Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain

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Recombinant adeno-associated viruses (rAAVs) are commonly used vehicles for in vivo gene transfer1–6. However, the tropism repertoire of naturally occurring AAVs is limited, prompting a search for novel AAV capsids with desired characteristics7–13. Here we describe a capsid selection method, called Cre recombination–based AAV targeted evolution (CREATE), that enables the development of AAV capsids that more efficiently transduce define Cre-expressing cell populations in vivo. We use CREATE to generate AAV variants that efficiently and widely transduce the adult mouse central nervous system (CNS) after intravenous injection. One variant, AAV-PHP.B, transfers genes throughout the CNS with an efficiency that is at least 40-fold greater than that of the current standard, AAV9 (refs. 14–17), and transduces the majority of astrocytes and neurons across multiple CNS regions. In vivo, it transduces human neurons and astrocytes more efficiently than does AAV9, demonstrating the potential of CREATE to produce customized AAV vectors for biomedical applications.

AAVs are the preferred vehicles for many in vivo gene transfer applications to nondividing cell populations and are proving safe in clinical trials18–20. However, therapeutic applications have been limited by inefficient transduction of target cell populations. A prime example is gene transfer to the CNS; the transduction efficiency of currently available vectors in the mouse CNS after intravenous administration is at least an order of magnitude lower than in the liver21. The transduction efficiency of rAAVs can be enhanced by creating AAV capsid libraries and selecting for variants with more desirable characteristics8–13. This approach, however, has not generated more effective gene delivery vehicles for global CNS transduction. Because the highly selective blood-brain barrier (BBB) and the cellular heterogeneity of the CNS present challenges for gene transfer to the CNS through the vasculature, we reasoned that it would be beneficial to provide selective pressure for capsids that cross the BBB and functionally transduce specific cell types. To meet this need, we devised CREATE—a Cre recombination–dependent approach to selectively recover capsids that transduce predefined Cre-expressing target cell populations (Fig. 1a).

CREATE uses an rAAV capsid genome (rAAV-Cap-in-cis-lox) that couples a full-length AAV cap gene, controlled by regulatory elements from the AAV rep gene (Fig. 1b and Online Methods), with a Cre-invertible switch (Fig. 1b). By building capsid libraries within the rAAV-Cap-in-cis-lox backbone and delivering the virus libraries to animals with Cre expression in a defined cell population, the system enables the selective amplification and recovery of sequences that have transduced the target population (Fig. 1c). Because the rAAV-Cap-in-cis-lox genome lacks a functional rep gene, rep must be provided in trans for virus production. For this purpose, we modified an AAV2/9 Rep-Cap plasmid to eliminate capsid protein expression by inserting in-frame stop codons within the reading frame for each capsid protein, VP1–VP3 (Fig. 1d). These stop codons do not alter the amino acid sequence of the assembly-activating protein (AAP), which is expressed from an alternative reading frame within the cap gene22. This split rAAV-Cap-in-cis-lox genome and Rep-AAP AAV helper system efficiently generates rAAV (Fig. 1e) and is the foundation of the CREATE selection system, which enables capsid sequence recovery from genetically defined Cre-expressing cell populations within heterogeneous tissue samples (see Supplementary Fig. 1).

Within the rAAV-Cap-in-cis-lox acceptor genome, we generated a library of AAV variants by inserting 7 amino acids of randomized sequence (7-mer) between amino acids 588 and 589 (the VP1 position) of the AAV9 capsid (Fig. 1f,g). To select for vectors that crossed the BBB and transduced cells throughout the CNS, we administered the capsid library intravenously to adult GFAP-Cre mice, which express Cre in astrocytes23. One week later, we isolated DNA from brain and spinal cord tissue and recovered capsid sequences by PCR from viral genomes that had undergone Cre-mediated recombination. We cloned the entire library of recovered cap sequences back into the rAAV-Cap-in-cis-lox acceptor genome to generate the library GFAP1 and randomly chose 13 clones for sequencing. All tested sequences recovered from the GFAP1 plasmid library were unique, and therefore we used the GFAP1 library to generate a second virus library (GFAP2) and performed an additional round of selection in GFAP-Cre mice. After the second selection, several variants were enriched (Supplementary Fig. 2a) and showed enhanced CNS transduction (Supplementary Fig. 2b). We chose the most enriched variant, AAV-PHP.B, which represented 25% of recovered library sequences and encodes the 7-mer sequence TLAVPFK, for further tropism evaluation in vivo.

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AAV-PHP.B and AAV9 capsids were used to package a single-stranded (ss) GFP reporter vector driven by the ubiquitous CAG promoter (ssAAV-CAG-GFP). Both AAV-PHP.B and AAV9 produced virus with similar efficiencies (Supplementary Fig. 2c). We next administered 1 × 10^{12} vector genomes (vg) of either vector to 6-week-old mice by intravenous injection and assessed transduction by GFP.
expression 3 weeks later. Our data show that AAV-PHP.B transduced the entire adult CNS with high efficiency, as indicated by imaging GFP immunohistochemistry (IHC) (Fig. 2a) or native eGFP fluorescence in several brain regions (Fig. 2b), spinal cord (Fig. 2c) and retina (Fig. 2d). Using PARS-based CLARITY for whole-body tissue clearing24, we imaged native eGFP fluorescence through cleared sections of tissue from the spinal cord (Fig. 2c), cortex and striatum (Fig. 2e). These three-dimensional (3D) renderings (also see Supplementary Videos 1–3) further demonstrate the broad and efficient CNS transduction with the AAV-PHP.B vector. Outside the CNS, the cellular level tropism of AAV-PHP.B and AAV9 appeared similar in several organs, with the exception of the pancreas, where the efficiency of transduction by AAV-PHP.B was lower than with AAV9 (Fig. 2f).

To quantify the efficiency of gene transfer to the CNS and peripheral organs by AAV-PHP.B as compared with AAV9, we measured the number of viral genomes present in several brain regions and organs 25 d after injection (Fig. 2g). AAV-PHP.B provided significantly greater gene transfer than AAV9 to each of the CNS regions examined: cortex (40-fold), striatum (92-fold), thalamus (76-fold), cerebellum (41-fold) and spinal cord (75-fold). Vector genome biodistribution outside the CNS showed that AAV-PHP.B transferred genes to the pancreas and adrenal gland less efficiently than did AAV9 (Fig. 2g). No significant differences were found between the two vectors in the liver, heart, skeletal muscle and kidneys. When these data were considered together with the CNS biodistribution data, in all CNS areas except the cerebellum, the number of viral genomes detected in mice treated with AAV-PHP.B was similar to that measured in the liver, an organ efficiently transduced by AAV9 (refs. 21,25), and greater than that observed in other organs. In contrast, AAV9-mediated gene transfer to each of the examined CNS regions was at least 120-fold lower than in the liver. Therefore, although the tropism of AAV-PHP.B is not CNS specific, the enhanced gene transfer characteristics of this vector are CNS specific.

AAV9 preferentially transduces astrocytes when delivered intravenously to adult mice and nonhuman primates, but it also transduces neurons in several regions14,26. To examine the cell types transduced by AAV-PHP.B, we analyzed the localization of GFP with several cell-type markers. Owing to the highly efficient transduction, individual GFP-expressing astrocytes were difficult to discern in mice that received 1 × 10^{12} vg of AAV-PHP.B (Fig. 2a), but they could be more easily identified morphologically by their highly ramified processes in animals that received tenfold less virus (Fig. 2b,a) and by colocalization with IHC for GFAP (Fig. 3a). In addition to astrocytes, AAV-PHP.B transduced CC1+ oligodendrocytes (Fig. 3b) and NeuN+ neurons throughout the brain (Fig. 3c) as well as several neuronal subtypes including midbrain tyrosine hydroxylase (TH)+ dopaminergic neurons (Fig. 3d), calbindin+ cerebellar Purkinje cells (Fig. 3e) and several interneuron populations (Supplementary Fig. 3a–d). AAV-PHP.B also transduced CD31+ endothelial cells (Supplementary Fig. 3e) but did not appear to transduce Iba1+ microglia (Supplementary Fig. 3f,g). The paucity of GFP+ microglia seen after intravenous AAV-PHP.B delivery is consistent with previous reports of rare or nonexistent AAV-mediated gene expression in this cell population14,27–30. We have observed native GFP expression throughout the brain.

**Figure 3** AAV-PHP.B transduces multiple CNS cell types more efficiently than AAV9. (a–e) AAV-PHP.B transduces astrocytes, oligodendrocytes and neurons. Images show GFP expression 3 weeks after intravenous injection of 1 × 10^{11} vg (a) or 1 × 10^{12} vg (b–e) of ssAAV-PHP.B:CAG-GFP into adult mice. (a) MIP image of GFP (green) and GFAP (magenta) IHC in the hippocampus. (b) GFP and CC1 (magenta) IHC in the cortex. Numbered boxes highlight examples of double-positive cells. Corresponding single-channel images are shown at right. Asterisks highlight cells without detectable GFP expression. (c) GFP and NeuN IHC in the indicated brain region. (d) MIP image of GFP fluorescence and TH IHC in the midbrain. (e) GFP and calbindin (Calb) IHC in the cerebellum. (f) Quantification of the percentage of cells positive for NLS-GFP in the indicated brain region 3 weeks after injection. (g) AAV-PHP.B transduces ChAT+ spinal motor neurons. Images show native NLS-GFP fluorescence and IHC for ChAT in whole transverse spinal cord sections (left) or ventral horn MIP image (right). The percentage of ChAT+ neurons that expressed NLS-GFP in each spinal cord region is given ± the 95% confidence interval. For quantification, n = 5 per group; mean ± s.d; all pairs of AAV9 versus AAV-PHP.B means were found to be different (*** P < 0.001) unpaired t tests corrected for multiple comparisons by the Holm-Sidak method. Scale bars, 20 µm (a,b,d), 50 µm (c,g, right), 200 µm (e) and 1 mm (g, left).
Figure 4 AAV-PHP.A exhibits efficient transduction of CNS astrocytes and reduced tropism for peripheral organs. (a–c,e) Images show GFP expression 3 weeks after intravenous injection of 3 × 10^{11} of ssAAV9:CAG-GFP or ssAAV-PHP.A:CAG-GFP into adult mice. (a,b) Representative images of GFP IHC. (c) GFP IHC (green) and GFAP IHC (magenta) in the hippocampus. Numbered boxes highlight examples of GFP^+GFAP^+ cells. Corresponding single-channel images are shown on the right. (d) 2.5 × 10^{11} vg of ssAAV-PHP.A:CAG-NLS-GFP was injected intravenously into adult mice. Graphs show quantification of the percentage of Aldh1L1^+ (blue) and NeuN^+ cells (orange) positive for NLS-GFP. (e) Native GFP expression in the liver (green) and tissue autofluorescence (magenta). (f) AAV biodistribution in the brain (left) and peripheral organs (right) 25 d after intravenous injection of 1 × 10^{11} vg into adult mice. (e,f) n = 4 per group; mean ± s.d.; *P < 0.05, **P < 0.01, ***P < 0.001, unpaired t-tests corrected for multiple comparisons by the Holm-Sidak method. Scale bars, 1 mm (a); 100 μm (b); 50 μm (c,e).

lasting over a year after administration of AAV-PHP.B, suggesting that AAV-PHP.B can provide long-term, CNS-directed transgene expression (Supplementary Fig. 4).

We next quantified the fractions of several cell types transduced by AAV-PHP.B as compared to AAV9. To facilitate reliable individual cell counting, we constructed a vector expressing a nuclear-localized GFP (NLS-GFP) under the control of the CAG promoter, ssAAV-CAG-NLS-GFP. A single injection of 2 × 10^{12} vg/mouse of ssAAV-PHP.B:CAG-NLS-GFP transduced the majority of Aldh1L1^+ astrocytes (Fig. 3f and Supplementary Fig. 5a) and NeuN^+ neurons (Fig. 3f and Supplementary Fig. 5b), as well as a modest fraction of Olig2^+ oligodendrocyte lineage cells (Fig. 3f and Supplementary Fig. 5c), across all brain regions examined. In all cases, AAV-PHP.B provided significantly enhanced transduction as compared to the same dose of AAV9. Notably, AAV-PHP.B also transduced over 94% of Chat^+ motor neurons throughout the spinal cord (Fig. 3g), 91.4 ± 1.6% of TH^+ midbrain dopaminergic neurons (Supplemental Fig. 5d) and 91.7 ± 5.8% of calbindin^+ Purkinje cells (n = 5). In sum, adult intravenous administration of AAV-PHP.B efficiently targets multiple neuronal and glial cell types in the mouse CNS.

The method used to identify AAV-PHP.B selects only for transduction of the target cell population; it does not necessarily select for specificity. Nevertheless, in a separate trial in GFAP-Cre mice, we identified (after two rounds of in vivo selection) another AAV capsid variant, AAV-PHP.A, with the 7-mer sequence, YTLSQGW, that exhibits both more efficient and selective CNS astrocyte transduction (Fig. 4a–d) and reduced tropism for the liver (Fig. 4e,f) and other peripheral organs (Fig. 4g) as compared to AAV9. The increase in specificity for gene transfer to the CNS over the liver provided by AAV-PHP.A versus AAV9 is 400- to 1,200-fold, resulting from a combination of enhanced adult CNS gene transfer (2.6- to 8-fold more depending on the specific region) and reduced liver gene transfer (152-fold). Two other variants enriched in this trial (Supplementary Fig. 2a) did not show enhanced GFP expression in CNS neurons or glia as compared with AAV9.

To determine whether AAV-PHP.A and AAV-PHP.B can also transduce human neural cells, we tested them on cortical neurons and astrocytes derived from human induced pluripotent stem cells (hiPSCs) using a 3D differentiation method. hiPSC lines from two individuals were differentiated into 3D cerebral cortex–like structures (cortical spheroids) and maintained in vitro for up to 200 d. Aged cortical spheroids contain superficial- and deep-layer cortical excitatory neurons and up to 20% astrocytes. In dissociated cortical spheroids that were exposed to the three viruses in monolayer, AAV-PHP.B more efficiently transduced both GFAP-expressing astrocytes and MAP2-expressing neurons than did either AAV9 or AAV-PHP.A (Supplementary Fig. 6; two-way ANOVA, P < 0.01, n = 3). In addition, all three viruses were capable of transducing intact 3D cortical spheroids (Supplementary Fig. 6c).

Using CREATE, we have developed new AAV variants that enable efficient, widespread gene transfer to the adult mouse CNS after intravenous administration. An important advantage of this system is that it introduces selective pressure for capsids that mediate efficient intracelular trafficking and conversion of the single-stranded viral genome to persistent double-stranded DNA forms necessary for long-term transduction (only the double-stranded DNA genomes should serve as substrates for Cre). This additional selective pressure
for functional capsids may have contributed to the identification of AAV-PHP.A and the AAV-PHP.B variants in independent trials after only two rounds of in vivo selection. In comparison, many previous in vivo and in vitro AAV capsid selection methods have applied 3–10 rounds of selection to identify capsid variants with enhanced properties.\(^9\)\(^{13}\)\(^{32}\)

In principle, CREATE could be applied to develop AAV capsid variants that target defined, Cre-expressing cell types in any organ. Thus, it could be used not only in transgenic animals, as shown here, but also to develop AAV variants that target (i) specific Cre\(^+\) cell types in spheroid cultures\(^31\) or organoid cultures; (ii) cells made Cre\(^+\) in nontransgenic animals by, for example, viral injections that achieve population-\(^+\), projection-\(^35\), or activity-based Cre expression\(^34\)\(^{35}\); or (iii) Cre\(^+\) human cells in human-mouse chimera animals. Given the reported AAV tropism differences between animal models and humans\(^11\), selection schemes that use human Cre\(^+\) cells in vivo, cellspecific Cre expression in three-dimensional hiPSC-derived cellular models, or future Cre transgenic marmosets\(^36\) may be desirable for developing improved vectors for clinical applications. In addition, the success of AAV-based gene therapies, especially those requiring systemic delivery, can be hindered by the presence of neutralizing AAV capsid antibodies in the human population\(^37\)–\(^39\). By using CREATE together with exposure of AAV libraries to pooled human sera, one could envision simultaneously selecting for capsids with retained or enhanced transduction characteristics that are also less susceptible to antibody-mediated neutralization.

A limitation of CREATE and other reported capsid selection methods is that it is difficult to predict, beyond an increase in target cell transduction efficiency, what characteristics the enriched variants will have before they are tested. In our two trials for astrocyte targeting, we identified several variants (AAV-PHP.B, AAV-PHP.B2 and AAV-PHP.B3; Supplementary Fig. 2) that provide broad CNS transduction of both neurons and glia, and one (AAV-PHP.A) that provides selectively more efficient astrocyte transduction. Identification of capsids with distinct properties from the same selection scheme was expected given that the recovery method we used selected for astrocyte transduction rather than for any specific intermediate step(s), such as brain vascular association, BBB transcytosis or astrocyte binding and internalization. Therefore, capsid variants that are more efficient at any of these intermediate steps should be recovered in our selection process. Indeed, by immunostaining for capsids we found that, unlike AAV9, both AAV-PHP capsids readily localized to the brain vasculature shortly after intravenous administration (Supplementary Fig. 7a,b). In addition, by 24 h after administration, significantly more GFP-expressing cells were observed along the brain vasculature of mice that received AAV-PHP.B than in those that received AAV9 or AAV-PHP.A (Supplementary Fig. 7c,d). Considered together with the transduction characteristics of AAV-PHP.B and AAV-PHP.A in vivo (Figs. 2–4) and in human neural cultures (Supplementary Fig. 6), these data suggest that although both AAV-PHP vectors more efficiently associate with the brain vasculature, they may differ in subsequent cell-type-specific entry or transport step(s).

In the future, CREATE could be used with next-generation sequencing to better predict the characteristics of the recovered sequences before testing the variants individually. Sequencing the entire pool of variants recovered from the Cre-expressing target cells along with the unselected virus library should enable quantification of the relative extent of enrichment of each recovered sequence. Furthermore, sequencing capsids recovered from multiple Cre-expressing or non-Cre-expressing populations could provide a means to perform both positive and negative selection in multiple cell types in a single experiment. Such in vivo–in silico selection approaches should increase the power of CREATE to enhance gene transfer to the CNS and other difficult-to-target cell populations.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** GenBank: AAV capsid sequences, KU056473, KU056474, KU056475 and KU056476.

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**AUTHOR CONTRIBUTIONS**

R.E.D. designed and performed experiments, analyzed data, prepared figures and wrote the manuscript. P.L.P., B.P.S., S.R.K., A.B. and K.Y.C. performed experiments, virus production and characterization. W.-L.W. performed tissue processing and IHC. B.Y. assisted with tissue clearing and imaging. N.H. and S.P.P. performed the experiments with human cells, analyzed the data, and prepared the associated figure and text. V.G. helped with study design and data analysis, manuscript and figure preparation and supervised the project. All authors edited and approved the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Plasmids. The rAAV-Cap-in-cis-lox genome plasmid contains three main flanking elements from AAV2 ITRs: (i) an mCherry expression cassette, which comprises a 398-bp fragment of the human UBC gene upstream of the mCherry cDNA followed by a synthetic polyadenylation sequence43; (ii) the AAV9 capsid gene and regulatory sequences, which includes the AAV5 p41 promoter sequence (residues 1680–1974 of GenBank: AF085716.1) (refs. 41,42); and splicing sequences taken from the AAV2 rep gene; and (iii) a Cre-dependent switch, which is made from a SV40 polyadenylation sequence (pa) flanked by inverted lox71 and lox66 sites43 (Fig. 1b). The rAAV-Cap-in-cis-lox genome plasmid was further modified to introduce two unique restriction sites, XbaI and AgeI, within the capsid sequence. These sites flank the region (amino acids 450–592) that is replaced by the randomized library fragment. The introduction of the XbaI site introduces a K49R mutation, which does not have an overt effect on vector production or transduction. The mutations required to insert the AgeI site are silent. For the rAAV-ΔCap-in-cis acceptor plasmid used for the capsid library cloning, the coding region between the XbaI and AgeI sites was removed to prevent virus production from the acceptor plasmid lacking the library fragment.

As a template for the library fragment, we PCR amplified the region spanning the XbaI and AgeI sites of the modified AAV9. This sequence was modified to remove a unique EarI restriction site and insert a unique KpnI site (both silent mutations) to create the xE fragment. The modified xE fragment was TA cloned into pCRII (Life Technologies) to generate pCRII-9Cap-xE. Eliminating the EarI site provided a second method that could be used, if necessary, to selectively digest contaminating (AAV9) capsid sequences recovered by PCR, but not digest the library-derived sequences. We did not find it necessary to use this digestion step. Using the rAAV-ΔCap-in-cis acceptor for cloning the libraries and taking standard PCR precautions (e.g., UV treating reagents and pipettors) was sufficient to prevent contamination.

The AAV2/9 REP-AAP helper plasmid was constructed by introducing five stop codons into the coding sequence of the VP repeat protein, which is encoded within an alternative reading frame.

Several rAAV genomes were used in this study. Each is constructed within a single strand (ss) rAAV genome with a reporter driven by the ubiquitous CMV-β-Actin-intron-β-Globin hybrid promoter (CAG). For simplicity, the vector descriptions have been abbreviated in the text. ssAAV-CAG-GFP refers to ssAAV-CAG-eGFP-2A-MgL2-WPRE-SV40 polyA. ssAAV-CAG-NLS-GFP refers to ssAAV-CAG-NLS-GFP-WPRE-SV40 polyA, which was constructed by inserting the nuclear localization sequence of a human growth hormone polysomal polyA signal. The mNeonGreen44 was modified with the membrane targeting sequence PKKKRKV at both the N- and C-termini of GFP. ssAAV-CAG-mNeonGreen-f refers to ssAAV-CAG-mNeonGreen-f-WPRE with a human growth hormone polyA signal. The mNeonGreen44 was modified with the membrane targeting (farnesylation and palmitoylation signals) sequence c-h-Ras45.

Capsid library generation. The random 7-mer library fragment (inserted between amino acids 588 and 589) was generated by PCR using Q5 Hot Start High-Fidelity DNA Polymerase (NEB; M0493), primers XF and 7xMNN and pCRII-9Cap-xE as a template. A schematic showing the approximate primer bind-

In vivo selection. For the selections in GFAP-Cre mice, 1 × 1011 vg of the capsid libraries were injected intravenously into adult Cre+ mice of either sex. Seven to eight days post-injection, mice were euthanized and the brain and spinal cord were collected. Vector DNA was recovered from one hemisphere of the brain and half of the spinal cord using 4·5 ml of Trizol (Life Technologies; 15596). To purify viral DNA, the upper aqueous fraction was collected according to the manufacturer’s extraction protocol. We found that the aqueous fraction contains a significant portion of the viral DNA genomes as well as RNA. RNA was then digested by treatment with 1 ul. of RNase A (Qiagen) at 37 °C overnight. Next, a two-step PCR amplification strategy was used to selectively recover cap sequences from Cre-recombined genomes. The first amplification step preferentially amplifies Cre-recombined rAAV-Cap-in-cis-lox sequences using the primers 9Cap-cdf (see Supplementary Fig. 1). The PCR was performed for 20–26 cycles of 95 °C for 20 sec, 60 °C for 20 sec and 72 °C for 30 sec using Q5 Hot Start High-fidelity DNA Polymerase. The PCR product was then diluted 1:10 or 1:100 and then used as a template for a second Cre-independent PCR reaction using primers XF and AR (Supplementary Fig. 1c). The second PCR generated the fragment that was cloned back into the rAAV-Cap-in-cis acceptor plasmid as described above. 1 µl of the Gibson Assembly reactions were then diluted 1:10 and transformed into SURE2 competent cells (Agilent; 200152) to generate individual clones for sequencing.

Variants that showed evidence of enrichment were cloned into an AAV Rep-Cap plasmid and transformed into DH5α competent cells (NEB). The novel AAV Rep-Cap variants, or AAV2/9 Rep-Cap as a control, were then evaluated using one of the reporter genomes described above.

Vector biodistribution. Six-week-old female C57Bl/6 mice were injected intravenously with 1 × 1011 vg of the ssAAV-CAG-GFP vector packaged into the indicated AAV capsid. Animals were randomly assigned to groups. 25 days after injection, the mice were euthanized and tissues and indicated brain regions were collected and frozen at −80 °C. DNA was isolated from the tissue samples using QIA Quick DNA Blood and Tissue kit. Vector genomes were detected using PCR primers that bind to the WPRE element and were normalized to mouse genomes using primers specific to the mouse glucagon gene. Absolute quantification was performed using serial dilutions of linearized plasmid standards of known concentration46. One randomly chosen animal injected with AAV-PHP.B was removed from the bio-distribution study for histological analysis.
Tissue preparation, immunohistochemistry and imaging. Mice were anesthetized with Nembutal and transcardially perfused with 0.1 M phosphate buffer (PB) at room temperature (RT) at pH 7.4 and then with freshly prepared, ice-cold 4% paraformaldehyde (PFA) in PB. Brains were post-fixed in 4% PFA overnight and then sectioned by vibratome or cryoprotected and sectioned by cryostat. IHC was performed on floating sections with primary and secondary antibodies in PBS containing 10% goat or donkey serum and 0.5% Triton X-100 (no detergent was used for GAD67 staining). Primary antibodies used were mouse anti-AAV capsid (1:20; American Research Products, 03-65158, clone B1), rabbit anti-GFP (1:1000; Invitrogen, A11122), chicken anti-GFP (1:1,000; Abcam, ab13970), mouse anti-CC1 (1:200; Calbiochem, OP80), rabbit anti-GFAP (1:1,000; Dako, Z0334), mouse anti-NeuN (1:500; Millipore, AB377), rabbit anti-Iba1 (1:500; Biocare Medical, CP290), mouse anti-Calbindin D28K (1:200; Sigma, CB-955), rabbit anti-Calretinin (1:1,000; Chemicon, AB5054), mouse anti-GADD67 (1:1,000; Millipore, MB-AB406), guinea pig anti-MAP (1:1,000; Synaptic Systems, 880004), mouse anti-Parvalbumin (1:1,000; Sigma), Tyrosine Hydroxylase (1:1,000, Aves) and rabbit anti-CD31 (1:50; Abcam, ab28364). Primary antibody incubations were performed for 16–24 hrs at RT. The sections were then washed and incubated with secondary Alexa-conjugated antibodies (1:1,000; Invitrogen) for 2–16 hrs. For capsid detection with the B1 antibody that recognizes an internal epitope, floating sections were treated with 2 M HCl for 15 minutes at 37 °C and then washed extensively with PBS prior to incubation with the primary antibody. For some images, the 16-bit green channel (GFP) gamma was adjusted to enable visualization (without oversaturation) of both low and high GFP expressing cells present within the same field of view. In all cases, changes to gamma or contrast as well as microscope and laser settings remained consistent across sets of images. Images were taken with a Zeiss LSM 780 confocal microscope fitted with the following objectives: Fluar 5x/0.25 M27 Plan-Apochromat 10×/0.45 M27 (working distance 2.0 mm), Plan-Apochromat 25×/0.8 Imm Corr DIC M27 multi-immersion and LD C-Apochromat 40×/1.1 W Korr and Plan-Apochromat 100×/1.46 Oil DIC objectives. 3D MIP images and supplementary videos were generated with Imaris (Bitplane).

Quantification of cell type–specific transduction. Six-week-old female mice were randomly assigned to groups and injected intravenously with 2 × 1012 vg ssAAV-CAG-NLS-GFP packaged into AAV9, AAV-PHP.B or AAV-PHP.A. Animals per group was pre-established; no animals were excluded from the experiment. Spheroids derived from two healthy control individuals were grown on inactivated mouse embryonic fibroblast feeder cells in the following medium: DMEM/F12, Knockout Serum 20%, 1 mM non-essential amino acids (1:100), Glutamax (1:200), β-mercaptoethanol (0.1 mM), penicillin and streptomycin (1:100) (Life Technologies), and 10–15 ng/mL FGFR2 (R&D Systems). Cultures were regularly tested and maintained mycoplasma free. Colonies of iPSCs were detached intact with dispase (0.35 mg/mL, Invitrogen) and transferred into low-attachment plates in iPSC medium supplemented with dorsomorphin (5 μM, Sigma) and SB-431542 (10 μM, Tocris), and the medium was changed daily. On day six of in vitro differentiation, neural spheroids were transferred to neural medium (NM; Neurobasal A, B27 without vitamin A, Glutamax (1:100), penicillin and streptomycin; Life Technologies), which was supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL) until day 24, and then supplemented with BDNF (20 ng/mL) and NT3 (20 ng/mL) from day 25 to 42. From day 43 onwards, cortical spheroids were maintained in NPC medium, which was changed every 4 days.

Dissociation and viral infection of cortical spheroids. For enzymatic dissociation and culture in monolayer, cortical spheroids at day 170–200 of in vitro differentiation (two independent neural differentiations of one iPSC line from one individual and one differentiation of an iPSC line from another individual) were incubated with Accutase (Innovative Cell Technologies) for 25 min at 37 °C, washed three times with NM and gently triturated with a P-200 pipette. Cells were plated on polystyrene and laminin coated glass coverslip (15 μm) at ~300,000 cells/well and maintained in NPC media supplemented with BDNF (20 ng/mL) and NT3 (20 ng/mL) for the first 24 hrs, and then maintained in NM without growth factors.

Cultures grown on coverslips were infected with each of the viruses at a titer of 1 × 109 vg/well and fixed 5 days later with 4% paraformaldehyde (PFA) for 10 min. For immunocytochemistry, cells were permeabilized with 0.2% Triton X-100 for 10 min and blocked with 10% goat serum in PBS for 1 hr. Coverslips were then incubated with antibodies diluted in blocking solution for 2 hr. Nuclei were visualized with Hoechst 33258 (Life Technologies, 1:10,000).

Cells were imaged with a Zeiss M1 Axioscope using a 40× objective. The proportion of GFP+ cells colabeled with either GFAP or MAP2 was quantified in images of 10 random fields per coverslip for each experimental condition. Results presented are the average of two separate dissociation and infection experiments.

To infect intact 3D cultures with AAVs, single human cortical spheroids at day 197 of in vitro differentiation were transferred overnight into 1.5 ml Eppendorf tubes containing 6 × 109 vg/400 μL in NPC media, and were fixed 7 days later in 4% PFA overnight. Fixed spheroids were then transferred into 30% sucrose for 24 hrs, embedded in O.C.T. (Fisher Scientific) and cut at 14 μm sections. For immunohistochemistry, sections were blocked with 10% goat serum in PBS containing 0.3% Triton-X100 for 1 hr. Images were collected with a Leica TCS SP8 confocal microscope.

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