Direct and Retrograde Transduction of Nigral Neurons with AAV6, 8, and 9 and Intraneuronal Persistence of Viral Particles

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Abstract

Recombinant adeno-associated viral (AAV) vectors of serotypes 6, 8, and 9 were characterized as tools for gene delivery to dopaminergic neurons in the substantia nigra for future gene therapeutic applications in Parkinson's disease. While vectors of all three serotypes transduced nigral dopaminergic neurons with equal efficiency when directly injected to the substantia nigra, AAV6 was clearly superior to AAV8 and AAV9 for retrograde transduction of nigral neurons after striatal delivery. For sequential transduction of nigral dopaminergic neurons, the combination of AAV9 with AAV6 proved to be more powerful than AAV8 with AAV6 or repeated AAV6 administration. Surprisingly, single-stranded viral genomes persisted in nigral dopaminergic neurons within cell bodies and axon terminals in the striatum, and intact assembled AAV capsid was enriched in nuclei of nigral neurons, 4 weeks after virus injections to the substantia nigra. 6-Hydroxydopamine (6-OHDA)-induced degeneration of dopaminergic neurons in the substantia nigra reduced the number of viral genomes in the striatum, in line with viral genome persistence in axon terminals. However, 6-OHDA-induced axonal degeneration did not induce any transsynaptic spread of AAV infection in the striatum. Therefore, the potential presence of viral particles in axons may not represent an important safety issue for AAV gene therapy applications in neurodegenerative diseases.

Introduction

PARKINSON'S DISEASE (PD) IS CHARACTERIZED by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) resulting in severe motor impairment. To date, most gene therapeutic approaches to PD have involved AAV-mediated transgene delivery to the striatum, the target projection area of nigral dopaminergic neurons. Indeed, the striatum has been the prime site for vector delivery in PD therapies aiming at dopamine restoration through expression of dopamine-synthesizing enzymes in striatal neurons (During *et al.*, 1998; Wang *et al.*, 2002; Eberling *et al.*, 2008; Christine *et al.*, 2009; Jarraya *et al.*, 2009; Muramatsu *et al.*, 2010; Valles *et al.*, 2010), and also in gene therapeutic approaches involving neurotrophic factors such as glial cell-derived neurotrophic factor (GDNF) or neurturin (Kordower *et al.*, 2000; Eslamboli *et al.*, 2005; Eberling *et al.*, 2009; Marks *et al.*, 2010). The protective effect of neurotrophic factors released by transduced striatal neurons relies on their uptake by dopaminergic fibers in the striatum and retrograde axonal transport to the cell soma in the SNpc. In a recent double-blind phase 2 clinical trial for PD, AAV2-mediated intraputamin delivery of neurturin did not improve the

primary outcome measurement in virus-injected compared to sham-operated PD patients on the unified PD rating scale at 12 months but showed significant improvement in motor off scores at 18 months (Marks *et al.*, 2010). The authors of the study attributed the delay of the effect to the inefficiency with which neurturin became retrogradely transported from the striatum to the substantia nigra (SN). This finding contrasted with prior experience from studies with young, aged, and parkinsonian (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated) monkeys, who all displayed efficient retrograde neurturin transport to the SN (Bartus *et al.*, 2011b). Therefore, the axons of diseased neurons in advanced PD patients may transport neurotrophins poorly. Interestingly, AAV2 has recently been demonstrated to undergo primarily anterograde and not retrograde axonal transport in the basal ganglia (Ciesielska *et al.*, 2011). Nevertheless, even with the striato-nigral route of viral transport still intact in PD, potential neurturin release from either medium spiny neuron terminals or from anterogradely transduced cells in the SN pars reticulata did not prove very efficient in PD patients.

Overall, these observations highlight the importance of alternative modes of gene delivery for the nigrostriatal system. Along these lines, a recent study has already

demonstrated feasibility of AAV2-neurturin delivery to the SN without causing weight loss and has also demonstrated feasibility of nigral targeting in macaque monkeys through proper virus up-scaling (Bartus *et al.*, 2011a). Also, potential gene therapeutic approaches aimed at the degradation or modification of α -synuclein, reduction of oxidative or mitochondrial damage, or alteration of metabolic activity in nigral dopaminergic neurons require expression of therapeutic genes directly in neurons of the SNpc.

We therefore compared three different AAV serotypes for their capacity to transduce nigral dopaminergic neurons. The tropism of AAV vectors is mainly determined by viral capsid pseudotyping. AAV2 is currently the most frequently used serotype in gene therapy for the central nervous system (Christine *et al.*, 2009; Marks *et al.*, 2010; Diaz-Nido, 2010; Fitzsimons *et al.*, 2010; Muramatsu *et al.*, 2010; Valles *et al.*, 2010), a major drawback being the widespread pre-existing immunity in the human population (72%) as a result of natural exposure to wild-type AAV2 (Boutin *et al.*, 2010). Although the brain is generally regarded as an immune-privileged site, peripheral pre-immunization with wild-type AAV2 completely prevented striatal neuron transduction with AAV2 but not AAV5 (Peden *et al.*, 2004). Similarly, high titers of neutralizing antibodies reduced transgene expression in rats after striatal AAV injection (Sanftner *et al.*, 2004). AAV binding to heparin has been postulated as a prerequisite for dendritic cell infection and development of persisting immunity (Vandenberghe *et al.*, 2006; Lu and Song, 2009). We therefore focused our study on two non-heparin-binding serotypes, AAV8 (Gao *et al.*, 2002) and AAV9 (Gao *et al.*, 2004). Antibodies recognizing AAV8 and 9 were found to be less prevalent in the human population than antibodies to AAV1 or 2, and more importantly, seropositive individuals generally exhibited very low titers of neutralizing antibodies to AAV8 and AAV9 (Boutin *et al.*, 2010). Furthermore, transduction of dopaminergic neurons in the SNpc with AAV8 and AAV9 has been described to be more effective than with AAV2, with regard to both the percentage of transduced tyrosine hydroxylase (TH)-positive cells and the level of transgene expression (Klein *et al.*, 2008; McFarland *et al.*, 2009). We further included AAV6 (Rutledge *et al.*, 1998) in our study, which has previously been reported to have retrograde transduction properties (Towne *et al.* 2010, 2011; Salegio *et al.*, 2013). AAV6 could therefore be used for simultaneous expression of neurotrophic factors in the striatum and substantia nigra after striatal injections, provided that in the future diagnosis of PD can be accomplished early enough to allow retrograde transport of AAV6 in the nigrostriatal projections.

In the present study, we quantified the efficiency of AAV serotypes 6, 8, and 9 to transduce nigral dopaminergic neurons via either direct nigral injection or striatal administration relying on retrograde transport to the SNpc. We also compared the sequential transduction of nigral neurons with serotype combinations. We further evaluated two safety aspects of viral gene delivery, viral persistence and potential transgene spread, in the context of neurodegeneration.

Material and Methods

Cloning of viral transfer plasmids

Vector pAAV-MCS (Agilent Technologies, Inc., Santa Clara, CA), containing the AAV2 inverted terminal repeats, a

CMV promoter, a β -globin intron, a multiple cloning site, and the human growth hormone poly adenylation sequence (hGH polyA) was used for reporter gene transfer. The coding sequences for enhanced green fluorescent protein (EGFP) and DsRed2 were retrieved from plasmids pEGFP-N1 (Clontech Laboratories, Inc., Mountain View, CA) and pDsRed2 (Clontech Laboratories, Inc.), respectively, and were inserted into the pAAV-MCS multiple cloning site via standard procedures. The fusion protein between the nuclear localization signal (NLS) of the Simian Virus 40 Large T antigen and the fluorescent reporter EGFP was produced by polymerase chain reaction (PCR), using the plasmid pEGFP-N1 as a template and the primers 5'-GCT AGG GAA TTC GTC ATG GCT CCA AAA AAG AAG AGA AAG GTA ATG GTG AGC AAG GGC GAG GAG CTG-3' and 5'-GGT TAG AAG CTT TTA CTT GTA CAG CTC GTC CAT GCC-3'. The NLS EGFP PCR product was then inserted into the poly-linker of vector pAAV-MCS via *EcoRI* and *HindIII*. The three resulting reporter gene transfer vectors are hereafter referred to as pAAV CMV EGFP, pAAV CMV DsRed, and pAAV CMV NLS EGFP.

Purification and titration of recombinant AAV2/6, AAV2/8, and AAV2/9 vectors

For production of recombinant AAV, HEK 293 cells were seeded on a surface of 2500 cm² and were co-transfected with 500 μ g of one of the above transfer plasmids pAAV CMV EGFP, pAAV CMV DsRed, or pAAV CMV NLS EGFP in combination with 1 mg of helper plasmid, expressing the AAV serotype-specific cap genes, the AAV2 rep gene, and the adenoviral helper functions. For production of AAV2/6, the helper plasmid pDP6 was used (Grimm *et al.*, 2003). The helper plasmids pDP8rs.gck and pDP9rs.gck (kindly provided by Dr. J. Kleinschmidt, Deutsches Krebsforschungszentrum, Heidelberg, Germany) were generated by exchange of the cap gene in pDF5 (Grimm *et al.*, 2003) with the AAV8 or AAV9 cap genes (Gao *et al.*, 2002, 2004) and were used for production of AAV2/8 and AAV2/9 particles, respectively. Cell lysates of all serotypes were subjected to iodixanol gradient purification, which in the case of AAV6 was followed by affinity chromatography over HiTrap Heparin columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and in the cases of AAV8 and AAV9 by anion exchange chromatography over HiTrap Q-FF columns (GE Healthcare Bio-Sciences AB). Virus was concentrated in phosphate-buffered saline (PBS) with Centricon Plus-20 filtration devices (regenerated cellulose, 100,000 MWCO, Millipore, Billerica, MA) to a final volume of approximately 100 μ L, resulting in titers of $(1-5) \times 10^{13}$ vector genomes (vg)/mL. Purity of the virus preparations was verified in Coomassie gels.

Vector genome (vg) titers of AAV6, AAV8, and AAV9 preparations were determined by real-time PCR (qPCR) using the Rotor-GeneTM Probe PCR kit (Qiagen Inc., Valencia, CA). Virus concentrate was diluted 10^4 - to 10^5 -fold, and 3 μ L of diluted virus was mixed in a 24- μ L reaction with Rotor-Gene master mix containing HotStarTaq Plus DNA Polymerase and with a primer and probe set annealing to the β -globin intron (0.4 μ M forward primer [5'-CGTGCCAAGAGTGACGTAAG-3'], 0.4 μ M reverse primer [5'-TGGTGCAAAGAGGCATGTA-3'], 0.2 μ M TaqMan probe [5'-FAM-TTGCCCTGAAAGAAAGAGATTAGGGAA-BHQ-1-3']). Samples were run in a

Corbett Rotor-Gene RG-3000 cycler (Qiagen GmbH, Hilden, Germany) using the following program: one cycle of 20 min at 95°C for capsid denaturation, followed by 40 cycles of 3 sec at 95°C and 10 sec at 60°C. A qPCR standard curve for vg quantification was established in parallel with defined copy numbers of the pAAV-MCS plasmid, covering a range of 6×10^3 to 6×10^7 single-stranded plasmid templates/reaction. Acquired data were analyzed with Rotor-Gene 6.1.90 software (Qiagen GmbH), applying slope correction and dynamic tube normalization and setting the threshold for cycle measurements to 0.01.

Stereotaxic injection of AAV vectors to the rat SN and striatum

Adult female Sprague-Dawley rats (Charles River Laboratories, L'Arbresle Cedex, France) weighing approximately 230 g were housed on a 12 hr light/dark cycle, with *ad libitum* access to food and water, in accordance with Swiss legislation and the European Community Council directive (86/609/EEC) for the care and use of laboratory animals. For stereotaxic injections, animals were anesthetized with a mixture of ketamine (75 mg/kg, intraperitoneal [i.p.]) and xylazine (10 mg/kg i.p.), placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and virus infusions were performed with a 34-gauge needle hooked up to a 10 μ L Hamilton syringe that was driven by an automatic pump (CMA Microdialysis AB, Kista, Sweden). For unilateral AAV delivery to the right SN, 1.9×10^{10} vg were injected in a volume of 2 μ L at a flow rate of 0.2 μ L/min to a single site at coordinates anterior-posterior (AP), -5.2 mm; medio-lateral (ML), -2.0 mm; and dorso-ventral (DV), -7.8 mm (relative to the bregma).

For comparison of retrograde transport properties of AAV, 8.2×10^{10} vg was injected at a flow-rate of 0.2 μ L/min to two sites in the right striatum in a volume of 2.5 μ L each at coordinates: AP, +0.5 mm; ML, -3.0 mm; DV, -6.0 mm; and AP, +0.5 mm; ML, -3.0 mm; DV, -4.5 mm.

The same two sites within the right striatum were targeted for AAV6 injections in the combined nigral and striatal delivery paradigm, and a total of 4.0×10^{10} vg was administered per striatal hemisphere. The needle was left in place for 5 min after completion of viral infusions and was slowly retracted thereafter.

Stereotaxic injection of 6-hydroxydopamine to the medial forebrain bundle

6-Hydroxydopamine (6-OHDA) was diluted in a solution of 0.4% ascorbic acid and 0.9% NaCl to a final concentration of 5 μ g/ μ L. To induce dopaminergic neuron degeneration in the previously AAV-injected SN, 6-OHDA was delivered to the ipsilateral medial forebrain bundle: using a 34-gauge needle connected to a Hamilton syringe and an automatic pump, 4 μ L of the 6-OHDA solution was injected at a flow rate of 1 μ L/min to a single site at coordinates AP, -1.9 mm; ML, -1.9 mm (relative to the bregma); and DV, -7.2 mm.

Tissue processing and immunohistochemistry

Rats were deeply anesthetized with an overdose of pentobarbital and perfused transcardially with 120 mM phosphate buffer (pH 7.4), followed by 4% ice-cold parafor-

maldehyde (PFA) in 120 mM phosphate buffer (pH 7.4). Brains were removed and postfixed for an additional 90 min in 4% PFA solution and then transferred to 25% sucrose in PBS (pH 7.4). Sections were cut with a sliding microtome (SM2400; Leica, Nussloch, Germany) in the coronal plane at a thickness of 25 μ m. Immunohistochemistry was performed on free-floating sections. The following primary antibodies were used in this study: anti-TH (rabbit IgG, 1:500 in fluorescence, 1:800 in light level immunohistochemistry; AB152, Millipore AG, Zug, Switzerland), anti-GFP (mouse IgG, 1:500; A-11120, Molecular Probes, Invitrogen AG, Basel, Switzerland), anti-GFP (rabbit IgG fraction, 1:1000; A-11122, Molecular Probes, Invitrogen AG), anti-Olig-2 (oligodendrocyte transcription factor 2; purified rabbit Ig, 1:500; AB9610 Millipore AG), anti-NeuN (neuronal nuclei, clone A60, mouse IgG1, 1:500; MAB377 Millipore AG), anti-ionized calcium binding adaptor molecule1 (Iba-1, purified rabbit Ig, 1:500; 019-19741, Wako Chemicals GmbH, Neuss, Germany), anti-glial fibrillary acidic protein (GFAP, purified Ig fraction from rabbit serum; 1:1000; Z0334, Dako Schweiz, AG, Baar, Switzerland), anti-AAV1-intact particle (clone ADK1a, mouse IgG, 1:20; BM5093, Acris Antibodies GmbH, Herford, Germany).

For immunofluorescence labeling, sections were washed three times in PBS, blocked with 10% normal goat or donkey serum in PBS, 0.1% Triton X-100 for 1 hr at room temperature. Sections were then incubated overnight at 4°C with the primary antibody diluted in blocking buffer, washed three times with PBS, and then incubated for 2 hr at room temperature with Alexa Fluor-488 (Molecular Probes, Invitrogen AG) or Cy3 (Jackson ImmunoResearch, Suffolk, UK) conjugated secondary antibodies diluted 1:500 and 1:1000 in PBS, respectively. Following additional washes in PBS, sections were mounted to glass slides and coverslipped with MO-WIOL mounting solution.

For light level immunohistochemistry, endogenous peroxidase activity was quenched with 0.1% phenylhydrazine in PBS for 1 hr at 37°C, sections were then washed three times with PBS, blocked, and incubated with primary antibody overnight at 4°C as already described. Sections were subsequently incubated for 2 hr at room temperature with biotinylated goat anti-rabbit or horse anti-mouse (rat preadsorbed) antibodies (IgG (H+L), 1:200, Vector Laboratories Inc., Burlingame, CA) followed by exposure to avidin-biotin-peroxidase complex for 45 min at room temperature and development of the signal in a 3,3'-diaminobenzidine reaction. Sections were mounted on glass slides, dehydrated, and coverslipped with Eukitt.

For Olig-2 labeling, tissue sections were incubated for 10 min in antigen retrieval buffer (20 mM Tris HCl [pH 9], 136 mM NaCl) at 95°C and then washed three times in PBS prior to blocking and antibody administration.

Quantification of transduced nigral dopaminergic neurons

Rats that had been injected with equal genome copy numbers of AAV CMV EGFP to the SN (1.9×10^{10} vg) or the striatum (8.2×10^{10} vg) were sacrificed 4 weeks after viral delivery. A series of every 12th section through the SN (seven sections/animal) was colabeled with anti-GFP and anti-TH antibodies in combination with Alexa Fluor-488- and Cy3-coupled secondary antibodies, respectively. To

determine the percentage of EGFP-positive dopaminergic neurons in the SN after AAV injection, 8-bit images were separately acquired in the 488- and 594-nm channels with a 10× objective on a Leica DM5500 microscope, using a motorized stage to assemble individual tiles to a mosaic covering the entire structure of the SN. Metamorph 7.5 software (Molecular Devices, Downingtown, PA) was then used to mark individual TH-positive, nigral dopaminergic neurons in the 594-nm channel and to then activate the 488-nm channel in merged images to identify EGFP-expressing neurons among these prelabeled, TH-positive neurons.

For analysis of the retrograde AAV transport capacity after vector injection to the striatum, the total number of EGFP-positive, TH-expressing neurons was determined in a series of every 12th section through the SN.

Sequential transduction of individual neurons in the SN was evaluated 8 weeks after injection of AAV vectors to the SN (1.9×10^{10} vg) and 4 weeks after AAV injection to the striatum (4.0×10^{10} vg). Images of a series of every 12th section through the SN were acquired in the 488- and the 594-nm channels with a 10× objective on an Olympus AX70 microscope (Olympus Schweiz AG, Volketswil, Switzerland) and overlaid with Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA). The total number of retrogradely transduced, DsRed-positive neurons in the SN was determined. We further analyzed whether RFP-positive nigral neurons expressed EGFP at the same time.

Detection of intact AAV6 capsid in infected HEK293 cells

HEK293T cells were seeded at a density of 25,000 cells/cm² on Poly-D Lysine (100 µg/mL) and laminin (20 µg/mL) coated Lab-Tek® Permanox™ Chamber Slides (Nalge Nunc International, Rochester, NY). The following day, cells were infected with 8.9×10^7 transducing units (TU) of AAV6 CMV EGFP in 0.8-cm² wells, corresponding to a multiplicity of infection of approximately $(1-2) \times 10^3$. Forty-eight hours after virus infection, cells were fixed with 4% PFA for 20 min, rinsed with PBS three times and blocked for 1 hr at room temperature with 10% normal goat serum and 0.25% Triton X-100 in PBS. Cells were then incubated for 1.5 hr at room temperature with ADK1a antibody diluted 1:50 in blocking buffer. Following PBS washes, slides were exposed to Alexa Fluor-594 coupled goat anti-mouse antibody diluted 200-fold in 1% normal goat serum and 0.25% Triton X-100 in PBS, washed, and embedded in Mowiol.

Detection of single-stranded viral DNA by in situ hybridization

To detect minus strand recombinant AAV genomes in tissue sections by *in situ* hybridization, 4 weeks after AAV injection to the SN, a digoxigenin-labeled EGFP sense probe was produced. The EGFP coding sequence was excised from plasmid pEGFP-N1 (Clontech Laboratories, Inc.) and cloned into pBluescript II SK (Agilent Technologies, Inc.). One microgram of the resulting pBluescript II SK EGFP plasmid was linearized and used to synthesize the EGFP sense probe in a 20-µL *in vitro* transcription reaction with T7 RNA polymerase (DIG RNA Labeling KIT [Sp6/T7], Roche Diagnostics GmbH, Mannheim, Germany). Digoxigenin incorporation into the sense probe was verified in a dot blot, using the Dig

Nucleic Acid Detection Kit in combination with the Dig Wash and Block Buffer Set (Roche Diagnostics GmbH).

For *in situ* hybridization, PFA (4%)-fixed brain sections were subjected to a 90-min treatment with 40 µg/mL RNase A in 10 mM Tris (pH 7.6) and 0.5 mM EDTA at 37°C. *In situ* hybridization was carried out according to standard protocols in an automated Ventana Discovery® XT machine (F. Hoffmann La-Roche Ltd., Basel, Switzerland): Sections were postfixed with RiboPrep solution (Roche Diagnostics AG, Rotkreuz, Switzerland) for 4 min at room temperature, incubated with RiboClear solution (Roche Diagnostics) for 12 min at 37°C, and subsequently treated with ISH Protease 3 (0.02 units alkaline protease activity/mL, Roche Diagnostics) for 16 min at 37°C. The denatured digoxigenin-labeled probe was diluted 1500-fold in RiboHybe buffer (Roche Diagnostics) and slides were incubated for 6 hr at 60°C with 100 µL of diluted probe. After three washes in 2× SSC at 60°C for 8 min each, sections were postfixed with RiboFix solution (Roche Diagnostics). For detection of the hybridization signal, slides were incubated for 30 min with alkaline phosphatase coupled antidigoxigenin antibody, diluted 1:2000 in PBS, 1% bovine serum albumin (Roche Diagnostics). The signal was developed in a colorimetric reaction for 6 hr using NBT/BCIP (Roche Diagnostics) as a substrate.

Quantification of vg copies in the SN and striatum by qPCR

To quantify AAV infection of the nigrostriatal projection, rats were bilaterally injected in the SN with 1.9×10^{10} vg of AAV6, AAV8, or AAV9 CMV EGFP. Noninjected rats served as negative controls. Animals were sacrificed 4 weeks after virus delivery. A short transcatheter perfusion with ice-cold phosphate buffer was performed to remove blood from the circulation, and SN and striatum were immediately dissected and collected separately for both hemispheres. Tissue samples were frozen on dry ice and stored at -80°C until further processing.

Total DNA was extracted from tissue using the NucleoSpin® Genomic DNA from Tissue kit (Macherey-Nagel AG, Oensingen, Switzerland) according to the manufacturer's instructions. Briefly, tissue was digested with proteinase K (2 mg/mL) for 75 min at 56°C and RNase A was added to a final concentration of 2 mg/mL to degrade RNA during a 5-min incubation step at room temperature. After tissue lysis, DNA was purified over NucleoSpin Tissue columns and eluted with 100 µL 5 mM Tris HCl (pH 8.5). This first DNA extract contained rat genomic DNA as well as single- and double-stranded viral DNA. Half of the eluate was further subjected to S1 nuclease digest (0.03 U/µL, Promega, Madison, WI) in 50 mM sodium acetate (pH 4.5), 280 mM NaCl, and 4.5 mM ZnSO₄ for 30 min at 37°C to remove single-stranded viral DNA. S1 nuclease was subsequently inactivated at 90°C for 15 min. DNA was purified a second time over NucleoSpin Tissue columns and eluted with 100 µL 5 mM Tris HCl (pH 8.5).

The number of vg templates was determined by qPCR in RNA-free DNA extracts of SN and striatum before (total viral DNA) and after S1 nuclease treatment (double-stranded viral DNA only) and was normalized to the number of host cells contained within the sample. As already described for the titration of recombinant viruses, a primer and probe

(TaqMan) set annealing to the human β -globin intron within the recombinant viral DNA was used for quantification of vg. To determine the number of host cells within the sample, a second qPCR amplification was performed using a primer and probe set annealing to the 3'-UTR of rat β -actin within genomic DNA (two alleles per cell, RNA digested beforehand, see prior description) and giving rise to a 70-bp PCR product (forward primer 5'-GCGCTTTTGACTCAAGGATTAA-3' [0.8 μ M], reverse primer 5'-GGGATGTTTGCTCAACCAA-3' [0.8 μ M], TaqMan probe 5'-FAM-CGGTCGCTTCACCGTTCCAGTT-TAMRA-3' [0.2 μ M]). All qPCR amplifications were set up with the Rotor-Gene Probe PCR kit (Qiagen Inc.) in a volume of 24 μ L and contained 3 μ L of nigral or striatal DNA extracts and the above primer and probe sets at the indicated concentrations. Samples were run in a Corbett Rotor-Gene RG-3000 cycler (Qiagen GmbH) using the following program: 1 cycle at 95°C for 180 sec, followed by 40 cycles at 95°C for 3 sec and 60°C for 10 sec. Acquired data were analyzed with Rotor-Gene 6.1.90 software (Qiagen GmbH) as previously described. The number of vg and rat β -actin copies per reaction were determined by means of standard curves established in parallel with defined numbers of pAAV-MCS plasmid, and rat genomes and thus β -actin alleles, respectively.

Viral genome copies were also quantified by qPCR in the striatum of rats, 10 weeks after unilateral AAV6 CMV NLS EGFP injections to the SN and 6 weeks after 6-OHDA administration to the ipsilateral medial forebrain bundle in a subgroup of animals. For simultaneous quantification of the vg in the striatum and evaluation of dopaminergic neuron degeneration in the SN, DNA extractions as well as immunohistochemistry were performed on every 12th PFA-fixed tissue section. For qPCR, striata were dissected from 10 sections per animal, and pooled for the right (injected) and left (noninjected) hemisphere separately. Total DNA (genomic and viral) was extracted from pooled striatal sections using the QIAamp[®] DNA FFPE Tissue kit (Qiagen Inc.) according to the manufacturer's instructions. Briefly, tissue sections were digested with proteinase K (2 mg/mL) in tissue lysis buffer for 1 hr at 56°C, followed by a 1-hr incubation step at 90°C to partially revert formaldehyde modifications of DNA. After RNA degradation with 1 mg/mL RNase A for 2 min at room temperature, DNA was purified over QIAamp MinElute columns and eluted in a volume of 20 μ L (Qiagen Inc.). Three microliters of the fixed tissue DNA eluate were used in each 24 μ L of the qPCR amplification to quantify the vg copy number per cell as described above for DNA extracts from fresh frozen tissue.

DNase I protection assay to detect encapsidated AAV genomes in vivo

The percentage of encapsidated and thus DNase I-resistant vg was determined in AAV-injected SN by comparison of vg numbers in nigral tissue samples before and after DNase I treatment. Rats received intranigral injections with 1.8×10^7 TU of AAV6 and were sacrificed 8 weeks later. After a brief transcardial perfusion with ice-cold PBS, nigral tissue was excised and frozen at -80°C until further processing. Each SN was minced in 50 μ L 10 mM Tris HCl, pH 8, with a minimortar. As control, noninjected SNs were prepared in the same way and mixed with 1.8×10^7 TU of intact AAV6 from

the same viral preparation used for intranigral injections, to verify protection of encapsidated vg from DNase I degradation. To establish the efficiency of naked AAV genome degradation by DNase I, suspensions of noninjected SNs were mixed with 1.8×10^7 TU of AAV6 after destruction of viral capsids by a 20-min heat denaturation at 95°C. Samples from all three groups were then subjected in parallel to three consecutive freeze-thaw cycles, with alternating incubations in dry-ice/ethanol and 37°C water baths. CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) was added to a final concentration of 0.5%, and samples were incubated for 30 min at 37°C to release AAV particles from cell nuclei. After 2-fold dilution with 10 mM Tris HCl (pH 8), samples were incubated for 10 min at room temperature with 2 mg/mL RNase A (740505, Macherey-Nagel AG). $10 \times$ DNase I buffer was added to a final concentration of 40 mM Tris HCl, pH 8, 10 mM MgSO₄, and 1 mM CaCl₂. All samples were split in half to perform DNase I digests with one fraction, but not the other. Optimal DNase I digestion of AAV decapsidated genomes, with minimal degradation of encapsidated genomes, was obtained with 30-min digests at 37°C using 0.3 U DNase I (M6101, Promega). After digestion, DNase I was inactivated by addition of $10 \times$ STOP solution to a final concentration of 2 mM EGTA, pH 8, and heating to 65°C for 15 min. After addition of 200 μ L of T1 buffer to each sample, digests with 2 mg/mL proteinase K were performed for 90 min at 56°C (NucleoSpin Tissue, Macherey-Nagel AG) to release viral DNA from capsids. Subsequently, DNA was purified by extraction with 25:24:1 phenol:chloroform:isoamyl alcohol followed by ethanol precipitation in the presence of 20 μ g of herring sperm DNA. DNA was resuspended in 100 μ L of H₂O, and samples were diluted 100-fold prior to determination of vg copy numbers by qPCR with the above described β -globin primer and probe set. The number of vg detected in the DNase I-treated relative to the non-DNase I-treated portion of each sample was expressed as percentage of protected vg copies.

Statistical analysis

Data are expressed as mean $\pm$ SEM. The alpha level of significance was set at $p < 0.05$.

Group differences between AAV serotypes with regard to the number and percentage of EGFP-expressing nigral neurons were assessed by one-way ANOVA followed by *post hoc* analysis (Newman-Keuls test). The same statistical tests were also employed to detect group differences in the sequential AAV transduction of dopaminergic neurons.

The vg copy numbers/cell were quantified for the SN and striatum for both hemispheres separately, and serotype-specific differences were determined by one-way ANOVA, followed by *post hoc* analysis (Fisher test).

For the percentage of DNase I-protected AAV genomes that remained in the SN, group differences were established by ANOVA followed by *post hoc* Fisher test.

Results

AAV6, 8, and 9 directly injected to the SNpc transduce dopaminergic neurons with equal efficiency

Preparations of AAV6, AAV8, and AAV9, expressing the EGFP reporter under the control of the CMV promoter were used for comparison of nigral neuron transduction

efficiencies between these three serotypes. Virus preparations were adjusted to equal genome titers, and 1.9×10^{10} vg was unilaterally injected into the rat SN. Four weeks after viral delivery, transduced nigral neurons were identified by EGFP reporter gene expression. The percentage of transduced, TH-positive dopaminergic neurons did not significantly differ between AAV serotypes (AAV6: $47.5 \pm 1.4\%$; AAV8: $40.3 \pm 7.5\%$; and AAV9: $50.5 \pm 2.4\%$; Fig. 1). Therefore, all three serotypes transduced dopaminergic neurons in the SNpc with equal efficiency. Transgene expression was exclusively found in neurons following AAV6 and 9 injections. However, a significant number of oligodendrocytes expressing EGFP were noticed near the needle tract following AAV8 injections to the SN (see Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/hum).

None of the three serotypes caused an inflammatory response, as concluded from the absence of microglial and astrocytic activation in Iba-1 and GFAP immunofluorescent labelings, respectively (Supplementary Fig. S2), and from the absence of cellular infiltrates around the injection site in cresyl violet stains (Supplementary Fig. S3). In summary,

AAV serotypes 6, 8, and 9 were equally suited for the transduction of dopaminergic neurons via direct injections to the SNpc.

AAV6 is more potent than AAV8 and AAV9 in retrograde transduction of nigral dopaminergic neurons

We next analyzed the capacity of AAV serotypes 6, 8, and 9 for retrograde transduction of nigral dopaminergic neurons after vector injections to the striatum. 8.2×10^{10} vg of AAV6, AAV8, or AAV9 CMV EGFP were injected to two sites in the striatum within the same hemisphere and animals were sacrificed 4 weeks later.

EGFP expression in striatal sections revealed that viral spread of the heparin-binding AAV serotype 6 was limited to approximately $300 \mu\text{m}$ around the injection site (Fig. 2a), whereas EGFP-positive cells could be detected throughout the entire striatum after injection of AAV9 (Fig. 2c). The diffusion range of AAV8 was intermediate between AAV6 and AAV9 (Fig. 2b). As concluded from fluorescence intensity, the EGFP expression level in the injection site was

FIG. 1. Comparison of the efficiency of AAV6, 8, and 9 to transduce dopaminergic neurons in the SN. Rats were unilaterally injected to the SN with 1.9×10^{10} vg of AAV6 CMV EGFP ($n=5$) (a–c), AAV8 CMV EGFP ($n=4$) (d–f), or AAV9 CMV EGFP ($n=5$) (g–i) and sacrificed 4 weeks later. (a–i) Coronal sections through the SN. Transduced nigral dopaminergic neurons were identified by cellular colocalization of EGFP reporter (a, d, g) with TH immunofluorescent label (b, e, h) in overlays of images (c, f, i). (j) Quantification of EGFP-positive dopaminergic neurons in the SN. The percentage of transduced dopaminergic neurons did not differ between AAV serotypes 6, 8, and 9. AAV, adeno-associated virus; SN, substantia nigra; vg, vector genomes; EGFP, enhanced green fluorescent protein; TH, tyrosine hydroxylase.

highest for AAV6 (Fig. 2a) and thus inversely correlated with the diffusion range. After striatal injections with AAV8 and AAV9, EGFP-labeled axons of medium spiny neuron origin could be detected throughout the entire SN pars reticulata (Figs. 2e, 2f). By contrast, only part of the pars reticulata was covered with EGFP-filled axons after striatal injections with AAV6, again reflecting restricted diffusion inside the striatum (Fig. 2d).

AAV6 and AAV9 vectors injected in the striatum displayed a pure neuronal tropism, as demonstrated by colocalization of EGFP with the neuronal marker NeuN (Supplementary Fig. S4). AAV8 not only infected neurons (Supplementary Fig. S4), but EGFP was also colocalized with the Olig2 oligodendrocyte marker in white matter tracts within the striatum (Supplementary Fig. S1). Oligodendrocyte infection by AAV8 was likewise detected in corpus callosum along the needle tract (Supplementary Fig. S1). Neither AAV6, 8, nor 9 displayed astocytic transduction after striatal injections (Supplementary Fig. S5).

Retrograde transduction of nigral dopaminergic neurons after injection of recombinant AAV6, 8, or 9 to the striatum was assessed by quantification of the number of EGFP-expressing, TH-positive neurons in the SNpc (Figs. 2g–2o). The number of nigral neurons retrogradely transduced by AAV6 was 1.4-fold higher than the number transduced by AAV9 and 3.75-fold higher than by AAV8 (Fig. 2p). Also, the intensity of EGFP fluorescence in retrogradely transduced nigral neurons was higher for AAV6 than for AAV8 or AAV9 (Figs. 2g, 2j, 2m), suggesting higher copy numbers per cell after AAV6 injections. Among the three serotypes analyzed, AAV6 thus proved to be the most efficient one for retrograde transduction of nigral dopaminergic neurons.

The combination of AAV9 with AAV6 allows for sequential dopaminergic neuron transduction

Repeated nigral neuron transduction might be a desirable feature for either the sequential administration of different therapeutic genes or boosting of long-term expression of a single transgene. Also, repeated transduction of nigral neurons could be used in experimental models to assess the functional interaction between two transgenes.

We therefore tested whether nigral neurons could be sequentially transduced with the AAV serotype 6 and whether the combination of AAV6 with a different serotype, either AAV8 or AAV9, would increase the efficiency of repeated transduction. We combined nigral with striatal injections to avoid the confounding effect of the local tissue reaction due to mechanical damage caused by repeated needle penetrations. AAV6, AAV8 or AAV9 CMV EGFP (1.9×10^{10} vg) was unilaterally injected to the SN. AAV6, the serotype identified as the most efficient with regard to retrograde transduction (Fig. 2p), was then used for delayed striatal injections. Four weeks after the initial nigral injection, 4.0×10^{10} vg of AAV6 CMV DsRed was injected into two sites within the ipsilateral striatum and the animals were sacrificed 4 weeks later.

For evaluation of sequential nigral neuron transduction, we determined the total number of nigral neurons positive for both EGFP and DsRed in every 12th section (Fig. 3a–3i). There was no absolute refractoriness of nigral neurons to repeated transduction with AAV6, since DsRed expression

could be detected in EGFP-positive neurons after sequential AAV6 administration (Fig. 3a–3c). Nevertheless, the combination of nigral AAV9 with striatal AAV6 (Fig. 3g–3i) proved to be twice as efficient for sequential transduction of nigral neurons, and the mean number of double-transduced neurons in the AAV8/AAV6 combination (Fig. 3d–3f) was increased 1.3-fold (not significant) over the AAV6/AAV6 combination (Fig. 3k).

We next analyzed whether, upon second virus exposure, there was an overall reduction of nigral neuron transduction or a shift towards previously nontransduced cells in the AAV6/AAV6 compared with the serotype alternation groups. The total number of DsRed-expressing cells turned out to be reduced, irrespective of EGFP expression, after repeated AAV6 administration compared with AAV9/AAV6 serotype alternation (Fig. 3j). By contrast, there was no significant difference between groups regarding the number of DsRed-positive, but EGFP-negative neurons that had solely been retrogradely transduced (Fig. 3l). These neurons accounted for only $14.4 \pm 3.9\%$, $20.9 \pm 6.9\%$, and $11.5 \pm 4.8\%$ of the total number of DsRed-expressing cells in the AAV6/AAV6, AAV8/AAV6, and AAV9/AAV6 groups, respectively (not significantly different). Therefore, more than 80% of the retrogradely transduced cells were indeed double infected. Together these results suggest that partial refractoriness to repeated AAV6 transduction is not a consequence of cell autonomous, innate immunity. Rather, a systemic response to AAV6 after the first exposure reduced the overall AAV6 infection rate in the second round of vector administration.

In summary, nigral injections with AAV9 followed by delayed striatal injections with AAV6 proved to be the most efficient combination for repeated transduction of nigral dopaminergic neurons.

Prior nigral neuron transduction with AAV does not affect retrograde axonal transport of AAV

To analyze whether the capability to retrogradely transport AAV from the striatum is affected in nigral dopaminergic neurons by prior infection with AAV, we unilaterally injected 1.9×10^{10} vg of AAV9 CMV GFP to the SN in one subset of animals, whereas the other subset remained naive. Four weeks later, all animals were injected with 8.2×10^{10} vg of AAV6 CMV DsRed to the ipsilateral striatum. We combined nigral AAV9 with striatal AAV6 injections to avoid any possible overlapping effect with pre-existing immunity in case of repeated injections with the same serotype. Rats were sacrificed at 4 weeks after striatal injections. No difference in the total number of retrogradely transduced, DsRed-expressing neurons in the SN was found between the two groups (Fig. 3m). Thus, retrograde axonal transport of AAV is not impaired by prior transduction of nigral dopaminergic neurons with AAV.

Single-stranded vg persist in the SN and striatum after nigral AAV injections

We had demonstrated earlier that the percentage of transduced nigral neurons did not differ between AAV serotypes 6, 8, and 9 after direct injections to the SNpc (Fig. 1j). We sought to further assess whether any difference may exist between these serotypes with regard to viral

entry into nigral neurons, trafficking inside the neuron, and delivery of transcriptionally active, double-stranded vg. Therefore, we next quantified both single- and double-stranded vg in the nigrostriatal system following AAV delivery to the SN.

The SN of rats was bilaterally injected with 1.9×10^{10} vg of AAV6, AAV8, or AAV9 CMV EGFP. Noninjected rats served as negative controls. Four weeks after vector injections, genomic DNA was extracted from either the SN or the striatum, and a subfraction of the DNA was further subjected to S1 nuclease digestion to remove single-stranded vector DNA. In both S1 and non-S1-treated samples, we determined by qPCR the number of vector DNA copies (primers annealing to the β -globin intron) and normalized to cellular genomic DNA (primers directed against β -actin).

In the SN, the number of vg templates per cell did not significantly differ between AAV serotypes, either in total DNA samples (AAV6: 754 ± 154 vg/cell; AAV8: 574 ± 134 vg/cell; and AAV9: 908 ± 192 vg/cell) or in S1-treated samples that contained double-stranded viral DNA exclusively (AAV6: 196 ± 50 vg/cell; AAV8: 108 ± 20 vg/cell; and AAV9: 212 ± 34 vg/cell; Fig. 4a). These results indicate that AAV6, 8, and 9 enter nigral neurons with similar efficiency and that second strand synthesis had occurred to a comparable extent for all three serotypes over the course of 4 weeks. Surprisingly, the majority of vector DNA persisted in the SN as single-stranded DNA; only 26% (AAV6), 19% (AAV8), and 23% (AAV9) of vg templates detected in the SN were resistant to S1 nuclease.

Vector DNA was likewise detected in the striatum. The total number of vg per cell was surprisingly high, but did not significantly differ between the AAV6, 8, and 9 with 62 ± 14 , 42 ± 8 , and 88 ± 40 vg/cell, respectively (Fig. 4b). Vector DNA in the striatum was predominantly single-stranded, and viral DNA was reduced to background levels after S1 nuclease treatment (Fig. 4b). At the same time, EGFP-expressing cells could be detected in the striatum (Fig. 4c–4e), especially in case of AAV6 injections to the SN (Fig. 4c). These cells likely represent medium spiny neurons retrogradely transduced via their axon terminals in the SN pars reticulata and therefore contain double-stranded vg. The density of transduced neurons in the striatum was highest for AAV6 (Fig. 4c), followed by AAV9 (Fig. 4e), and was poorest for AAV8 (Fig. 4d), with barely any detectable EGFP-positive cells in the striatum.

Nevertheless, the striatal vg number per cell after nigral AAV8 injections exceeded 40 copies and was not significantly different from the vg number per cell measured after AAV6 injections. Therefore, medium spiny neurons cannot represent the sole source of striatal viral DNA. The continued presence of predominantly single-stranded vector DNA in the striatum could instead be due to the persistence of vg in axon terminals of transduced nigral dopaminergic neurons.

To confirm the presence of single-stranded viral DNA by a second method, we performed *in situ* hybridization on midbrain sections, using an EGFP sense probe to detect minus strand recombinant vector DNA and prevent detection of potentially remaining transcribed EGFP mRNA. A hybridization signal was clearly revealed in the virus-injected SN (Figs. 4g, 4h), but not in the uninjected side (Fig. 4f), further confirming the persistence of single-stranded viral DNA in nigral neurons. The sensitivity of *in situ* hybridization was not sufficient to detect the 10-fold lower levels of single-stranded DNA in the striatum.

When considering the total number of vg originally delivered to the SN (1.9×10^{10} vg) and extrapolating the detected number of vg to the entire SN and striatum, we found that more than 98.5% of AAV had been cleared 4 weeks after vector injections. Approximately 0.5% of injected AAV6 vg, 1.4% of AAV8 vg, and 0.6% of AAV9 vg were still present in the SN, and approximately 0.1% in the striatum. Therefore, we further investigated whether these vg could be contained in functional capsids and considered the possibility that remaining viral particles may traffic along the nigrostriatal axonal fibers and distally transduce cells in the brain.

Intact, assembled AAV capsids are enriched in the nucleus of nigral neurons after AAV6 injections to the SN

We next tested whether the continued presence of single-stranded vector DNA in nigral neurons was accompanied by the presence of viral capsids indicative of intact recombinant AAV particles. The monoclonal antibody ADK1a specifically recognizes intact assembled, but not native dissociated capsid proteins of AAV1, and cross-reacts with AAV6 (Kuck *et al.*, 2007). We confirmed that the ADK1a antibody detects AAV6 capsids in the periphery of HEK293T cells, 48 h after infection with recombinant AAV6 CMV

FIG. 2. Comparison of the capacity of AAV6, 8, and 9 to retrogradely transduce dopaminergic neurons in the SN. Rats were unilaterally injected at two sites within the striatum with a total of 8.2×10^{10} vg of AAV6 CMV EGFP ($n=4$), AAV8 CMV EGFP ($n=5$), or AAV9 CMV EGFP ($n=5$). Animals were sacrificed 4 weeks later. (a–c) Coronal sections through the injected striatum and (d–o) coronal sections through the ipsilateral SN. Diffusion of the heparin-binding AAV serotype 6 was restricted to approximately 300 μ m around the injection site (a), whereas AAV9 spreads throughout the entire striatum (c). AAV8 displayed an intermediary spread range (b). AAV6 and AAV9 exhibited a pure neuronal transduction pattern; see magnification boxes in (a) and (c), also see Supplementary Fig. S4. EGFP expression after AAV8 injection could be found in cells with neuronal morphology [white arrow in magnification box in (b)], as well as in small diameter cells within white matter tracts of the striatum [white arrow heads in magnification box in (b), also see Supplementary Fig. S1]. The limited diffusion range of AAV6 within the striatum was likewise reflected by the small area within the SN pars reticulata harboring EGFP-positive axons of medium spiny neuron origin (d). By contrast, EGFP-filled axons were found throughout the entire SN pars reticulata after AAV8 (e) or AAV9 (f) injections to the striatum [SNpc in (d–f) is outlined by TH-positive dopaminergic neurons in red]. Retrogradely transduced dopaminergic neurons in the SN were identified in overlays (i, l, o) of sections labeled with EGFP (g, j, m) and TH (h, k, n), 4 weeks after AAV6 (g–i), AAV8 (j–l), or AAV9 (m–o) injections to the ipsilateral striatum [for cellular colocalization of markers see magnified inserts in (g–o)]. (p) Quantification of the number of EGFP- and TH-labeled neurons in the SN. AAV6 was superior in retrograde transduction of nigral dopaminergic neurons to AAV8 and AAV9, and AAV9 was more efficient in retrograde transduction than AAV8 (ANOVA, Newman-Keuls test, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$). SNpc, substantia nigra pars compacta.

EGFP (Figs. 5a–5c). We then proceeded to immunohistochemistry on midbrain sections from rats injected with 1.9×10^{10} vg of AAV6 CMV EGFP to the SN. Four weeks post-injection, ADK1a immunofluorescent signal could be detected in EGFP-expressing cells of the SN. Intact AAV6

capsids were specifically enriched in the nuclei of transduced cells as revealed by colabeling with the nuclear marker 4',6-diamidino-2-phenylindole (Figs. 5e–5h). A predominantly nuclear ADK1a labeling was likewise found in transduced striatal neurons, 4 weeks after striatal injections with AAV6

FIG. 3. Comparison of different AAV serotype combinations for the sequential transduction of dopaminergic neurons in the SN. Rats were unilaterally injected in the SN with 1.9×10^{10} vg of AAV6 CMV EGFP ($n=5$) (a–c), AAV8 CMV EGFP ($n=4$) (d–f), or AAV9 CMV EGFP ($n=5$) (g–i). Four weeks later, 4.0×10^{10} vg of AAV6 CMV DsRed was administered at two sites of the ipsilateral striatum. Rats were sacrificed 8 weeks after the first virus injection. (a–i) Coronal sections through the SN. Transduced cells resulting from injections of AAV serotypes 6, 8, or 9 to the SN could be identified by EGFP reporter gene expression (a, d, g) and retrogradely transduced nigral neurons resulting from delayed striatal AAV6 injections could be detected by DsRed expression (b, e, h). Nigral neurons that underwent transduction twice in sequence thus express EGFP and DsRed and could be identified as yellow cells in image overlays (c, f, i). (j–l) Quantification of the number of nigral neurons expressing DsRed (j), DsRed and EGFP (k), and DsRed but not EGFP (l) in the SN. The combination of nigral AAV9 with striatal AAV6 injections proved to be more efficient for sequential transduction of nigral dopaminergic neurons than the combination of nigral AAV8 with striatal AAV6 injections or injection of AAV6 to both SN and striatum (ANOVA, Newman-Keuls test, $*p < 0.05$). (m) The SN of rats was unilaterally injected with 1.9×10^{10} vg of AAV6 CMV EGFP ($n=5$) or remained uninjected ($n=6$). Four weeks later, all rats received striatal injections of 8.2×10^{10} vg of AAV6 CMV DsRed. No difference in the number of retrogradely transduced, DsRed-expressing neurons in the SN was detected between the two groups 8 weeks after the initial nigral injection (every 12th section analyzed).

vector (Figs. 5i–5l). These findings suggest that viral capsids are only partially cleared from transduced neurons up to 1 month after injection and accumulate in neuronal nuclei.

DNase I resistant viral genomes persist in the substantia nigra

We next addressed the question whether the remaining single-stranded vg in the SN were packaged in viral capsids and would therefore be protected from degradation by

DNase I. Eight weeks after injection of 1.8×10^7 TU of AAV6 to the SN, nigral tissue was subjected to freeze–thaw cycles and detergent treatment to break open cell nuclei and liberate viral capsids. To assess the percentage of encapsidated vg, we determined by qPCR the ratio of vg detected following DNase I digestion (encapsidated vg only) to vg detected in the absence of DNase I (total vg). To verify the resistance of intact particles to DNase I treatment, non-injected SN was mixed with 1.8×10^7 TU of intact AAV6. In this condition, we indeed found that 84.1% of vg were still

FIG. 4. Single-stranded viral genomes persisted in the SN and striatum after AAV6, 8, or 9 injections in the SN. (a, b) 1.9×10^{10} vg of AAV6 CMV EGFP, AAV8, or AAV9 was bilaterally injected into the rat SN. The number of double-stranded vg templates (S1 nuclease treated, black bars) and total vg templates per cell (non-S1-treated, hatched bars) were determined in samples from SN and striatum. While the number of vg in the SN and striatum did not differ between serotypes, copy numbers in the SN (a) were significantly reduced for each serotype after S1 nuclease digest ($n=4$ for AAV6 and AAV8, $n=3$ for AAV9; ANOVA, Fisher test, $*p<0.05$; $**p<0.01$; $***p<0.001$). In the striatum (b), the proportional reduction of vg templates after S1 nuclease digest was even more pronounced than in the SN ($n=4$ for AAV6 and AAV8, $n=3$ for AAV9; ANOVA, Fisher-test, $*p<0.05$; $**p<0.01$; $***p<0.001$). (c–e) coronal sections through the striatum, showing retrograde transduction following injection of AAV6 (c), AAV8 (d), or AAV9 (e) to the SN. When using AAV6 as a gene delivery vehicle, scattered EGFP-expressing neurons could be detected within the dense network of EGFP-filled dopaminergic axons (c), fewer EGFP-labeled cells were seen after AAV9 injections (e) and barely any EGFP-expressing cells were found after AAV8 injections to the SN (d). (f–h) Coronal sections through rat SN, 4 weeks after unilateral injection of 1.9×10^{10} vg of AAV6 CMV EGFP. *In situ* hybridization was performed on RNase-treated sections with digoxigenin-labeled EGFP sense probe. Minus strand vg could be specifically detected in the SN on the injected (g) and higher magnification in (h), but not the uninjected side (f).

detected after DNase I digestion (Fig. 5o). Therefore, approximately 16% of the loss of detected vg can be attributed to the viral suspension itself and/or to the experimental procedure. When the same amount of AAV particles were heat pre-denatured and mixed with noninjected SN tissue extract, only 32.9% of vg remained detectable following DNase I treatment, demonstrating effective nuclease activity on viral genomes released from the AAV capsid.

In the AAV-injected SN, 67.8% of vg were protected from DNase I degradation, which was significantly different from the proportion of DNase I-resistant vg when capsids were heat pre-denatured to release viral DNA (Fig. 5o). By contrast, the proportion of DNase I-protected AAV genomes in the injected SN did not differ from the condition in which intact capsids were mixed with SN tissue extracts. Nevertheless, the observed partial degradation of vg in the injected

SN might reflect the breakdown of decapsidated vg copies successfully incorporated in the nuclei of transduced neurons. Altogether, these results suggest that a significant proportion of single-stranded viral genomes detected weeks after AAV injection to the SN remains encapsidated and is therefore resistant to DNase I treatment.

No spread of viral transduction is observed in the striatum following 6-OHDA-induced degeneration of AAV6-infected nigrostriatal dopaminergic axons

The continued presence of single-stranded vg as well as intact AAV capsids in nigral dopaminergic neurons prompted us to investigate whether infectious recombinant virus could persist and be released from infected neurons upon degeneration. As described above, the number of single-stranded viral

FIG. 5. Intact viral capsids and DNase I-protected viral genomes remained present in the nigrostriatal system, weeks after AAV injections. **(a–d)** Antibody ADK1a, recognizing assembled native AAV1 capsid was tested for its capacity to detect AAV6 intact capsid in HEK-293T cells, 48 h after infection with recombinant AAV6 CMV EGFP. **(a)** native EGFP fluorescence in transduced HEK293T cells. **(b)** Immunofluorescence labeling with ADK1a antibody. **(c)** Overlay of **(a)** and **(b)** demonstrating AAV6 viral particle detection in the periphery of transduced cells by antibody ADK1a. No signal was detected when infected cells were incubated with secondary antibody only **(d)**. **(e–h)** Coronal sections through rat SN, 4 weeks after injection with 1.9×10^{10} vg of AAV6 CMV EGFP. Co-immunofluorescence labeling with anti-GFP antibody **(e)**, ADK1a antibody **(f)**, and the nuclear marker DAPI **(g)** showed accumulation of AAV6 intact capsid in the nucleus of nigral neurons [see overlay in **(h)**]. No signal was detected with ADK1a antibody in the noninjected SN **(m)**. Likewise, intact AAV6 particles persist in striatal neurons, 4 weeks after striatal AAV6 injections **(i–l)**. Nuclear accumulation of AAV6 assembled capsid was demonstrated in overlays **(l)** of GFP **(i)**, ADK1a **(j)**, and DAPI **(k)** immunofluorescence labelings. No ADK1a signal was detected in the uninjected side **(n)**. **(o)** Detection of DNase I-protected AAV genomes in the rat SN, 8 weeks after injection with 1.8×10^7 TU of AAV6 ($n=3$). In parallel, noninjected SN was mixed with 1.8×10^7 TU of the same viral preparation, either intact ($n=3$) or after capsid denaturation through heat treatment ($n=3$). The graph indicates the ratio of vg measured in DNase I-treated samples to untreated samples, indicative of the proportion of DNase-resistant, encapsidated vg (ANOVA, Fisher test, $*p < 0.05$; $**p < 0.01$). DAPI, 4',6-diamidino-2-phenylindole.

DNA detected in the striatum after AAV injections to the SN suggests the presence of recombinant virus in terminals of nigral dopaminergic neurons. We therefore analyzed whether the selective 6-OHDA-induced degeneration of AAV-infected nigral dopaminergic neurons would lead to the release of recombinant AAV in the striatum and result in transduction of striatal neurons.

To this end, rats were unilaterally injected to the SN with 1.9×10^{10} vg of AAV6 expressing EGFP fused to a nuclear localization signal (NLS EGFP) to decrease fluorescent labeling within the axonal compartment, and thereby facilitate the identification of transduced cells in the striatum. Four

weeks after AAV6 vector injection, 6-OHDA was administered to the ipsilateral medial forebrain bundle in a subgroup of animals. Six weeks thereafter, we determined the number of vg in the striatum and assessed the transduction of striatal neurons by GFP immunohistochemistry in both 6-OHDA-lesioned and intact rats.

Proper targeting of AAV6 CMV NLS EGFP to the SN was verified by immunohistochemical detection of NLS EGFP reporter (Figs. 6a, 6e). In animals that had received AAV injections exclusively, NLS EGFP expression was found throughout the intact SN (Fig. 6a). Despite some leakage of NLS EGFP into striatal fibers (Figs. 6c, 6i, 6j) at 10 weeks

FIG. 6. 6-Hydroxydopamine (6-OHDA)-induced neurodegeneration of virus-infected SN did not trigger release of infectious AAV in the striatum. AAV6 CMV NLS-EGFP was unilaterally injected to the SN ($n=14$). In half of the animals, 6-OHDA was administered to the ipsilateral medial forebrain bundle, 4 weeks later. Ten weeks after virus injection, NLS-EGFP (a, c, e, g, i–l) and TH (b, d, f, h) expression were immunohistochemically detected in coronal brain sections through the SN (a, b, e, f) and striatum (c, d, g–l). In rats only injected with AAV6 NLS-EGFP (a–d, i, j), EGFP expression could be detected in the SNpc (a) and striatum (c, i, j). No TH marker loss was evident in the SN (b) and striatum (d). In rats with delayed 6-OHDA administration (e–h, k, l), there was a selective elimination of neurons expressing EGFP in the SNpc (e) and a loss of EGFP-positive fibers in the striatum (g, k, l). TH immunostaining was completely lost both in the SNpc (f) and striatum (h), confirming nigrostriatal degeneration. (a–h) Scale bars = 500 μ m. (i–l) EGFP labeling in the striatum. Note the presence of EGFP-positive axons (i, j), which were completely eliminated following 6-OHDA intoxication (k, l). In contrast, the number of retrogradely transduced EGFP-positive neurons in the striatum remained similar in both intact and lesioned animals; compare (i) and (k). (j, l) Magnifications of boxes in (i) and (k), showing retrogradely transduced neurons in the striatum. Scale bars = 50 μ m. (m) The number of vg copies detected in the striatum ipsilateral to AAV injection was significantly reduced following 6-OHDA lesioning ($n=7$, black bars), as compared to nonlesioned animals ($n=7$, hatched bars), in line with clearance of vg from nigral neuron terminals (ANOVA, Fisher test, $*p<0.05$; $***p<0.001$).

post-vector injection, axonal EGFP labeling was low enough to clearly distinguish transduced medium spiny neurons expressing NLS EGFP (Figs. 6i, 6j). In rats injected with 6-OHDA after nigral vector injections, remaining NLS EGFP expression was restricted to few cells outside the SN (Fig. 6e) and TH immunostaining confirmed the complete degeneration of nigral dopaminergic neurons (Fig. 6f). Accordingly, a complete loss of EGFP- and TH-positive dopaminergic fibers was observed in the ipsilateral striatum (Figs. 6g, 6h, 6k, 6l), demonstrating the elimination of dopaminergic axons projecting to the striatum.

Despite the total elimination of nigral dopaminergic neurons with 6-OHDA, we did not observe any increase in the density of EGFP-expressing neurons in the striatum (compare representative Figs. 6i, 6j and 6k, 6l), therefore speaking against the release of infectious viral particles from degen-

erating dopaminergic neurons following exposure to the 6-OHDA toxin.

To verify that the vg copies detected in the striatum by qPCR were in fact contained within the dopaminergic compartment, we next assessed whether 6-OHDA lesions following nigral injections of AAV6 changed the number of vg copies present in the striatum (Fig. 6m). Six weeks after 6-OHDA intoxication, the number of vg templates in the striatum was significantly reduced from 32 ± 4 vg/cell genome to 10 ± 2 vg/cell genome.

In summary, we found that 6-OHDA-induced degeneration of dopaminergic axons leads to the elimination of vg copies from the striatum, confirming the presence of vector DNA within striatal dopaminergic axon terminals at 4 weeks post-injection. We did not detect newly transduced, postsynaptic medium spiny neurons in the striatum as a

consequence of 6-OHDA-induced degeneration. This is an important finding with regard to the application of AAV vectors as gene delivery vehicles for the treatment of neurodegenerative disease: induced axonal degeneration did not result in spread of AAV and thus did not lead to undesired transgene expression in projection areas of infected neurons.

Discussion

In this study we characterized AAV serotypes 6, 8, and 9 for their capacity to transduce dopaminergic neurons in the SN via two different routes of delivery and analyzed sequential transduction of nigral neurons with different serotype combinations. We further evaluated viral persistence and potential spread of AAV to target projection areas of transduced neurons in the context of neurodegeneration.

We found that transduction of nigral neurons was equally efficient for AAV serotypes 6, 8, and 9, 4 weeks after direct injections of virus to the SN. Neither the percentage of transduced dopaminergic neurons in the SN nor the number of double-stranded vg per cell differed among these three serotypes. Efficient transduction of dopaminergic neurons in the SN with recombinant AAV serotypes 6, 8, and 9 has previously been reported (Klein *et al.* 2006, 2008; Dusanich *et al.*, 2009; Van der Perren *et al.*, 2011). Comparison of AAV serotypes 5, 6, 7, and 8 for transduction of rat nigral dopaminergic neurons using synapsin promoter driven GFP reporter gene expression yielded no difference between these serotypes with regard to the percentage of GFP-expressing TH-positive neurons (Korecka *et al.*, 2011). In a study by Klein *et al.* (2008) investigating AAV transduction kinetics, the onset of transgene expression was found to be faster for AAV9 than for AAV8, as evidenced by the higher AAV9 transgene expression level, 1 and 2 weeks after viral injections to the SN. However, the expression level of AAV8-delivered transgene increased over time and converged with the AAV9 expression level after 4 weeks (Klein *et al.*, 2008), in line with data from our study, in which equal amounts of double-stranded recombinant viral DNA were detected in AAV8- and AAV9-infected nigral neurons at this time point.

For retrograde transduction of nigral neurons after virus injections to the striatum, AAV6 proved to be the most efficient serotype, followed by AAV9 and AAV8. Highly efficient retrograde axonal transport to spinal cord motor neurons has previously been reported for AAV6 after intramuscular injections in mice and nonhuman primates (Towne *et al.* 2010, 2011). More recently, retrograde transport of AAV6 to the SNpc has also been demonstrated after striatal injections (Salegio *et al.*, 2013). Retrograde transduction of neurons in the SN involves AAV uptake via dopaminergic axon terminals in the striatum. After injection of equal amounts of AAV (vg), highest local virus concentrations were achieved in the striatal injection site with the heparin-binding AAV serotype 6, due to its limited diffusion capacity. The non-heparin-binding AAV serotypes 8 and 9, by contrast, spread throughout the entire striatum. High local threshold concentrations of virus thus seem to be more critical to successful retrograde transduction of nigral neurons than homogenous distribution of AAV throughout the striatum. This observation likely reflects the enormous degree of dopaminergic axon arborization and overlapping innervation in the striatum (Matsuda *et al.*, 2009). The su-

perior retrograde transport properties of AAV9 compared with AAV8 might be explained by the difference in cellular tropism between these two serotypes. Within the adult striatum, AAV8 transduced neurons as well as oligodendrocytes, which is in line with earlier reports by Lawlor *et al.* (2009). AAV9, after striatal injections displayed a neuronal, but not astrocytic or oligodendrocytic tropism, also previously reported for mice after striatal or cortical injections of AAV9 (Cearley and Wolfe, 2006; Foust *et al.*, 2009). In contrast, AAV9 tail vein injections in adult mice and injections into the internal carotid artery or into the cisterna magna in nonhuman primates, resulted in a predominantly astrocytic tropism (Foust *et al.*, 2009; Samaranch *et al.*, 2012). The vascular route of delivery necessitates blood-brain barrier crossing where AAV first encounters astrocytic endfeet upon entry into the brain parenchyma, which might explain the preferential astrocytic infection. This is not the case after intraparenchymal injections, where AAV9 clearly favors neuronal transduction. The amount of AAV potentially available for presentation on neuronal surfaces is thus higher for AAV9 than for AAV8 with its mixed neuron/oligodendrocyte transduction profile, which might facilitate AAV9 uptake into presynaptic dopaminergic axon terminals and subsequent retrograde transport to the SN.

We further analyzed whether individual nigral neurons could be repeatedly transduced and whether the efficiency of sequential transduction could be increased by switching AAV serotypes between injections. Although we did not observe a general refractoriness of previously AAV6-transduced neurons to a second AAV6 transduction, the combination of AAV9 with AAV6 proved to be twice as efficient as repeated administration of AAV6. We did not find evidence for elimination of double-transduced nigral neurons, or for the activation of intracellular innate defense mechanisms that would have specifically blocked a second transduction with the same AAV serotype. The lower efficiency in the second round of transduction with repeated AAV6 administration is therefore likely due to a weak AAV6-specific immune response that, upon second exposure, eliminates virus prior to uptake into axon terminals of nigral dopaminergic neurons.

The brain is generally regarded as an immune-privileged site, nevertheless, weak immunization, characterized by the presence of neutralizing, serotype-specific antibodies has been reported after sequential striatal injections with AAV2 (Peden *et al.*, 2004). Interestingly, brain dendritic cells have recently been described that differentiate from resident microglia and are capable of stimulating naïve CD4⁺ T cells in an antigen-dependent fashion (Gottfried-Blackmore *et al.*, 2009). The immunogenicity of AAV seems to be determined by its capacity to bind heparan sulfate proteoglycan patches at the surface of dendritic cells (Vandenberghe *et al.*, 2006; Lu and Song, 2009). It is therefore conceivable that the heparin-binding serotype AAV6 elicited a dendritic cell-mediated AAV6-specific immune response leading to reduced transduction of nigral neurons upon a second exposure to this serotype.

When using viral vectors for gene transfer, viral clearance remains an important safety issue. While more than 98.5% of AAV vg originally injected into the SN had been cleared by 4 weeks, we unexpectedly found that significant amounts of vg persisted in the single-strand conformation within the SN (>450 vg/cell, see Fig. 4a), a finding further confirmed by

in situ hybridization. We also detected intact, assembled AAV6 capsid within the nuclei of transduced neurons in the SN and found that significant amounts of DNase I-resistant AAV6 vg remained in the SN, weeks after viral injection, thus suggesting the continued presence of encapsidated vg. To break down nonprotected AAV genomes in our assay we used DNase I, which degrades double-stranded, as well as single-stranded DNA, instead of the single-strand specific S1 nuclease, for the following reasons: (1) DNase I also digests nonprotected, episomal double-stranded genomes that emerge from second-strand synthesis in the process of nigral cell transduction and (2) AAV genomic single-stranded DNA can be of plus or minus strand polarity and can thus anneal to form double-stranded genomes outside the capsid. In contrast to S1 nuclease, DNase I can degrade these two DNA species and clearly identifies the presence of encapsidated, nuclease-resistant vg.

One drawback of our assay was the incomplete digestion of single-stranded AAV genomes released from heat-denatured capsids (32.9% of vg remaining) because DNase I concentration was chosen to spare encapsidated vg. Nevertheless, the 67.8% of protected vg in the injected SN turned out to be significantly higher than the 32.9% vg remaining after incomplete degradation, demonstrating the presence of capsid-enclosed vg, 8 weeks after viral injections. Together these findings support the hypothesis that uncoating takes place only after entry of intact virus into the nucleus. This hypothesis is further supported by the observation that nuclear injection of capsid-specific antibodies nearly completely prevented rep gene expression in wild-type AAV2-infected cells (Sonntag *et al.*, 2006). Translocation of AAV to the nucleus requires the exposure of a nuclear localization signal at the N-terminus of VP1 capsid protein during viral passage through the endosome (Kelkar *et al.*, 2006; Sonntag *et al.*, 2006; Johnson and Samulski, 2009; Johnson *et al.*, 2010). Interestingly, empty AAV capsids do not undergo the necessary conformational changes and are therefore excluded from entry into the nucleus (Johnson and Samulski, 2009).

To date, it largely remains elusive how intracellular AAV is cleared. Several *in vitro* studies point to ubiquitination, followed by proteasomal degradation as a possible mechanism. In these studies proteasome inhibition led to an increased number of AAV genomes per cell and accumulation of intact recombinant AAV within the nucleus, which resulted in an increased transduction efficiency (Douar *et al.*, 2001; Denby *et al.*, 2005; Johnson and Samulski, 2009; Johnson *et al.*, 2010). The protection of nuclear AAV capsid from degradation suggests low proteasomal activity within the nucleus of nigral dopaminergic neurons. According to a different study, proteasomal inhibition could also improve AAV transduction efficiency without simultaneously increasing the levels of virion DNA inside the cell (Yan *et al.*, 2002). The authors suggested that ubiquitination of AAV capsid might represent a positive signal for virus particle disassembly in the process of uncoating rather than a general degradation signal. It is thus conceivable that the remaining AAV particles that we detected in the nucleus of nigral dopaminergic neurons had escaped ubiquitination and could thus not disassemble to liberate viral DNA for transduction, which would also explain the continued presence of single-stranded viral DNA in the SN. Uncoating, in addition to the

formation of double-stranded vector DNA, might therefore represent a major bottleneck in AAV transduction.

Four and 10 weeks after viral injections to the SN, we found that single-stranded viral DNA was also present in the striatal axonal compartment and could be eliminated following 6-OHDA-induced degeneration. A comparable amount of single-stranded vector genomes per cell in the striatum was also detected in our laboratory 4 months after injection of AAV6 into the substantia nigra, suggesting that vg do not become cleared in the intact dopaminergic fiber. In fact, persistence of intact AAV particles in synapses has been reported for dogs and nonhuman primates for up to 6 years post subretinal administration of AAV serotypes 2, 4, and 5 (Stieger *et al.*, 2009). Interestingly, AAV particles were detected in presynaptic as well as postsynaptic structures of the retina by transmission electron microscopy in combination with immunogold labeling (Stieger *et al.*, 2009), suggesting transsynaptic spread of AAV. Further evidence for axonal and transsynaptic transport of AAV comes from reports stating the presence of vector DNA along the visual pathway after intravitreal and subretinal injection of AAV2/2 in dogs and rats (Provost *et al.*, 2005; Jacobson *et al.*, 2006). Furthermore, GFP reporter gene expression could be found in neurons of the geniculate nucleus after subretinal injection of AAV2/8 to the contralateral eye in dogs (Stieger *et al.*, 2008).

We therefore further addressed the question of whether persisting recombinant AAV particles were still infectious and could be released from neurons of the nigrostriatal system upon degeneration, and might thus lead to undesired virus spread throughout the brain, an important safety issue for gene therapeutic treatment of neurodegenerative disease. Following 6-OHDA intoxication, we did not find any increase in the density of transduced striatal neurons. This observation argues against AAV release and spread during the process of nigral neuron degeneration, and instead implies two possible conclusions: the capsids containing single-stranded viral DNA were not intact capsids and therefore were no longer infectious, or infectious AAV particles became cleared during degeneration of nigral neurons. Either way, 6-OHDA-induced nigral neuron degeneration did not unleash release of infectious AAV from axon terminals in the striatum and therefore did not lead to undesired spread of transgene expression.

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References

- Bartus, R.T., Brown, L., Wilson, A., *et al.* (2011a). Properly scaled and targeted AAV2-NRTN (neurturin) to the substantia nigra is safe, effective and causes no weight loss: support for nigral targeting in Parkinson's disease. *Neurobiol Dis* 44, 38–52.
- Bartus, R.T., Herzog, C.D., Chu, Y., *et al.* (2011b). Bioactivity of AAV2-neurturin gene therapy (CERE-120): differences between Parkinson's disease and nonhuman primate brains. *Mov Disord* 26, 27–36.
- Boutin, S., Monteilhet, V., Veron, P., *et al.* (2010). Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Hum Gene Ther* 21, 704–712.
- Cearley, C.N., and Wolfe, J.H. (2006). Transduction characteristics of adeno-associated virus vectors expressing cap serotypes 7, 8, 9, and Rh10 in the mouse brain. *Mol Ther* 13, 528–537.
- Christine, C.W., Starr, P.A., Larson, P.S., *et al.* (2009). Safety and tolerability of putaminal AADC gene therapy for Parkinson disease. *Neurology* 73, 1662–1669.
- Ciesielska, A., Mittermeyer, G., Hadaczek, P., *et al.* (2011). Anterograde axonal transport of AAV2-GDNF in rat basal ganglia. *Mol Ther* 19, 922–927.
- Denby, L., Nicklin, S.A., and Baker, A.H. (2005). Adeno-associated virus (AAV)-7 and -8 poorly transduce vascular endothelial cells and are sensitive to proteasomal degradation. *Gene Ther* 12, 1534–1538.
- Diaz-Nido, J. (2010). NLX-P101, an adeno-associated virus gene therapy encoding glutamic acid decarboxylase, for the potential treatment of Parkinson's disease. *Curr Opin Investig Drugs* 11, 813–822.
- Douar, A.M., Poulard, K., Stockholm, D., and Danos, O. (2001). Intracellular trafficking of adeno-associated virus vectors: routing to the late endosomal compartment and proteasome degradation. *J Virol* 75, 1824–1833.
- During, M.J., Samulski, R.J., Elsworth, J.D., *et al.* (1998). In vivo expression of therapeutic human genes for dopamine production in the caudates of MPTP-treated monkeys using an AAV vector. *Gene Ther* 5, 820–827.
- Dusonchet, J., Bensadoun, J.C., Schneider, B.L., and Aebischer, P. (2009). Targeted overexpression of the parkin substrate Pael-R in the nigrostriatal system of adult rats to model Parkinson's disease. *Neurobiol Dis* 35, 32–41.
- Eberling, J.L., Jagust, W.J., Christine, C.W., *et al.* (2008). Results from a phase I safety trial of hAADC gene therapy for Parkinson disease. *Neurology* 70, 1980–1983.
- Eberling, J.L., Kells, A.P., Pivrotto, P., *et al.* (2009). Functional effects of AAV2-GDNF on the dopaminergic nigrostriatal pathway in parkinsonian rhesus monkeys. *Hum Gene Ther* 20, 511–518.
- Eslamboli, A., Georgievska, B., Ridley, R.M., *et al.* (2005). Continuous low-level glial cell line-derived neurotrophic factor delivery using recombinant adeno-associated viral vectors provides neuroprotection and induces behavioral recovery in a primate model of Parkinson's disease. *J Neurosci* 25, 769–777.
- Fitzsimons, H.L., Riban, V., Bland, R.J., Wendelken, J.L., *et al.* (2010). Biodistribution and safety assessment of AAV2-GAD following intrasubthalamic injection in the rat. *J Gene Med* 12, 385–398.
- Foust, K.D., Nurre, E., Montgomery, C.L., *et al.* (2009). Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nat Biotechnol* 27, 59–65.
- Gao, G., Vandenberghe, L.H., Alvira, M.R., *et al.* (2004). Clades of adeno-associated viruses are widely disseminated in human tissues. *J Virol* 78, 6381–6388.
- Gao, G.P., Alvira, M.R., Wang, L., *et al.* (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci U S A* 99, 11854–11859.
- Gottfried-Blackmore, A., Kaunzner, U.W., Idoyaga, J., *et al.* (2009). Acute in vivo exposure to interferon-gamma enables resident brain dendritic cells to become effective antigen presenting cells. *Proc Natl Acad Sci U S A* 106, 20918–20923.
- Grimm, D., Kay, M.A., and Kleinschmidt, J.A. (2003). Helper virus-free, optically controllable, and two-plasmid-based production of adeno-associated virus vectors of serotypes 1 to 6. *Mol Ther* 7, 839–850.
- Jacobson, S.G., Acland, G.M., Aguirre, G.D., *et al.* (2006). Safety of recombinant adeno-associated virus type 2-RPE65 vector delivered by ocular subretinal injection. *Mol Ther* 13, 1074–1084.
- Jaraya, B., Boulet, S., Ralph, G.S., *et al.* (2009). Dopamine gene therapy for Parkinson's disease in a nonhuman primate without associated dyskinesia. *Sci Transl Med* 1, 2ra4.
- Johnson, J.S., and Samulski, R.J. (2009). Enhancement of adeno-associated virus infection by mobilizing capsids into and out of the nucleolus. *J Virol* 83, 2632–2644.
- Johnson, J.S., Li, C., Diprimio, N., *et al.* (2010). Mutagenesis of adeno-associated virus type 2 capsid protein VP1 uncovers new roles for basic amino acids in trafficking and cell-specific transduction. *J Virol* 84, 8888–8902.
- Kelkar, S., De, B.P., Gao, G., *et al.* (2006). A common mechanism for cytoplasmic dynein-dependent microtubule binding shared among adeno-associated virus and adenovirus serotypes. *J Virol* 80, 7781–7785.
- Klein, R.L., Dayton, R.D., Leidenheimer, N.J., *et al.* (2006). Efficient neuronal gene transfer with AAV8 leads to neurotoxic levels of tau or green fluorescent proteins. *Mol Ther* 13, 517–527.
- Klein, R.L., Dayton, R.D., Tatom, J.B., *et al.* (2008). Tau expression levels from various adeno-associated virus vector serotypes produce graded neurodegenerative disease states. *Eur J Neurosci* 27, 1615–1625.
- Kordower, J.H., Emborg, M.E., Bloch, J., *et al.* (2000). Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science* 290, 767–773.
- Korecka, J.A., Schouten, M., Eggers, R., *et al.* (2011). Comparison of AAV serotypes for gene delivery to dopaminergic neurons in the substantia nigra. In *Viral Gene Therapy*. Xu, K., ed. InTech. pp 205–224.
- Kuck, D., Kern, A., and Kleinschmidt, J.A. (2007). Development of AAV serotype-specific ELISAs using novel monoclonal antibodies. *J Virol Methods* 140, 17–24.
- Lawlor, P.A., Bland, R.J., Mouravlev, A., *et al.* (2009). Efficient gene delivery and selective transduction of glial cells in the mammalian brain by AAV serotypes isolated from nonhuman primates. *Mol Ther* 17, 1692–1702.
- Lu, Y., and Song, S. (2009). Distinct immune responses to transgene products from rAAV1 and rAAV8 vectors. *Proc Natl Acad Sci U S A* 106, 17158–17162.
- Marks, W.J., Jr., Bartus, R.T., Siffert, J., *et al.* (2010). Gene delivery of AAV2-neurturin for Parkinson's disease: a double-blind, randomised, controlled trial. *Lancet Neurol* 9, 1164–1172.
- Matsuda, W., Furuta, T., Nakamura, K.C., *et al.* (2009). Single nigrostriatal dopaminergic neurons form widely spread and highly dense axonal arborizations in the neostriatum. *J Neurosci* 29, 444–453.

- McFarland, N.R., Lee, J.S., Hyman, B.T., and McLean PJ. (2009). Comparison of transduction efficiency of recombinant AAV serotypes 1, 2, 5, and 8 in the rat nigrostriatal system. *J Neurochem* 109, 838–845.
- Muramatsu, S., Fujimoto, K., Kato, S., *et al.* (2010). A phase I study of aromatic L-amino acid decarboxylase gene therapy for Parkinson's disease. *Mol Ther* 18, 1731–1735.
- Peden, C.S., Burger, C., Muzyczka, N., and Mandel RJ. (2004). Circulating anti-wild-type adeno-associated virus type 2 (AAV2) antibodies inhibit recombinant AAV2 (rAAV2)-mediated, but not rAAV5-mediated, gene transfer in the brain. *J Virol* 78, 6344–6359.
- Provost, N., Le Meur, G., Weber, M., *et al.* (2005). Biodistribution of raav vectors following intraocular administration: evidence for the presence and persistence of vector DNA in the optic nerve and in the brain. *Mol Ther* 11, 275–283.
- Rutledge, E.A., Halbert, C.L., and Russell DW. (1998). Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. *J Virol* 72, 309–319.
- Salegio, E.A., Samaranch, L., Kells, A.P., *et al.* (2013). Axonal transport of adeno-associated viral vectors is serotype-dependent. *Gene Ther* 20, 348–352.
- Samaranch, L., Salegio, E.A., San Sebastian, W., *et al.* (2012). Adeno-associated virus serotype 9 transduction in the central nervous system of nonhuman primates. *Hum Gene Ther* 23, 382–389.
- Sanftner, L.M., Suzuki, B.M., Doroudchi, M.M., *et al.* (2004). Striatal delivery of rAAV-hAADC to rats with preexisting immunity to AAV. *Mol Ther* 9, 403–409.
- Sonntag, F., Bleker, S., Leuchs, B., *et al.* (2006). Adeno-associated virus type 2 capsids with externalized VP1/VP2 trafficking domains are generated prior to passage through the cytoplasm and are maintained until uncoating occurs in the nucleus. *J Virol* 80, 11040–11054.
- Stieger, K., Colle, M.A., Dubreil, L., *et al.* (2008). Subretinal delivery of recombinant AAV serotype 8 vector in dogs results in gene transfer to neurons in the brain. *Mol Ther* 16, 916–923.
- Stieger, K., Schroeder, J., Provost, N., *et al.* (2009). Detection of intact rAAV particles up to 6 years after successful gene transfer in the retina of dogs and primates. *Mol Ther* 17, 516–523.
- Towne, C., Schneider, B.L., Kieran, D., *et al.* (2010). Efficient transduction of non-human primate motor neurons after intramuscular delivery of recombinant AAV serotype 6. *Gene Ther* 17, 141–146.
- Towne, C., Setola, V., Schneider, B.L., and Aebischer, P. (2011). Neuroprotection by gene therapy targeting mutant SOD1 in individual pools of motor neurons does not translate into therapeutic benefit in fALS mice. *Mol Ther* 19, 274–283.
- Valles, F., Fiandaca, M.S., Eberling, J.L., *et al.* (2010). Qualitative imaging of adeno-associated virus serotype 2-human aromatic L-amino acid decarboxylase gene therapy in a phase I study for the treatment of Parkinson disease. *Neurosurgery* 67, 1377–1385.
- Van der Perren, A., Toelen, J., Carlon, M., *et al.* (2011). Efficient and stable transduction of dopaminergic neurons in rat substantia nigra by rAAV 2/1, 2/2, 2/5, 2/6.2, 2/7, 2/8 and 2/9. *Gene Ther* 18, 517–527.
- Vandenbergh, L.H., Wang, L., Somanathan, S., *et al.* (2006). Heparin binding directs activation of T cells against adeno-associated virus serotype 2 capsid. *Nat Med* 12, 967–971.
- Wang, L., Muramatsu, S., Lu, Y., *et al.* (2002). Delayed delivery of AAV-GDNF prevents nigral neurodegeneration and promotes functional recovery in a rat model of Parkinson's disease. *Gene Ther* 9, 381–389.
- Yan, Z., Zak, R., Luxton, G.W., *et al.* (2002). Ubiquitination of both adeno-associated virus type 2 and 5 capsid proteins affects the transduction efficiency of recombinant vectors. *J Virol* 76, 2043–2053.

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