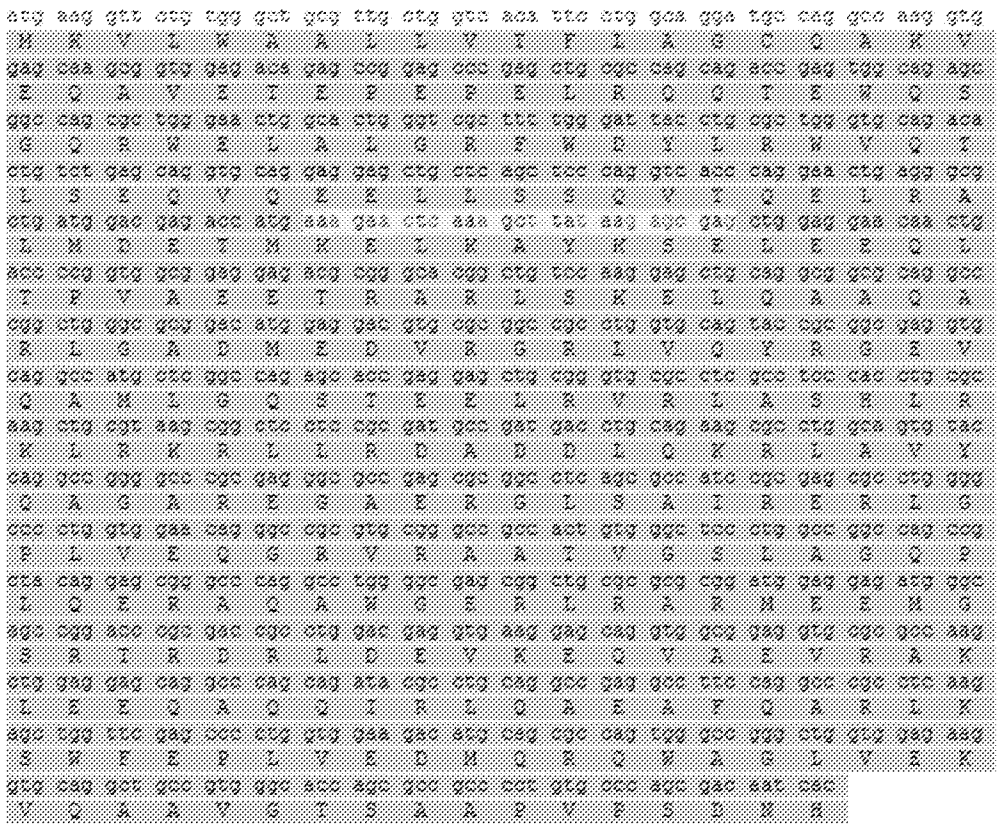
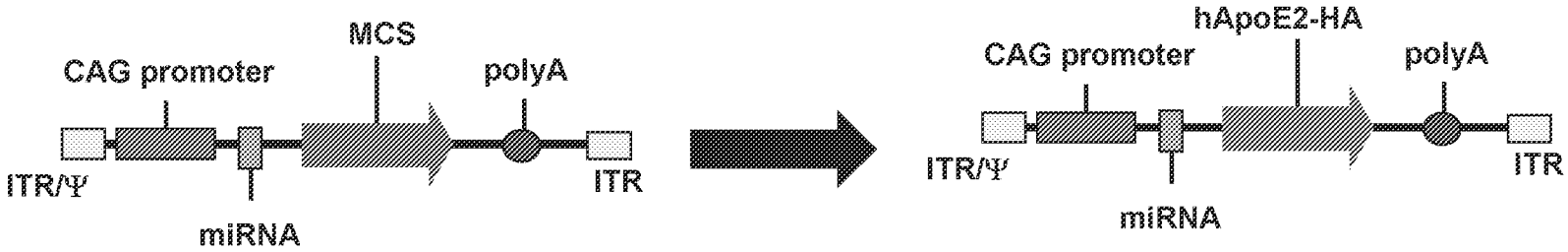


Figure 28. Insertion of hApoE2-HA

Strategy for Generating pAAV-miRNA-APOE-HA

- Clone APOE2 into the pAAV with miRAPOE4



- BsaI-BmgBI fragment successfully PCR amplified (~250 bp)
- BsaI-BmgBI is cloned into the pAAV-miRNA with the strongest knockdown

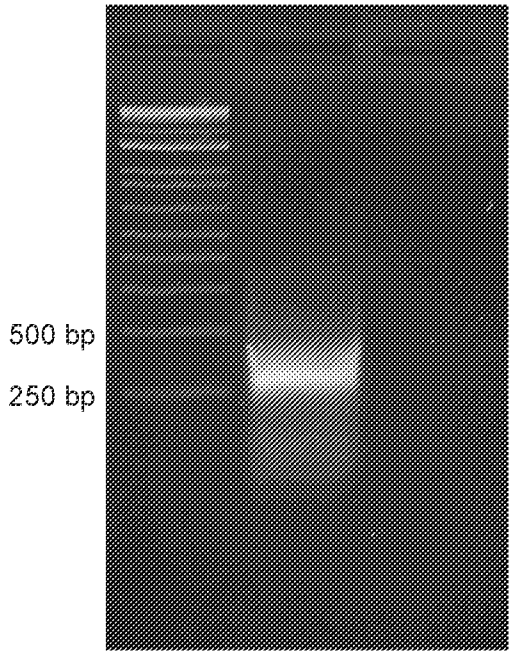


Figure 29. *In Vivo* Studies

- Breeding P301S (Jax) mice with TRE4 mice
- AAV9 preparation with confirmed miRs

Table I. Mouse Proof of Principle Study

Mouse model (genotype)[2]	Vector	Administration dose and route	Parameters to be evaluated
P301S/E4	AAV9-hAPOE2	10^{11} gc, via intracisternal (IC) route	• Behavior and neurologic assessment (monthly) • Brain expression of hAPOE4 and hAPOE2 • Soluble and insoluble Aβ levels • Assessment of phosphorylated tau and total tau levels in brain homogenates • CNS assessment of neurofibrillary tangles • Microglial activation • Pathology of hippocampus and cortex
	AAV9-hAPOE2-mirAPOE4		
	AAV9-mCherry-mirAPOE4		
	AAV9-null		
	No therapy (PBS)	None (PBS, IC)	
C57Bl/6 (controls)	No therapy (PBS)	None (PBS, IC)	
2 strains	**5 vectors**	**1 route**	**Necropsy @ 3 and 9 months of age**

Figure 30. All Controls

