

Novel neurotropic recombinant adeno-associated virus particles**Field of the invention**

The present invention relates to the fields of medicine, molecular biology and gene therapy. In particular, the invention relates to neurotropic recombinant adeno-associated virus (rAAV) particles, novel variants thereof, methods of manufacturing these rAAV particles, and use of these rAAV particles in the treatment of Central Nervous System (CNS) diseases.

Background of the invention

Recombinant adeno-associated viruses (rAAV) have been developed into a preferred platform for gene therapy. Yet, the success of rAAV-based therapeutic strategies is limited, amongst others, by nonspecific tropism. Specifically, developing novel recombinant AAV capsids which are able to specifically transduce cells in the central nervous system (CNS) is one of the most difficult challenges in the field of rAAV-based gene therapy for CNS diseases. The inefficiency of crossing the blood-brain barrier (BBB), in combination with high-level sequestration and transduction of peripheral organs, narrows the therapeutic window and thus limits applications in the CNS for rAAV-based vectors. For that reason, several further rAAV capsids have been engineered in recent years resulting in CNS-aimed capsid variants with different tropisms.

For example, Hanlon et al. (Mol Ther Methods Clin Dev. 2019, 15:320-332) and Nonnenmacher et al., (Mol Ther Methods Clin Dev. 2020, 20:366-378) report variant rAAV serotype 9 (rAAV9) capsids, with increased CNS transduction efficiency compared to parental rAAV9. However, these variant rAAV9 capsids still transduce peripheral organs such as the liver, skeletal muscle and the heart, with similar efficiencies as parental rAAV9. Pulicherla et al. (Mol Ther. 2011, 19(6):1070-8) report the generation of variant rAAV9 capsids using random mutagenesis of residues within a surface-exposed region of the major AAV9 capsid proteins. Their capsid variants were classified into three functional subgroups, with respect to parental rAAV9, displaying: (i) a functionally defective phenotype with decreased transduction efficiency across multiple tissues; (ii) a selective decrease in liver transduction, or (iii) a similar transduction profile. Colle et al. (Human Molecular Genetics. 2010, 19(1):147-58) were able to develop an rAAV serotype 2 – rAAV serotype 5 hybrid (rAAV2-5) capsid with good transduction when injected intracerebrally in non-human primates. However, even when injected intracerebrally, a considerable amount of vector gene copies were found in liver cells and spleen cells. It is clear that there remains a need for further improved rAAV capsids that efficiently transduce cells in the CNS while showing reduced transduction in peripheral organs.

An additional challenge faced in rAAV-based gene therapies are post-treatment immune responses observed after transduction of cells, as is, among others, described by Ronzitti et al. (Ronzitti et al., Front. Immunol. 2020, 11:1-13) and Muhuri et al. (Muhuri et al., J Clin Invest. 2021 131(1):e143780). Such immune responses include both innate immune responses, elicited from the rAAV-derived pathogen-associated molecular patterns (PAMP), and adaptive immune responses, directed against rAAV capsid-derived epitopes. Both types of immune responses may influence the efficiency of rAAV-based gene therapy.

It is an object of the present invention to provide rAAV5 particles that specifically transduce cells in the CNS and display decreased post-treatment immune responses.

Summary of the invention

5 In one aspect, the invention provides a recombinant adeno-associated (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising:

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- 10 ii) an expression cassette flanked by at least one AAV inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease.

In one aspect, the nucleic acid molecule encodes at least one of an RNA and a polypeptide.

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In one aspect, an rAAV5 particle for use according to the invention may comprise an additional amino acid, preferably an alanine or valine, inserted at a position corresponding to between positions 1 and 2 of wild type AAV5 VP1 capsid protein.

20 In one aspect, an rAAV5 particle for use according to the invention comprises an exchange of the N-terminal region corresponding to the first 136 N-terminal amino acids of wild type AAV5 VP1 capsid protein with the N-terminal region corresponding to the first 137 N-terminal amino acids of wild type AAV serotype 2 (AAV2) VP1 capsid protein.

25 In one aspect, the present invention provides a composition comprising an rAAV5 particle of the invention and an excipient, preferably for intrathecal (cisternal or lumbar), intracerebroventricular, intracerebral, intrastratial, intraputamina, intra-cerebrospinal fluid (CSF) and intraparenchymal administration, and/or intranasal instillation.

30 In one aspect, the present invention provides a method for manufacturing a recombinant adeno-associated virus serotype 5 (rAAV5) particle, the method comprising the steps of: I) culturing a host cell comprising:
i) an expression construct for the expression of a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule
35 encoding at least one product having an effect in the CNS disease, the mutated capsid protein further comprising: a) an additional amino acid, preferably an alanine or valine, inserted at a position corresponding

to between positions 1 and 2 of wild type AAV5 VP1 capsid protein or b) an exchange of the N-terminal region corresponding to the first 136 N-terminal amino acids of wild type AAV5 VP1 capsid protein with the N-terminal region corresponding to the first 137 N-terminal amino acids of wild type AAV serotype 2 (AAV2) VP1 capsid protein, said host cell preferably further comprising a nucleotide sequence encoding a parvoviral replication (Rep) protein under conditions such that the rAAV5 particle is produced; and, II) recovery of the rAAV5 particle, preferably wherein recovery of the rAAV5 particle in step II) comprises at least one of: affinity-purification of the particle using an immobilised anti-AAV antibody, preferably a single chain camelid antibody or a fragment thereof, or filtration over a filter having a nominal pore size of 30 - 70 nm.

In one aspect, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle comprising: i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease, wherein the rAAV5 particle further comprises: a) an additional amino acid, preferably an alanine or valine, inserted at a position corresponding to between positions 1 and 2 of wild type AAV5 VP1 capsid protein; or b) an exchange of the N-terminal region corresponding to the first 136 N-terminal amino acids of wild type AAV5 VP1 capsid protein with the N-terminal region corresponding to the first 137 N-terminal amino acids of wild type AAV serotype 2 (AAV2) VP1 capsid protein.

Description of the invention

The present invention relates to gene therapy, in particular to rAAV5 particles with high CNS tropism and lower immunogenicity, when compared to state of the art rAAV5 particles. rAAV5 particles are currently being employed and investigated as a vehicle for gene therapy, both for systemic diseases as well as for diseases of the CNS. The amino acid sequence of the capsid of rAAV5 particles can be, and has been optimized in multiple ways, e.g. to optimize their manufacturability and expression in insect cells (Urabe *et al.*, Journal of Virology, 2006, 80(4):1874-85). Furthermore, Afione et al. (Journal of Virology, 2015, 89(3):1660-72) conducted a mutagenesis assay to find determinants for increased transduction efficiency as well as cell tropism. The data of this study are also described in US patent 10,081,659. In the abovementioned study by Afione et al., it was found that a mutation of the methionine (M) amino acid at position 569 of AAV5 viral protein 1 (VP1) resulted in almost complete abolishment of transduction in multiple different cell types *in vitro*. The authors also showed that an rAAV particle comprising the mutant rAAV5 capsid was unable to transduce cells of the salivary gland *in vivo*. Hence, Afione et al. concluded that the mutation at position M569 results in complete inhibition of transduction of all cell types. The present inventors surprisingly found that rAAV5 particles comprising the mutation at position M569 are able to

transduce cells of the CNS significantly more efficiently and specifically *in vivo*. Furthermore, it was surprisingly found that rAAV5 particles comprising the mutation at position M569 induce a reduced immune response compared to parental rAAV5 virus particles.

Recombinant Adeno-Associated Virus (AAV) serotype 5 particle

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The present invention provides a recombinant adeno-associated (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising:

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1);
- and
- ii) an expression cassette flanked by at least one AAV inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease.

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It is described herein that such rAAV5 particles are highly specific for transduction of cells of the CNS *in vivo*. Given the fact that Afione et al. described that rAAV5 particles having the M569V mutation were unable to transduce all tested cell types *in vitro*, including a human glioblastoma cell line (SF-268), this is surprising.

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Therefore, in one embodiment, there is provided an rAAV5 particle as described herein, with increased transduction to the CNS when compared to parental rAAV5.

In one embodiment, there is provided an rAAV5 particle as described herein, with reduced transduction efficiency of liver cells when compared to parental rAAV5.

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In a further embodiment, there is provided an rAAV5 particle as described herein, with increased transduction to the CNS and reduced transduction efficiency of liver cells, when compared to parental rAAV5.

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In a further embodiment, there is provided an rAAV5 particle as described herein, with increased transduction efficiency as compared to parental rAAV5 at sites distal of the injection site.

The term "transduction" as used herein refers to the process of transfer of the viral vector into a host cell. Upon primary transduction, the viral vector may be transferred from a viral particle into a host cell. Upon secondary or further transduction, a viral vector and/or a viral vector-derived expression cassette may be transferred from a first host cell to a second host cell and from the second host cell to further host cells. This transfer may be mediated by extracellular vesicles, or exosomes, or other cell to cell contacts. The

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skilled person is aware of methods to measure transduction. Exemplary methods as described herein include but are not limited to measurement of vDNA using PCR-based assays, or measurement of the expression of a reporter gene, such as Green Fluorescent Protein (GFP).

5 As described above, host immune responses, among which neutralizing antibodies, pose a hurdle in AAV-based gene therapy. Afione et al. describe that rAAV5 M569V particles are neutralized to a lesser extent by a polyclonal serum raised against wild type AAV5 as compared to the parental rAAV5. These immune pathways include, but are not limited to: "Inflammation mediated by chemokine and cytokine signalling pathway", "T-cell activation pathway", "Interleukin signalling pathway", and "Toll-like receptor pathway".

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In one embodiment, there is provided an rAAV5 particle as described herein, which induces less upregulation of multiple immune pathways involved in both innate and adaptive immune responses upon administration in the CNS as compared to parental rAAV5.

15 In a further embodiment, the immune pathways include, but are not limited to: "Inflammation mediated by chemokine and cytokine signalling pathway", "T-cell activation pathway", "Interleukin signalling pathway", and "Toll-like receptor pathway".

The skilled person is aware of methods to determine upregulation of immune pathways, including but not limited to: immunohistochemistry-based analysis. For example, ionized calcium-binding adapter 1 (IBA-1), is known to one of skill in the art to be a marker of inflammation.

25 It may be anticipated that an rAAV5 particle which transduces cells of the CNS with high efficiency, while showing less off-target effects may also have increased efficacy and safety. Due to the high efficiency of transduction of cells of the CNS, lower GC numbers may lead to more effective treatment. Lower GC numbers per subject may reduce the cost of goods associated with treatment.

In one embodiment, there is provided an rAAV5 particle as described herein with increased efficiency of transgene expression as compared to parental rAAV5.

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The skilled person is aware of methods to determine transgene expression, including but not limited to the measurement of any resulting protein expression, or associated labelling, or PCR, as described herein.

35 Further benefits may also include, but are not limited to, a decrease in the need for immunosuppressants before, during and/or after treatment. This may significantly decrease the burden on the patient, and further decrease the costs of the treatment.

The term "recombinant adeno-associated (rAAV) 5 particle" as used herein refers to an rAAV5 particle having a mutated capsid as compared to wild type AAV5 particles, meaning that the amino acid sequence of the capsid is non-naturally occurring. As used herein, the term "non-naturally occurring" when used in reference to an rAAV5 particle means that the rAAV5 particle has at least one mutation that is not normally found in a naturally occurring AAV5 particles, including wild-type AAV5 particles. Mutations include but are not limited to, modifications introducing additions of nucleic acids, nucleic acid deletions, nucleic acid substitutions, or alterations of the AAV5 capsid proteins, such as additions, deletions, or substitutions of amino acids in the capsid proteins.

The term "rAAV5 particle" as used herein may be interchangeably be denominated by the term "rAAV5 virion" or simply "rAAV5", and entails the capsid as well as the cargo of the capsid (i.e. what is carried within the capsid). The terms "parental rAAV5 particle", and "parental rAAV5" as used herein, pertain to recombinant AAV5 particles having a non-naturally occurring nucleic acid sequence and, optionally, a non-naturally occurring capsid amino acid sequence, but which have the native amino acid methionine at a position corresponding to M569 of wild type AAV5 VP1 capsid protein. In some embodiments, the term rAAV5 particle as used herein may refer to a hybrid rAAV5 particle. A hybrid rAAV5 particle may comprise capsid proteins derived from at least two different AAV serotypes. An exemplary hybrid rAAV5 particle comprises an exchange of the N-terminal region corresponding to the first 136 N-terminal amino acids of wild type AAV5 VP1 capsid protein with the N-terminal region corresponding to the first 137 N-terminal amino acids of wild type AAV serotype 2 (AAV2) VP1 capsid protein. Such a hybrid rAAV5 particle may be referred to herein as rAAV2/5 as well as rAAV5. As such, the terms rAAV5 and rAAV2/5 used herein may be used interchangeably.

The term "wild type" as used herein takes its known meaning in the art. Examples of the use of this term include "wild type AAV5 particle", "wild type AAV5 VP1 capsid protein" and "wild type AAV5" as used and refer to an AAV5 particle, an AAV5 VP1 capsid protein or an AAV5, respectively, as they may occur in nature. Wild type AAV5 VP1 capsid protein is herein understood to comprise, consist of, or to be comprised within the amino acid sequence of SEQ ID NO: 1 (AAV5 VP1 capsid protein; UniProt® Database Accession No. Q9YIJ1 / Q9YIJ1_9VIRU; NCBI Reference Sequence. YP_068409.1). The designation of all amino acid positions in the rAAV5 VP1 capsid proteins and variants thereof as used herein is with respect to the wild type AAV5 VP1 capsid subunit amino acid sequence numbering of SEQ ID NO: 1. Corresponding positions in amino acid sequences other than SEQ ID NO: 1 are identified using sequence alignment, preferably using algorithms and settings that are familiar to the person skilled in the art.

“Sequence identity” can be determined by alignment of peptide or nucleotide sequences using global or local alignment algorithms, depending on the length of the sequences. Sequences of similar lengths are preferably aligned using global alignment algorithms (e.g. Needleman Wunsch) which align the sequences optimally over the entire length, while sequences of substantially different lengths are preferably aligned using local alignment algorithms (e.g. Smith Waterman). Sequences may then be referred to as “substantially identical” or “essentially similar” when they (when optimally aligned by for example the programs GAP or BESTFIT using default parameters) share at least a certain minimal percentage of sequence identity. Alternatively, percentage similarity or identity may be determined by searching against public databases, using algorithms such as FASTA, BLAST, etc. Thus, the nucleic acid and protein sequences of the present invention can further be used as a “query sequence” to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the BLASTn and BLASTx programs (version 2.0) of Altschul, et al. (J. Mol. Biol. 1990, 215:403—10).

In one embodiment the present invention provides the use of a recombinant adeno-associated (rAAV) serotype 5 particle, the rAAV5 particle comprising:

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
 - ii) an expression cassette flanked by at least one AAV inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in a Central Nervous System (CNS) disease,
- in the manufacture of a medicament for the treatment of the CNS disease.

In another embodiment the present invention provides a method of treating a Central Nervous System (CNS) disease by administering a recombinant adeno-associated (rAAV) serotype 5 particle, the rAAV5 particle comprising:

- i) a mutated capsid protein comprising anon-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one AAV inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease.

Diseases of the Central Nervous System (CNS)

The terms “Central Nervous System” and “CNS”, as used herein refer to both the brain and the spinal cord. A “disease of the CNS” or “CNS disease” or “CNS disorder” as referred to herein, is defined herein as a disease wherein the brain and/or spinal cord do not exert their function as compared to the brain and/or spinal cord of a healthy individual. The terms “disease of the CNS” or “CNS disease” or “CNS disorder”

may be used interchangeably. Diseases that also affect the brain and/or spinal cord next to having different symptoms can also be a “disease of the CNS”, or “CNS disease”. Such diseases include, for instance, neurological diseases which affect both the central nervous system and the peripheral nervous system.

- 5 In one embodiment, the brain and/or spinal cord function is impaired as compared to a healthy individual in a CNS disease as defined herein.

In a preferred embodiment, the CNS disease is caused by a genetic defect.

- 10 In a further embodiment, the genetic defect is a mutation in a gene and/or accumulation of dysfunctional or non-functional proteins when compared to a healthy individual.

In a further embodiment, the mutation in a gene results in an increase in protein aggregation. In still a further embodiment, the increase in protein aggregation comprises a dysfunctional or non-functional protein.

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- Exemplary CNS diseases include, but are not limited to, the diseases listed in Table 1. In a preferred embodiment, the CNS disease is selected from: Parkinson’s Disease (PD); Parkinson disease 1, autosomal resistant (PARK1); Parkinson disease 4, autosomal dominant (PARK4); Multiple System Atrophy (MSA); Lewy Body Dementia (LBD); Huntington’s Disease (HD); Spinocerebellar Ataxia 1 (SCA1); Spinocerebellar Ataxia 2 (SCA2); Spinocerebellar Ataxia 3 (SCA3); Primary Tauopathies; Pick’s Disease (PiD); Progressive Supranuclear Palsy (PSP); Corticobasal Degeneration (CBD); Argyrophilic Grain Disease (AGD); Secondary Tauopathies; Chronic Traumatic Encephalopathy (CTE); Alzheimer’s Disease (AD); Frontotemporal Dementia (FTD); Amyotrophic Lateral Sclerosis (ALS); Temporal Lobe Epilepsy (TLE); refractory Temporal Lobe Epilepsy (rTLE); Focal Cortical Dysplasia (FCD); Spinal Muscular Atrophy 1 (SMA1; Werdnig-Hoffmann disease); Spinal Muscular Atrophy 2 (SMA2, Dubowitz disease); Spinal Muscular Atrophy 3 (SMA3, Kugelberg-Welander disease); Spinal Muscular Atrophy 4 (SMA4); Gaucher Disease (GD); Mucopolysaccharidosis 3A (MPS3A); Mucopolysaccharidosis 2 (MPS2); Mucopolysaccharidosis 1 (MPS1); Ceroid Lipofuscinosis, Neuronal, 1 (CLN1); Immunodeficiency 78 with autoimmunity and developmental delay (IMD78); Ceroid Lipofuscinosis, Neuronal, 3 (CLN3); Batten Disease; Ceroid Lipofuscinosis, Neuronal, 5 (CLN5); Ceroid Lipofuscinosis, Neuronal, 6 (CLN6); Ceroid Lipofuscinosis, Neuronal, 7 (CLN7); Ceroid Lipofuscinosis, Neuronal, 8 (CLN8); Canavan Disease (CAND); Ubiquitin-positive Frontotemporal Dementia (UP-FTD); Ceroid Lipofuscinosis, Neuronal, 11 (CLN11); Angelman Syndrome (AS); Rett Syndrome (RTT); GM1-Gangliosidosis 1 (GM1G1); GM1-Gangliosidosis 2 (GM1G2); GM1-Gangliosidosis 3 (GM1G3); Giant Axonal Neuropathy 1; Autosomal Recessive (GAN1); Citrullinemia (CTLN1); GM2-Gangliosidosis 1 (GM2G1); Tay-Sachs Disease; Fragile X Syndrome (FXS); Niemann-Pick Disease A (NPDA); Niemann-Pick Disease C (NPDC); Krabbe Disease (KRB); Mucopolysaccharidosis 1H (MPS1H); Epilepsy Metachromatic Leukodystrophy (MLD); Multiple Sulfatase
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Deficiency (MSD); Ceroid Lipofuscinosis, Neuronal, 2 (CLN2); Friedreich Ataxia (FRDA); Tuberous Sclerosis; Aromatic L-Amino Acid Decarboxylase Deficiency (AADCD); Pompe Disease; Mucopolysaccharidosis 3B (MPS3B); Alexander disease (ALXDRD); Dup15q syndrome; Developmental and epileptic encephalopathy 79 (DEE79); Epilepsy, childhood absence 5 (ECA5); Developmental and epileptic encephalopathy (DEE43); Prader-Willi syndrome (PWS); Phelan-McDermid Syndrome (PHMDS); PMS); Dravet Syndrome (DRVT); Lafora Disease (LD); Episodic Ataxia 2 (EA2); Alternating Hemiplegia of Childhood 1 (AHC1).

The term "treatment" as used herein specifies medical care given to an individual having a disease or a disorder. As a result of treatment, symptoms of the disease or disorder may be slowed down when compared to when no treatment would be given. It is preferred that the symptoms of the disease or disorder are halted and/or do not progress. It is more preferred that the symptoms of the disease or disorder are alleviated.

In one embodiment, the present invention provides a recombinant adeno-associated (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising:

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one AAV inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease,

wherein the CNS disease is selected from Table 1

In one embodiment, the present invention provides a recombinant adeno-associated (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising:

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one AAV inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the one or more CNS diseases,

wherein the CNS disease is selected from: Parkinson's Disease (PD); Parkinson disease 1, autosomal resistant (PARK1); Parkinson disease 4, autosomal dominant (PARK4); Multiple System Atrophy (MSA); Lewy Body Dementia (LBD); Huntington's Disease (HD); Spinocerebellar Ataxia 1 (SCA1); Spinocerebellar Ataxia 2 (SCA2); Spinocerebellar Ataxia 3 (SCA3); Primary Tauopathies; Pick's Disease (PiD); Progressive Supranuclear Palsy (PSP); Corticobasal Degeneration (CBD); Argrophilic Grain Disease (AGD);

Secondary Tauopathies; Chronic Traumatic Encephalopathy (CTE); Alzheimer's Disease (AD); Frontotemporal Dementia (FTD); Amyotrophic Lateral Sclerosis (ALS); Temporal Lobe Epilepsy (TLE); refractory Temporal Lobe Epilepsy (rTLE); Focal Cortical Dysplasia (FCD); Focal Cortical Dysplasia 2 (FCORD2); Smith-Kingsmore Syndrome (SKS); Spinal Muscular Atrophy 1 (SMA1; Werdnig-Hoffmann disease); Spinal Muscular Atrophy 2 (SMA2, Dubowitz disease); Spinal Muscular Atrophy 3 (SMA3, Kugelberg-Welander disease); Spinal Muscular Atrophy 4 (SMA4); Gaucher Disease (GD); Mucopolysaccharidosis 3A (MPS3A); Mucopolysaccharidosis 2 (MPS2); Mucopolysaccharidosis 1 (MPS1); Ceroid Lipofuscinosis, Neuronal, 1 (CLN1); Immunodeficiency 78 with autoimmunity and developmental delay (IMD78); Ceroid Lipofuscinosis, Neuronal, 3 (CLN3); Batten Disease; Ceroid Lipofuscinosis, Neuronal, 5 (CLN5); Ceroid Lipofuscinosis, Neuronal, 6 (CLN6); Ceroid Lipofuscinosis, Neuronal, 7 (CLN7); Ceroid Lipofuscinosis, Neuronal, 8 (CLN8); Canavan Disease (CAND); Ubiquitin-positive Frontotemporal Dementia (UP-FTD); Ceroid Lipofuscinosis, Neuronal, 11 (CLN11); Angelman Syndrome (AS); Intellectual Developmental Disorder, X-linked, Syndromic 13 (MRXS13); Rett Syndrome (RTT); Autism, X-linked 3 (AUTSX3); Encephalopathy, Neonatal Severe, due to MECP2 Mutations (ENS-MECP2); Intellectual Developmental Disorder, X-linked, Syndromic, Lubs type (MRXSL); GM1-Gangliosidosis 1 (GM1G1); GM1-Gangliosidosis 2 (GM1G2); GM1-Gangliosidosis 3 (GM1G3); Giant Axonal Neuropathy 1; Autosomal Recessive (GAN1); Citrullinemia (CTLN1); GM2-Gangliosidosis 1 (GM2G1); Tay-Sachs Disease; Fragile X Syndrome (FXS); Fragile X tremor/ataxia Syndrome (FXTAS); Cerebral Cavernous Malformations 4 (CCM4); Epilepsy, familial focal, with variable foci 1 (FFEVF1); Niemann-Pick Disease A (NPDA); Niemann-Pick Disease C (NPDC); Krabbe Disease (KRB); Mucopolysaccharidosis 1H (MPS1H); Epilepsy Metachromatic Leukodystrophy (MLD); Multiple Sulfatase Deficiency (MSD); Ceroid Lipofuscinosis, Neuronal, 2 (CLN2); Friedreich Ataxia (FRDA); Tuberous Sclerosis; Aromatic L-Amino Acid Decarboxylase Deficiency (AADCD); Pompe Disease; Mucopolysaccharidosis 3B (MPS3B); Alexander disease (ALXDRD); Dup15q syndrome ; Developmental and epileptic encephalopathy 79 (DEE79); Epilepsy, childhood absence 5 (ECA5); Developmental and epileptic encephalopathy (DEE43); Prader-Willi syndrome (PWS); Phelan-McDermid Syndrome (PHMDS; PMS), Dravet Syndrome (DRVT); Lafora Disease (LD); Epilepsy, progressive myoclonic 2 (EPM2); Familial Hemiplegic Migraine 3 (FHM3); Generalized epilepsy with febrile seizures plus 2 (GEFSP2); Intractable childhood epilepsy with generalized tonic-clonic seizures (ICEGTC); Febrile seizures, familial, 3A (FEB3A); Developmental and epileptic encephalopathy 6B (DEE6B); Familial Hemiplegic Migraine 1 (FHM1); Episodic ataxia 2 (EA2); Developmental and epileptic encephalopathy 42 (DEE42); Familial Hemiplegic Migraine 2 (FHM2); Alternating hemiplegia of childhood 1 (AHC1); Fetal akinesia, respiratory insufficiency, microcephaly, polymicrogyria and dysmorphic facies (FARIMPD); Developmental and epileptic encephalopathy 98 (DEE98), preferably Parkinson's Disease (PD); Parkinson disease 1, autosomal resistant (PARK1); Parkinson disease 4, autosomal dominant (PARK4); Multiple System Atrophy (MSA); Lewy Body Dementia (LBD); Huntington's Disease (HD); Spinocerebellar Ataxia 1 (SCA1); Spinocerebellar Ataxia 2 (SCA2); Spinocerebellar Ataxia 3 (SCA3); Primary Tauopathies; Pick's Disease (PiD); Progressive Supranuclear

- Palsy (PSP); Corticobasal Degeneration (CBD); Argyrophilic Grain Disease (AGD); Secondary Tauopathies; Chronic Traumatic Encephalopathy (CTE); Alzheimer's Disease (AD); Frontotemporal Dementia (FTD); Amyotrophic Lateral Sclerosis (ALS); Temporal Lobe Epilepsy (TLE); refractory Temporal Lobe Epilepsy (rTLE); Focal Cortical Dysplasia (FCD); Spinal Muscular Atrophy 1 (SMA1; Werdnig-Hoffmann disease); Spinal Muscular Atrophy 2 (SMA2, Dubowitz disease); Spinal Muscular Atrophy 3 (SMA3, Kugelberg-Welander disease); Spinal Muscular Atrophy 4 (SMA4); Gaucher Disease (GD); Mucopolysaccharidosis 3A (MPS3A); Mucopolysaccharidosis 2 (MPS2); Mucopolysaccharidosis 1 (MPS1); Ceroid Lipofuscinosis, Neuronal, 1 (CLN1); Immunodeficiency 78 with autoimmunity and developmental delay (IMD78); Ceroid Lipofuscinosis, Neuronal, 3 (CLN3); Batten Disease; Ceroid Lipofuscinosis, Neuronal, 5 (CLN5); Ceroid Lipofuscinosis, Neuronal, 6 (CLN6); Ceroid Lipofuscinosis, Neuronal, 7 (CLN7); Ceroid Lipofuscinosis, Neuronal, 8 (CLN8); Canavan Disease (CAND); Ubiquitin-positive Frontotemporal Dementia (UP-FTD); Ceroid Lipofuscinosis, Neuronal, 11 (CLN11); Angelman Syndrome (AS); Rett Syndrome (RTT); GM1-Gangliosidosis 1 (GM1G1); GM1-Gangliosidosis 2 (GM1G2); GM1-Gangliosidosis 3 (GM1G3); Giant Axonal Neuropathy 1; Autosomal Recessive (GAN1); Citrullinemia (CTLN1); GM2-Gangliosidosis 1 (GM2G1); Tay-Sachs Disease; Fragile X Syndrome (FXS); Niemann-Pick Disease A (NPDA); Niemann-Pick Disease C (NPDC); Krabbe Disease (KRB); Mucopolysaccharidosis 1H (MPS1H); Epilepsy Metachromatic Leukodystrophy (MLD); Multiple Sulfatase Deficiency (MSD); Ceroid Lipofuscinosis, Neuronal, 2 (CLN2); Friedreich Ataxia (FRDA); Tuberous Sclerosis; Aromatic L-Amino Acid Decarboxylase Deficiency (AADCD); Pompe Disease; Mucopolysaccharidosis 3B (MPS3B); Alexander disease (ALXDRD); Dup15q syndrome; Developmental and epileptic encephalopathy 79 (DEE79); Epilepsy, childhood absence 5 (ECA5); Developmental and epileptic encephalopathy (DEE43); Prader-Willi syndrome (PWS); Phelan-McDermid Syndrome (PHMDS; PMS); Dravet Syndrome (DRV1); Lafora Disease (LD); Episodic Ataxia 2 (EA2); Alternating Hemiplegia of Childhood 1 (AHC1).
- 25 In one aspect the present invention provides the use of a recombinant adeno-associated (rAAV) serotype 5 particle in the manufacture of a medicament for the treatment of a CNS disease selected from Table 1, the rAAV5 particle comprising:
- 30 i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one AAV inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the Central Nervous System (CNS) disease.
- 35 In a further embodiment the present invention provides a method of treating a Central Nervous System (CNS) disease by administering a recombinant adeno-associated (rAAV) serotype 5 particle, the rAAV5 particle comprising:

- 5 i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one AAV inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease,
- wherein the CNS disease is selected from Table 1.

Table 1 Genes, proteins, and associated CNS diseases

Gene name (synonyms); HGNC reference	Protein name (alternative names); abbreviation(s); UniProtKB reference	Associated CNS disease
SNCA (NACP; PARK1); HGNC: 11138	a-Synuclein (Non-A beta component of AD amyloid; Non-A4 component of amyloid precursor (NACP)); a-Syn; UniProtKB: P37840	Parkinson's Disease (PD); Parkinson disease 1, autosomal resistant (PARK1); Parkinson disease 4, autosomal dominant (PARK4); Multiple System Atrophy (MSA); Lewy Body Dementia (LBD)
LRRK2; HGNC:18618	Leucine-rich repeat serine/threonine- protein kinase 2; LRRK2; UniProtKB: A0A2R8Y4F8	Parkinson's Disease (PD); Multiple System Atrophy (MSA); Lewy Body Dementia (LBD)
TPPP (TPPP1); HGNC: 24164	Tubulin polymerization-promoting protein (25 kDa brain-specific protein 1; TPPP/p25; p24; p25-alpha); TPPP; UniProtKB: O94811	Parkinson's Disease (PD); Multiple System Atrophy (MSA); Lewy Body Dementia (LBD)
HTT (HD; IT15); HGNC: 4851	Huntingtin (Huntington disease protein (HD protein)); HTT; UniProtKB: P42858	Huntington's Disease (HD)
MSH3 (DUC1; DUG); HGNC: 7326	MutS homolog 3 (Divergent upstream protein (DUP); mismatch repair protein 1 (MRP1); MSH3; UniProtKB: P20585	Huntington's Disease (HD); Spinocerebellar Ataxia's (SCA1; SCA2; SCA3)
MAPT (MAPTL; MTBT1; TAU); HGNC: 6893	Microtubule-associated protein tau (Neurofibrillary tangle protein; Paired helical filament-tau (PHF-tau)); MAPT; UniProtKB: P10636	Primary Tauopathies; Pick's Disease (PiD); Progressive Supranuclear Palsy (PSP); Corticobasal Degeneration (CBD); Argyrophilic Grain Disease (AGD); Secondary Tauopathies;

		Chronic Traumatic Encephalopathy (CTE)
APOE; HGNC: 613	Apolipoprotein E isoform 4; APOE4; UniProtKB: A0A0S2Z3B1	Alzheimer's Disease (AD)
ATXN1 (ATX1; SCA1); HGNC: 10548	Ataxin-1 (Spinocerebellar ataxia type 1 protein); ATXN1; UniProtKB: P54253	Spinocerebellar Ataxias (SCA1; SCA2; SCA3)
ATXN2 (ATX2; SCA2; TNRC13); HGNC: 10555	Ataxin-2 (Spinocerebellar ataxia type 2 protein; Trinucleotide repeat-containing gene 13 protein); ATXN2; UniProtKB: Q99700	Spinocerebellar Ataxias (SCA1; SCA2; SCA3); Frontotemporal Dementia
ATXN3 (ATX3; MJD; MJD1; SCA3); HGNC: 7106	Ataxin-3 (Machado-Joseph disease protein 1; Spinocerebellar ataxia type 3 protein); ATXN3; UniProtKB: P54252	Spinocerebellar Ataxias (SCA1; SCA2; SCA3)
TARDBP (TDP43); HGNC: 11571	TAR DNA-binding protein 43; TDP-43; UniProtKB: Q13148	Amyotrophic Lateral Sclerosis (ALS); Frontotemporal Dementia
UBQLN2 (N4BP4; PLIC2); HGNC: 12509	Ubiquilin-2 (Chap1; DSK2 homolog Protein linking IAP with cytoskeleton 2 (PLIC-2; hPLIC-2); Ubiquitin-like product; Chap1/Dsk2); UniProtKB: Q9UHD9	Amyotrophic Lateral Sclerosis (ALS); Frontotemporal Dementia (FTD)
C9orf72 (DENND9; DENNL72); HGNC: 28337	Guanine Nucleotide Exchange Factor C9orf72; UniProtKB: Q96LT7	Amyotrophic Lateral Sclerosis (ALS); Frontotemporal Dementia (FTD)
GRIK2 (GLUR6); HGNC: 4580	Glutamate receptor ionotropic, kainate 2 (Excitatory amino acid receptor 4 (EAA4); Glutamate receptor 6 (GluR-6; GluR6)); GluK2; UniProtKB: Q13002	Temporal Lobe Epilepsy (TLE), refractory Temporal Lobe Epilepsy (rTLE)
FUS (TLS); HGNC: 4010	RNA-binding protein FUS (75 kDa DNA-pairing protein; Oncogene FUS; Oncogene TLS; POMp75; Translocated in liposarcoma protein); UniProtKB: P35637	Amyotrophic Lateral Sclerosis; Frontotemporal Dementia (FTD)
SOD1 (ALS; ALS1); HGNC: 11179	Superoxide Dismutase; UniProtKB: X5D5A9	Amyotrophic Lateral Sclerosis (ALS)
SQSTM1 (ORCA; OSIL); HGNC: 11280	Sequestosome-1 (EBI3-associated protein of 60 kDa (EBIAP; p60); Phosphotyrosine-independent ligand for the Lck SH2	Amyotrophic Lateral Sclerosis (ALS); Frontotemporal Dementia (FTD)

	domain of 62 kDa; Ubiquitin-binding protein p62); UniProtKB: Q13501	
ANG (RNASE5); HGNC: 483	Angiogenin (ribonuclease 5 (RNase 5)); UniProtKB: P03950	Amyotrophic Lateral Sclerosis (ALS)
FAN1 (KIAA1018; MTMR15); HGNC: 29170	Fanconi-associated nuclease 1 (FANCD2/FANCI-associated nuclease 1; hFAN1; Myotubularin-related protein 15); UniProtKB: Q9Y2M0	Huntington's Disease (HD)
IL1B (IL1F2); HGNC: 5992	Interleukin-1 beta (Catabolin); IL-1 beta; UniProtKB: P01584	Inflammatory diseases; Inflammation
TNF (TNFA; TNFSF2); HGNC: 11892	Tumor Necrosis Factor (Tumor necrosis factor alpha; cachectin; Tumor necrosis factor ligand superfamily member 2); UniProtKB: P01375	Inflammatory diseases; Inflammation
LTA (TNFB; TNFSF1); HGNC: 6709	Lymphotoxin-alpha (TNF-beta; tumor necrosis factor ligand superfamily member 1); LT-alpha; UniProtKB: P01374	Inflammatory diseases; Inflammation
IL1a (IL1F1); HGNC: 5991	Interleukin-1 alpha (Hematopoietin-1); IL-1 alpha; UniProtKB: P01583	Inflammatory diseases; Inflammation
C1QA; HGNC: 1241 C1QB; HGNC: 1242 C1QC; HGNC: 1243	Complement Component C1q (subunits A, B and C) ; C1q; UniProtKB: P02745, P02746, P02747	Inflammatory diseases; Inflammation
IL1RN (IL1F3; IL1RA); HGNC: 6000	Interleukin-1 Receptor Antagonist Protein (ICIL-1RA; IL1 inhibitor); IL-1Ra; IRAP; IL-1RNs; UniProtKB: P18510	Inflammatory diseases; Inflammation
IL12A; HGNC: 5969 IL12B; HGNC: 5970	Interleukin-12; IL-12; UniProtKB: O60595 and A0A0C5PS98	Inflammatory diseases; Inflammation
IL23A (SGRF); HGNC: 15488 IL12B; HGNC: 5970	Interleukin-23; IL-23; UniProtKB: Q9NPF7 and A0A0C5PS98	Inflammatory diseases; Inflammation
IL17A (CTLA8; IL17); HGNC: 5981	Interleukin-17 (cytotoxic T-lymphocyte-associated antigen 8 (CTLA-8); IL-17; IL-17A; UniProtKB: Q16552	Inflammatory diseases; Inflammation

IL6 (IFNB2); HGNC: 6018	Interleukin-6 (B-cell stimulatory factor 2 (BSF-2); CTL differentiation factor (CDF); Hybridoma growth factor; Interferon beta-2 (IFN-beta-2)); IL-6; UniProtKB: P05231	Inflammatory diseases; Inflammation
GDNF; HGNC: 4232	Glial cell line-derived neurotrophic factor (astrocyte-derived trophic factor (ATF)); hGDNF; UniProtKB: P39905	Parkinson's Disease (PD)
SMN1 (SMN; SMNT); HGNC: 11117 SMN2 (SMNC); HGNC: 11118	Survival motor neuron protein (component of gems 1, gemin-1); UniProtKB: Q16637	Spinal Muscular Atrophy 1 (SMA1, Werdnig- Hoffmann disease), Spinal Muscular Atrophy 2 (SMA2, Dubowitz disease), Spinal Muscular Atrophy 3 (SMA3, Kugelberg- Welander disease), Spinal Muscular Atrophy 4 (SMA4).
GBA1 (GBA; GC; GLUC); HGNC: 4177	Lysosomal acid glucosylceramidase (acid beta-glucosidase; glucocerease; beta-glucocerebrosidase (Beta-GC); beta-glucosylceramidase 1; cholesterol glucosyltransferase (SGTase)); Lysosomal acid GCase; UniProtKB: P04062	Gaucher Disease (GD)
SGSH (HSS); HGNC: 10818	N-sulphoglucosamine sulphohydrolase (sulphoglucosamine sulfamidase; sulphamidase); UniProtKB: P51688	Mucopolysaccharidosis 3A (MPS3A)
IDS (IDS); HGNC: 5389	Iduronate 2-sulfatase (alpha-L-iduronate sulfate sulfatase (idursulfatase)); UniProtKB: 22304	Mucopolysaccharidosis 2 (MPS2)
IDUA; HGNC: 5391	Alpha-L-iduronidase; UniProtKB: P35475	Mucopolysaccharidosis 1 (MPS1)
PPT1 (CLN1; PPT); HGNC: 9325	Palmitoyl-protein thioesterase 1 (palmitoyl-protein hydrolase 1); PPT-1; UniProtKB: P50897	Ceroid Lipofuscinosis, Neuronal, 1 (CLN1)
TPP2; HGNC: 12016	Tripeptidyl-peptidase 2 (Tripeptidyl aminopeptidase; Tripeptidyl-peptidase II (TPP-II)); TPP-2; UniProtKB: P29144	Immunodeficiency 78 with autoimmunity and developmental delay (IMD78)

CLN3 (BTS); HGNC: 2074	Battenin (battenin disease protein; protein CLN3); UniProtKB: Q13286	Ceroid Lipofuscinosis, Neuronal, 3 (CLN3); Batten disease
CLN5; HGNC: 2076	Ceroid-lipofuscinosis neuronal protein 5 (Ceroid-lipofuscinosis neuronal protein 5, secreted protein); Protein CLN5; UniProtKB: O75503	Ceroid Lipofuscinosis, Neuronal, 5 (CLN5)
CLN6; HGNC: 2077	Ceroid-lipofuscinosis neuronal protein 6; Protein CLN6; UniProtKB: Q9NWW5	Ceroid Lipofuscinosis, Neuronal, 6 (CLN6)
MFSD8 (CLN7); HGNC: 28486	Major facilitator superfamily domain- containing protein 8 (Ceroid-lipofuscinosis neuronal protein 7); UniProtKB: Q8NHS3	Ceroid Lipofuscinosis, Neuronal, 7 (CLN7)
CLN8 (C8orf61); HGNC: 2079	Protein CLN8; UniProtKB: Q9UBY8	Ceroid Lipofuscinosis, Neuronal, 8 (CLN8)
ASPA (ACY2; ASP); HGNC: 756	Aspartoacylase (aminoacylase-2 ACY-2); UniProtKB: P45381	Canavan Disease (CAND)
GRN; HGNC: 4601	Progranulin (acroganin; epithelin precursor; glycoprotein of 88 kDa (GP88; Glycoprotein 88); granulin precursor; PC cell-derived growth factor (PCDGF); proepithelin (PEPI); PGRN; UniProtKB: P28799	Ubiquitin-positive Frontotemporal Dementia (UP-FTD); Ceroid Lipofuscinosis, Neuronal, 11 (CLN11)
MECP2; HGNC: 6990	Methyl-CpG-binding protein 2; MeCP-2 protein, MeCP2; UniProtKB: P51608	Intellectual Developmental Disorder; X-linked, Syndromic 13 (MRXS13); Rett Syndrome (RTT); Autism, X-linked 3 (AUTSX3); Encephalopathy, Neonatal Severe, due to MECP2 Mutations (ENS- MECP2); Intellectual Developmental Disorder, X-linked, Syndromic, Lubs type (MRXSL), Angelman syndrome (AS)
GLB1 (ELNR1); HGNC: 4298	Beta-galactosidase (acid beta-galactosidase (Lactase); elastin receptor 1); UniProtKB: P16278	GM1-Gangliosidosis 1 (GM1G1); GM1- Gangliosidosis 2 (GM1G2); GM1- Gangliosidosis 3 (GM1G3);

GAN (GAN1; KLHL16); HGNC: 4137	Gigaxonin (Kelch-like protein 16); UniProtKB: Q9H2C0	Giant Axonal Neuropathy 1, Autosomal Recessive (GAN1)
ASS1 (ASS); HGNC: 758	Argininosuccinate synthase; (Citrulline-aspartate ligase); UniProtKB: P00966	Citrullinemia (CTLN1)
HEXA; HGNC: 4878	Beta-hexosaminidase subunit alpha (Beta-N-acetylhexosaminidase subunit alpha (Hexosaminidase subunit A); N-acetyl-beta-glucosaminidase subunit alpha); UniProtKB: P06865	GM2-Gangliosidosis 1 (GM2G1), Tay-Sachs Disease
FMR1; HGNC: 3775	Fragile X messenger ribonucleoprotein 1 (Fragile X messenger ribonucleoprotein (FMRP); Protein FMR-1); UniProtKB: Q06787	Fragile X Syndrome (FXS); Fragile X tremor/ataxia Syndrome (FXTAS)
SMPD1 (ASM); HGNC: 11120	Sphingomyelin phosphodiesterase (acid sphingomyelinase (aSMase); UniProtKB: P17405	Niemann-Pick Disease A (NPDA)
NPC1; HGNC: 7897	NPC intracellular cholesterol transporter 1 (Niemann-Pick C1 protein); UniProtKB: O15118	Niemann-Pick Disease C (NPDC)
GALC; HGNC: 4115	Galactocerebrosidase (Galactocerebroside beta- galactosidase; Galactosylceramidase; Galactosylceramide beta- galactosidase); GALCerase; UniProtKB: P54803	Krabbe Disease (KRB)
IDUA; HGNC: 5391	Alpa-L-iduronidase; UniProtKB: P35475	Mucopolysaccharidosis 1H (MPS1H)
NPY; HGNC: 7955	Pro-neuropeptide Y; UniProtKB: P01303	Epilepsy
NPY2R; HGNC: 7957	Neuropeptide Y receptor type 2 (NOPY-Y2 receptor (Y2 receptor); NPY2-R; UniProtKB: P49146	Epilepsy
ARSA; HGNC: 713	Arylsulfatase A (Cerebroside-sulfatase); ASA; UniProtKB: 15289	Metachromatic Leukodystrophy (MLD); Multiple Sulfatase Deficiency (MSD)
TPP1 (CLN2); HGNC: 2073	Tripeptidyl-peptidase 1 (Cell growth-inhibiting gene 1 protein; Lysosomal pepstatin-insensitive protease (LPIC); Tripeptidyl aminopeptidase; Tripeptidyl-peptidase I (TPP-I)); UniProtKB: O14773	Ceroid Lipofuscinosis, Neuronal, 2 (CLN2)

FXN (FRDA; X25); HGNC: 3951	Frataxin (Friedreich ataxia protein (Fxn)); UniProtKB: Q16595	Friedreich Ataxia (FRDA)
TSC1 (KIAA0243; TSC); HGNC: 12362	Hamartin (tuberous sclerosis 1 protein); UniProtKB: Q92574	Tuberous sclerosis
TSC2 (TSC4); HGNC: 12363	Tuberin (Tuberous sclerosis 2 protein); UniProtKB: P49815	Tuberous sclerosis
DCC (AADC); HGNC: 2719	Aromatic-L-amino-acid decarboxylase (DOPA) decarboxylase (DCC)); AADC; UniProtKB: P20711	Aromatic L-Amino Acid Decarboxylase Deficiency (AADCD)
GAA; HGNC: 4065	Lysosomal alpha-glucosidase (acid maltase; aglucosidase alfa); UniProtKB: P10253	Pompe Disease
NAGLU (UFHSD1); HGNC: 7632	Alpha-N-acetylglucosaminidase (N-acetyl-alpha-glucosaminidase (NAG)); UniProtKB: P54802	Mucopolysaccharidosis 3B (MPS3B)
GFAP HGNC: 4235	Glial fibrillary acidic protein; GFAP; UniProtKB: P14136	Alexander disease (ALXDRD)
UBE3A (E6AP; EPVE6AP; HPVE6A); HGNC: 12496	Ubiquitin-protein ligase E3A (E6AP ubiquitin-protein ligase 1; HECT-type ubiquitin transferase E3A; Human papillomavirus E6-associated protein; Oncogenic protein-associated protein E6-AP; Renal carcinoma antigen NY-REN-54); UniProtKB: Q05086	Angelman Syndrome (AS); Dup15q syndrome
GABRA5; HGNC: 4079	Gamma-aminobutyric acid receptor subunit alpha-5; (GABA(A) receptor subunit alpha-5); UniProtKB: P31644	Dup15q syndrome; Developmental and epileptic encephalopathy 79 (DEE79)
GABRB3; HGNC: 4083	Gamma-aminobutyric acid receptor subunit beta-3; (GABA(A) receptor subunit beta-3); UniProtKB: P28472	Dup15q syndrome, Epilepsy, childhood absence 5 (ECA5); Developmental and epileptic encephalopathy (DEE43)
GABRG3; HGNC: 4088	Gamma-aminobutyric acid receptor subunit gamma-3; (GABA(A) receptor subunit gamma- 3); UniProtKB: Q99928	Dup15q syndrome
SNRPN (HCERB3, SMN); HGNC: 11164	Small nuclear ribonucleoprotein- associated protein N; (Sm protein D (Sm-D); Sm protein N (Sm-N; SmN); Tissue-specific-splicing protein) snRNP-N; UniProtKB: P63162	Prader-Willi syndrome (PWS)
NDN HGNC: 7675	Necdin; UniProtKB: Q99608	Prader-Willi syndrome (PWS)

BDNF; HGNC: 1033	Brain-derived neurotropic factor; (Abrineurin); BDNF; UniProtKB: P23560	Prader-Willi syndrome (PWS)
SHANK3 (KIAA1650; PROSAP2; PSAP2); HGNC: 14294	SH3 and multiple ankyrin repeat domains protein 3 (proline-rich synapse-associated protein 2 (ProSAP2); Shank3; UniProtKB: QBYB0	Phelan-McDermid Syndrome (PHMDS; PMS)
MTOR (FRAP, FRSP1, FRAP2, RAFT1, RAFT2); HGNC: 3942	Serine/threonine-protein kinase mTOR (FK506-binding protein 12-rapamycin complex-associated protein 1; FKBP12-rapamycin complex- associated protein; Mammalian target of rapamycin (mTOR); Mechanistic target of rapamycin; Rapamycin and FKBP12 target 1; Rapamycin target protein 1); UniProtKB: P42345	Focal Cortical Dysplasia (FCD); Focal Cortical Dysplasia 2 (FCORD2); Smith-Kingsmore Syndrome (SKS)
PIK3CA; HGNC: 8975	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform (Phosphatidylinositol 4,5- bisphosphate 3-kinase 110 kDa catalytic subunit alpha (PtdIns-3- kinase subunit p110-alpha; p110alpha); Phosphoinositide 3- kinase alpha; Phosphoinositide-3- kinase catalytic alpha polypeptide; Serine/threonine protein kinase PIK3CA); PI3-kinase subunit alpha; PI3K-alpha; PI3Kalpha; PtdIns-3-kinase subunit alpha; UniProtKB: P42336	Focal Cortical Dysplasia (FCD); Cerebral Cavernous Malformations 4 (CCM4)
PIK3R2 HGNC: 8980	Phosphatidylinositol 3-kinase regulatory subunit beta (Phosphatidylinositol 3-kinase 85 kDa regulatory subunit beta (PI3-kinase subunit p85-beta; PtdIns-3-kinase regulatory subunit p85-beta)); PI3-kinase regulatory subunit beta; PI3K regulatory subunit beta; PtdIns- 3-kinase regulatory subunit beta; UniProtKB: O00459	Focal Cortical Dysplasia (FCD)
AKT3 (PKBG); HGNC: 393	RAC-gamma serine/threonine-protein kinase (Protein kinase Akt-3; Protein kinase B gamma (PKB gamma); RAC-PK- gamma; STK-2); UniProtKB: Q9Y243	Focal Cortical Dysplasia (FCD)

DEPDC5 (KIAA0645); HGNC: 18423	GATOR complex protein DEPDC5 (DEP domain-containing protein 5); UniProtKB: O75140	Focal Cortical Dysplasia (FCD); Epilepsy, familial focal, with variable foci 1 (FFEVF1)
EPM2A; HGNC: 3414	Laforin (Glucan phosphatase; Glycogen phosphatase; Lafora PTPase (LAFPTPase); UniProtKB: O95278	Epilepsy, progressive myoclonic 2 (EPM2); Lafora Disease (LD)
NHLRC1 (EPM2B); HGNC: 21576	E3 ubiquitin-protein ligase NHLRC1 (Malin; NHL repeat-containing protein 1; RING-type E3ubiquitin transferase NHLRC1); UniProtKB: Q6VVB1	Epilepsy, progressive myoclonic 2 (EPM2); Lafora Disease (LD)
SCN1A (NAC1; SCN1); HGNC: 10585	Sodium channel protein type 1 subunit alpha (Sodium channel protein brain I subunit alpha; Sodium channel protein type I subunit alpha; Voltage- gated sodium channel subunit alpha Nav1.1); UniProtKB: P35498	Dravet Syndrome (DRV1); Familial Hemiplegic Migraine 3 (FHM3); Generalized epilepsy with febrile seizures plus 2 (GEFSP2); Intractable childhood epilepsy with generalized tonic-clonic seizures (ICEGTC); Febrile seizures, familial, 3A (FEB3A); Developmental and epileptic encephalopathy 6B (DEE6B)
CACNA1A (CACH4; CACN3; CACNL1A4); HGNC: 1388	Voltage-dependent P/Q-type calcium channel subunit alpha-1A (Brain calcium channel I; Calcium channel, L type, alpha-1 polypeptide isoform 4; Voltage-gated calcium channel subunit alpha Cav2.1); UniProtKB: O00555	Spinocerebellar ataxia 6 (SCA6); Familial Hemiplegic Migraine 1 (FHM1); Episodic ataxia 2 (EA2); Developmental and epileptic encephalopathy 42 (DEE42)
ATP1A2 (KIAA0778); HGNC: 800	Sodium/potassium-transporting ATPase subunit alpha 2 (Sodium pump subunit alpha-2); Na(+)/K(+) ATPase alpha-2 subunit; UniProtKB: P50993	Familial Hemiplegic Migraine 2 (FHM2); Alternating hemiplegia of childhood 1 (AHC1); Fetal akinesia, respiratory insufficiency, microcephaly, polymicrogyria and dysmorphic facies (FARIMPD); Developmental and epileptic encephalopathy 98 (DEE98)

Table 1: The left column provides gene names and HGNC denominations. In the middle column proteins that are expressed from the gene in the left column are further specified. The right column indicates CNS diseases which are associated with variations and/or deletions in the genes and associated proteins of the left and middle column.

The term "gene" as used herein refers to a DNA fragment comprising a region (transcribed region), which is transcribed into an RNA molecule (e.g. an mRNA) in a cell, wherein the DNA fragment is operably linked to suitable regulatory regions (e.g. a promoter). A gene will usually comprise several operably linked fragments, such as a promoter, a 5' leader sequence, a coding region and a 3'-nontranslated sequence (3'-end) comprising a polyadenylation site. "Expression of a gene" refers to the process wherein a DNA region which is operably linked to appropriate regulatory regions, particularly a promoter, is transcribed into an RNA, which is biologically active, i.e. which is capable of being translated into a biologically active protein, polypeptide or peptide. Variations in the nucleic acid sequence of a gene may form the cause of a CNS disease. Such variations include deletions of part of the DNA sequence that constitutes the gene, or insertions, inversions, substitutions as well as mutations of single nucleotides. The HGNC denominations provided for the genes listed in Table 1 reflect the nucleotide sequences of genes that do not have deletions, insertions, inversions, substitutions and/or mutations. Upon introduction of variations in these genes, the associated diseases may occur. These variations may or may not be specific, i.e. may be a specific mutation causing the gene to encode a highly pathogenic protein, or any mutation leading to a loss of function and/or expression of the protein encoded by the gene.

Variations in a gene may result in loss (partly or completely) of expression of the protein that it encodes or may render the protein that it encodes functional, non-functional, dysfunctional or hyper-functional. In case a protein remains functional, the protein is able to fulfill its function at the same rate as a protein expressed from a gene without variations in the nucleotide sequence. A functional protein as defined herein does not have any pathogenic effects. In case variation in a gene renders a protein non-functional, the protein does not display any functionality at all. In case a variation in a gene renders a protein dysfunctional, the protein may display decreased functionality compared to a protein expressed from a gene without variations. In case a variation in a gene renders a protein hyper-functional, the protein may display increased functionality compared to a protein expressed from a gene without variations. Additionally a variation in a gene may cause the protein to have a pathogenic effect. Any change in expression or functionality, abrogation of functionality or pathogenic effect may contribute to the symptoms of the CNS disease.

Expression cassette

Wild type AAV particles carry a single stranded DNA genome, which consists of two open reading frames carrying the replicase genes and the capsid genes, Rep and Cap, and is flanked by two inverted terminal repeats (ITRs). In recombinant AAV particles, the single stranded DNA genome is replaced with an expression cassette.

The term "expression cassette" as used herein describes the functional unit capable of affecting expression of a transgene, or sequence encoding a product to be expressed. The coding sequence is operably linked to the appropriate expression control sequences, which at least comprises a suitable transcription regulatory sequence and optionally, 3' transcription termination signals. In addition, the term "expression

cassette" as used herein described the functional unit capable of affecting expression of a transgene, or sequence encoding one or more products to be expressed. The sequence encoding one or more products to be expressed may be operably linked to the appropriate expression control sequences, which may comprise a suitable transcription regulatory sequence and optionally, 3' transcription termination signals.

5 Additional factors necessary or helpful in affecting expression may also be present, such as expression enhancer elements, and would be known to the skilled person.

An expression cassette as used herein may be flanked by at least one ITR. In one embodiment, the expression cassette is flanked by two AAV ITR nucleotide sequences, preferably whereby the expression

10 cassette is located in between the two AAV ITR nucleotide sequences. In another embodiment, the expression cassette is flanked by one ITR engineered with two D regions, wherein preferably the expression cassette is located on either side of the engineered ITR.

Typically, the nucleic acid molecule comprising an expression cassette and at least one ITR as used herein

15 is 5,000 nucleotides (nt) or less in length. The skilled person is aware that the maximum AAV packaging limit is understood to be 5.5 kilobasepairs (kbp). However, in other embodiments, wherein oversized nucleic acid constructs are used, e.g. more than 5,000 nt in length, or even more than the maximum AAV packaging limit of 5.5 kbp may also still allow the generation of rAAV particles and are therefore not excluded.

20

Promoter

In one embodiment, the expression cassette as described herein comprises a transcription regulatory sequence and/or promoter. In a further embodiment, the transcription regulatory sequence and/or promoter is mammalian cell compatible, preferably the transcription regulatory sequence and/or promoter is operably

25 linked to the nucleotide sequence encoding for at least one product.

As used herein, the term "transcription regulatory sequence" refers to a nucleic acid fragment that functions to control the transcription of one or more coding nucleotide sequences. For example, a "promoter" and is a transcription regulatory sequence that is located upstream with respect to the direction of transcription of the transcription initiation site of the coding nucleotide sequence and is structurally identified by the

30 presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but not limited to transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one of skill in the art to act directly or indirectly to regulate the amount of transcription from the promoter. A "constitutive" promoter is a promoter that is active under most physiological and developmental conditions. An "inducible" promoter is

35 a promoter that is physiologically or developmentally regulated, e.g. by the application of a chemical inducer or biological entity. Many mammalian cell compatible promoters are known in the art (Sambrook and

Russell, Molecular Cloning: A laboratory Manual. 3rd Edition, Cold Spring Harbor Laboratory Press, New York, (2001) 1.31-31.42 and 31.59-31.61.).

5 In one embodiment, the promoter is a constitutive or ubiquitous promoter that is broadly expressed in many cell-types. Exemplary constitutive or ubiquitous promoters include, but are not limited to the Cytomegalovirus (CMV) promoter, the CMV early enhancer/chicken β -actin (CAG) promoter, the chicken β -actin (CBA) promoter and the phosphoglycerate kinase-1 (PGK) promoter..

10 In a further embodiment, the promoter is an inducible, tissue-specific, cell-type-specific, or cell cycle-specific promoter.

In a preferred embodiment, the promoter is a neuron-specific promoter. Exemplary neuron-specific promoters include, but are not limited to: a neuron-specific enolase (NSE) promoter, platelet-derived growth factor (PDGF) promoter, platelet-derived growth factor B-chain (PDGF-(3) promoter, synapsin or synapsin-1 (Syn or Syn-1) promoter, methyl-CpG binding protein 2 (MeCP2) promoter, Ca⁺/calmodulin-dependent protein kinase II (CaMKII) promoter, metabotropic glutamate receptor 2 (mGluR2) promoter, neurofilament light (NFL) or heavy (NFH) promoter, β -globin minigene n β 2 promoter, preproenkephalin (PPE) promoter, enkephalin (Enk) promoter and excitatory amino acid transporter 2 (EAAT2) promoter.

20 In a further embodiment, the promoter is an astrocyte-specific promoter. Exemplary astrocyte-specific promoters include, but are not limited to: glial fibrillary acidic protein (GFAP) and EAAT2 promoters.

In a further embodiment, the promoter is an oligodendrocyte-specific promoter. An exemplary oligodendrocyte-specific promoter includes, but is not limited to the myelin basic protein (MBP) promoter.

25 In a particularly preferred embodiment, the promoter for expression of transgene in the peripheral and/or central nervous system is the CBh promoter (Gray et al., Hum. Gene Ther. 2011, 22:1143–1153).

Transgene

30 In one embodiment, the expression cassette as described herein, comprises a transgene. A “transgene” or “sequence encoding a product to be expressed” as referred to herein can be defined as a nucleotide sequence which comprises a coding nucleotide sequence coding for at least one product. As also mentioned above, in one embodiment, the transgene is operably linked to appropriate expression control sequences. Such expression control sequences may comprise a suitable transcription regulatory sequence or promoter and optionally, 3' transcription termination signals. Therefore, in one embodiment, the expression control sequence as defined herein comprises a suitable transcription regulatory sequence or promoter and optionally, 3' transcription termination signals.

The rAAV5 particles for use according to the invention may comprise an expression cassette as defined above, which in turn may comprise a transgene, wherein the transgene may comprise a nucleic acid molecule encoding at least one product having an effect in an CNS disease.

Therefore, in one embodiment, there is provided an rAAV5 particle as described herein, comprising an expression cassette.

In a further embodiment, there is provided an rAAV5 particle as described herein, comprising an expression cassette comprising a transgene.

In a further embodiment, there is provided an rAAV5 particle as described herein, comprising an expression cassette comprising a transgene, wherein the transgene comprises a nucleic acid molecule encoding at least one product having an effect on an CNS disease.

Product

As introduced above, a transgene incorporated in an rAAV5 particle for use according to the invention may comprise a coding nucleotide sequence coding for at least one product. The term "product" in relation to a transgene as used herein refers to a nucleic acid molecule encoding at least one RNA molecule and/or encoding at least one polypeptide, or a combination thereof. A transgene according to the invention may comprise a range of different products, such as a plurality of the same product, but also a combination of different products. Such products include, but are not limited to, RNA molecules and polypeptides, as well as combinations thereof. As is also described above, rAAV particles, and more specifically also rAAV5 particles are often employed to deliver, for example, nucleic acid sequences encoding therapeutic molecules, including RNA molecules and polypeptides. Although differences in capsids do influence the tropism of the various rAAV serotypes, these capsid differences do not greatly affect the capacity of the various rAAV serotypes. Hence, the rAAV5 particles for use according to the invention will be able to transfer rAAV particles of different serotypes and having different capsid structures. The products that can be encoded by the transgene incorporated in the rAAV5 particles for use include, but are not limited to the RNA molecules and polypeptides that are described in further detail below.

RNA molecules

The term "RNA molecule" as used herein takes its regular meaning in the art. In one embodiment, the RNA molecule as disclosed herein has an effect in a CNS disease, more preferably it has a therapeutic effect. In a preferred embodiment, an RNA molecule described herein slows down the progress of a CNS disease. In a further embodiment, an RNA molecule as described herein, slows down the deterioration of the condition of a subject suffering from a CNS disease.

In one embodiment, an RNA molecule as described herein eliminates one or more symptoms of a CNS disease.

5 In a preferred embodiment, an RNA molecule described herein halts the CNS disease and prevents the CNS disease from progressing. In such an embodiment, an RNA molecule as described herein stops and prevents the further deterioration of the condition of a subject suffering from a CNS disease.

10 In a further embodiment, an RNA molecule as described herein improves the condition of a subject suffering from a CNS disease.

In one embodiment, an RNA molecule described herein includes, but is not limited to: RNA interference (RNAi) molecules and antisense oligonucleotides (ASOs).

15 RNAi molecules are RNA molecules that are capable of RNA interference such as, e.g. an miRNA (microRNA) or an siRNA (short interfering RNA). In general RNA molecules as used herein serve to silence, or negatively influence, or inhibit gene expression.

20 RNA molecules are particularly useful products to be included in an rAAV5 particle for use according to the invention in case the CNS disease involves the (over)expression of a protein resulting in disease symptoms and/or the expression of a pathogenic or dysfunctional protein. In such cases, RNA molecules, such as RNAi molecules, can be applied to suppress the process of gene expression leading towards the production of the pathogenic or dysfunctional protein.

RNA interference (RNAi)

25 RNA molecules involved in RNAi can sequence-specifically suppress gene expression, e.g. by decreasing the levels of specific mRNA-molecules. In the process of RNAi, the RNA molecule encoded by the nucleic molecule is cleaved by Drosha (i.e. a class 2 ribonuclease III enzyme) into a short hairpin RNA (shRNA) or a long hairpin RNA (lhRNA) in the nucleus of the target cell. At the end of this step, the shRNA and lhRNA has lost the flanking regions at the 5' and 3' ends of the RNA. Subsequently, the shRNA and lhRNA is
30 exported to the cytoplasm of the cell. Here, the cleaved RNA is further cleaved by Argonaute-2 (AGO-2) of the RNA-induced silencing complex (RISC), wherein the passenger RNA sequence of the cleaved RNA is trimmed off (i.e. cleaved) by poly(A)-specific ribonuclease (PARN). The other strand of the cleaved RNA is called a guide strand (i.e. a guide sequence). The guide strand comprising the sequence substantially complementary to the target RNA sequence, as described above, is not processed and/or cleaved by AGO-
35 2. The guide strand generated by the above-described process is then able to bind to a complementary mRNA sequence, ultimately leading towards cleavage of the complementary mRNA, thus interfering with gene expression, thereby reducing protein levels.

In one embodiment, the product comprised in an rAAV5 particle for use according to the invention is an RNA comprising two strands that are complementary to each other and of which one of the strands (passenger strand) is cleaved. A particularly preferred embodiment provides a miR-451 scaffold or a combination of a miR-144 and a miR-451 scaffold for expression of an RNAi substantially complementary to an RNA transcribed from a gene related to CNS disease as described above. Such miR-451 and miR-144 scaffolds are described in WO201133889, which is incorporated by reference herein. Further miR-451 and miR-144 scaffolds are described in WO2016102664, WO2020053258, WO2020104295 and WO2022268835, which are incorporated by reference herein. The miR-451 scaffold induces RNA interference (RNAi); particularly, the RNAi is induced by the guide strand of this scaffold. Processing of the pri-miRNA and pre-miRNA giving rise to miR-451 does not result in a passenger strand because the processing is different from the canonical miRNA processing pathway (Cheloufi et al., Nature 2010, 465(7298):584-9, and Yang et. al., Proc Natl Acad Sci USA. 2010, 107:15163-8). Thereby, the use of miR-451 can prevent or reduce the possibility of having unwanted potential off-targeting by passenger strands. The use of a miR-144 scaffold, upstream of the miR-451 scaffold improves the biogenesis of the miR-451 hairpin, thereby increasing the amount of the guide strand incorporated in the miR-451 scaffold.

Additionally, the miR-144 can have a mutation that further improves guide strand processing of the guide strand derived from the miR-415 scaffold. In one example, a scaffold comprising one guide RNA incorporated in miR-451 which may be preceded by a miR144 mutant on the 5' end, can decrease expression of the target mRNA as well as protein expression.

As described herein, scaffolds may comprise multiple different combinations of a miR-144 and miR-451 hairpin. The scaffolds may further comprise multiple guide sequences which can either be the same or different. In this way, multiple miRNAs expressing the same or different guide sequences can be expressed from a single nucleic acid molecule. The expression of multiple miRNAs from one nucleic acid molecule, wherein the miRNAs have either the same or a different sequence, allows for efficient inhibition of mRNA as well as protein levels

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising:

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease,

wherein the at least one product comprises at least one RNA molecule, preferably an RNA molecule that is substantially complementary to a part of a gene selected from Table 1, preferably fully complementary to a part of the coding sequence of a gene selected from Table 1.

5 It is understood that “substantially complementary” in this context means that it is not required to have all the nucleotides of the guide sequence and the target sequence to be base paired, i.e. to be fully complementary, or all the nucleotides of the guide sequence and the target sequence to be base paired. As long as the second RNA is capable of inducing RNA interference to thereby sequence-specifically target a sequence comprising the target RNA sequence, such substantial complementarity is contemplated in
10 accordance with the invention.

In one embodiment, the RNA molecule as described herein preferably is substantially complementary to a part of a gene selected from Table 1, preferably fully complementary to a part of the coding sequence of a gene selected from Table 1.

15

In some embodiments, the RNA molecule is substantially complementary to a part of a gene selected from Table 1, wherein the part of the gene selected from Table 1 has at least one deletion, insertion, inversion, substitution and/or mutation as compared to the HGNC denomination provided for the gene in Table 1.

In some embodiments the RNA molecule is substantially complementary to a part of the coding sequence
20 of a gene selected from Table 1, wherein the part of the coding sequence of the gene selected from Table 1 has at least one deletion, insertion, inversion, substitution and/or mutation as compared to the HGNC denomination provided for the gene in Table 1. In such an embodiment, the RNA molecule is not substantially complementary to a part of a gene selected from Table 1 having the sequence provided by HGNC denomination referenced in Table 1.

25

In a preferred embodiment, the at least one deletion, insertion, inversion, substitution and/or mutation as compared to the HGNC denomination causes a CNS disease. More preferably, the CNS disease is listed in Table 1.

30 Where herein reference is made to “coding sequence of a gene”, it is understood that reference is made to the sequence of a gene that codes for the spliced mRNA. This means that the coding sequence does not comprise intronic sequences that are removed during splicing of the mRNA, and furthermore does not comprise 3' and 5' UTRs.

35 In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising

i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and

5 ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease,

wherein the at least one product comprises a first RNA molecule and second RNA molecule, wherein:

- 10 a) the first RNA molecule comprises a first guide sequence substantially complementary to a first target sequence in a first transcript encoded by a first gene;
- b) the second RNA molecule comprises a second guide sequence substantially complementary to a second target sequence in a second transcript encoded by a second gene;
- c) the first RNA and the second RNA molecule each comprise a hairpin; and
- d) the first RNA molecule comprises SEQ ID NO. 2 and the second RNA molecule comprises SEQ ID NO. 3; and variants thereof.

15

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising

- 20 i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease,

wherein the at least one product comprises a first, a second, and a third RNA molecule, wherein:

- 25 a) the first RNA molecule comprises a first guide sequence substantially complementary to a first target sequence in a first transcript encoded by a first gene;
- b) the second RNA molecule comprises a second guide sequence substantially complementary to a second target sequence in a second transcript encoded by a second gene;
- c) the first RNA and the second RNA molecule each comprise a hairpin; and
- 30 d) the first RNA molecule comprises SEQ ID NO. 2 and the second and third RNA molecule comprise SEQ ID NO. 3; and variants thereof.

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising

- 35 i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and

ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease,

wherein the at least one product comprises a first RNA molecule and second RNA molecule, wherein:

- 5 a) the first RNA molecule comprises a first guide sequence substantially complementary to a first target sequence in a first transcript encoded by a first gene;
- b) the second RNA molecule comprises a second guide sequence substantially complementary to a second target sequence in a second transcript encoded by a second gene;
- c) the first RNA and the second RNA molecule each comprise a hairpin; and
- 10 d) the first RNA molecule comprises SEQ ID NO. 4 and the second RNA molecule comprises SEQ ID NO. 3; and variants thereof.

Polypeptides

The term "polypeptide" or "protein" as used herein takes its regular meaning in the art. In some embodiments, a polypeptide or protein as described herein has an effect in a CNS disease, more preferably a therapeutic effect.

In one embodiment, a polypeptide or protein as described herein slows down the progress of a CNS disease.

20 In a further embodiment, a polypeptide or protein as described herein slows down the deterioration of the condition of a subject suffering from a CNS disease.

In one embodiment, a polypeptide or protein as described herein eliminates one or more symptoms of a CNS disease.

25 In a preferred embodiment, a polypeptide or protein as described herein halts the CNS disease and prevents the CNS disease from progressing. In such an embodiment, a polypeptide or protein as described herein stops and prevents the further deterioration of the condition of a subject suffering from a CNS disease.

30 In a further embodiment, a polypeptide or protein as described herein improves the condition of a subject suffering from a CNS disease.

35 In one embodiment, a polypeptide or protein as described herein is encoded by a nucleic acid molecule comprised in the transgene.

In a further embodiment, a polypeptide and/or protein as described herein is selected from the group including, but not limited to: wild type proteins, binding moieties and DNA- and/or RNA-editing enzymes.

5 Polypeptides and proteins are useful products to be included in an rAAV5 particle for use according to the invention in case the CNS disease involves reduced expression, or absence, of the wild type protein or the expression of a dysfunctional protein. In such cases, polypeptides and proteins, such as DNA- and/or RNA-editing enzymes, may be applied to suppress the process of gene expression leading towards the production of a dysfunctional protein. In addition, binding moieties can be applied to specifically bind to a dysfunctional protein, leading towards the clearance of the dysfunctional protein. Furthermore, a
10 polypeptide or protein, such as a wild type protein, can substitute the function of the absent or dysfunctional protein.

In one embodiment, polypeptides and proteins as described herein are encoded by a transgene included in an rAAV5 particle for use according to the invention. Such an embodiment is particularly useful in case
15 the CNS disease involves the expression of a non-functional protein.

In a further embodiment, a polypeptide or protein as described herein, including, but not limited to a wild type protein, substitutes the function of a non-functional protein.

Wild type proteins

20 In some embodiments, the product is a wild type protein. In a further embodiment, the product is a wild type protein comprising an at least partially a codon optimized nucleotide sequence.

In a further embodiment the wild type protein is completely codon optimized.

In one embodiment, the transgene comprises a nucleic acid molecule comprising the full length wild type coding sequence of a wild type protein, or the transgene comprises a nucleic acid molecule comprising the
25 full length wild type nucleotide sequence of a gene encoding a wild type protein.

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising

30 i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1);
and

ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding an at least partial wild type protein, preferably a full length wild type protein having an effect in the CNS
35 disease.

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising

- 5 i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding a protein selected from Table 1.

10 In one embodiment, the wild type protein as described herein is encoded by a nucleic acid molecule substantially complementary to at least part of a gene selected from Table 1. In a further embodiment, the wild type protein is encoded by a nucleic acid molecule substantially complementary to least part of a gene selected from Table 1, wherein preferably, the gene selected from Table 1 has the sequence provided by HGNC denomination referenced in Table 1.

15

Binding moieties

In some embodiments, the transgene included in an rAAV5 capsid for use according to the invention comprises a nucleic acid molecule coding for a binding moiety. The term "binding moiety" as used herein refers to a polypeptide having the capacity to specifically bind to a certain tertiary protein structure, or

20 epitope. This tertiary protein structure or epitope may be part of a wild type protein, but it may also be part of a protein that is found to be mutated and/or has a different conformation in a CNS disease. Binding moieties include but are not limited to, monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies formed from at least two intact antibodies), and antibody fragments (e.g., diabodies) so long as they exhibit a desired biological activity (e.g., " functional"). Examples of

25 antibody fragments may include, but are not limited to Fab, Fab', F (ab')₂, bispecific F(ab')₂, trispecific F(ab)₃, Fv fragments, single chain variable fragments (scFv), scFv-Fc, VHH or single domain antibody (sdAb); minibodies, diabodies, bispecific diabodies, trispecific triabodies, linear antibodies, single-chain antibody molecules, hclgG, IgNAR, V-NAR, DARPin, affibody, affilin, adnectin or monobody, affitin, repebody, fynomer, alphabody, avimer, atrimer, centyrin, pronectin or anticalin; and multispecific antibodies

30 formed from antibody fragments. Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen binding site. In a preferred embodiment, the binding moiety is a full length antibody, Fab, F(ab')₂ or scFv. The term " full length antibody" as used herein refers to an antibody containing two heavy and two light chains. Each of the chains contains constant (C) and variable (V) regions, which can be broken down into domains designated CH1, CH2, CH3, VH, and

35 CL, VL.

In a preferred embodiment, a binding moiety as used herein is comprised of at least a heavy chain and/or a light chain of an antibody. In a further embodiment, the transgene comprises a nucleic acid sequence encoding a heavy chain and/or a light chain of an antibody. The heavy chain and/or light chain are expressed and assembled to form a binding moiety in cells transduced by the rAAV5 capsids of the invention. In one embodiment, the binding moiety is specific for a tertiary protein structure or epitope that is associated with a CNS disease, or specific for a CNS disease. Exemplary protein structures include, but are not limited to, neurofibrillary tangles (NFTs), formed by hyperphosphorylated tau protein, and alpha-synuclein cross-sheets.

The present inventors have shown that rAAV5 particles may be used to efficiently express binding moieties *in vivo*. Furthermore, binding moieties that were expressed using rAAV5 particles are shown to be both secreted and functional.

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding a binding moiety having an effect in the CNS disease, preferably a full length antibody, Fab, F(ab')₂ or scFv.

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding a binding moiety that specifically binds to a protein that is encoded by a gene selected from Table 1, preferably a full length antibody, Fab, F(ab')₂ or scFv.

In one embodiment, a binding moiety as described herein specifically binds to a protein that is encoded by a gene selected from Table 1. Preferably the binding moiety specifically binds to a protein that is encoded by a gene selected from Table 1, wherein the gene selected from Table 1 has at least one deletion, insertion, inversion, substitution and/or mutation as compared to the HGNC denomination provided for the

gene in Table 1. In a preferred embodiment, the at least one deletion, insertion, inversion, substitution and/or mutation as compared to the HGNC denomination causes a CNS disease. In a more preferred embodiment, the CNS disease is listed in Table 1. In a still more preferred embodiment, the binding moiety specifically binds to a protein encoded by a gene selected from Table 1, wherein the gene has at least one deletion, insertion, inversion, substitution and/or mutation as compared to the HGNC denomination provided for the gene in Table 1, but does not bind to a protein encoded by a gene selected from Table 1 having the sequence provided by HGNC denomination referenced in Table 1.

DNA- and/or RNA-editing enzymes

In some embodiments, a transgene incorporated in an rAAV5 particle for use according to the invention comprises a nucleic acid molecule coding for a DNA- and/or RNA-editing enzyme. The term "DNA- and/or RNA-editing enzyme" as used herein refers to a polypeptide having the capacity to specifically induce targeted DNA double-stranded breaks and/or RNA double and single stranded breaks. Such double stranded breaks allow for site-specific genomic modifications. DNA- and/or RNA-editing enzymes include clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9), transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs) (Gaj et al. Cold Spring Harb Perspect Biol. 2016, 8(12):a023754). ZFNs comprise a large family of nucleic acid binding proteins. Most ZFNs that have been described and developed up to date specifically bind and induce strand breaks in double stranded DNA. Some ZFN enzymes can specifically bind single stranded RNA molecules, such as pre-mRNA and mRNA molecules, eventually leading towards the destabilization and degradation of such RNA molecules (Brown, Current Opinion in Structural Biology, 2005, 15-94-8).

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding a DNA- and/or RNA-editing enzyme, preferably a Zinc Finger Nuclease (ZFN).

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and

ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding a Zinc Finger Nuclease (ZFN).

- 5 In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising
- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- 10 ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding a Zinc Finger Nuclease (ZFN) that specifically binds to a nucleotide sequence that is substantially complementary to at least part of a gene selected from Table 1.

In a preferred embodiment, the Zinc Finger Nuclease (ZFN) as described herein specifically binds to a nucleotide sequence that is substantially complementary to a part of a gene selected from Table 1, wherein the part of the gene selected from Table 1 has at least one deletion, insertion, inversion, substitution and/or mutation as compared to the HGNC denomination provided for the gene in Table 1. In one embodiment, the ZFN does not bind to a nucleotide sequence that is substantially complementary to a part of a gene selected from Table 1 having the sequence provided by HGNC denomination referenced in Table 1. In a preferred embodiment, the at least one deletion, insertion, inversion, substitution and/or mutation as compared to the HGNC denomination causes a CNS disease, preferably, the CNS disease is listed in Table 1.

Combinations

In one embodiment, a transgene incorporated in an rAAV5 particle for use according to the invention encodes more than one product, preferably up to 2, 3, 4, 5, 6, 7, 8, 9, or 10 products. In one embodiment, the 2, 3, 4, 5, 6, 7, 8, 9, or 10 products may be the same, such as 2, 3, 4, 5, 6, 7, 8, 9 or 10 RNA molecules or 2, 3, 4, 5, 6, 7, 8, 9 or 10 polypeptides. In one embodiment, the 2, 3, 4, 5, 6, 7, 8, 9, or 10 RNA molecules may all comprise the same nucleic acid sequence. In an alternative embodiment, the 2, 3, 4, 5, 6, 7, 8, 9, or 10 RNA molecules have different nucleic acid sequences. All options in between may also be envisaged.

30 An exemplary transgene may comprise 5 RNA molecules, of which 2 RNA molecules have the same sequence and the three other RNA molecules all have different sequences. In one example, multiple RNA molecules which are encoded by one transgene can together efficiently and specifically decrease mRNA levels as well as protein levels. A further exemplary transgene may comprise 5 polypeptides, of which 2 polypeptides have the same sequence and the three other polypeptides all have different sequences.

Alternatively, in one embodiment, the 2, 3, 4, 5, 6, 7, 8, 9, or 10 products may comprise both at least one RNA molecule and at least one polypeptide. This is particularly useful in case the CNS disease is caused by a dysfunctional or non-functional protein. In one embodiment the transgene encodes at least one RNA molecule and a binding moiety. The at least one RNA molecule decreases gene expression causing lower
5 levels of the dysfunctional or non-functional protein to be produced, while the binding moiety binds to the dysfunctional or non-functional protein, leading to the degradation of the protein and possibly therewith any symptoms associated with the CNS disease. A further exemplary transgene encodes at least one RNA molecule and a wild type protein. This is particularly useful in case a dysfunctional protein is produced. The
10 at least one RNA molecules decreases expression of the dysfunctional associated with a CNS disease, thus decreasing the symptoms of the CNS disease. At the same time, the wild type protein restores the healthy function of the protein.

In one embodiment, the transgene comprises 1 product, wherein the product is an RNA molecule, or a polypeptide chosen from: a binding moiety, a wild type protein, or a Zinc Finger nuclease (ZFN). In another embodiment, the transgene comprises 2 products, wherein the 2 products are 2 RNA molecules having the
15 same sequence, 2 RNA molecules having different sequences, 1 RNA molecule and 1 binding moiety, 1 RNA molecule and 1 wild type protein, 1 RNA and 1 Zinc Finger nuclease (ZFN), 1 binding moiety and 1 wild type protein, or 1 ZFN and 1 wild type protein. The present invention may also allow for delivery of a transgene encoding a binding moiety and a protein the brain. Therefore, in one embodiment, a transgene encoding a binding moiety and a protein are expressed, preferably in the brain. In a further embodiment,
20 the transgene encoding a binding moiety is a vectorized antibody.

In another embodiment, the transgene comprises 3 products, wherein the 3 products are 3 RNA molecules having the same sequence, 3 RNA molecules having three different sequences, 3 RNA molecules wherein
25 2 RNA molecules have the same sequence and 1 RNA molecule has a different sequence, 2 RNA molecules having the same sequence and one binding moiety, 2 RNA molecules having the same sequence and one wild type protein, 2 RNA molecules having the same sequence and one Zinc Finger nuclease (ZFN), 2 RNA molecules having two different sequences and one binding moiety, 2 RNA molecules having two different sequences and one wild type protein, 2 RNA molecules having two different sequences and one ZFN, 1
30 RNA molecule and 2 binding moieties, 1 RNA molecule and 2 wild type proteins, 1 RNA molecule and 2 ZFNs, 1 RNA molecule and 1 binding moiety and 1 wild type protein, 1 RNA molecule and 1 binding moiety and 1 ZFN, or 1 RNA molecule and 1 wild type protein and 1 ZFN.

In another embodiment, the transgene comprises 4 products, wherein the 4 products are 4 RNA molecules having the same sequences, 3 RNA molecules having the same sequence and 1 RNA molecule having a
35 different sequence, 2 RNA molecules having the same sequence and 2 RNA molecules having a different sequence, 3 RNA molecules having the same sequence and 1 binding moiety, 3 RNA molecules having the same sequence and 1 wild type protein, 3 RNA molecules having the same sequence and 1 Zinc Finger

nuclease (ZFN), 2 RNA molecules having the same sequence and 1 RNA molecule having a different sequences and 1 binding moiety, 2 RNA molecules having the same sequence and 1 RNA molecule having a different sequences and 1 wild type protein, 2 RNA molecules having the same sequence and 1 RNA molecule having a different sequences and 1 ZFN, 3 RNA molecules having 3 different sequences and 1 binding moiety, 3 RNA molecules having 3 different sequences and 1 wild type protein, 3 RNA molecules having 3 different sequences and 1 ZFN, 2 RNA molecules having different sequences and 2 different binding moieties, 2 RNA molecules having different sequences and 2 different wild type proteins, 2 RNA molecules having different sequences and 2 different ZFNs, 2 RNA molecules having different sequences and 1 binding moiety and 1 wild type protein, 2 RNA molecules having different sequences and 1 binding moiety and 1 ZFN, 2 RNA molecules having different sequences and 1 wild type protein and 1 ZFN, 2 RNA molecules having the same sequence and 2 binding moieties, 2 RNA molecules having the same sequence and 2 wild type proteins, 2 RNA molecules having the same sequence and 2 ZFNs, 2 RNA molecules having the same sequence and 1 binding moiety and 1 wild type protein, 2 RNA molecules having the same sequence and 1 binding moiety and 1 ZFN or 2 RNA molecules having the same sequence and 1 wild type protein and 1 ZFN.

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one RNA molecule and at least one polypeptide having an effect in the CNS disease.

In a further embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least two RNA molecules and at least two polypeptides having an effect in the CNS disease.

In a preferred embodiment, a polypeptide having an effect in a CNS disease specifically binds to a protein that is expressed from a gene selected from Table 1. Similarly, an RNA molecule having an effect in a

CNS disease is substantially complementary to a part of a gene selected from Table 1, preferably the RNA molecule is fully complementary to at least a part of the coding sequence of a gene selected from Table 1.

5 *Method of administration*

An rAAV5 particle for use according to the invention can be administered to the CNS using different methods and administration sites. Exemplary administration methods include intrathecal (cisternal or lumbar), intracerebroventricular, intracerebral, intrastriatal, intraputamina, intra-CSF, and/or intraparenchymal administration, and/or intranasal instillation. The rAAV5 particles may be injected in one
10 all at the same time, or in more than one injection. Additional methods of administration include intranasal instillation (Gau et al, Gene Ther., 2011, 18(5):432-6). Once administered, the rAAV5 particles may be transported towards other sites in the brain, for instance via retrograde axonal transport (Surdyka and Figiel, BioTechnologia (Pozn). 2021; 102(4):473-8). Therefore, in one embodiment, the method of administration is intrathecal (cisternal or lumbar), intracerebroventricular, intracerebral, intra-
15 CSF, and/or intraparenchymal administration, and/or intranasal instillation. In a preferred embodiment, the administration is intraputamina.

Dosage

Different dosages can be envisaged for an rAAV5 particle for use according to the invention to constitute
20 an effective amount. The effective amount of active agent(s) used to practice the present invention for treatment varies depending upon the manner of administration, administration scheme, the age, body weight, and general health of the subject. Ultimately, the attending physician will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount, which may be determined for instance as genome copies per kilogram (GC/kg), as GC per dose or as GC per
25 subject/patient. In the present invention, the dose is expressed as GC per subject.

In connection with the administration of a drug which, in the context of the present invention, is "effective against" a disease or condition indicates that administration in a clinically appropriate manner results in a beneficial effect for at least a statistically significant fraction of patients, such as an improvement of
30 symptoms, a cure, a reduction in at least one disease sign or symptom, extension of life, improvement in quality of life, or other effect generally recognized as positive by medical doctors familiar with treating the particular type of disease or condition.

In one embodiment, an effective amount of rAAV5 particles according to the present invention comprises
35 $1 \times 10^{10} - 1 \times 10^{16}$, preferably $3 \times 10^{10} - 3 \times 10^{15}$, more preferably $1 \times 10^{11} - 1 \times 10^{15}$, most preferably $3 \times 10^{11} - 3 \times 10^{14}$ GC per subject. These dosage ranges are envisaged for administering to adults and adolescents (≥ 12 years of age). For subjects < 12 years of age the dose may be adjusted based on brain weight, which

is, e.g., for newborns about 20 – 25% in comparison to an adult brain. For a one year old, the brain weight is about 60 – 70% of adult brain weight. Brain weight reaches adult values (about 1.45 kg) between 10 and 12 years of age. Such an amount of GC may be administered all at once, but may also be divided over separate administrations. Separate administrations may be take place at same point in time, for instance
5 by administering the rAAV5 particles at different sites. Separate administrations may also take place at different time points, for example with a week, one month, or a year in between two or more separate administrations. In case the dose is divided over separate administrations, the total dose may remain within the range of 1×10^{10} – 1×10^{16} , preferably 3×10^{10} – 3×10^{15} , more preferably 1×10^{11} – 1×10^{15} , most preferably 3×10^{11} – 3×10^{14} GC per subject. As mentioned above, rAAV5 particles for use according to the invention
10 transduce cells in the CNS with high efficiency. Due to the high efficiency it is envisioned that comparatively low GC numbers per subject can be used to reach the same effect in a CNS disease. It is advantageous to have lower GC numbers per dose, as this will reduce the costs of goods per subject to be treated. In particular if dosed locally, e.g., by intraputamenal or intraparenchymal administration, dosages in the lower range are deemed sufficient, whereas when dosed more systemically (in the CNS), e.g., intra-CSF,
15 dosages in the upper range may be required.

Composition

In one embodiment, there is provided a composition comprising an rAAV5 particle as described herein and a suitable excipient, such as a buffer, stabilizer, antioxidant etc. In preferred embodiments the compositions
20 are used for treatment of (human) subjects. For that purpose there is provided, in one embodiment a pharmaceutical composition comprising an rAAV5 particle as described herein and at least one pharmaceutically acceptable carrier. In the case of rAAV-mediated gene delivery vehicles a pharmaceutical composition typically comprises physiological buffers for buffering the composition to a pH of about 7.3-7.5. Exemplary physiological buffers are: phosphate buffered saline (PBS), or
25 tris(hydroxymethyl)aminomethane (Tris). Furthermore, a pharmaceutical composition may comprise further stabilizing agents such as e.g. sucrose, mannitol, trehalose, cyclodextrin, sodium chloride, potassium chloride, hydroxypropyl-beta-cyclodextrin (HP-BCD), or polysorbate. In a preferred embodiment, the pharmaceutical composition is suitable for administration in the CNS. More preferably the pharmaceutical composition is suitable for administration in the CNS using intrathecal, lumbar, intracerebroventricular,
30 intracerebral, intraatrial, intraputamenal, intra-CSF and/or intraparenchymal administration, or intranasal instillation. Moreover, the pharmaceutical composition should be suitable to accommodate a dose of 1×10^{10} – 1×10^{16} , preferably 3×10^{10} – 3×10^{15} , more preferably 1×10^{11} – 1×10^{15} , most preferably 3×10^{11} – 3×10^{14} GC in a volume suitable for administration in the CNS. Such volumes range between 0.5 and 10 millilitres (mL), preferably between 1 and 7 mL, more preferably between 2 and 4 mL. The indicated volume is per subject
35 and may be divided over both hemispheres if applicable, i.e., 0.25 – 5 mL, preferably between 0.5 – 3.5 mL, more preferably between 1 – 2 mL per hemisphere. If dosed intra-CSF, higher volumes, such as volumes above 10 mL can be used when a similar amount of CSF is removed prior to administration, in

order to avoid pressure build-up in the CNS. Typically, if administered at a site where one cannot withdraw CSF, such as intraparenchymal, for instance, several injections of low volume (e.g., about 100 µl per injection) are administered at different sites in order to avoid pressure build-up of the tissue surrounding the administration site. Such administration can for instance be about 15 injections of about 100 µl per injection per hemisphere. It is within the skilled person's ability to select a suitable volume for a particular administration site.

In one embodiment, the present invention provides a pharmaceutical composition for use in the treatment of a Central Nervous System (CNS) disease comprising at least one pharmaceutically acceptable carrier and a recombinant adeno-associated virus (rAAV) serotype 5 particle, the rAAV5 particle comprising:

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease.

In one embodiment, the present invention provides a pharmaceutical composition comprising at least one pharmaceutically acceptable carrier and a recombinant adeno-associated virus (rAAV) serotype 5 particle, the rAAV5 particle comprising:

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease.

Further modifications to the capsid

A capsid protein variant according to the invention can be optimized for production in insect cells as described, for instance in WO2007/046703, WO2015/137802, and WO2019/016349, which are hereby incorporated by reference in their entirety. Hence, in addition to the mutation at position M569 of the VP1 capsid protein, the capsid of an rAAV5 particle for use according to the present invention may comprise at least one further mutations. Therefore, in one embodiment, in addition to the mutation at position M569 of the wild type AAV5 VP1 capsid protein, the capsid of a particle for use according to the present invention may comprise at least one further mutation.

In some embodiments wherein the rAAV5 particle is produced in insect cells, a suboptimal translation initiation codon is used, wherein the suboptimal translation initiation codon is selected from CTG, ACG,

TTG or GTG. In some embodiments, the suboptimal translation initiation codon is followed by an additional second codon encoding an amino acid selected from alanine, glycine, valine, aspartic acid and glutamic acid, preferably valine or alanine. These variations in nucleic acid sequence may benefit stoichiometry of the VP1, VP2 and VP3 capsid proteins when the rAAV5 particle of the invention is produced in insect cells, which may further increase infectivity of the rAAV5 particles.

In one embodiment, an rAAV5 particle for use according to the invention may comprise an additional amino acid, preferably an alanine or valine, inserted at a position corresponding to between positions 1 and 2 of wild type AAV5 VP1 capsid protein.

rAAV5 particles having such a mutation which are produced using baculoviruses and insect cells have been shown to have improved infectivity compared to rAAV5 particles which lack this mutation, as is described in WO2015/137802.

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising:

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease,

wherein the rAAV5 particle comprises:

- a) an additional amino acid, preferably an alanine or valine, inserted at a position corresponding to between positions 1 and 2 of wild type AAV5 VP1 capsid protein.

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease,

wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 5.

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1);
- 5 and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease,

wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 6, 8 or 29.

An rAAV5 particle for use according to the invention may comprise an exchange of the N-terminal region corresponding to the first 136 N-terminal amino acids of wild type AAV5 VP1 capsid protein with the N-terminal region corresponding first 137 N-terminal amino acids of wild type AAV serotype 2 (AAV2) VP1 capsid protein. Urabe et al. (Urabe et al., Journal of Virology, 2006, 80:1874-85) reported that exchanging the first 136 N-terminal amino acids of VP1 of AAV5 with the first 137 N-terminal amino acids of VP1 of AAV2 leads to more efficient recombinant-baculovirus-based production of infectious rAAV5 particles in insect cells. rAAV5 particles comprising an exchange of the N-terminal region corresponding to the first 136 N-terminal amino acids of wild type AAV5 VP1 capsid protein with the N-terminal region corresponding first 137 N-terminal amino acids of wild type AAV serotype 2 (AAV2) VP1 capsid protein may herein also be referred to as rAAV2/5 particles.

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising:

- 25 i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1);
- and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease,

further comprising an exchange of the N-terminal region corresponding to the first 136 N-terminal amino acids of wild type AAV5 VP1 capsid protein with the N-terminal region corresponding to the first 137 N-terminal amino acids of wild type AAV serotype 2 (AAV2) VP1 capsid protein.

35 In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- 5 ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease,

wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 7.

- 10 In one embodiment, a capsid protein variant as described herein has at least one mutation that confers a phenotype of increased transduction of the CNS as compared to a control capsid protein not having the mutation(s).

- In one embodiment, a phenotype of increased transduction of the CNS displays an increase in CNS transduction of at least 2, 3, 5, 10, 20, 50, or 100-fold as compared to a control capsid not having the mutation.
- 15

In one embodiment, an rAAV5 particle according to the invention comprises at least one mutation that confers a phenotype of decreased liver transduction as compared to a control capsid protein not having the mutation(s).

- 20 In some embodiments, a phenotype of decreased liver transduction in the liver i displays a decrease in liver transduction of 2, 3, 5, 10, 20, 50, 100 or 200-fold as compared to a control capsid not having the mutation. The skilled person is aware of methods to measure transduction of the CNS and/or liver. Exemplary methods as described herein are measurement of vDNA using PCR-based assays, or measurement of expression of a reporter gene such as Green Fluorescent Protein (GFP).

- 25 In one embodiment, the present invention provides a recombinant adeno-associated (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising:

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- 30 ii) an expression cassette flanked by at least one AAV inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in a CNS disease,

- wherein the rAAV5 particle shows increased transduction in the CNS and decreased transduction in the liver, and/or wherein the rAAV5 particle shows reduced inflammation and/or reduced microglial activation
- 35 in the CNS.

Preferably the increased transduction in the CNS, decreased transduction in the liver and/or reduced inflammation and/or reduced microglial activation in the CNS is compared to the level of transduction in liver and/or inflammation and/or microglial activation in the CNS that is induced by a parental rAAV5 particle. Methods to determine the level of transduction in the liver and the CNS are known by the person skilled in the art. Briefly, transduction of a capsid protein variant as compared to a control capsid protein can be determined by administering rAAV5 particles comprising the capsid protein variant to a non-human animal, for example directly to the CNS, and determining the transduction profile (i.e. distribution) of the rAAV5 particles over various tissues of interest in the animal, and comparing the transduction profile of rAAV5 particles comprising the capsid protein variant with the transduction profile of control rAAV5 particles, under otherwise identical conditions.

In one embodiment, the transduction profile of an rAAV5 particle as described herein is determined 2, 3, 4, 5 or 6 weeks post-administration.

In one embodiment, the transduction profile of an rAAV5 particle as described herein is determined in a non-human animal, preferably wherein the non-human animal is a mouse or other research animal, such as, but not limited to, a rabbit, a rat, a mini-pig, or a non-human primate.

As will be understood, the tissues of interest in determining the transduction profile, depend on the desired tropism and de-targeting of the capsid protein variant. For improved CNS tropism and liver de-targeting, the tissues of interest will at least include CNS (brain) and liver but can include further major organs such as heart, lung, and skeletal muscle. For a more specific determination of the CNS transduction profile various sections of the brain can be examined separately, such as frontal brain, caudal brain and cerebellum. Methods for determining transduction profiles are known in the art per se and are for example described in Pulicherla et al. (Mol Ther. 2011, 19(6):1070-8), Hanlon et al. (Mol Ther Methods Clin Dev. 2019, 15:320-332), Nonnenmacher et al., (Mol Ther Methods Clin Dev. 2020, 20:366-378) herein below and in the Examples below.

Similarly, methods to determine the level of inflammation and/or microglial activation in the CNS are known by the person skilled in the art. Briefly, inflammation levels can be compared using transduction with a capsid protein variant as compared to a control capsid protein can be determined by administering an rAAV5 particle comprising the capsid protein variant to a non-human animal, for example directly to the CNS, and determining the level of inflammation and/or microglial activation in the CNS and preferably comparing the inflammation level induced by rAAV5 particles comprising the capsid protein variant with the inflammation levels induced by control rAAV5 particles under otherwise identical conditions.

In one embodiment, the inflammation level in a subject having received an rAAV5 particle according to the invention is determined 2, 3, 4, 5 or 6 weeks post-administration.

5 In one embodiment, the inflammation level induced by administration of an rAAV5 particle according to the invention is determined in a non-human animal, preferably wherein the non-human animal is a mouse or other research animal, such as, but not limited to, a rabbit, a rat, a mini-pig or a non-human primate.

10 For a more specific determination of inflammation levels and/or microglial activation, various sections of the brain can be examined separately, such as frontal brain, caudal brain and cerebellum. Methods for determining inflammation levels are known in the art per se and are for example described herein. Methods for determining microglial activation in the CNS are known in the art per se and are for example described by Jurga et al. (Frontiers in Cellular Neuroscience, 2020, 14(198):1-18).

Method of production of rAAV5 particle variants

15 In one aspect there is provided a method for producing an rAAV5 particle as described herein, comprising capsid protein variants as described herein. In a further embodiment, the method comprises the steps of: a) culturing a host cell as herein defined above under conditions such that the rAAV5 vector is produced; and, b) optionally, one or more of recovery, purification and formulation of the rAAV5 vector.

20 In general, suitable methods for producing an rAAV5 particle as described herein in mammalian or insect host cells, and means therefore (such as expression constructs for expression of AAV rep proteins), are described, for mammalian cells in: Clark et al. (Hum. Gene Ther. 1995, 6, 1329-134), Gao et al. (Hum. Gene Ther. 1998, 9, 2353-2362), Inoue and Russell (J. Virol. 1998, 72, 7024-7031), Grimm et al. (Hum. Gene Ther. 1998, 9, 2745-2760), Xiao et al. (J. Virol. 1998, 72, 2224-2232) and Judd et al. (Mol Ther Nucleic Acids. 2012; 1: e54), and for insect cells in: Urabe et al. (Hum. Gene Ther. 2002, 13:1935-1943),
25 WO2007/046703, WO2007/148971, WO2009/014445, WO2009/104964, WO2011/122950, WO2013/036118, WO2015/137802, WO2019/016349, WO2021/198508, WO2021/198510 and WO2022/253955, all of which are incorporated herein in their entirety.

30 AAV sequences that may be used as described herein for the production of a rAAV5 particle of the invention in insect cells can be derived from the genome of any AAV serotype. Generally, the AAV serotypes have genomic sequences of significant homology at the amino acid and the nucleic acid levels, provide an identical set of genetic functions, and produce virions which are essentially physically and functionally equivalent, and replicate and assemble by practically identical mechanisms. For the genomic sequence of
35 the various AAV serotypes and an overview of the genomic similarities see e.g. GenBank Accession number U89790; GenBank Accession number J01901; GenBank Accession number AF043303; GenBank Accession number AF085716; Chlorini et al. (J. Vir. 1997, 71: 6823-33); Srivastava et al. (J. Vir. 1983,

45:555-64); Chlorini *et al.* (J. Vir. 1999, 73:1309-1319); Rutledge *et al.* (J. Vir. 1998, 72:309-319); and Wu *et al.* (J. Vir. 2000, 74: 8635-47). Preferably the AAV ITR sequences for use in the context of the present invention are derived from AAV1, AAV2, AAV4 and/or AAV7. Likewise, the Rep (Rep78/68 and Rep52/40) coding sequences are preferably derived from AAV1, AAV2, AAV4 and/or AAV7.

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AAV Rep and ITR sequences are particularly conserved among most serotypes. The Rep78 proteins of various AAV serotypes are e.g. more than 89% identical and the total nucleotide sequence identity at the genome level between AAV2, AAV3A, AAV3B, and AAV6 is around 82% (Bantel-Schaal *et al.*, J. Virol., 1999, 73(2):939-947). Moreover, the Rep sequences and ITRs of many AAV serotypes are known to efficiently cross-complement (i.e., functionally substitute) corresponding sequences from other serotypes in production of AAV particles in mammalian cells. US2003148506 reports that AAV Rep and ITR sequences also efficiently cross-complement other AAV Rep and ITR sequences in insect cells. Modified "AAV" sequences also can be used in this context, e.g. for the production of rAAV vectors in insect cells. Such modified sequences e.g. include sequences having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more nucleotide and/or amino acid sequence identity (e.g., a sequence having about 75-99% nucleotide sequence identity) to an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12 or AAV13 ITR or Rep can be used in place of wild-type AAV ITR or Rep sequences.

20 Mammalian cell lines for producing AAV vectors are selected from among any mammalian species, including, without limitation, cells such as A549, WEHI, 3T3, 10T1/2, BHK, MDCK, COS 1, COS 7, BSC 1, BSC 40, BMT 10, VERO, WI38, HeLa, a HEK 293 cell (which express functional adenoviral E1), Saos, C2C12, L cells, HT1080, HepG2 and primary fibroblast, hepatocyte and myoblast cells derived from mammals including human, monkey, mouse, rat, rabbit, and hamster. The selection of the mammalian species providing the cells is not a limitation of this disclosure; nor is the type of mammalian cell, i.e., fibroblast, hepatocyte, tumour cell. Mammalian cell lines for producing AAV vectors in particular include a broad range of HEK293 cell lines, of which the HEK293T cell line is preferred.

30 Insect cell lines for producing rAAV particles can be any cell line that is suitable for the production of heterologous proteins. Preferably the insect cell allows for replication of baculoviral vectors and can be maintained in culture, more preferably in suspended culture. In a preferred embodiment, the insect cell allows for replication of recombinant parvoviral vectors, including rAAV vectors. For example, the cell line used can be from *Spodoptera frugiperda*, *Drosophila*, or mosquito, e.g., *Aedes albopictus* derived cell lines. Preferred insect cells or cell lines are cells from the insect species which are susceptible to baculovirus infection, including e.g. S2 (CRL-1963, ATCC), Se301, SeZD2109, SeUCR1, Sf9, Sf900+, Sf21, BTI-TN-5B1-4, MG-1, Tn368, HzAm1, Ha2302, Hz2E5, High Five (Invitrogen, CA, USA) and *expresSF+*[®] (US 6,103,526; Protein Sciences Corp., CT, USA).

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- The rAAV5 particles in the supernatant can be recovered and/or purified using suitable techniques which are known to those of skill in the art. For example, monolith columns (e.g., in ion exchange, affinity or IMAC mode), chromatography (e.g., capture chromatography, fixed method chromatography, and expanded bed chromatography), centrifugation, filtration and precipitation, can be used for purification and concentration. These methods may be used alone or in combination. In one embodiment, capture chromatography methods, including column-based or membrane-based systems, are utilized in combination with filtration and precipitation. Suitable precipitation methods, e.g., utilizing polyethylene glycol (PEG) 8000 and NH₄SO₄, can be readily selected by one of skill in the art. Thereafter, the precipitate can be treated with benzonase and purified using suitable techniques. In addition, recovery may preferably comprises the step of affinity-purification of the rAAV5 particle using an anti-AAV antibody, preferably an immobilised antibody. The anti-AAV antibody preferably is a monoclonal antibody. A particularly suitable antibody is a single chain camelid antibody or a fragment thereof as e.g. obtainable from camels or llamas (see e.g. Muyldermans, Biotechnol. 2001, 74: 277-302). The antibody for affinity-purification of rAAV5 preferably is an antibody that specifically binds an epitope on an rAAV5 capsid protein, whereby preferably the epitope is an epitope that is present on capsid protein of more than one AAV serotype. E.g. the antibody may be raised or selected on the basis of specific binding to AAV2 capsid but at the same time also it may also specifically bind to rAAV5 particles.
- In one embodiment, the present invention provides a method for manufacturing a recombinant adeno-associated virus serotype 5 (rAAV5) particle, the method comprising the steps of:
- I) culturing a cell comprising:
 - i) an expression construct for expression of a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
 - ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease, the mutated capsid protein further comprising:
 - a) an additional amino acid, preferably an alanine or valine, inserted at a position corresponding to between positions 1 and 2 of wild type AAV5 VP1 capsid protein or
 - b) an exchange of the N-terminal region corresponding to the first 136 N-terminal amino acids of wild type AAV5 VP1 capsid protein with the N-terminal region corresponding to the first 137 N-terminal amino acids of wild type AAV serotype 2 (AAV2) VP1 capsid protein,

said host cell preferably further comprising a nucleotide sequence encoding a parvoviral replication (Rep) protein under conditions such that the rAAV5 particle is produced; and,
II) recovery of the rAAV5 particle, preferably wherein recovery of the rAAV5 particle in step II) comprises at least one of: affinity-purification of the particle using an immobilised anti-AAV
5 antibody, preferably a single chain camelid antibody or a fragment thereof, or filtration over a filter having a nominal pore size of 30 - 70 nm.

In one embodiment the present invention provides a method according to the invention for manufacturing a recombinant adeno-associated virus (rAAV) serotype 5 particle,
10 wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 6.

In one embodiment the present invention provides a method according to the invention for manufacturing a recombinant adeno-associated virus (rAAV) serotype 5 particle,
15 wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 7.

In one embodiment the present invention provides a method according to the invention for manufacturing a recombinant adeno-associated virus (rAAV) serotype 5 particle,
20 wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 8.

In one embodiment the present invention provides a method according to the invention for manufacturing a recombinant adeno-associated virus (rAAV) serotype 5 particle,
25 wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 29.

In one embodiment, the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle comprising:
30 i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1);
and
ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one
35 product having an effect in the CNS disease,
wherein the rAAV5 particle comprises:

- a) an additional amino acid, preferably an alanine or valine, inserted at a position corresponding to between positions 1 and 2 of wild type AAV5 VP1 capsid protein; or
- b) an exchange of the N-terminal region corresponding to the first 136 N-terminal amino acids of wild type AAV5 VP1 capsid protein with the N-terminal region corresponding to the first 137 N-terminal amino acids of wild type AAV serotype 2 (AAV2) VP1 capsid protein.

In one embodiment the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle comprising:

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in a CNS disease,
- wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 6.

In one embodiment the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle comprising:

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in a CNS disease,
- wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 7.

In one embodiment the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle comprising:

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in a CNS disease,

wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 8.

In one embodiment the present invention provides a recombinant adeno-associated virus (rAAV) serotype 5 particle comprising:

- i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in a CNS disease,

wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 29.

15 *Expression construct*

In one embodiment, the present invention provides an expression construct for expression of an rAAV5 particle according to the invention. The expression construct for expression of the capsid protein(s) is an insect cell-compatible vector or a mammalian cell-compatible vector. A "mammalian cell-compatible vector" is understood to be a nucleic acid molecule capable of productive transformation or transfection of a mammalian cell or cell line. Mammalian cell-compatible vectors are well-known in the art. An "insect cell-compatible vector" is understood to be a nucleic acid molecule capable of productive transformation or transfection of an insect or insect cell. Exemplary insect cell-compatible vectors include plasmids, linear nucleic acid molecules, and recombinant viruses, such as baculoviruses. Any vector can be employed as long as it is insect cell-compatible. The mammalian or insect cell-compatible vector may integrate into the cell's genome but the presence of the vector in the cell need not be permanent and transient episomal vectors are also included. The vectors can be introduced by any means known, for example by chemical treatment of the cells, electroporation, or transduction. The expression construct will be introduced into a suitable host cell and be able to affect expression of the coding sequence in an in vitro cell culture of the host cell. A preferred expression construct will be suitable for expression of viral proteins and/or nucleic acids, particularly recombinant parvoviral proteins and/or nucleic acids, such as baculoviral vectors for expression of parvoviral proteins and/or nucleic acids in insect cells.

In one embodiment, the vector is a viral vector or a plasmid. In a more preferred embodiment, the viral vector is an insect cell-compatible vector, preferably is a baculovirus. In one embodiment, the nucleic acid construct is a baculovirus-expression vector (BEV). It is well-known that baculovirus-expression vectors are particularly suitable for the transfer of nucleic acids to insect cells and methods for their use are described for example in: Summers and Smith, 1986, "A Manual of Methods for Baculovirus Vectors and Insect

Culture Procedures", Texas Agricultural Experimental Station Bull. No. 7555, College Station, Tex.; Luckow, 1991, In Prokop et al., "Cloning and Expression of Heterologous Genes in Insect Cells with Baculovirus Vectors' Recombinant DNA Technology and Applications", 97-152; King and Possee, 1992, "The baculovirus expression system", Chapman and Hall, United Kingdom; O'Reilly, Miller, and Luckow, 1992, "Baculovirus Expression Vectors: A Laboratory Manual", New York; Freeman and Richardson, 1995, "Baculovirus Expression Protocols", Methods in Molecular Biology, volume 39; US 4,745,051; US2003148506; and WO 03/074714.

In one embodiment, there is provided at least one expression construct comprising separate expression cassettes for each of the VP1, VP2 and VP3 capsid proteins, which capsid proteins, independently may be a variant as described herein. In one embodiment, there is provided at least one expression construct comprising a separate expression cassette for a VP1 capsid protein and a separate expression cassette for the VP2 and VP3 proteins, wherein the VP1 capsid protein and/or both VP2 and VP3 capsid proteins, may be variants as described herein. The various expression cassettes for the different capsid protein variants as described herein can be present together on a single expression construct/nucleic acid molecule, or one or more of the expression cassettes for the different capsid proteins can be present on two or three separate expression constructs/nucleic acid molecules, each comprising one of the expression cassettes for the different capsid proteins. As will be understood, the use of separate expression cassettes for one or more of the different capsid proteins allows to produce AAV vectors with an above-described modification/mutation in only some of capsid proteins, e.g. only in VP1 and not in VP2 and VP3 or vice versa, or it allows to produce AAV vectors with different above-described modifications/mutations in the different capsid proteins. Expression constructs for separate expression of the various capsid proteins in mammalian cells are e.g. disclosed in Judd et al. (Mol Ther Nucleic Acids. 2012; 1: e54). Expression constructs for separate expression of the various capsid proteins in insect cells are disclosed in WO2022/253955.

In another embodiment, there is provided an expression construct comprising a single expression cassette for expression of all three of the VP1, VP2 and VP3 capsid protein variants as described herein, preferably from a single coding sequence. Expression constructs for expression of all three of the VP1, VP2 and VP3 capsid proteins from a single expression cassette in mammalian cells are e.g. disclosed in Clark et al. (Hum. Gene Ther. 1995, 6, 1329-134), Gao et al. (Hum. Gene Ther. 1998, 9, 2353-2362), Inoue and Russell (J. Virol. 1998, 72, 7024-7031), Grimm et al. (Hum. Gene Ther. 1998, 9, 2745-2760) and Xiao et al. (J. Virol. 1998, 72, 2224-2232). Expression constructs for expression of all three of the VP1, VP2 and VP3 capsid proteins from a single expression cassette in insect cells are e.g. disclosed in Urabe et al. (Hum. Gene Ther. 2002, 13:1935-1943), WO2007/046703, WO2015/137802 and WO2019/016349. As will be understood, expression of all three of the VP1, VP2 and VP3 capsid protein variants as described herein,

from a single coding sequence (in a single expression cassette), allows to produce AAV vectors comprising mutations/modifications as described herein in all three of its VP1, VP2 and VP3 capsid proteins.

5 In one embodiment, an expression construct for expression of an rAAV5 particle according to the invention comprises a nucleic acid comprising a suboptimal translation initiation codon for the VP1 capsid protein, wherein the suboptimal translation initiation codon is selected from CTG, ACG, TTG or GTG. In some
embodiments, the suboptimal translation initiation codon is followed by an additional second codon
encoding an amino acid selected from alanine, glycine, valine, aspartic acid and glutamic acid, preferably
valine or alanine.

10 In one embodiment, the present invention provides an expression construct for expression of an rAAV5 particle according to the invention, wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 6.

15 In one embodiment, the present invention provides an expression construct for expression of an rAAV5 particle according to the invention, wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 7.

In one embodiment, the present invention provides an expression construct for expression of an rAAV5 particle according to the invention, wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 8.

20 In one embodiment, the present invention provides an expression construct for expression of an rAAV5 particle according to the invention, wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 29.

Host cell comprising rAAV5 particle variants

25 In one embodiment, the present invention provides a host cell comprising a nucleic acid molecule or expression construct for expression of the modified rAAV5 particle variants as described herein. In one embodiment, the host cell is suitable for the production of rAAV particles. Accordingly the host cell is a host cell that is amenable to in vitro culture, preferably at large scale. Host cell that are suitable for the production of rAAV particles are well-known in the art and will typically be a mammalian or an insect cell line.
30 Mammalian cell lines for producing rAAV particles are selected from among any mammalian species, including, without limitation, cells such as A549, WEHI, 3T3, 10T1/2, BHK, MDCK, COS 1, COS 7, BSC 1, BSC 40, BMT 10, VERO, WI38, HeLa, a HEK 293 cell (which express functional adenoviral E1), Saos, C2C12, L cells, HT1080, HepG2 and primary fibroblast, hepatocyte and myoblast cells derived from mammals including human, monkey, mouse, rat, rabbit, and hamster. The selection of the mammalian
35 species providing the cells is not a limitation of this disclosure; nor is the type of mammalian cell, i.e.,

fibroblast, hepatocyte, tumor cell. Mammalian cell lines suitable for producing rAAV particles in particular include a broad range of HEK293 cell lines, of which the HEK293T cell line is preferred.

5 In one embodiment, the present invention provides a host cell comprising a nucleic acid molecule or expression construct for expression of an rAAV5 particle according to the invention, wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 6.

10 In one embodiment, the present invention provides a host cell comprising a nucleic acid molecule or expression construct for expression of an rAAV5 particle according to the invention, wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 7.

15 In one embodiment, the present invention provides a host cell comprising a nucleic acid molecule or expression construct for expression of an rAAV5 particle according to the invention, wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 8.

20 In one embodiment, the present invention provides a host cell comprising a nucleic acid molecule or expression construct for expression of an rAAV5 particle according to the invention, wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 29.

25 Description of the figures

Figure 1. A) Design of exemplary miRNA candidate scaffolds. B) Endogenous mRNA lowering results by miRNA candidate scaffolds.

Figure 2. Human α -syn protein levels in striatum in *in vivo* Proof of Concept study in rAAV5-Syn rat model.

30 Figure 3. A) Vectorized antibody (vAb) design. B) Dose dependent response of an exemplary rAAV5-delivered anti-II-1 β antibody. Three different doses were administered to RAK KO mice, 5E12 gc/kg or vAb Low, 2.5E13 gc/kg or vAb Mid and 1.25E14gc/kg or vAb High dose.

35 Figure 4. Vectorized anti-II-1 β , expressed in RAK KO mice, is able to suppress II-1 β signaling ex-vivo in HEK-293Blue II-1 β cells.

Figure 5. Antibody levels, as measured by Mesoscale Discover (MSD), in the brain tissue samples from in vivo study 1. The antibody levels in A) striatum and B) cortex C) serum.

Figure 6. A) Activity of secreted alkaline phosphatase delivered by either parental rAAV5 or rAAV5 M569V in Huh7 cells. SEAP activity was measured 24 and 48 hours post infection. B) Transduction efficiency of parental rAAV5 versus rAAV5 M569V into HEK239T cells as determined by flow cytometry detecting eGFP. C) Transduction efficiency of parental rAAV5 particles versus rAAV5 M569V particles in iPSC-derived cerebral organoids as determined by flow cytometry detecting eGFP. D) anti-AAVR (AAV receptor) antibody (5 ug/ml) neutralization assay represented as fold-change compared to a non-targeting isotype control (5 ug/ml). Both parental rAAV5 and rAAV5 M569V displayed a 3-4 fold reduction in transduction efficiency upon AAVR neutralization. E) Transduction efficiency of parental rAAV5 particles and rAAV5 M569 particles with neuraminidase treated and untreated cells. NanoLuciferase activity is expressed as relative light units (RLU).

Figure 7. A) Immunohistological detection of rAAV-transgene eGFP via anti-eGFP antibody-HRP staining of coronal brain sections. The darker shade in the bottom four sections, representing brains of animals transduced with rAAV5 M569V, reflects higher eGFP expression. B) Molecular analysis via qPCR using transgene specific primer-probe combination on isolated total DNA of liver tissue of animals injected with PBS, parental rAAV5 or rAAV5 M569V (n=4).

Figure 8. A) rAAV vDNA distribution 1 month after intraputaminial injection in minipig brain. B) eGFP transgene mRNA quantification by RT-qPCR 1 month after intraputaminial injection in minipig brain C) Immunohistological detection of rAAV transgene eGFP via anti-eGFP antibody-HRP staining of minipig half-brain slices 1 month after the injections (n=3). The darker staining in the lower three half-brain slices, representing rAAV5 M569V, indicates higher transduction efficiency. D) Exemplary comparison between brain slices after section: eGFP expression is visible with the naked eye in brain slices of animals injected with rAAV5 M569V.

Figure 9. Inflammation related genes represented as transcripts per million (TPM) for parental rAAV5- or rAAV5 M569V-injected animals (n=3).

Figure 10. A) Immunohistochemical analysis of IBA-1 (ionized calcium-binding adapter molecule 1) in brain slices obtained from minipigs injected with either parental rAAV5 (lower three pictures) or rAAV5 M569V (upper three pictures) B) Quantification of lesion and inflammation area in the samples from (A) on the left, and quantification of IBA-1 positive area within the region of interest in the samples from (A) on the right.

Figure 11. Single nuclei staining of brain tissue punches from 3 minipigs injected with parental rAAV5 and 3 minipigs injected with rAAV5 M569V. The upper six panels in the upper left corner show cell clusters of nine distinct cell types for three different brain punches per minipig treated with rAAV5 M569V (upper three panels) and a minipig treated with parental rAAV5 (lower three panels). The punches were taken from three different brain regions – white matter (WM), caudate nucleus, and cerebral cortex cingulate. The six panels in the upper right corner specifically show representation of B-cells for three different brain punches, the six panels in the lower left corner show representation of T-cells and the six panels in the lower right corner show the representation of microglia and perivascular macrophages in three different brain punches for both capsids.

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Figure 12. Visualization of Non-Human Primate (NHP) brain punches obtained from NHP-derived brain slices.

Figure 13. Vector DNA (vDNA) quantification in brain punches, liver tissue and spinal cord of NHPs. Levels of vDNA are expressed as genome copies per microgram DNA that was obtained from the tissue samples and brain punches. The numbers provided on the x-axis refer to brain punch sites visualized (in part) in Figure 12 and further detailed in Table 9.

Figure 14. MicroRNA quantification in brain punches, liver tissue and spinal cord of NHPs. Levels of alpha synuclein messenger RNA (SNCA mRNA)-binding miRNA (miSNCA) are expressed as miSNCA copy numbers per microgram of RNA obtained from the tissue samples and brain punches. The numbers provided on the x-axis refer to brain punch sites visualized (in part) in Figure 12 and further detailed in Table 9.

Figure 15. Relative SNCA messenger RNA (mRNA SNCA) levels measured in brain punches, liver tissue and spinal cord of NHPs. The relative SNCA mRNA levels are expressed as a percentage of the average SNCA mRNA levels that were measured in vehicle-treated (control) animals. The numbers provided on the x-axis refer to brain punch sites visualized (in part) in Figure 12 and further detailed in Table 9.

Figure 16. Relative alpha synuclein levels measured in brain punches and liver of NHPs. The alpha synuclein protein levels are expressed as percentage of the average alpha synuclein levels from vehicle treated (control) animals. The numbers provided on the X-axis refer to the brain punch sites visualized (in part) in Figure 12 and further detailed in Table 9 and 10.

Figure 17. vDNA, mRNA and protein levels in striatum from in vivo study. A) vDNA concentrations of the different samples expressed as gc/μg. B) mRNA concentrations of huAPOE variant in the different samples expressed as mRNA copies per μg total RNA measured. C) mRNA concentrations of scFv anti-Tau

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antibody of the different samples expressed as mRNA copies per μg total RNA measured. D) Protein concentrations of the huAPOE variant in the different samples, expressed as $\mu\text{g}/\text{mg}$ tissue. E) Protein concentrations of the scFv anti-Tau antibody in the different samples, expressed as $\mu\text{g}/\text{mg}$ tissue.

5 **Examples**

RNA interference

SNCA miRNA guide strand design

10 The miSNCA (miRNA guide strands) were designed to target common RNA sequences of the most common alpha synuclein (SNCA) mRNA variant; SNCA140, SNCA126, SNCA112 and SNCA98, which encode pathogenic protein variants prone to aggregation. For designing a miRNA that is targeting all of these variants, thereby lowering the levels of the mRNA variants having a pathogenic effect, miRNAs in regions that are common with all of the major mRNA variants of SNCA were designed. The target regions of the SNCA mRNA sequences are a portion of the exon 2, exon 4 and exon 6. The most common single
15 nucleotide polymorphism (SNP) outside of these exons, namely A30P, was avoided to be included in the guide RNAs.

The miSNCA guides were selected based on the following criteria: the miRNA guide sequence should not include a stretch of >4 G, >4 C, >5 A and >5 T nt, a GC content between 30% and 70%, < 4000 predicted
20 off-target genes of the miRNA seed sequence for SNCA targeting guides and < 5000 predicted off-target genes of the miRNA seed sequence for SNCA targeting guides by using siSPOTR analysis (<https://sispotr.icts.uiowa.edu/sispotr/tools/lookup/evaluate.html>) and pre-miRNA sequence folding energy between -44 and -55 kcal/mole. To generate a negative control, two scramble guides were selected for *in vitro* testing.

25 The selected miSNCA guides meet the following criteria: conservation with monkey SNCA gene sequence (*Macaca mulatta*, NCBI accession number NC_041768.1), the miRNA guide sequence should not include a stretch of >4 G or >4 C nt, a GC content between 20% and 70%, a GC seed content between 40% and 70%, pre-miRNA sequence folding energy between -45 and -55 kcal/mole and no matching with
30 endogenous miRNA seeds.

Guide sequences were incorporated into human pri-miRNA miR-451 scaffold sequences and the mFold program (<http://unafold.rna.albany.edu/?q=mfold>) was used with standard settings to determine whether the candidates are folded into the secondary structures.

35 The SNCA Scaffold 1) consists of the miR-144 hairpin/scaffold combined with one mir-451 downstream hairpin/scaffold. The SNCA Scaffold 2 consists of the miR-144 hairpin combined with two or more mir-451

downstream scaffold. Placement of the miR144 hairpin is always at the 5' most compared to the miR451 hairpin sequence(s) (Figure 1A).

Transfections and endogenous a-synuclein lowering

5 To evaluate endogenous a-synuclein lowering by miSNCA candidates, HEK293T cells were used. For these assays the HEK293T cells (1 or 5x10⁵cells/well) were plated into 24 or 6-well tissue culture-treated plates respectively. The cells were transfected with varying amounts (50-100-200-1000ng) of plasmid carrying miSNCA candidates using Lipofectamine 2000 or Lipofectamine 3000 (Thermo Fisher Scientific). Each transfection was performed in triplicates. The cells were collected two days after transfection. The cell
10 samples were analyzed for the mRNA levels of SNCA and a-synuclein protein levels. The experiments were repeated at least three times.

Proof of Concept study in rAAV- α-synuclein rat model

This study is designed to assess the ability of two miRNAs targeting SNCA mRNA (encoding a-synuclein)
15 to protect dopaminergic function in the rAAV1/2-hA53T-aSyn rat model of Parkinson's disease. On day 1 (D1), rAAV1/2 human A53T a-synuclein (rAAV1/2-hA53T- a-Syn), in combination with one of 4 other rAAV5 vectors, is administered unilaterally into the right substantia nigra according to stereotaxic techniques. Groups are indicated in Table 2.

20 **Table 2**

Group	Viral vector -1	Viral vector -2	NN
	intra-SN, 2 µl volume		
1	D1: rAAV1/2-EV	D14: rAAV5-unrelated miR	12
2	D1: rAAV1/2-hA53T-aSyn	D14: rAAV5-unrelated miR (for scaffold 1)	12
3	D1: rAAV1/2-hA53T-aSyn	D14: rAAV5-unrelated miR (for Scaffold 2)	12
4	D1: rAAV1/2-hA53T-aSyn	D14: rAAV5-miSNCA2	12
5	D1: rAAV1/2-hA53T-aSyn	D14: rAAV5-miSNCA5	12
6	D1: rAAV1/2-hA53T-aSyn	D14: rAAV5-miSNCA15	12

7	D1: rAAV1/2-hA53T-aSyn	D14: rAAV5-miSNCA5+15 low dose	12
8	D1: rAAV1/2-hA53T-aSyn	D14: rAAV5-miSNCA5+15 mid dose	12
9	D1: rAAV1/2-hA53T-aSyn	D14: rAAV5-miSNCA5+15 high dose	12

Table 2. Groups of in vivo study. rAAV1/2-hA53T-aSyn = rAAV1/2 human A53T a-synuclein, 3 x; rAAV1/2-EV = empty a-Syn vector; D15, viral vector 2; Group 1 to 6, and Group 9, 3 x 10¹³ gc/ml; Group 7, 6 x 10¹² gc/ml; Group 8, 1.2 x 10¹² gc/ml; SN = substantia nigra; D = day. All groups are sequential injections of rAAVs on D1 and D14.

On D57, animals were sacrificed for post-mortem assessments. Blood, samples were collected, processed and stored as required.

Primary endpoints of the study included: assessment of forelimb asymmetry (via cylinder test); quantification of striatal dopamine and metabolite levels (via LC-MS/MS); quantification of dopamine transporter (via autoradiography); and quantification of striatal transgene-derived aSyn levels (via ELISA). Optional endpoints included: quantification of tyrosine hydroxylase positive (TH+ve) cells in the substantia nigra with and without co-expression with human aSyn (via double label immunofluorescence).

Sacrifice and sample collection were done as follows: animals were deeply anaesthetized with isoflurane and then sacrificed via exsanguination by way of transcardial perfusion with ice-cold 0.9 % saline containing 0.2 % heparin. Brains were placed, ventral up, into an ice-cold stainless steel rat brain matrix and were first cut in the coronal plane at the level of the hypothalamus. The rostral portion of the brain, including the entire striatum, was immediately frozen in isopentane chilled to -42 °C and later sectioned for DAT autoradiography and dissected for the quantification of levels of dopamine and metabolites of dopamine (HVA and DOPAC) by LC-MS/MS and levels of human aSyn by ELISA. Tissues were stored in a locked freezer at -80 °C. Additional regions of interest (including additional striatal dissections) were collected according to Table 3 for molecular assays.

Table 3. Brain regions of interest and corresponding assays and sample handling.

Region of interest	Assay	Buffers used	Format
striatum	autoradiography	None, cryosectioned	on-slide
striatum	LCMS	See LCMS method	supernatant

		below	
striatum	ELISA	RIPA, protease and phosphatase inhibitors	share aliquots between assays
striatum	TR-FRET	RIPA, protease and phosphatase inhibitors	
striatum	Q-PCR (DNA) RT-QPCR (miRNA, RNA)	None, cryosectioned and dissected	In tube

The remainder of the caudal portion of the brain, including the mesencephalon, was immersed in 4 % paraformaldehyde (PFA) for 48 hours for fixation followed by cryoprotection in graded sucrose solutions (15 to 30 % sucrose). Tissue prepared in this manner was used for quantification of dopamine neuron numbers in the substantia nigra pars compacta (SNc) via immunohistochemistry of tyrosine hydroxylase and unbiased stereology.

Results – RNA interference

Example 1

Endogenous SNCA expression is lowered in cells transfected with Scaffold 1 and 2 constructs

The miSNCA5, miSNCA15 and miSNCA2 Scaffold 1 (SEQ ID NO: 9-11, respectively) and Scaffold 2 constructs (miSNCA2+5, miSNCA5+15, miSNCA5+5, miSNCA5+15+2; SEQ ID NO: 12-15, respectively) and control miRNAs for Scaffolds 1 and 2 (SEQ ID NO: 16-18) single (CTR1), double (CTR1+CTR2) or triple scrambled miRNAs (CTR1+CTR2+CTR3)) were chosen for testing the knockdown of SNCA expression in cells. Transfection of 50 ng of miRNA plasmid resulted in a decrease of SNCA mRNA expression of 60-40% by all miSNCA Scaffold 2 candidates tested (Figure 1B). A similar decrease in protein expression is expected.

Example 2

miSNCA lowers SNCA protein level *in vivo* in rAAV-Syn rat model

Different rAAV5-miSNCA Scaffold 1 and rAAV5-miSNCA Scaffold 2 candidates were tested in the rAAV-Syn rat model. The rAAV5-miSNCA vectors recovered the disease phenotype in the rAAV-Syn rat model, in motor phenotype, molecular and neurochemical alterations.

To determine the *in vivo* proof of concept of lowering SNCA levels, and the relation to motor phenotype improvement, a rat PD model was used (rAAV1/2-hA53T-aSyn; viral-induced overexpression of human A53T a-syn). The right substantia nigra (SN) received unilateral injections of the disease-inducing rAAV1/2-hSNCA vector, followed by administration of the different therapeutic vectors (rAAV5-miSNCA) and respective controls miCTR1 or miCTR1+CTR2 Empty vector (EV_CTR) was used as control for the disease

model. In all the injected groups, at the single dose used, vDNA was detected in the striatum of the rAAV5 injected brain hemispheres. In the last three rAAV5-miSNCA Scaffold 2 groups with low-mid and high doses, dose dependent vDNA levels were detected, while in the control hemispheres (left striatum), vDNA levels were below the lower limit of quantification (LLOQ) (not shown). The miSNCA expression led to lowering at the protein level reflected by reduced α -syn protein levels in all rAAV5-miSNCA treated groups with respect to controls, as measured by ELISA (Figure 2).

Vectorized antibodies

Antibody design

A vectorized antibody based on a modified anti-II-1 β antibody from the public domain was designed (SEQ ID NO: 19). The heavy chain protein subunit-encoding DNA sequence (SEQ ID NO: 20) and light chain protein subunit-encoding DNA sequence (SEQ ID NO: 21) were connected with a Furin cleavage site (F2A; SEQ ID NO: 22) a linker (SEQ ID NO: 23) and a 2A cleavage site (2A; SEQ ID NO: 24) to ensure assembly of heavy and light chain (Figure 3A). Additionally, the vectorized antibody design contained a DNA sequence that encodes human growth hormone leader (hGH-L and HGH-H; SEQ ID NO: 25 and 26, respectively) (Figure 3A) as a signal peptide for efficient secretion. A lambda constant light chain region was used. In addition, two rAAV- vAb candidates having alpha-synuclein specificity were designed (vec-Ab-PRX002 (SEQ ID NO: 27) and vec-Ab-MEDI1341 (SEQ ID NO: 28)) and evaluated in wild type mice.

rAAV5 vectors

rAAV material was generated by Sirion Biotech GmbH (Germany). pRC plasmids and pLTR plasmids were provided by uniQure. In brief, 10-stack CellFactories were used for culturing of HEK293T cells. Cells were transfected with pRC, pLTR and pHelper using PEI as transfection reagent. After 48 hours at 37C and 5% CO₂ rAAV was harvested from cell pellets by 0.1% Triton lysis and viral particles purified from crude lysates using POROS CaptureSelect AAV-X. Genome-containing capsids were enriched by iodixanol gradient centrifugation. Purified virus was subsequently concentrated using Amicon ULTRA 15 filter units and formulated in PBS + 0.001% Pluronic. Viral Titers were determined by qPCR on LightCycler 480 using ITR specific PCR primers. Capsid integrity was confirmed by SDS-PAGE electrophoresis and genome integrity by electrophoresis on 0.8% agarose gels.

Electrochemiluminescence ELISA for quantification of antibodies from in vivo samples

For measuring the amount of vectorized antibody (vAb) in *in vitro* samples (mouse serum), an MSD (Mesoscale Discovery) assay was developed. Recombinant human II-1 β (Biolegend, 579408) was coated on MSD Multi-Array 96 Plates (Mesoscale Discovery). Serum samples were added to the plate and vectorized antibody was detected using Sulfo-tag Anti-HU/NHP IgG antibody (MSD, D20JL-6) and MSD gold read buffer A (Mesoscale Discovery). Detection of electrochemiluminescence (ECL) signal was done using the MESO QuickPlex SQ 120MM instrument (Mesoscale Discovery). To quantify the amount of vAb

in the samples, purified vAb was used to create a standard line between 2-2000 ng antibody/mL. A sigmoidal curve was fitted and ECL signal from samples were interpolated to calculate the vAb serum concentration, expressed as µg/mL.

5 *HEKBlue-Il-1 β reporter cell assay*

To further confirm vAb functionality, neutralization assays were performed using HEKBlue-Il-1 β reporter cells (InvivoGen, hkb-il1bv2). This cell line is modified to secrete embryonic alkaline phosphatase (SEAP) under an Il-1 β receptor. Binding of Il-1 β to these cells will induce NF- κ B activation and subsequent production of SEAP. SEAP levels in the medium are assessed using QUANTI-Blue.

10 HEKBlue cells were maintained using selective antibiotics Normocin (InvivoGen) and Zeocin (InvivoGen) to keep a pure cell culture. For experiments, cells were seeded in 96-well Clear Flat Bottom Polystyrene TC-treated Microplates (Corning) with a seeding density of 50,000 cells/well. Cells were allowed to attach to the wells for at least 3 hours. Serum samples were diluted in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific) containing 5 ng/mL recombinant Human Il-1 β (Biolegend, 579408). Serum
15 samples were diluted in a serial dilution starting from 4 µg vAb/mL. Vectorized antibody levels in serum were based on MSD assay as described above. For negative controls, serum from negative mice was used, as well as an irrelevant human IgG (human anti-alpha synuclein, FJ2030), produced at FairJourney biologics was used. As positive control, purified vAb was spiked in negative control serum. Samples were incubated for 15 minutes at RT to allow vAb binding to Il-1 β and added to the cells overnight at 37 °C and
20 5% CO₂. To quantify SEAP levels in the medium, QUANTI-Blue solution (InvivoGen) was added together with cell culture medium to Nunc Maxisorp 96 Well Plates (Thermo Fisher Scientific, 456537) for 30 minutes at 37 °C with in between mixing. SEAP levels were determined by measuring absorbance at 600 nm with a Glomax instrument; higher OD600 values indicating higher SEAP concentration.

25 *In vivo study – Dose-range study vAb in RAG2 KO mice*

In this study, the dose-dependent expression and safety of expressing vectorized antibodies after i.v. injection was evaluated. A total of 4 treatment groups of B6 RAG2 immunodeficient mice (C57BL/6NRj-Rag2tm1Ciphe/Rj; Janvier Labs, France) 7-8 weeks of age were employed according to the set-up below, see Table 4. rAAV-vAb was administered intravenously (i.v.) through a single lateral tail vein injection (t =
30 0). The group injected with only formulation buffer served as control groups.

Table 4. Study groups for the dose-range vAb study

Group	Genotype	No. of mice	Compound name	Dose AAV (gc/kg)	Dosing	In-life phase (weeks)
1	RAG2	5	vAb	5.00E+12	i.v.	8
2	RAG2	5	vAb	2.50E+13	i.v.	8
3	RAG2	5	vAb	1.25E+14	i.v.	8
4	RAG2	4	Formulation buffer	-	i.v.	8

Serum samples were collected and prepared using a standard serum preparation procedure at baseline (pre-dose, Day -1), once weekly after dosing and at termination. At termination, a larger amount (as much as possible) of blood was collected from all mice in a regular microcentrifuge tube.

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In vivo study – Dose-range study vAb in wild type mice

In this study, the dose-dependent expression and safety of expressing vectorized antibodies after direct brain injection was evaluated. The two rAAV- vAb candidates specific for alpha-synuclein (vec-Ab-PRX002 and vec-Ab-MEDI1341) were evaluated in wild type mice. A total of 7 treatment groups with 7 to 10 animals per group (total N=77) were used. On Day 1, all animals received a 2 x 3µl bilateral injection of rAAV5- vAb -PRX002 or rAAV5- vAb -MEDI1341 into the striatum at three different doses (high, medium and low). The two groups injected with only formulation buffer or an empty rAAV5 vector at medium dose served as control groups. Groups are indicated in Table 5.

15 **Table 5. Study groups for the dose-range vec-Ab study**

Group number	Number of Animals	Test Article	Titer (gc/mL)	Dose (gc/brain)	Collection Protocol (animals per type of analysis)
1	7	Formulation Buffer	NA	NA	3 Molecular Analysis 4 IHC/FISH
2	10	Empty Vector, mid dose	7.0x10 ¹²	4.2x10 ¹⁰	6 Molecular Analysis 4 IHC/FISH
3	10	rAAV5-vec-Ab-MEDI1341 , high dose	7.42x10 ¹³	4.45x10 ¹¹	6 Molecular Analysis 4 IHC/FISH
4	10	rAAV5-vec-Ab-MEDI1341 , mid dose	1.44x10 ¹³	8.64x10 ¹⁰	6 Molecular Analysis 4 IHC/FISH
5	10	rAAV5-vec-Ab-MEDI1341 , low dose	2.56x10 ¹²	1.54x10 ¹⁰	6 Molecular Analysis 4 IHC/FISH
6	10	rAAV5-vec-Ab-PRX002 , high dose	5.37x10 ¹³	3.22x10 ¹¹	6 Molecular Analysis 4 IHC/FISH
7	10	rAAV5-vec-Ab-PRX002 , mid dose	9.13x10 ¹²	5.48x10 ¹⁰	6 Molecular Analysis 4 IHC/FISH
8	10	rAAV5-vec-Ab-PRX002 , low dose	1.49x10 ¹²	8.94x10 ⁹	6 Molecular Analysis 4 IHC/FISH

Seventy-seven (77) male C57Bl/6J mice (Stock 000664) were purchased by PsychoGenics from Jax laboratory (8-9 weeks of age). Mice were group housed (3-4/cage) at PsychoGenics after health status clearance. Mice were assigned unique identification numbers and housed in polycarbonate OptiMICE cages. Each animal received a unique identification number, examined, manipulated and weighed prior to study initiation to ensure adequate health and suitability. During the course of the study 12/12 light/dark

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cycles was maintained. The room temperature was maintained between 20 and 23° C with a relative humidity maintained around 50%. Chow and water was provided ad libitum for the duration of the study.

5 Mice were anesthetized with isoflurane and stereotactically injected bilaterally with test articles into the right and left forebrain to the dorsal neostriatum [3µL per hemisphere; 6µL per brain] (coordinates: AP +0.8mm, ML +/- 1.8mm, DV -3.0 mm). Injections were performed with a 10µl syringe (Hamilton, NV) connected to a 30G metal cannula using PE50 plastic tubing (PlasticsOne). Injections occurred at a rate of 0.5µl/min with the needle left in place for 5 minutes following administration at each target. Pre- and post-operative analgesics was administered in accordance with veterinarian recommendations.

10 Animals were body weighed once per week and was checked for survival twice per day for the duration of the study, including weekends and holidays. Experimental animals were sub mandibular bled at four time-points as shown in Table 6. Briefly, whole blood samples were processed for plasma using K2-EDTA tubes, flash frozen and stored at -80°C until processing. For each time point, a single aliquot of approximately 25 µl was collected.

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Table 6. Blood sample collection times

Study Timelines (days)	-14	-7	0	7	14	21	28	35	42	57
Animals Arrived	X									
Bilateral Striatal injections			X							
Bleeds (Sub mandibular)		X			X		X		X	
Terminal Biological Sample Collection										X

On day 57, animals were anesthetized with isoflurane and CSF, plasma, liver and brain samples were harvested. CSF was collected from all animals. Briefly, the fur and skin from the neck was removed with surgical scissors (the mouse was not be decapitated). The tissue surrounding the base of the skull and brain stem was carefully removed via blunt dissection with minimal bleeding. Once the meninges were exposed at the base of the skull, in the area of the foremen magnum, a 27G butterfly needle was held perpendicular to the skull and inserted laterally into the cerebrospinal fluid (CSF) space. Samples were centrifuged for 10 minutes at 2000 g, at 4°C before freezing. Samples were stored in 0.5mL tubes at -80°C until use.

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After CSF collections, whole blood (approximately 400µl) was collected via closed cardiac puncture using a 1ml syringe with 25-gauge needle prior to transcardial perfusion. Whole blood was processed for plasma

by using K2-EDTA tubes. A single 200µl aliquot of plasma was collected from each animal. Plasma samples were frozen on dry ice and stored at -80°C.

Animals were then flush perfused with saline until blood was fully cleared and tissue was harvested as described below: after the completion of the perfusion protocol, the liver was harvested and snap frozen (two vials with 100mg of liver tissue from the left lateral lobe and one vial with the rest of the liver). Next, the whole brain was removed, weighed and micro-dissected into the frontal cortex, caudal cortex, striatum, cerebellum and the rest of the brain. The structures from both hemispheres were pooled into one vial and snap frozen.

Fixed samples for IHC/FISH (4 animals/group): After the completion of the perfusion protocol, the liver was harvested and post-fixed in 4% paraformaldehyde solution in a 30mL vial for a minimum of 48 hours. Next, the brains were immediately harvested and placed into a 30mL vial with 4% paraformaldehyde, the tissue to fixative ratio was at least 1:20. After 24 hours, the brain was dissected into two. Both pieces were placed back into 4% formaldehyde for a minimum of 12 hours and maximum of 24 hours.

After 48 hours, both the liver and brain pieces were then transferred to 70% ethanol, stored at 4°C until paraffin embedding.

Results

Example 3

Vectorized anti-II-1β is detected in serum of RAK KO mice

As shown in Figure 3B, rAAV5-delivered anti-II-1β antibody is expressed in RAK KO mice. Already after three weeks all the groups reached >10ug/ml of anti-II-1β in serum with a continuous concentration increase over the in live phase of 8 weeks. Highest concentrations measured in the high group was 950ug/ml to 1500ug/ml. In the low group, concentrations were in the range of 25ug/ml up to 80ug/ml after 8 weeks, which already in a low administered dose could result in a potential therapeutic level for anti-II-1β. With the in-vivo study a safe and dose-dependent expression of vAb was established after i.v. injection in RAK2-Ko mice. The antibodies are expressed and secreted efficiently over a period of 8 weeks and can be found in the serum at relevant therapeutic levels which might help to establish a steady delivery of the therapy, avoiding peaks associated with passive antibody therapy.

Example 4

Vectorized anti-II-1β suppresses II-1 β signaling in vitro

In addition, the vectorized anti-II-1β was able to suppress II-1β signaling in vitro up to 1µg/ml before going back to baseline at 0.5ug/ml when added to the HEK293Blue II-1β cell line (Figure 4). This is in line with the positive control where purified, vectorized anti-II-1β was spiked in RAK2 KO mouse serum. As negative

control an irrelevant human IgG (human anti-alpha synuclein, FJ2030), produced at FairJourney biologics was used which did not lead to any signal decrease. This shows that rAAV5-delivered anti-IL-1 β is functional.

Example 5

5 Vectorized antibodies are expressed in brain regions

To address whether vAb can be expressed in brain regions, rAAV5 was used as a vector, which was delivered via intrastriatal administration. Antibody expression levels and histopathology were assessed after rAAV5-vAb administration to the striatum in adult wild type mice. Vectorized antibodies were detected both in striatal and cortical samples (Figure 5A and 5B). In the high-dose treatment groups (Group 3 and 10 Group 6) the antibody could also be detected in the serum samples (Figure 5C). Baseline serum samples from these group of animals were all undetected. The data in Figure 5C is presented in log scale to better visualize the differences between measurements.

rAAV5 particles: parental rAAV5 (rAAV5 WT) vs rAAV5 M569V

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Plasmid generation

pRC5, encoding the rAAV5 capsid, was obtained commercially (Cell Biolabs). pRC5 M569V was generated by site-directed mutagenesis PCR. In brief, pRC5 wild-type was amplified by Q5 Polymerase (NEB) using mutagenesis primers SEK001 and SEK002. After confirmation of correct amplicon size by 1% agarose gel 20 electrophoresis and SYBR safe DNA visualization, the amplicon was extracted from the gel and purified using NucleoSpin Gel and PCR purification kit. Purified PCR amplicon was enzymatically treated using a site-directed mutagenesis kit (NEB) prior to transformation into NEB stable *E. coli* cells. Individual clones were picked and plasmid DNA extracted from overnight cultures. Sequence identity of mutagenized *cap* sequence was confirmed by sanger sequencing and plasmid integrity by restriction digestion analysis.

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rAAV generation

rAAV material was generated by Sirion Biotech GmbH (Germany). pRC plasmids and pLTR plasmids were provided by uniQure. In brief, 10-stack CellFactories were used for culturing of HEK293T cells. Cells were transfected with pRC, pLTR and pHelper using PEI as transfection reagent. After 48 hours at 37°C and 5% 30 CO₂ rAAV was harvested from cell pellets by 0.1% Triton lysis and viral particles purified from crude lysates using POROS CaptureSelect AAV-X. Genome-containing capsids were enriched by iodixanol gradient centrifugation. Purified virus was subsequently concentrated using Amicon ULTRA 15 filter units and formulated in PBS + 0.001% Pluronic. Viral Titers were determined by qPCR on LightCycler 480 using ITR specific PCR primers. Capsid integrity was confirmed by SDS-PAGE electrophoresis and genome integrity 35 by electrophoresis on 0.8% agarose gels.

Mouse study

All experiments were carried out according to the guidelines for the care and use of experimental animals. Male C57Bl6 were bi-laterally injected into the striatum with a volume of 3 μ l (virus stock concentration of 2e13 GC/ml) per hemisphere, resulting in a total dose of 1.3e11 genome copies per animal. Animals were sacrificed 4 weeks post-injection and brain and liver tissues extracted. Brain tissues were fixated in PFA and subsequently paraffin embedded for histological sectioning and staining. Liver tissues were snap frozen and subsequently pulverized for extraction of DNA and RNA. Sectioned brain tissues were prepared for antibody-based staining using HRP-conjugated anti-eGFP antibodies.

MiniPig Study

All experiments were carried out according to the guidelines for the care and use of experimental animals and approved by the State Veterinary Administration of the Czech Republic. Male minipigs 6–9 months old were used. General anesthesia of minipigs was induced followed by intravenous ear cannulation and intubation. Artificial ventilation and isoflurane/nitrous oxide anesthesia were used during the rest of the procedure. The head of animals was mounted into a stereotaxic localizer box. Exact coordinates for AAV constructs putamen injections as well as the injection devices (syringe, CED needle, etc.) were determined in previous similar experiments with Libechov minipigs of the same age. Craniotomy and dural incision were performed by oscillation saw, neurosurgery mallet, and chisel. rAAV particles as well as formulation buffer were delivered by a 500- μ L Hamilton syringe with 34G beveled NanoFil needle (World Precision Instruments; NF34BV) with 185 μ m optical density (OD), placed into the Injection Robot (Neurostar, Germany) and mounted onto stereotaxic localizer box. Single injection of rAAV particles or formulation buffer was administered to each animal, with injection rate increasing every minute until reaching the final volume of 50 μ L per putamen (first min: 1 μ L/min, second min: 2 μ L/min, etc.). The skull was closed by a bone graft and cement, periosteum stitching, and skin by suturing. Perioperative as well as postoperative antibiotic and analgesic were administrated.

At the end of the experiments the animals were anesthetized and then euthanized by an overdose of propofol. Immediately after overdosing, the pigs were transcardially perfused with 10 L of ice-cold PBS. The entire brain was removed from the skull and placed into a brain matrix and coronally sliced into 3–4 mm blocks. Each brain slice were split into the left and right hemisphere. The right hemisphere brain slices were post-fixed by immersion in 4% paraformaldehyde (PFA) in PBS, followed by sucrose (30%, with azide) cryopreservation, cryostat cutting (20–30 μ m), for histological examination and immunohistochemistry. From the left hemisphere brain slices, a total 46 brain punches were taken for biomolecular analysis. A sample from three sections of spinal cord and from the liver was also collected. The samples (liver, brain punches, and spinal cord transversal sections) for molecular analysis were immediately snap-frozen in liquid nitrogen and stored at –80°C.

Tissue punches were crushed using CryoPrep System (Covaris, Woburn, MA, USA), and powder was divided for RNA and DNA analyses. For combined genomic DNA and total RNA isolation, 10 mg of powdered tissue was used with the AllPrep DNA/RNA 96-well kit (QIAGEN, Germany). Purified genomic DNA was spectroscopically quantified and DNA concentrations normalized for each sample. Primer/Probe combinations specific for the CAG promoter were used to quantify amplification of the transgene in a TaqMan qPCR (Thermo Fisher Scientific, Waltham, MA, USA) on ABI 7500 FAST real-time PCR system (Applied Biosystems, Waltham, MA, USA). The amount of vector DNA was calculated from a plasmid standard curve, which was taken along on the same qPCR plate. Results were reported as GC per microgram of genomic DNA.

For quantification of transgene mRNA transcripts, AllPrep-isolated total RNA was spectroscopically quantified and RNA concentrations normalized for each sample. After normalization, RNA samples were DNase I treated in order to remove residual contaminating DNA. cDNA was synthesized from total RNA using Maxima Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). Primer/Probe combinations specific for the eGFP transgene sequence were used to quantify amplification of the transgene in a TaqMan qPCR (Thermo Fisher Scientific, Waltham, MA, USA) on ABI 7500 FAST real-time PCR system (Applied Biosystems, Waltham, MA, USA). The amount of transgene transcript was calculated from a plasmid standard curve, which was taken along on the same qPCR plate. Results were reported as transcript copies per microgram of total RNA.

IBA-1 IHC

Cryopreserved brain slices were provided as described above. IBA-1 was detected and quantified using Rabbit anti-IBA-1 (Cat. #234003, polyclonal, Synaptic Systems GmbH).

Single nuclei sequencing

Single nuclei sequencing was performed by SingulOmics Corporation, New York, US. Nuclei were extracted from frozen minipig brain tissue punches from frontal lobe white matter, caudate nucleus and cingulate cortex. Regions from three animals were pooled to obtain ~5000 nuclei per sample. 3' single-cell gene expression libraries (Next GEM v3.1) were generated using the 10x Genomics Chromium system. Subsequently, the libraries underwent sequencing, producing approximately 400 million paired-end 150-base reads per sample on the Illumina NovaSeq platform. The sequencing data were then analyzed against the minipig reference genome (GCA_000003025.6) with the incorporation of the eGFP transgene.

Secreted Alkaline Phosphatase Activity Assay

Huh7 cells were seeded with 1e5 cells per well in 48-well plates 24 hours prior to rAAV transduction. After 24 hours, seeding medium was replaced with Adenovirus Ad5-supplemented DMEM at MOI 30. Parental rAAV5 particles (rAAV5 WT) or rAAV5 M569V particles were added to culture medium at MOI of 1e5

genome copies per cell. Culture medium was sampled after 24 and 48 hours respectively and phosphatase activity determined using Phospha-Light™ SEAP Reporter Gene Assay System (Thermo Fisher).

Transduction Efficiency via eGFP flowcytometric detection

- 5 HEK293T cells were seeded with 1e5 cells per well in 48-well plates 24 hours prior to rAAV transduction. After 24 hours, seeding medium was replaced with Adenovirus Ad5-supplemented DMEM at MOI 30. Parental rAAV5 particles (rAAV5 WT) or rAAV5 M569V particles were added to culture medium at MOI of 1e5 or 1e6 genome copies per cell. After 48 hrs cells were physically dislodged from wells using dPBS. Dislodged cells were collected in 2 ml Eppendorf tubes and subsequently washed twice with dPBS.
- 10 stained with LIVE/DEAD stain (Thermo Fisher) prior to flow cytometric analysis. Number of eGFP positive cells in viable cell population was measured using a flow cytometer (BD Biosciences).

Organoid long-term culture

- iPSC-derived brain organoids were provided by the Amsterdam Medical Center (AMC) and maintained in
- 15 maintenance medium for the course of the experiment. Two brain organoids were deposited per well in a 12-well plate and subsequently infected with either parental rAAV5 (rAAV5 WT) particles or rAAV5 M569V particles at MOI of 5e5 GC/cell. Organoids were first enzymatically and then physically dissociated after 1, 2 and 3 weeks post-injection. Single cells were stained using LIVE/DEAD viability stain and subsequently number of eGFP positive cells determined among viable cell population using a flow cytometer (BD
- 20 Biosciences).

Neuraminidase assay

- HEK293T cells were seeded with a seeding density of 1e5 cells/well in 48-well plates 24 hours prior to neuraminidase treatment rAAV transduction. After 24 hours, seeding medium was replaced with
- 25 Adenovirus Ad5-supplemented DMEM at MOI 30 and incubated for 2 hours at 37°C. Adenovirus-supplemented medium was removed and 1x Glycobuffer supplemented with 10 U Neuraminidase A (New England Biolabs) added to assigned wells. Cells were incubated for 90 minutes at 37°C after which neuraminidase was removed from wells and DMEM-FBS supplemented with either parental rAAV5 particles (rAAV5 WT) or rAAV5 M569V particles (MOI 1e5 gc/cell). Cells were incubated for 48 hours at 37°C after
- 30 which culture medium was sampled and secreted nanoLuciferase activity measured using a NanoLuc® Luciferase activity kit (Promega).

Antibody-mediated AAVR neutralization Assay

- Huh7 cells were seeded with a seeding density of 1e5 cells/well in 48-well plates 24 hours prior to rAAV
- 35 transduction. After 24 hours, Anti-AAVR antibody (ab105385) or IgG isotype control (both from Abcam, Cambridge, CA) were incubated with cells at concentrations ranging from 0 to 5 µg/ml in DMEM media for 1 hr at 4°C. Following incubation, cells were co-transduced with Adenovirus 5 (MOI 30) and either parental

rAAV5 particles (rAAV5 WT) or rAAV5 M569V particles (both MOI 1e5) and incubated for 48 hours at 37°C. Subsequently culture medium was sampled and secreted nanoLuciferase activity measured using a NanoLuc® Luciferase activity kit (Promega).

5 **Results**

Example 6

Transduction capacity of rAAV5 M569V capsid is impaired and *in vitro* and independent of SIA as attachment glycan.

10 An *in vitro* experiment was performed to recapitulate the results of Afione et al. (2015, J Virol. 89(3):1660-72) and reduced transduction efficiency of rAAV5 M569V could indeed be observed, but only by 5 to 10-fold when compared with parental rAAV5 (rAAV5 WT) (Figures 6A and 6B). The reduced efficiency could also be observed in prolonged culture study using iPSC-derived cerebral organoids (Figure 6C). Nevertheless, dose and time-dependent increment of reporter signals could still be observed, indicating the
15 intact functionality of the rAAV5 M569V capsid asides from the potential switch of attachment factor. However, the main entry receptor remained intact, as antibody blockage of cellular AAVR would still further abrupt the capsid transduction (Figure 6D). In order to assess whether the M569V mutation in rAAV5 M569V capsid causes a loss of SIA-dependency, HEK293T cells were pre-treated with Sialidase, an enzyme catalyzing the cleavage of glycan chains, before being transduced with either parental rAAV5
20 (rAAV5 WT) or rAAV5 M569V. Indeed, transient removal of sialic acid from target cells resulted in a 2-4 fold reduction of parental rAAV5 (rAAV5 WT) transduction, while rAAV5 M569V was not affected and displayed same transduction levels between transiently sialidase treated and untreated cells (Figure 6E). This finding confirms the observation by Afione et al. that rAAV5 M569V displays SIA-independent capsid functionality.

25

Example 7

Intrastriatal delivery of rAAV5 M569V capsid demonstrates enhanced mouse brain transduction with reduced peripheral off targeting

In order to compare parental rAAV5 (rAAV5 WT) and rAAV5 M569V *in vivo*, the parental rAAV5 and rAAV5
30 M569V capsid were each administered to four C57BL/6 mice via intrastriatal injection at a dose of 1.3e11 genome copies per animal. After 4 weeks in-life the animals were sacrificed for extraction of brain and liver tissues. By immunohistological detection of the eGFP protein in the brain, we surprisingly found increased transduction with rAAV5 M569V compared to parental rAAV5 (Figure 7A). The brains of the animals injected with rAAV5 M569V displayed a more homogeneous and widespread distribution of transduced cells. In
35 addition, liver targeting of parental rAAV5 and rAAV5 M569V capsids was assessed by molecular analysis quantifying the presence of vector DNA. Intriguingly, the rAAV5 M569V vector copy quantification (in GC/μg DNA) in liver tissue showed a 200-fold reduction compared to parental rAAV5 (Figure 7B).

Example 8**Intraputaminal delivery of rAAV5 M569V capsid conjointly demonstrates enhanced minipig brain transduction**

5 Based on the findings obtained in mice we sought to assess whether the phenotype of enhanced transduction efficiency could be translated to large animals. Minipigs were studied according to a previous internal study by Evers et al. (Mol Ther. 2018 Sep 5;26(9):2163-2177). The pigs were intraparenchymally injected into the putamen with either parental rAAV5 or rAAV5 M569V at a dose of 2×10^{12} genome copies per animal (1×10^{12} GCs/hemisphere). Following an in life-phase of 4 weeks the animals were sacrificed for the

10 extraction of brain and liver tissues. Transduction efficiency in terms of vDNA levels was determined via molecular analysis of brain tissues (Figure 8A) and expression of eGFP was quantified using RT-qPCR (Figure 8B) as well as immunohistochemical detection (Figure 8D). Analysis of vector copy numbers in tissue punches across the brain showed on average a 10-fold increase of vDNA/ μ g total DNA for rAAV5 M569V compared to parental rAAV5 (Figure 8A). In agreement with vector copy quantification results, RT-

15 qPCR quantification of eGFP mRNA demonstrated a significant difference in transgene transcript levels between tissue punches of animals injected with parental rAAV5 and animals injected with rAAV5 M569V. In punches closer to the injection site (indicated by arrows in Figure 8A and 8B), tissues derived from animals that received rAAV5 M569V expressed up to a 100-fold higher transcript levels compared to tissues derived from animals that received parental rAAV5. On average, transcript levels were 10-fold higher in

20 tissue derived from animals that were injected with rAAV5 M569V compared to animals that were injected with parental rAAV5 (Figure 8B). Of note, expression of eGFP could already be observed with the naked eye in brain slices derived from animals that had received rAAV5 M569V, whereas this was not the case for brain slices derived from animals that had received parental rAAV5 (Figure 8C). This observation led to the expectation that eGFP would be expressed in higher levels in brain tissue derived from animals injected

25 with rAAV5 M569V. This was indeed confirmed using immunohistochemical analysis, which also displayed more distinct and higher eGFP levels across the brains of animals that had received rAAV5 M569V-injected (Figure 8D).

Intraputaminal delivery of rAAV5 M569V capsid exhibits a favorable immune activation profile compared to parental rAAV5

30 In order to obtain information about the immune reaction raised upon injection with rAAV5 M569V, a tissue punch of the injection site was used for extraction of total RNA which was subsequently sequenced for transcriptome analysis. Differential gene expression analysis was performed on transcriptome data of injection site punches either from parental rAAV5 or rAAV5 M569V injected animals. In total, 235 genes

35 were found to be upregulated in parental rAAV5 injected brain tissues when compared to rAAV5 M569V tissues, which could be confidently assigned to 133 known pathways. Of those 235 genes ~20% were related to inflammation and immune responses (Figure 9). In contrast, only 11 genes appeared upregulated

in rAAV5 M569V injected brain tissue of which none were related to immune responses. These results suggest that the rAAV5 M569V capsid is less immunogenic than the parental rAAV5.

The decreased immunogenicity of rAAV5 M569V compared to parental rAAV5 in minipigs was further confirmed using an ionized calcium-binding adapter (IBA)-1 staining (Figure 10A and B) as well as single nuclei sequencing (Figure 11). IBA-1 is an inflammatory marker found in activated macrophages, and was prominently stained in the lower three panels of Figure 10A. These three panels represent brain slices of three individual minipigs that were injected with parental rAAV5. Of note, the upper three panels, representing brain slices of three individual minipigs that were injected with rAAV5 M569V, show little to no IBA-1 staining (Figure 10A). This is confirmed in Figure 10B, where in the left graph the total lesion and inflammation area is plotted, and in the right graphs the percentage of IBA-1 positive staining in the region of interest. In both graphs of Figure 10B brain slices of rAAV5 M569V score comparable to vehicle-treated animals, whereas brain slices of animals that received parental rAAV5 do show signs of inflammation.

In addition, single nuclei sequencing was performed. This was done with the aim to assess the abundance of immune cells in the minipig frontal lobe white matter, caudate nucleus, and cingulate cortex. The resulting cell clusters were categorized into nine distinct cell types (upper left 6 panels of Figure 11), including B-cells (upper right six panels of Figure 11), T-cells (lower left 6 panels of Figure 11), and microglia/perivascular macrophages (lower right 6 panels of Figure 11). Notably, immune-related cell types were found to be more prevalent in minipigs that were injected with parental rAAV5 (bottom three panels for each cell type) compared to those injected with rAAV5 M569V (top three panels for each cell type) across all three brain regions. Further analysis of the clusters revealed a substantial increase, with up to 10-fold more T-cells, 14-fold more B-cells, and a 2-fold higher abundance of microglia in animals that were injected with parental rAAV5-injected compared to animals injected with rAAV5 M569V (data not shown). Taken together these findings suggest that the rAAV5 M569V capsid exhibits lower immunogenicity compared to the parental rAAV5 capsid.

Non-Human Primate Study

The goal of this study was to investigate the feasibility, safety, and biodistribution of rAAV2/5-miSNCA and rAAV2/5-M569V-miSCNA in non-human primates after MRI-guided intra-putamen injections. This study provides information on e.g. the biodistribution of vector DNA and miRNA in brain and off-target organs as well as immune responses raised against the different capsids.

As part of this study, fifteen cynomolgus monkeys received bilateral injections into the putamen using real-time MRI guided injections and ClearPoint Neuro Smartflow Cannulas. The study complies with all applicable sections of the current version of the Final Rules of the Animal Welfare Act Regulations (9 CFR), and the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 8th edition.

Table 7 – study design NHP study

Group	Number of Animals	Vehicle / Test Article	Nominal Dose Level (gc/brain)	Nominal Dose Concentration (gc/mL)	Volume Per Hemisphere (µL)	Necropsy Study Day
1	1M/2F	Vehicle	0.00	0.00	Putamen: 150	57 ± 3
2	2M/1F	rAAV2/5	1.1E12	2.0E12	Putamen: 150	57 ± 3
3	1M/2F	rAAV2/5	4.8E12	1.0E13	Putamen: 150	57 ± 3
4	2M/1F	rAAV2/5	2.3E13	5.0E13	Putamen: 150	57 ± 3
5	2M/1F	rAAV2/5-M569V	2.0E12	1.0E13	Putamen: 150	57 ± 3

In-life observations and measurements included clinical observations, body weight, and clinical pathology evaluations as described elsewhere in this protocol. Samples were collected for biodistribution, antibody analysis, and cytokine analysis. On Study Day 57 ± 3, the animals were euthanized, and selected tissues were harvested for histopathology and biodistribution evaluation.

Dose administration was performed using real-time MRI guided injections targeting the putamen (bilateral). Prior to dose administration, the animals received one dose of dexamethasone (1-2mg/kg) to prevent swelling of the brain. An occipital approach was used for targeting the putamen. The two hemispheres were injected simultaneously. The dose was administered via a calibrated MRI-compatible infusion pump (Harvard Apparatus Model PHD 2000 Infuse/Withdraw). The dosing syringe containing dosing solution was attached directly to the cannula. The dose volume was administered by convection enhanced delivery. The following clinical pathology tests were conducted on available samples and as survival allows.

Whole blood samples were collected from an appropriate blood vessel into a lithium heparin vacutainer tube for PBMC isolation pre-study and prior to necropsy. For the non-terminal collection, ~6 mL of blood was collected. For the terminal collection, ~10 mL of blood was collected while the animal is under anaesthesia for necropsy.

Cerebrospinal Fluid (CSF) was collected using a Cisterna Magna Tap, at a volume of 0.75 mL at scheduled collection intervals, and was stored on ice.

Table 8 – parameters studies per sample type

Sample Type	Parameters Evaluated
Hematology	- Red blood cell count - Hematocrit - Hemoglobin - Mean corpuscular hemoglobin - Mean corpuscular hemoglobin - Platelet count - Mean Platelet Volume - White blood cell count (absolute and differential) - Reticulocytes count (absolute and

Sample Type	Parameters Evaluated	
	concentration (calculated) • Mean corpuscular volume	differential
Clinical Chemistry	• A/G (albumin/globulin) ratio (calculated) • Alanine aminotransferase • Albumin • Alkaline phosphatase • Aspartate aminotransferase • Calcium • Creatinine • Electrolytes (sodium, potassium, chloride)	• Gamma glutamyl transferase • Globulin (calculated) • Glucose • Phosphorus • Total bilirubin • Total cholesterol • Total protein • Triglycerides • Urea nitrogen
CSF Total Cell Count and Chemistry	• Color and clarity • Erythrocyte count • Leukocyte count (total and differential) • Total protein • Glucose	• Albumin • Calcium • Chloride • Sodium • Potassium • Phosphate

Whole blood samples as well as CSF samples were used to measure GFAP, Tau and Nfl protein levels using an S-Plex assay. Serum cytokine analysis was performed using the U-plex kit measuring IFN- γ , IL-1 β , IL-6, IL-8, MCP-1, TNF- α and YKL-40 (Mesoscale). Additionally, IgG specific rAAV5 capsid response analysis was performed.

All animals were euthanized and perfused. The animals were sedated per SOP-SRG-001, administered an intravenous bolus of heparin sodium (200 IU/kg), and euthanized with an IV overdose of Pentobarbital (e.g., Fatal-Plus®). The animals were then perfused via the left cardiac ventricle with chilled 0.001% sodium nitrite in saline.

Tissues designated for macroscopic evaluation and collection were saved in 10% neutral buffered formalin (NBF), unless otherwise stated. Testes and epididymides designated for microscopic evaluation were saved in modified Davidson's fixative. After 24 – 48 hours, the tissues were transferred to 10% NBF. Brain slices designated for microscopic evaluation were saved in 10% NBF for 24 $\pm$ 4 hours, then transferred to 70% ethanol and stored refrigerated (2-8°C) until shipment for paraffin embedding. Paraffin embedding occurred within 7 days of collection. Tissue samples designated for biodistribution analyses (DNA, miRNA, and protein) and RNA Extraction (miRNA, mRNA) were frozen on dry ice and stored in an ultra-low temperature freezer.

Following removal from the skull, the brain was placed in a chilled brain matrix and sliced coronally at approximately 4 mm slice thickness. Each brain slice was hemisected. The brain slices from the left hemisphere were placed in labelled cassettes and saved in 10% NBF.

Brain slices from the right hemisphere were laid out on a chilled surface for biodistribution, alpha synuclein protein measurement and RNA Extraction punch sample collection. Photographs were taken prior to and after tissue punch collection. Each sample was collected using a new biopsy punch, weighed, placed in a labelled vial, frozen on dry ice, and stored in an ultra-low temperature freezer. The residual brain slice, following sample collection was placed in a labelled cassette and stored in an ultra-low temperature freezer until shipment.

A total of 42 brain punches was taken from the right hemisphere brain slices. A list of all the brain punches taken is provided in Table 9. Punches of 3 mm or 5mm were taken from each brain region listed in Table 9. 5mm punches were taken as single punches. Of the 3mm punches, two were taken from the brain regions provided in Table 9 and labelled as "A" and "B". "A" punches were used for measuring alpha synuclein (α -syn) protein levels and "B" punches were used for isolation of RNA/DNA and eventual molecular read outs (vector DNA, mRNA and microRNA).

Table 9 - Brain punches taken from the right hemisphere

Brain slice #	Punch Number	Limbic system	Brain region (L2)	Brain region (L3)
2	1	Cerebral cortex	Frontal Lobe	Inferior cingulate gyrus (IFG)
3	2	Cerebral cortex	Frontal Lobe	Superior frontal gyrus (SFG)
3	3	Cerebral cortex	Frontal Lobe	Inferior cingulate gyrus (IFG)
3	4A+B	Cerebral cortex	Frontal Lobe	Anterior cingulate gyrus (ACgG)
4	5	Cerebral cortex	Frontal Lobe	Anterior cingulate gyrus (ACgG)
4	6	White matter	White matter	White matter
4	7A+B	Cerebral cortex	Frontal Lobe	Precentral gyrus (PrG) (= primary motor cortex)
5	8A+B	The basal ganglia	The corpus striatum	Head of caudate nucleus (HCd)
5	9A+B	The basal ganglia	The corpus striatum	Putamen
5	10A+B	Limbic system	The basal ganglia	Nucleus accumbens (Acb)
5	11	Cerebral cortex	Temporal Lobe	Superior temporal gyrus (STG)
5	12	Cerebral cortex	Temporal Lobe	Periamygdaloid area (PA)
6	13A+B	The basal ganglia	The corpus striatum	Head of caudate nucleus (HCd)
		White matter	White matter	internal capsule (IC)
6	14A+B	The basal ganglia	The corpus striatum	Putamen

6	15A+B	The basal ganglia	The basal ganglia	Lateral Globus Pallidus (LGP)
6	16A+B	Cerebral cortex	Lateral sulcus	Insula (= the insular cortex)
6	17A+B	Cerebral cortex	Frontal Lobe	Precentral gyrus (PrG) (= primary motor cortex)
6	18A+B	Limbic system	Mesial temporal lobe	Amygdala (Amg)
7	19	White matter	White matter	White matter
7	20	Cerebral cortex	Lateral sulcus	Insula (= the insular cortex)
7	21A+B	The basal ganglia	The corpus striatum	Putamen
7	22A+B	The basal ganglia	The basal ganglia	Globus Pallidus
8	23	Cerebral cortex	Frontal Lobe	Precentral gyrus (PrG) (= primary motor cortex)
8	24	White matter	White matter	White matter
8	25A+B	The basal ganglia	The corpus striatum	Putamen
8	26A+B	Limbic system	Thalamus	Thalamus
8	27A+B	Midbrain	The basal ganglia	Substantia Nigra
8	28A+B	Limbic system	Hippocampus	Hippocampus
9	29A+B	Cerebral cortex	Parietal Lobe	Supramarginal gyrus (SMG) (Brodmann area 40)
9	30A+B	Limbic system	Thalamus	Pulvinar nuclei
9	31	Midbrain	The basal ganglia	Mesencephalic nuclei
9	32	Temporal lobe	Hippocampus	Hippocampus
10	33	Cerebral cortex	Parietal Lobe	Superior parietal lobule (SPL) (contains Brodmann's areas 5 and 7)
10	34	Cerebral cortex	Parietal Lobe	Supramarginal gyrus (SMG) (Brodmann area 40)
10	35	Midbrain	Tectal plate	Superior colliculus (SC) Inferior colliculus (IC)
10	36A+B	crf5	Pons	Pons (ventral)
11	37A+B	Brainstem	Medulla	Medulla
12	38	Cerebellum	Cerebellar cortex	Cerebellar cortex
12	39	Brainstem	Brainstem	Brainstem
13	40A+B	Cerebellum	Cerebellar nuclei	Dentate Nucleus
13	41A+B	Cerebellum	Cerebellar cortex	Cerebellar cortex
13	42A+B	Brainstem	Brainstem	Brainstem

DNA and RNA extraction from NHP tissue

Tissue punches were crushed using CryoPrep System (Covaris, Woburn, MA, USA), and powder was divided for RNA and DNA analyses. For combined genomic DNA and total RNA isolation, 10 mg of powdered tissue was used with the AllPrep DNA/RNA 96-well kit (QIAGEN, Germany). Purified genomic DNA was spectroscopically quantified and DNA concentrations normalized for each sample. Primer/Probe

combinations specific for the CAG promoter were used to quantify amplification of the transgene in a TaqMan qPCR (Thermo Fisher Scientific, Waltham, MA, USA) on ABI 7500 FAST real-time PCR system (Applied Biosystems, Waltham, MA, USA). The amount of vector DNA was calculated from a plasmid standard curve, which was taken along on the same qPCR plate. Results were reported as GC per microgram of genomic DNA. For quantification of transgene mRNA transcripts, AllPrep-isolated total RNA was spectroscopically quantified and RNA concentrations normalized for each sample. After normalization, RNA samples were DNase I treated in order to remove residual contaminating DNA. cDNA was synthesized from total RNA using Maxima Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). Primer/Probe combinations specific for the eGFP transgene sequence were used to quantify amplification of the transgene in a TaqMan qPCR (Thermo Fisher Scientific, Waltham, MA, USA) on ABI 7500 FAST real-time PCR system (Applied Biosystems, Waltham, MA, USA). The amount of transgene transcript was calculated from a plasmid standard curve, which was taken along on the same qPCR plate. Results were reported as transcript copies per microgram of total RNA.

qPCR method for quantification of vector genome copy levels in NHP tissue and biofluid samples

After extraction, the concentration of the DNA samples was normalized to ensure equal input for the quantitative polymerase chain reaction (qPCR). TaqMan assay with specific primers (Fw: 5' - CAT CGG CGC TAT GCT TCC T- 3' (SEQ ID NO: 30); Rev: 5' - GTG TCT CAT CGC AAA CTT ACA GTA TAT G-3' (SEQ ID NO: 31) and probe (5' - /56-FAM/TGA AAT CCC AGC CAG GG-MGBNFQ - 3' (SEQ ID NO: 32)) for the vector DNA was used for absolute quantification with TaqMan™ Fast Advanced Master Mix (Applied Biosystems, P/N 4444557 or equivalent part number). For absolute quantification, 8 dilutions (108, 107, 106, 105, 104, 103, 100 and 50 copies) of the linearized plasmid (Genewiz, Lot no.: 90-792660955-1) were prepared. The qPCR was performed using a QuantStudio™ 7 Flex System (ThermoFisher). The lower limit of quantification (LLOQ) was defined as 50 genome copies per 1000 ng of gDNA per reaction.

Stem-loop RT-qPCR method for quantification of Vector-derived miSNCA15 in NHP tissues and biofluid samples

After extraction, RNA concentrations were normalized to ensure equal input prior to a two-step reverse transcription qPCR (RT-qPCR). RNA was treated with DNase I and reverse transcribed with miSNCA15 RT primer (Custom TaqMan Small RNA Assay, Applied Biosystems, P/N 4398988, assay ID CT2W72H) using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, P/N 4366596 or equivalent) following the suppliers' protocol. The synthesized cDNA was diluted to 5 ng of RNA equivalent per reaction and TaqMan™ Fast Advanced Master Mix (Applied Biosystems, P/N 44445657) was used with primers/probe (Custom TaqMan Small RNA Assay, Applied Biosystems, P/N 4398988, assay ID CT2W72H) for qPCR reaction. For absolute quantification, 11 dilutions (108, 107, 106, 105, 104, 103, 100, 50, 25, 10, 5 copies) of the miSNCA15 22nt Synthetic RNA Oligo (GenScript) (5'- UUG CCC AAC UGG UCC UUU UUG A -3' (SEQ ID NO: 33)) were prepared. The qPCR was performed using a QuantStudio™

7 Flex System (ThermoFisher). The lower limit of quantification (LLOQ) was defined as 25 copies of miSCNA15 RNA oligo per 5 ng RNA per reaction.

RT-dPCR method for quantification of endogenous SNCA mRNA expression levels in NHP tissues and biofluids samples

Similar to described above, RNA concentrations were normalized to ensure equal input prior to a one-step reverse transcription dPCR (RT-dPCR). RNA samples were treated with DNase I. The SNCA mRNA levels were then measured with QIAcuity Digital PCR System (Qiagen) using QIAcuity OneStep Advanced Probe Kit (Qiagen P/N 250131 or 250132) and SNCA TaqMan Gene Expression Assay primer and probe kit (Applied Biosystems P/N 4351368, Assay ID Mf02793033_m1). The lower limit of quantification (LLOQ) was defined as 151 copies per 100 ng of RNA per reaction.

Protein quantification

A subset of “A” brain punches that were analyzed for the alpha synuclein protein levels is listed in Table 10. Briefly, proteins were extracted from frozen punches by addition of RIPA Lysis buffer into each tissue punch and subsequent agitation using Qiagen TissueLyzer. The crude lysates were then clarified by centrifugation and the supernatant containing soluble proteins was used to measure alpha synuclein with an electrochemiluminescence ELISA kit from MSD from Meso Scale Discovery (U-PLEX Human α -Synuclein Kit; catalog number K151WKK). Next, the alpha synuclein levels were extrapolated using the standard curve generated in each plate. The total protein concentration was measured using a BCA (Bicinchoninic Acid) assay. In order to obtain a normalized value for each measurement, similar amounts of total protein were added into each MSD well.

Table 10 Brain punches that were analyzed for alpha synuclein protein

Punch # on punching scheme and the graph	Corresponding brain region	25
8	Head of caudate nucleus (HCd)	
9	Putamen	
10	Nucleus accumbens (Acb)	
13	Head of caudate nucleus (HCd) + internal capsule (IC)	30
14	Putamen	
22	Globus Pallidus	
25	Putamen	
26	Thalamus	
27	Substantia Nigra (same)	
28	Hippocampus	
29	(SMG) (Brodmann area 40) (same)	35

The spinal cord with associated dorsal root ganglia (DRG) attached was divided into four segments (cervical, thoracic, lumbar, and sacral). An approximate 1 cm section from the rostral portion of each segment was collected. The DRGs were removed from each 1 cm section to yield at least 2 paired DRGs from each section. At least 2 DRGs from the left side of each 1 cm section and at least 2 DRGs from the right side of each 1 cm section were combined and saved for biodistribution. Combined DRGs were weighed, frozen on dry ice, and stored in an ultra-low temperature freezer. The residual 1 cm spinal cord section was saved for biodistribution. The samples were weighed, frozen on dry ice, and stored in an ultra-low temperature freezer. The caudal portion from each segment (DRGs remain intact) was designated for histopathology and saved in 10% NBF.

Samples (approximately 50 - 100 mg or entire tissue) of the adrenal gland (left), heart (left ventricle), kidney (left), liver (left lobe), lung (left caudal lobe), submandibular lymph nodes (left), deep cervical lymph nodes (left), ovary (left), spleen, and testis (left) were saved for biodistribution. The samples were weighed, frozen on dry ice, and stored in an ultra-low temperature freezer. One sample (approximately 50 - 100 mg) of the liver (left lobe) is saved for RNA extraction. The samples were weighed, frozen on dry ice, and stored in an ultra-low temperature freezer.

All brain slices were embedded in paraffin within 7 days of necropsy. Sections 5 – 8 of the brain (left hemisphere) from all animals were sectioned using a 5 micron block advance and stained with H&E. The remaining slices were retained in paraffin blocks.

The spinal cord from all animals was trimmed to include within the paraffin block a transverse and oblique section from each of the cervical, thoracic, lumbar, and sacral regions and stained with H&E.

DRGs with nerve roots [one pair per cervical, thoracic, lumbar, and sacral region] were embedded in paraffin, sectioned longitudinally using a 5- micron block advance, and stained with H&E.

All peripheral tissues scheduled for microscopic evaluation (including macroscopic lesions) were trimmed, embedded in paraffin, sectioned using a 5 micron block advance, and stained with H&E.

Example 9

Intrapataminal delivery of rAAV2/5 M569V capsid demonstrates enhanced NHP brain transduction vDNA

Based on the findings obtained in mice and minipigs it was assessed whether the phenotype of enhanced transduction efficiency could be translated to non-human primates (NHP). Fifteen cynomolgus monkeys were intraparenchymally injected into the putamen with either parental rAAV2/5 (low, mid, or high dose) or rAAV2/5 M569V (2E12 gc/animal). As also provided in Table 7, the low dose of parental rAAV2/5

constituted 1.1E12 gc/animal, mid dose 4.8E12 gc/animal and the high dose 2.3E13/animal. The dose of rAAV2/5 M569V (2.0E12 gc/animal) was positioned between the low dose (1.1E12 gc/animal) and the mid dose (4.8E12 gc/animal) of parental rAAV2/5. Following an in life-phase of approximately 57 days, the animals were sacrificed. Transduction efficiency (vDNA) was determined by molecular analysis of punches
5 derived from specific brain tissues (Figure 12 and 13). Analysis of vector copy numbers in tissue punches across the brain, expressed as genome copies per microgram of tissue (gc/ μ g), showed that rAAV2/5 M569V vector DNA vector copy numbers were slightly higher or comparable to the mid dose of the parental rAAV2/5 at punch sites close to the site of injection (putamen, punch number 25b) (Figure 13). Such sites include e.g. tissue punch numbers 17b, 21b, 22b, and 25b – 30b (Figure 13). These results suggest an
10 increased transduction efficiency, because the rAAV2/5 M569V dose was approximately 2-fold lower compared to the parental rAAV2/5 mid dose, yet gc/ μ g is higher or comparable. This indicates that the trend in increase induction efficiency which was previously established in minipigs (Figure 8A) also holds true for NHP.

miRNA

Expression of microRNA from the viral vector delivered either by parental rAAV2/5 or rAAV2/5 M569V was quantified using RT-qPCR (Figure 14). In punch numbers 13b, 16b, 17, 22b and 26b-30b miRNA levels in animals injected with rAAV2/5 M569V were slightly higher or comparable to the mid dose of the parental rAAV2/5. Interestingly, in punch numbers 26b, 27b and 28b, the rAAV2/5 M569V condition showed levels
20 of microRNA comparable to, or even higher than, the high dose parental rAAV2/5 (Figure 14). This means that even with an approximately 10-fold lower dose, a comparable or higher expression of the miRNA was obtained.

mRNA

Functionality of the miRNA was determined using an RT-dPCR assay, quantifying alpha synuclein mRNA levels (Figure 15). The average alpha synuclein mRNA level found in vehicle-treated animals was used as a reference (100%). It was found that alpha synuclein mRNA levels were most prominently decreased around the injection site (e.g. punches 15, 21 and 22). At these sites, animals injected with rAAV2/5 M569V showed mRNA levels lower than or similar to the brain punches obtained from animals that received an
30 approximately 10-fold higher dose of parental rAAV2/5.

Protein

Total alpha synuclein protein levels were determined using an ELISA-based assay (Figure 16). The average alpha synuclein protein level found in vehicle-treated animals was used as a reference (100%). Alpha
35 synuclein protein levels were most distinctly decreased in punch number 22, as well as in punch number 9, 26 and 29. At these sites, animals injected with rAAV2/5 M569V showed protein levels lower than or

similar to the brain punches obtained from animals that received an approximately 10-fold higher dose of parental rAAV2/5 (punch number 22), or a 2-fold higher dose (punch numbers 9, 26 and 29).

Taken together, the above described results suggest that using rAAV2/5 M569V more efficient transduction is obtained in terms of vDNA itself as well as the product expressed from the vDNA (miRNA). This translates into more efficient downregulation of the target mRNA, resulting in less expression of the protein encoded by the mRNA. Of note, the difference in transduction efficiency between parental rAAV2/5 and rAAV2/5 M569V is less pronounced in terms of vDNA, and more pronounced in the product expressed from the vDNA.

Mouse study

In this study, the expression of ApoE2-Jac and scFv anti-Tau from a single vector was evaluated after direct brain injection. A total of 5 treatment groups with 6 animals per group (total N=24) of male wild-type mice (C57BL/6J) 9-10 weeks of age were employed according to the set-up below, see Table 11. At day 0, rAAV5 was administered through bilateral intrastriatal (i.s.) injections. The group injected with only formulation buffer served as control group.

Table 11. Study groups for the in vivo pilot study

Group	Genotype	No. of mice	Compound name	Dose AAV (gc/animal)	Dosing	In-life phase (weeks)
1	WT	6	Formulation Buffer	N/A	i.s.	4
3	WT	6	rAAV5-anti-Tau scFv + APOE2-Jac	5.00E+10	i.s.	4
7	WT	6	rAAV5-scFV anti-Tau	5.00E+10	i.s.	4
8	WT	6	rAAV5-APOE2-Jac	5.00E+10	i.s.	4
9	WT	6	rAAV5-APOE2-Jac + anti-Tau scFv	5.00E+10	i.s.	4

Male wild type C57BL/6J mice arrived at the facility (uniQure, Groningen, Netherlands) at age of 8 weeks. Mice had an acclimatization period of one week prior to start of the experiment. Mice were group housed (3 males/cage) in Tecniplast (Green Line) IVC cages with a 12h:12h light:dark cycle (lights on 7AM, lights off 7PM). Temperature in the rooms was maintained at 20-23°C with a relative humidity of ~50%. Nesting material, a cardboard tube and wooden blocks were added to each cage as enrichment. Mice were weighed on the day before the start of the procedure and 1x per week. Chow and water were provided ad libitum. Animals received an RFID identification chip (Somark Innovations) in the tail for animal identification over the course of the study.

Mice were anesthetized with isoflurane. An automated stereotax (RWD71000, RWD) was used to inject drug products to target the striatum bilaterally. Animals were placed in a stereotaxic device and the head was levelled with a maximal height difference between Bregma and Lambda of 0.1 mm. A volume of 3 µL

was injected per hemisphere with a total volume of 6 μ L per brain. Injections were performed with an autoclavable 10 μ l syringe (701 RN, Hamilton) connected to an RN NEEDLE ((33G / 15mm / point style 4 with a 25 degree bevel) Hamilton). Injections occurred at a rate of 0.2 μ l/min with the needle left in place for at least 5 minutes following administration at each target. The needle was retracted at a rate of 0.05 mm/sec.

Animals underwent 4 mandibular vein (50 μ l) bleedings and 1 terminal bleeding. The first bleeding occurred prior to the start of the rAAV administration and subsequently at week1-week4 after administration. Serum samples were collected and prepared using a standard serum preparation procedure.

On day 28, animals were anesthetized with (5% induction, 2-3% maintenance) isoflurane in oxygen and CSF, plasma, liver and brain samples were harvested.

CSF was collected under anaesthesia. A stereotaxic device (RWD71000, RWD) was used to stereotaxically withdraw CSF through the cisterna magna. CSF collection was performed with a 10 μ l syringe (Hamilton) connected to a 30G metal Kel/F hub needle (Hamilton) at a rate of 2.0 μ l/min. After CSF collection, terminal blood was collected (>400-500 μ L) via closed cardiac puncture using a 1ml syringe with 30G needle. Whole blood was processed for plasma by using K2-EDTA tubes. A single 200 μ l aliquot of plasma was collected from each animal. Plasma samples were frozen on dry ice and stored at -80°C.

After terminal blood collection, animals were perfused. A 25G winged infusion set with protector (terumo) was attached to a peristaltic pump and used to puncture the heart. Animals were perfused with ice-cold saline (5mL/min for 6-10 minutes) until blood is fully cleared. After completion of the perfusion protocol, the head was cut with a pair of scissors and kept on ice, while the liver was harvested and snap frozen. Next, the whole brain was removed, weighed and micro-dissected into the frontal cortex, caudal cortex, striatum, hippocampus, cerebellum and rest of the brain. The structures from both hemispheres were pooled into one vial and snap frozen with liquid nitrogen.

rAAV5 vectors

The expression cassettes carrying the different constructs were subcloned into ITR containing plasmids generating the different rAAV5 vectors (Table 12).

Table 12. List of ITR containing plasmids generated

Transgene
Single chain Fv UCB 0107 + ApoE2b (D1)
ApoE2b + single chain Fv UCB 0107 (D2)
Single chain Fv UCB 0107
ApoE2b

All the above-listed plasmids carry a CMV enhancer and CBA promoter, an intron to support the promoter activity, the protein and antibody to be expressed and the polyA sequence.

5 rAAV5 harbouring the expression cassettes were produced by infecting SF+ insect cells (Protein Sciences Corporation, Meriden, Connecticut, USA) with two Baculoviruses, encoding Rep and Cap or Transgene. This was followed by a standard protein purification procedure on a fast protein liquid chromatography system (AKTA Explorer, GE 30 Healthcare) using AVB Sepharose (GE Healthcare). The titer was determined using qPCR.

10 **Molecular and Biochemical Assays**

DNA/RNA isolation from in vivo samples

For DNA and RNA isolation, AllPrep Mini Kit from Qiagen (Catalog no. 80204) was used in all experiments described, following manufacturer's protocol. RLT Plus lysis buffer was applied to pulverized animal tissue. The tissue suspension was then applied to Lysing Matrix D tubes with Matrix D beads (MP Biomedicals 15 116913100) and homogenized using TissueLyser system (Qiagen). The DNA and RNA were then isolated from this extract by applying it first to the DNA binding column from All Prep DNA/RNA mini kit. The flow-through from the DNA column was then added to the RNA binding column. After centrifugation- and washing steps, DNA and RNA was eluted from the corresponding columns. This way, DNA and RNA was isolated from the same sample. DNA and RNA quantity and integrity were determined by Nanodrop.

20

Protein extraction from in vivo samples

For protein isolation from in vivo samples, MSD Tris Lysis Buffer (Mesoscale Discovery, R60TX-2) containing EDTA-free protease inhibitor (Roche) was applied to the pulverized animal tissue. The tissue suspension was then applied to Lysing Matrix D tubes with Matrix D beads (MP Biomedicals 116913100) and homogenized using TissueLyser system (Qiagen). After centrifugation the extract was used for total protein measurement using Pierce™ BCA Protein Assay Kits (Thermo Fisher Scientific, 23225) and antibody and protein detection using MSD assays.

25

Vector DNA quantification from in vivo samples

30 Vector genome copies were quantified by using TaqMan qPCR assay (Thermo Fisher Scientific) with primers and probes against the CMV enhancer region of the vector, and primers and probes against mouse β -actin for housekeeping gene control (Table 13). The quantification (GC/ μ g DNA) was done using a linear plasmid to generate a standard curve with a dilution range of this plasmid. This standard curve was used to calculate the vector DNA copy number from DNA, isolated from in vivo tissue samples.

35

Table 13. Primer sequences for vDNA Taqman assays

qPCR assay	Forward primer	Reverse primer	Probe sequence
CMV enhancer	AGTAACGCCAATAGGGACTTTC (SEQ ID NO: 34)	GGCGTACTTGGCATATGATACA (SEQ ID NO: 35)	TTACGGTAAACTGCCCCACTTGCA (SEQ ID NO: 36)
Mouse β-actin	GACACTGGCACAGCCAACTTT (SEQ ID NO: 37)	GTGTCTACACCGCGGGAAATG (SEQ ID NO: 38)	CTAGCGTGTAGACTCT (SEQ ID NO: 39)

RT-qPCR assays for mRNA quantification from in vivo samples

cDNA synthesis was performed using the Maxima First Strand DNA Synthesis kit (Thermo Fisher Scientific) for RT-qPCR, according to manufacturer’s protocol. For the quantification of ApoE2 and scFv anti-Tau mRNA levels, RT-qPCR Taqman assays were used, consisting of a set of primers and probes designed against scFv anti-Tau and against human ApoE (Table 14). Probes against ApoE carried a CY5 reporter and probes against scFv anti-Tau carried an FAM reporter, allowing for simultaneous quantification of both transgenes in the same sample. The quantification (RNA copies/μg total RNA) was done using a dilution range of linearized plasmid to generate a standard curve. This standard curve was used to calculate the transgene copy number from in vivo samples.

Table 14. Primer sequences for RT-qPCR of in vivo samples

qPCR assay	Forward primer	Reverse Primer	Probe sequence
scFv anti-Tau	GTGTGCCTGATCGGTTTAGT (SEQ ID NO: 40)	TGAGTGGCCTGGAAACAATAG (SEQ ID NO: 41)	TACACACCCACATCTTCGGCCTC (SEQ ID NO: 42)
ApoE	GGAAGTGGAGGGCGCTGATG (SEQ ID NO: 43)	GCCTGCAGCTCCTTGGAC (SEQ ID NO: 44)	TCGGAAGTGGAGGAACAAGTGAAC (SEQ ID NO: 45)

Electrochemiluminescence ELISA for quantification of antibodies from in vivo samples

Two MSD (Mesoscale Discovery) assays were developed for measuring the amount of scFv and ApoE in in vivo tissue samples. For scFv detection, recombinant human Tau (Abcam, ab84700) was coated on MSD Multi-Array 96 Plates (Mesoscale Discovery). Tissue protein extracts were normalized for total protein concentration and added to the plate. scFv antibody was detected using mouse anti-His (BioXCell, 6-His), followed by Sulfo-tag labelled anti-mouse antibody (MSD, R32AC-1) and MSD gold read buffer A (Mesoscale Discovery). For ApoE detection, mouse anti-human ApoE (Mabtech, 83712-3-250) was coated on MSD Multi-Array 96 Plates. Normalized tissue protein extracts were added to the plate and ApoE protein was detected using biotinylated mouse anti-ApoE (Mabtech, 3712-6-250), followed by Sulfo-tag labelled streptavidin (MSD, R32AD-1) and MSD gold read buffer A. Detection of electrochemiluminescence (ECL) signal was done using the MESO QuickPlex SQ 120MM instrument (Mesoscale Discovery). To quantify the amount of scFv in the samples, purified scFv anti-Tau (custom produced by FairJourney Biologics) was used to create a standard line between 1-1000 ng antibody/mL. To quantify ApoE levels, a standard line of

recombinant human ApoE2 (Peprotech, 350-12) between 2-2000 ng protein/mL was used. A sigmoidal curve was fitted and ECL signal from samples were interpolated to calculate the scFv and ApoE levels, expressed as ng/mg total protein.

5 **Example 10**

rAAV5 comprising a viral vector encoding both a vectorized antibody and a human protein (D1 and D2) were provided to mice using intrastriatal injection in order to test whether both the human protein as well as the vectorized antibody would be expressed efficiently. All constructs that were tested exhibited consistent vector DNA concentrations of 10^7 in the mouse striatum, indicating successful delivery and presence of the genetic material (Figure 17A). The control, represented by the formulation buffer, showed background signals, which were to be expected. In terms of human APOE2-Jac mRNA concentration, the construct encoding for huAPOE2-Jac only reached the highest concentration ($\sim 4 \times 10^7$ copies/ μ g total RNA) compared to the other conditions. Constructs expressing both huAPOE2-Jac and scFv anti-Tau (D1 and D2) showed a 2-fold reduction in mRNA levels for APOE2-Jac compared to the APOE2-Jac only condition (Figure 17B). scFv anti-Tau mRNA concentration was highest in the construct comprising scFv anti-Tau upstream from APOE2-Jac (D1) ($\sim 1 \times 10^9$ copies/ μ g total RNA). For the scFv anti-Tau a 2.6 fold lower mRNA expression was found, and for the construct harbouring APOE2-Jac upstream from scFv anti-Tau (D2) a 3.2-fold reduction was found (Figure 17C). The protein concentrations of APOE2-Jac mirrored the results observed for mRNA levels, indicating consistency between transcription and translation processes (Figure 17D). Similar to APOE2-Jac, the protein concentrations of scFv anti-Tau antibodies mirrored the mRNA expression levels, reinforcing the consistency between transcription and translation for this construct (Figure 17E).

Overall, the results demonstrate successful rAAV5-mediated delivery and expression of genetic constructs in the mouse striatum. While some constructs exhibited higher expression levels than others, the consistency between mRNA and protein concentrations suggests efficient translation processes of both the vectorized antibody and the therapeutic protein. This experiment highlights efficient rAAV5-mediated delivery and robust expression of both a vectorized antibody and human (therapeutic) protein from a single viral vector in brain tissue.

Claims

1. A recombinant adeno-associated virus (rAAV) serotype 5 particle for use in the treatment of a central nervous system (CNS) disease, the rAAV5 particle comprising:
 - 5 i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
 - ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one
- 10 product having an effect in the CNS disease.
- 2 An rAAV5 particle for use according to claim 1, wherein the CNS disease is selected from Table 1.
3. An rAAV5 particle for use according to claim 1 or 2, wherein the nucleic acid molecule encodes at
- 15 least one of an RNA molecule and a polypeptide.
4. An rAAV5 particle for use according any one of claims 1-3, wherein the rAAV5 particle is administered via intrathecal (cisternal or lumbar), intracerebroventricular, intracerebral, intraatrial, intraputaminar, intra-cerebrospinal fluid (CSF) and/or intraparenchymal administration, and/or through
- 20 intranasal instillation.
5. An rAAV5 particle for use according to any one of claims 1 – 4, wherein the rAAV5 particle is administered in a dose ranging between 1×10^{10} – 1×10^{16} , preferably 3×10^{10} – 3×10^{15} , more preferably 1×10^{11} – 1×10^{15} , most preferably 3×10^{11} – 3×10^{14} genome copies (GC) per subject.
- 25
6. An rAAV5 particle for use according to any one of claims 1 – 5, wherein the mutated capsid protein further comprises:
 - a) an additional amino acid, preferably an alanine or valine, inserted at a position corresponding to between positions 1 and 2 of wild type AAV5 VP1 capsid protein.
- 30
7. An rAAV5 particle for use according to any one of claims 1 – 5, wherein the mutated capsid protein further comprises an exchange of the N-terminal region corresponding to the first 136 N-terminal amino acids of wild type AAV5 VP1 capsid protein with the N-terminal region corresponding to the first 137 N-terminal amino acids of wild type AAV serotype 2 (AAV2) VP1 capsid protein.
- 35

8. An rAAV5 particle for use according to any one of claims 1 - 6, wherein the mutated capsid protein variant comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 6, 8 or 29.
- 5 9. An rAAV5 particle for use according to any one of claims 1 – 5 or 7, wherein the mutated capsid protein variant comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 7.
- 10 10. An rAAV5 particle for use according to any one of claims 1 – 9, wherein the rAAV5 particle does not comprise wild type AAV VP1, viral protein 2 (VP2) or viral protein 3 (VP3) capsid protein.
11. A composition comprising an rAAV5 particle for use as defined in any one of claims 1 – 11 and an excipient, preferably for intrathecal (cisternal or lumbar), intracerebroventricular, intracerebral, intrastriatal, intraputamina, intra-CSF and intraparenchymal administration and/or intranasal instillation.
- 15 12. A method for manufacturing a recombinant adeno-associated virus serotype 5 (rAAV5) particle, the method comprising the steps of:
- I) culturing a host cell comprising:
- i) an expression construct for expression of a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1); and
- ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease, the mutated capsid protein further comprising:
- a) an additional amino acid, preferably an alanine or valine, inserted at a position corresponding to between positions 1 and 2 of wild type AAV5 VP1 capsid protein or
- b) an exchange of the N-terminal region corresponding to the first 136 N-terminal amino acids of wild type AAV5 VP1 capsid protein with the N-terminal region corresponding to the first 137 N-terminal amino acids of wild type AAV serotype 2 (AAV2) VP1 capsid protein,
- said host cell preferably further comprising a nucleotide sequence encoding a parvoviral replication (Rep) protein under conditions such that the rAAV5 particle is produced; and,
- 35 II) recovery of the rAAV5 particle, preferably wherein recovery of the rAAV5 particle in step II) comprises at least one of: affinity-purification of the particle using an immobilised anti-AAV

antibody, preferably a single chain camelid antibody or a fragment thereof, or filtration over a filter having a nominal pore size of 30 - 70 nm.

13. The method of claim 12, wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 6, 7, 8, or 29.

14. A recombinant adeno-associated virus (rAAV) serotype 5 particle comprising:
i) a mutated capsid protein comprising a non-native amino acid, preferably a valine, at a position corresponding to M569 of wild type adeno-associated virus serotype 5 (AAV5) viral protein 1 (VP1);
and
ii) an expression cassette flanked by at least one adeno-associated virus (AAV) inverted terminal repeat (ITR), comprising a transgene comprising a nucleic acid molecule encoding at least one product having an effect in the CNS disease,

wherein the rAAV5 particle further comprises:

a) an additional amino acid, preferably an alanine or valine, inserted at a position corresponding to between positions 1 and 2 of wild type AAV5 VP1 capsid protein; or
b) an exchange of the N-terminal region corresponding to the first 136 N-terminal amino acids of wild type AAV5 VP1 capsid protein with the N-terminal region corresponding to the first 137 N-terminal amino acids of wild type AAV serotype 2 (AAV2) VP1 capsid protein.

15. The rAAV5 particle according to claim 14, wherein the rAAV5 particle comprises an amino acid sequence with more than at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO: 6, 7, 8, or 29.