

(19) **DANMARK**

(10) **DK/EP 3472183 T5**



Patent- og
Varemærkestyrelsen

(12) **Rettet oversættelse af
europæisk patentskrift**

-
- (51) Int.Cl.: **C 07 K 14/015 (2006.01)** **A 61 K 48/00 (2006.01)** **C 12 N 7/01 (2006.01)**
- (45) Oversættelsen bekendtgjort den: **2024-09-09**
- (80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2023-08-09**
- (86) Europæisk ansøgning nr.: **17734575.8**
- (86) Europæisk indleveringsdag: **2017-06-15**
- (87) Den europæiske ansøgnings publiceringsdag: **2019-04-24**
- (86) International ansøgning nr.: **US2017037781**
- (87) Internationalt publikationsnr.: **WO2017218842**
- (30) Prioritet: **2016-06-15 US 201662350361 P** **2016-10-05 US 201662404585 P**
- (84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**
- (73) Patenthaver: **Howard Hughes Medical Institute, , 4000 Jones Bridge Road, Chevy Chase, Maryland 20815-6789, USA**
The Regents of the University of California, 111 Franklin Street, 5th Floor, Oakland, CA 94607-5200, USA
- (72) Opfinder: **DUDMAN, Joshua, 19700 Helix Drive, Ashburn, Virginia 20147, USA**
HANTMAN, Adam, 19700 Helix Drive, Ashburn, Virginia 20147, USA
LOOGER, Loren, 19700 Helix Drive, Ashburn, Virginia 20147, USA
RITOLA, Kimberly, 19700 Helix Drive, Ashburn, Virginia 20147, USA
SCHAFFER, David, 19700 Helix Drive, Ashburn, Virginia 20147, USA
VISWANATHAN, Sarada, 19700 Helix Drive, Ashburn, Virginia 20147, USA
TERVO, Dougal Gowanlock Robinson, 19700 Helix Drive, Ashburn, Virginia 20147, USA
HWANG, Bum-Yeol, 303 Deerfield Dr., Moraga, California 94556, USA
KARPOVA, Alla, 19700 Helix Drive, Ashburn, Virginia 20147, USA
- (74) Fuldmægtig i Danmark: **Patrade A/S, Ceresbyen 75, 6., 8000 Århus C, Danmark**
- (54) Benævnelse: **VARIANT ADENO-ASSOCIEREDE VIRUS OG FREMGANGSMÅDER TIL ANVENDELSE**
- (56) Fremdragne publikationer:
WO-A1-2015/038958
WO-A1-2015/121501
WO-A1-2016/054554
WO-A2-2006/119150
LOCHRIE M A ET AL: "Adeno-associated virus (AAV) capsid genes isolated from rat and mouse liver genomic DNA define two new AAV species distantly related to AAV-5", VIROLOGY, ELSEVIER, AMSTERDAM, NL, vol. 353, no. 1, 15 September 2006 (2006-09-15), pages 68-82, XP024896378, ISSN: 0042-6822, DOI: 10.1016/J.VIROL.2006.05.023 [retrieved on 2006-09-15]
ALEXANDER BELLO ET AL: "Novel Adeno-associated Viruses Derived From Pig Tissues Transduce Most

Fortsættes ...

Major Organs in Mice", SCIENTIFIC REPORTS, vol. 4, 22 October 2014 (2014-10-22), page 6644, XP055349487, DOI: 10.1038/srep06644

E A SALEGIO ET AL: "Axonal transport of adeno-associated viral vectors is serotype-dependent", GENE THERAPY, vol. 20, no. 3, 1 March 2013 (2013-03-01), pages 348-352, XP055304195, GB ISSN: 0969-7128, DOI: 10.1038/gt.2012.27

DOMINIK F. ASCHAUER ET AL: "Analysis of Transduction Efficiency, Tropism and Axonal Transport of AAV Serotypes 1, 2, 5, 6, 8 and 9 in the Mouse Brain", PLOS ONE, vol. 8, no. 9, 27 September 2013 (2013-09-27), page e76310, XP055408666, DOI: 10.1371/journal.pone.0076310

TERVO D GOWANLOCK R ET AL: "A Designer AAV Variant Permits Efficient Retrograde Access to Projection Neurons", NEURON, vol. 92, no. 2, 19 October 2016 (2016-10-19), pages 372-382, XP029778113, ISSN: 0896-6273, DOI: 10.1016/J.NEURON.2016.09.021

TERVO D GOWANLOCK R ET AL: "96: A Designer AAV Variant Permits Efficient Retrograde Access to Projection Neurons", MOLECULAR THERAPY; ASGCT ANNUAL MEETING: COMPLETE LISTING OF ACCEPTED ABSTRACTS FOR PRESENTATION AT ASGCT'S 20TH ANNUAL MEETING, ACADEMIC PRESS, US; WASHINGTON, DC, USA, vol. 25, no. 5, Suppl.1, 1 May 2017 (2017-05-01), page 46, XP009500358, ISSN: 1525-0016

LOCHRIE M A ET AL: "Adeno-associated virus (AAV) capsid genes isolated from rat and mouse liver genomic DNA define two new AAV species distantly related to AAV-5", VIROLOGY, ELSEVIER, AMSTERDAM, NL, vol. 353, no. 1, 15 September 2006 (2006-09-15), pages 68 - 82, XP024896378, ISSN: 0042-6822, [retrieved on 20060915], DOI: 10.1016/J.VIROL.2006.05.023

ALEXANDER BELLO ET AL: "Novel Adeno-associated Viruses Derived From Pig Tissues Transduce Most Major Organs in Mice", SCIENTIFIC REPORTS, vol. 4, 22 October 2014 (2014-10-22), pages 6644, XP055349487, DOI: 10.1038/srep06644

E A SALEGIO ET AL: "Axonal transport of adeno-associated viral vectors is serotype-dependent", GENE THERAPY, vol. 20, no. 3, 1 March 2013 (2013-03-01), GB, pages 348 - 352, XP055304195, ISSN: 0969-7128, DOI: 10.1038/gt.2012.27

DOMINIK F. ASCHAUER ET AL: "Analysis of Transduction Efficiency, Tropism and Axonal Transport of AAV Serotypes 1, 2, 5, 6, 8 and 9 in the Mouse Brain", PLOS ONE, vol. 8, no. 9, 27 September 2013 (2013-09-27), pages e76310, XP055408666, DOI: 10.1371/journal.pone.0076310

TERVO D GOWANLOCK R ET AL: "A Designer AAV Variant Permits Efficient Retrograde Access to Projection Neurons", NEURON, vol. 92, no. 2, 19 October 2016 (2016-10-19), pages 372 - 382, XP029778113, ISSN: 0896-6273, DOI: 10.1016/J.NEURON.2016.09.021

TERVO D GOWANLOCK R ET AL: "96: A Designer AAV Variant Permits Efficient Retrograde Access to Projection Neurons", MOLECULAR THERAPY; ASGCT ANNUAL MEETING: COMPLETE LISTING OF ACCEPTED ABSTRACTS FOR PRESENTATION AT ASGCT'S 20TH ANNUAL MEETING, ACADEMIC PRESS, US; WASHINGTON, DC, USA, vol. 25, no. 5, Suppl.1, 1 May 2017 (2017-05-01), pages 46, XP009500358, ISSN: 1525-0016

DESCRIPTION

TECHNICAL FIELD

[0001] This disclosure generally relates to variant adeno-associated viruses and methods of making and using.

BACKGROUND

[0002] Brain functions such as perception, cognition, and the control of movement depend on the coordinated action of large-scale neuronal networks, which are composed of local circuit modules that perform specific computations and are linked together by long-range connections that distribute the results of these computations. Such long range connections are formed by specialized projection neurons that often include several intermingled classes, each projecting to a different downstream target within the network. Projection neurons have also been implicated in the spread of several neurodegenerative diseases from spatially localized sites of onset. Thus, the ability to selectively target specific classes of projection neurons for transgene delivery (e.g. for activity monitoring/manipulation or genome editing for targeted gene knock-outs or repair of pathological mutations) will be important both for gaining insights into how large-scale networks contribute to brain function and, in the long-run, for therapeutic intervention in neurodegenerative diseases.

[0003] Viral vectors constitute an important class of tools for introducing transgenes into specific neuronal populations, and are by far the best option for genetic access to target projection neurons through entry at axonal terminals and retrograde transport of their payload to the cell nuclei. A number of naturally evolved neurotropic viruses exhibit retrograde spread as part of their lifecycle, including rabies, poliovirus and herpes simplex virus (HSV), among others. Of these, rabies virus is particularly neuro-invasive and quickly propagates through the nervous system through transcellular transfer. However, its potential for both biological investigation and gene therapy is hampered by excessive virulence, though progress is being made towards reducing its toxicity. In addition to naturally neurotropic strains, many other viruses can infect neurons when administered directly to the nervous system, with "pseudorabies" (SuHV1, actually a herpesvirus), adenoviruses and lentiviruses used most commonly in animal research. Canine adenovirus-2 (CAV-2) displays the best infectivity and retrograde transport in this class of viruses and has increasingly become the reagent of choice for accessing projection neurons. CAV-2, however, mediates only modest levels of transgene expression, displays potential for toxicity and is currently not easily compatible with scalable, robust production for the generation of clinical-grade or even large animal studies. Thus, the development of a non-toxic, readily manufactured viral vector that affords flexible packaging of different transgenes, is robustly internalized and retrogradely transported by axons, and supports long-term, high-level payload expression remains a pressing need.

[0004] Lochrie et al., 2006, Virology, 353:68-82 describes adeno-associated virus capsid genes isolated from rat and mouse liver genomic DNA define two AAV species distantly related to AAV-5. Bello et al., 2014, Scientific Reports, 4:6644 describes adeno-associated viruses derived from pig tissues transduce most major organs in mice. WO 2016/054554 A1 describes recombinant adeno-associated viruses having distinct tissue targeting capabilities. WO 2006/119150 A2 describes viral capsid proteins with increased targeting to neurons and increased retrograde transport. WO 2015/121501 A1 describes a recombinant AAV vector. Salegio et al., 2013, Gene Therapy, 20:348-352 describes axonal transport of adeno-associated viral vectors is serotype-dependent. Aschauer et al., 2013, Plos One, 8:e76310 describes analysis of transduction efficiency, tropism and axonal transport of AAV serotypes 1, 2, 5, 6, 8 and 9 in the mouse brain. WO 2015/038958 A1 describes methods of selective screening, various targeting proteins and sequences, as well as methods of their use.

SUMMARY

[0005] Efficient retrograde access to projection neurons for the delivery of sensors and effectors constitutes an important and enabling capability for circuit dissection. Such approach also would be useful for gene therapy, including the treatment of neurodegenerative disorders characterized by pathological spread through functionally connected and highly distributed networks. Viral vectors, in particular, are powerful gene delivery vehicles for the nervous system, but all available tools suffer from inefficient retrograde transport or limited clinical potential. To address this need, *in vivo* directed evolution was applied to engineer potent retrograde functionality into the capsid of adeno-associated virus (AAV) - a vector that has shown promise in neuroscience research and the clinic. The variant described herein, referred to as rAAV2-retro, permits robust retrograde access to projection neurons with efficiency comparable to classical synthetic retrograde labeling reagents, and enables high expression level that is sufficient for functional circuit interrogation and *in vivo* genome editing using CRISPR/Cas9 in targeted neuronal populations.

[0006] The invention is as set out in the claims. The viral capsid proteins described in the following paragraphs, insofar not falling within the scope of the claims, are not part of the invention.

[0007] In one aspect, a viral capsid protein is provided that includes a sequence selected from the group consisting of xxDxTKx (SEQ ID NO:1) and xDxTKxx (SEQ ID NO:2), wherein the viral capsid protein has an amino acid sequence selected from the group consisting of SEQ ID NO: 6, 8, 10, 12, 14, 18, 22, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 54, 56, 60, 64, 66, 68, 70, 72, 74, 76, and 78. The viral capsid protein can have at least 95% sequence identity to SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78. The viral capsid protein can have the sequence shown in SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78. The viral capsid protein can be encoded by a nucleic acid having at least 95% sequence identity to SEQ ID NO: SEQ ID NO: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, or 77. The viral capsid protein can be encoded by a nucleic acid having the sequence shown in SEQ ID NO: SEQ ID NO: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, and 77.

[0008] In still another aspect, a viral particle is provided that includes a viral capsid protein as described herein. In some embodiments, the viral particle exhibits a preference for retrograde movement. In some embodiments, the viral particle possesses retrograde transport capability.

[0009] In some embodiments, a viral particle as described herein further includes a nucleic acid encoding a payload. In some embodiments, the nucleic acid encoding a payload includes a promoter sequence operably linked to a coding sequence that encodes the payload. Representative promoter sequences include, without limitation, synapsin-1, CMV, GFAP, CAG, CaMKII, MBP, EF1alpha, TRE and mDlx. In some embodiments, the coding sequence encoding the payload is selected from the group consisting of a protein-coding gene and an inhibitory RNA nucleic acid. In some embodiments, the inhibitory RNA nucleic acid is an antisense oligonucleotide, an siRNA, or an RNAi.

[0010] In some embodiments, the payload is an effector protein. Representative effector proteins include, without limitation, a recombinase (e.g., Cre or Flp), a gene editing system (e.g., CRISPR/Cas9, TALEN, zinc finger nuclease), optogenetic reagents (activators (e.g., channelrhodopsin or variants thereof) or inhibitors (e.g., halorhodopsin or Arch)), chemogenetic reagents (e.g., activator/inhibitor versions of the DREADD or PSAM/PSEM systems), activators and/or inhibitors of cell system pathways, and enzymes for control of epigenetics.

[0011] In some embodiments, the payload is an optical reporter construct. Representative optical reporter constructs include, without limitation, GCaMP6 (s, m, or f), a fluorophore (e.g., green fluorescent protein (GFP), enhanced GFP (EGFP), red fluorescent protein (RFP), yellow fluorescent protein (YFP), tdTomato), a color-flipping construct (e.g., a payload that expresses one reporter in one cell population and a different reporter in another population), Glucose Sensors, jRCaMP, jRGECO, and CaMPARI, voltage indicators, secondary messengers, receptor signalers, transcription reporters, epigenetic reporters, and neuromodulator reporters.

[0012] In some embodiments, the payload is a viral protein. A representative viral protein is the rabies G protein. In some embodiments, the viral protein is a protein that complements the function of a virus other than the AAV or a protein that relates to cellular and transcellular transport.

[0013] In some embodiments, the coding sequence encoding the payload is a therapeutic gene. In some embodiments, the therapeutic gene is for treatment of a neurodegenerative disorder. In some embodiments, the therapeutic gene is HSP104 for treatment of Alzheimer's disease or other diseases with toxic protein aggregates. In some embodiments, the therapeutic gene is frataxin for treatment of Friedreich's ataxia. In some embodiments, the therapeutic gene is lysosomal glucocerebrosidase (GBA) for treatment of Parkinson's disease. In some embodiments, the therapeutic gene is polyQ-binding protein for treatment of Huntington's disease. In some embodiments, the therapeutic gene is survival motor neuron 1 for treatment of Spinal muscular atrophy, amyotrophic lateral sclerosis (ALS), autism, dementia, peripheral neuropathy, schizophrenia, or retinal degeneration.

[0014] In some embodiments, the payload is a therapeutic moiety. A representative therapeutic moiety is an antibody or fragment thereof. A representative therapeutic moiety is an immunomodulatory protein. A representative therapeutic moiety is a RNA interference molecule.

[0015] In some embodiments, the viral particles described herein exhibit up to two orders of magnitude greater retrograde access to cortico-pontine projection neurons than canine adenovirus-2 (CAV-2). In some embodiments, the retrograde access to cortico-pontine projection neurons or afferents to the dorsomedial striatum (DMS) is comparable to the synthetic tracer, Fluoro-Gold fluorescent beads.

[0016] Also described is a method of delivering a payload to one or more neurons. Such a method typically includes contacting one or more neurons with a variant adeno-associated virus (AAV) that includes a payload packaged therein, wherein the variant AAV includes a capsid protein comprising a sequence selected from the group consisting of xxDTKx (SEQ ID NO: 1) and xDTKxx (SEQ ID NO:2). In some embodiments, the variant AAV exhibits retrograde movement in the neurons.

[0017] Another aspect is a variant adeno-associated virus (AAV) comprising a payload packaged therein, for use in a method of treatment of a neurodegenerative disorder, the method comprising contacting one or more neurons with said variant AAV, wherein said variant AAV comprises a capsid protein comprising a sequence selected from the group consisting of xxDTKx (SEQ ID NO: 1) and xDTKxx (SEQ ID NO:2), wherein the variant AAV has an amino acid sequence selected from the group consisting of SEQ ID NO: 6, 8, 10, 12, 14, 18, 22, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 54, 56, 60, 64, 66, 68, 70, 72, 74, 76, and 78.

[0018] In some embodiments, the neurons are projection neurons. In some embodiments, the neurons are in a subject (e.g., in the central nervous system (CNS) in a subject). In some embodiments, the subject is a human. In some embodiments, the subject is a non-human (e.g., a primate, a rodent, a reptile, or a bird). The contacting step can be in cell culture. In some embodiments, the contacting step is *in vivo* via intracranial, intraspinal or intramuscular injection.

[0019] 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 the methods and compositions of matter belong.

DESCRIPTION OF DRAWINGS

[0020]

Figure 1 shows the directed evolution of rAAV2-retro. Panel A of Figure 1 is a schematic of the directed evolution procedure. Plasmid libraries containing variant AAV cap genes previously generated by error prone PCR, peptide insertion, randomization of loop regions, and DNA shuffling were packaged and injected into substantia nigra or deep cerebellar nuclei. 3 weeks later, striatal or hindbrain tissue, respectively, was removed, viral genomes isolated, and selected cap genes amplified and packaged for the next selection round. Panel B of Figure 1 shows the intermingled subpopulations of cortical neurons projecting to striatum (CP: caudate-putamen), thalamus and superior colliculus (SC) retrogradely labeled by injecting rAAV2-retro carrying different fluorescent proteins in the corresponding axonal field.

Figure 2 show results from experiments to quantify retrograde transport efficiency. Panel A of Figure 2 shows cortico-pontine tract labeled via basal pontine injection of rAAV2-retro [Top panel of Panel A shows a schematic of the experiment; consistency of targeting and injection quality were monitored by co-injecting AAV1-CAG-EGFP. Bottom panel of Panel A shows the level of expression 3 weeks after injection. Scale bar: 1 mm]. Panel B of Figure 2 shows the quantification assay design [Top panel of Panel B shows a schematic of the experiment; arrow indicates expected nuclear GFP labeling in cortical neurons of the cortico-pontine tract. Middle panel of Panel B shows a representative image of an AAV2-injected brain. Bottom panel of Panel B shows a representative image of rAAV2retro-injected brain. Scale bar: 1 mm]. Panel C of Figure 2 shows a schematic of the semi-automated quantification procedure. Fluorescent nuclei (green) were automatically detected and counted along a manually drawn line that traced the length of cortical layer V (black). Panel D of Figure 2 shows the retrograde transport efficiency for different AAV serotypes and for canine adenovirus-2 (CAV-2). Error bars represent the SEM. See also Figure 8.

Figure 3 show results from experiments to demonstrate the generality of retrograde transport afforded by rAAV2-retro. Panel A of Figure 3 are representative images showing extensive labeling in the main input structures to the dorsal striatum including cortex (image #1), amygdala (image #3), and thalamus (image #4). Panel B of Figure 3 shows a schematic of automated whole-brain quantification of retrograde labeling. Brains of Rosa26-LSL-H2B-GFP injected with rAAV2-retro hSyn1-Cre were imaged to visualize DAPI-stained nuclei and green fluorescence from H2B-GFP-expressing nuclei. The green channel is used to detect labeled neurons; the blue channel is aligned to the Nissl images from the Allen Brain Institute's standard mouse brain. The alignment permits detected neurons to be assigned to different regions using the annotation provided by the brain atlas. Scale bar: 1.25 mm. Panel C of Figure 3 shows whole-brain quantification of retrograde labeling out of a small region of the dorso-medial striatum. Abbreviations for the different brain areas are given according to the Allen Brain Atlas. Arrow highlights the SNC. Error bars represent the SEM.

Figure 4 is data from experiments in which the rAAV2-retro system was combined with Cre driver lines. Panel A of Figure 4 shows a schematic of the experiment. rAAV2-retro carrying a Cre-dependent color-flipping fluorescent reporter was injected into the striatum of a cortical layer V-specific Cre-line. Panel B of Figure 4 shows the two cortico-striatal pathways differentially labeled through a Cre-dependent inversion of the reporter in one pathway, but not the other.

Figure 5 is data from experiments showing that rAAV2-retro supports sufficient transgene expression for functional circuit interrogation. Panel A of Figure 5 is a schematic of the experiment. Expression of calcium indicator GCaMP6f is restricted to cortico-pontine neurons using localized injection of rAAV2-retro into basal pons. Panel B of Figure 5 is a cross-section of the brain showing GCaMP6f expression throughout the cortico-pontine tract. Panel C of Figure 5 shows the maximum projection of a two-photon calcium image showing layer V pyramidal tract somas and apical dendrites. Panel D of Figure 5 shows the activity of 89 ROIs during a single hand reach repetition (broken line denotes the tone "go" signal). Panel E and Panel F of Figure 5 are two examples of single cortico-pontine neurons during 40 consecutive trials (same animal as in Panel B and Panel D of Figure 5).

Figure 6 is data showing that the rAAV2-retro system enables *in vivo* genome editing using CRISPR/Cas9. Panel A of Figure 6 is a schematic of the experiment [Top panel of Panel A shows that the rAAV2-retro system was used to deliver *Staphylococcus aureus* Cas9 (SaCas9) - single guide RNA combination engineered to ablate expression of tdTomato. Bottom panel of Panel A shows that the rAAV2-retro carrying the SaCas9-anti-tdTomato payload was injected into the basal pons of mice expressing tdTomato from a single genomic locus in layer V neurons]. Panel B of Figure 6 shows representative images from brain sections of animals that received the CRISPR/Cas9 system targeted against tdTomato or carrying a non-targeted guide. SaCas9 was epitope tagged, permitting identification of retrogradely labeled neurons (green channel). Upward arrows show the expected labeling following successful ablation of tdTomato; Downward arrows show the expected labeling if tdTomato expression is unaffected. Panel C of Figure 6 shows the efficiency of ablation. Error bars represent the SEM.

Figure 7 is data showing that rAAV2-retro mediates efficient access to projection neurons in the rat (the data shown in Figure 7 is related to the data shown in Figure 1). Panel A of Figure 7 is a schematic of the injection. Separate lots of rAAV2-retro expressing EGFP or tdTomato were injected in the striatum or superior colliculus respectively.

Panels B-E of Figure 7 show projection neurons in various brain regions accessed through these localized injections and imaged 3 weeks following virus delivery.

Figure 8 are representative images of brains from animals injected with various AAV serotypes in the basal pons, showing that no naturally-occurring AAV serotypes match rAAV2-retro performance in cortico-pontine circuit (the data shown in Figure 8 is related to the data shown in Figure 2).

Figure 9 is data showing reduced heparin affinity for rAAV2-retro compared to its parental serotype AAV2 (the data shown in Figure 9 is related to the data shown in Figure 1). Panel A of Figure 9 is a schematic of the heparin binding assay. Panel B of Figure 9 shows a fraction of virus eluted with increasing concentrations of NaCl following loading in 150 mM NaCl. The load fraction represents the virus recovered in the column flow-through after sample loading in 150 mM NaCl. Error bars represent the SD.

DETAILED DESCRIPTION

[0021] Recombinant adeno-associated viruses (rAAVs) have emerged as an effective platform for *in vivo* gene therapy, as they mediate high-level transgene expression, are non-toxic, and evoke minimal immune responses. These properties were at the core of the decision to grant the first full regulatory approval of any gene therapy treatment to rAAV-mediated restoration of lipoprotein lipase deficiency (see, for example, Gaudet et al., 2010, *Atherosclerosis Suppl.*, 11:55-60). rAAVs hold great promise in clinical trials for a range of neurological disorders, and they constitute some of the most widespread vectors in neuroscience research. Since the original discovery that AAV can undergo retrograde transport (Kaspar et al., 2002, *Mol. Ther.*, 5:50-56), rAAVs have afforded some degree of retrograde access to projection neurons in select circuits, but their natural propensity for retrograde transport is low, hampering efforts to address the role of projection neurons in circuit computations or disease progression.

[0022] This disclosure describes a new rAAV variant (rAAV2-retro) that, in addition to its regular ability to infect neuronal cell bodies at the site of exposure, is robustly internalized by axons and mediates retrograde access to projection neurons with efficiency comparable to classical retrograde labeling reagents such as synthetic dyes. The rAAV2-retro gene delivery system described herein can be used on its own or in conjunction with Cre recombinase driver lines to achieve long-term, high-level transgene expression that is sufficient for effective functional interrogation of neural circuit function, as well as for genome editing in targeted neuronal populations.

[0023] Retrograde transport shuttles molecules and/or organelles away from axon termini toward the cell body. Retrograde axonal transport is mediated by cytoplasmic dynein, and is used, for example, to send chemical messages and endocytosis products headed to endolysosomes from the axon back to the cell. Operating *in vivo* at average speeds of approximately 2 $\mu\text{m}/\text{sec}$, fast retrograde transport can cover 10-20 centimeters per day. Fast retrograde transport returns used synaptic vesicles and other materials to the soma and informs the soma of conditions at the axon terminals. Thus, as used herein, "retrograde" transport refers to movement in an axon toward its cell body.

Adeno-Associated Virus (AAV) Nucleic Acid and Polypeptide Sequences

[0024] As described herein, consensus sequences were identified that, when present in an AAV capsid protein, result in a profound enhancement in the efficacy of retrograde transport. Those consensus sequences are xxDxTKx (SEQ ID NO:1) or xDxTKxx (SEQ ID NO:2). To demonstrate the effectiveness of the consensus sequences described herein for imparting retrograde transport capability, seventeen different capsid sequences, each containing a consensus sequence as described herein, were generated and shown to exhibit a preference for retrograde movement in neurons. The nucleic acid sequence of those 40 capsid sequences are shown in SEQ ID NO: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, and 77, and the encoded capsid polypeptides, each containing one of the consensus sequences described herein, are shown in SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, and 78, respectively.

[0025] In addition to the capsid polypeptides having the sequences shown in SEQ ID NOs: SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, and 78, polypeptides are provided that have at least 95% sequence identity (e.g., at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity) to the capsid polypeptides having the sequences shown in SEQ ID NOs: SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, and 78. Similarly, in addition to the nucleic acid molecules having the sequences shown in SEQ ID NOs: SEQ ID NO: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, and 77, nucleic acid molecules are provided that have at least 95% sequence identity (e.g., at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity) to the nucleic acid molecules having the sequences shown in SEQ ID NOs: SEQ ID NO: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, and 77.

[0026] In calculating percent sequence identity, two sequences are aligned and the number of identical matches of nucleotides or amino acid residues between the two sequences is determined. The number of identical matches is divided by the length of the aligned region (i.e., the number of aligned nucleotides or amino acid residues) and multiplied by 100 to arrive at a percent sequence identity value. It will be appreciated that the length of the aligned region can be a portion of one or both sequences up to the full-length size of the shortest sequence. It also will be appreciated that a single sequence can align with more than one other sequence and hence, can have different percent sequence identity values over each aligned region.

[0027] The alignment of two or more sequences to determine percent sequence identity can be performed using the algorithm described by Altschul et al. (1997, *Nucleic Acids Res.*, 25:3389-3402) as incorporated into BLAST (basic local alignment search tool) programs, available at ncbi.nlm.nih.gov on the World Wide Web. BLAST searches can be performed to determine percent sequence identity between a sequence (nucleic acid or amino acid) and any other sequence or portion thereof aligned using the Altschul et al. algorithm. BLASTN is the program used to align and compare the identity between nucleic acid sequences, while BLASTP is the program used to align and compare the identity between amino acid sequences. When utilizing BLAST programs to calculate the percent identity between a sequence and another sequence, the default parameters of the respective programs generally are used.

[0028] Vectors containing nucleic acid molecules that encode polypeptides also are provided. Vectors, including expression vectors, are commercially available or can be produced by recombinant technology. A vector containing a nucleic acid molecule can have one or more elements for expression operably linked to such a nucleic acid molecule, and further can include sequences such as those encoding a selectable marker (e.g., an antibiotic resistance gene), and/or those that can be used in purification of a polypeptide (e.g., 6xHis tag). Elements for expression include nucleic acid sequences that direct and regulate expression of nucleic acid coding sequences. One example of an expression element is a promoter sequence. Expression elements also can include one or more of introns, enhancer sequences, response elements, or inducible elements that modulate expression of a nucleic acid molecule. Expression elements can be of bacterial, yeast, insect, mammalian, or viral origin and vectors can contain a combination of expression elements from different origins. As used herein, operably linked means that elements for expression are positioned in a vector relative to a coding sequence in such a way as to direct or regulate expression of the coding sequence.

[0029] A nucleic acid molecule, e.g., a nucleic acid molecule in a vector (e.g., an expression vector, a viral vector) can be introduced into a host cell. The term "host cell" refers not only to the particular cell(s) into which the nucleic acid molecule has been introduced, but also to the progeny or potential progeny of such a cell. Many suitable host cells are known to those skilled in the art; host cells can be prokaryotic cells (e.g., *E. coli*) or eukaryotic cells (e.g., yeast cells, insect cells, plant cells, mammalian cells). Representative host cells can include, without limitation, A549, WEHI, 3T3, 10T1/2, BHK, MDCK, COS 1, COS 7, BSC 1, BSC 40, BMT 10, VERO, WI38, HeLa, 293 cells, Saos, C2C12, L cells, HT1080, HepG2 and primary fibroblast, hepatocyte and myoblast cells derived from mammals including human, monkey, mouse, rat, rabbit, and hamster. Methods for introducing nucleic acid molecules into host cells are well known in the art and include, without limitation, calcium phosphate precipitation, electroporation, heat shock, lipofection, microinjection, and viral-mediated nucleic acid transfer (e.g., transduction).

[0030] With respect to polypeptides, "purified" refers to a polypeptide (i.e., a peptide or a polypeptide) that has been separated or purified from cellular components that naturally accompany it. Typically, the polypeptide is considered "purified" when it is at least 70% (e.g., at least 75%, 80%, 85%, 90%, 95%, or 99%) by dry weight, free from the polypeptides and naturally occurring molecules with which it is naturally associated. Since a polypeptide that is chemically synthesized is, by nature, separated from the components that naturally accompany it, a synthetic polypeptide is considered "purified," but further can be removed from the components used to synthesize the polypeptide (e.g., amino acid residues). With respect to nucleic acid molecules, "isolated" refers to a nucleic acid molecule that is separated from other nucleic acid molecules that are usually associated with it in the genome. In addition, an isolated nucleic acid molecule can include an engineered nucleic acid molecule such as a recombinant or a synthetic nucleic acid molecule.

[0031] Polypeptides can be obtained (e.g., purified) from natural sources (e.g., a biological sample) by known methods such as DEAE ion exchange, gel filtration, and/or hydroxyapatite chromatography. A purified polypeptide also can be obtained, for example, by expressing a nucleic acid molecule in an expression vector or by chemical synthesis. The extent of purity of a polypeptide can be measured using any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. Similarly, nucleic acid molecules can be obtained (e.g., isolated) using routine methods such as, without limitation, recombinant nucleic acid technology (e.g., restriction enzyme digestion and ligation) or the polymerase chain reaction (PCR; see, for example, PCR Primer: A Laboratory Manual, Dieffenbach & Dveksler, Eds., Cold Spring Harbor Laboratory Press, 1995). In addition, isolated nucleic acid molecules can be chemically synthesized.

Methods of Making Virus Particles that Exhibit a Preference for Retrograde Movement in Neurons

[0032] Once a capsid polypeptide has been produced, or once a nucleic acid molecule has been generated and expressed to produce a capsid polypeptide, the polypeptide can be assembled into a virus particle using, for example, a packaging host cell. The components of a virus particle (e.g., rep sequences, cap sequences, inverted terminal repeat (ITR) sequences) can be introduced, transiently or stably, into a packaging host cell using one or more vectors. While the capsid polypeptide of a virus particle can contain a consensus sequence as described herein, the remaining components can be from one or more known AAV serotypes (e.g., AAV2, AAV8, etc.).

[0033] Such virus particles can be purified using routine methods. As used herein, "purified" virus particles refer to virus particles that are removed from components in the mixture in which they were made such as, but not limited to, viral components (e.g., rep sequences, cap sequences), packaging host cells, and partially- or incompletely-assembled virus particles.

[0034] Once assembled, the virus particles can be screened for, e.g., the ability to replicate; gene transfer properties; receptor binding ability; and/or seroprevalence in a population (e.g., a human population). Determining whether a virus particle can replicate is routine in the art and typically includes infecting a host cell with an amount of virus particles and determining if the virus particles increase in number over time. Determining whether a virus particle is capable of performing gene transfer also is routine in the art and typically includes infecting host cells with virus particles containing a transgene (e.g., a detectable transgene such as a reporter gene). Following infection and clearance of the virus, the host cells can be evaluated for the presence or absence of the transgene. Determining whether a virus particle binds to its receptor is routine in the art, and such methods can be performed *in vitro* or *in vivo*.

[0035] Determining the seroprevalence of a virus particle is routinely performed in the art and typically includes using an immunoassay to determine the prevalence of one or more antibodies in samples (e.g., blood samples) from a particular population of individuals. Seroprevalence is understood in the art to refer to the proportion of subjects in a population that is seropositive (i.e., has been exposed to a particular pathogen or immunogen), and is calculated as the number of subjects in a population who produce an antibody against a particular pathogen or immunogen divided by the total number of individuals in the population examined. Immunoassays are well known in the art and include, without limitation, an immunodot, Western blot, enzyme immunoassays (EIA), enzyme-linked immunosorbent assay (ELISA), or radioimmunoassay (RIA). Similarly, several methods to determine the extent of neutralizing antibodies in a serum sample are available. For example, a neutralizing antibody assay measures the titer at which an experimental sample contains an antibody concentration that neutralizes infection by 50% or more as compared to a control sample without antibody. See, also, Fisher et al. (1997, *Nature Med.*, 3:306-12); and Manning et al. (1998, *Human Gene Ther.*, 9:477-85).

Methods of Using Viruses that Exhibit a Preference for Retrograde Movement in Neurons

[0036] A virus or portion thereof as described herein can be used in a number of research and/or therapeutic applications. For example, a virus or portion thereof as described herein can be used in human or animal medicine for gene therapy (e.g., in a vector or vector system for gene transfer) or for vaccination (e.g., for antigen presentation). More specifically, a virus or portion thereof as described herein can be used for gene addition, gene augmentation, genetic delivery of a polypeptide therapeutic, genetic vaccination, gene silencing, genome editing, gene therapy, RNAi delivery, cDNA delivery, mRNA delivery, miRNA delivery, miRNA sponging, genetic immunization, optogenetic gene therapy, transgenesis, DNA vaccination, or DNA immunization.

[0037] A host cell can be transduced or infected with a virus or portion thereof *in vitro* (e.g., growing in culture) or *in vivo* (e.g., in a subject, e.g., a human or a non-human). Host cells that can be transduced or infected with a virus or portion thereof *in vitro* are described herein; host cells that can be transduced or infected with a virus or portion thereof *in vivo* include, without limitation, projection neurons (e.g., cortico-pontine projection neurons, sympathetic projection neurons, central nervous system projection neurons), afferents to the dorsomedial striatum (DMS), spinal cortical/luminal neurons, pre-cerebellar neurons, inputs to basal ganglia, pre-thalamic neurons, motor neurons, sensory neurons, or other neurons or cells of the central nervous system.

[0038] A virus or portion thereof as described herein can be modified to include a payload. A payload typically includes at least one nucleic acid (e.g., a promoter sequence operably linked to a coding sequence that encodes the payload). In certain instances, a payload can be an inhibitory nucleic acid. Inhibitory nucleic acids are known in the art and include, for example, antisense oligonucleotides, short interfering RNAs (siRNAs), and RNA interference (RNAi) molecules. In certain instances, a payload can be one or more protein-coding genes. Without limitation, protein-coding genes include an optical reporter construct, a therapeutic gene, or an effector protein.

[0039] Many types of optical reporter constructs are known in the art, and the following list is meant to be representative and not exhaustive. For example, optical reporter constructs include, without limitation, GCaMP6 (GCaMP6s, GCaMP6m, or GCaMP6f; see WO 2014/059154), a fluorophore (e.g., green fluorescent protein (GFP), enhanced GFP (EGFP), red fluorescent protein (RFP), yellow fluorescent protein (YFP), tdTomato), a color-flipping construct (e.g., a payload that expresses one reporter in one cell population and a different reporter in another population), Glucose Sensors (e.g., US 2015/0111222), iRCaMP (U.S. Patent No. 9,644,007), iRGECO (e.g., U.S. Patent No. 9,644,007), CaMPARI (e.g., U.S. Patent No. 9,518,996), voltage indicators, secondary messengers, receptor signalers, transcription reporters, epigenetic reporters, and neuromodulator reporters.

[0040] Therapeutic genes also are known in the art and will be dependent upon the particular disease or disorder being treated. For example, a therapeutic gene can be for the treatment of a neurodegenerative disorder. Representative therapeutic genes (or the encoded polypeptide) and their associated neurodegenerative disorders include, without limitation, HSP104 for the treatment of Alzheimer's disease, Ataxin (e.g., frataxin) for the treatment of Friedreich's ataxia, lysosomal glucocerebrosidase (GBA) for the treatment of Parkinson's disease, polyQ-binding protein for the treatment of Huntington's disease, survival motor neuron 1 for the treatment of spinal muscular atrophy, and Amyotrophic lateral sclerosis (ALS), Autism, Dementia, peripheral neuropathy, schizophrenia, and retinal degeneration. In some instances, the payload may be a therapeutic moiety (as opposed to a gene). Therapeutic moieties include, for example, an antibody or fragment thereof, an immunomodulatory protein, or a RNA interference (RNAi) molecule.

[0041] As used herein, an "effector" protein refers to any type of protein that imparts an effect on a cell or the contents thereof (e.g., nucleic acids, proteins, organelles or

processes involving any of the above). For example, effector proteins include, without limitation, recombinases (e.g., Cre or Flp), gene editing systems (e.g., CRISPR/Cas9, TALEN, zinc finger nuclease), optogenetic reagents (activators (e.g., channelrhodopsin or variants thereof) or inhibitors (e.g., halorhodopsin or Arch)), chemogenetic reagents (e.g., activator/inhibitor versions of the DREADD or PSAM/PSEM systems), activators and/or inhibitors of cell system pathways, and enzymes for control of epigenetics.

[0042] It would be appreciated that it might be desirable to deliver a viral protein that complements or inhibits the function of a virus (e.g., a virus other than AAV). For example, a viral protein that is an entry receptor (e.g., the rabies G protein, a protein that relates to cellular and/or transcellular transport).

[0043] Any number of promoters can be used to drive the sequence encoding the payload. Constitutive promoters are known in the art, as are tissue-specific promoters (e.g., neuron-specific promoters). Simply by way of example, the promoter can be a synapsin-1, CMV, GFAP, CAG, CaMKII, MBP, EF1alpha, mDlx, or TRE promoter.

[0044] A virus or portion thereof, usually suspended in a physiologically compatible carrier, can be administered to a subject (e.g., a human or a non-human subject (e.g., a primate, a rodent, a reptile, or a bird)). Suitable carriers include saline, which may be formulated with a variety of buffering solutions (e.g., phosphate buffered saline), lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, and water. The virus or portion thereof is administered in sufficient amounts to transduce or infect the cells and to provide sufficient levels of gene transfer and expression to provide a therapeutic benefit without undue adverse effects. Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, intracranial, intraspinal or intramuscular injection. Additional routes of administration include, for example, orally, intranasally, intratracheally, by inhalation, intravenously, intraocularly, subcutaneously, intradermally, transmucosally, or by other routes of administration. Routes of administration can be combined, if desired.

[0045] The dose of the virus or portion thereof administered to a subject will depend primarily on factors such as the condition being treated, and the age, weight, and health of the subject. For example, a therapeutically effective dosage of a virus or portion thereof to be administered to a human subject generally is in the range of from about 0.1 ml to about 10 ml of a solution containing concentrations of from about 1×10^1 to 1×10^{12} genome copies (GCs) of viruses (e.g., about 1×10^3 to 1×10^9 GCs). Transduction and/or expression of a transgene can be monitored at various time points following administration by DNA, RNA, or protein assays. In some instances, the levels of expression of the transgene can be monitored to determine the frequency and/or amount of dosage.

[0046] Significantly, the AAV-retro particles described herein exhibit up to two orders of magnitude greater retrograde access to cortico-pontine projection neurons than, for example, canine adenovirus-2 (CAV-2), and the retrograde access to certain neurons (e.g., cortico-pontine projection neurons or afferents to the dorsomedial striatum (DMS)) is comparable to that observed with a synthetic tracer such as Fluoro-Gold® fluorescent beads.

[0047] In accordance with the present invention, there may be employed conventional molecular biology, microbiology, biochemical, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. The invention will be further described in the following examples.

EXAMPLES

Example 1-Experimental Procedures

[0048] All procedures were performed in accordance with protocols approved by the Janelia Research Campus and the University of California Berkeley Institutional Animal Care and Use Committees.

Example 2-Library Generation and Viral Production

[0049] Four previously generated viral libraries were used at the start of the directed evolution procedure: 1) a random mutagenesis library generated by subjecting the AAV2 cap gene (encoding viral proteins VP1-3 and assembly-activating protein (AAP)) to error prone PCR (Maheshri et al., 2006, Nat. Biotechnol., 24:198-204); 2) library of AAV2 cap gene variants containing 7-mer peptide inserts between N587 and R588 (Müller et al., 2003, Nat. Biotechnol., 21:1040-6); 3) library of AAV2 cap gene variants containing randomized loop regions (Koerber et al., 2009, Mol. Ther., 17:2088-95); and 4) a DNA shuffling library generated from wild-type AAV1, AAV2, AAV4, AAV5, AAV6, AAV8 and AAV9 cap gene sequences (Koerber et al., 2008, Mol. Ther., 16:1703-9). Each pool of mutant DNA had been originally sub-cloned into the replication-competent AAV packaging plasmid to create a viral plasmid library that, when packaged into AAV virions, can be selected for any new property or function. The replication-competent AAV system incorporates the mutant cap gene into the viral payload, and thus, the genotype of each variant is linked to its phenotype. Capsid sequences of the desired property can then be recovered by DNA sequence analysis of the encapsulated AAV genome.

[0050] The four replication-competent AAV libraries were packaged by calcium phosphate transient transfection of HEK293-T cells followed by viral harvest, iodixanol gradient centrifugation, and Amicon filtration (Maheshri et al., 2006, Nat. Biotechnol., 24:198-204).

Example 3-In Vivo Virus or Tracer Injections

[0051] For localized *in vivo* viral delivery, mice or rats were anaesthetized with isoflurane (~2% by volume in O₂; SurgiVet, Smiths Medical) and a small hole was drilled in the skull above the requisite injection site (see Table 1). For some injection sites, several injections were made at different depths (see Table 1). For viral injections, ~50-100 nl (mice) or 250-500 nl (rats) of virus-containing solution was slowly injected at each depth into the tissue. For tracer injections, 50 nl of 5% Fluoro-gold (Fluorochrome, Denver, CO) in 0.9% NaCl, or 100 nl of retro-beads (LumaFluor, Durham, NC) diluted 1:1 in 0.9% NaCl were injected at the same set of sites for each injection target. Injections were done with a pulled glass pipette (broken and beveled to 25-30 µm (outside diameter); Drummond Scientific, Wiretrol II Capillary Microdispenser) back-filled with mineral oil. A fitted plunger was inserted into the pipette and advanced to displace the contents using a hydraulic manipulator (Narashige, MO-10).

[0052] Retraction of the plunger was used to load the pipette with virus. The injection pipette was positioned with a Sutter MP-285 manipulator.

Table 1. Coordinates Used in This Study

Injection target	Injection coordinates, mm *
mouse SNr	A/P: -3.5; M/L: 1.5; D/V: -4.0 and -4.5
mouse cerebellum	A/P: 7.1; M/L: 1.4; D/V: -0.8 and -0.5
mouse dorso-medial striatum	A/P: 0.5; M/L: 1.5; D/V: -2.5 and -2.3
mouse pontine nucleus (BPN)	A/P: 0.40; M/L: 0.40; D/V: -5.50, -5.75, and -6.0
rat dorso-medial striatum	A/P 2.16; M/L 1.7; D/V -4.3
rat superior colliculus	A/P -6.0; M/L ±1.8; D/V -5.45
* All A/P coordinates are given with respect to Bregma.	

Example 4-Library Selection and Evolution

[0053] The four mutant viral libraries were pooled and injected either into the SNr or into the cerebellum of adult (6-8 week old) wild-type C57/Bl6J mice (either sex; Charles River). Three weeks following injections, striatal or hindbrain tissue was accordingly removed, DNA was extracted, and virions that had successfully reached the remote retrograde target tissue were PCR-amplified and re-cloned into rCAV packaging plasmid to create a new replication competent AAV library for the next round of selection. After three selection steps, the rescued cap genes were randomly mutated by error prone PCR using 5'-ACG CGG AAG CTT CGA TCA ACT ACG CAG-3' (SEQ ID NO:79) and 5'-AGA CCA AAG TTC AAC TGA AAC GAA TTA AAC GG-3' (SEQ ID NO:80) as forward and reverse primers, respectively.

[0054] Two additional *in vivo* selection rounds were then performed. Individual isolates were sequenced after rounds 4 and 5 to evaluate the degree of library enrichment (Table 2).

Table 2. Convergence of Peptide Insert Sequences for Variants Isolated in Rounds 4 and 5

Clone	Clone SEQ ID NO (nucleic acid / protein)	Loop	Loop SEQ ID NO	E36G	E67A	V182	S207G	N382D	N408S	T567A	Q598L	I648V	V708I	M235L	F284Y	5492	N496	other
4R-H-3	3/4	LAISDQTKHA	81										x					
4R-H-4	5/6	LAISDQTKHA	81							x			x				D	L59P
4R-H-5	7/8	LAKDQTKSTA	82						x				x					
4R-H-9	9/10	LANQDYTKTA	83													P		
4R-H-10	11/12	LAISDQTKHA	81										x			Y		
4R-H-12	13/14	LANQDYTKTA	83	x									x					
4R-H-15	15/16	LAISDQTKHA	81										x					
4R-H-16	17/18	LAHDITKNIA	84						x				x					
4R-H-17	19/20	LAHDITKNIA	84						x				x					
4R-5D-2	21/22	LAHDITKNIA	84		x		x		x				x					
4R-5D-3	23/24	LAHDITKNIA	84						x				x					
4R-5D-10	25/26	LAISDQTKHA	81						x				x					
4R-5D-11	27/28	LANQDYTKTA	83			E							x			P		
4R-5D-12	29/30	LAISDQTKHA	81					x					x					Q186R, D594N
4R-5D-15	31/32	LADQDYTKTA	85										x	x	x			
4R-5D-16	33/34	LAISDQTKHA	81			E			x				x					D69E
4R-5D-17	35/36	LAISDQTKHA	81			E							x				Y	
4R-5D-18		LAHDITKNIA	84						x				x					
5R-Hind1	37/38	LAQPDATKNA	86		x	I							x					
5R-Hind2	39/40	LAHDITKNIA	84		x		x						x					E12V/K 137E
5R-Hind3	41/42	LANQDYTKTA	83		x								x					R585G, S662C
5R-Hind6	43/44	LADQDYTKTA	85					x					x					
5R-Hind8	45/46	LANQDYTKTA	83		x								x					
5R-Hind10	47/48	LADQDYTKTA	85	x					x				x					
5R-Hind11	49/50	LANQDYTKTA	83	x					x				x					N335K
5R-Hind12	51/52	LAHDITKNIA	84						x				x					
5R-Hind14	53/54	LAISDQTKHA	81										x					S423N
5R-Hind15	55/56	LAISDQTKHA	81										X					N214D
5R-	57/58	LAHDITKNIA	84						x				x					

Clone	Clone SEQ ID NO (nucleic acid / protein)	Loop	Loop SEQ ID NO	E36G	E67A	V182	S207G	N382D	N408S	T567A	Q598L	I648V	V708I	M235L	F284Y	5492	N496	other
Hind16																		
5R- Hind17	59/60	LADQDYTKTA	85		x				x				x					
5R- Hind18	61/62	LAISDQTKHA	81										X					
5R- Hind19	63/64	LAHDITKNIA	84		x		x		x				x					H38L,E 147A
5R- Hind22	65/66	LANQDYTKTA	83	x									x			P		N227D, N 5875
5R- Hind23	67/68	LAHDITKNIA	84	x	x				x				x					
5R- Ce1	69/70	LANQDYTKTA	83		x								x			P		D41G,K 105E,G 163D
5R- Ce5	71/72	LADQDYTKTA	85							x			x					
5R- Ce6	73/74	LAQPDATKNA	86		x		x						x					P29A
5R- Ce8	75/76	LAKDQTKSTA	82										x					
5R- Ce9	77/78	LANQDYTKTA	83		x		x		x				x			P		

[0055] Seventeen variants were chosen for secondary screening at the end of round 5, and the corresponding cap gene sequences were re-cloned into an rAAV helper plasmid. Individual high-titer preps of the parental wild type AAV2 and of each of the chosen 17 mutant variants carrying a CMV-EGFP payload were then performed by Vector BioLabs, Inc (Philadelphia, PA). The CMV promoter is typically weak in neurons, and thus, this secondary screen provided a stringent test of the efficiency of retrograde transport. The individual AAV variants were injected either into the cerebellum or into the globus pallidus. After 3 weeks, endogenous, unamplified EGFP fluorescence was visualized in the regions expected to be labeled if retrograde transport was efficient. Mutant 5R-Hind6 (SEQ ID NO:43 encoding SEQ ID NO:44) displayed the strongest retrograde transport in both circuits, and was thus chosen for further analysis and dubbed rAAV2-retro.

Example 5-Heparin Binding Assay

[0056] The heparin affinity of AAV2-retro and wild-type AAV2 were analyzed as previously described (Jang et al., 2011, Mol. Ther., 19:667-75). Briefly, approximately 10^{11} purified genomic particles were loaded onto a 1 mL HiTrap heparin column (GE Healthcare Sciences, Piscataway, NJ) previously equilibrated with 150 mM NaCl and 50 mM Tris at pH 7.5. Elution was then performed by increasing the concentration of NaCl in steps of 50 mM up to a final concentration of 950 mM, followed by a wash with 1M NaCl. A small fraction of each elution was used to infect HEK293T cells, and the percentage of GFP positive cells was quantified 48 hours post-infection using a Guava EasyCyte 6HT flow cytometer (EMD/Millipore).

Example 6-Payloads Used in the Study

[0057] For all subsequent experiments, the CMV promoter was replaced with a promoter known to be more robust in adult neurons. Cre recombinase and the GCaMP6f calcium sensor were driven by the human Synapsin-1 (hSyn1) promoter. All of the fluorophores were driven by the CAG promoter, and the color-flipping construct was driven by the EF1- α promoter.

Example 7-Virus Production for the Quantification of Retrograde Efficiency

[0058] hSyn-Cre payload was packaged using AAV1, AAV2, AAV5, AAV8, AAV9, DJ and AAV2-retro capsids at the Janelia Viral Shared Resource. All seven virus preparations were processed in parallel, and were titer matched before *in vivo* injections. All lots were diluted to the lowest measured titer (1.3×10^{12} GC/ml), and each virus was injected into the right pontine nucleus of three adult Rosa26Lox-STOP-LoxH2B-GFP mice (He et al., 2012, Neuron, 73:35-48).

Example 8-Histology

[0059] Animals were sacrificed three weeks following virus injections, at which point the brains were harvested and the right hemisphere was sagittally sectioned at a thickness of 50 μ m. The sections were mounted in VECTASHIELD Antifade Mounting Medium containing DAPI (Vector Laboratories), and imaged using a P-E Panoramic slide scanner (3D Histech) using a 20x objective and FITC and DAPI filters.

Example 9-Retrograde Transport Quantification

[0060] Images obtained with the Panoramic slide scanner were stitched and then analyzed using custom software written in Matlab (Mathworks) to detect the GFP labeled nuclei across the cortex. A region of interest (ROI) was manually drawn around the cortex to isolate the area in the image for automated cell counting. To enhance the detection of nuclei, the image was then convolved with a "Mexican Hat" kernel comprising the difference of two Gaussians (26.00 μ m variance and 3.25 μ m variance). Image noise was reduced using a median filter, and basic peak detection was then performed.

Example 10-Analysis of the Generality of Retrograde Transport

[0061] Rosa26-LSL-H2B-GFP mice were injected with 25 nl of rAAV2-retro hSyn1-Cre in the dorsal striatum. Three weeks after injection, coronally-sectioned brains were imaged using a Panoramic Scanner to visualize DAPI stained nuclei and green fluorescence from H2B-GFP expressing nuclei. The green channel was convolved with a difference of two Gaussians, and peaks were then detected as local maxima on these threshold images using custom functions written in Matlab. The blue channel of each section was aligned to the Nissl images from the Allen Brain Institute's standardized mouse brain atlas using custom analysis routines written with the help of the Matlab Image Processing Toolbox. The annotated regions from the ABI's mouse brain atlas were then used to assign detected neurons in aligned sections to specific brain regions.

[0062] It was noted that finite precision of the reference atlas together with anatomical variability of individual brains limit the robustness of this semi-automated process to prominent afferent inputs.

Example 11-Imaging of Neuronal Population Activity *In Vivo* Following Retrograde Delivery of GCaMP6f

[0063] Seven adult mice were anesthetized with isoflurane (2%) and placed in a stereotactic frame (Kopf Instruments; Tujunga, CA) on a 37°C heating pad. The scalp and periosteum over the skull were removed, a layer of UV-curing OptiBond adhesive (Kerr; Orange, CA) was applied, and a custom-made headpost (Osborne and Dudman, 2014, PLoS One, 9(2):e89007) was affixed with dental cement. AAV2-retro carrying a hSynGCaMP6f payload was injected into the BPN (3.9 mm posterior and 0.4 mm lateral to Bregma, depths 5.8, 5.6 and 5.4 mm, 100 nl at each depth) using a Nanoliter 2010 injector (WPI). A cranial window (one 170 µm-thick pane of laser-cut glass, 2 mm diameter) was placed over the primary motor cortex (centered on 0.7 mm anterior and 1.6 mm lateral to Bregma).

[0064] Following surgery, injections of ketoprofen (5 mg/kg) and buprenorphine (0.1 mg/kg; Henry Schein Animal Health; Melville, NY) were administered subcutaneously. Mice were allowed to recover for 1 week following surgery and then were imaged briefly under a 2-photon microscope to assess virus expression. All animals had visually identified GCaMP6f expressing cells in layer V of M1 one-week post injection. Then the animals were habituated to head fixation in a custom-built apparatus and trained to retrieve a food pellet as previously described (Guo et al., 2014, Nat. Med., 20:130-8).

[0065] GCaMP6f was excited at 920 nm (typically 20-40 mW at the back aperture) with a Ti: Sapphire laser (Chameleon, Coherent) and imaged through a Nikon 16x, 0.8-N.A. objective. Emission light passed through a 565 DCXR dichroic filter (Chroma Technology) and an ET525/70m-2p filter (Chroma Technology) and was detected by a GaAsP photomultiplier tube (10770PB-40, Hamamatsu). Images (512 × 512 pixels) were acquired at ~30 Hz with resonating scanners using ScanImage software.

Example 12-CRISPR/Cas9 Genome Editing

[0066] The CMV promoter in pAAV-CMV-SaCas9-empty (Slaymaker et al., 2016, Science, 351:84-8) was replaced with hSyn1 to generate pAAV-hSyn1-SaCas9-empty. Oligonucleotides encoding sgRNA protospacer sequences were custom ordered, phosphorylated, hybridized and ligated into the BsaI restriction sites of pAAV-hSyn1-SaCas9-empty to generate pAAV-hSyn1-SaCas9-tdTomato-1 to -10. Oligonucleotide sequences used were:

tdTomato sgRNA 1 Fwd: CAC CGC AAG GGC GAG GAG GTC ATC A (SEQ ID NO:87)
 tdTomato sgRNA 1 Rev: AAA CTG ATG ACC TCC TCG CCC TTG C (SEQ ID NO:88)
 tdTomato sgRNA 2 Fwd: CAC CGT GGA GGG CTC CAT GAA CGG CC (SEQ ID NO:89)
 tdTomato sgRNA 2 Rev: AAA CGG CCG TTC ATG GAG CCC TCC AC (SEQ ID NO:90)
 tdTomato sgRNA 3 Fwd: CAC CGA GGA CGG CGG CCA CTA CCT GG (SEQ ID NO:91)
 tdTomato sgRNA 3 Rev: AAA CCC AGG TAG TGG CCG CCG TCC TC (SEQ ID NO:92)
 tdTomato sgRNA 4 Fwd: CAC CGA CAA CAA CAT GGC CGT CAT CA (SEQ ID NO:93)
 tdTomato sgRNA 4 Rev: AAA CTG ATG ACG GCC ATG TTG TTG TC (SEQ ID NO:94)
 tdTomato sgRNA 5 Fwd: CAC CGA AGG ACG GCG GCC ACT ACC TGG (SEQ ID NO:95)
 tdTomato sgRNA 5 Rev: AAA CCC AGG TAG TGG CCG CCG TCC TTC (SEQ ID NO:96)
 tdTomato sgRNA 6 Fwd: CAC CGA CAA CAA CAT GGC CGT CAT CA (SEQ ID NO:97)
 tdTomato sgRNA 6 Rev: AAA CTG ATG ACG GCC ATG TTG TTG TC (SEQ ID NO:98)
 tdTomato sgRNA 7 Fwd: CAC CGG TCA CCT TCA GCT TGG CGG T (SEQ ID NO:99)
 tdTomato sgRNA 7 Rev: AAA CAC CGC CAA GCT GAA GGT GAC C (SEQ ID NO:100)
 tdTomato sgRNA 8 Fwd: CAC CGC CGT ACA TGA ACT GGG GGG A (SEQ ID NO:101)
 tdTomato sgRNA 8 Rev: AAA CTC CCC CCA GTT CAT GTA CGG (SEQ ID NO:102)
 tdTomato sgRNA 9 Fwd: CAC CGT CTT GTA ATC GGG GAT GTC GG (SEQ ID NO:103)
 tdTomato sgRNA 9 Rev: AAA CCC GAC ATC CCC GAT TAC AAG AC (SEQ ID NO:104)
 tdTomato sgRNA 10 Fwd: CAC CGC CGT CCT GCA GGG AGG AGT C (SEQ ID NO:105)
 tdTomato sgRNA 10 Rev: AAA CGA CTC CTC CCT GCA GGA CGG C (SEQ ID NO:106)

[0067] The ability of each oligo to direct genome editing was first evaluated *in vitro*. Neuro2A cells were transfected with 800 ng of pAAV-hSyn1-SaCas9-tdTomato-1 to -10, 100 ng of pAAV-FLEX-CAGtdTomato and 100 ng of pAAV-CAG-EGFP using polyethylenimine. At 72 hrs after transfection, cells were harvested and ~70,000 EGFP positive Neuro2A cells were isolated by fluorescence activated cell sorting (FACS) using a BD Influx Sorter (BD Biosciences). Genomic DNA was then extracted, and the frequency of tdTomato gene modification was evaluated by the Surveyor nuclease assay (Integrated DNA Technologies) as previously described (Cong et al., 2013, Science, 339:819-23). sgRNA 7 - one of two that appeared to direct two cleavage events within the tdTomato sequence - was packaged into retro-AAV2 and used for *in vivo* genome editing.

[0068] ~100 nl (5×10^{13} vector genomes (vg)/ml) of AAV2-retro-hSyn1-SaCas9-tdTomato or AAV2retro-hSyn1-SaCas9-empty was then injected into the BPN of Rbp4-Cre × tdTomato mice as described above. Six weeks after injections, brains were harvested and 40 µm-thick coronal sections were cut and stained against the HA-tagged Cas9 (anti-HA antibody C29F4 from Cell Signaling, diluted 1:1600; secondary antibody: donkey anti-mouse Alexa Fluor 488 (1:250; Jackson ImmunoResearch) and against the NeuN neuronal marker (anti-NeuN antibody A60 from Millipore, diluted 1:250; secondary antibody: donkey anti-rabbit Alexa Fluor 647 (1:500; ThermoFisher, A-31573)).

Following antibody staining, the sections were mounted onto slides with VECTASHIELD Antifade Mounting Medium containing DAPI (Vector Laboratories) and visualized using a Zeiss Axio Observer A1 inverted microscope (Zeiss). Quantification of immunostaining was performed using ImageJ analysis software (NIH).

Example 13-Directed Evolution of rAAV2-retro

[0069] To engineer novel rAAVs with enhanced retrograde transport, an *in vivo* directed evolution approach was designed that enriched for rAAV capsid variants that were efficiently transported to cell bodies of neurons sending long-range projections to the site of virus injection in the mouse brain (Figure 1A). To maximize the likelihood of recovering a variant with the desired properties, a diverse mixture of previously described libraries of rAAV cap variants (Koerber et al., 2008, Mol. Ther., 16:1703-9; Koerber et al., 2009, Mol. Ther., 17:2088-95; Koerber et al., 2006, Nat. Protocols, 1:701-6; Müller et al., 2003, Nat. Biotechnol., 21: 1040-6) was used as starting material. Viral particles were packaged in a way that linked each variant capsid to the AAV genome containing the corresponding cap gene, and the final pool of capsid variants included point mutants, variants with a random 7-mer peptide insertion into the region AAV2 utilizes to bind its co-receptor, heparan sulfate, and random chimeras of capsid gene sequences from seven parental serotypes (Figure 1A). To identify variants with broad retrograde tropism, two independent populations of projection neurons were targeted: striatal GABAergic neurons projecting to substantia nigra pars reticulata (SNr) and glutamatergic hindbrain neurons projecting to the cerebellar cortex. Three weeks following injection of the entire pool of rAAV variants into the SNr or cerebellum (one injection per animal), striatal or hindbrain tissue, respectively, was removed, the cap sequences were recovered by PCR and virus was repackaged (Figure 1B). After two more selection steps, error-prone PCR was performed to further diversify the library, followed by two final *in vivo* selection steps.

[0070] 30 cap variants were sequenced after the 4th round of evolution, and the majority originated from the insertion library and contained exogenous 7-mer peptides between N587 and R588 of the wild-type AAV2 VP1 capsid gene. Intriguingly, all of the recovered inserts were of the form *L*AxxDxTKxA (SEQ ID NO: 107) or *L*AxDxTKxxA (SEQ ID NO: 108) (where the bold, italicized residues are from the insert); mutations elsewhere in the sequence were also enriched (Table 2). A further 22 clones were sequenced after the 5th round of evolution, and with this additional round of evolution, all sequences were AAV2 mutants with *L*AxxDxTKxA (SEQ ID NO: 107) / *L*AxDxTKxxA (SEQ ID NO: 108) insertions (Table 2), demonstrating a marked degree of further convergence. Such convergence suggested that specific peptide insertions into the heparin-binding loop were largely responsible for the retrograde functionality, with potential secondary contributions from the other sites.

[0071] A secondary screen on seventeen isolated variants possessing different combinations of peptide insertions and point mutations in the capsid sequence was examined next. To apply a high level of stringency, the chosen variants were packaged with enhanced green fluorescent protein (EGFP) transgene driven by the CMV promoter, which is typically weak in neurons. For each capsid variant, its ability to deliver sufficient payload to cell bodies in key afferent regions to permit the detection of non-antibody-amplified EGFP signal three weeks after injection was evaluated. The clone (insert LADQDYTKA (SEQ ID NO:85) + V708I + N382D) that displayed the strongest retrograde transport in two independent circuits in this secondary screen (cortex to globus pallidus and inferior olive/basal pontine nucleus to cerebellum) was chosen for further analysis and dubbed rAAV2-retro. When two additional promoters more commonly used in rodent *in vivo* studies were assessed (CAG - Figure 1B, or human Synapsin-1, data not shown), remarkable retrograde labeling efficiency was observed with this rAAV variant in a range of different circuits in mice and rats (Figure 1B and Figure 7). Swapping the 7-mer insertion to one of the other recovered sequences, or adding additional point mutations identified in the screen, did not lead to further increases in retrograde transport.

Example 14-Efficient Retrograde Access to Projection Neurons

[0072] Within the descending motor pathways, the cortico-pontine tract is remarkably convergent, and is known to contribute over 95% of the afferents to the basal pontine nuclei (BPN). This pathway thus represents a particularly advantageous system for quantifying the efficiency of viral uptake and retrograde transport by axonal terminals of projection neurons. Indeed, injection of rAAV2-retro into the BPN resulted in dense labeling of layer V neurons (Figure 2A), consistent with past tracing studies (Legg et al., 1989, J. Comp. Neurol., 286(4):427-41).

[0073] Next, the efficacy of retrograde transport in the cortico-pontine circuit for rAAV2-retro vs. several commonly used AAV serotypes was compared under identical infection and processing conditions (Figure 2B-D). To ensure quantification accuracy, and to eliminate the possible confound of cell-to-cell variability in transgene expression level, the AAV was used to deliver Cre recombinase in Rosa26-Lox-STOP-Lox-H2B-GFP transgenic mice (He et al., 2012, Neuron, 73:35-48). It has been shown that even a low concentration of the Cre enzyme is sufficient to turn on the expression of such a Cre-dependent cassette, and the stringent nuclear localization of the histone-fused reporter affords unambiguous identification of infected cells, without confounding neuropil signal.

[0074] A semi-automated analysis procedure was used to calculate the linear density of infected cortical projection neurons in imaged sagittal sections from mouse brains harvested three weeks after local virus injection in the BPN (Figure 2C). For animals infected with rAAV2, minimal cortical GFP expression was observed (linear density 0.98 ± 0.20 neurons/mm, mean $\pm$ sem, n=5; Figures 2B, middle panel). In contrast, and in agreement with the earlier observation (Figure 2A), a dense layer of GFP-positive layer V projection neurons could be observed throughout the rostro-caudal axis of the cortex in rAAV2-retro-injected animals (linear density 130.11 ± 11.08 neurons/mm, n=4; Figure 2B, lower panel). None of the other commonly used AAV serotypes, nor canine adenovirus-2, matched the retrograde efficiency of the engineered rAAV2-retro variant (linear density AAV1: 0.05 ± 0.04 , AAV2: 0.98 ± 0.2 , AAV5: 2.38 ± 1.24 , AAV8: 1.43 ± 1.43 , AAV9: 1.98 ± 0.86 , DJ: 24.82 ± 14.32 , CAV-2: 5.56 ± 4.13 , n=3 to 5 each; Figure 2D, Figure 8). Furthermore, the density of cortical projection neurons labeled by rAAV2-retro was comparable to that achieved with Fluoro-Gold (Schmued and Fallon, 1986, Brain Res., 377:147-54), a robust synthetic retrograde tracer (linear density 81.03 ± 11.08 neurons/mm, n= 3). Thus, rAAV2-retro exhibits up to two orders of magnitude enhancement over existing serotypes in retrograde access to cortico-pontine projection neurons, and rivals the efficacy of synthetic retrograde tracers.

Example 15-Generality of Retrograde Functionality

[0075] Whether the retrograde functionality of rAAV2-retro extended to other circuits was examined next, specifically by characterizing the extent to which it labeled various afferents to the dorsomedial striatum (DMS) - a part of the basal ganglia that receives long-range inputs from a variety of cortical and subcortical areas. It was found that the efficacy of rAAV2-retro-mediated retrograde access to the strongest afferent inputs into DMS - cortex, thalamus and amygdala - was comparable to that of fluorescent beads classically used for retrograde tracing (Figure 3A). To provide an unbiased estimate for the number of retrogradely labeled neurons in all brain areas known to provide significant long-range input to the DMS, an algorithm was developed that assigned any detected fluorescent label in an imaged section of the mouse brain to specific brain areas by aligning the section to the annotated Allen Brain Atlas (Figure 3B-C). Quantitative analysis (Figure 3C) revealed that strong retrograde labeling was found in a vast majority of regions that have been previously reported to send prominent projections to the striatum (Pan et al., 2010, Front Neuroanat., 4: 147). In one noticeable exception, only modest labeling was observed in the substantia nigra pars compacta, despite it being the source of a strong dopaminergic input to the DMS (Figure 3C, arrow at cell count for SNC). A small subset of projection neuron classes was found to be similarly refractory to retrograde access by rAAV2-retro in some of the other circuits tested (Table 3; note that all other AAV serotypes tested also failed to label these projections). Despite these exceptions, rAAV2-retro is broadly applicable within the central nervous system.

Table 3. rAAV2-retro Efficiency in Various Circuits. Low efficiency rather than absence of retrograde transport in cortico-thalamic and cortico-collicular projections is suggested by efficient labeling of projection neurons following local delivery of Cre-dependent payloads to projection neuron cell bodies

Injection location	Location of Projection Neurons Targeted	Labeling efficiency	transgene
mouse mPFC (IL/PL)	MEC	strong	Cre
mouse mPFC (IL/PL)	LEC	strong	Cre

Injection location	Location of Projection Neurons Targeted	Labeling efficiency	transgene
mouse mPFC (IL/PL)	contra mPFC	strong	Cre
mouse mPFC (IL/PL)	mediodorsal thalamus	moderate	Cre
mouse mPFC (IL/PL)	nucleus reuniens of thalamus	moderate	Cre
mouse mPFC (IL/PL)	insular cortex	strong	Cre
mouse mPFC (IL/PL)	hippocampus- ventral CA1	strong	Cre
mouse mPFC (IL/PL)	AcG	strong	Cre
OFC	MEC	strong	Cre
OFC	LEC	Strong	Cre
OFC	contra OFC	strong	Cre
OFC	insular cortex	strong	Cre
OFC	hippocampus- ventral CA1	moderate	Cre
dorsal CA 1	MEC	strong	Cre
dorsal CA2	LEC	strong	Cre
dorsal CA3	CA3	weak	Cre
dorsal CA4	nucleus reuniens of thalamus	weak	Cre
amygdala	mPFC	strong	Cre
lateral hypothalamus	mPFC	strong	Cre
dorsomedial striatum	mPFC	strong	Cre
ventral striatum (NAcc)	mPFC	strong	Cre
MEC	mPFC	moderate	FLPo
mPFC	ventral CA1	moderate	FLPo
mPFC	MEC	moderate	FLPo
subiculum	nucleus accumbens	moderate	Cre
subiculum	ventral hippocampus	strong	Cre
subiculum	intereanterior thalamic nucleus	moderate	Cre
subiculum	retrosplenial cortex	strong	Cre
subiculum	lateral entorhinal cortex	strong	Cre
subiculum	medial entorhinal cortex	strong	Cre
superior colliculus	layer 5 in visual cortex	very weak	Gcamp6s
superior colliculus	layer 5 in visual cortex	strong	Cre
dLGN	layer 6 in visual cortex	very weak	Gcamp6s
dLGN	layer 6 in visual cortex	strong	Cre
visual cortex	contralateral visual cortex	strong	Cre
visual cortex	contralateral visual cortex	strong	GFP
visual cortex	contralateral visual cortex	weak	Gcamp6s
visual cortex	contralateral visual cortex	strong	tdtomato
visual cortex	LGN	strong	tdtomato
visual cortex	claustrum	strong	tdtomato
visual cortex	cingulate	moderate	tdtomato
visual cortex	LM	strong	tdtomato
dorso-lateral striatum	M1	strong	tdtomato
dorso-lateral striatum	M1	moderate	GFP
dorso-lateral striatum	M2	strong	tdtomato
dorso-lateral striatum	somatosensory cortex	strong	tdtomato
dorso-lateral striatum	basolateral amygdala	strong	tdtomato
dorso-lateral striatum	insular cortex	strong	tdtomato
dorso-lateral striatum	entorhinal cortex	strong	tdtomato
dorso-lateral striatum	Globus Pallidus	moderate	tdtomato
dorso-lateral striatum	thalamus	moderate	tdtomato
dorso-lateral striatum	intralaminar thalamic nuclei	strong	GFP
dorso-lateral striatum	raphe nucleus	very weak	tdtomato
dorso-lateral striatum	VTA	weak	tdtomato
basolateral amygdala	taste cortex	strong	flex-tdtomato
forelimb motor cortex	motor thalamus	weak	GFP
nucleus tractus solitarius	lateral amygdala	moderate	tdtomato
spinal cord	cortex	strong	flex-tdtomato
spinal cord	cortex	strong	GFP
spinal cord, lamina IX	spinal cord: presynaptic inhibitory neurons	strong	flex-tdtomato
muscle	spinal motor neurons	moderate	Cre
muscle	proprioceptor neurons	strong	Cre

Example 16-Retrograde Access to Genetically Defined Neuronal Populations

[0076] It also was determined whether the retrograde functionality of rAAV2-retro could be combined with the specificity of Cre transgenic lines to enable the interrogation of specific classes of projection neurons. Specifically, experiments were performed to determine whether rAAV2-retro and Cre transgenic lines could be combined to segregate two functionally distinct long-range connections that run in parallel between two brain areas. Projections from cerebral cortex to the striatum arise primarily from neurons in layer V but some neurons in layers II and III also provide striatal inputs. It has been suggested that inputs to the striatum from different cortical layers constitute separate pathways, with neurons in layer V projecting to the patch compartment of the striatum, and ones in layers II and III to the matrix. The interdigitated nature of patch and matrix micro-compartments makes it difficult to selectively target these pathways for functional interrogation with the rAAV2-retro system alone. It was therefore explored whether the two inputs could be segregated by combining rAAV2-retro with a transgenic line that expresses Cre recombinase in all layer V neurons (Gerfen et al., 2013, *Neuron*, 80:1368-83), but not in neurons in layers II and III (Figure 4A).

[0077] To highlight both pathways in the same experiment, a Cre-dependent color-flipping payload was chosen, which expresses tdTomato in the absence of Cre, but inverts to drive the expression of EGFP in Cre-positive cells (Saunders et al., 2012, *Front Neural Circuits*, 6:47). When rAAV2-retro carrying this payload was injected into the dorso-medial striatum of a layer V-specific Cre driver line, Rbp4_KL100 Cre (Gerfen et al., 2013, *Neuron*, 80: 1368-83), only those layer V neurons projecting to the striatum expressed EGFP. Furthermore, the layer II and III corticostriatal pathway was clearly distinguishable by the expression of tdTomato (Figure 4B). In accordance with the topographic nature of the cortico-striatal projection, highly localized injections of the virus into the dorsomedial striatum led to a labeling of correspondingly small sections of the cortex. The layer V and the layer II and III cortico-striatal populations, however, were always co-labeled (Figure 4B, bottom panel), suggesting that the two pathways from the same cortical area traverse through the neighboring matrix and patch micro-compartments within striatum.

[0078] While this experiment highlighted both pathways, choosing a "Cre-on" or a "Cre-off" payload will permit selective access to one at the exclusion of the other. This example, therefore, highlights the added specificity of circuit interrogation that can be achieved by combining a highly efficient retrograde virus with available Cre (or Flip) driver lines.

Example 17-Using rAAV2-retro for Circuit Interrogation and Gene Manipulation

[0079] The utility of rAAV2-retro for circuit interrogation will depend on its ability to mediate high levels of expression of genetically encoded indicators and effectors. The ability to monitor neural activity in defined classes of projection neurons was first assessed through rAAV2-retro-mediated expression of GCaMP6f (Chen et al., 2013, *Nature*, 499:295-300) (Figure 5A-C). Using *in vivo* two-photon Ca^{2+} imaging, dendritic and somatic Ca^{2+} transients were detected in the primary motor cortex as early as 7 days after viral delivery to the BPN (Figure 5D). The temporal profile of Ca^{2+} signals reflected the structure of a cued reaching task, with the signal in many cortico-pontine neurons being tightly linked to the "go" cue (Figure 5E-F) (Li et al., 2015, *Nature*, 519:51-6). Repeated recordings from identified neurons were possible both across trials within a session early in the expression time course (Figure 5E, F), and across many behavioral sessions for over two months' post-infection. Thus, rAAV2-retro confers the ability to express sensors in projection neurons at levels sufficient for imaging, creating many new opportunities for deciphering the contributions of specific projections to circuit computations.

[0080] Finally, the utility of rAAV2-retro was evaluated for delivery of effectors, such as the CRISPR/Cas9 gene editing system, to projection neurons (Figure 6). Specifically, the *Staphylococcus aureus* Cas9 (SaCas9 (Slaymaker et al., 2016, *Science*, 351:84-8)) and a single guide RNA designed to ablate the expression of tdTomato were packaged into rAAV2-retro. Delivery of rAAV2-retro-SaCas9-antitdTomato to the BPN of animals expressing tdTomato in cortical layer V excitatory neurons resulted in suppression of tdTomato expression in $88.6 \pm 0.7\%$ of SaCas9-expressing layer V neurons (Figure 6B, bottom panel and Figure 6C, $n = 3$). In contrast, delivery of a non-targeted SaCas9 did not lead to any discernible changes, with only $4.4 \pm 3.2\%$ of cells displaying potential reduction in tdTomato expression (Figure 6B, top panel, and Figure 6C, $n = 3$). Furthermore, tdTomato expression remained unaffected in layer V neurons that were inaccessible via pontine injection. The rAAV2-retro system thus permits efficient gene modification selectively in neurons projecting to specific areas of interest.

[0081] Collectively, these observations establish rAAV2-retro as an effective reagent to genetically access projection neurons for functional interrogation of neural circuits and, in the long run, for possible therapeutics.

Example 18-Discussion

[0082] Recombinant adeno-associated viruses can greatly facilitate the functional dissection of mammalian neural circuits, and hold promise for therapeutic intervention in disorders of the nervous system. Directed evolution has been used to endow the AAV capsid with the additional capacity for efficient retrograde access to projection neurons in many circuits. The newly engineered rAAV2-retro offers up to two orders of magnitude enhancement in retrograde transport compared to commonly used AAV serotypes, matching the efficacy of synthetic retrograde tracers in many circuits. The level of transgene expression achieved with rAAV2-retro via retrograde access is ample for interrogating neural circuit function, as well as for targeted manipulations of the neuronal genome. Thus, by enabling selective monitoring and manipulation of projection neurons connecting different brain areas, rAAV2-retro-based tools are poised to provide insights into how large-scale networks enable brain function, and hold promise for therapeutic intervention in diseases characterized by progressive large-scale network dysfunction.

[0083] The markedly increased efficacy of retrograde access afforded by rAAV2-retro compared to its parental serotype AAV2 may have been enabled through an insertion-mediated disruption of the native binding site for heparan sulfate and/or through the creation of a new binding surface that incorporates the inserted peptide. This variant does have reduced heparin affinity (Figure 9), which could decrease virus sequestration in the extracellular matrix of the synaptic cleft and enhance local vector spread, as has been observed with AAV1 and AAV6. However, the resulting increase in vector spread alone cannot explain the efficacy of retrograde transport, as other inserted 7-mer sequences disrupt heparin binding similarly but do not affect retrograde transport. Furthermore, AAV5 and AAV9 do not bind heparin, yet their retrograde transport efficiency is similar to that of AAV2. In support of the alternative explanation, the peptide insertions selected in the original selection (*L*AxxDxTKxXa (SEQ ID NO: 107) / *L*AxDxTKxxXa (SEQ ID NO: 108)) share the same overall composition, differing simply in the register of the conserved motif. The engineered peptide insertion might support enhanced binding to an existing cellular co-factor in the AAV pathway (e.g., the recently identified common AAV receptor), or it might create a novel interaction with the cellular machinery - a cell-surface receptor and/or a component of the vesicular trafficking or nuclear entry pathway.

[0084] Despite the multiple-orders-of-magnitude improvement in retrograde transport that rAAV2-retro offers over existing serotypes, a small set of projection neuron classes appears refractory to efficient retrograde infection by this newly evolved rAAV variant (Table 3). It should be noted, however, that other AAV serotypes fail to label these projections as well. Whether the expression level of the critical cellular factor that interfaces with this novel AAV variant in these neurons is just extremely low - a conclusion supported for the cortico-thalamic and cortico-collicular projections, for instance, by the observation that rAAV2-retro could still deliver sufficient Cre recombinase to cell bodies to direct high level of expression for locally delivered Cre-dependent payloads (Table 3, highlighted entries) - or whether the factor is missing entirely, remains to be determined in each case. Additional future capsid modifications to this or other variants could enable enhanced retrograde transduction of these neuron classes.

[0085] The rAAV2-retro vector system provides an important addition to the genetic toolkit for dissecting neural circuit function, since gaining access to individual classes of projection neurons will be a critical enabling step in elucidating how local circuit dynamics and large-scale network function are coordinated. Local circuit computations are increasingly thought to depend on the dynamics of the entire neuronal population within a particular local circuit module. How these dynamics map onto the different classes of projection neurons - and thus what information is passed on to the different downstream targets - remains unresolved for most circuits. rAAV2-retro based vectors, alone or in combination with specific Cre transgenic lines, permit genetic access to specific populations of projections neurons. In turn, rAAV2-retro carrying rabies G glycoprotein can be used to trans-complement the newly developed non-toxic conditional rabies vector for access to presynaptic micro-circuits impinging on particular classes of projection neurons. The resulting ability to selectively monitor and manipulate activity of individual projection neuron classes and their local microcircuits should provide

insight into how projection neurons translate the local circuit dynamics for their respective large-scale networks.

[0086] rAAV2-retro also holds promise for therapeutic intervention, with several possible applications. For example, in situations where pathology impacts large volumes of neural tissue - such as Alzheimer's or lysosomal storage diseases - multiple injections pose a safety risk and may be insufficient to achieve the needed levels of transduction. However, a small number of injections in strategic locations can enable vector dispersal over large volumes (e.g., cortico-pontine tract from the point of convergence in the BPN), or difficult-to-access tissue (e.g., spinal motor neurons from the muscle). Furthermore, large-scale functional networks have been implicated in the spread of many neurodegenerative disorders from their spatially localized onset. A prominent emerging view posits that deposition of abnormal protein assemblies in vulnerable neuronal populations triggers a pathological cascade of aberrant neuronal activity within, and disintegration of, large-scale functional networks, ultimately leading to failure of neurological functions. Intriguingly, the affected neural networks appear capable of transiently overcoming the aberrant dynamics early in the disease, as patients with many neurodegenerative diseases often display periods of dramatic improvement. Thus, early intervention aimed at slowing down the spread of aggregates from the cortical origin of pathology may be sufficient to stabilize, or even restore, cognitive function. From this perspective, subcortically-projecting neurons in the mesocortical regions where pathological protein aggregates first appear, in both Alzheimer's and Parkinson's disorders, constitute an attractive intervention target. Accessing those projection neurons with rAAV2retro-based tools designed, for instance, to introduce a mutation that is capable of arresting further aggregation in cis or to deliver chaperones capable of disassembling the aggregates has the potential to slow down the progression of the most debilitating, cognitive, symptoms. Evaluating the efficiency and long-term safety of rAAV2-retro reagents in non-human primates will pave the path to their eventual consideration for these, and other, gene therapy approaches.

[0087] It is to be understood that, while the methods and compositions of matter have been described herein in conjunction with a number of different aspects, the foregoing description of the various aspects is intended to illustrate the methods and compositions of matter.

REFERENCES CITED IN THE DESCRIPTION

Cited references

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- [WO2016054654A1](#) [0084]
- [WO2006118160A2](#) [0084]
- [WO2015121601A1](#) [0084]
- [WO2015038938A1](#) [0084]
- [WO2014058154A](#) [0039]
- [US20150111222A](#) [0039]
- [US9644007B](#) [0039] [0039]
- [US9518096B](#) [0039]

Non-patent literature cited in the description

- LOCHRIE et al.Virology, 2006, vol. 353, 68-82 [0004]
- BELLO et al.Scientific Reports, 2014, vol. 4, 6644- [0004]
- SALEGIO et al.Gene Therapy, 2013, vol. 20, 348-352 [0004]
- ASCHAUER et al.Plos One, 2013, vol. 8, e76310- [0004]
- GAUDET et al.Atherosclerosis Supplem., 2010, vol. 11, 55-60 [0021]
- KASPAR et al.Mol. Ther., 2002, vol. 5, 50-56 [0021]
- ALTSCHUL et al.Nucleic Acids Res., 1997, vol. 25, 3389-3402 [0022]
- PCR Primer: A Laboratory ManualCold Spring Harbor Laboratory Press19950000 [0011]
- FISHER et al.Nature Med., 1997, vol. 3, 306-12 [0035]
- MANNING et al.Human Gene Ther., 1998, vol. 9, 477-85 [0035]
- MAHESHRI et al.Nat. Biotechnol., 2006, vol. 24, 198-204 [0048] [0060]
- MÜLLER et al.Nat. Biotechnol., 2003, vol. 21, 1040-6 [0048] [0060]
- KOERBER et al.Mol. Ther., 2009, vol. 17, 2088-95 [0048] [0060]
- KOERBER et al.Mol. Ther., 2008, vol. 16, 1703-9 [0048] [0060]
- JANG et al.Mol. Ther., 2011, vol. 19, 667-75 [0060]
- HE et al.Neuron, 2012, vol. 73, 35-48 [0060] [0073]
- OSBORNEDUDMANPlos One, 2014, vol. 9, 2e89007- [0063]
- GUO et al.Nat. Med., 2014, vol. 20, 130-8 [0084]
- SLAYMAKER et al.Science, 2016, vol. 351, 84-8 [0060] [0060]
- CONG et al.Science, 2013, vol. 339, 819-23 [0087]
- KOERBER et al.Nat. Protocols, 2006, vol. 1, 701-6 [0060]
- LEGG et al.J. Comp. Neurol., 1989, vol. 286, 4427-41 [0072]
- SCHMUEDFALLONBrain Res., 1986, vol. 377, 147-54 [0074]
- PAN et al.Front Neuroanat., 2010, vol. 4, 147- [0075]
- GERFEN et al.Neuron, 2013, vol. 80, 1368-83 [0078] [0077]
- SAUNDERS et al.Front Neural Circuits, 2012, vol. 6, 47- [0077]
- CHEN et al.Nature, 2013, vol. 499, 295-300 [0079]
- LI et al.Nature, 2015, vol. 519, 51-6 [0079]

Patentkrav

1. Viralt capsidprotein omfattende en sekvens udvalgt blandt gruppen bestående af xxDxTKx (SEQ ID NO:1) og xDxTKxx (SEQ ID NO:2), hvor det virale capsidprotein har en aminosyresekvens udvalgt blandt gruppen bestående af SEQ ID NO: 6, 8, 10, 12, 14, 18, 22, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 54, 56, 60, 64, 66, 68, 70, 72, 74, 76 og 78.
2. Viral partikel omfattende det virale capsidprotein ifølge krav 1.
3. Viral partikel ifølge krav 2, yderligere omfattende en nukleinsyre, der koder for en payload; foretrukket hvor nukleinsyren, der koder for en payload, omfatter en promotorsekvens, der er operabelt forbundet med en kodende sekvens, der koder for payload.
4. Viral partikel ifølge krav 3, hvor promotoren er udvalgt blandt gruppen bestående af synapsin-1, CMV, GFAP, CAG, CaMKII, MBP, EF1alpha, TRE og mDlx.
5. Viral partikel ifølge krav 4, hvor den kodende sekvens, der koder for payload, er udvalgt blandt gruppen bestående af et proteinkodende gen og en inhiberende RNA-nukleinsyre; foretrukket hvor den inhiberende RNA-nukleinsyre er et antisense-oligonukleotid, et siRNA eller et RNAi.
6. Viral partikel ifølge krav 3, hvor payload er:
 - i) et effektorprotein; foretrukket hvor effektorproteinet er udvalgt blandt gruppen bestående af en rekombinase (f.eks. Cre eller Flp), et genredigeringsystem (f.eks. CRISPR/Cas9, TALEN, zinkfinger-nuklease), optogenetiske reagenser (aktivatorer (f.eks. kanalrhodopsin) eller varianter deraf) eller inhibitorer (f.eks. halorhodopsin eller Arch)), kemogenetiske reagenser (f.eks. aktivator/inhibitor-versioner af DREADD- eller PSAM/PSEM-systemerne), aktivatorer og/eller inhibitorer af celledystemveje og enzymer til kontrol af epigenetik;
 - ii) en optisk reporterkonstruktion; foretrukket hvor den optiske reporterkonstruktion er udvalgt blandt gruppen bestående af GCaMP6 (s, m eller f), en fluorofor (f.eks. grønt fluorescerende protein (GFP), forstærket GFP (EGFP), rødt fluorescerende protein (RFP), gult fluorescerende protein (YFP), tdTomato), en farve-flipping-konstruktion (f.eks. en payload, der udtrykker en reporter i en cellepopulation og en anden reporter i en anden population), Glucose Sensorer, jRCaMP, jRGECO og CaMPARI, spændingsindikatorer, sekundære messengers, receptorsignalere, transkriptionsreportere, epigenetiske reportere og neuromodulatorreportere; eller

iii) et viralt protein; foretrukket hvor det virale protein enten er rabies G-proteinet eller et protein, der komplementerer funktionen af en anden virus end AAV'en eller et protein, der relaterer til cellulær og transcellulær transport.

5 **7. Viral partikel ifølge krav 3, hvor den kodende sekvens, der koder for payload, er et terapeutisk gen; foretrukket hvor det terapeutiske gen er til behandling af en neurodegenerativ lidelse; for eksempel hvor det terapeutiske gen er:**

- 10 i) HSP104 til behandling af Alzheimers sygdom eller andre sygdomme med toksiske proteinaggregater;
- ii) frataxin til behandling af Friedreichs ataksi;
- iii) lysosomal glucocerebrosidase (GBA) til behandling af Parkinsons sygdom;
- iv) polyQ-bindende protein til behandling af Huntingtons sygdom; eller
- 15 v) overlevelseshypokretin 1 til behandling af spinal muskelatrofi, amyotrofisk lateral sklerose (ALS), autisme, demens, perifer neuropati, skizofreni eller retinal degeneration.

8. Viral partikel ifølge krav 3, hvor payload er en terapeutisk del; foretrukket hvor den terapeutiske del er:

- 20 i) et antistof eller fragment deraf;
- ii) et immunmodulerende protein; eller
- iii) et RNA-interferensmolekyle.

25 **9. Viral partikel ifølge et hvilket som helst af kravene 2 til 8, hvor den retrograde adgang til cortico-pontine projektionsneuroner eller afferenter til dorsomedial striatum (DMS) er sammenlignelig med det syntetiske sporstof, Fluoro-Gold fluorescerende perler.**

30 **10. Variant adeno-associeret virus (AAV) omfattende en payload pakket deri, til anvendelse i en fremgangsmåde til behandling af en neurodegenerativ lidelse, fremgangsmåden omfatter at bringe en eller flere neuroner i kontakt med varianten AAV, hvor varianten AAV omfatter en capsidprotein omfattende en sekvens udvalgt blandt gruppen bestående af xxDxTKx (SEQ ID NO:1) og xDxTKxx (SEQ ID NO:2), hvor varianten AAV har en aminosyresekvens udvalgt blandt gruppen bestående af SEQ ID NO: 6, 8, 10, 12, 14, 18, 22, 26, 28, 30, 32, 34, 36, 38,**

35 **40, 42, 44, 46, 48, 50, 54, 56, 60, 64, 66, 68 70, 72, 74, 76 og 78.**

11. Variant adeno-associeret virus (AAV) til anvendelse ifølge krav 10, hvor neuronerne er projektionsneuroner.

12. Variant adeno-associeret virus (AAV) til anvendelse ifølge krav 10 eller krav 11, hvor neuronerne er i et individ; foretrukket hvor neuronerne er i centralnervesystemet (CNS) i et individ.

5 **13.** Variant adeno-associeret virus (AAV) til anvendelse ifølge et hvilket som helst af kravene 10 til 12, hvor individet er et menneske.

10 **14.** Variant adeno-associeret virus (AAV) til anvendelse ifølge krav 10 til 12, hvor individet er et ikke-menneske; helst hvor det ikke-menneskelige individ er udvalgt fra en primat, en gnaver, et krybdyr og en fugl.

15. Variant adeno-associeret virus (AAV) til anvendelse ifølge et hvilket som helst af kravene 10 til 14, hvor kontakttrinnet er in vivo via intrakraniell, intraspinal eller intramuskulær injektion.

15 **16.** Viralt capsidprotein ifølge krav 1, hvor det virale capsidprotein har en aminosyresekvens ifølge SEQ ID NO: 44.

17. Variant adeno-associeret virus (AAV) til anvendelse ifølge krav 10, hvor det virale capsidprotein har en aminosyresekvens af SEQ ID NO: 44.

DRAWINGS

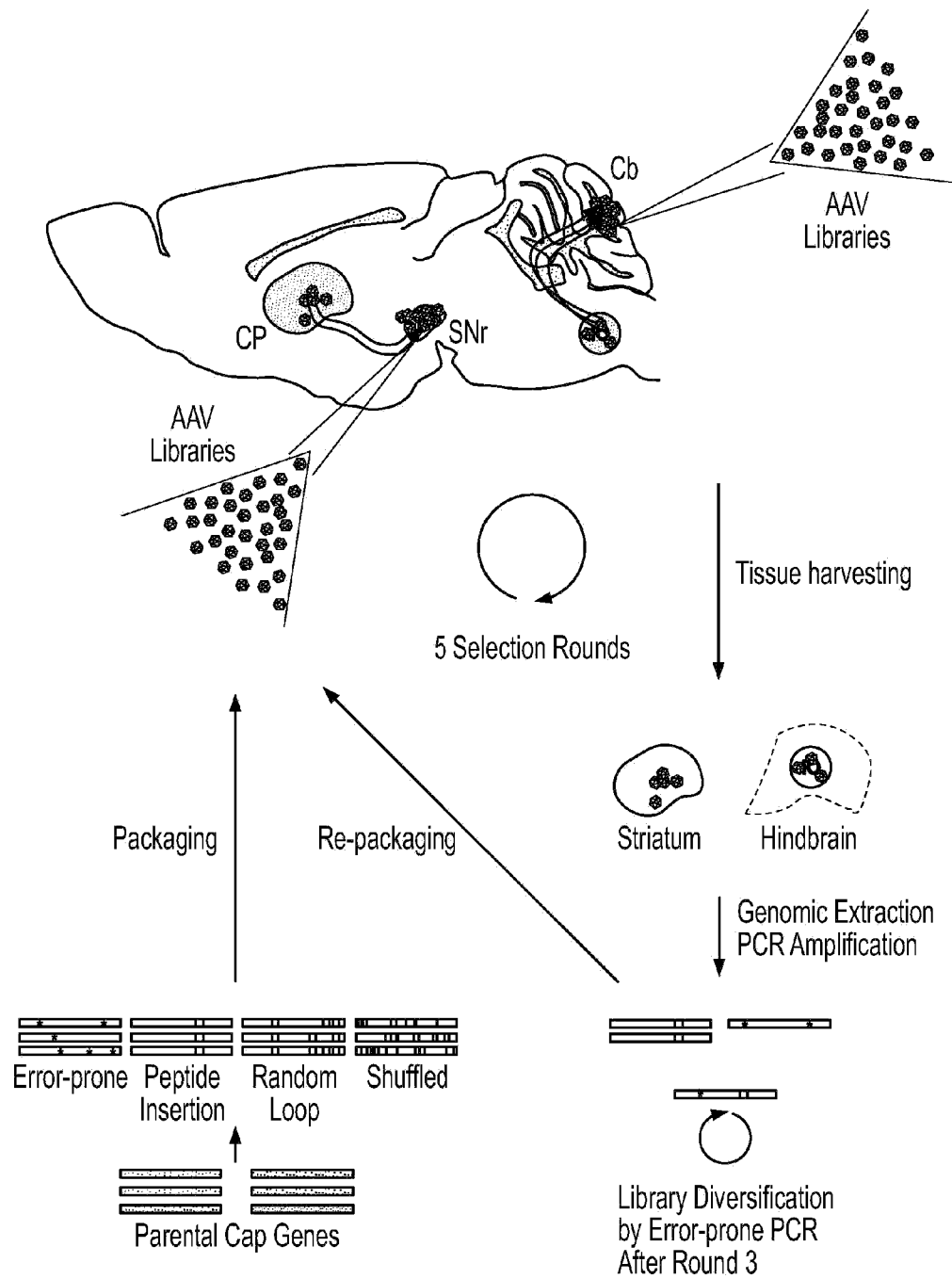


FIG. 1A

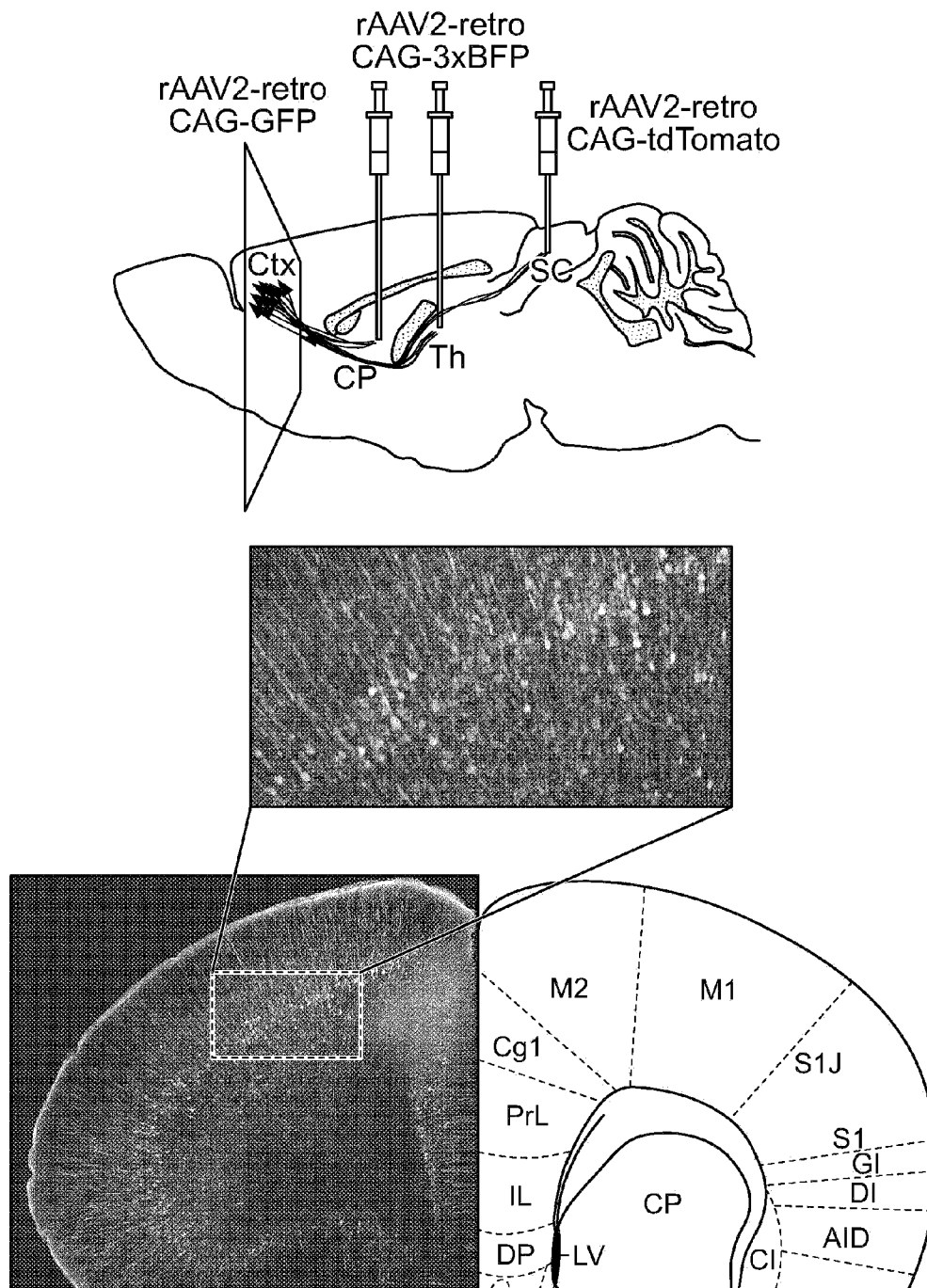


FIG. 1B

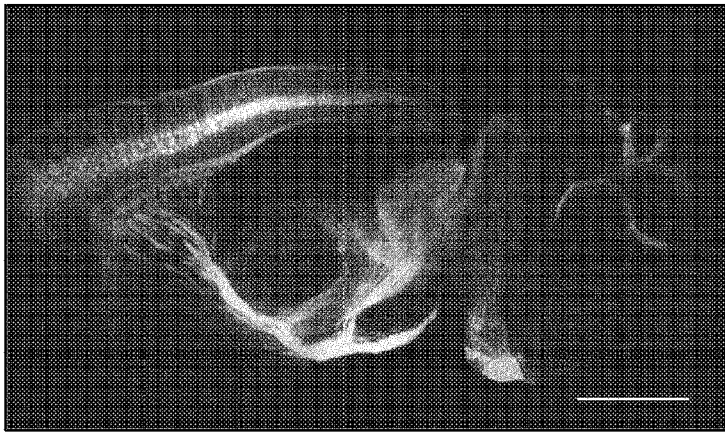
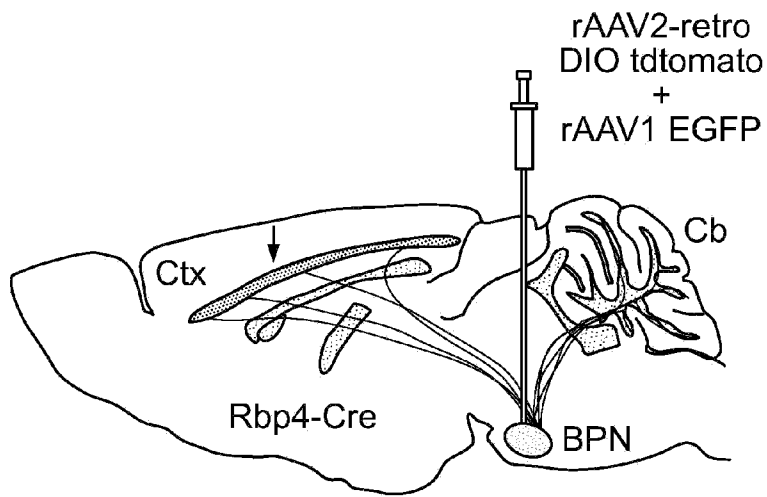


FIG. 2A

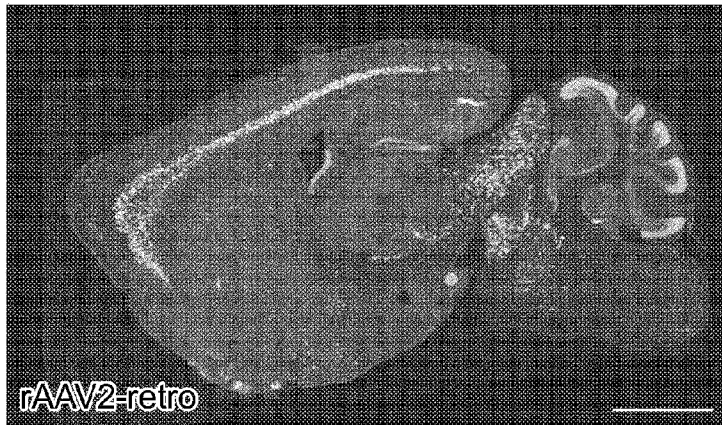
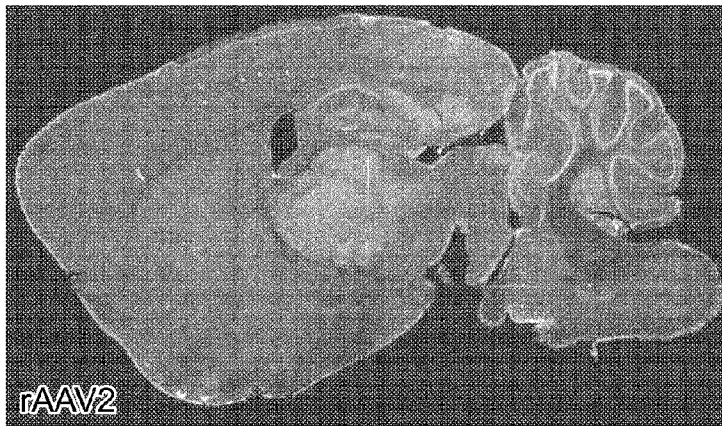
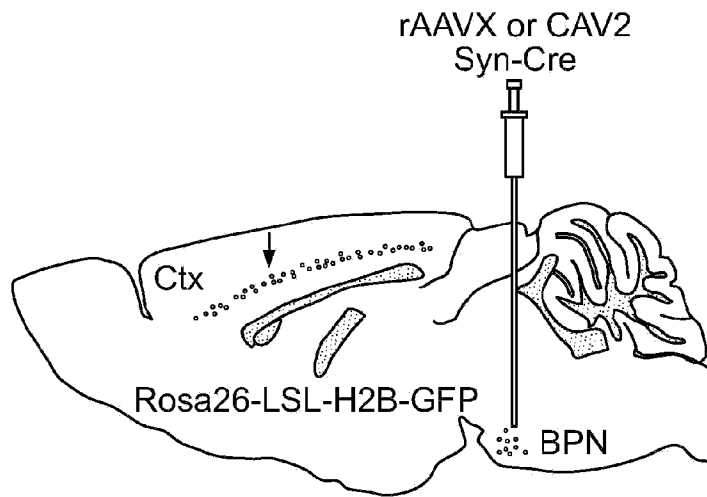


FIG. 2B

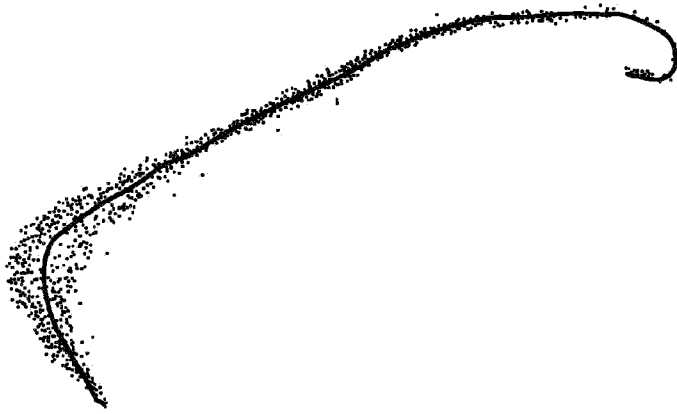


FIG. 2C

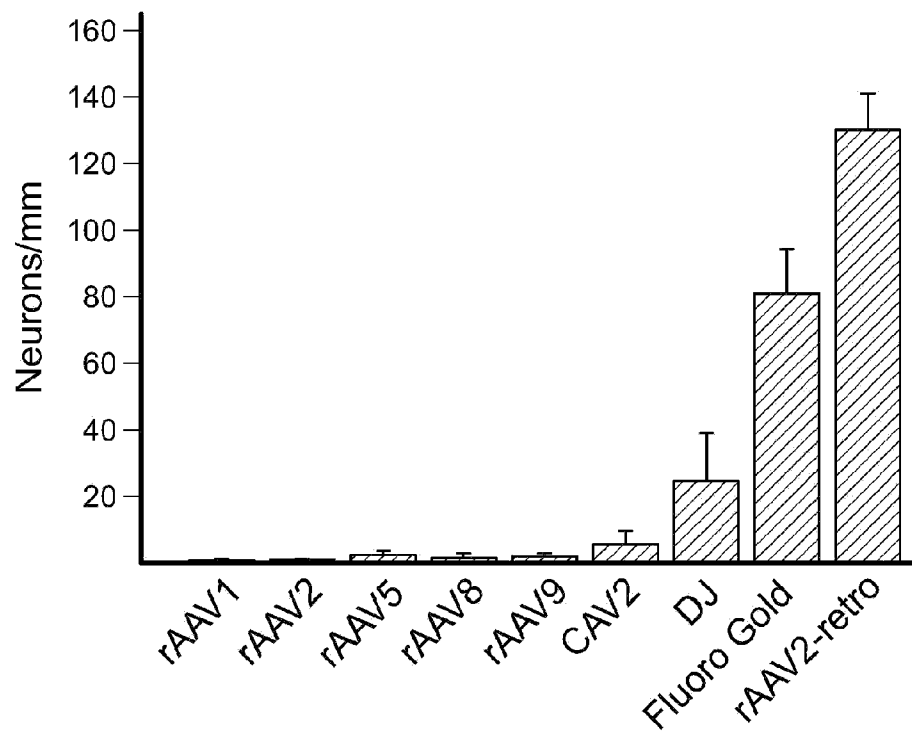


FIG. 2D

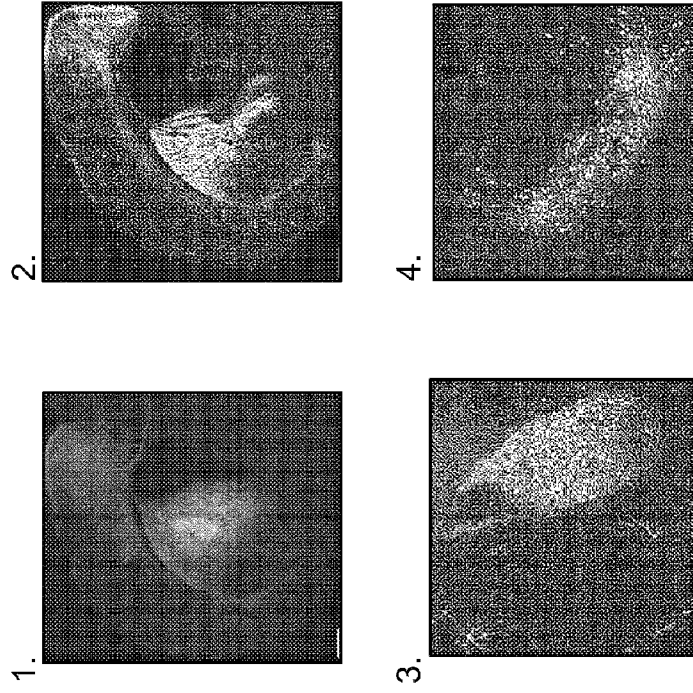
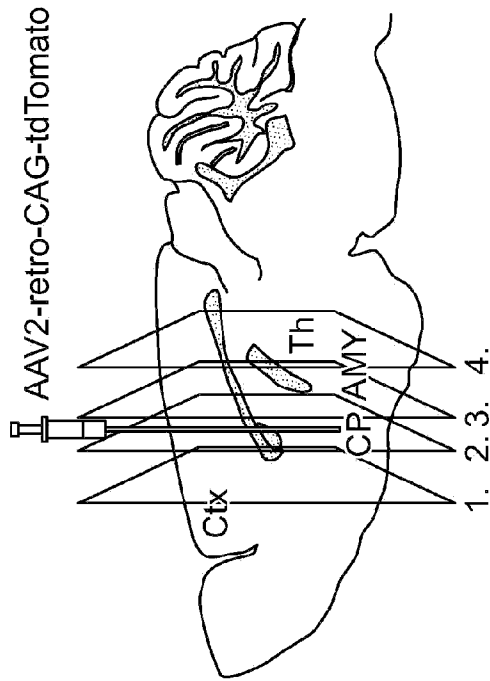


FIG. 3A



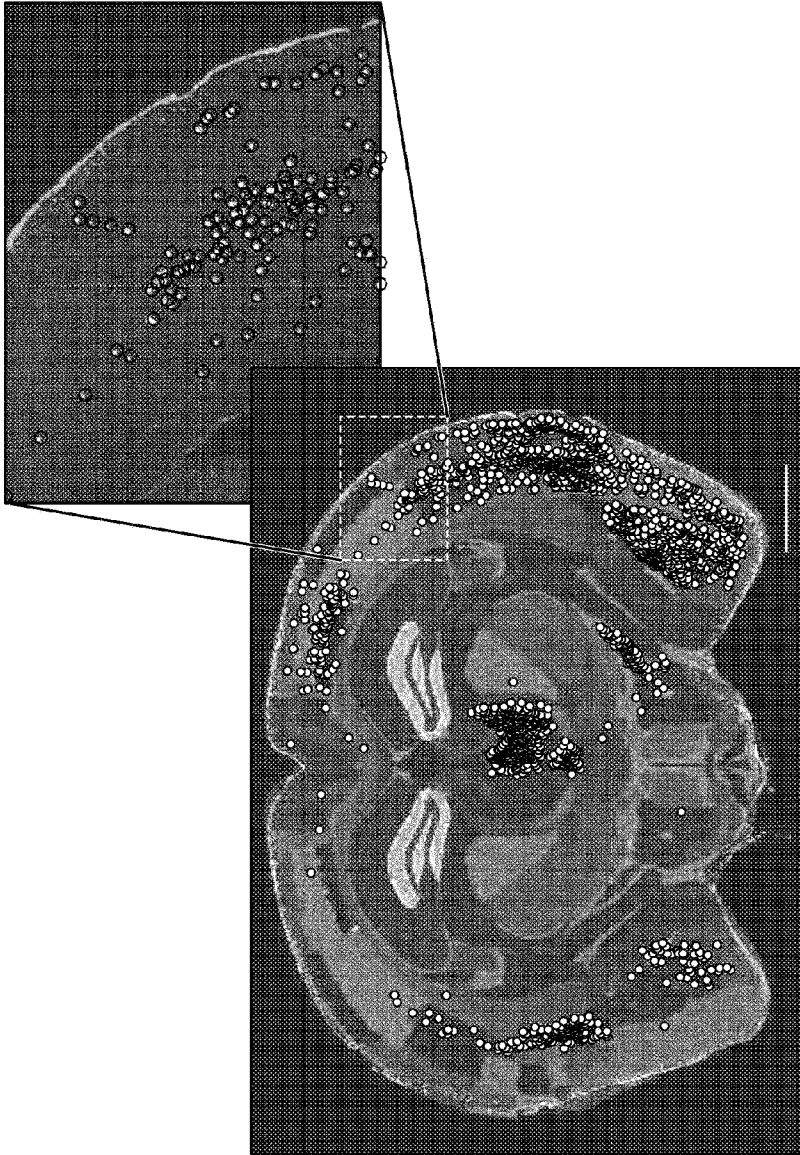


FIG. 3B

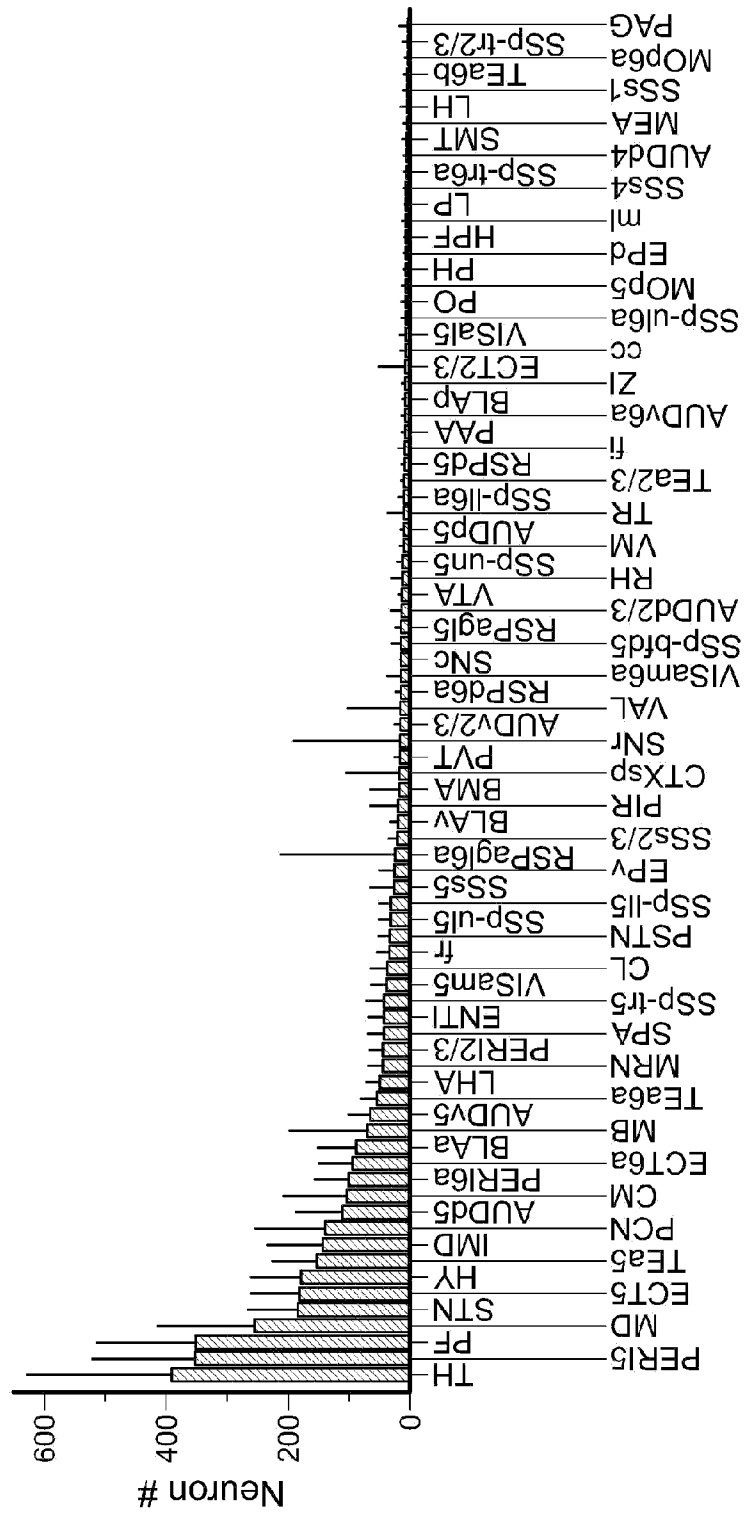


FIG. 3C

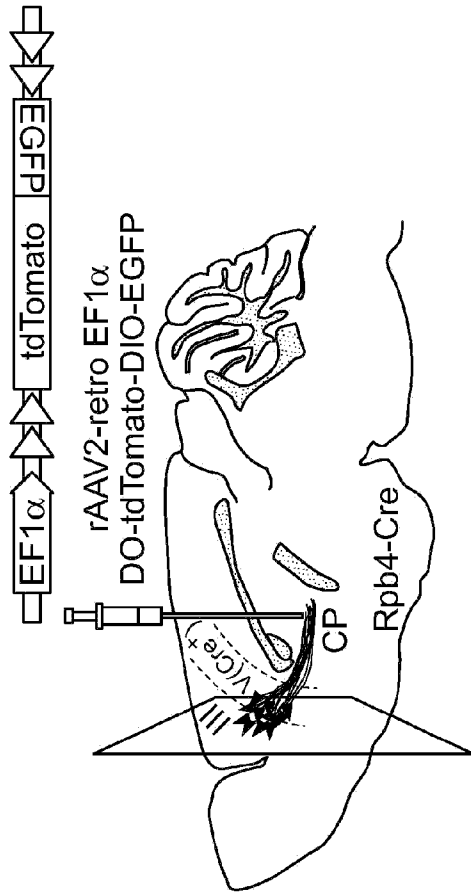


FIG. 4A

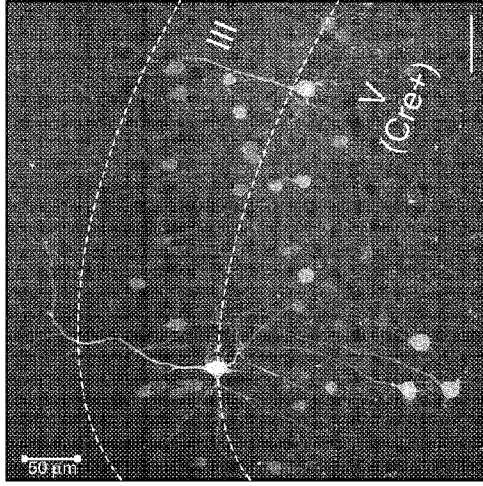


FIG. 4B

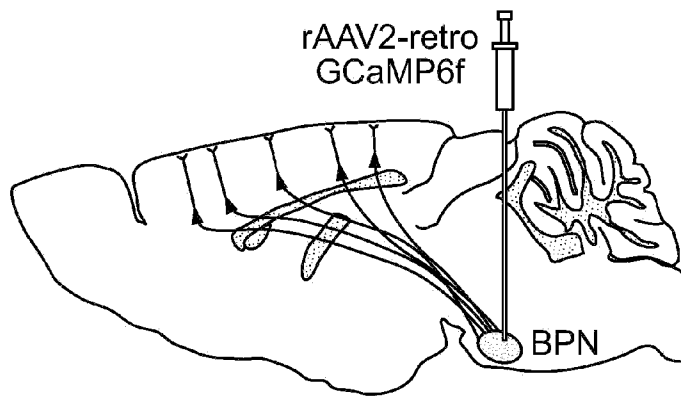


FIG. 5A

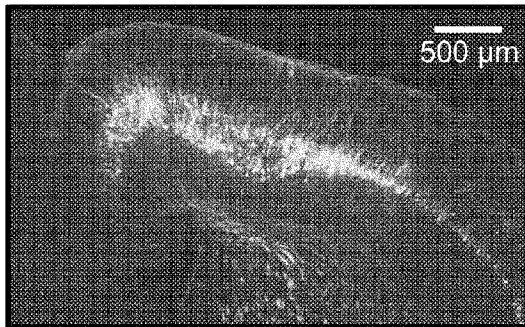


FIG. 5B

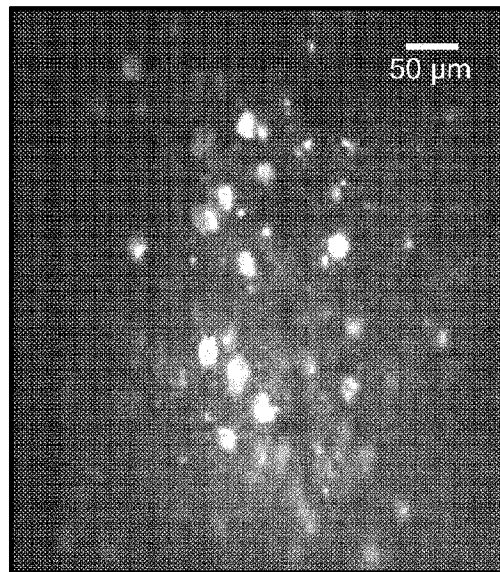


FIG. 5C

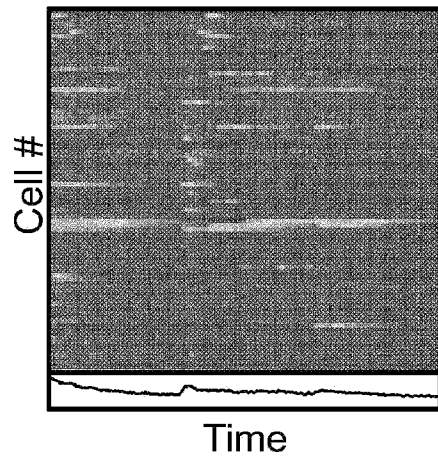


FIG. 5D

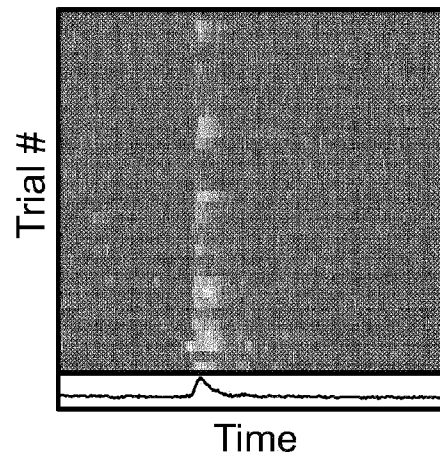


FIG. 5E

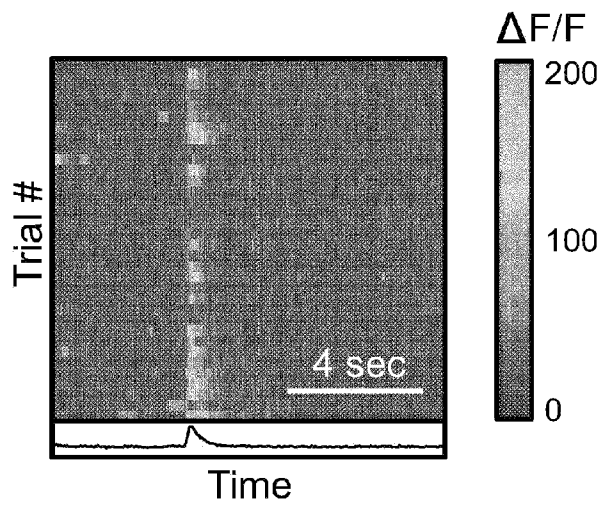


FIG. 5F

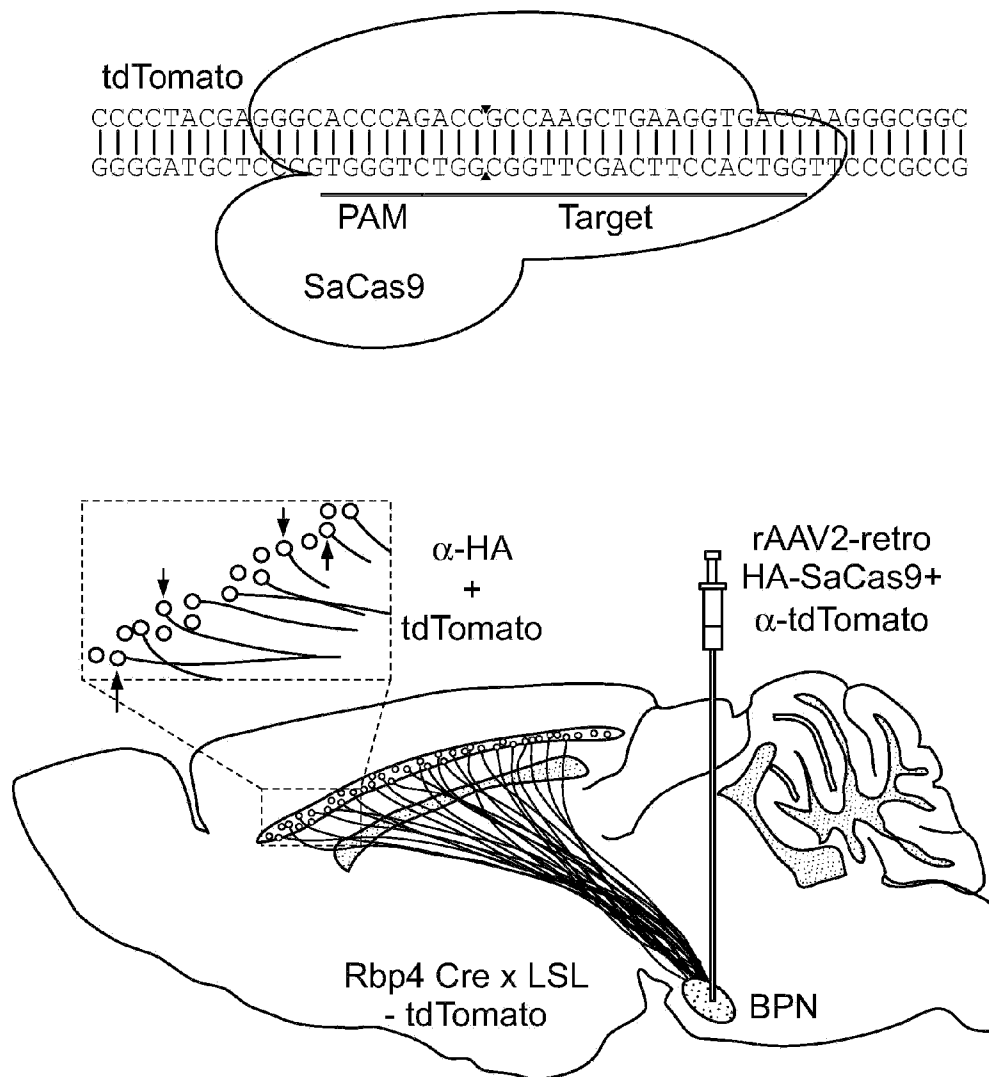


FIG. 6A

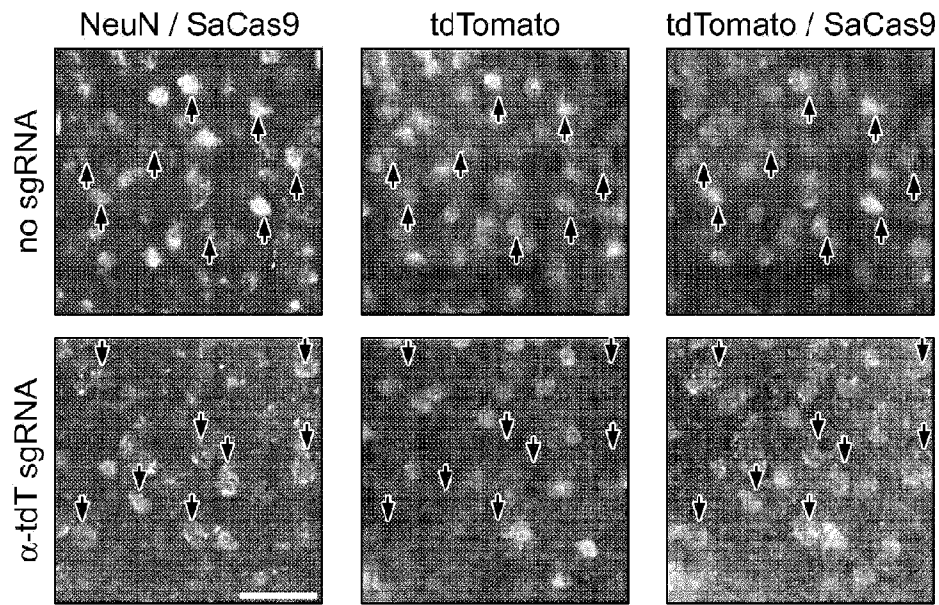


FIG. 6B

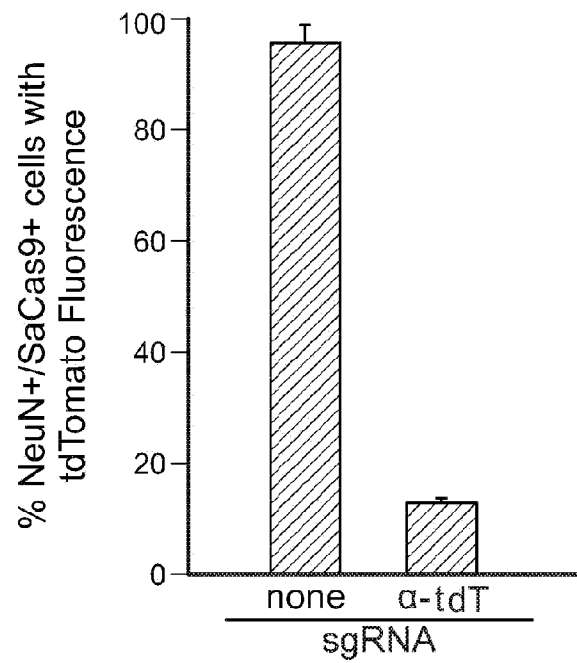


FIG. 6C

rAAV2-retro-CAG-GFP
rAAV2-retro-CAG-tdTomato

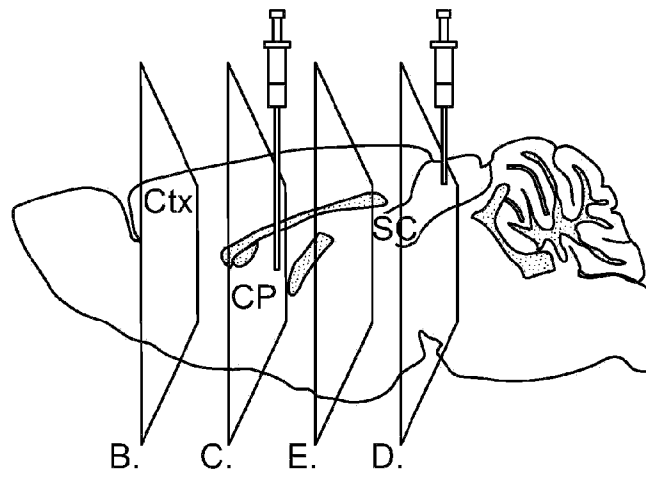


FIG. 7A

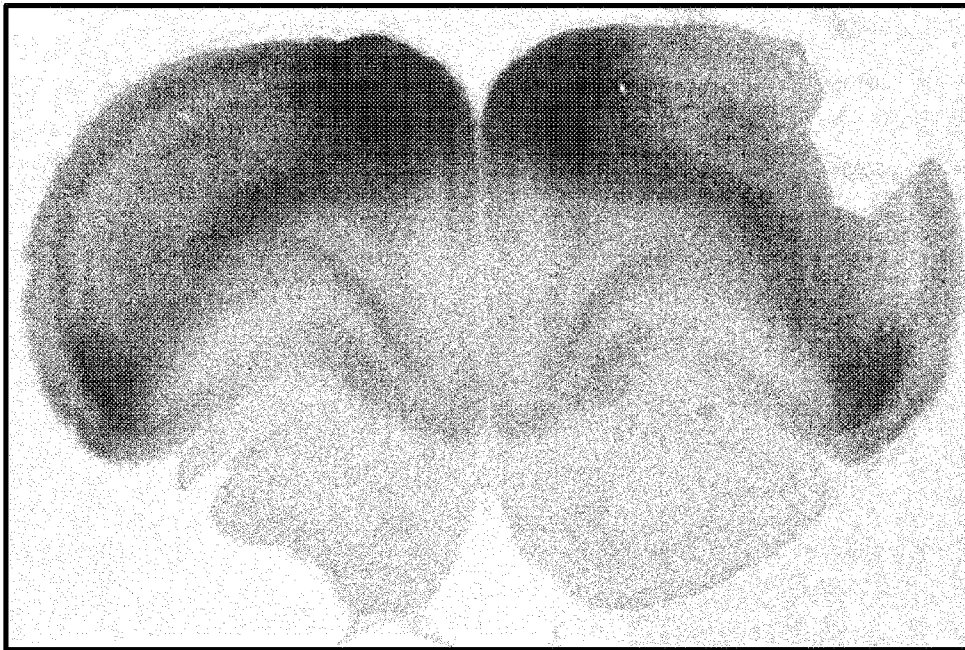


FIG. 7B



FIG. 7C

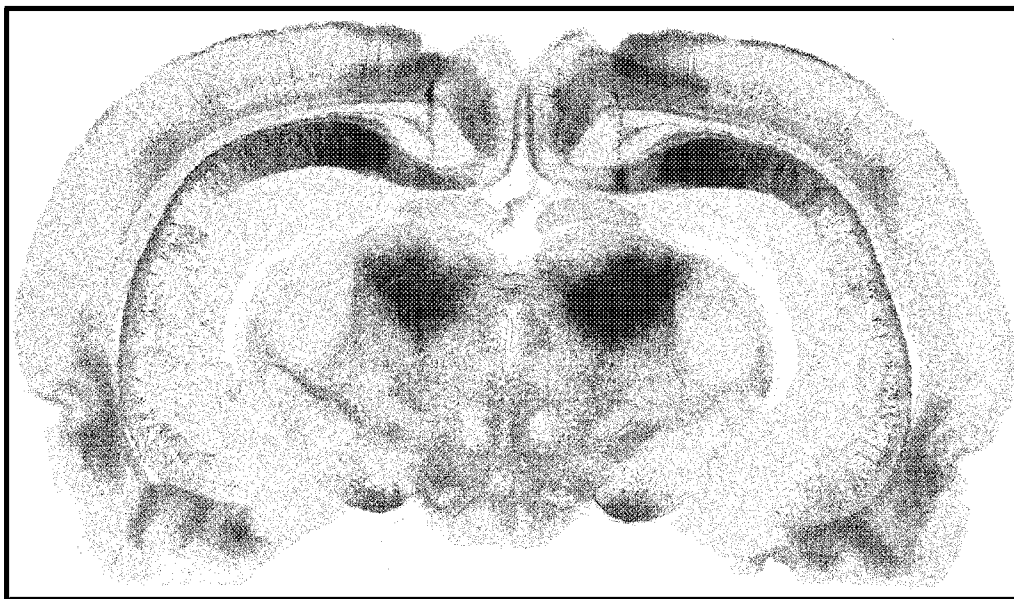


FIG. 7D

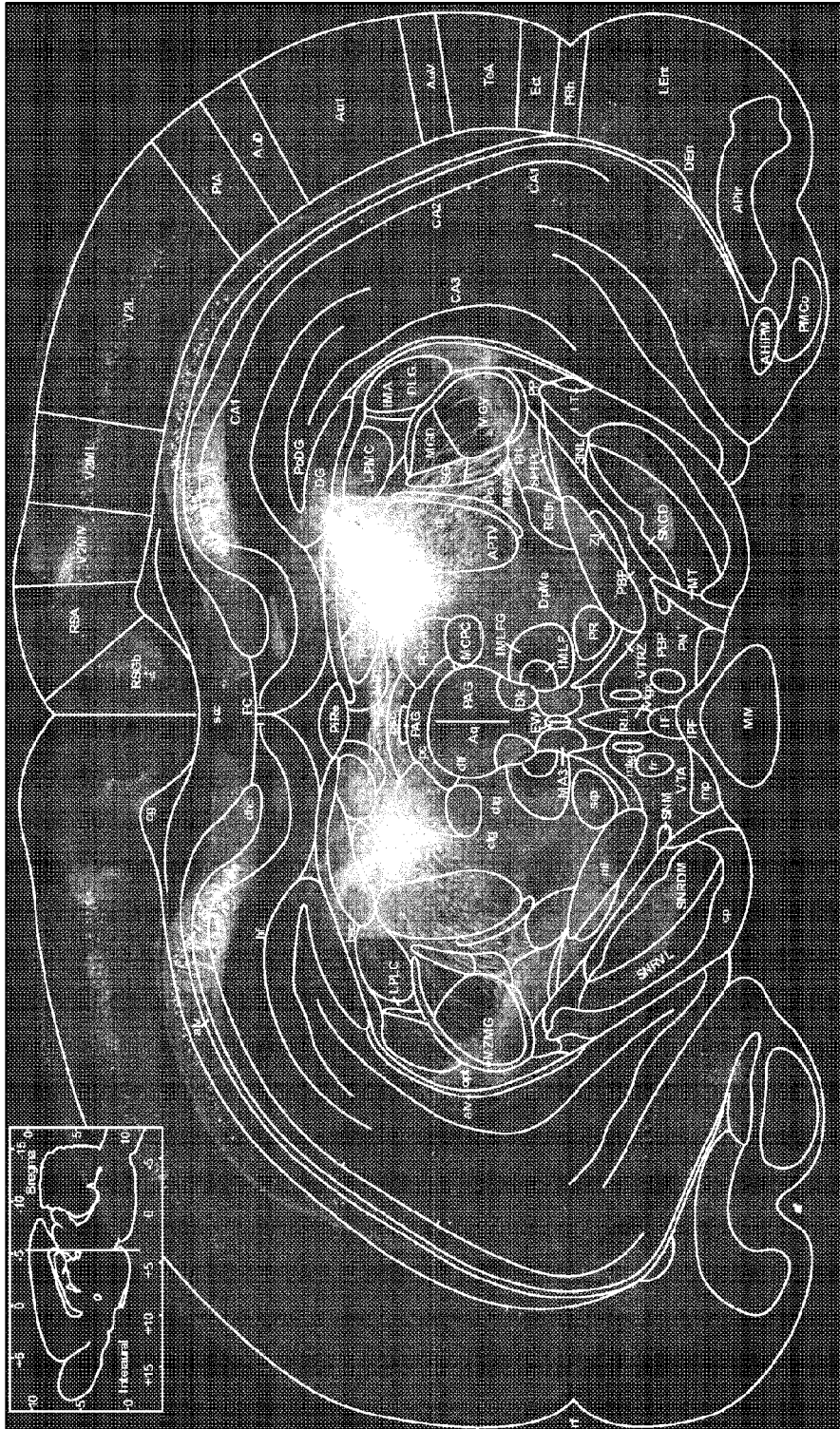


FIG. 7E

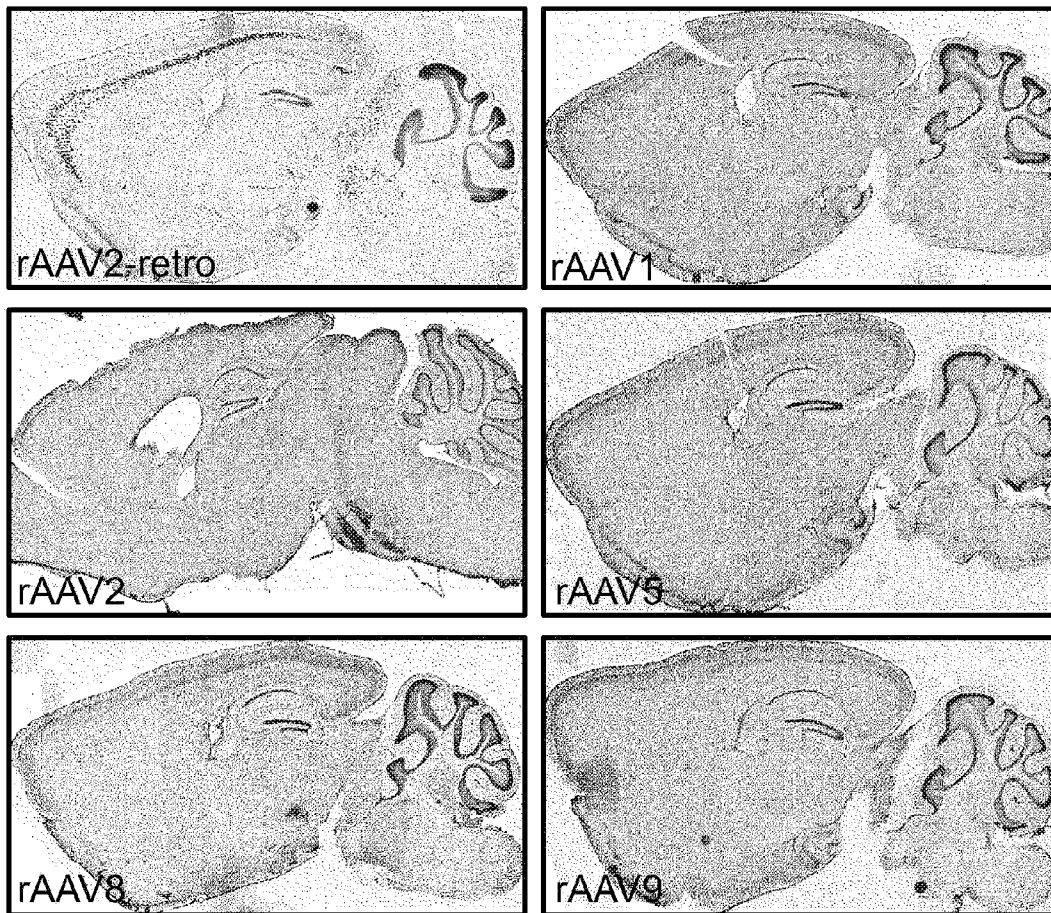


FIG. 8

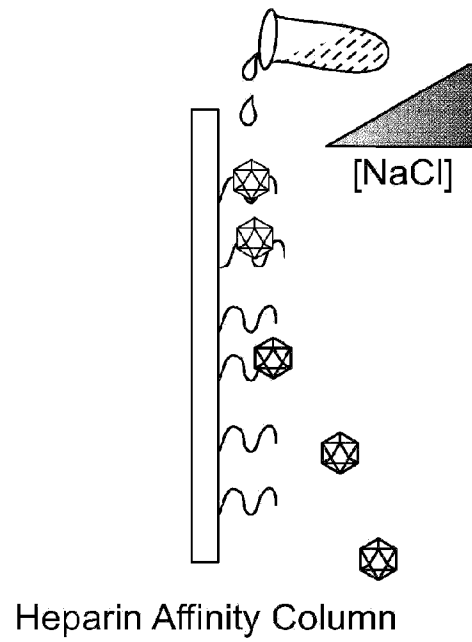


FIG. 9A

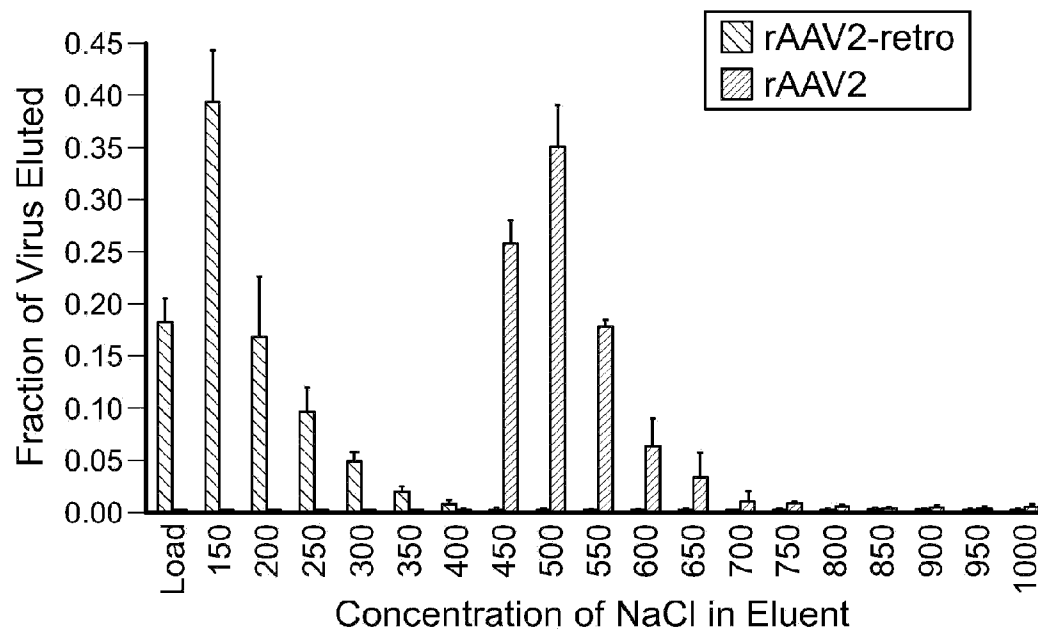


FIG. 9B

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.

