Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 3105381 A1 2020/01/16

(21) 3 105 381

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2019/07/11
(87) Date publication PCT/PCT Publication Date: 2020/01/16
(85) Entrée phase nationale/National Entry: 2020/12/29
(86) N° demande PCT/PCT Application No.: US 2019/041386
(87) N° publication PCT/PCT Publication No.: 2020/014471
(30) Priorité/Priority: 2018/07/11 (US62/696,422)

(51) Cl.Int./Int.Cl. *C07K 14/015* (2006.01), *C12N 15/864* (2006.01)

(71) Demandeur/Applicant:
THE BRIGHAM AND WOMEN'S HOSPITAL, INC., US

(72) Inventeur/Inventor:
BEI, FENGFENG, US

(74) Agent: SMART & BIGGAR LLP

(54) Titre : PROCEDES ET COMPOSITIONS POUR L'ADMINISTRATION D'AGENTS A TRAVERS LA BARRIERE HEMATO-ENCEPHALIQUE

(54) Title: METHODS AND COMPOSITIONS FOR DELIVERY OF AGENTS ACROSS THE BLOOD-BRAIN BARRIER

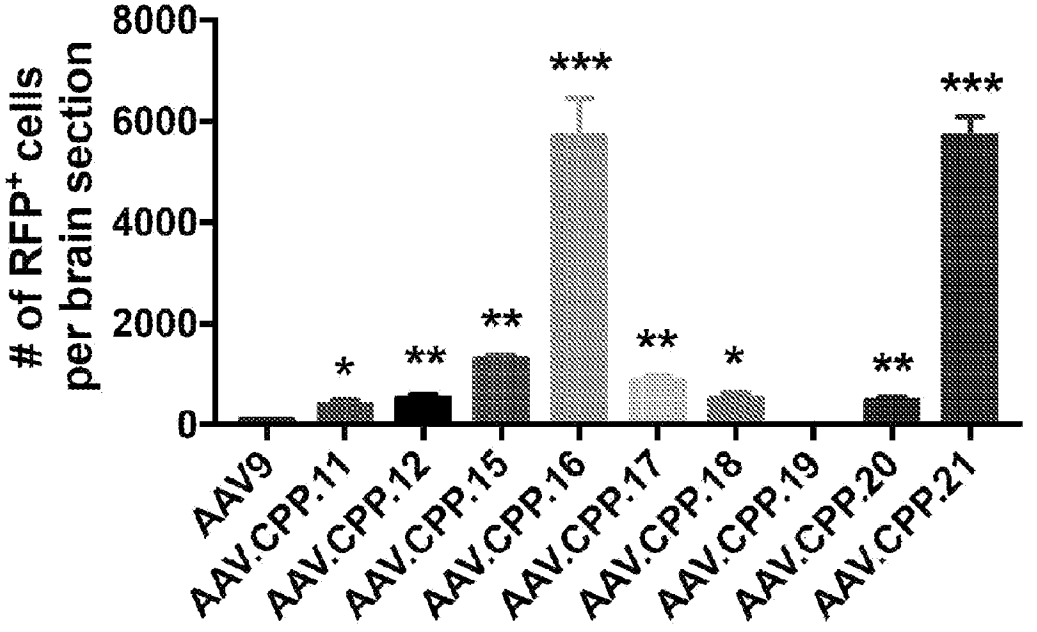


FIG. 3C

(57) Abrégé/Abstract:
The present invention is based on the development of artificial targeting sequences that enhance permeation of agents into cells and across the blood brain barrier, compositions comprising the sequences, and methods of use thereof. Provided herein is an AAV comprising a capsid protein comprising a targeting sequence and a transgene, preferably a therapeutic or diagnostic transgene. Further, provided herein are methods of delivering a transgene to a cell, the method comprising contacting the cell with an AAV or fusion protein described herein.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 January 2020 (16.01.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/014471 A1

(51) International Patent Classification:
C07K 14/015 (2006.01) *C12N 15/864* (2006.01)

(21) International Application Number:
PCT/US2019/041386

(22) International Filing Date:
11 July 2019 (11.07.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/696,422 11 July 2018 (11.07.2018) US

(71) Applicant: **THE BRIGHAM AND WOMEN'S HOSPITAL, INC.** [US/US]; 75 Francis Street, Boston, Massachusetts 02115 (US).

(72) Inventor: **BEI, Fengfeng**; 75 Francis Street, Boston, Massachusetts 02115 (US).

(74) Agent: **DEYOUNG, Janice Kugler** et al.; Fish & Richardson P.C., P.O. Box 1022, Minneapolis, Minnesota 55440-1022 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title:** METHODS AND COMPOSITIONS FOR DELIVERY OF AGENTS ACROSS THE BLOOD-BRAIN BARRIER

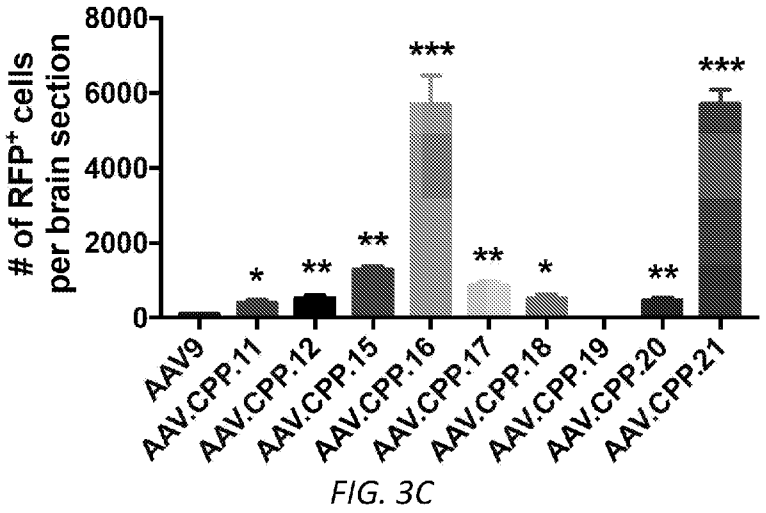


FIG. 3C

(57) **Abstract:** The present invention is based on the development of artificial targeting sequences that enhance permeation of agents into cells and across the blood brain barrier, compositions comprising the sequences, and methods of use thereof. Provided herein is an AAV comprising a capsid protein comprising a targeting sequence and a transgene, preferably a therapeutic or diagnostic transgene. Further, provided herein are methods of delivering a transgene to a cell, the method comprising contacting the cell with an AAV or fusion protein described herein.

WO 2020/014471 A1

METHODS AND COMPOSITIONS FOR DELIVERY OF AGENTS ACROSS THE BLOOD-BRAIN BARRIER

CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Application Serial No. 62/696,422, filed on July 11, 2018. The entire contents of the foregoing are incorporated herein by reference.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on July 11, 2019, is named 29618-0200WO1_SL.txt and is 58,834 bytes in size.

TECHNICAL FIELD

Described herein are sequences that enhance permeation of agents across the blood brain barrier, compositions comprising the sequences, and methods of use thereof.

BACKGROUND

Delivery of therapeutic agents, including gene therapy reagents, is an impediment to development of treatments for a number of conditions. The blood-brain barrier (BBB) is a key obstacle for drug delivery to the mammalian central nervous system (CNS), particularly for delivery to the human brain, to treat conditions including neurodegenerative diseases such as Parkinson's disease; Alzheimer's disease; Huntington's disease; Amyotrophic lateral sclerosis; and Multiple sclerosis.

SUMMARY

The present invention is based on the development of artificial targeting sequences that enhance permeation of agents into cells and across the blood brain barrier.

Thus provided herein is an AAV capsid protein, e.g., an engineered AAV capsid protein, comprising a targeting sequence that comprises at least four contiguous amino acids from the sequence TVSALFK (SEQ ID NO:8); TVSALK

(SEQ ID NO:4); KLASVT (SEQ ID NO:83); or KFLASVT (SEQ ID NO:84). In some embodiments, the AAV capsid protein comprises a targeting sequence that comprises at least five contiguous amino acids from the sequence TVSALK (SEQ ID NO:4); TVSALFK (SEQ ID NO:8); KLASVT (SEQ ID NO:83); or KFLASVT (SEQ ID NO:84). In some embodiments, the AAV capsid protein comprises a targeting sequence that comprises at least six contiguous amino acids from the sequence TVSALK (SEQ ID NO:4); TVSALFK (SEQ ID NO:8); KLASVT (SEQ ID NO:83); or KFLASVT (SEQ ID NO:84).

In some embodiments, the AAV is AAV9; other AAV as known in the art (e.g., AAV1, 2, 3, 4, 5, 6, 7, 8 and variants thereof and others as known in the art or described herein) can also be used.

In some embodiments, the AAV capsid protein comprises AAV9 VP1 (e.g., SEQ ID NO:85).

In some embodiments, the targeting sequence is inserted in the capsid protein at a position corresponding to between amino acids 588 and 589 of SEQ ID NO:85.

Also provided herein are nucleic acids encoding the AAV capsid proteins comprising a targeting sequence as described herein.

In addition, provided herein is an AAV comprising a capsid protein comprising a targeting sequence as described herein. In some embodiments, AAV further comprises a transgene, preferably a therapeutic or diagnostic transgene. Therapeutic transgenes can include, e.g., cDNAs that restore protein function, guide RNA for gene editing, RNA, or miRNA.

Also provided herein are targeting sequences comprising V[S/p][A/m/t/]L (SEQ ID NO:79), TV[S/p][A/m/t/]L (SEQ ID NO:80), TV[S/p][A/m/t/]LK (SEQ ID NO:81), or TV[S/p][A/m/t/]LFK. (SEQ ID NO:82). In some embodiments, the targeting sequence comprises VPALR (SEQ ID NO:1); VSALK (SEQ ID NO:2); TVPALR (SEQ ID NO:3); TVSALK (SEQ ID NO:4); TVPMLK (SEQ ID NO:12); TVPTLK (SEQ ID NO:13); FTVSALK (SEQ ID NO:5); LTVSALK (SEQ ID NO:6); TVSALFK (SEQ ID NO:8); TVPALFR (SEQ ID NO:9); TVPMLFK (SEQ ID NO:10) or TVPTLFK (SEQ ID NO:11). Also provided are fusion proteins comprising the targeting sequences linked to a heterologous (e.g., non-AAV VP1) sequence, and AAV capsid proteins (e.g., AAV9 VP1) comprising the targeting

sequence. In some embodiments, the targeting sequence is inserted in a position corresponding to amino acids 588 and 589 of SEQ ID NO:85.

Additionally provided herein are nucleic acids encoding the targeting sequences, fusion proteins or AAV capsid proteins described herein, as well as AAV comprising the capsid proteins comprising a targeting sequence. In some embodiments, the AAV further comprises a transgene, preferably a therapeutic or diagnostic transgene. Therapeutic transgenes can include, e.g., cDNAs that restore protein function, guide RNA for gene editing, RNA, or miRNA.

Further, provided herein are methods of delivering a transgene to a cell, the method comprising contacting the cell with an AAV or fusion protein described herein. In some embodiments, the cell is in a living subject, e.g., a mammalian subject. In some embodiments, the cell is in a tissue selected from the brain, spinal cord, dorsal root ganglion, heart, or muscle, and a combination thereof. In some embodiments, the cell is a neuron (optionally a dorsal root ganglion neuron), astrocyte, cardiomyocyte, or myocyte.

In some embodiments, the subject has a neurodegenerative disease, epilepsy; stroke; spinocerebellar ataxia; Canavan's disease; Metachromatic leukodystrophy; Spinal muscular atrophy; Friedreich's ataxia; X-linked centronuclear myopathy; Lysosomal storage disease; Barth syndrome; Duchenne muscular dystrophy; Wilson's disease; or Crigler-Najjar syndrome type 1. In some embodiments, the neurodegenerative disease is Parkinson’s disease; Alzheimer’s disease; Huntington's disease; Amyotrophic lateral sclerosis; and Multiple sclerosis.

In some embodiments, the subject has a brain cancer, and the method includes administering an AAV encoding an anti-cancer agent. In some embodiments, the anti-cancer agent is HSV.TK1, and the method further comprises administering ganciclovir.

In some embodiments, the cell is in the brain of the subject, and the AAV is administered by parenteral delivery (e.g., via intravenous, intraarterial, subcutaneous, intraperitoneal, or intramuscular delivery); intracerebral; or intrathecal delivery (e.g., via lumbar injection, cisternal magna injection, or intraparenchymal injection).

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in

the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

Figs. 1A-1B depict an exemplary strategy of engineering AAV9 by inserting cell-penetrating peptides (CPPs) into its capsid. Fig. 1A is a 3D model of an AAV9 virus. Individual CPP inserted into the capsid between amino acids 588 and 589 (VP1 numbering) will be displayed at the 3-fold axis where receptor binding presumably occurs. Fig. 1B illustrate the method of individual AAV production. Three plasmids including pRC (engineered or not), pHelper and pAAV are co-transfected into HEK 293T cells, with AAVs harvested and purified using iodixanol gradient.

Figs. 2A-2B depict representative images of mouse brain sections and their quantitative analysis after intravenous administration of low-dose candidate AAVs. Mice with mixed genetic background are used. Candidate AAVs differs in their inserted CPPs (see Table 3), but all express nuclear red fluorescent protein (RFP) as reporter. Candidate AAVs with low production yields are excluded for further screening. The dose of AAV is 1×10^{10} vg (viral genome) per animal. Each white dot in Fig. 2A represents a RFP-labeled cell. In Fig. 2B, * $P< 0.05$, vs. AAV9, ANOVA.

Figs. 2C-2D depict representative images of mouse brain sections and their quantitative analysis after intravenous administration of AAV.CPP.11 and AAV.CPP.12 in a repeat experiment. AAV.CPP.11 and AAV.CPP.12 contain CPPs BIP1 and BIP2 respectively (see Table 3). The doses of the AAVs are increased to 1×10^{11} vg per animal. Candidate AAVs express nuclear red fluorescent protein (RFP) as reporter. Each white dot in Fig. 2C represents a RFP-labeled cell. In Fig. 2D, * $P< 0.05$, ** $P< 0.01$, vs. AAV9, ANOVA.

Fig. 3A depicts the optimization of the BIP targeting sequence in order to further engineer AAV9 towards better brain transduction. BIP1 (VPALR, SEQ ID NO:1), which enables AAV9 to transduce brain more efficiently (as in AAV.CPP.11),

is derived from the protein Ku70 in rats. Human, mouse and rat Ku70 proteins differ in their exact amino acid sequences. BIP2 (VSALK, SEQ ID NO:2) as in AAV.CPP.12 is a "synthetic" peptide related to BIP1. Further engineering focuses on the VSALK sequence in the hope of minimizing species specificity of final engineered AAV. To generate new targeting sequence, amino acids of interest are added to the VSALK sequence, and in other cases positions of individual amino acids are switched. All new BIP2-derived sequences are again inserted into the AAV9 capsid to generate new candidate AAVs for screening. Sequences appearing in order are SEQ ID NOs: 69, 70, 71, 1-6, 72, 7, and 8.

Figs. 3B-3C depict representative images of mouse brain sections and their quantitative analysis after intravenous administration of more candidate AAVs. All candidate AAVs express nuclear red fluorescent protein (RFP) as reporter. The dose of AAV is 1×10^{11} vg per animal. Each white dot in Fig. 3B represents a RFP-labeled cell. AAV.CPP.16 and AAV.CPP.21 were identified as top hits with their robust and widespread brain transduction. In Fig. 3C, * $P< 0.05$, ** $P< 0.01$, *** $P< 0.001$, vs. AAV9, ANOVA.

Fig. 3D depicts quantitative analysis of transduction efficiency in the liver after intravenous administration of candidate AAVs. Percentage of transduced liver cells is presented. The dose of AAV is 1×10^{11} vg per animal. *** $P< 0.001$, vs. AAV9, ANOVA.

Figs. 4A-4E depict screening of selected candidate AAVs in an in vitro spheroid model of human blood-brain barrier. Fig. 4A illustrates the spheroid comprising human microvascular endothelial cells, which forms a barrier at the surface, and human pericyte and astrocytes inside the spheriod. Candidate AAVs were assessed for their ability to penetrate from the surrounding medium into the inside of the spheroid and to transduce the cells inside. Fig. 4B-4D shows images of AAV9, AAV.CPP.16 and AAV.CPP.21 treated spheroids. Fig. 4E shows relative RFP intensity of different AAV treated spheroids. *** $P< 0.001$, vs. AAV9, ANOVA.

Figs. 5A-5B depict representative images of brain sections and their quantitative analysis after intravenous administration of AAV9, AAV.CPP.16 and AAV.CPP.21 in C57BL/6J inbred mice. All candidate AAVs express nuclear red fluorescent protein (RFP) as reporter. The dose of AAV is 1×10^{12} vg per animal. Each

white dot in Fig. 5A represents a RFP-labeled cell. In Fig. 5B, * P< 0.05, *** P< 0.001, ANOVA.

Figs. 6A-6B depict representative images of brain sections and their quantitative analysis after intravenous administration of AAV9, AAV.CPP.16 and AAV.CPP.21 in BALB/cJ inbred mice. All candidate AAVs express nuclear red fluorescent protein (RFP) as reporter. The dose of AAV is 1×10^{12} vg per animal. Each white dot in Fig. 6A represents a RFP-labeled cell. In Fig. 6B, *** P< 0.001, ANOVA.

Figs. 7A-7B depict representative images of brain sections and their quantitative analysis after intravenous administration of high-dose AAV.CPP.16 and AAV.CPP.21 in C57BL/6J inbred mice. Both candidate AAVs express nuclear red fluorescent protein (RFP) as reporter. The dose of AAV is 4×10^{12} vg per animal. Each white dot in Fig. 7A represents a RFP-labeled cell. In Fig. 7B, * P< 0.05, Student test.

Fig. 8A shows AAV.CPP.16 and AAV.CPP.21 transduce adult neurons (labeled by a NeuN antibody) across multiple brain regions in mice including the cortex, midbrain and hippocampus. Transduced neurons are co-labeled by NeuN antibody and RFP. AAVs of 4×10^{12} vg were administered intravenously in adult C57BL/6J mice (6 weeks old).

Fig. 8B depicts that AAV.CPP.16 and AAV.CPP.21 show enhanced ability vs. AAV9 in targeting the spinal cord and motor neurons in mice. AAVs of 4×10^{10} vg were administered intravenously into neonate mice (1 day after birth). Motor neurons in the ventral horn of the spinal cord were visualized using CHAT antibody staining. Co-localization of RFP and CHAT signals suggests specific transduction of the motor neurons.

Fig. 9A depicts that AAV.CPP.16 shows enhanced ability vs. AAV9 in targeting the heart in adult mice. AAVs of 1×10^{11} vg were administered intravenously in adult C57BL/6J mice (6 weeks old). Percentage of RFP-labeled cells relative to all DAPI-stained cells is presented. * P< 0.05, Student test.

Fig. 9B depicts that AAV.CPP.16 shows enhanced ability vs. AAV9 in targeting the skeletal muscle in adult mice. AAVs of 1×10^{11} vg were administered intravenously in adult C57BL/6J mice (6 weeks old). Percentage of RFP-labeled cells relative to all DAPI-stained cells is presented. * P< 0.05, Student test.

Fig. 9C depicts that AAV.CPP.16 shows enhanced ability vs. AAV9 in targeting the dorsal root ganglion (DRG) in adult mice. AAVs of 1×10^{11} vg were

administered intravenously in adult C57BL/6J mice (6 weeks old). Percentage of RFP-labeled cells relative to all DAPI-stained cells is presented. * $P < 0.05$, Student test.

Fig. 10A depicts that AAV.CPP.16 and AAV.CPP.21 show enhanced ability vs. AAV9 to transduce brain cells in primary visual cortex after intravenous administration in non-human primates. 2×10^{13} vg/kg AAVs-CAG-AADC (as reporter gene) were injected intravenously into 3 months old cynomolgus monkeys with low pre-existing neutralizing antibody. AAV-transduced cells (shown in black) were visualized using antibody staining against AADC. Squared areas in the left panels are enlarged as in the right panels. AAV.CPP.16 transduced significantly more cells vs. AAV9. AAV.CPP.21 also transduced more cell vs. AAV9 although its effect was less evident in comparison with AAV.CPP.16.

Fig. 10B depicts that AAV.CPP.16 and AAV.CPP.21 show enhanced ability vs. AAV9 to transduce brain cells in parietal cortex after intravenous administration in non-human primates. 2×10^{13} vg/kg AAVs-CAG-AADC (as reporter gene) were injected intravenously into 3 months old cynomolgus monkeys with low pre-existing neutralizing antibody. AAV-transduced cells (shown in black) were visualized using antibody staining against AADC. Squared areas in the left panels are enlarged as in the right panels. AAV.CPP.16 transduced significantly more cells vs. AAV9. AAV.CPP.21 also transduced more cell vs. AAV9 although its effect was less evident in comparison with AAV.CPP.16.

Fig. 10C depicts that AAV.CPP.16 and AAV.CPP.21 show enhanced ability vs. AAV9 to transduce brain cells in thalamus after intravenous administration in non-human primates. 2×10^{13} vg/kg AAVs-CAG-AADC (as reporter gene) were injected intravenously into 3 months old cynomolgus monkeys with low pre-existing neutralizing antibody. AAV-transduced cells (shown in black) were visualized using antibody staining against AADC. Squared areas in the left panels are enlarged as in the right panels. AAV.CPP.16 transduced significantly more cells vs. AAV9. AAV.CPP.21 also transduced more cell vs. AAV9 although its effect was less evident in comparison with AAV.CPP.16.

Fig. 10D depicts that AAV.CPP.16 and AAV.CPP.21 show enhanced ability vs. AAV9 to transduce brain cells in cerebellum after intravenous administration in non-human primates. 2×10^{13} vg/kg AAVs-CAG-AADC (as reporter gene) were injected

intravenously into 3 months old cynomolgus monkeys with low pre-existing neutralizing antibody. AAV-transduced cells (shown in black) were visualized using antibody staining against AADC. Squared areas in the left panels are enlarged as in the right panels. Both AAV.CPP.16 and AAV.CPP.21 transduced significantly more cells vs. AAV9.

Figs. 11A-11B depict that AAV.CPP.16 and AAV.CPP.21 do not bind to LY6A. LY6A serves as a receptor for AAV.PHP.B and its variants including AAV.PHP.eB (as in US9102949, US20170166926) and mediates AAV.PHP.eB's robust effect in crossing the BBB in certain mouse strains (Hordeaux et al. Mol Ther 2019 27(5):912-921; Huang et al. 2019, dx.doi.org/10.1101/538421). Over-expressing mouse LY6A in cultured 293 cells significantly increases binding of AAV.PHP.eB to the cell surface (Fig. 11A). On the contrary, over-expressing LY6A does not increase viral binding for AAV9, AAV.CPP.16 or AAV.CPP.21 (Fig. 11B). This suggests AAV.CPP.16 or AAV.CPP.21 does not share LY6A with AAV.PHP.eB as a receptor.

Figs. 12A-12C depict that AAV.CPP.21 can be used to systemically deliver a therapeutic gene into brain tumor in a mouse mode of glioblastoma (GBM). As in Fig. 11A, intravenously administered AAV.CPP.21-H2BmCherry was shown to target tumor mass, especially the tumor expanding frontier. In Fig. 11B-11C, using AAV.CPP.21 to systemically deliver the "suicide gene" HSV.TK1 results in shrinkage of brain tumor mass, when combined with the pro-drug ganciclovir. HSV.TK1 turns the otherwise "dormant" ganciclovir into a tumor-killing drug. * $P< 0.05$, Student test.

Fig. 13 depicts that when injected locally into adult mouse brain, AAV.CPP.21 resulted in more widespread and robust transduction of brain tissue in comparison with AAV9. Intracerebral injection of AAVs (1×10^{11} vg) was performed in adult mice (>6 weeks old) and brain tissues were harvested and examined 3 weeks after AAV injection. ** $P< 0.01$, Student test.

DETAILED DESCRIPTION

Difficulties associated with delivery across the BBB have hindered development of therapeutic agents to treat brain disorders including cancer and neurodegenerative disorders. Adeno-associated virus (AAV) has emerged as an important research and clinical tool for delivering therapeutic genes to the brain, spinal cord and the eye; see, e.g., US9102949; US 9585971; and US20170166926.

However, existing AAVs including AAV9 have either limited efficiency in crossing the BBB, or only work in some non-primate species.

Through rational design and targeted screening on the basis of known cell-penetrating peptides (CPPs) (see, e.g., Gomez et al., *Bax-inhibiting peptides derived from Ku70 and cell-penetrating pentapeptides.* Biochem. Soc. Trans. 2007;35(Pt 4):797–801), targeting sequences have been discovered that, when engineered into the capsid of an AAV, improved the efficiency of gene delivery to the brain by up to three orders of magnitude. These methods were used to engineer one such AAV vector that dramatically reduced tumor size in an animal model of glioblastoma.

Targeting Sequences

The present methods identified a number of potential targeting peptides that enhance permeation through the BBB, e.g., when inserted into the capsid of an AAV, e.g., AAV1, AAV2, AAV8, or AAV9, or when conjugated to a biological agent, e.g., an antibody or other large biomolecule, either chemically or via expression as a fusion protein.

In some embodiments, the targeting peptides comprise sequences of at least 5 amino acids. In some embodiments, the amino acid sequence comprises at least 4, e.g., 5, contiguous amino acids of the sequences VPALR (SEQ ID NO:1) and VSALK (SEQ ID NO:2).

In some embodiments, the targeting peptides comprise a sequence of X_1 X_2 X_3 X_4 X_5, wherein:

(i) X_1, X_2, X_3, X_4 are any four non-identical amino acids of V, A, L, I, G, P, S, T, or M; and

(ii) X_5 is K, R, H, D, or E (SEQ ID NO:73).

In some embodiments, the targeting peptides comprise sequences of at least 6 amino acids. In some embodiments, the amino acid sequence comprises at least 4, e.g., 5 or 6 contiguous amino acids of the sequences TVPALR (SEQ ID NO:3), TVSALK (SEQ ID NO:4), TVPMLK (SEQ ID NO:12) and TVPTLK (SEQ ID NO:13).

In some embodiments, the targeting peptides comprise a sequence of X_1 X_2 X_3 X_4 X_5 X_6, wherein:

(i) X_1 is T;

(ii) X_2, X_3, X_4, X_5 are any four non-identical amino acids of V, A, L, I, G, P, S, T, or M; and

(iii) X_6 is K, R, H, D, or E (SEQ ID NO:74).

In some embodiments, the targeting peptides comprise a sequence of X_1 X_2 X_3 X_4 X_5 X_6, wherein:

(i) X_1, X_2, X_3, X_4 are any four non-identical amino acids from V, A, L, I, G, P, S, T, or M;

(ii) X_5 is K, R, H, D, or E; and

(iii) X_6 is E or D (SEQ ID NO:75).

In some embodiments, the targeting peptides comprise sequences of at least 7 amino acids. In some embodiments, the amino acid sequence comprises at least 4, e.g., 5, 6, or 7 contiguous amino acids of the sequences FTVSALK (SEQ ID NO:5), LTVSALK (SEQ ID NO:6), TVSALFK (SEQ ID NO:8), TVPALFR (SEQ ID NO:9), TVPMLFK (SEQ ID NO:10) and TVPTLFK (SEQ ID NO:11). In some other embodiments, the targeting peptides comprise a sequence of X_1 X_2 X_3 X_4 X_5 X_6 X_7, wherein:

(i) X_1 is F, L, W, or Y;

(ii) X_2 is T;

(iii) X_3, X_4, X_5, X_6 are any four non-identical amino acids of V, A, L, I, G, P, S, T, or M; and

(iv) X_7 is K, R, H, D, or E (SEQ ID NO:76).

In some embodiments, the targeting peptides comprise a sequence of X_1 X_2 X_3 X_4 X_5 X_6 X_7, wherein:

(i) X_1 is T;

(ii) X_2, X_3, X_4, X_5 are any four non-identical amino acids of V, A, L, I, G, P, S, T, or M;

(iii) X_6 is K, R, H, D, or E; and

(iv) X_7 is E or D (SEQ ID NO:77).

In some embodiments, the targeting peptides comprise a sequence of X_1 X_2 X_3 X_4 X_5 X_6 X_7, wherein:

(i) X_1, X_2, X_3, X_4 are any four non-identical amino acids of V, A, L, I, G, P, S, T, or M;

(ii) X_5 is K, R, H, D, or E;

(iii) X_6 is E or D; and

(iv) X_7 is A or I (SEQ ID NO:78).

In some embodiments, the targeting peptides comprise a sequence of V[S/p][A/m/t/]L (SEQ ID NO:79), wherein the upper case letters are preferred at that position. In some embodiments, the targeting peptides comprise a sequence of TV[S/p][A/m/t/]L (SEQ ID NO:80). In some embodiments, the targeting peptides comprise a sequence of TV[S/p][A/m/t/]LK (SEQ ID NO:81). In some embodiments, the targeting peptides comprise a sequence of TV[S/p][A/m/t/]LFK. (SEQ ID NO:82).

In some embodiments, the targeting peptide does not consist of VPALR (SEQ ID NO:1) or VSALK (SEQ ID NO:2).

Specific exemplary amino acid sequences that include the above mentioned 5, 6, or 7-amino acid sequences are listed in Table 1.

TABLE 1 - Targeting Sequences

SEQ ID NO:	Targeting Peptide Sequence
1.	VPALR
2.	VSALK
3.	TVPALR
4.	**TVSALK**
5.	FTVSALK
6.	LTVSALK
7.	TFVSALK
8.	**TVSALFK**
9.	TVPALFR
10.	**TVPMLFK**
11.	TVPTLFK
12.	**TVPMLK**
13.	TVPTLK
14.	VPMLK
15.	VPTLK
16.	**VPMLKE**
17.	VPTLKD
18.	VPALRD
19.	**VSALKE**
20.	**VSALKD**
21.	TAVSLK
22.	TALVSK
23.	TVLSAK
24.	TLVSAK
25.	TMVPLK
26.	TMLVPK
27.	TVLPMK

SEQ ID NO:	Targeting Peptide Sequence
28.	TLVPMK
29.	TTVPLK
30.	TTLVPK
31.	TVLPTK
32.	TLVPTK
33.	TAVPLR
34.	TALVPR
35.	TVLPAR
36.	TLVPAR
37.	TAVSLKE
38.	TALVSKE
39.	TVLSAKE
40.	TLVSAKE
41.	TMVPLKE
42.	TMLVPKE
43.	TVLPMKE
44.	TLVPMKE
45.	TTVPLKD
46.	TTLVPKD
47.	TVLPTKD
48.	TLVPTKD
49.	TAVPLRD
50.	TALVPRD
51.	TVLPARD
52.	TLVPARD
53.	TAVSLFK
54.	TALVSFK
55.	TVLSAFK
56.	TLVSAFK
57.	TMVPLFK
58.	TMLVPFK
59.	TVLPMFK
60.	TLVPMFK
61.	TTVPLFK
62.	TTLVPFK
63.	TVLPTFK
64.	TLVPTFK
65.	TAVPLFR
66.	TALVPFR
67.	TVLPAFR
68.	TLVPAFR

Targeting peptides including reversed sequences can also be used, e.g., KLASVT (SEQ ID NO:83) and KFLASVT (SEQ ID NO:84).

Targeting peptides disclosed herein can be modified according to the methods known in the art for producing peptidomimetics. See, e.g., Qvit et al., Drug Discov Today. 2017 Feb; 22(2): 454–462; Farhadi and Hashemian, Drug Des Devel Ther. 2018; 12: 1239–1254; Avan et al., Chem. Soc. Rev., 2014,43, 3575-3594; Pathak, et al., Indo American Journal of Pharmaceutical Research, 2015. 8; Kazmierski, W.M., ed., Peptidomimetics Protocols, Human Press (Totowa NJ 1998); Goodman et al., eds., Houben-Weyl Methods of Organic Chemistry: Synthesis of Peptides and Peptidomimetics, Thiele Verlag (New York 2003); and Mayo et al., J. Biol. Chem., 278:45746 (2003). In some cases, these modified peptidomimetic versions of the peptides and fragments disclosed herein exhibit enhanced stability *in vivo*, relative to the non-peptidomimetic peptides.

Methods for creating a peptidomimetic include substituting one or more, e.g., all, of the amino acids in a peptide sequence with D-amino acid enantiomers. Such sequences are referred to herein as "retro" sequences. In another method, the N-terminal to C-terminal order of the amino acid residues is reversed, such that the order of amino acid residues from the N-terminus to the C-terminus of the original peptide becomes the order of amino acid residues from the C-terminus to the N-terminus in the modified peptidomimetic. Such sequences can be referred to as "inverso" sequences.

Peptidomimetics can be both the retro and inverso versions, i.e., the "retro-inverso" version of a peptide disclosed herein. The new peptidomimetics can be composed of D-amino acids arranged so that the order of amino acid residues from the N-terminus to the C-terminus in the peptidomimetic corresponds to the order of amino acid residues from the C-terminus to the N-terminus in the original peptide.

Other methods for making a peptidomimetic include replacing one or more amino acid residues in a peptide with a chemically distinct but recognized functional analog of the amino acid, i.e., an artificial amino acid analog. Artificial amino acid analogs include β-amino acids, β-substituted β-amino acids ("β^3-amino acids"), phosphorous analogs of amino acids, such as ∀-amino phosphonic acids and ∀-amino phosphinic acids, and amino acids having non-peptide linkages. Artificial amino acids can be used to create peptidomimetics, such as peptoid oligomers (e.g., peptoid amide or ester analogues), β-peptides, cyclic peptides, oligourea or oligocarbamate peptides; or heterocyclic ring molecules. Exemplary retro-inverso targeting

peptidomimetics include KLASVT and KFLASVT, wherein the sequences include all D-amino acids. These sequences can be modified, e.g., by biotinylation of the amino terminus and amidation of the carboxy terminus.

AAVs

Viral vectors for use in the present methods and compositions include recombinant retroviruses, adenovirus, adeno-associated virus, alphavirus, and lentivirus, comprising the targeting peptides described herein and optionally a transgene for expression in a target tissue.

A preferred viral vector system useful for delivery of nucleic acids in the present methods is the adeno-associated virus (AAV). AAV is a tiny non-enveloped virus having a 25 nm capsid. No disease is known or has been shown to be associated with the wild type virus. AAV has a single-stranded DNA (ssDNA) genome. AAV has been shown to exhibit long-term episomal transgene expression, and AAV has demonstrated excellent transgene expression in the brain, particularly in neurons. Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.7 kb. An AAV vector such as that described in Tratschin et al., Mol. Cell. Biol. 5:3251-3260 (1985) can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al., Proc. Natl. Acad. Sci. USA 81:6466-6470 (1984); Tratschin et al., Mol. Cell. Biol. 4:2072-2081 (1985); Wondisford et al., Mol. Endocrinol. 2:32-39 (1988); Tratschin et al., J. Virol. 51:611-619 (1984); and Flotte et al., J. Biol. Chem. 268:3781-3790 (1993). There are numerous alternative AAV variants (over 100 have been cloned), and AAV variants have been identified based on desirable characteristics. In some embodiments, the AAV is AAV1, AAV2, AAV4, AAV5, AAV6, AV6.2, AAV7, AAV8, AAV9, rh.10, rh.39, rh.43 or CSp3; for CNS use, in some embodiments the AAV is AAV1, AAV2, AAV4, AAV5, AAV6, AAV8, or AAV9. As one example, AAV9 has been shown to somewhat efficiently cross the blood-brain barrier. Using the present methods, the AAV capsid can be genetically engineered to increase permeation across the BBB, or into a specific tissue, by insertion of a targeting sequence as described herein into the capsid protein, e.g., into the AAV9 capsid protein VP1 between amino acids 588 and 589.

An exemplary wild type AAV9 capsid protein VP1 (Q6JC40-1) sequence is as follows:

```
          10         20         30         40         50
MAADGYLPDW LEDNLSEGIR EWWALKPGAP QPKANQQHQD NARGLVLPGY
          60         70         80         90        100
KYLGPGNGLD KGEPVNAADA AALEHDKAYD QQLKAGDNPY LKYNHADAEF
         110        120        130        140        150
QERLKEDTSF GGNLGRAVFQ AKKRLLEPLG LVEEAAKTAP GKKRPVEQSP
         160        170        180        190        200
QEPDSSAGIG KSGAQPAKKR LNFGQTGDTE SVPDPQPIGE PPAAPSGVGS
         210        220        230        240        250
LTMASGGGAP VADNNEGADG VGSSSGNWHC DSQWLGDRVI TTSTRTWALP
         260        270        280        290        300
TYNNHLYKQI SNSTSGGSSN DNAYFGYSTP WGYFDFNRFH CHFSPRDWQR
         310        320        330        340        350
LINNNWGFRP KRLNFKLFNI QVKEVTDNNG VKTIANNLTS TVQVFTDSDY
         360        370        380        390        400
QLPYVLGSAH EGCLPPFPAD VFMIPQYGYL TLNDGSQAVG RSSFYCLEYF
         410        420        430        440        450
PSQMLRTGNN FQFSYEFENV PFHSSYAHSQ SLDRLMNPLI DQYLYYLSKT
         460        470        480        490        500
INGSGQNQQT LKFSVAGPSN MAVQGRNYIP GPSYRQQRVS TTVTQNNNSE
         510        520        530        540        550
FAWPGASSWA LNGRNSLMNP GPAMASHKEG EDRFFPLSGS LIFGKQGTGR
         560        570        580        590        600
DNVDADKVMI TNEEEIKTTN PVATESYGQV ATNHQSAQAQ AQTGWVQNQG
         610        620        630        640        650
ILPGMVWQDR DVYLQGPIWA KIPHTDGNFH PSPLMGGFGM KHPPPQILIK
         660        670        680        690        700
NTPVPADPPT AFNKDKLNSF ITQYSTGQVS VEIEWELQKE NSKRWNPEIQ
         710        720        730
YTSNYYKSNN VEFAVNTEGV YSEPRPIGTR YLTRNL     (SEQ ID
NO:85)
```

Thus provided herein are AAV that include one or more of the targeting peptide sequences described herein, e.g., an AAV comprising a capsid protein comprising a targeting sequence described herein, e.g., a capsid protein comprising SEQ ID NO:1 wherein a targeting peptide sequence has been inserted into the sequence, e.g., between amino acids 588 and 589.

In some embodiments, the AAV also includes a transgene sequence (i.e., a heterologous sequence), e.g., a transgene encoding a therapeutic agent, e.g., as described herein or as known in the art, or a reporter protein, e.g., a fluorescent protein, an enzyme that catalyzes a reaction yielding a detectable product, or a cell

surface antigen. The transgene is preferably linked to sequences that promote/drive expression of the transgene in the target tissue.

Exemplary transgenes for use as therapeutics include neuronal apoptosis inhibitory protein (NAIP), nerve growth factor (NGF), glial-derived growth factor (GDNF), brain-derived growth factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxlase (TH), GTP-cyclohydrolase (GTPCH), amino acid decorboxylase (AADC), aspartoacylase (ASPA), blood factors, such as β-globin, hemoglobin, tissue plasminogen activator, and coagulation factors; colony stimulating factors (CSF); interleukins, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, etc.; growth factors, such as keratinocyte growth factor (KGF), stem cell factor (SCF), fibroblast growth factor (FGF, such as basic FGF and acidic FGF), hepatocyte growth factor (HGF), insulin-like growth factors (IGFs), bone morphogenetic protein (BMP), epidermal growth factor (EGF), growth differentiation factor-9 (GDF-9), hepatoma derived growth factor (HDGF), myostatin (GDF-8), nerve growth factor (NGF), neurotrophins, platelet-derived growth factor (PDGF), thrombopoietin (TPO), transforming growth factor alpha (TGF-α), transforming growth factor beta (TGF-β), and the like; soluble receptors, such as soluble TNF-α receptors, soluble VEGF receptors, soluble interleukin receptors (e.g., soluble IL-1 receptors and soluble type II IL-1 receptors), soluble gamma/delta T cell receptors, ligand-binding fragments of a soluble receptor, and the like; enzymes, such as α-glucosidase, imiglucarase, β-glucocerebrosidase, and alglucerase; enzyme activators, such as tissue plasminogen activator; chemokines, such as IP-10, monokine induced by interferon-gamma (Mig), Groa/IL-8, RANTES, MIP-1α, MIP-1β, MCP-1, PF-4, and the like; angiogenic agents, such as vascular endothelial growth factors (VEGFs, e.g., VEGF121, VEGF165, VEGF-C, VEGF-2), transforming growth factor-beta, basic fibroblast growth factor, glioma-derived growth factor, angiogenin, angiogenin-2; and the like; anti-angiogenic agents, such as a soluble VEGF receptor; protein vaccine; neuroactive peptides, such as nerve growth factor (NGF), bradykinin, cholecystokinin, gastin, secretin, oxytocin, gonadotropin-releasing hormone, beta-endorphin, enkephalin, substance P, somatostatin, prolactin, galanin, growth hormone-releasing hormone, bombesin, dynorphin, warfarin, neurotensin, motilin, thyrotropin, neuropeptide Y, luteinizing hormone, calcitonin, insulin, glucagons, vasopressin, angiotensin II, thyrotropin-releasing hormone, vasoactive intestinal peptide, a sleep peptide, and the

like; thrombolytic agents; atrial natriuretic peptide; relaxin; glial fibrillary acidic protein; follicle stimulating hormone (FSH); human alpha-1 antitryp sin; leukemia inhibitory factor (LIF); transforming growth factors (TGFs); tissue factors, luteinizing hormone; macrophage activating factors; tumor necrosis factor (TNF); neutrophil chemotactic factor (NCF); nerve growth factor; tissue inhibitors of metalloproteinases; vasoactive intestinal peptide; angiogenin; angiotropin; fibrin; hirudin; IL-1 receptor antagonists; and the like. Some other examples of protein of interest include ciliary neurotrophic factor (CNTF); neurotrophins 3 and 4/5 (NT-3 and 4/5); glial cell derived neurotrophic factor (GDNF); aromatic amino acid decarboxylase (AADC); hemophilia related clotting proteins, such as Factor VIII, Factor IX, Factor X; dystrophin or nini-dystrophin; lysosomal acid lipase; phenylalanine hydroxylase (PAH); glycogen storage disease-related enzymes, such as glucose-6-phosphatase, acid maltase, glycogen debranching enzyme, muscle glycogen phosphorylase, liver glycogen phosphorylase, muscle phosphofructokinase, phosphorylase kinase (e.g., PHKA2), glucose transporter (e.g., GLUT2), aldolase A, β-enolase, and glycogen synthase; lysosomal enzymes (e.g., beta-N-acetylhexosaminidase A); and any variants thereof.

The transgene can also encode an antibody, e.g., an immune checkpoint inhibitory antibody, e.g., to PD-L1, PD-1, CTLA-4 (Cytotoxic T-Lymphocyte-Associated Protein-4; CD152); LAG-3 (Lymphocyte Activation Gene 3; CD223); TIM-3 (T-cell Immunoglobulin domain and Mucin domain 3; HAVCR2); TIGIT (T-cell Immunoreceptor with Ig and ITIM domains); B7-H3 (CD276); VSIR (V-set immunoregulatory receptor, aka VISTA, B7H5, C10orf54); BTLA 30 (B- and T-Lymphocyte Attenuator, CD272); GARP (Glycoprotein A Repetitions; Predominant; PVRIG (PVR related immunoglobulin domain containing); or VTCN1 (Vset domain containing T cell activation inhibitor 1, aka B7-H4).

Other transgenes can include small or inhibitory nucleic acids that alter/reduce expression of a target gene, e.g., siRNA, shRNA, miRNA, antisense oligos, or long non-coding RNAs that alter gene expression (see, e.g., WO2012087983 and US20140142160), or CRISPR Cas9/cas12a and guide RNAs .

The virus can also include one or more sequences that promote expression of a transgene, e.g., one or more promoter sequences; enhancer sequences, e.g., 5' untranslated region (UTR) or a 3' UTR; a polyadenylation site; and/or insulator

sequences. In some embodiments, the promoter is a brain tissue specific promoter, e.g.,a neuron-specific or glia-specific promoter. In certain embodiments, the promoter is a promoter of a gene selected to from: neuronal nuclei (NeuN), glial fibrillary acidic protein (GFAP), MeCP2, adenomatous polyposis coli (APC), ionized calcium-binding adapter molecule 1 (Iba-1), synapsin I (SYN), calcium/calmodulin-dependent protein kinase II, tubulin alpha I, neuron-specific enolase and platelet-derived growth factor beta chain. In some embodiments, the promoter is a pan-cell type promoter, e.g., cytomegalovirus (CMV), beta glucuronidase, (GUSB), ubiquitin C (UBC), or rous sarcoma virus (RSV) promoter. The woodchuck hepatitis virus posttranscriptional response element (WPRE) can also be used.

In some embodiments, the AAV also has one or more additional mutations that increase delivery to the target tissue, e.g., the CNS, or that reduce off-tissue targeting, e.g., mutations that decrease liver delivery when CNS, heart, or muscle delivery is intended (e.g., as described in Pulicherla et al. (2011) Mol Ther 19:1070-1078); or the addition of other targeting peptides, e.g., as described in Chen et al. (2008) Nat Med 15:1215-1218 or Xu et al., (2005) Virology 341:203-214 or US9102949; US 9585971; and US20170166926. See also Gray and Samulski (2011) "Vector design and considerations for CNS applications," in Gene Vector Design and Application to Treat Nervous System Disorders ed. Glorioso J., editor. (Washington, DC: Society for Neuroscience;) 1–9, available at sfn.org/~/media/SfN/Documents/Short%20Courses/2011%20Short%20Course%20I/2011_SC1_Gray.ashx.

Targeting Peptides as Tags/Fusions

The targeting peptides described herein can also be used to increase permeation of other (heterologous) molecules across the BBB, e.g., by conjugation to the molecule, or by expression as part of a fusion protein, e.g., with an antibody or other large biomolecule. These can include genome editing proteins or complexes (e.g., TALEs, ZFNs, Base editors, and CRISPR RNPs comprising a gene editing protein such as Cas9 or Cas12a, fused to a peptide described herein (e.g., at the N terminus, C terminus, or internally) and a guide RNA), in addition to therapeutic agents or reporters as described herein as well as those listed in Table 2. The fusions/complexes do not comprise any other sequences from Ku70, e.g., comprise heterologous non-Ku70 sequences, and are not present in nature.

In some embodiments, targeting sequences used as part of a non-AAV fusion protein do not comprise or consist of VPALR (SEQ ID NO:1) or VSALK (SEQ ID NO:2).

Methods of Use

The methods and compositions described herein can be used to deliver any composition, e.g., a sequence of interest to a tissue, e.g., to the central nervous system (brain), heart, muscle, or dorsal root ganglion or spinal cord (peripheral nervous system). In some embodiments, the methods include delivery to specific brain regions, e.g., cortex, cerebellum, hippocampus, substantia nigra, amygdala. In some embodiments, the methods include delivery to neurons, astrocytes, glial cells, or cardiomyocytes.

In some embodiments, the methods and compositions, e.g., AAVs, are used to deliver a nucleic acid sequence to a subject who has a disease, e.g., a disease of the CNS; see, e.g., US9102949; US 9585971; and US20170166926. In some embodiments, the subject has a condition listed in Table 2; in some embodiments, the vectors are used to deliver a therapeutic agent listed in Table 2 for treating the corresponding disease listed in Table 2. The therapeutic agent can be delivered as a nucleic acid, e.g. via a viral vector, wherein the nucleic acid encodes a therapeutic protein or other nucleic acid such as an antisense oligo, siRNA, shRNA, and so on; or as a fusion protein/complex with a targeting peptide as described herein.

TABLE 2 – Diseases

Examples of diseases	Tissue targeted	Therapeutic agent
Parkinson's disease	CNS	GDNF, AADC
Alzheimer's disease	CNS	Tau antibody, APP antibody
Huntington's disease	CNS	miRNA targeting HTT
Amyotrophic lateral sclerosis	CNS	shRNA targeting SOD
Multiple sclerosis	CNS	IFN-beta
Epilepsy	CNS	Neuropeptide Y
Stroke	CNS	IGF-1, osteopontin
Brain cancer	CNS	HSV.TK1, PD-1/PD-L1 antibody
Spinocerebellar ataxia	CNS	RNAi targeting ataxin
Canavan disease	CNS	ASPA
Metachromatic leukodystrophy	Nervous systems	ARSA, PSAP
Spinal muscular atrophy	Neuromuscular system	SMN1
Friedreich's ataxia	Nervous systems, heart	Frataxin
X-linked myotubular myopathy	Neuromuscular system	MTM1
Pompe disease	Lysosome (global including CNS)	GAA
Barth syndrome	Heart, muscle	TAZ
Duchenne muscular dystrophy	Muscle	dystrophin
Wilson's disease	Brain, liver	ATP7B
Crigler-Najjar syndrome type 1	Liver	UGT1A1

In some embodiments, the compositions and methods are used to treat brain cancer. Brain cancers include gliomas (e.g., glioblastoma multiforme (GBM)), metastases (e.g., from lung, breast, melanoma, or colon cancer), meningiomas, pituitary adenomas, and acoustic neuromas. The compositions include a targeting peptide linked to an anticancer agent, e.g., a "suicide gene" that induces apoptosis in a target cell (e.g., HSV.TK1, Cytosine Deaminase (CD) from Herpes simplex virus or *Escherichia coli*, or Escherichia coli purine nucleoside phosphorylase (PNP)/fludarabine; see Krohne et al., Hepatology. 2001 Sep;34(3):511-8; Dey and Evans, "Suicide Gene Therapy by Herpes Simplex Virus-1 Thymidine Kinase (HSV-TK)" (2011) DOI: 10.5772/18544) an immune checkpoint inhibitory antibody as known in the art or described herein. For example, an AAV vector comprising a targeting peptide as described herein can be used to deliver the "suicide gene" HSV.TK1 to a brain tumor. HSV.TK1 turns the otherwise "dormant" ganciclovir into a tumor-killing drug. Thus the methods can include systemically, e.g., intravenously, administering an AAV (e.g., AAV9) comprising a targeting peptide as described

herein and encoding HSV.TK1, and the pro-drug ganciclovir, to a subject who has been diagnosed with brain cancer.

Pharmaceutical Compositions and Methods of Administration

The methods described herein include the use of pharmaceutical compositions comprising the targeting peptides as an active ingredient.

Pharmaceutical compositions typically include a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" includes saline, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration.

Pharmaceutical compositions are typically formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intraarterial, subcutaneous, intraperitoneal intramuscular or injection or infusion administration. Delivery can thus be systemic or localized.

Methods of formulating suitable pharmaceutical compositions are known in the art, see, e.g., *Remington: The Science and Practice of Pharmacy*, 21st ed., 2005; and the books in the series *Drugs and the Pharmaceutical Sciences: a Series of Textbooks and Monographs* (Dekker, NY). For example, solutions or suspensions used for parenteral application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use can include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the

extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, aluminum monostearate and gelatin.

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

In one embodiment, the therapeutic compounds are prepared with carriers that will protect the therapeutic compounds against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Such formulations can be prepared using standard techniques, or obtained commercially, e.g., from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to selected cells with monoclonal antibodies to cellular antigens) can also be used as pharmaceutically

acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

The pharmaceutical compositions can be included in a kit, container, pack, or dispenser together with instructions for administration. For example, the compositions comprising an AAV comprising a targeting peptide as described herein and a nucleic acid encoding HSV.TK1 can be provided in a kit with ganciclovir.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Materials and Methods

The following materials and methods were used in the Examples below.

1. Generation of capsid variants

To generate the capsid variant plasmids, DNA fragments that encode the cell-penetrating peptides (Table 3) were synthesized (GenScript), and inserted into the backbone of the AAV9 Rep-cap plasmid (pRC9) between amino acid position 588 and 589 (VP1 amino acid numbering), using CloneEZ seamless cloning technology (GenScript). CPPs BIP1(VPALR, SEQ ID NO:1) and BIP2 (VSALK, SEQ ID NO:2), as well as their derivatives such as TVSALK (SEQ ID NO:4) in AAV.CPP.16 and TVSALFK (SEQ ID NO:8) in AAV.CPP.21, are derived from the Ku70 proteins, of which the sequences are provided as below:

```
Human Ku70  MSGWESYYKTEGDEEAEEEQEENLEASGDYKYSGRDSLIFLVDASKAMFESQSEDELTPF 60
Mouse Ku70  MSEWESYYKTEGEEEEEE--EESPDTGGEYKYSGRDSLIFLVDASRAMFESQGEDELTPF 58
Rat Ku70    MSEWESYYKTEGEEEEEE--EQSPDTNGEYKYSGRDSLIFLVDASRAMFESQGEDELTPF 58

Human Ku70  DMSIQCIQSVYISKIISSDRDLLAVVFYGTEKDKNSVNFKNIYVLQELDNPGAKRILELD 120
Mouse Ku70  DMSIQCIQSVYTSKIISSDRDLLAVVFYGTEKDKNSVNFKNIYVLQDLDNPGAKRVLELD 118
Rat Ku70    DMSIQCIQSVYTSKIISSDRDLLAVVFYGTEKDKNSVNFKSIYVLQDLDNPGAKRVLELD 118

Human Ku70  QFKGQQGQKRFQDMMGHGSDYSLSEVLWVCANLFSDVQFKMSHKRIMLFTNEDNPHGNDS 180
Mouse Ku70  QFKGQQGKKHFRDTVGHGSDYSLSEVLWVCANLFSDVQLKMSHKRIMLFTNEDDPHGRDS 178
Rat Ku70    RFKGQQGKKHFRDTIGHGSDYSLSEVLWVCANLFSDVQFKMSHKRIMLFTNEDDPHGNDS 178

Human Ku70  AKASRARTKAGDLRDTGIFLDLMHLKKPGGFDISLFYRDIISIAEDEDLRVHFEESSKLE 240
Mouse Ku70  AKASRARTKASDLRDTGIFLDLMHLKKPGGFDVSVFYRDIITTAEDEDLGVHFEESSKLE 238
Rat Ku70    AKASRARTKASDLRDTGIFLDLMHLKKRGGFDVSLFYRDIISIAEDEDLGVHFEESSKLE 238
```

Human Ku70 DLLRKVRAKETRKRALSRLKLKLNKDIVISVGIYNLVQKALKPPPIKLYRETNEPVKTKT 300
Mouse Ku70 DLLRKVRAKETKKRVLSRLKFKLGEDVVLMVGIYNLVQKANKPFPVRLYRETNEPVKTKT 298
Rat Ku70 DLLRKVRAKETKKRVLSRLKFKLGKDVALMVGVYNLVQKANKPFPVRLYRETNEPVKTKT 298

Human Ku70 RTFNTSTGGLLLPSDTKRSQIYGSRQIILEKEETEELKRFDDPGLMLMGFKPLVLLKKHH 360
Mouse Ku70 RTFNVNTGSLLLPSDTKRSLTYGTRQIVLEKEETEELKRFDEPGLILMGFKPTVMLKKQH 358
Rat Ku70 RTFNVNTGSLLLPSDTKRSLTFGTRQIVLEKEETEELKRFDEPGLILMGFKPMVMLKNHH 358

Human Ku70 YLRPSLFVYPEESLVIGSSTLFSALLIKCLEKEVAALCRYTPRRNIPPYFVALVPQEEEL 420
Mouse Ku70 YLRPSLFVYPEESLVSGSSTLFSALLTKCVEKEVIAVCRYTPRKNVSPYFVALVPQEEEL 418
Rat Ku70 YLRPSLFLYPEESLVNGSSTLFSALLTKCVEKEVIAVCRYTARKNVSPYFVALVPQEEEL 418

Human Ku70 DDQKIQVTPPGFQLVFLPFADDKRKMPFTEKIMATPEQVGKMKAIVEKLRFTYRSDSFEN 480
Mouse Ku70 DDQNIQVTPGGFQLVFLPYADDKRKVPFTEKVTANQEQIDKMKAIVQKLRFTYRSDSFEN 478
Rat Ku70 DDQNIQVTPAGFQLVFLPYADDKRKVPFTEKVMANPEQIDKMKAIVQKLRFTYRSDSFEN 478

Human Ku70 PVLQQHFRNLEALALDLMEPEQAVDLTLPKVEAMNKRLGSLVDEFKELVYPPDYNPEGKV 540
Mouse Ku70 PVLQQHFRNLEALALDMMESEQVVDLTLPKVEAIKKRLGSLADEFKELVYPPGYNPEGKV 538
Rat Ku70 PVLQQHFRNLEALALDMMESEQVVDLTLPKVEAIKKRLGSLADEFKELVYPPGYNPEGKI 538

Human Ku70 TKRKHDNEGSGSKRPKVEYSEEELKTHISKGTLG**KFTVPMLK**EACRAYGLKSGLKKQELL 600
Mouse Ku70 AKRKQDDEGSTSKKPKVELSEEELKAHFRKGTLG**KLTVPTLK**DICKAHGLKSGPKKQELL 598
Rat Ku70 AKRKADNEGSASKKPKVELSEEELKDLFAKGTLG**KLTVPALR**DICKAYGLKSGPKKQELL 598

Human Ku70 EALTKHFQD- 609 (SEQ ID NO:86)
Mouse Ku70 DALIRHLEKN 608 (SEQ ID NO:87)
Rat Ku70 EALSRHLEKN 608 (SEQ ID NO:88)

In addition, VP1 protein sequences for AAV9, AAV.CPP.16 and AAV.CPP.21 are provided as below:

AAV9 MAADGYLPDWLEDNLSEGIREWWALKPGAPQPKANQQHQDNARGLVLPGYKYLGPGNGLD 60
AAV.CPP16 MAADGYLPDWLEDNLSEGIREWWALKPGAPQPKANQQHQDNARGLVLPGYKYLGPGNGLD 60
AAV.CPP21 MAADGYLPDWLEDNLSEGIREWWALKPGAPQPKANQQHQDNARGLVLPGYKYLGPGNGLD 60

AAV9 KGEPVNAADAAALEHDKAYDQQLKAGDNPYLKYNHADAEFQERLKEDTSFGGNLGRAVFQ 120
AAV.CPP16 KGEPVNAADAAALEHDKAYDQQLKAGDNPYLKYNHADAEFQERLKEDTSFGGNLGRAVFQ 120
AAV.CPP21 KGEPVNAADAAALEHDKAYDQQLKAGDNPYLKYNHADAEFQERLKEDTSFGGNLGRAVFQ 120

AAV9 AKKRLLEPLGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQPAKKRLNFGQTGDTE 180
AAV.CPP16 AKKRLLEPLGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQPAKKRLNFGQTGDTE 180
AAV.CPP21 AKKRLLEPLGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQPAKKRLNFGQTGDTE 180

AAV9 SVPDPQPIGEPPAAPSGVGSLTMASGGGAPVADNNEGADGVGSSSGNWHCDSQWLGDRVI 240
AAV.CPP16 SVPDPQPIGEPPAAPSGVGSLTMASGGGAPVADNNEGADGVGSSSGNWHCDSQWLGDRVI 240
AAV.CPP21 SVPDPQPIGEPPAAPSGVGSLTMASGGGAPVADNNEGADGVGSSSGNWHCDSQWLGDRVI 240

AAV9 TTSTRTWALPTYNNHLYKQISNSTSGGSSNDNAYFGYSTPWGYFDFNRFHCHFSPRDWQR 300
AAV.CPP16 TTSTRTWALPTYNNHLYKQISNSTSGGSSNDNAYFGYSTPWGYFDFNRFHCHFSPRDWQR 300
AAV.CPP21 TTSTRTWALPTYNNHLYKQISNSTSGGSSNDNAYFGYSTPWGYFDFNRFHCHFSPRDWQR 300

AAV9 LINNNWGFRPKRLNFKLFNIQVKEVTDNNGVKTIANNLTSTVQVFTDSDYQLPYVLGSAH 360
AAV.CPP16 LINNNWGFRPKRLNFKLFNIQVKEVTDNNGVKTIANNLTSTVQVFTDSDYQLPYVLGSAH 360
AAV.CPP21 LINNNWGFRPKRLNFKLFNIQVKEVTDNNGVKTIANNLTSTVQVFTDSDYQLPYVLGSAH 360

AAV9 EGCLPPFPADVFMIPQYGYLTLNDGSQAVGRSSFYCLEYFPSQMLRTGNNFQFSYEFENV 420
AAV.CPP16 EGCLPPFPADVFMIPQYGYLTLNDGSQAVGRSSFYCLEYFPSQMLRTGNNFQFSYEFENV 420
AAV.CPP21 EGCLPPFPADVFMIPQYGYLTLNDGSQAVGRSSFYCLEYFPSQMLRTGNNFQFSYEFENV 420

AAV9 PFHSSYAHSQSLDRLMNPLIDQYLYYLSKTINGSGQNQQTLKFSVAGPSNMAVQGRNYIP 480
AAV.CPP16 PFHSSYAHSQSLDRLMNPLIDQYLYYLSKTINGSGQNQQTLKFSVAGPSNMAVQGRNYIP 480
AAV.CPP21 PFHSSYAHSQSLDRLMNPLIDQYLYYLSKTINGSGQNQQTLKFSVAGPSNMAVQGRNYIP 480

AAV9 GPSYRQQRVSTTVTQNNNSEFAWPGASSWALNGRNSLMNPGPAMASHKEGEDRFFPLSGS 540
AAV.CPP16 GPSYRQQRVSTTVTQNNNSEFAWPGASSWALNGRNSLMNPGPAMASHKEGEDRFFPLSGS 540
AAV.CPP21 GPSYRQQRVSTTVTQNNNSEFAWPGASSWALNGRNSLMNPGPAMASHKEGEDRFFPLSGS 540

AAV9 LIFGKQGTGRDNVDADKVMITNEEEIKTTNPVATESYGQVATNHQSAQ-------AQAQT 593
AAV.CPP16 LIFGKQGTGRDNVDADKVMITNEEEIKTTNPVATESYGQVATNHQSAQ**TVSAL-K**AQAQT 599
AAV.CPP21 LIFGKQGTGRDNVDADKVMITNEEEIKTTNPVATESYGQVATNHQSAQ**TVSALFK**AQAQT 600

AAV9 GWVQNQGILPGMVWQDRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGMKHPPPQILIKNTP 653
AAV.CPP16 GWVQNQGILPGMVWQDRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGMKHPPPQILIKNTP 659
AAV.CPP21 GWVQNQGILPGMVWQDRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGMKHPPPQILIKNTP 660

AAV9 VPADPPTAFNKDKLNSFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSNNVEF 713
AAV.CPP16 VPADPPTAFNKDKLNSFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSNNVEF 719
AAV.CPP21 VPADPPTAFNKDKLNSFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSNNVEF 720

AAV9 AVNTEGVYSEPRPIGTRYLTRNL 736 (SEQ ID NO:85)
AAV.CPP16 AVNTEGVYSEPRPIGTRYLTRNL 742 (SEQ ID NO:89)
AAV.CPP21 AVNTEGVYSEPRPIGTRYLTRNL 743 (SEQ ID NO:90)

2. Recombinant AAV production

Recombinant AAVs were packaged using standard three-plasmid co-transfection protocol (pRC plasmid, pHelper plasmid and pAAV plasmid). pRC9 (or its variant), pHelper and pAAV carrying a transgene (e.g. nucleus-directed RFP H2B-mCherry driven by an ubiquitous EF1a promoter) were co-transfected into HEK 293T cells using polyethylenimine (PEI, Polysciences). rAAVs vectors were collected from serum-free medium 72h and 120h post transfection and from cell at 120h post transfection. AAV particles in the medium were concentrated using a PEG-precipitation method with 8% PEG-8000 (wt/vol). Cell pellets containing viral particles were resuspended and lysed through sonication. Combined viral vectors from PEG-precipitation and cell lysates were treated with DNase and RNase at 37 °C for 30mins and then purified by iodixanol gradient (15%, 25%, 40% and 60%) with ultracentrifugation(VTi 50 rotor, 40,000 r.p.m, 18°C, 1h). rAAVs were then concentrated using Millipore Amicon filter unit (UFC910008, 100K MWCO) and formulated in Dulbecco's phosphate buffered saline (PBS) containing 0.001% Pluronic F68 (Gibco).

3. AAV titering

Virus titer was determined by measuring DNase-resistant genome copies using quantitative PCR. pAAV-CAG-GFP was digested with PVUII(NEB) to generate free ends for the plasmid ITRs, and was used for generating a standard curve. Virus samples were incubated with DNase I to eliminate contaminating DNA, followed by sodium hydroxide treatment to dissolve the viral capsid and to release the viral genome. Quantitative PCR was performed using an ITR Forward primer 5'-GGAACCCCTAGTGATGGAGTT (SEQ ID NO:91) and an ITR Reverse primer 5'-CGGCCTCAGTGAGCGA (SEQ ID NO:92). Vector titers were normalized to the rAAV-2 reference standard materials (RSMs, ATCC, cat No:VR-1616, Manassas, VA).

4. Administration of AAV in mice

For intravenous administration, AAV diluted in sterile saline (0.2 ml) was administered through tail vein injection in adult mice (over 6 weeks of age). Animals then survived for three weeks before being euthanized for tissue harvesting. For

intracerebral injection, AAV diluted in PBS (10 ul) was injected using a Hamilton syringe with coordinates from bregma: 1.0 mm right, 0.3 backward, 2.6 mm deep. All animal studies were performed in an AAALAC-accredited facility with IACUC approval.

5. Mouse tissue processing

Anesthetized animals were transcardially perfused with cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Tissues were post-fixed in 4% PFA overnight, and then immersed in 30% sucrose solutions for two days prior to embedding and snap-freezing in OCT. Typically, 80 um thick brain sections were cut for imaging of native fluorescence, 40um thick brain sections for IHC.

6. In vitro human BBB spheroid model

Hot 1% agarose (w/v, 50 ul) was added in a 96-well plate to cool/solidify. Primary human astrocytes (Lonza Bioscience), human brain microvascular pericytes (HBVP, ScienCell Research Laboratories) and human cerebral microvascular endothelial cells (hCMEC/D3; Cedarlane) were then seeded onto the agarose gel in a 1:1:1 ratio (1500 cells of each type). Cells were cultured at 37 °C in a 5% CO2 incubator for 48-72 hours to allow for spontaneous assembly of multicellular BBB spheroids. A multicellular barrier was reported to form at the periphery of the spheroid, mimicking the BBB. AAVs-H2B-mCherry were added to the culture medium, and 4 days later all spheroids were fixed using 4% PFA, transferred into a Nunc Lab-Tek II thin-glass 8-well chambered coverglass (Thermo Scientific), and imaged using a Zeiss LSM710 confocal microscope. The intensity of RFP signal inside the spheroids was examined and used as a “read-out”.

7. AAV administration in non-human primate (NHP)

All NHP studies were performed by a CRO in an AAALAC-accredited facility with IACUC approval. Cynomolgus monkeys were pre-screened for little or no pre-existing neutralizing antibody against AAV9 (titer of <1:5). AAV diluted in PBS/0.001%F68 was injected intravenously (via cephalic vein or femoral vein) using a peristaltic pump. 3 weeks later, animals were subject to transcardial perfusion with

PBS, followed by 4% PFA. Tissues were then collected and processed for paraffin embedding and sectioning.

8. Immunohistochemistry

Floating staining was performed for mouse tissue sections with primary antibodies diluted in PBS containing 10% donkey serum and 2% Triton X-100. Primary antibodies used include: chicken anti-GFP (1:1000); rabbit anti-RFP (1:1000); mouse anti-NeuN (1:500); rat anti-GFAP(1:500); Goat anti-GFAP(1:500); mouse anti-CD31(1:500). Secondary antibodies conjugated to fluorophores of Alexa Fluor 488, Alexa Fluor 555 or Alexa Fluor 647 were applied against the primary antibody's host species at a dilution of 1:200.

For paraffin sections of NHP tissue, DAB staining was performed to visualize cells transduced by AAV-AADC. Rabbit anti-AADC antibody (1:500, Millipore) was used as primary antibody.

9. AAV binding assay

HEK293T cells were cultured at 37 °C in a 5% CO2 incubator. One day after seeding of HEK293T cells in a 24-well plate at a density of 250,000 cells per well, a cDNA plasmid of LY6A was transiently transfected into the cells using a transfection mixture of 200ul DMEM (31053028; Gibco), 1 ug DNA plasmid and 3ug of PEI. 48 hours post transfection, cells were placed on ice to chill down for 10 mins. The medium was then changed with 500ul ice-cold serum-free DMEM medium containing rAAVs-mCherry at MOI of 10000. After incubating on ice for one hour, cells with presumably AAVs bound to their surface were washed with cold PBS for three times and were then subject to genomic DNA isolation. Cell-binding viral particles were quantified by using qPCR with primers specific to mCherry and normalized to HEK293T genomes using human GCG as reference.

10. Mouse model of glioblastoma

All experiments were performed in compliance with protocols approved by the Animal Care and Use Committees (IACUC) at the Brigham and Women's Hospital and Harvard Medical School. Syngeneic immuno-competent C57BL/6 female mice

weighing 20 +/- 1 g (Envigo) were used. GL261-Luc (100,000 mouse glioblastoma cells) resuspended in 2μL phosphate buffered saline (PBS) was injected intracranially using 10μl syringe with a 26-gauge needle (80075; Hamilton). A stereotactic frame was used to locate the implantation site (coordinates from bregma in mm: 2 right, 0.5 forward, at a depth of 3.5 into cortex). 7 days later, 200 ul AAV-HSV-TK1 (1E+12 viral genomes, IV) was administered once and ganciclovir (50 mg/kg) was administered daily for 10 days.

Example 1. Modification of AAV9 capsid

To identify peptide sequences that would enhance permeation of a biomolecule or virus across the blood brain barrier an AAV peptide display technique was used. Individual cell-penetrating peptides, as listed in Table 3, were inserted into the AAV9 capsid between amino acids 588 and 589 (VP1 numbering) as illustrated in FIG. 1A. The insertion was carried out by modifying the RC plasmid, one of the three plasmids co-transfected for AAV packaging; FIG. 1B shows an exemplary schematic of the experiments. Individual AAV variants were produced and screened separately. See Materials and Methods #1-3 for more details.

TABLE 3

	AAV	Name of CPP insert	Amino acid sequence of CPP	#	No. of CPP residues	Viral titer
Initial screening	AAV9	N/A	N/A		N/A	Normal
	AAV.CPP.1	SynB1	RGGRLSYSRRRFSTSTGR	93	18	Low
	AAV.CPP.2	L-2	HARIKPTFRRLKWKY KGKFW	94	20	Low
	AAV.CPP.3	PreS2-TLM	PLSSIFSRIGDP	95	12	Low
	AAV.CPP.4	Transportan 10	AGYLLGKINLKALAA LAKKIL	96	21	Low
	AAV.CPP.5	SAP	VRLPPPVRLPPPVRLPPP	97	18	Normal
	AAV.CPP.6	SAP(E)	VELPPPVELPPPVELPPP	98	18	Normal
	AAV.CPP.7	SVM3	KGTYKKKLMRIPLKGT	99	16	Low
	AAV.CPP.8	(PPR)3	PPRPPRPPR	100	9	Normal
	AAV.CPP.9	(PPR)5	PPRPPRPPRPPRPPR	101	15	Low
	AAV.CPP.10	Polyarginin e	RRRRRRRR	102	8	Low
	AAV.CPP.11	Bip1	VPALR	1	5	Normal
	AAV.CPP.12	Bip2	VSALK	2	5	Normal
	AAV.CPP.13	DPV15	LRRERQSRLRRERQSR	103	16	NA
	AAV.CPP.14	HIV-1 Tat	RKKRRQRRR	104	9	NA
Follow-up screening	AAV.CPP.15	Bip1.1	TVPALR (Rat)	3	6	Normal
	AAV.CPP.16	Bip2.1	TVSALK (Syn)	4	6	Normal
	AAV.CPP.17	Bip2.2	FTVSALK (Syn)	5	7	Normal
	AAV.CPP.18	Bip2.3	LTVSALK (Syn)	6	7	Normal
	AAV.CPP.19	Bip2.4	KFTVSALK (Syn)	72	8	Normal
	AAV.CPP.20	Bip2.5	TFVSALK (Syn)	7	7	Normal
	AAV.CPP.21	Bip2.6	TVSALFK (Syn)	8	7	Normal
	AAV.CPP.22	Bip2.6Rat	TVPALFR (Rat)	9	7	Normal

#, SEQ ID NO:

Syn, synthetic

Example 2. First Round of *in vivo* screening

AAVs expressing nuclear RFP (H2B-RFP) were injected intravenously in adult mice with mixed C57BL/6 and BALB/c genetic background. 3 weeks later, brain tissues were harvested and sectioned to reveal RFP-labelled cells (white dots in FIGs. 2A and 2C, quantified in FIGs. 2B and 2D, respectively). CPPs BIP1 and BIP2 were inserted into the capsids of AAV.CPP.11 and AAV.CPP.12, respectively. See Materials and Methods #4-5 for more details.

Example 3. Optimization of modified AAV9 capsids

AAV.CPP.11 and AAV.CPP.12 were further engineered by optimizing the BIP targeting sequences. BIP inserts were derived from the protein Ku70 (See FIG. 3A and Material/Methods #1 for full sequence). The BIP sequence VSALK, which is of "synthetic" origin, was chosen as a study focus to minimize potential species specificity of engineered AAV vectors. AAVs were produced and tested separately for brain transduction efficiency as compared with AAV9 (see FIGs. 3B-C). Percentages of cell transduction in the mouse liver 3 weeks after IV injection of some AAV variants delivering the reporter gene RFP are shown in FIG. 3D. See Materials and Methods #1-5 for more details.

Example 4. *In vitro* model - BBB permeation screening

Some of the AAV variants were screened for the ability to cross the human BBB using an *in vitro* spheroid BBB model. The spheroid contains human microvascular endothelial cells, which form a barrier at the surface, and human pericytes and astrocytes. AAVs carrying nuclear RFP as reporter were assessed for their ability to penetrate from the surrounding medium into the inside of the spheroid and to transduce the cells inside. FIG. 4A shows an experimental schematic. FIGs. 4B-D show results for wt AAV9, AAV.CPP.16, and AAV.CPP.21, respectively, those and other peptides are quantified in FIG. 4E. In this model, peptides 11, 15, 16, and 21 produced the greatest permeation into the spheroids. See Materials and Methods #6 for more details.

Example 5. *In vivo* BBB permeation screening

AAV.CPP.16 and AAV.CPP.21 were selected for further evaluation in an in vivo model, in experiments performed as described above for Example 2. All AAVs carried nuclear RFP as reporter. Both showed enhanced ability vs. AAV9 to transduce brain cells after intravenous administration in C57BL/6J adult mice (white dots in brain sections in FIG. 5A, quantified in FIG. 5B) and in BALB/c adult mice (white dots in brain sections in FIG. 6A, quantified in FIG. 6B).

High doses of AAV.CPP.16 and AAV.CPP.21 (4×10^{12} vg per mouse, administered IV) resulted in widespread brain transduction in mice. Both AAVs carried nuclear RFP as reporter (white dots in brain sections in FIG. 7A, quantified in FIG. 7B).

Example 6. *In vivo* distribution of modified AAVs

As shown in FIG. 8A, AAV.CPP.16 and AAV.CPP.21 preferentially targeted neurons (labeled by a NeuN antibody) across multiple brain regions in mice including the cortex, midbrain and hippocampus. Both AAVs carried nuclear RFP as a reporter.

AAV.CPP.16 and AAV.CPP.21 also showed enhanced ability vs. AAV9 in targeting the spinal cord and motor neurons in mice. All AAVs carry nuclear RFP as reporter and were administered intravenously into neonate mice (4×10^{10} vg). Motor neurons were visualized using CHAT antibody staining. Co-localization of RFP and CHAT signals in FIG. 8B suggested specific transduction of the motor neurons.

The relative abilities of AAV-CAG-H2B-RFP and AAV.CPP.16-CAG-H2B-RFP to transduce various tissues in mice was also evaluated. 1×10^{11} vg was injected intravenously. The number of cells transduced was normalized to the number of total cells labeled by DAPI nuclear staining. The results showed that AAV.CPP.16 was more efficient than AAV9 in targeting heart (FIG. 9A); skeletal muscle (FIG. 9B), and dorsal root ganglion (FIG. 9C) tissue in mice.

Example 7. BBB permeation in a non-human primate model

2×10^{13} vg/kg AAVs-CAG-AADC (as reporter gene) were injected intravenously into 3-month-old cynomolgus monkeys. AAV-transduced cells (shown in black) were visualized using antibody staining against AADC. As shown in FIGs. 10A-D, AAV.CPP.16 and AAV.CPP.21 showed enhanced ability vs. AAV9 to transduce brain cells after intravenous administration in non-human primates. AAV.CPP.16 transduced significantly more cells then wt AAV9 in the primary visual cortex (FIG. 10A), parietal cortex (FIG. 10B), thalamus (FIG. 10C), and cerebellum (FIG. 10D). See Materials and Methods #7-8 for more details.

Example 8. AAV.CPP.16 and AAV.CPP.21 do not bind to LY6A

LY6A serves as a receptor for AAV.PHP.eB and mediates AAV.PHP.eB's robust effect in crossing the BBB in certain mouse strains. Over-expressing mouse LY6A in cultured 293 cells significantly increased binding of AAV.PHP.eB to the cell surface (see FIG. 11A). On the contrary, over-expressing LY6A does not increase viral binding for AAV9, AAV.CPP.16 or AAV.CPP.21 (see FIG. 11B). This suggests

AAV.CPP.16 or AAV.CPP.21 does not share LY6A with AAV.PHP.eB as a receptor. See Materials and Methods #9 for more details.

Example 9. Delivering therapeutic proteins to the brain using AAV.CPP.21

AAV.CPP.21 was used to systemically deliver the "suicide gene" HSV.TK1 in a mouse model of brain tumor. HSV.TK1 turns the otherwise "dormant" ganciclovir into a tumor-killing drug. Intravenously administered AAV.CPP.21-H2BmCherry (FIG. 12A, bottom left and middle right panel) was shown to target tumor mass, especially the tumor expanding frontier. As shown in FIGs. 12B-C, using AAV.CPP.21 to systemically deliver the "suicide gene" HSV.TK1 resulted in shrinkage of brain tumor mass, when combined with the pro-drug ganciclovir. These results show that AAV.CPP.21 can be used to systemically deliver a therapeutic gene into brain tumor. See Materials and Methods #10 for more details.

Example 10. Intracerebral administration of AAV.CPP.21

In addition to systemic administration (such as in Example 2), an AAV as described herein was administered locally into the mouse brain. Intracerebral injection of AAV9-H2B-RFP and AAV.CPP.21-H2B-RFP (FIG. 13) resulted in more widespread and higher-intensity RFP signal in AAV.CPP.21-treated brain sections vs. AAV9-treated ones. See Materials and Methods #4 for more details.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. An AAV capsid protein comprising an amino acid sequence that comprises at least four contiguous amino acids from the sequence TVSALFK (SEQ ID NO:8); TVSALK (SEQ ID NO:4); KLASVT (SEQ ID NO:83); or KFLASVT (SEQ ID NO:84).

2. The AAV capsid protein of claim 1, comprising an amino acid sequence that comprises at least five contiguous amino acids from the sequence TVSALK (SEQ ID NO:4); TVSALFK (SEQ ID NO:8); KLASVT (SEQ ID NO:83); or KFLASVT (SEQ ID NO:84).

3. The AAV capsid protein of claim 1, comprising an amino acid sequence that comprises at least six contiguous amino acids from the sequence TVSALK (SEQ ID NO:4); TVSALFK (SEQ ID NO:8); KLASVT (SEQ ID NO:83); or KFLASVT (SEQ ID NO:84).

4. The AAV capsid protein of claims 1-3, wherein the AAV is AAV9.

5. The AAV capsid protein of claims 1-4, comprising AAV9 VP1.

6. The AAV capsid protein of claim 5, wherein the targeting sequence is inserted in a position corresponding to amino acids 588 and 589 of SEQ ID NO:85.

7. A nucleic acid encoding the AAV capsid protein of claims 1-6.

8. An AAV comprising the capsid protein of claims 1-6.

9. The AAV of claim 8, further comprising a transgene, preferably a therapeutic transgene.

10. A targeting sequence comprising V[S/p][A/m/t/]L (SEQ ID NO:79), TV[S/p][A/m/t/]L (SEQ ID NO:80), TV[S/p][A/m/t/]LK (SEQ ID NO:81), or TV[S/p][A/m/t/]LFK. (SEQ ID NO:82).

11. The targeting sequence of claim 10, wherein the targeting sequence comprises VPALR (SEQ ID NO:1); VSALK (SEQ ID NO:2); TVPALR (SEQ ID NO:3); TVSALK (SEQ ID NO:4); TVPMLK (SEQ ID NO:12); TVPTLK (SEQ ID

NO:13); FTVSALK (SEQ ID NO:5); LTVSALK (SEQ ID NO:6); TVSALFK (SEQ ID NO:8); TVPALFR (SEQ ID NO:9); TVPMLFK (SEQ ID NO:10) or TVPTLFK (SEQ ID NO:11).

12. A fusion protein comprising the targeting sequence of claims 10-11, and a heterologous sequence.

13. An AAV capsid protein comprising the targeting sequence of claims 10-11.

14. The AAV capsid protein of claim 13, comprising AAV9 VP1.

15. The AAV capsid protein of claim 14, wherein the targeting sequence is inserted in a position corresponding to amino acids 588 and 589 of SEQ ID NO: .

16. A nucleic acid encoding the targeting sequence, fusion protein or AAV capsid protein of claims 10-16.

17. An AAV comprising the capsid protein of claims 13-15.

18. The AAV of claim 17, further comprising a transgene, preferably a therapeutic transgene.

19. A method of delivering a transgene to a cell, the method comprising contacting the cell with the AAV of claims 1-9, 17, or 18.

20. The method of claim 19, wherein the cell is a neuron (optionally a dorsal root ganglion neuron), astrocyte, cardiomyocyte, or myocyte.

21. The method of claim 19, wherein the cell is in a living subject.

22. The method of claims 19, wherein the subject is a mammalian subject.

23. The method of claims 20 to 22, wherein the cell is in a tissue selected from the brain, spinal cord, dorsal root ganglion, heart, or muscle, and a combination thereof.

24. The method of claim 23, wherein the subject has a neurodegenerative disease, epilepsy; stroke; spinocerebellar ataxia; Canavan's disease; Metachromatic leukodystrophy; Spinal muscular atrophy; Friedreich's ataxia; X-linked

centronuclear myopathy; Lysosomal storage disease; Barth syndrome; Duchenne muscular dystrophy; Wilson's disease; or Crigler-Najjar syndrome type 1.

25. The method of claim 24, wherein the neurodegenerative disease is Parkinson's disease; Alzheimer's disease; Huntington's disease; Amyotrophic lateral sclerosis; and Multiple sclerosis.

26. The method of claim 23, wherein the subject has a brain cancer, and the method includes administering an AAV encoding an anti-cancer agent.

27. The method of claim 26, wherein the anti-cancer agent is HSV.TK1, and the method further comprises administering ganciclovir.

28. The method of any of claims 22-27, wherein the cell is in the brain of the subject, and the AAV is administered by parenteral delivery; intracerebral; or intrathecal delivery.

29. The method of claim 28, wherein the parenteral delivery is via intravenous, intraarterial, subcutaneous, intraperitoneal, or intramuscular delivery.

30. The method of claim 28, wherein the intrathecal delivery is via lumbar injection, cisternal magna injection, or intraparenchymal injection.

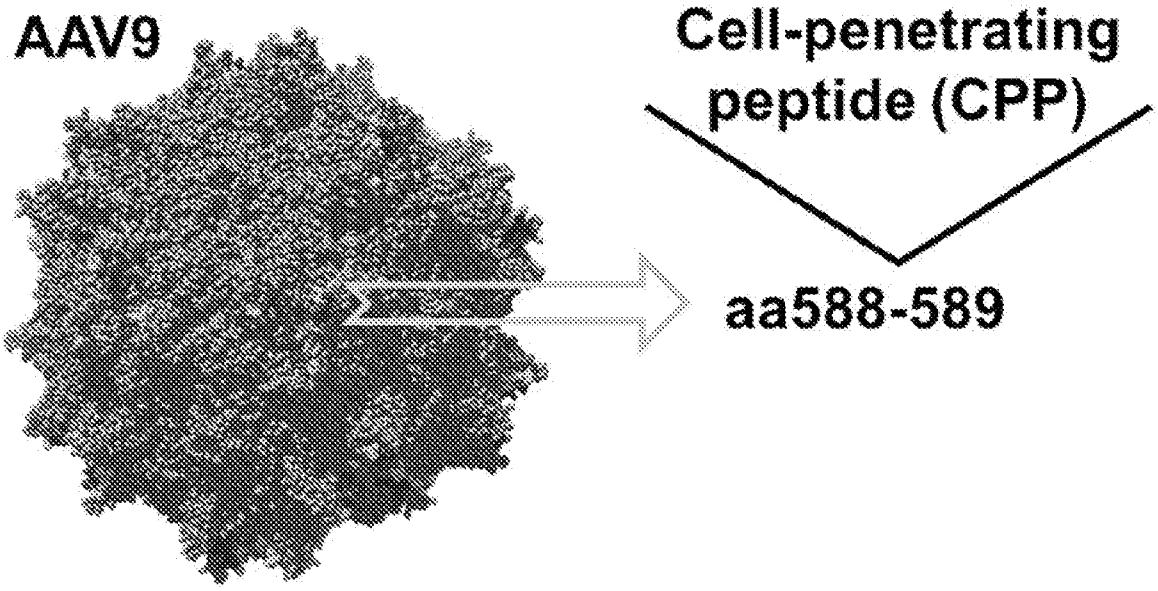

AAV9
Cell-penetrating peptide (CPP)
aa588-589

FIG. 1A

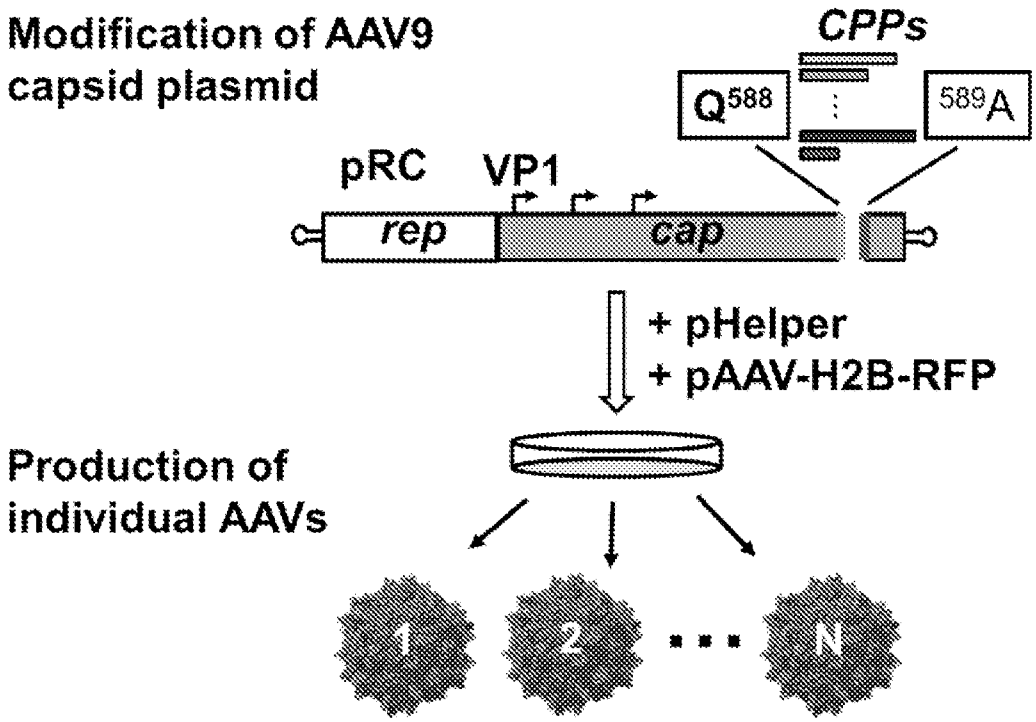

Modification of AAV9 capsid plasmid
CPPs
Q588
589A
pRC
VP1
rep
cap
+ pHelper
+ pAAV-H2B-RFP
Production of individual AAVs
1
2
N

FIG. 1B

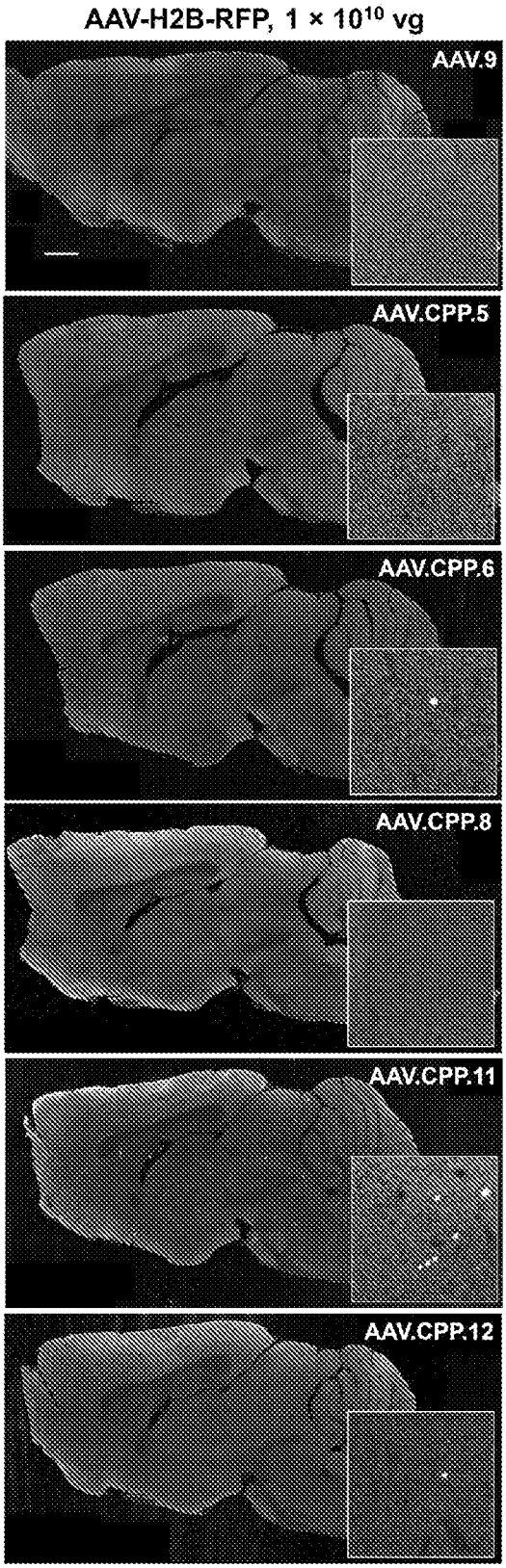

AAV-H2B-RFP, 1 × 10^10 vg
AAV.9
AAV.CPP.5
AAV.CPP.6
AAV.CPP.8
AAV.CPP.11
AAV.CPP.12

FIG. 2A

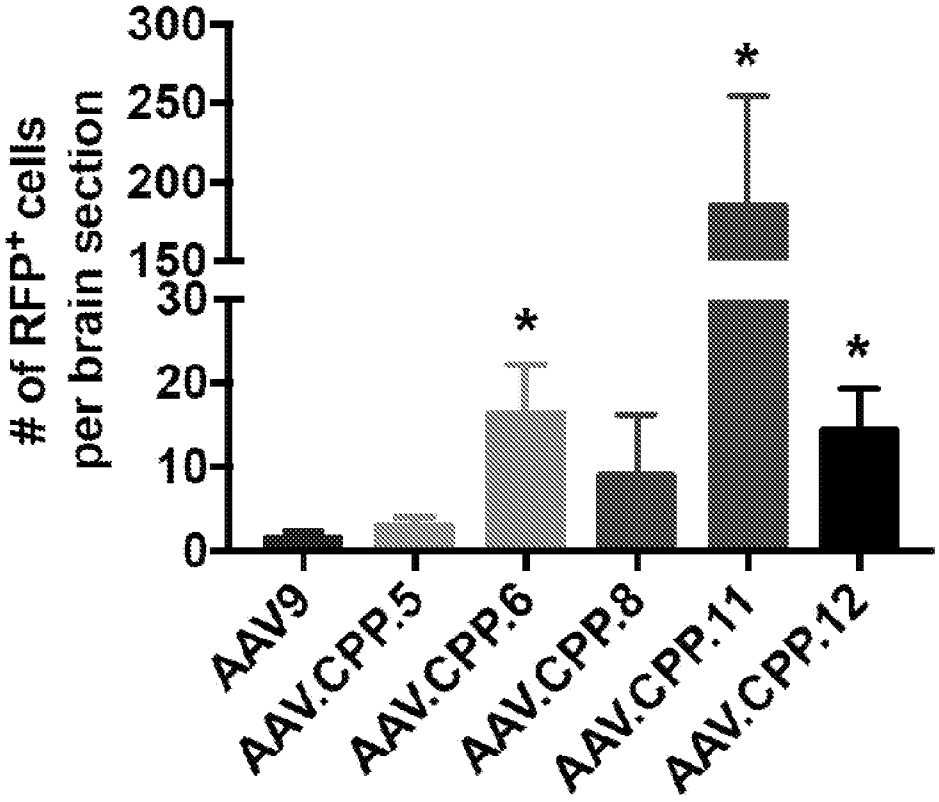

# of RFP+ cells per brain section
300
250
200
150
30
20
10
0
*
*
*
AAV9
AAV.CPP.5
AAV.CPP.6
AAV.CPP.8
AAV.CPP.11
AAV.CPP.12

FIG. 2B

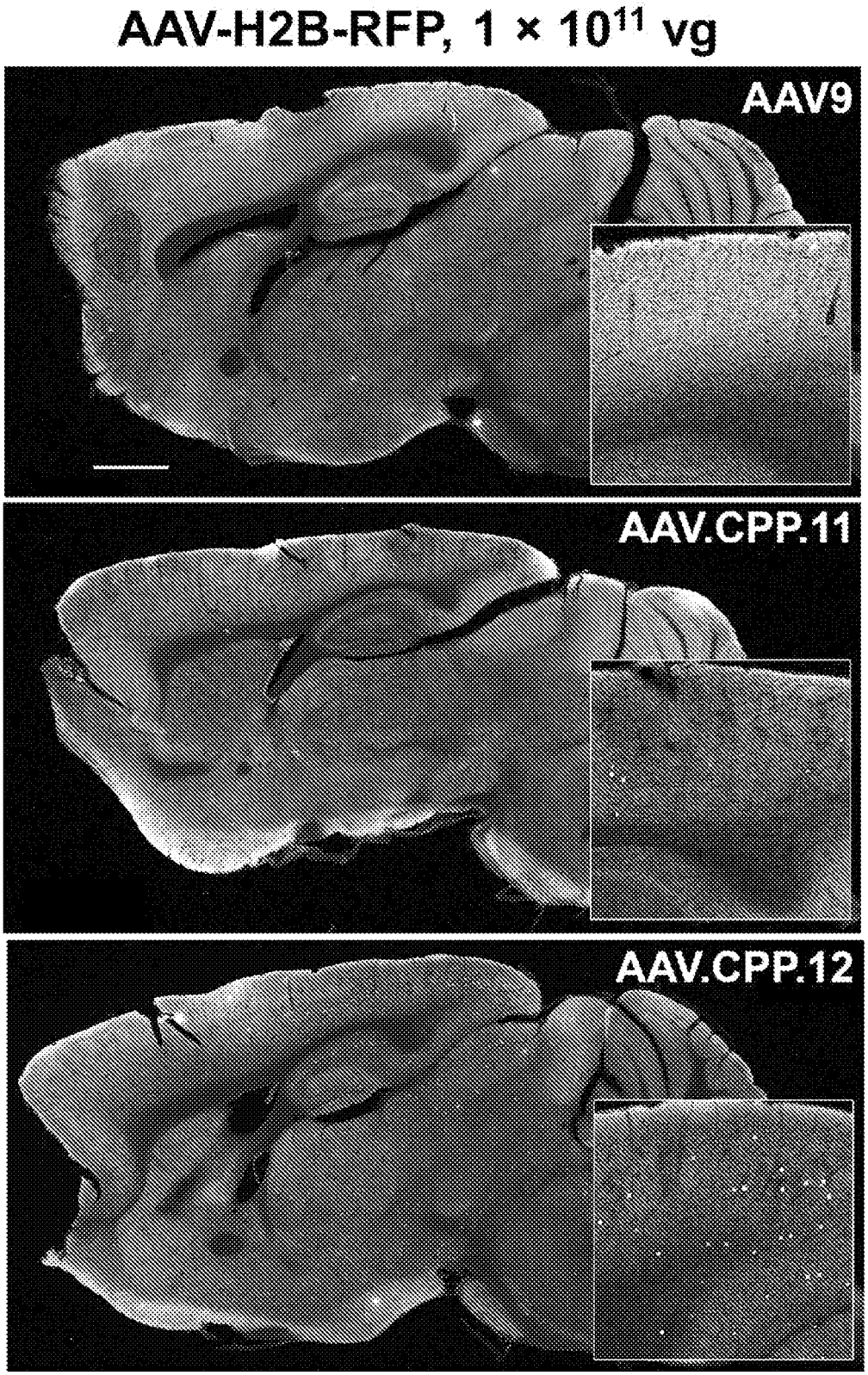


FIG. 2C

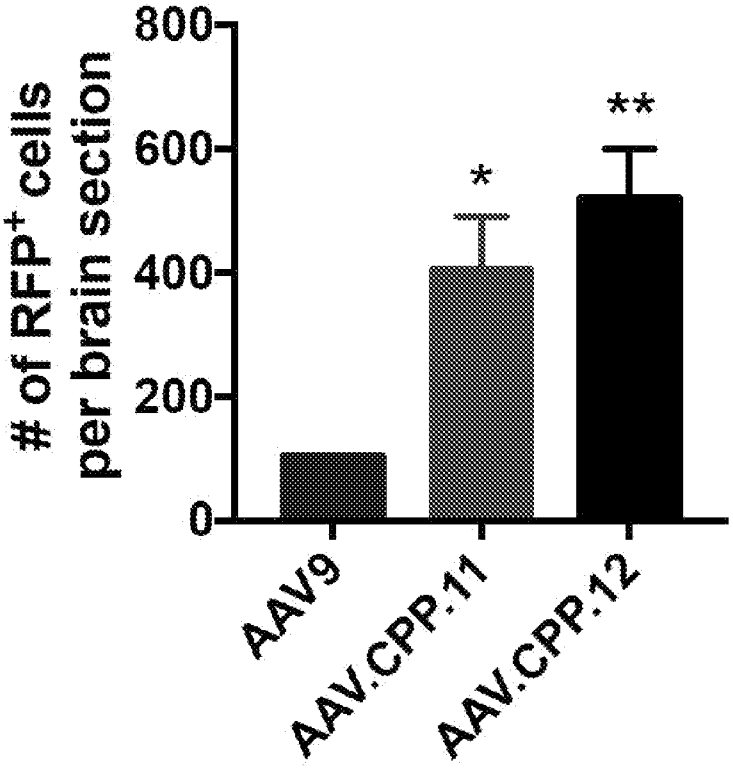

# of RFP+ cells per brain section
800
600
400
200
0
*
**
AAV9
AAV.CPP.11
AAV.CPP.12

FIG. 2D

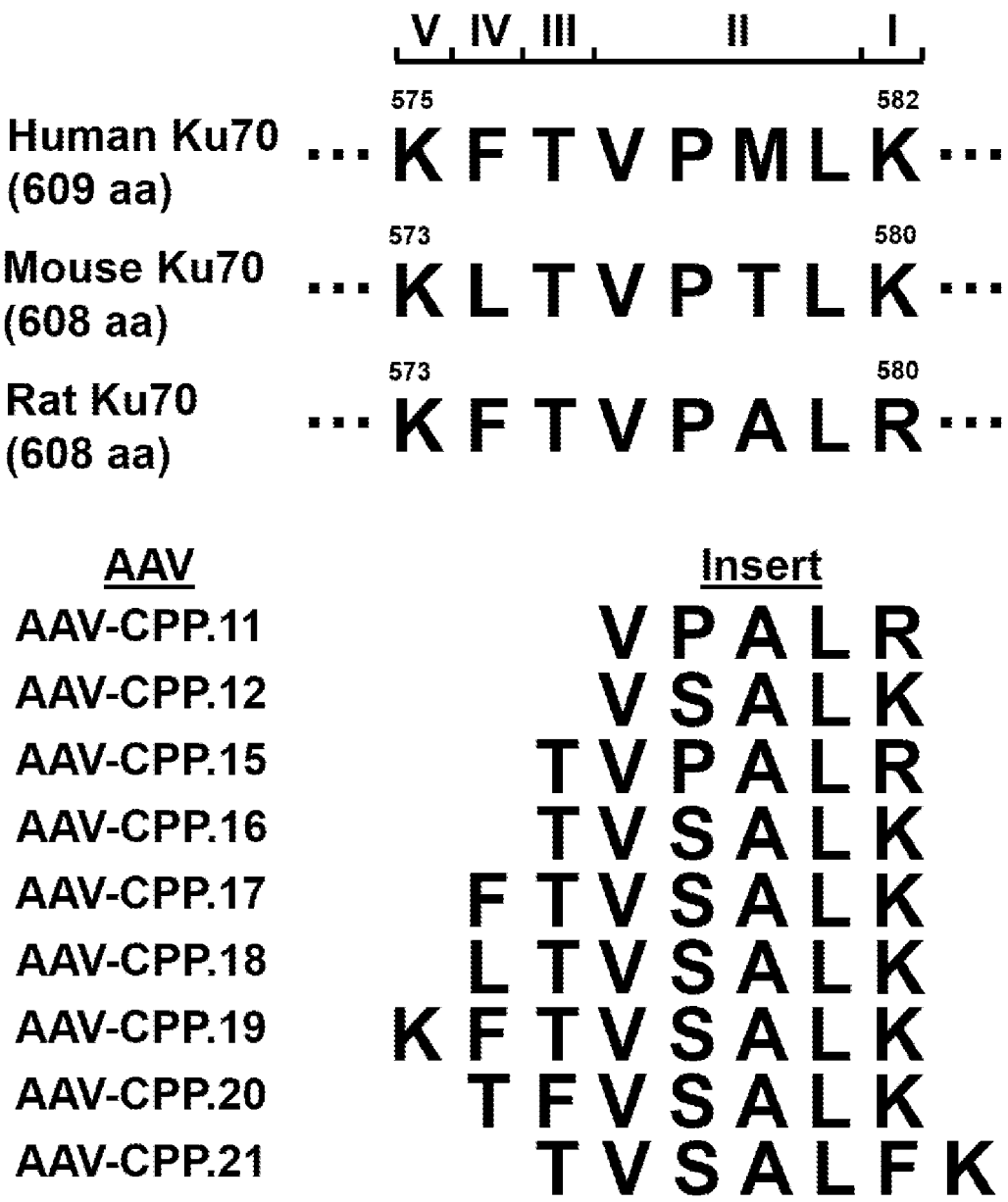

V IV III II I
575
582
Human Ku70 (609 aa) ... K F T V P M L K ...
573
580
Mouse Ku70 (608 aa) ... K L T V P T L K ...
573
580
Rat Ku70 (608 aa) ... K F T V P A L R ...
AAV
Insert
AAV-CPP.11 V P A L R
AAV-CPP.12 V S A L K
AAV-CPP.15 T V P A L R
AAV-CPP.16 T V S A L K
AAV-CPP.17 F T V S A L K
AAV-CPP.18 L T V S A L K
AAV-CPP.19 K F T V S A L K
AAV-CPP.20 T F V S A L K
AAV-CPP.21 T V S A L F K

FIG. 3A

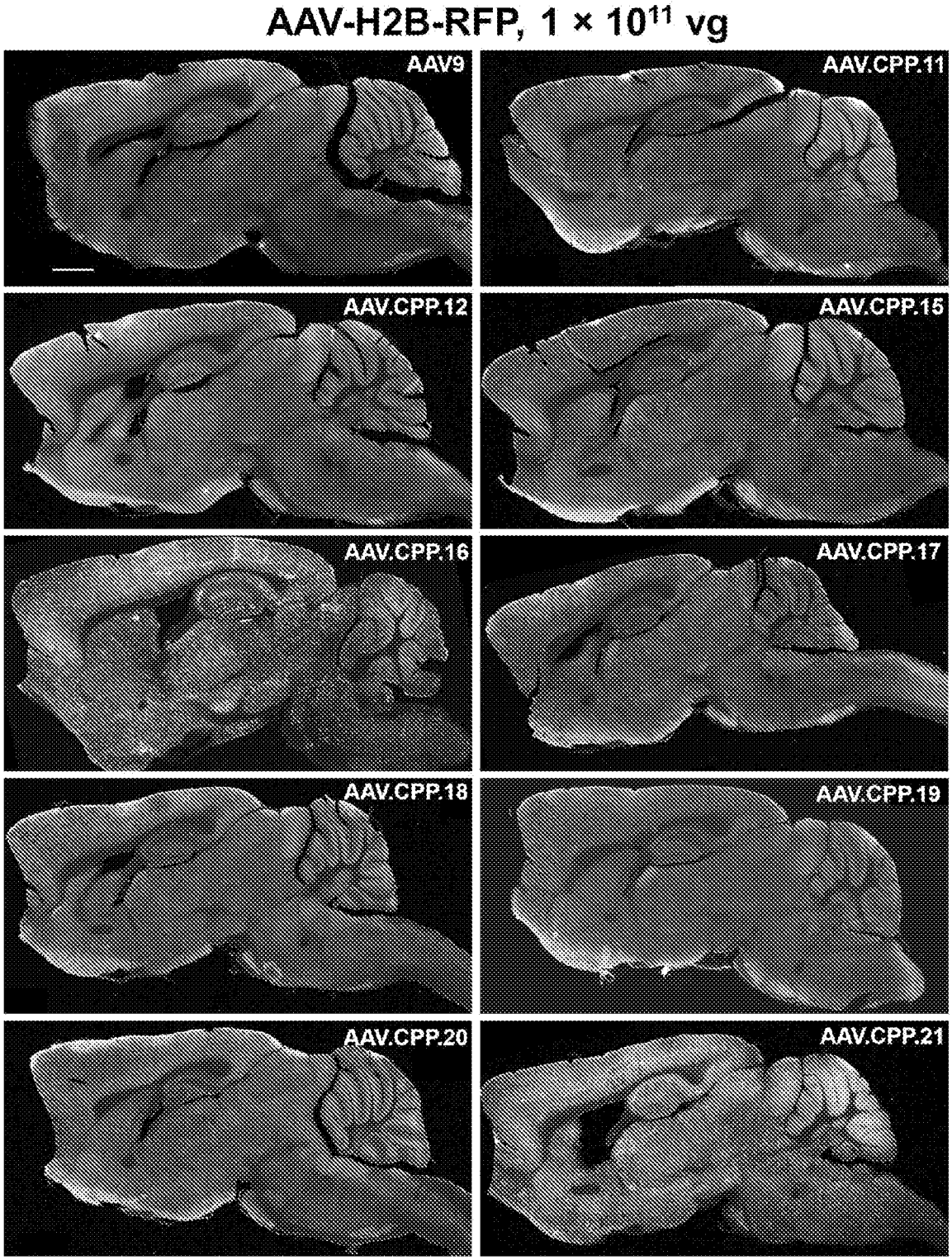

AAV-H2B-RFP, 1 × 10^11 vg
AAV9
AAV.CPP.11
AAV.CPP.12
AAV.CPP.15
AAV.CPP.16
AAV.CPP.17
AAV.CPP.18
AAV.CPP.19
AAV.CPP.20
AAV.CPP.21

FIG. 3B

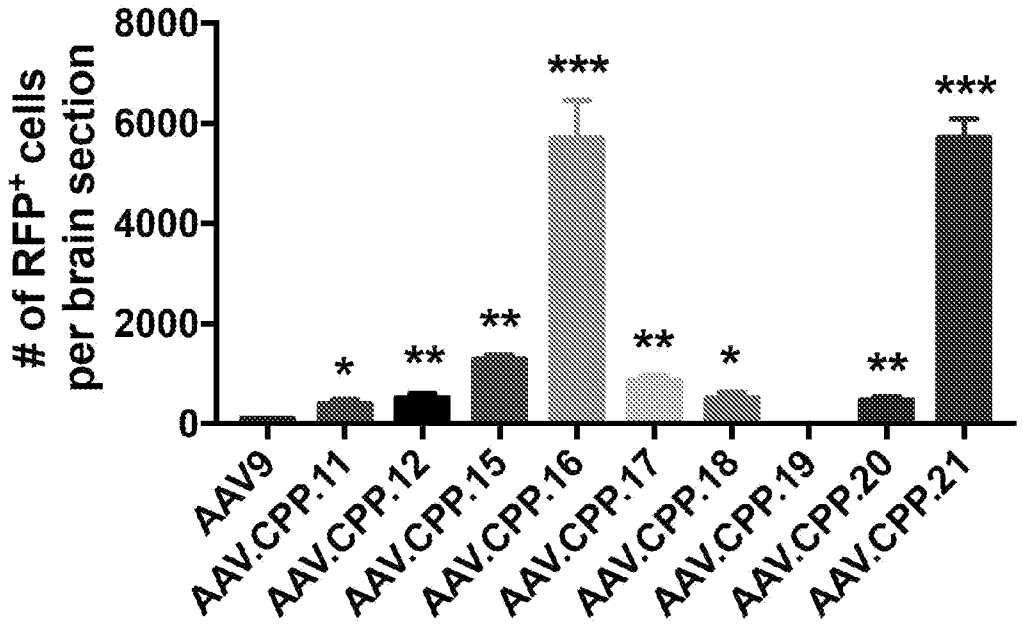

# of RFP+ cells per brain section
8000
6000
4000
2000
0
*
**
**
***
**
*
**
***
AAV9
AAV.CPP.11
AAV.CPP.12
AAV.CPP.15
AAV.CPP.16
AAV.CPP.17
AAV.CPP.18
AAV.CPP.19
AAV.CPP.20
AAV.CPP.21

FIG. 3C

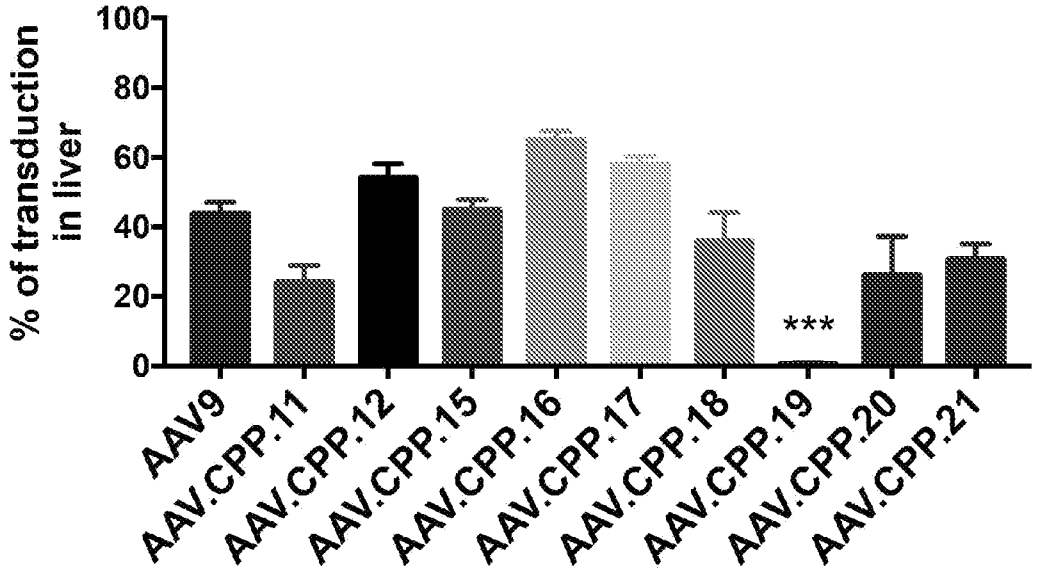

% of transduction in liver
100
80
60
40
20
0
***
AAV9
AAV.CPP.11
AAV.CPP.12
AAV.CPP.15
AAV.CPP.16
AAV.CPP.17
AAV.CPP.18
AAV.CPP.19
AAV.CPP.20
AAV.CPP.21

FIG. 3D

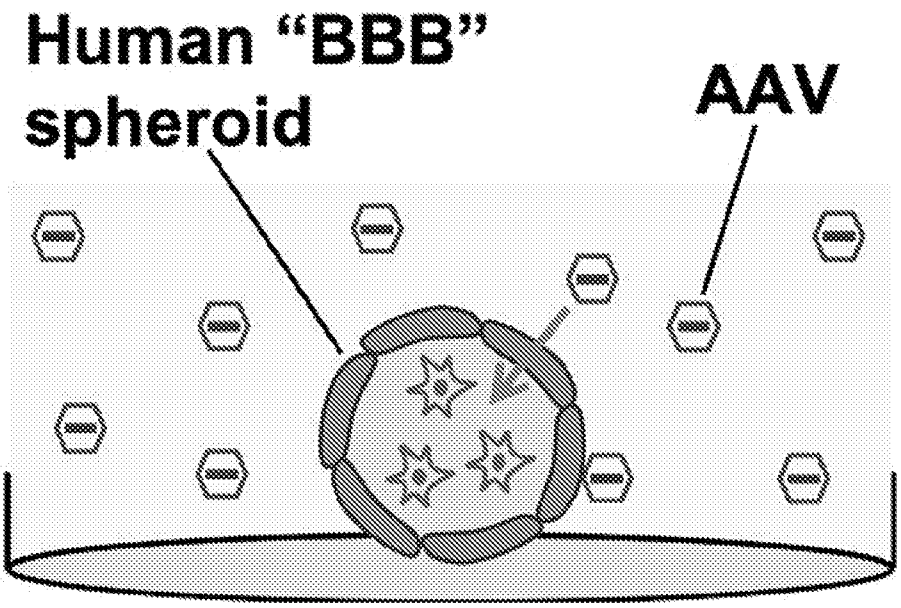


FIG. 4A

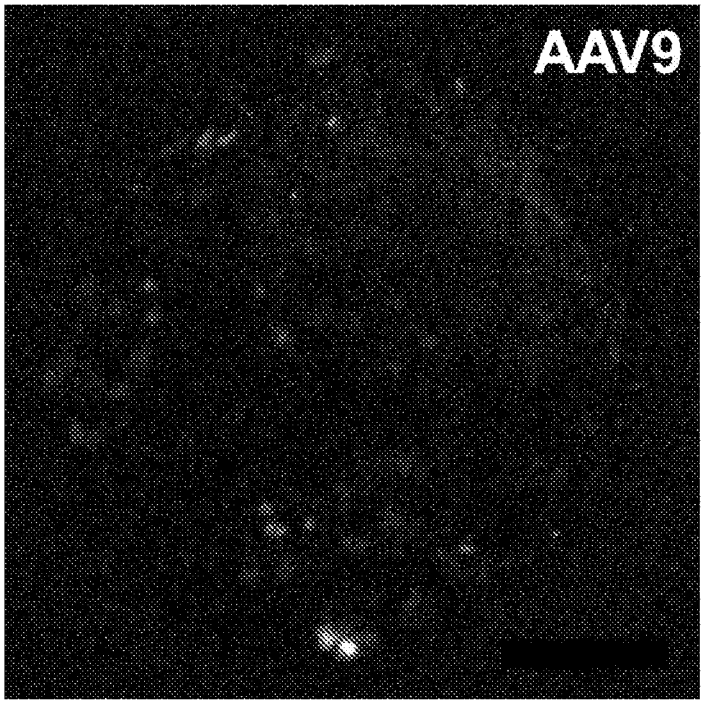


FIG. 4B

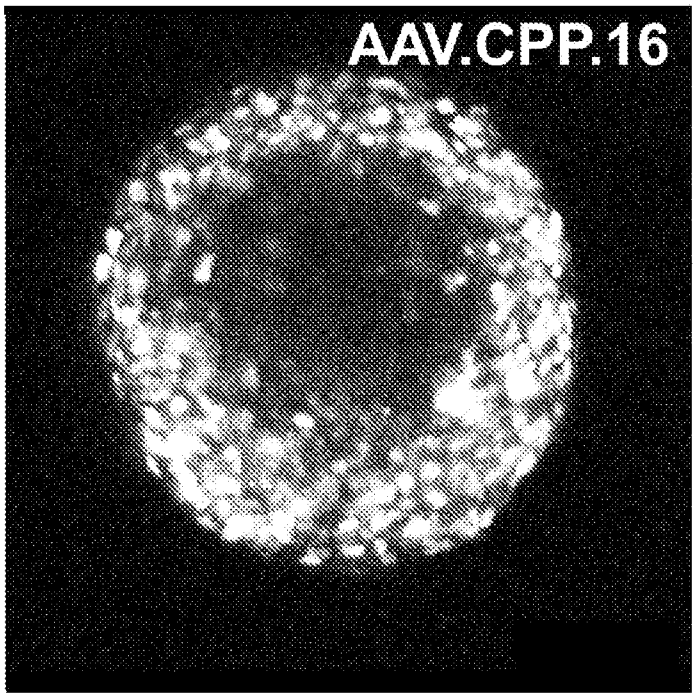


FIG. 4C

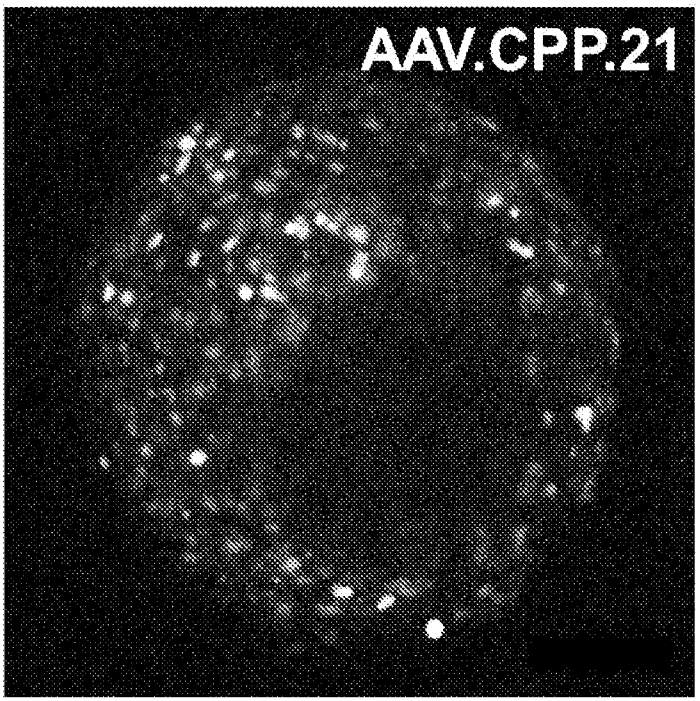


FIG. 4D

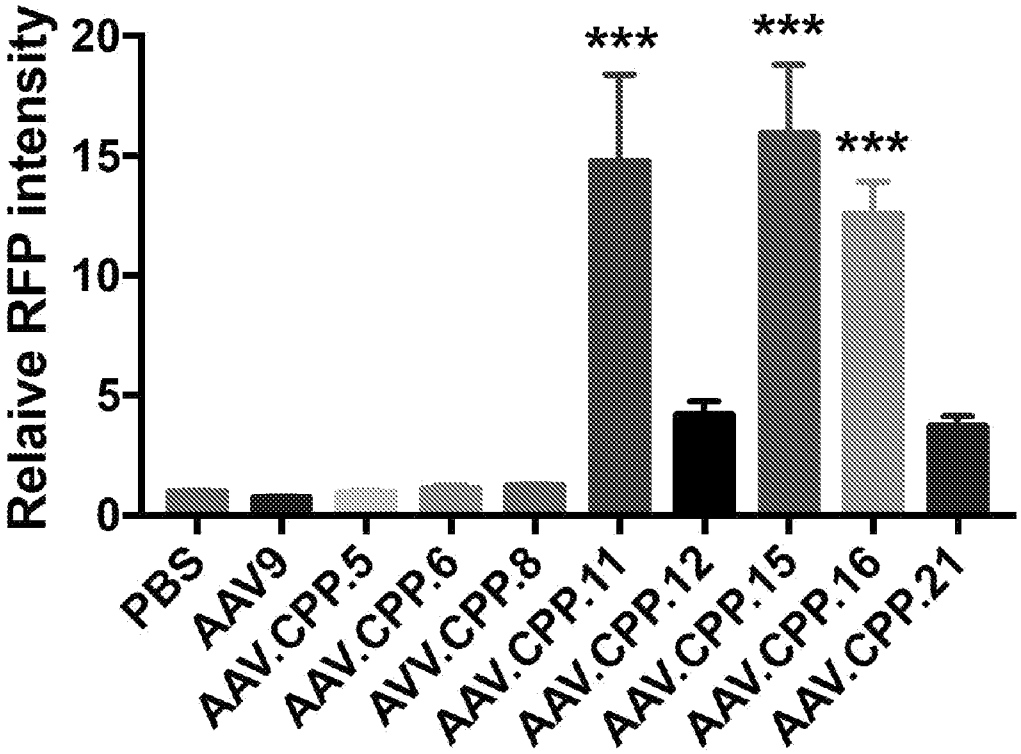

Relaive RFP intensity
20
15
10
5
0
***
***
***
PBS
AAV9
AAV.CPP.5
AAV.CPP.6
AVV.CPP.8
AAV.CPP.11
AAV.CPP.12
AAV.CPP.15
AAV.CPP.16
AAV.CPP.21

FIG. 4E

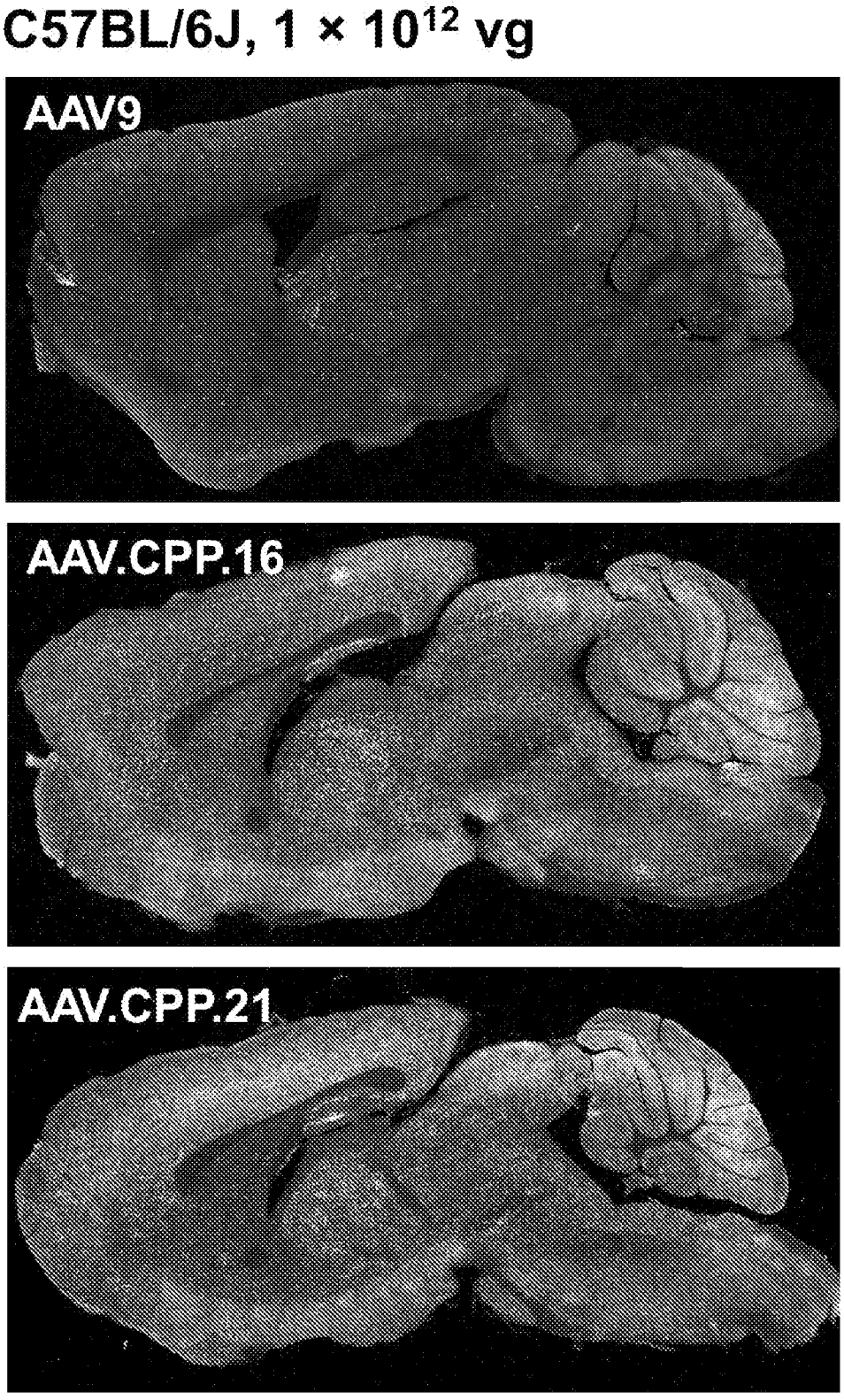


FIG. 5A

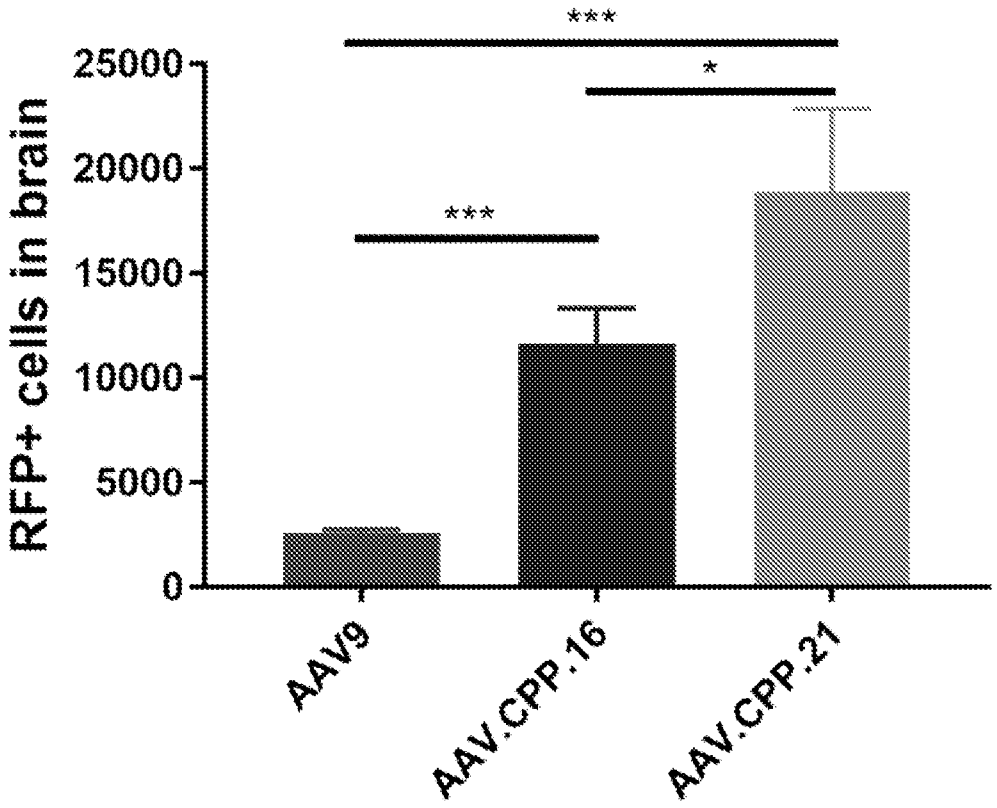

***
*
***
25000
20000
15000
10000
5000
0
RFP+ cells in brain
AAV9
AAV.CPP.16
AAV.CPP.21

FIG. 5B

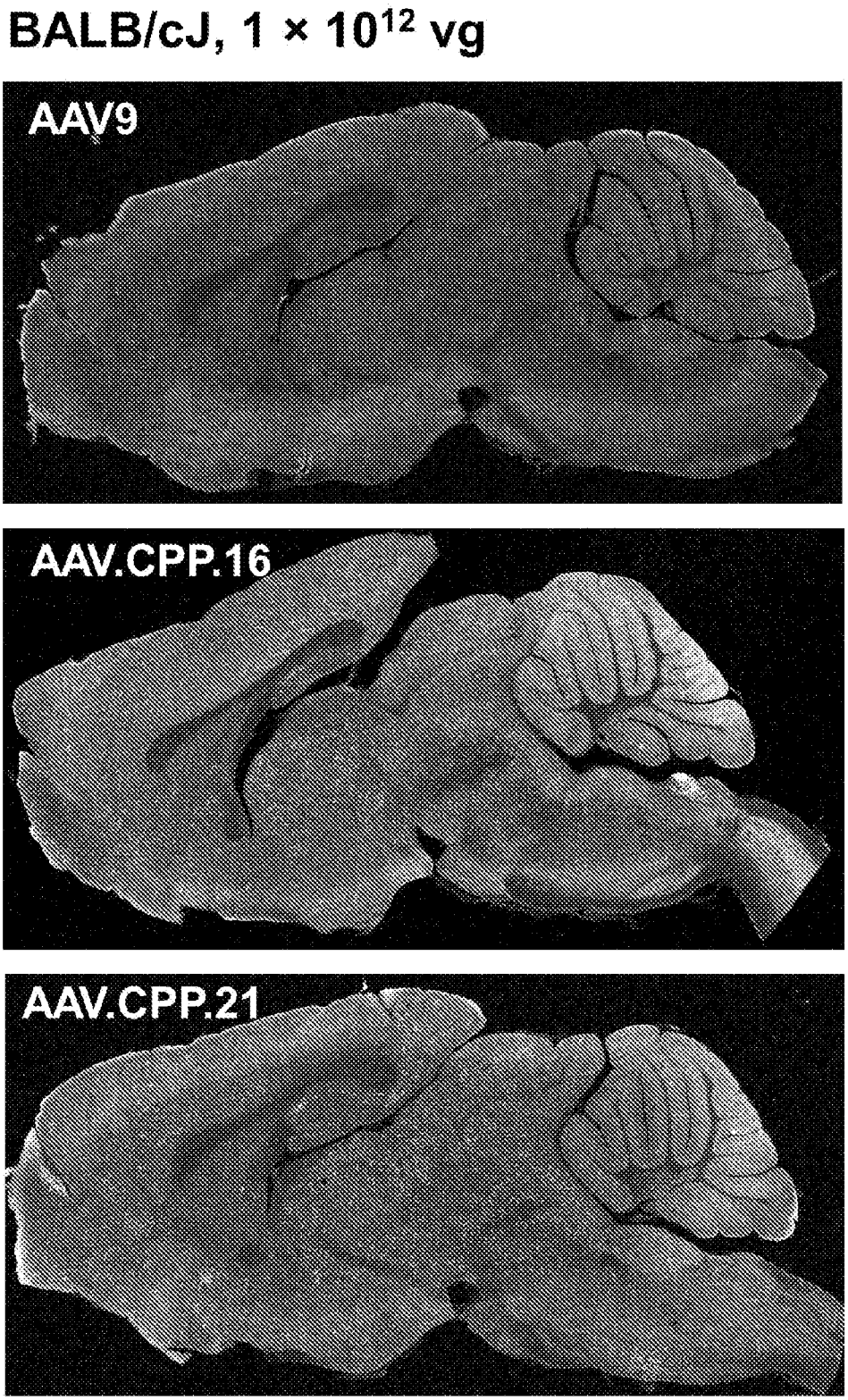


FIG. 6A

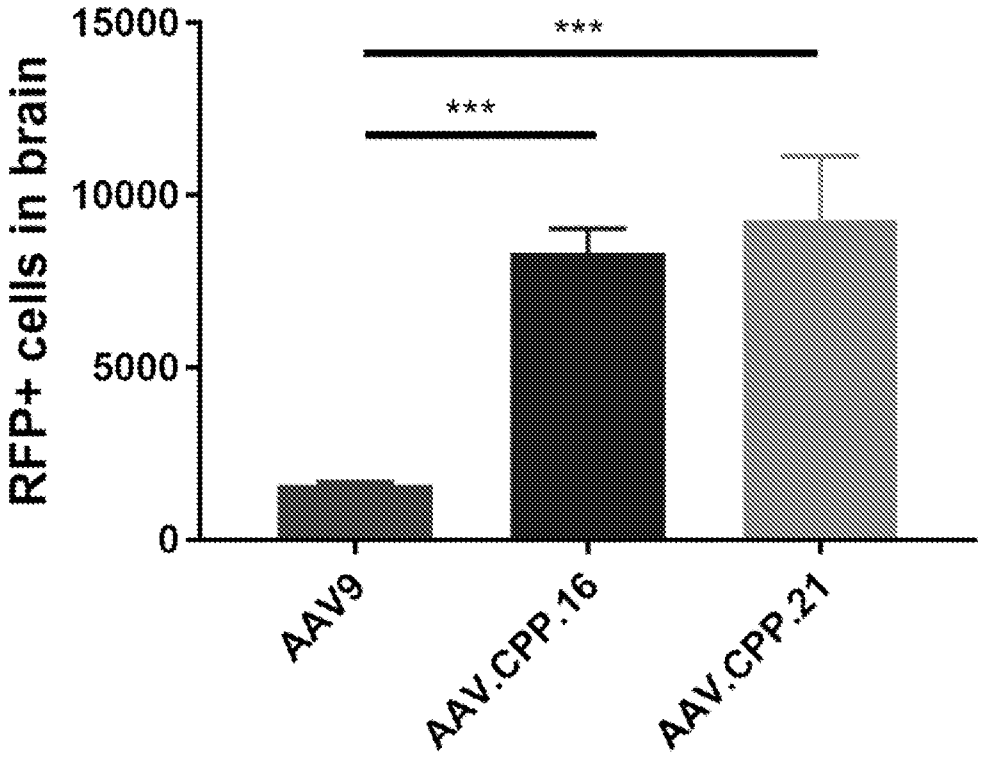

15000
10000
5000
0
RFP+ cells in brain
***
***
AAV9
AAV.CPP.16
AAV.CPP.21

FIG. 6B

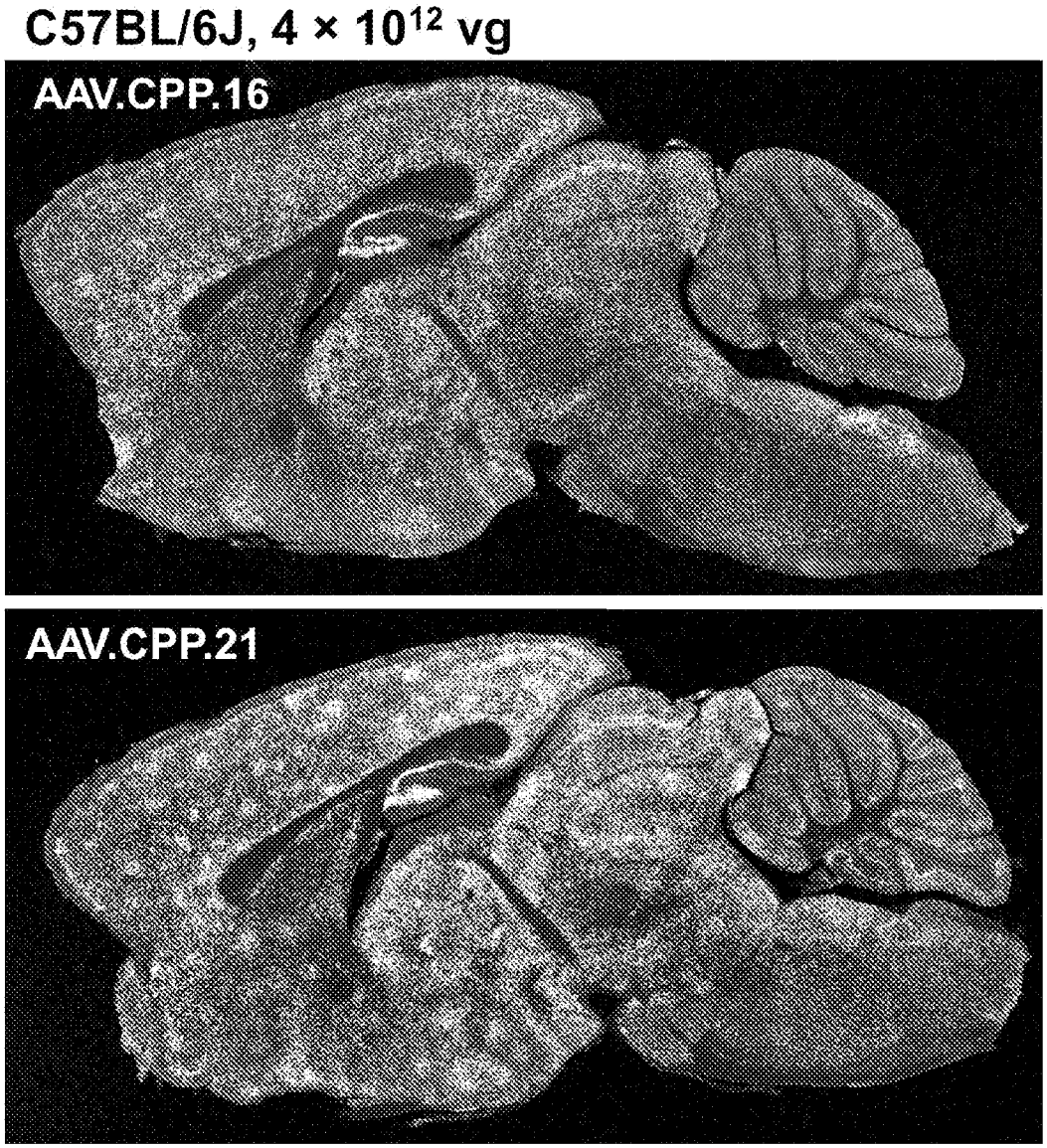


FIG. 7A

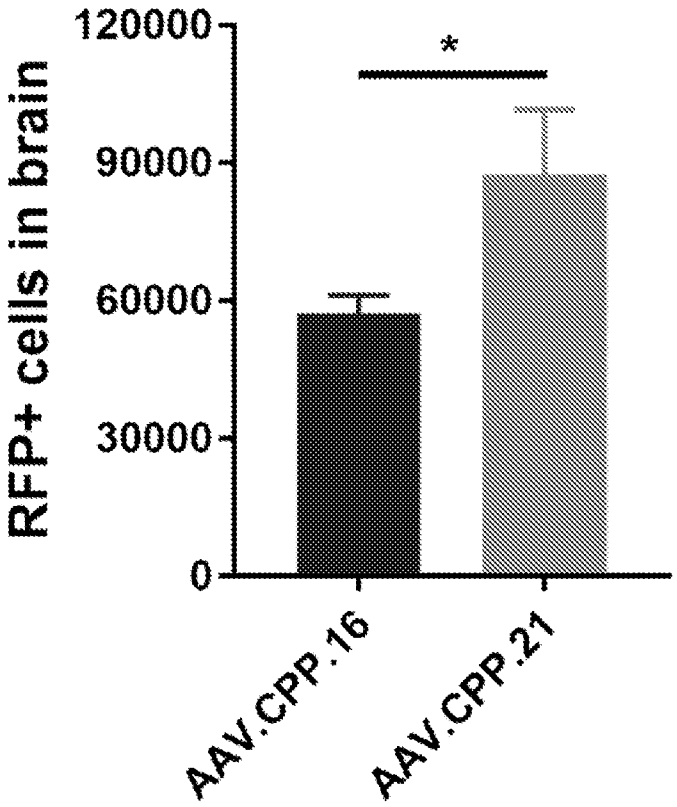

120000
90000
60000
30000
0
RFP+ cells in brain
*
AAV.CPP.16
AAV.CPP.21

FIG. 7B

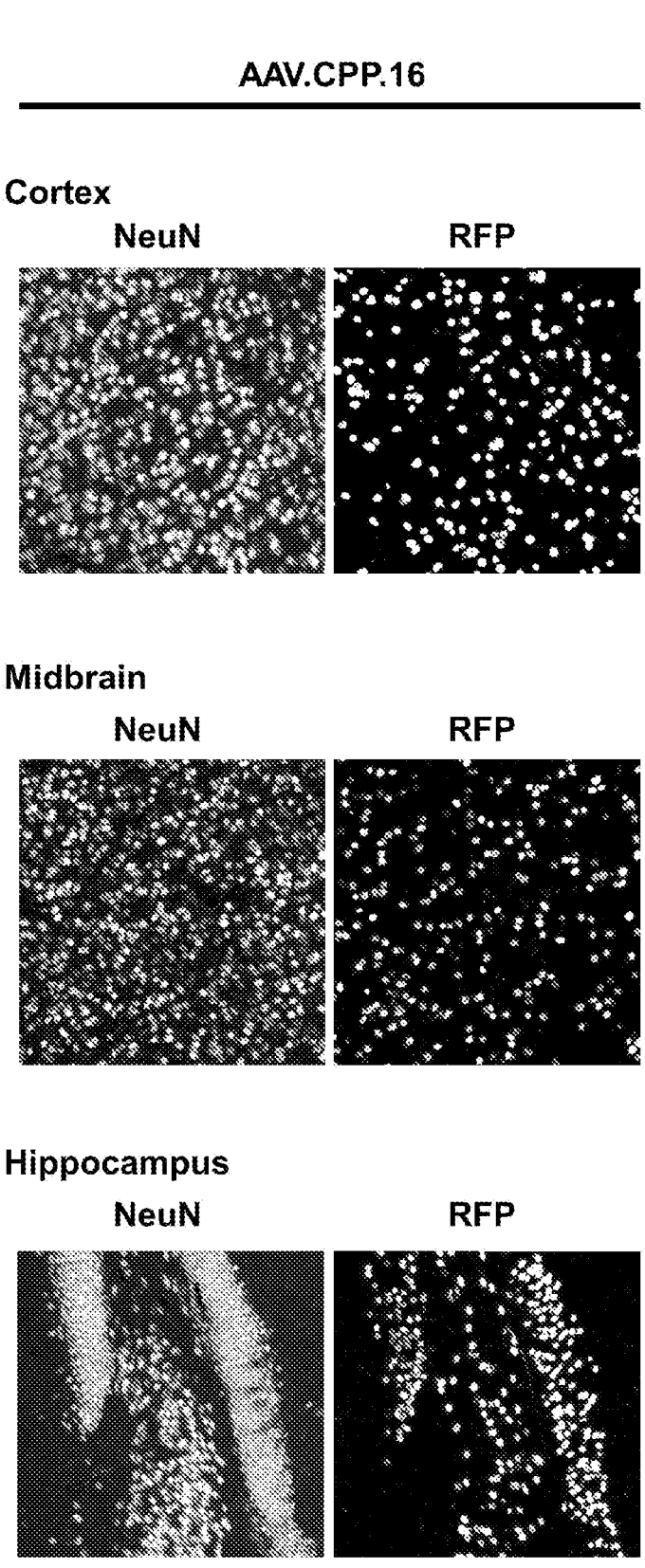
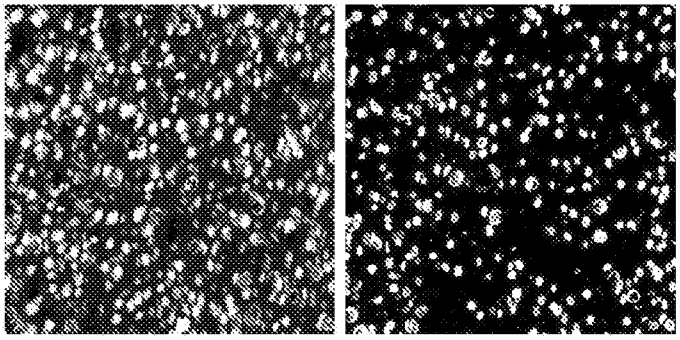

AAV.CPP.16
Cortex
NeuN
RFP
Midbrain
NeuN
RFP
Hippocampus
NeuN
RFP

FIG. 8A

AAV.CPP. 21

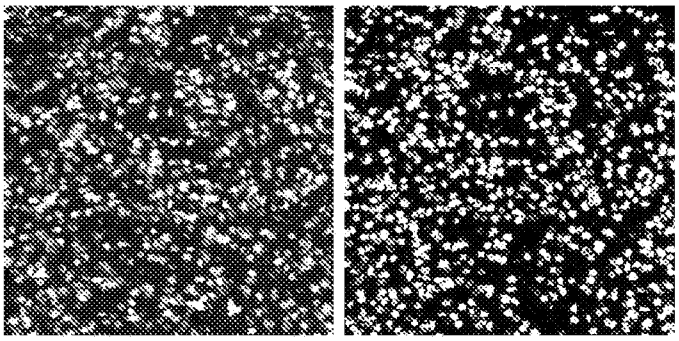
Cortex

NeuN RFP



Midbrain

NeuN RFP



Hippocampus

NeuN RFP

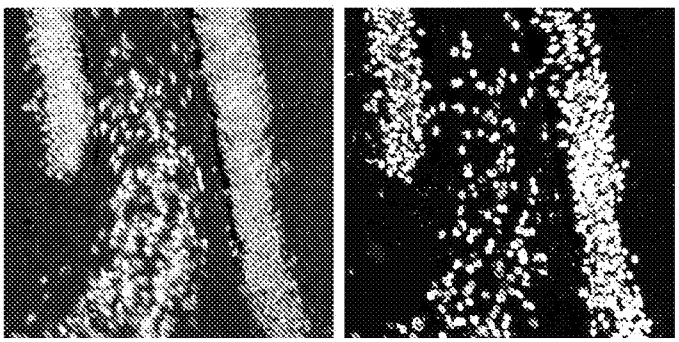


FIG. 8A, continued

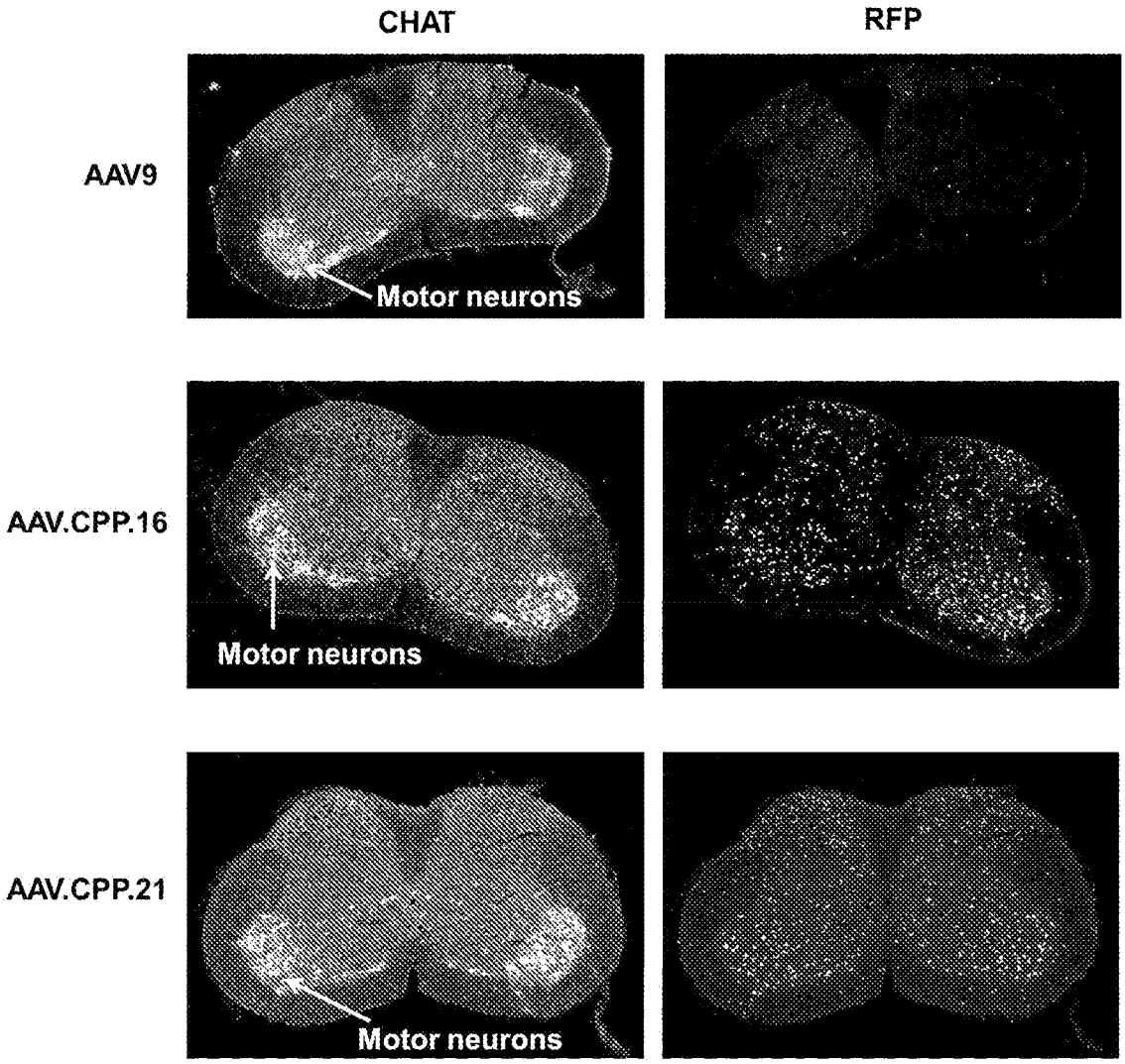

CHAT
RFP
AAV9
Motor neurons
AAV.CPP.16
Motor neurons
AAV.CPP.21
Motor neurons

FIG. 8B

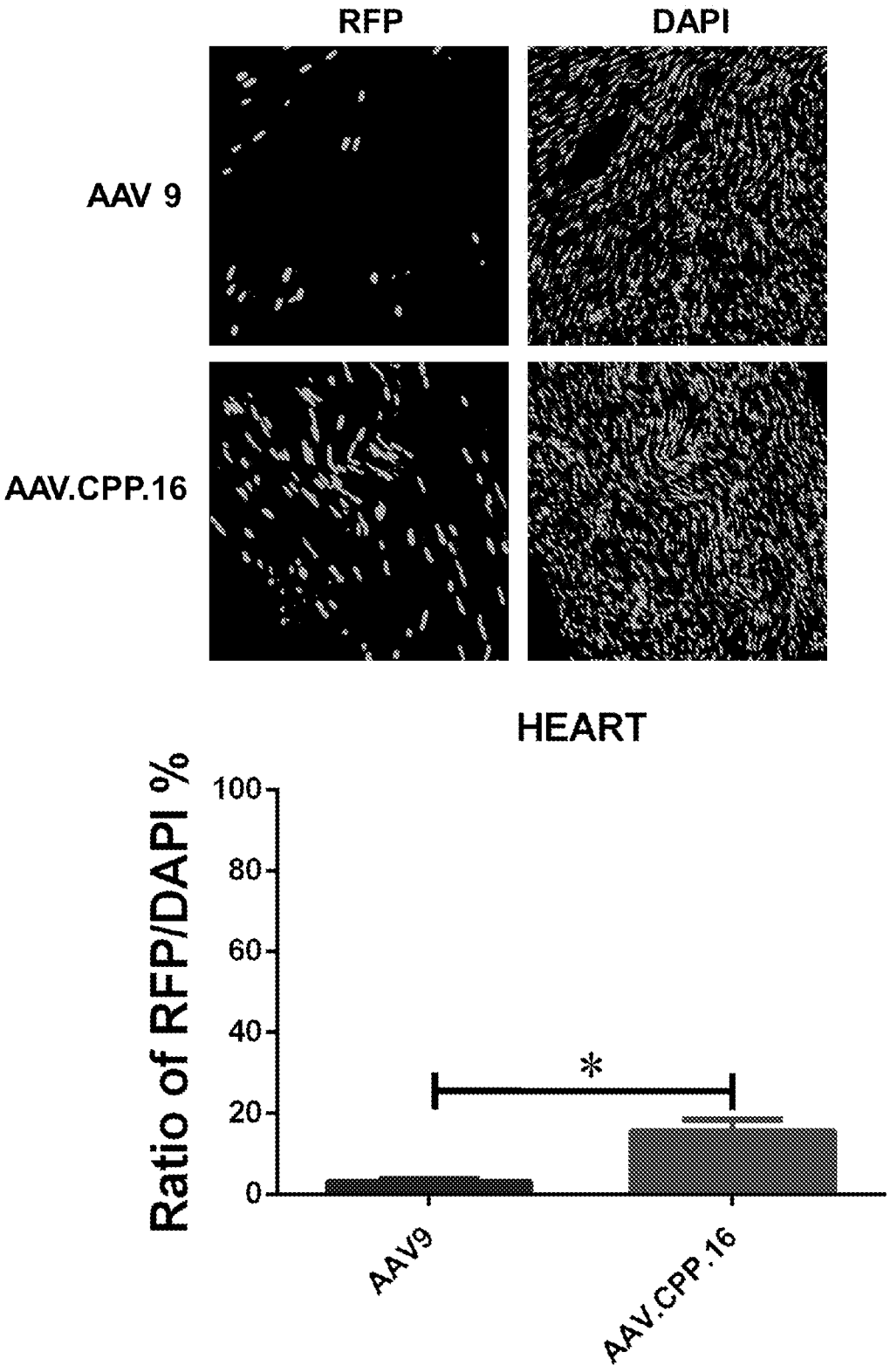

RFP
DAPI
AAV 9
AAV.CPP.16
HEART
Ratio of RFP/DAPI %
100
80
60
40
20
0
*
AAV9
AAV.CPP.16

FIG. 9A

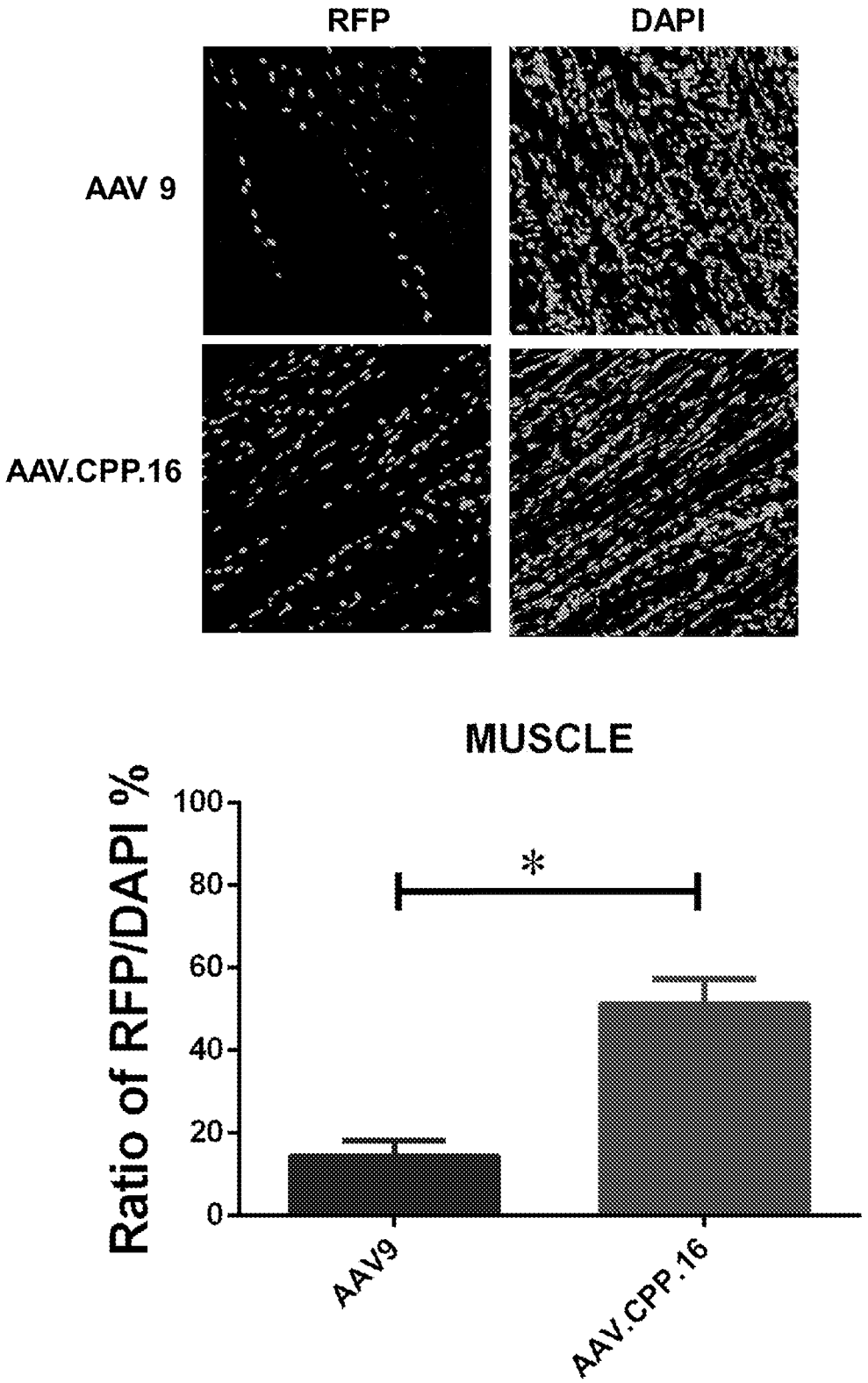

RFP
DAPI
AAV 9
AAV.CPP.16
MUSCLE
Ratio of RFP/DAPI %
100
80
60
40
20
0
*
AAV9
AAV.CPP.16

FIG. 9B

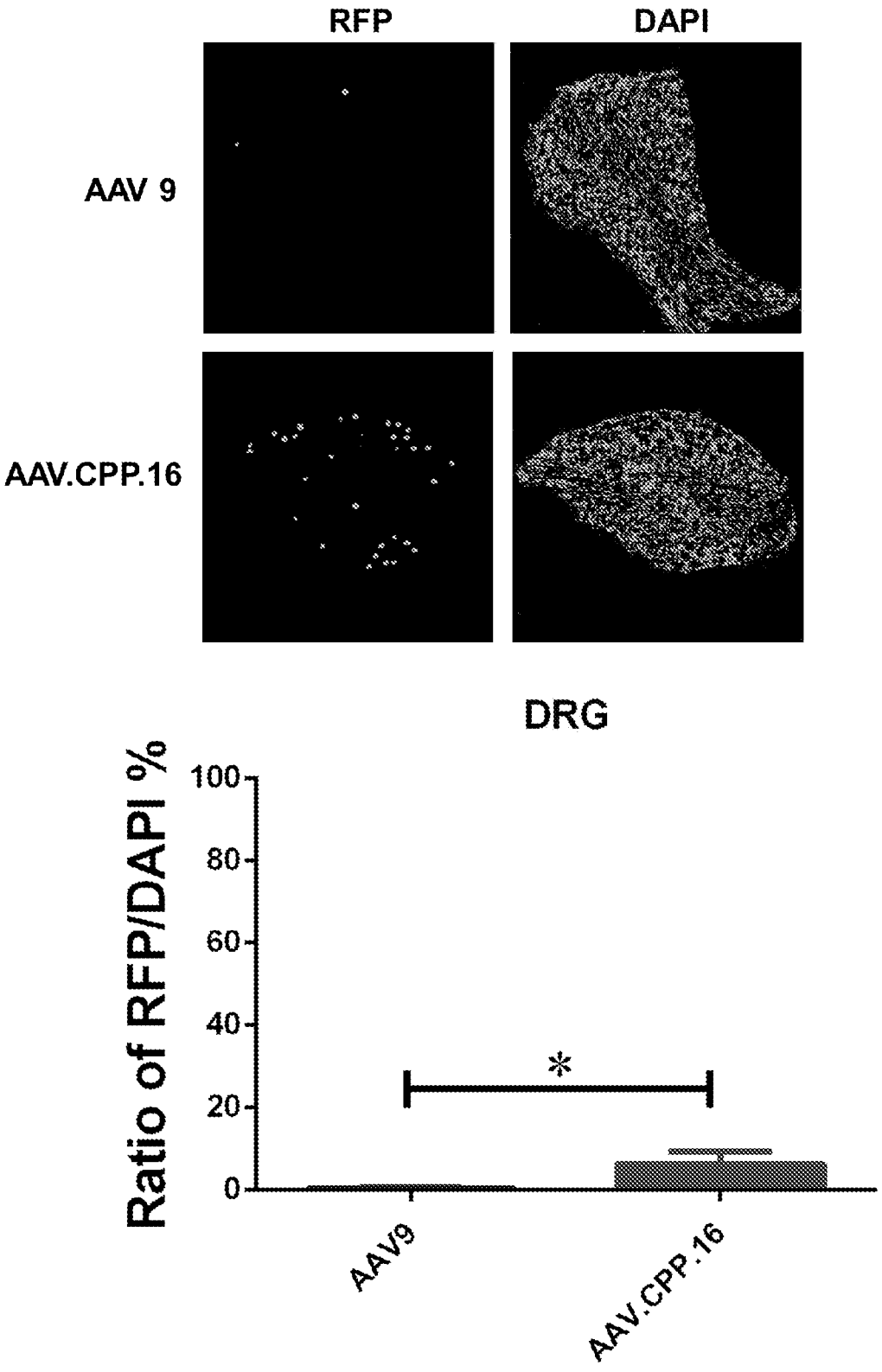

RFP
DAPI
AAV 9
AAV.CPP.16
DRG
Ratio of RFP/DAPI %
100
80
60
40
20
0
*
AAV9
AAV.CPP.16

FIG. 9C

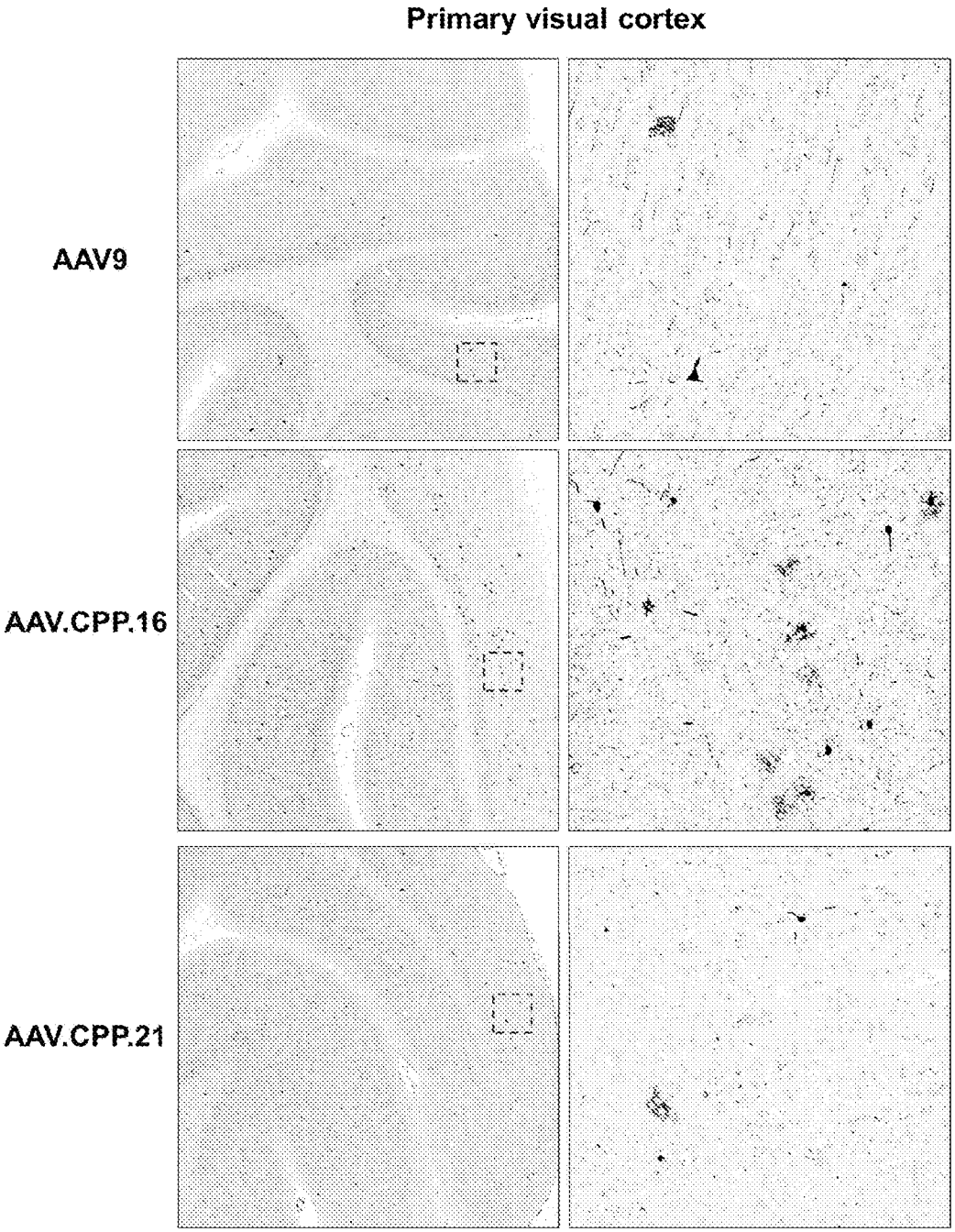


FIG. 10A

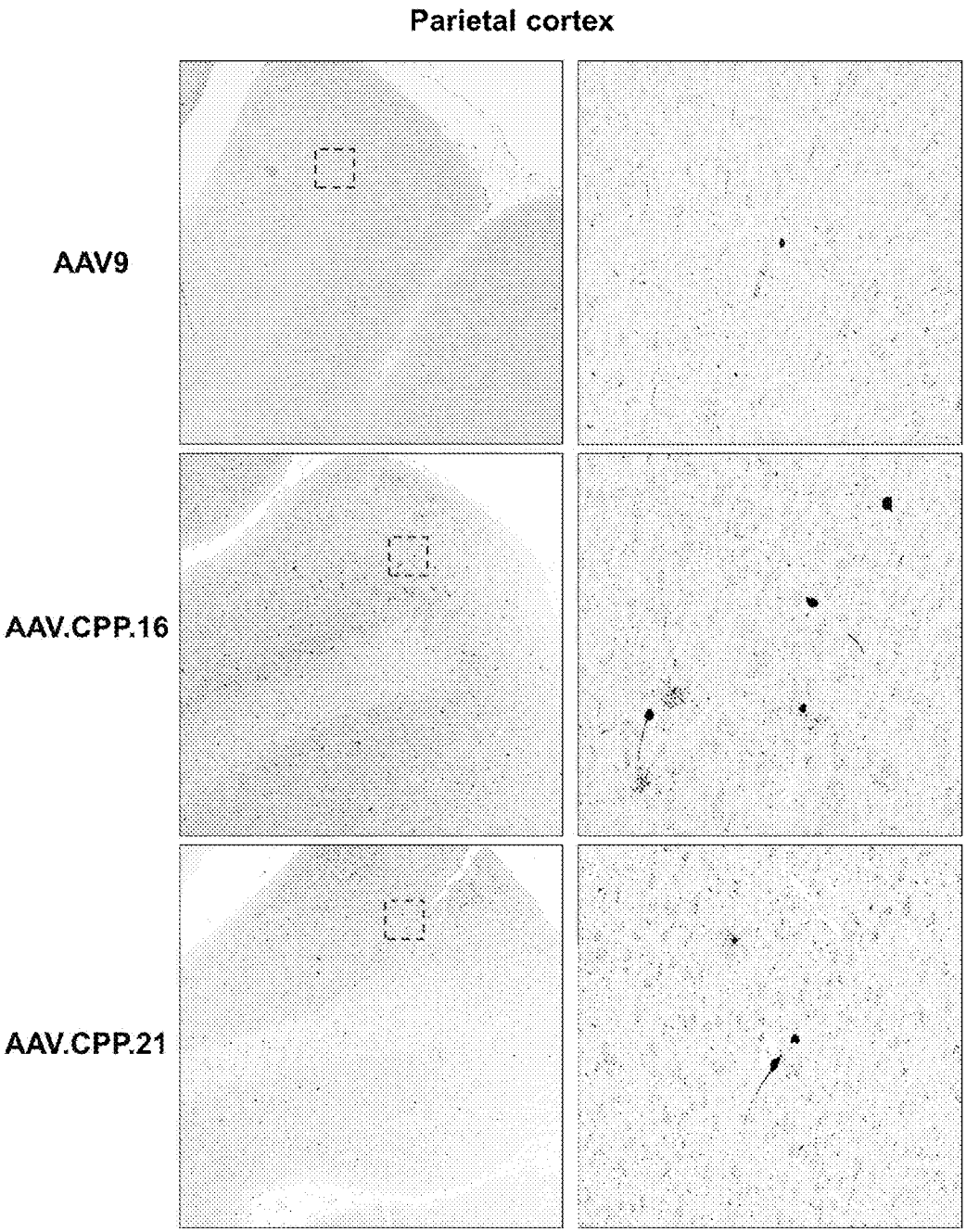


FIG. 10B

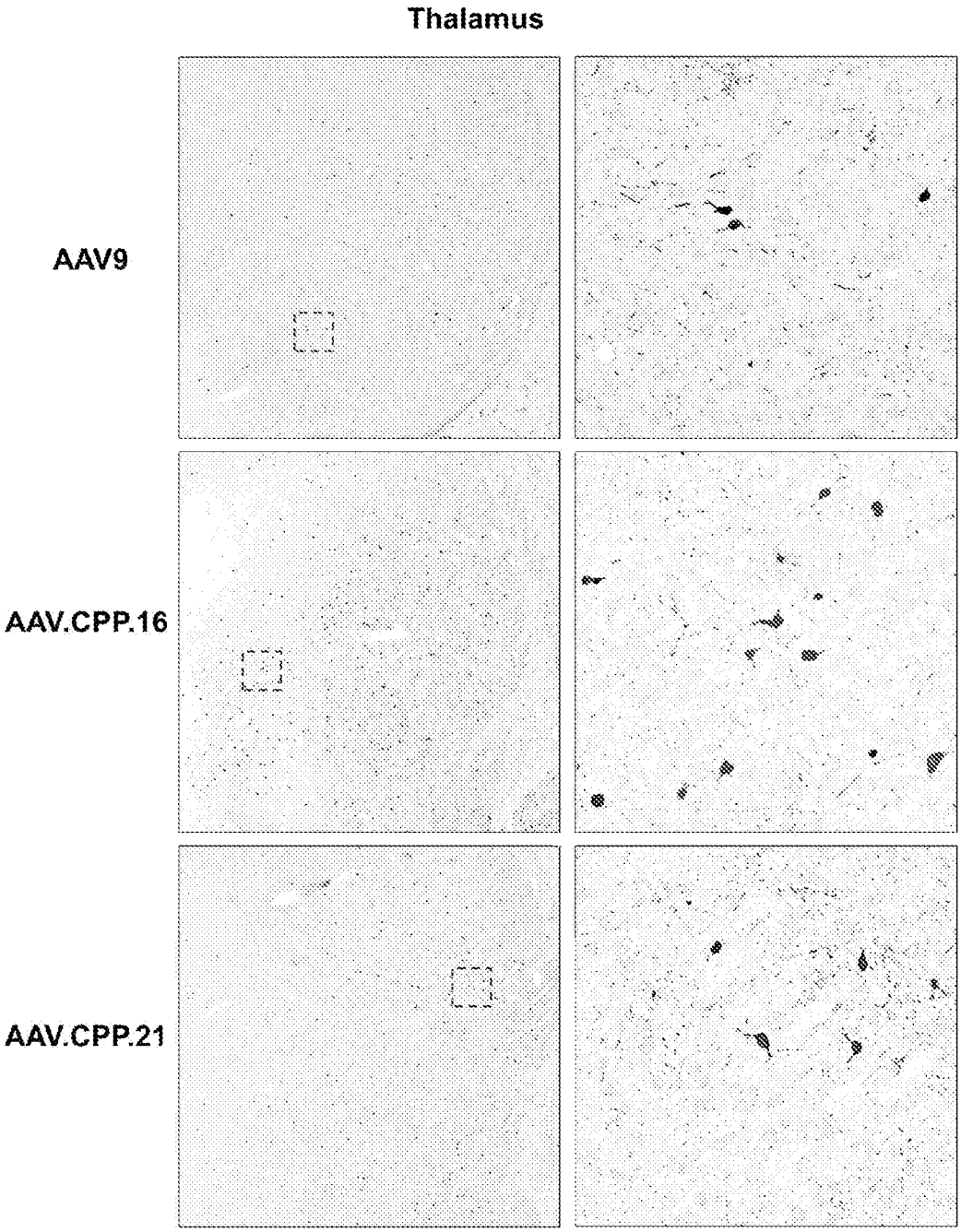


FIG. 10C

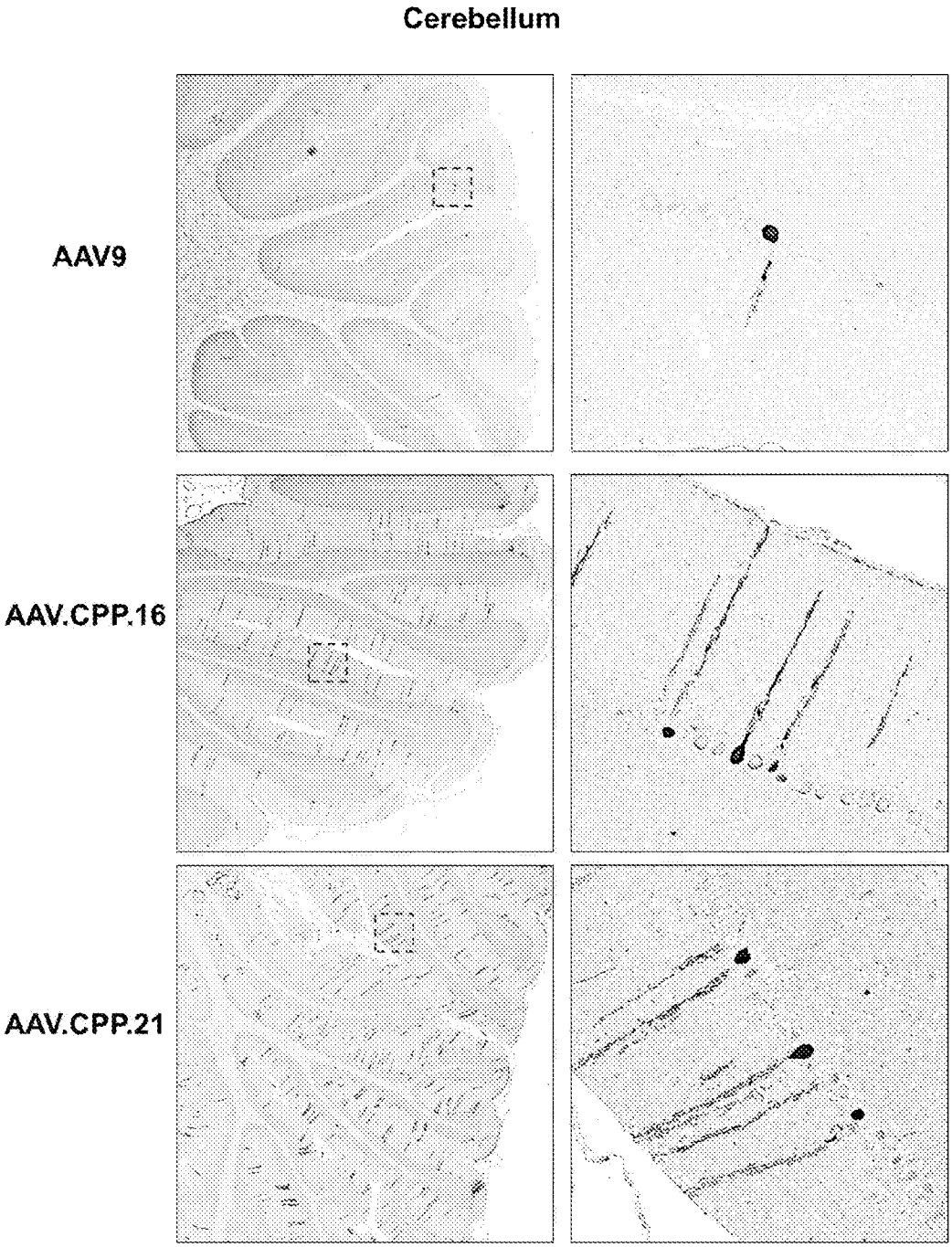


FIG. 10D

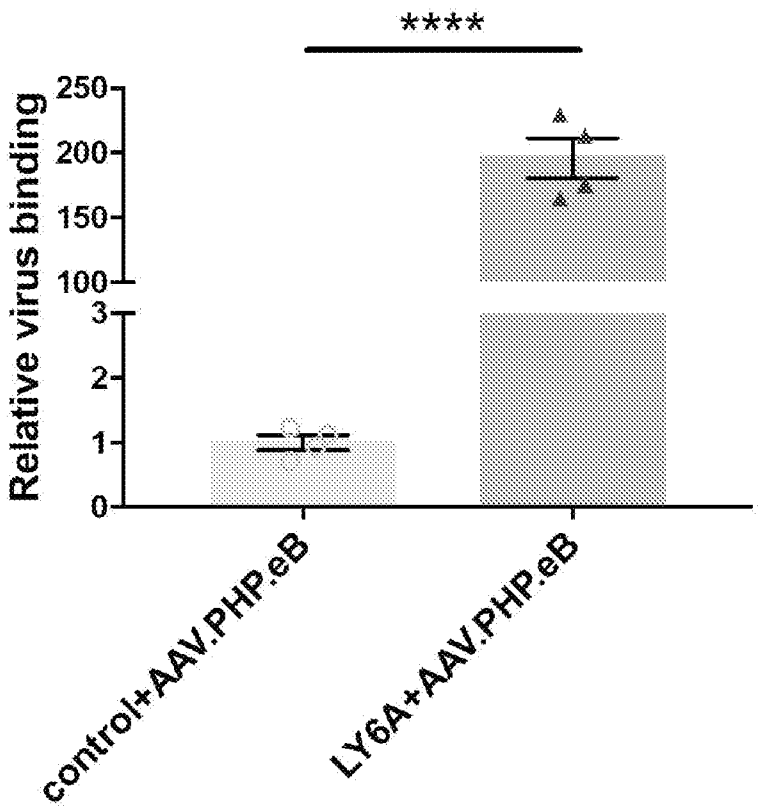


FIG. 11A

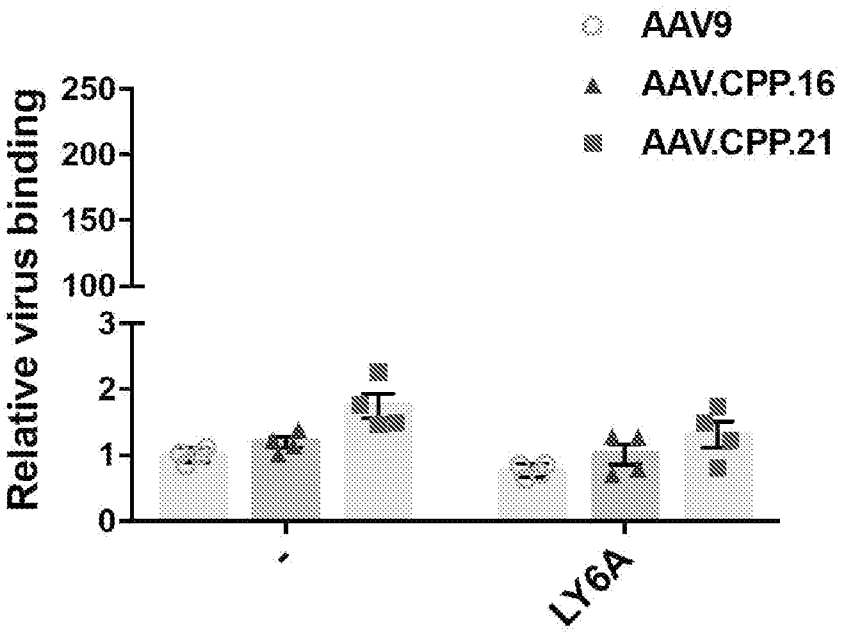


FIG. 11B

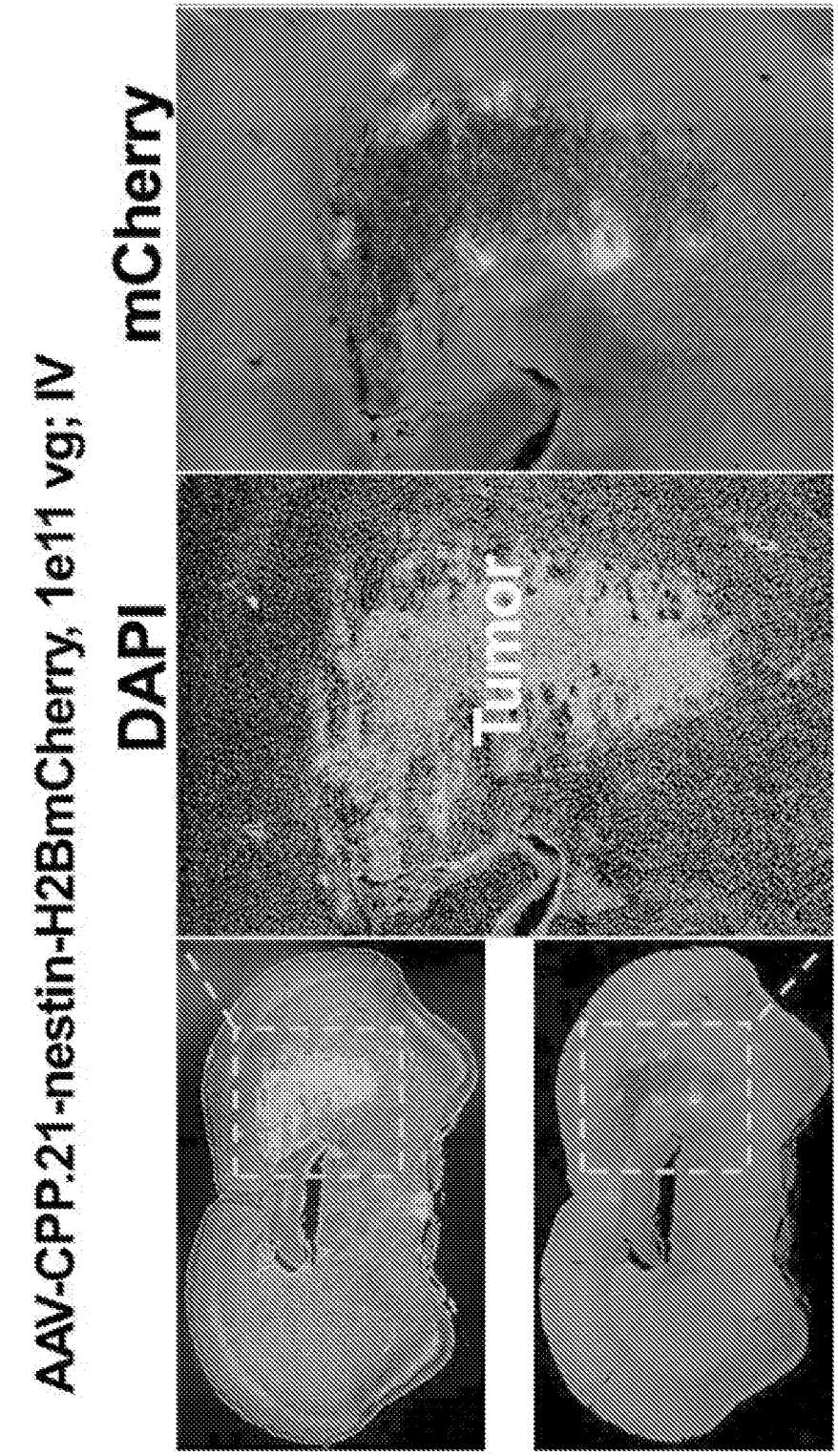

AAV-CPP.21-nestin-H2BmCherry, 1e11 vg; IV
DAPI
mCherry
Tumor
GL261 mouse tumor model

FIG. 12A

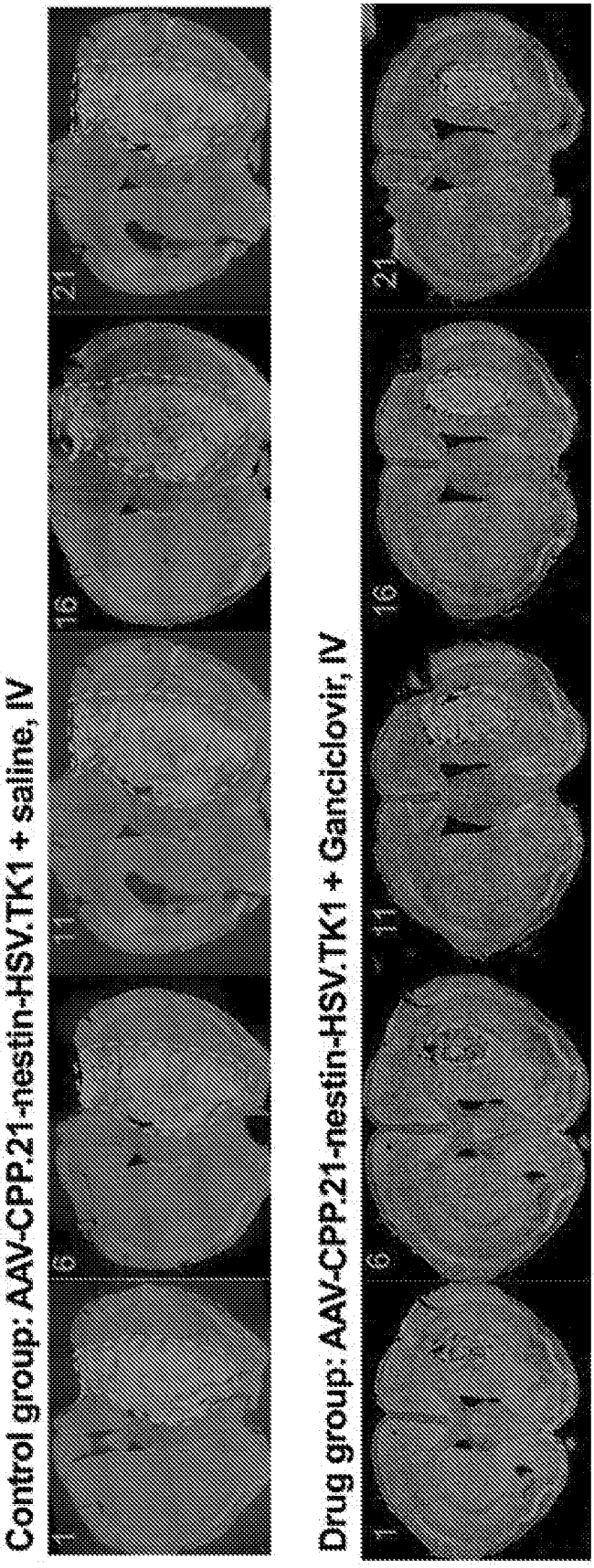

Control group: AAV-CPP.21-nestin-HSV.TK1 + saline, IV
Drug group: AAV-CPP.21-nestin-HSV.TK1 + Ganciclovir, IV
1
6
11
16
21

FIG. 12B

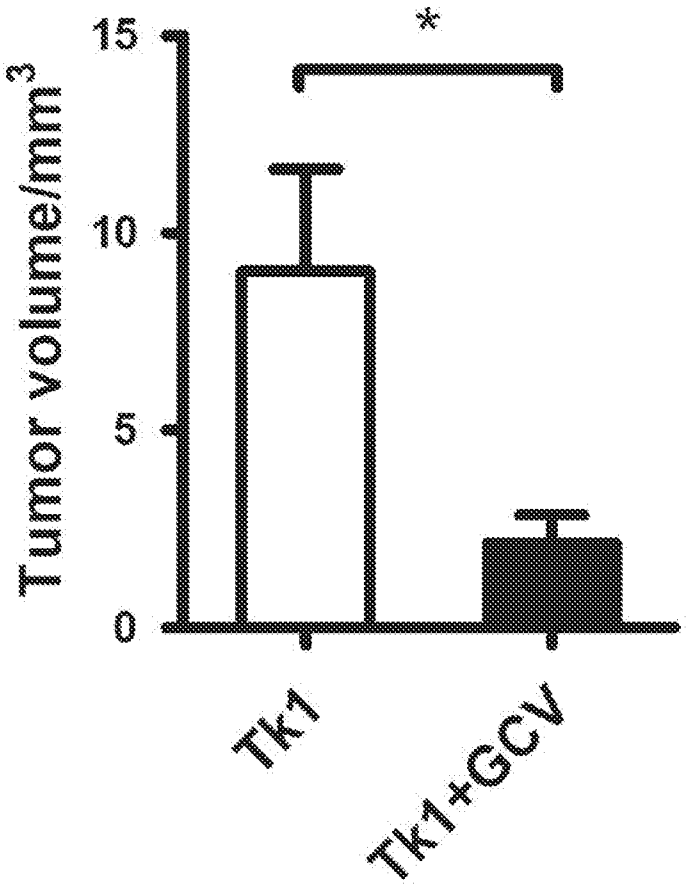


FIG. 12C

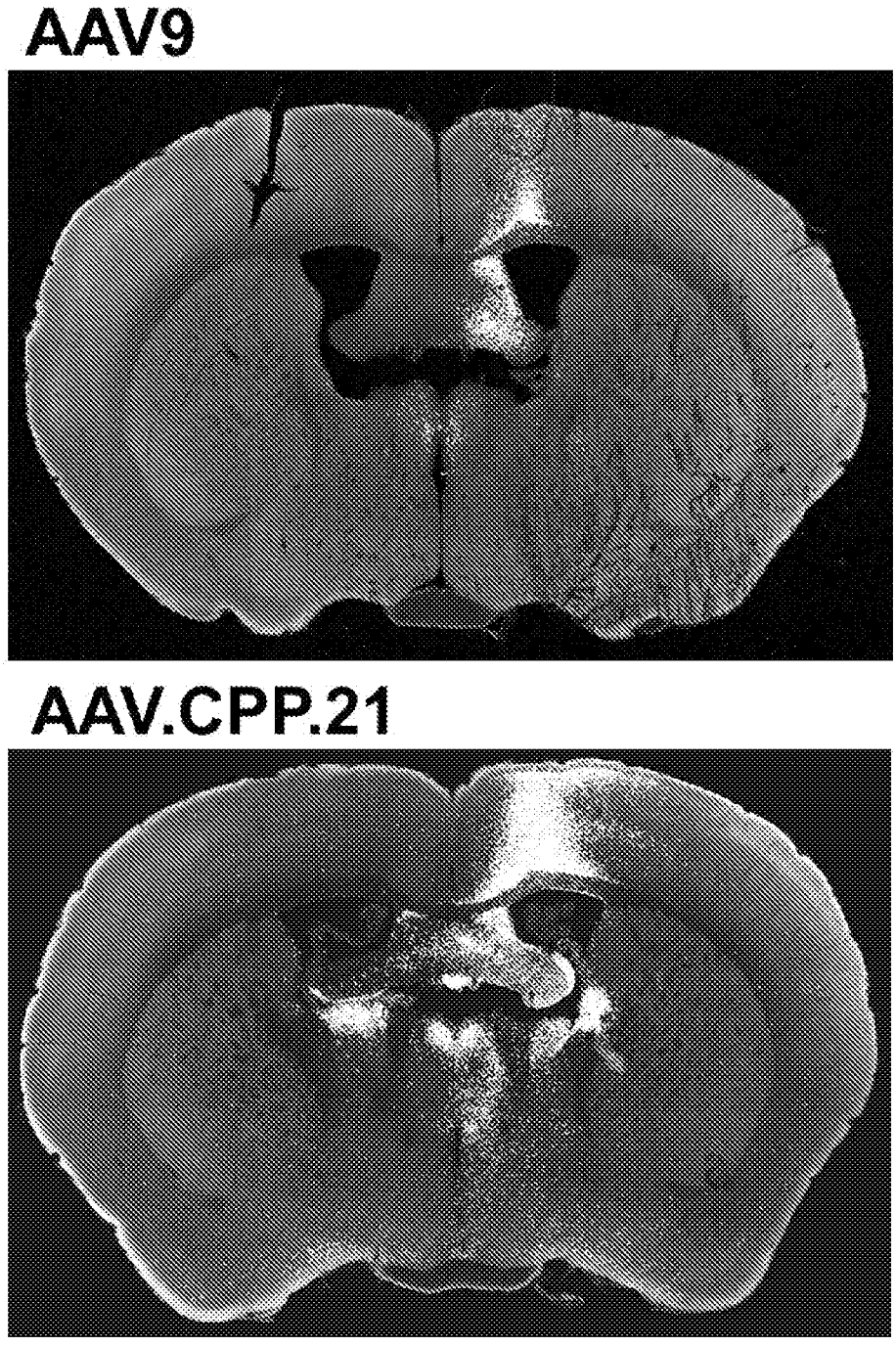


FIG. 13

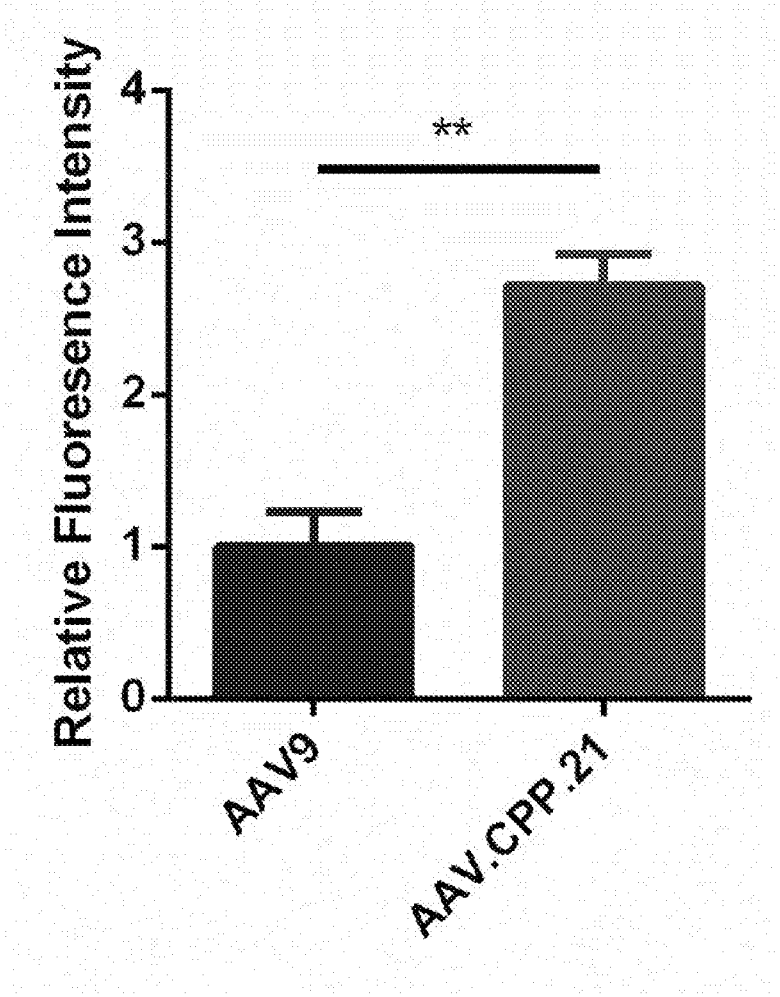


FIG. 13, continued

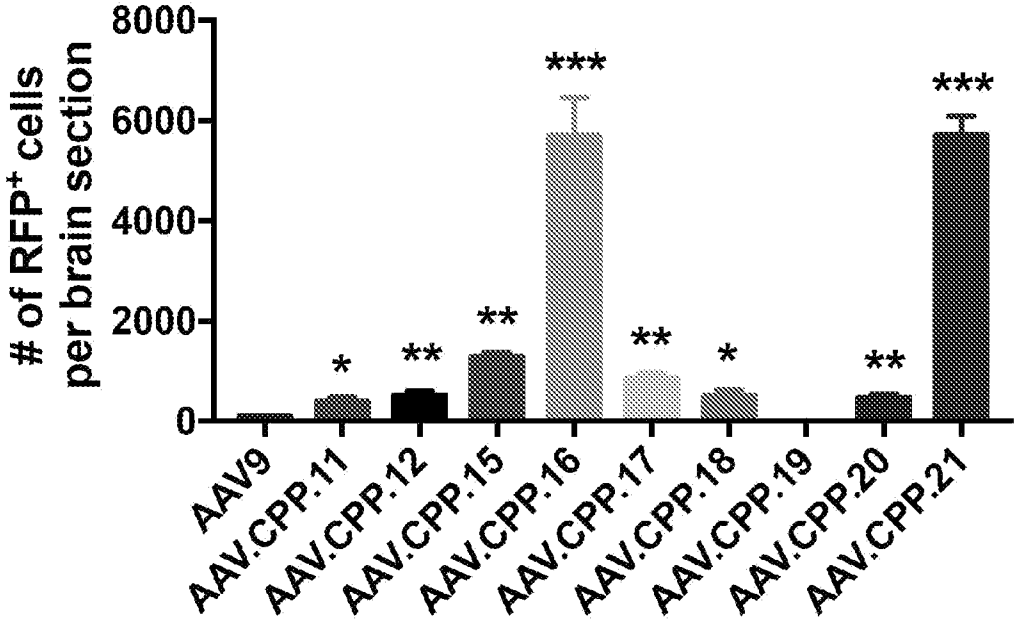

# of RFP+ cells per brain section
8000
6000
4000
2000
0
*
**
**
***
**
*
**
***
AAV9
AAV.CPP.11
AAV.CPP.12
AAV.CPP.15
AAV.CPP.16
AAV.CPP.17
AAV.CPP.18
AAV.CPP.19
AAV.CPP.20
AAV.CPP.21

FIG. 3C