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# Adeno-Associated Virus Toolkit to Target Diverse Brain Cells

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## Keywords

targeted brain delivery, systemic delivery, adeno-associated virus, AAV, viral capsid engineering, viral cargo

## Abstract

Recombinant adeno-associated viruses (AAVs) are commonly used gene delivery vehicles for neuroscience research. They have two engineerable features: the capsid (outer protein shell) and cargo (encapsulated genome). These features can be modified to enhance cell type or tissue tropism and control transgene expression, respectively. Several engineered AAV capsids with unique tropisms have been identified, including variants with enhanced central nervous system transduction, cell type specificity, and retrograde transport in neurons. Pairing these AAVs with modern gene regulatory elements and state-of-the-art reporter, sensor, and effector cargo enables highly specific transgene expression for anatomical and functional analyses of brain cells and circuits. Here, we discuss recent advances that provide a comprehensive (capsid and cargo) AAV toolkit for genetic access to molecularly defined brain cell types.

## Contents

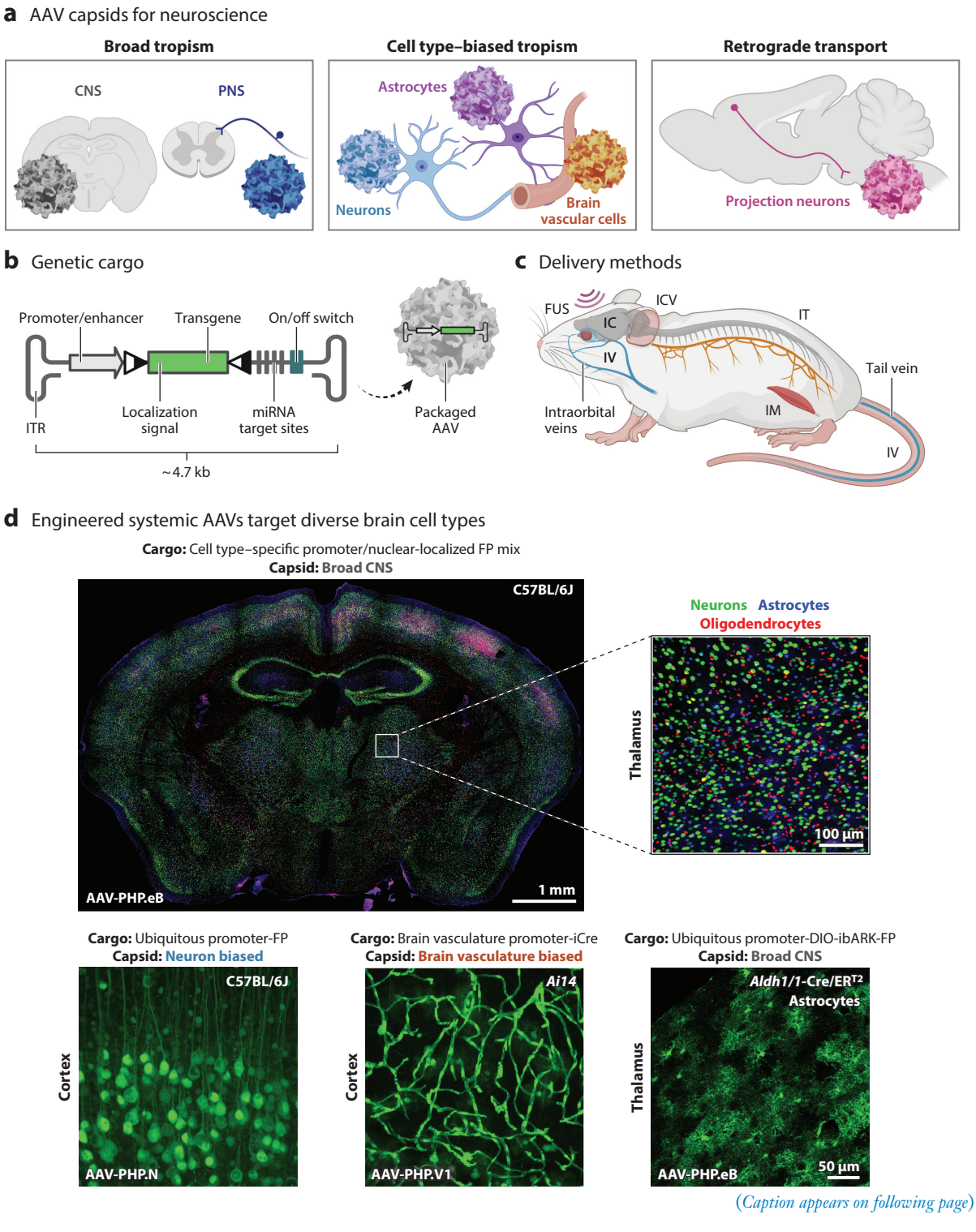


## 1. INTRODUCTION

Recombinant adeno-associated viruses (AAVs) are prominent gene delivery vehicles and the vector of choice for neuroscience research (Bedbrook et al. 2018, Haery et al. 2019) and for gene therapies for neurological disorders (Deverman et al. 2018, Hudry & Vandenberghe 2019). AAVs are well suited for transgene delivery due to their ability to transduce dividing and nondividing cells for long-term transgene expression in humans and animal models, including mice, rats, and nonhuman primates (NHPs) (Srivastava 2016). Naturally occurring serotypes have been widely used for delivery of reporters, sensors, and effectors to analyze brain structure and function, but their limited efficiency and precision often necessitates invasive routes of administration and complex cargo design strategies. One of the main challenges for advancing gene delivery to the central nervous system (CNS) is generating precisely targeted, minimally invasive vectors that maximize cargo transgene capacity. The maturation of AAVs as cell type– and tissue-specific vectors has involved engineering both the viral capsid, by identifying variants with more specific and efficient entry into the brain (**Figure 1*a***) (see Section 2 below), and the viral genome, by identifying short and specific gene regulatory elements (within the 4.7-kb size limit) to control transgene expression (**Figure 1*b***) (see Section 4 below). These efforts are mutually beneficial and readily combined to create an AAV toolbox for targeted gene delivery to the brain (**Figure 1*c*,*d***). Here, we discuss recently engineered AAV capsids and cargo for genetic access to diverse brain cell types.

## 2. ENGINEERING AAVs FOR THE BRAIN

While many naturally occurring AAV serotypes exist, their tropisms are broad and largely overlapping. Many serotypes broadly transduce neurons and glia in the brain after direct intraparenchymal injection (Aschauer et al. 2013, Watakabe et al. 2015). A subset (the most studied of which is AAV9) can cross the blood-brain barrier (BBB) (albeit inefficiently, especially in adults) to transduce brain cells after intravenous delivery (Ellsworth et al. 2019, Foust et al. 2009,




(Caption appears on following page)

**Figure 1** (*Figure appears on preceding page*)

AAV toolkit for targeted gene delivery to the brain. The AAV (*a*) capsid, (*b*) cargo, and (*c*) delivery method determine cell type– or region-specific transgene expression. (*a*) Several engineered capsids with unique tropisms are now available. These include variants with broad CNS or PNS transduction, cell type specificity, or retrograde transport in neurons (**Supplemental Table 1**). (*b*) Genetic cargo can be customized to control transgene expression. Gene regulatory elements [promoters and enhancers (**Supplemental Table 2**), miRNA target sites, and recombination target sequences, denoted by *triangles*] facilitate tissue or cell type specificity. An on/off genetic switch can be used for expression control. The packaging capacity of AAVs is ~4.7 kb. (*c*) Viral delivery methods include FUS, IC, ICV, IM, IT, and IV delivery. In mice, IV injections are typically administered in the lateral tail vein or retro-orbital sinus. IT injections are administered in the cisterna magna or lumbar spine. (*d*) Engineered systemic AAVs target diverse brain cell types. (*Top*) Widespread and targeted gene delivery to the brain after a single intravenous injection of AAV-PHP.eB. Nuclear-localized fluorescent reporters were targeted to neurons (*green*), astrocytes (*blue*), or oligodendrocytes (*red*) using the hSyn1, GFAP, or MBP promoters, respectively. (*Bottom*, *left*, and *middle*) Cell type–biased AAV capsids enforce unique tropisms with ubiquitous or cell type–specific promoters. AAV-PHP.N (neuron biased) and AAV-PHP.V1 (brain vasculature biased) (both identified in Ravindra Kumar et al. 2020) are shown. (*Bottom*, *right*) AAV-PHP.eB restricts transgene expression to astrocytes in astrocyte-specific *Aldh1l1*-Cre/ER$^{T2}$ mice (Nagai et al. 2021). Panel adapted with permission from Nagai et al. (2021); copyright 2021 Elsevier. The promoters used in panel *d* (*bottom*) include the ubiquitous CAG promoter and the brain vasculature–specific Ple261 promoter. Panels *a–c* adapted from images created with BioRender.com. Abbreviations: AAV, adeno-associated virus; CAG, synthetic promoter containing the cytomegalovirus early enhancer element, the promoter, first exon, and first intron of the chicken beta-actin gene and the splice acceptor from the rabbit beta-globin gene; CNS, central nervous system; DIO, double-floxed inverted orientation; FP, fluorescent protein; FUS, focused ultrasound; GFAP, glial fibrillary acidic promoter; hSyn1, human synapsin I promoter; IC, intracranial; iCre; improved Cre recombinase; ICV, intracerebroventricular; ibARK, inhibitory peptide from beta-adrenergic receptor kinase 1; IM, intramuscular; IT, intrathecal; ITR, inverted terminal repeat; IV, intravenous; MBP, myelin basic protein promoter; miRNA, microRNA; PNS, peripheral nervous system.

**Supplemental Material >**

Gray et al. 2011b, Yang et al. 2014). However, both strategies have significant drawbacks. Intracranial injections are invasive and result in poor coverage of large or dispersed brain regions, cells, and circuits. Intracranial injections can also produce a spatial gradient of transgene expression due to diffusion of viral particles from the injection site (Watakabe et al. 2015). Intravenous injections require high viral doses and result in transduction of peripheral organs (Gray et al. 2011b, Yang et al. 2014, Zincarelli et al. 2008), including the liver, a site of potential toxicity at high doses (Feldman et al. 2020, Hinderer et al. 2018). Intra–cerebrospinal fluid (CSF) delivery (e.g., by intrathecal injection) bypasses the BBB but is moderately invasive and lacks specificity (Gray et al. 2013, Hinderer et al. 2014). Potent, targeted, noninvasive vectors are needed to realize the promise of AAVs as research tools and human gene therapies.

To enhance and refine AAV tropisms, the mutational tolerance of AAV capsids has been extensively studied. Permissive sites for rational or random amino acid (AA) substitution and insertion have been identified (Adachi et al. 2014, Boucas et al. 2009, Girod et al. 1999, Maheshri et al. 2006, Ogden et al. 2019, Perabo et al. 2006, Rabinowitz et al. 1999, Vandenberghe et al. 2009), and methods for serotype-shuffled chimeric capsid construction have been developed (Grimm et al. 2008; Ho et al. 2013; Koerber et al. 2008, 2009; Li et al. 2008). Diverse strategies for generating and screening libraries of capsid variants for altered tropism through rounds of selection in cell culture or in vivo have yielded an ever-expanding array of engineered AAV capsids for studying the brain (**Supplemental Table 1**). The promise of these strategies is fourfold: (*a*) that recalcitrant cell populations may be targeted, (*b*) that transgene capacity is maximized (by reducing reliance on lengthy gene regulatory elements), (*c*) that immune reactions may be minimized (by, for example, reducing uptake by antigen-presenting cells), and (*d*) that dosage may be reduced (by redirecting off-target uptake to the cells under study).

## 2.1. CREATEing AAVs for Efficient, Noninvasive, and Targeted Gene Delivery to the Mouse Brain

Cre recombination–based AAV targeted evolution (CREATE) can be used to screen libraries of capsid variants for those capable of transducing specific cells of transgenic Cre-expressing

animals (Deverman et al. 2016). Successful variants are identified by Cre-dependent polymerase chain reaction amplification of the variants' Lox site–containing genomes (**Figure 2**, steps 1–3). CREATE identified variants of AAV9 (containing a randomized 7-mer peptide insertion between AA588–589) with enhanced CNS (AAV-PHP.B) or peripheral nervous system (AAV-PHP.S) transduction (Chan et al. 2017, Deverman et al. 2016). AAV-PHP.B and its matured variant AAV-PHP.eB, with further enhanced CNS transduction endowed by modification of insertion-adjacent residues (Chan et al. 2017), have been widely used for CNS applications (**Supplemental Table 3**). These vectors, for the first time, enabled noninvasive and broad genetic access to the adult mouse nervous system.

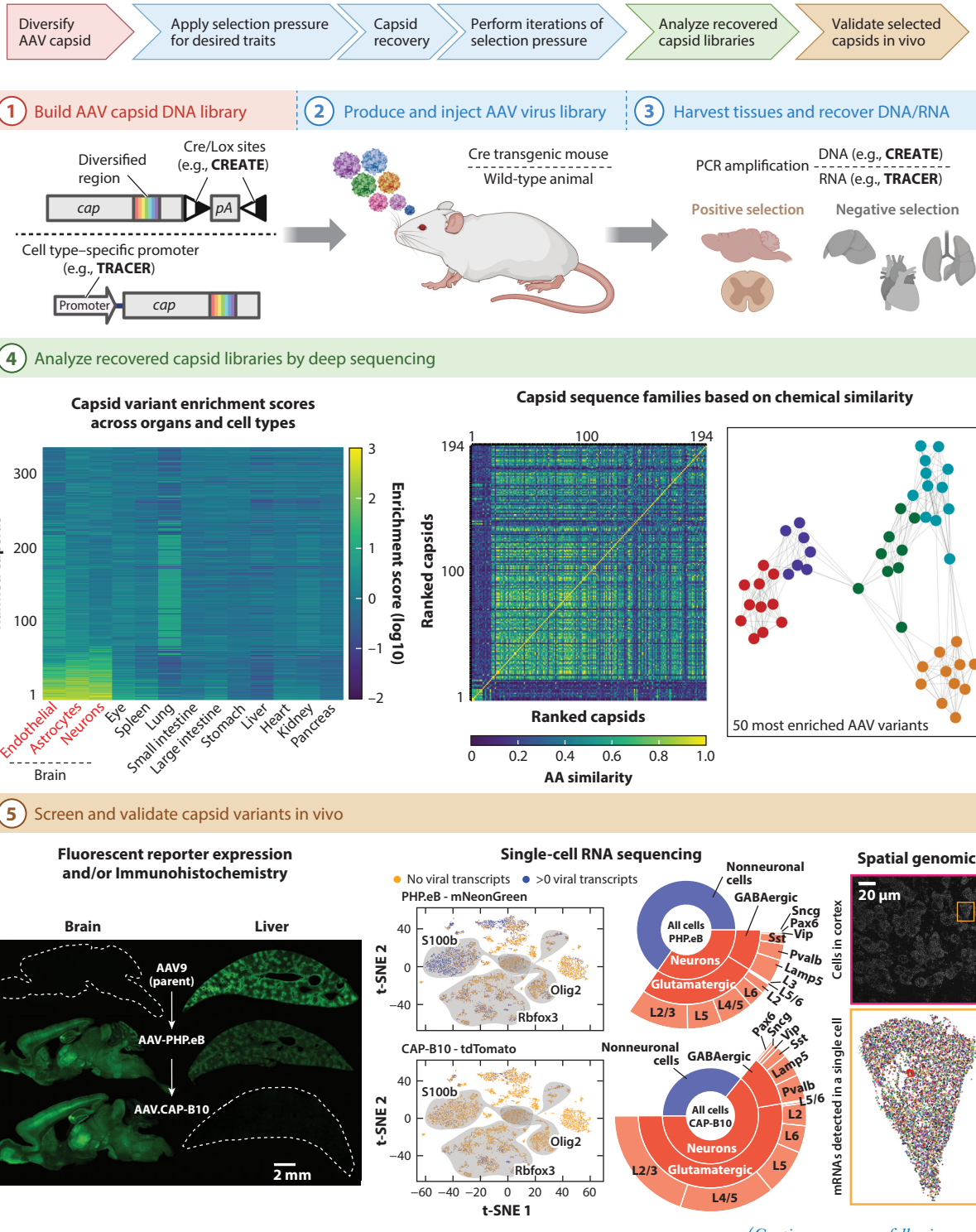
Supplemental Material >

CREATE was not optimized for two important AAV engineering goals, however: selecting for cell type specificity and selecting for mechanistic diversity. An improved methodology, multiplexed-CREATE (M-CREATE) (Ravindra Kumar et al. 2020), tracks every capsid variant's production and performance in parallel across multiple different tissues and cell types using next-generation sequencing while accounting for the experimental biases that can accumulate over the selection process (many of which are rigorously analyzed by de Alencastro et al. 2020) (**Figure 2**, step 4, left). This approach looks beyond the most enriched individual variants and identified cell type–specific AAVs such as AAV-PHP.N for neurons and AAV-PHP.V1 for vascular cells (**Figure 1*d***). Diverse sequence families with enhanced CNS tropisms upon systemic injection were also found (**Figure 2**, step 4, middle and right), including several, termed AAV-PHP.C, that retain enhanced CNS transduction across genetically diverse mouse strains (**Figure 3*a***).

The power of iterative capsid engineering was demonstrated by the maturation of AAV-PHP.B into the more efficient AAV-PHP.eB and the more cell type–selective AAV-PHP.N. Tropism refinement can occur through reengineering the AA588 insert location (as above) or by incorporating new sites. M-CREATE selection of random substitutions in the AA452-458 loop of AAV-PHP.eB (in mice) revealed AAV.CAP-B10, which transduces neurons in the CNS with greatly reduced targeting of astrocytes, oligodendrocytes, and peripheral organs in mice and marmosets (Goertsen et al. 2022) (**Figure 2**, step 5, left, and **Figure 3*b***). This strategy is also compatible with serotype-shuffled capsids as the capsid AAV-ie (for enhanced efficiency in the inner ear) was developed by inserting the AAV-PHP.eB 9-mer loop into AAV-DJ (Tan et al. 2019). However, the PHP.B insertion loop fails to alter BBB penetrance of AAV1 (Martino et al. 2021) and AAV5 (Pietersz et al. 2021).

## 2.2. Complementary Modern Methods for AAV Capsid Engineering

Several other groups have also generated novel AAV capsids for the brain (**Supplemental Table 1**). In 2016, the Schaffer and Karpova groups (Tervo et al. 2016) identified rAAV2-retro for enhanced retrograde transport in neurons by inserting a 10-mer peptide between AA587–588 of the AAV2 capsid gene. In 2018, a strategy similar to CREATE was published by the Schaffer group (Ojala et al. 2018), who used serotype shuffled libraries to identify SCH9 for enhanced neural stem cell targeting in the subventricular zone. In 2019, the Maguire group (Hanlon et al. 2019) published the iTransduce methodology to identify BBB-crossing AAVs, in which mutated AAVs deliver Cre under the control of a cell type–specific promoter to induce fluorescent protein expression in transgenic mice. This allowed functional capsid recovery by cell sorting and identified AAV-F, whose enhanced CNS transduction is retained in both C57BL/6J and BALB/cJ mice (**Figure 3*a***). In 2019, the Bjorklund lab (Davidsson et al. 2019) developed the BRAVE method, whereby peptides rationally designed from proteins with affinity to synapses formed a DNA barcoded capsid insertion library. BRAVE was used to identify the AAV2 variant MNM004 with enhanced retrograde transport in dopamine neurons. Nonnenmacher et al. (2021) developed the




(Caption appears on following page)

**Figure 2** (*Figure appears on preceding page*)

Engineering systemic AAVs for the brain. A simplified pipeline for the discovery of AAV capsids with unique tropisms includes the following steps. (①) Build an AAV capsid DNA library. This is done by diversifying the capsid sequence (*rainbow*) using PCR. CREATE methods (Chan et al. 2017, Deverman et al. 2016, Ravindra Kumar et al. 2020) use Cre/Lox sites for capsid recovery in Step 3. (②) Produce the diversified AAV virus library in HEK293T cells. Intravenously inject the AAV virus library in transgenic or wild-type animals, depending on the capsid selection method. Cell type–specific Cre-expressing transgenic mice are used in CREATE, and wild-type mice are used in TRACER (Nonnenmacher et al. 2021). (③) After 2–3 weeks, harvest tissues of interest and recover DNA (e.g., CREATE) or RNA (e.g., TRACER) using PCR amplification. (④) Analyze recovered capsid libraries by deep sequencing. (*Left*) A heatmap of capsid variant enrichment scores (as defined in Ravindra Kumar et al. 2020) across organs and cell types is displayed for the subset of library variants that are strongly enriched in the brain. Heatmap adapted from Ravindra Kumar et al. (2020). (*Right*) Plotting the chemical similarity between capsid variant insert sequences (ranked capsids) as a heatmap (correlation matrix) and clustering (each variant is a *dot* in the plot) reveals multiple distinct capsid sequence families (*colored clusters*) based on AA properties. (⑤) Screen and validate capsid variants in vivo. (*Left*) The traditional approach uses fluorescent reporter expression. Image adapted from Goertsen et al. (2022). (*Middle*, *right*) High-throughput profiling of viral vectors. (*Middle*) scRNA-seq of mice injected with AAV-PHP.eB and AAV.CAP-B10 reveals biases across cortical cell types and subtypes (Brown et al. 2021). Shaded areas in the t-SNE plots show clusters of cells (*yellow* and *blue dots*) with high expression of Rbfox3 (neurons), S100b (astrocytes), or Olig2 (oligodendrocytes). Starburst plots illustrate the cell type distributions of AAV-PHP.eB and AAV.CAP-B10. t-SNE plots were adapted from Brown et al. (2021) (CC BY 4.0). (*Right*) Spatial genomics methods can detect thousands of RNA transcripts in intact brain tissue. Reconstruction of RNAs detected in a single brain cell is shown. Panel adapted with permission from Eng et al. (2019); copyright 2019 Springer Nature. Steps 1–3 adapted from images created with BioRender.com. Abbreviations: AA, amino acid; AAV, adeno-associated virus; CREATE, Cre recombination–based AAV targeted evolution; HEK293T, human embryonic kidney cell line; PCR, polymerase chain reaction; scRNA-seq, single-cell RNA sequencing; TRACER, tropism redirection of AAV by cell type–specific expression of RNA.

TRACER method to identify systemic AAVs in wild-type mice (opening the door to stringent selections in species for which transgenics are not available). This method employs an RNA selection with cell type–specific promoters directing expression of the capsid variant in vivo, thereby allowing functional capsid recovery by RNAseq (**Figure 2**, steps 1–3). Validating this method with an AAV9 AA588–589 insertion library, TRACER selections affirmed M-CREATE's findings, with diverse capsid sequence families demonstrating enhanced CNS tropism in mice.

## 2.3. Toward Engineering AAV Tropisms for Molecularly Defined Cell Types

Supplemental Material >

While several AAVs with CNS-specific tropisms are now available (**Supplemental Table 1**), much work remains to assemble a full armamentarium of AAV tools for neuroscience research. Modern viral capsid selection experiments generate hundreds to thousands of AAV variants, most of which remain largely uncharacterized because determining AAV tropism through immunohistochemistry is low throughput and lacks resolution across diverse brain cell types (**Figure 2**, step 5, left). To facilitate fast identification and detailed characterization of already-engineered AAVs, and to engineer new AAV capsids for molecularly defined cell types, high-throughput capsid profiling methods are needed. Sequencing methods for barcoded vector genomes present one potential solution (Brown et al. 2021, Davidsson et al. 2019, Kondratov et al. 2021, Öztürk et al. 2021, Weinmann et al. 2020). In 2020, the Grimm lab (Weinmann et al. 2020) combined DNA and RNA barcoding with next-generation sequencing to monitor the performance of a small library of previously identified AAV variants across different tissues in vivo. The method (which revealed a muscle-tropic AAV) is compatible with any capsid and target and could accelerate the discovery of CNS-tropic variants. To thoroughly mine the rich data sets from M-CREATE selections, a single-cell RNA sequencing–based method can be used to inspect transcriptomes of brain cells transduced with pools of barcoded AAV vectors (Brown et al. 2021). This method revealed previously unknown biases for molecularly defined neuronal cell types between AAV-PHP.eB and AAV.CAP-B10 in mice (**Figure 2**, step 5, middle). These differences in transduction specificity highlight the importance of mining past, current, and future selections for engineered AAVs with novel molecularly defined cell type tropisms. Spatial genomics methods present another possible solution for rapid

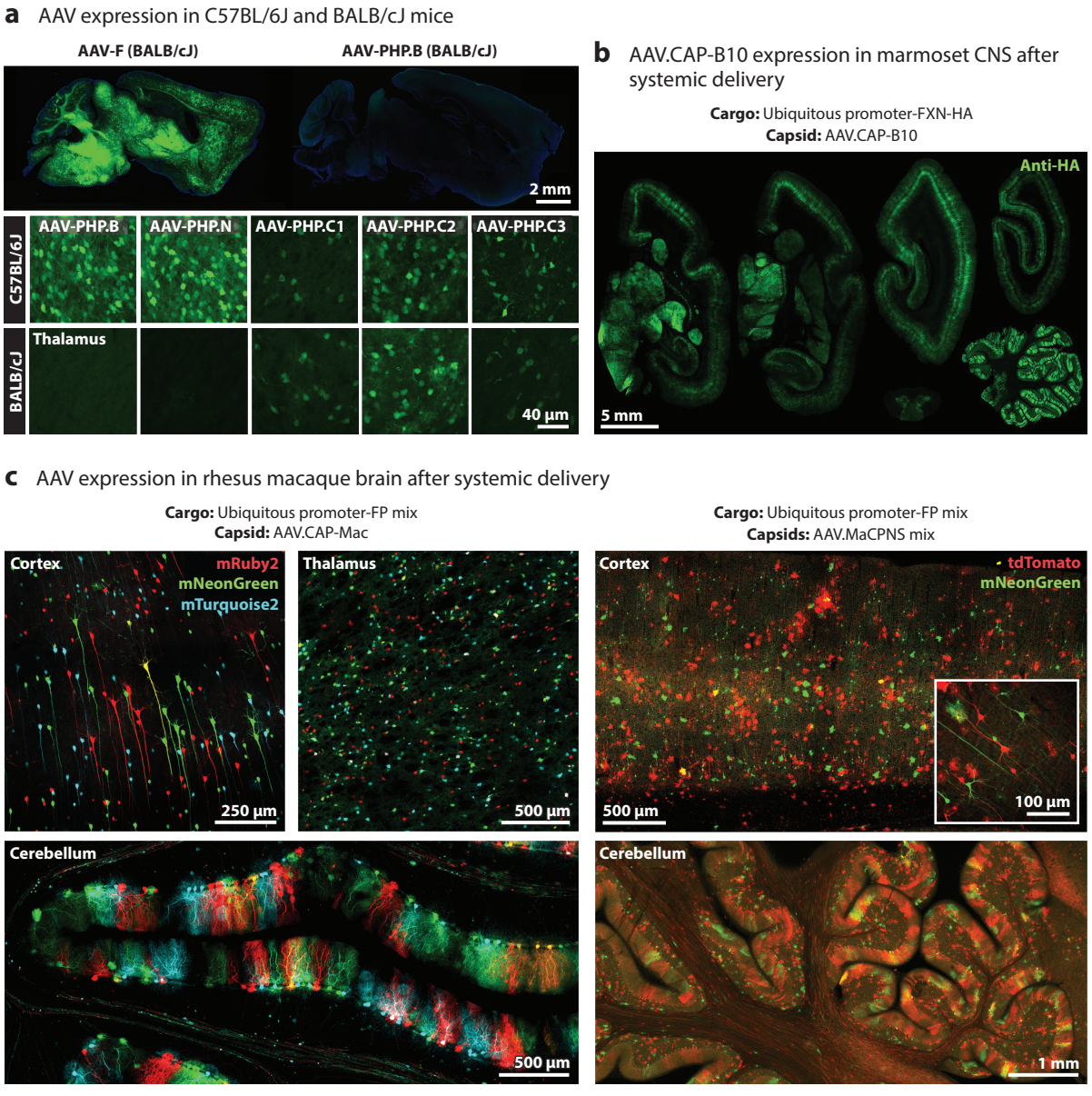



**Figure 3**

Utility of engineered systemic AAVs across mouse strains and in NHPs. (*a*) AAV variants from different sequence families show unique transduction properties in C57BL/6J and BALB/cJ mice (Hanlon et al. 2019, Ravindra Kumar et al. 2020). Top panel adapted with permission from Hanlon et al. (2019) and the American Society of Gene and Cell Therapy (CC BY-NC-ND 4.0). Bottom panel adapted from Ravindra Kumar et al. (2020). (*b*) The enhanced CNS transduction of AAV.CAP-B10 (identified in mice) is conserved in adult marmosets (Goertsen et al. 2022), as shown in these fluorescent images. Panel adapted from Goertsen et al. (2022). (*c*) AAV expression in the rhesus macaque brain after systemic delivery. (*Left*) AAV.CAP-Mac (identified in Chuapoco et al. 2022) preferentially transduces neurons in the infant macaque brain after intravenous administration. (*Right*) AAV.MaCPNS1 and AAV.MaCPNS2 (identified in Chen et al. 2022) transduce neurons and astrocytes in the infant macaque brain. The promoter used in panels *b* and *c* is the ubiquitous CAG promoter. Abbreviations: AAV, adeno-associated virus; CAG, synthetic promoter containing the cytomegalovirus early enhancer element, the promoter, first exon, and first intron of the chicken beta-actin gene and the splice acceptor from the rabbit beta-globin gene; CNS, central nervous system; FXN, human frataxin; HA, hemagglutinin; NHP, nonhuman primate.

and precise AAV variant characterization. The hybridization method signal amplification by exchange reaction fluorescence in situ hybridization (SABER-FISH) allows AAV genomes to be detected in situ (S.K. Wang et al. 2020), and sequential fluorescence in situ hybridization (seqFISH+) achieves multiplexed transcript detection and molecular cell typing in brain tissue (Eng et al. 2019) (**Figure 2**, step 5, right). In situ variant pool screens could reveal finer region- and cell type–specific tropisms, including specificity for neuronal subtypes or microglia, the immune cells in the brain that may be resistant to AAV transduction (Maes et al. 2019, S.K. Wang et al. 2020).

## 3. AAVs FOR DIVERSE ANIMAL MODELS

To enable broader adoption of gene delivery tools for research and eventual therapeutic use, AAV capsids and cargo must work in genetically diverse backgrounds within and across model organisms. Several variables, including age (Chakrabarty et al. 2013, Foust et al. 2009), sex (Maguire et al. 2013), strain (He et al. 2019, Hordeaux et al. 2018), and species (Hordeaux et al. 2018, Matsuzaki et al. 2018, Watakabe et al. 2015), can affect AAV brain transduction. Here, we address questions about the use of AAV serotypes across diverse animal models.

### 3.1. Do Genetic Factors Affect AAV Brain Transduction in Mice?

The genetic background of laboratory mice can influence AAV transduction properties in the brain (He et al. 2019, Hordeaux et al. 2018). The enhanced CNS transduction of AAV-PHP.B and AAV-PHP.eB, for example, is absent in select mouse strains, including BALB/cJ mice (Batista et al. 2019; Challis et al. 2019; Hordeaux et al. 2018, 2019; Huang et al. 2019; Matsuzaki et al. 2019) (**Figure 3*a***). Several groups identified the molecular origin of this phenotype to be strain-dependent polymorphisms in the murine-restricted protein Ly6a, which AAV-PHP.B and AAV-PHP.eB use as a receptor to cross the BBB (Batista et al. 2019, Hordeaux et al. 2019, Huang et al. 2019). At low doses, this strain-specific trend holds for AAV-PHP.V1 and AAV-PHP.N, newly identified members of the AAV-PHP.B sequence family (Ravindra Kumar et al. 2020) (**Figure 3*a***). AAV serotypes from different families, however, do not show strain-specific differences in brain transduction (Hanlon et al. 2019, Nonnenmacher et al. 2021, Ravindra Kumar et al. 2020). AAV-PHP.Cs (Ravindra Kumar et al. 2020) and AAV-F (Hanlon et al. 2019) retain enhanced brain transduction in BALB/cJ mice (**Figure 3*a***). These studies suggest that capsid variants from different sequence families have distinct mechanisms of BBB entry. Testing AAV serotypes in mouse strains from diverse genetic backgrounds (e.g., using immunohistochemistry or high-throughput capsid profiling methods) may reveal the molecular mechanisms underlying differences in BBB crossing.

Biological factors such as age and sex can also affect AAV transduction properties in mice. Systemic injection of AAV9 leads to higher transduction of the brain in female mice compared to male mice (Maguire et al. 2013), and lower liver transduction has been reported in females compared to males (Maguire et al. 2013, Rumachik et al. 2020). Brain cell type preferences and BBB entry can also depend on age at injection (Chakrabarty et al. 2013, Foust et al. 2009). More work is needed to determine the effects of age, sex, and other factors [e.g., vector production platforms (Rumachik et al. 2020) and disease state] on the transduction properties of AAV serotypes and whether observed differences translate to other vertebrate species, including NHPs.

### 3.2. Do AAVs Engineered in Mice Translate to Primate Species?

It depends on the vector and delivery route. AAV-PHP.B vectors, which work in many strains of mice (Batista et al. 2019, Challis et al. 2019, Hordeaux et al. 2019, Huang et al. 2019, Matsuzaki

et al. 2019) and rats (Challis et al. 2019, Chatterjee et al. 2021, Dayton et al. 2018, Jackson et al. 2016), do not outperform AAV9 after intravenous delivery in NHPs (Hordeaux et al. 2018, Matsuzaki et al. 2018). These results are consistent with the fact that Ly6a is absent in primates (Loughner et al. 2016). In macaques, intrathecal delivery (which bypasses the BBB) leads to greater CNS transduction by AAV-PHP.B compared to intravenous delivery (Liguore et al. 2019) [and AAV-PHP.eB outperforms AAV-PHP.B after intrathecal administration (Arotcarena et al. 2021)], but more studies are needed to understand whether these vectors perform better than AAV9 (Gray et al. 2013, Hinderer et al. 2014, Samaranch et al. 2012). Although systemic AAV-PHP.B vectors do not translate to NHPs, other capsid variants show enhanced tropism conservation in primate species. AAV2-HBKO, a modified AAV2 vector with enhanced cortical and striatal expression (Sullivan et al. 2018), shows neuron-biased transduction after intracranial injections in both mice (Sullivan et al. 2018) and macaques (Naidoo et al. 2018). Two systemic variants that evolved from AAV-PHP.eB (AAV.CAP-B10 and AAV.CAP-B22, both identified in mice) retain enhanced CNS transduction and cell type bias in marmosets (**Figure 3*b***), with four- and twofold increases over the number of neurons transduced by AAV9, respectively (Goertsen et al. 2022). The vector AAV.CAP-B10 shows high neuronal tropism and reduced targeting to the liver in marmosets [systemically delivered AAV9 mainly transduces astrocytes in the macaque CNS (Gray et al. 2011b, Samaranch et al. 2012), and the AAV9-like vector AAVhu68 can cause liver damage after high-dose systemic administration to NHPs (Hinderer et al. 2018)]. AAV.MaCPNS1 and AAV.MaCPNS2 were also evolved in mice and translate to NHPs (Chen et al. 2022) (**Figure 3*c***). Another AAV variant, rAAV2-retro (also identified in mice) (Tervo et al. 2016), shows robust retrograde transport in the macaque brain following intracranial injections (Weiss et al. 2020). These studies suggest that capsid selection platforms in rodents can be used prior to screening or validating novel AAVs in NHPs. RNA-based selections such as TRACER (in which cell type–specific promoters drive expression of capsid variants in wild-type mice) (**Figure 2**, steps 1–3) do not require transgenic animals (Nonnenmacher et al. 2021) and could be used to identify AAV capsids directly in NHPs (Tabebordbar et al. 2021). Recently, a multispecies selection strategy in NHPs identified AAV.CAP-Mac, an engineered systemic AAV variant for brain-wide delivery in Old World primates (Chuapoco et al. 2022) (**Figure 3*c***). Human neurosurgical brain tissue or region-specific brain organoids (Miura et al. 2020) are also promising systems in which to identify region- and cell-specific tropisms for clinical translation.

## 4. REGULATED AAV CARGO FOR THE BRAIN

Cell type specificity can also be achieved through discovery and engineering of gene regulatory elements that endow transgene specificity via promoters, enhancers, and microRNA (miRNA) target sites (**Figures 1*b*** and **4**).

### 4.1. Promoters and Enhancers

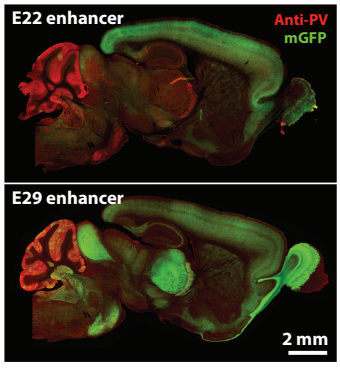
Supplemental Material >

Several brain cell type–specific promoters and enhancers to drive transgene expression have been identified and validated for use in AAVs (**Supplemental Table 2**). Most of these elements are less than 3 kb (compatible with the limited genetic payload of AAVs) and can be paired with broadly tropic capsids to restrict transcription to defined brain cells and regions (**Figures 1*d*** and **4*a***).

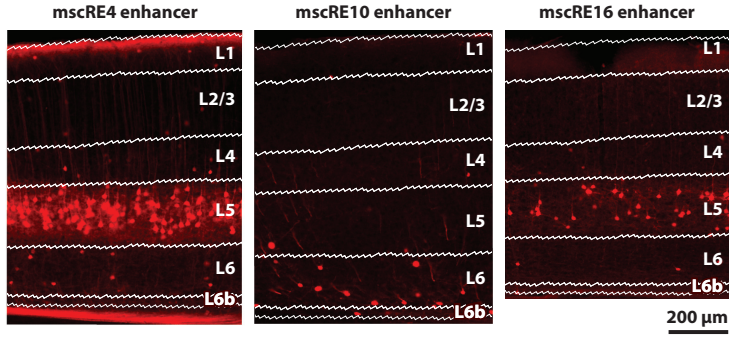
Promoters can drive AAV expression in diverse brain cell types, including neurons, glia, and vascular cells (**Figure 1*d***). Neuronal cell type–specific expression can be achieved with promoters for catecholaminergic cells (Chan et al. 2017), serotonergic cells (de Leeuw et al. 2016), Purkinje cells in the cerebellum (de Leeuw et al. 2016, Nitta et al. 2017), and parvalbumin-expressing GABAergic interneurons throughout the brain (Hoshino et al. 2021), among other subtypes

**a** Enhancer-driven cell subtype– and brain region–specific expression

**Cargo:** PV-specific enhancer-FP
**Capsid:** AAV-PHP.eB (IV)



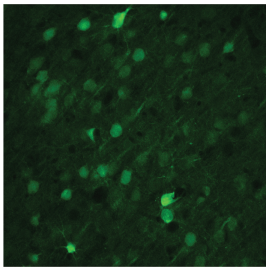

**Cargo:** Cortical cell subclass enhancer-FlpO (*Ai65F* reporter mouse)
**Capsid:** AAV-PHP.eB (IV)




**b** miRNA-mediated cell type–restricted expression

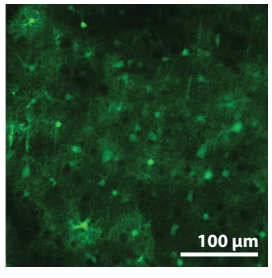
**Neurons**

**Cargo:** Ubiquitous promoter-FP-miR-204
**Capsid:** AAV-PHP.eB (IV)



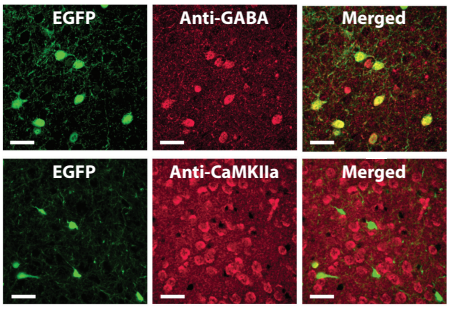
**Astrocytes**

**Cargo:** Ubiquitous promoter-FP-miR-708
**Capsid:** AAV-PHP.eB (IV)




**Interneurons**

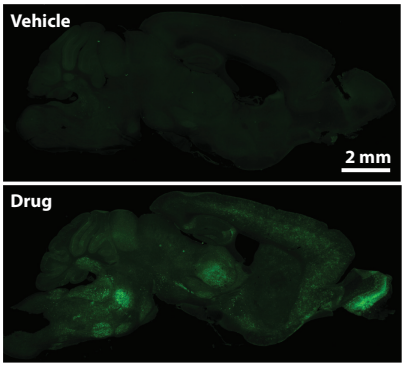
**Cargo:** Neuron-specific promoter-FP-miRs
**Capsid:** AAV9 (IC)




**c** Temporal control of transgene expression

**Drug-inducible splicing switch ($X^{on}$)**

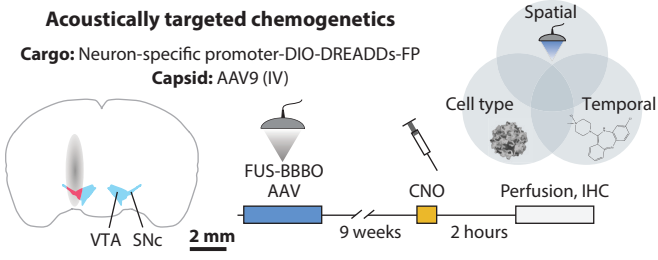
**Cargo:** Ubiquitous promoter-$X^{on}$-FP
**Capsid:** AAV-PHP.eB (IV)

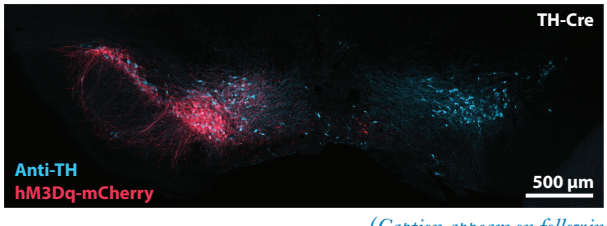



**d** Spatial control of transgene expression

**Acoustically targeted chemogenetics**

**Cargo:** Neuron-specific promoter-DIO-DREADDs-FP
**Capsid:** AAV9 (IV)




*(Caption appears on following page)*

**Figure 4** (*Figure appears on preceding page*)

AAV cargo for spatial, cell type, and temporal specificity. (*a*,*b*) Broadly tropic capsids can deliver targeted transgene expression by incorporating gene regulatory elements into the AAV genome. (*a*) Several cell subtype– and brain region–specific enhancers have been discovered (Graybuck et al. 2021, Vormstein-Schneider et al. 2020) (**Supplemental Table 2**). These elements can be delivered intravenously with AAV-PHP.eB to achieve regional and cell type specificity. Panel *a* (*left*) adapted with permission from Vormstein-Schneider et al. (2020); copyright 2020 Springer Nature. Panel *a* (*right*) adapted with permission from Graybuck et al. (2021); copyright 2021 Elsevier. (*b*) miRNA target sites can restrict expression to different cell types (e.g., neurons or astrocytes) (Challis et al. 2019) or subtypes (e.g., cortical interneurons) (Keaveney et al. 2018) after intravenous or intracranial injections, respectively. Scale bar for cortical interneuron images on right = 35 μm. Panel *b* (*right*) adapted with permission from Keaveney et al. (2018) and Elsevier (CC BY-NC-ND 4.0). (*c*) Temporal control of transgene expression can be achieved with a drug-inducible splicing switch ($X^{on}$) (Monteys et al. 2021). Panel *c* adapted with permission from Monteys et al. (2021); copyright 2021 Springer Nature. (*d*) Spatial control of transgene expression can be achieved with FUS-BBBO (Szablowski et al. 2018). Panel *d* adapted with permission from Szablowski et al. (2018); copyright 2018 Springer Nature. Abbreviations: AAV, adeno-associated virus; CaMKIIa, calcium/calmodulin-dependent protein kinase II alpha; CNO, clozapine *N*-oxide; DIO, double-floxed inverted orientation; DREADD, designer receptor exclusively activated by designer drugs; FlpO, mouse codon-optimized Flp recombinase; FP, fluorescent protein; FUS-BBBO, focused ultrasound blood-brain barrier opening; GABA, gamma aminobutyric acid; hM3Dq, Gq-coupled human M3 muscarinic DREADD receptor; IC, intracranial; IHC, immunohistochemistry; IV, intravenous; miRNA, microRNA; PV, parvalbumin; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

**Supplemental Material >**

(Mehta et al. 2019). Several promoters have been validated for cell type specificity after systemic injection of engineered AAV vectors in wild-type mice (Challis et al. 2019, Chan et al. 2017, Hoshino et al. 2021, Nitta et al. 2017).

Enhancers (short genomic regions that regulate transcription) can also drive transgene expression from AAV vectors. When combined with a minimal promoter, enhancers are capable of brain region– and cell type–specific labeling across vertebrate species. The mDlx enhancer, for example, restricts expression to forebrain GABAergic interneurons in zebra finches, rodents, NHPs, and human cells (Dimidschstein et al. 2016) and maintains regional and cell type specificity when delivered intravenously to mice using AAV-PHP.eB (Chan et al. 2017). Modern genomic methods (e.g., open chromatin analysis) have been leveraged to find new enhancers and generate viral tools. Many subtype- and region-specific enhancers are now available (Graybuck et al. 2021, Hrvatin et al. 2019, Mich et al. 2021, Nair et al. 2020, Rubin et al. 2020, Vormstein-Schneider et al. 2020) (**Supplemental Table 2**). These include regulatory elements that label excitatory and inhibitory subpopulations with high specificity after systemic or local injection of the broadly tropic AAV-PHP.eB vector in mice (Graybuck et al. 2021, Mich et al. 2021, Nair et al. 2020, Vormstein-Schneider et al. 2020) (**Figure 4*a***). Several enhancer elements also function in primate species (Dimidschstein et al. 2016, Mich et al. 2021, Vormstein-Schneider et al. 2020). Continued efforts to identify and validate enhancers (especially elements with increased efficiency after retro-orbital injection in mice) will greatly expand the AAV toolkit.

Promoter and enhancer strength and specificity hinge on many factors, including the vector dose, animal model, delivery method, and capsid. Capsid-promoter interactions (in which the AAV capsid influences the promoter's ability to drive transgene expression) have been reported in the CNS of rats (Powell et al. 2020) and NHPs (Bohlen et al. 2020) and should be considered when testing viral tools in vivo. Some ubiquitous promoters can drive stronger AAV expression than can cell type– or tissue-specific promoters. To achieve strong, selective expression in the brain, a ubiquitous promoter can be cloned upstream of a floxed transgene and delivered to cell type–specific Cre or Flp recombinase–expressing mice (Morabito et al. 2017). Several new driver lines targeting diverse cortical and subcortical cell populations were recently generated and could be used for this purpose (Daigle et al. 2018). Alternatively, enhancer viruses expressing an exogenous recombinase or transcription factor can be delivered intravenously to transgenic reporter mice for strong labeling (Graybuck et al. 2021) (**Figure 4*a***). A stronger minimal promoter and/or

multiple enhancer copies may also increase transgene expression from enhancer AAVs (Graybuck et al. 2021, Mich et al. 2021). Lastly, the transgene itself can be engineered to compensate for low-level expression (e.g., due to low per-cell genome copy number after systemic delivery). Reporters (Campbell et al. 2020), sensors (Broussard et al. 2018, Patriarchi et al. 2018, Shemesh et al. 2020, Unger et al. 2020), and effectors (Bedbrook et al. 2019, Gong et al. 2020) continue to be modified for improved expression, sensitivity, and targeting.

### 4.2. miRNA Target Sites for Improved Specificity and Safety

Cell type specificity can also be achieved by leveraging endogenously expressed miRNAs to suppress transgene expression in off-target tissues and cell types. miRNAs are small (~22 nt), noncoding RNA molecules that inhibit mRNA expression by binding complementary target sequences in the 3′ untranslated region (UTR). Cloning miRNA target sites into the 3′-UTR of the AAV genome (**Figure 1*b***) can reduce transgene expression in off-target cells where complementary miRNAs are expressed. When paired with strong promoters (as opposed to larger and often weaker cell type–specific promoters), this can drive a high level of transgene expression in on-target cells. For example, AAV-PHP.eB (a broadly tropic capsid) can deliver targeted transgene expression to neurons or astrocytes by incorporating miRNA target sites into its genome (Challis et al. 2019) (**Figure 4*b***). A similar approach was used to confine expression to cortical interneurons by repressing expression in excitatory cells after cortical injections with AAV9 (Keaveney et al. 2018) (**Figure 4*b***). Tissue-specific miRNA sites can be included in the transgene cassette to decrease expression in peripheral organs, including the liver and heart after systemic delivery to the CNS (Xie et al. 2011). miRNA detargeting strategies can also reduce transgene-related toxicity (Hordeaux et al. 2020b, Sinnett et al. 2021) and immune responses (Muhuri et al. 2021, Xiao et al. 2019). For example, miRNAs specifically expressed in dorsal root ganglion neurons ablate transgene expression in, and toxicity of, these cells after intra-CSF injections in NHPs (Hordeaux et al. 2020b). miRNAs expressed in immune cell populations suppress transgene-specific immune responses after intramuscular delivery (Muhuri et al. 2021, Xiao et al. 2019) and allow redosing the same mice with a different AAV serotype (Xiao et al. 2019). Hundreds of miRNAs have been identified (Isakova et al. 2020); however, their effects on tissue and cell type specificity, alone and in combination, have not been determined. Continued validation of miRNA target sites (e.g., using high-throughput AAV characterization methods) is needed to annotate target site specificities and assemble a library of target site combinations capable of restricting transgene expression to defined brain cell types and regions.

### 4.3. Spatiotemporal Control of Transgene Expression or Function

Genetic switches can be included in the AAV genome to regulate expression levels in vivo (**Figure 1*b***). Small RNA-based switches (riboswitches) can be placed in the 3′-UTR of the transgene cassette to turn on expression after delivery of an antisense oligonucleotide (Tickner & Farzan 2021, Zhong et al. 2020). Light or chemicals can also be used to hone expression. $X^{on}$, a tunable drug-induced splicing switch, was recently developed to control gene therapy expression levels (Monteys et al. 2021). When packaged into AAV-PHP.eB, $X^{on}$ enables brain-targeted delivery and gene editing (**Figure 4*c***). An off switch (e.g., for therapeutic cargo) would be useful if complications arise following AAV delivery, or if transient gene expression is needed for therapeutic rescue. Spatiotemporal control can be achieved with light-inducible recombinases (Yao et al. 2020) or chemogenetics (Roth 2016). Acoustically targeted chemogenetics (Szablowski et al. 2018) combines focused ultrasound for BBB opening and viral vector delivery (Thévenot et al. 2012) with chemogenetics for regional, cell type, and temporal specificity (**Figure 4*d***). Further

development of expression control systems could allow precise and conditional modulation of transgenes (including therapeutic genes) to increase the safety and efficacy of AAV-delivered therapeutics in both basic and translational research.

## 5. OUTLOOK: AAV-BASED THERAPEUTICS AND GENOME EDITING

### 5.1. Gene Therapy

The development of AAV as a therapeutic delivery vector for genetic diseases has been underway for decades (Samulski & Muzyczka 2014). In recent years, this effort has yielded clear signs of success in treating metabolic, hematologic, and neuromuscular disorders. US Food and Drug Administration approvals of Luxturna for *RPE65*-associated retinal dystrophy (Russell et al. 2017) and Zolgensma for spinal muscular atrophy (Mendell et al. 2017) demonstrate that AAV-based therapies can be effective in the CNS (Zhu et al. 2021). The initial success rates of clinical programs as measured by translation from investigational new drug to new drug application have been higher than for other modalities (Kuzmin et al. 2021). Notably, trial outcomes for neurological (Flotte et al. 2022) and ophthalmological (Sahel et al. 2021) indications have been similarly favorable. AAV-based optogenetic therapy, for example, recently achieved partial visual restoration in a blind patient (Sahel et al. 2021) and could pave the way for adopting other improved opsins (Bedbrook et al. 2019, Gong et al. 2020, Marshel et al. 2019) for circuit therapies.

As the clinical toolkit is further developed, toxicities associated with AAVs and their genetic cargo warrant attention. Serious adverse events and death have been reported in high-dose AAV gene therapy clinical trials (High-Dose AAV Deaths 2020). Preclinical work, however, can inform the field about safety concerns to increase therapeutic potential. In NHPs, clinically relevant titers of AAV (delivered either intravenously or intrathecally to the CNS) can lead to dorsal root ganglion toxicity (Hinderer et al. 2018, Hordeaux et al. 2020a), and, in piglets, severe ataxia (Hinderer et al. 2018). In mice, intracranial injections of AAV can ablate neural progenitor cells in the hippocampus (Johnston et al. 2021) and disrupt dendritic complexity and synaptic transmission in the cortex (Suriano et al. 2021). Understanding the mechanisms underlying toxicity [e.g., transgene overexpression (Hordeaux et al. 2020b, Van Alstyne et al. 2021) or immunodetection of the AAV genome (Suriano et al. 2021)], and how they may manifest clinically, will inform future therapies. AAV-induced toxicity can occur days to weeks after viral administration and persist for months postinjection in both rodents and NHPs (Hordeaux et al. 2020a, Johnston et al. 2021, Van Alstyne et al. 2021). Therefore, extended time points are needed in preclinical experiments to anticipate and mitigate complications in patients. In conjunction with resolving issues related to toxicity, improved injection targeting and, goal depending, vector spread and specificity are also needed to advance the clinical toolkit for brain disease (Castle et al. 2020).

Recent progress in identifying and characterizing new AAV variants and gene regulatory elements will facilitate clinical development of gene therapies in important ways. Screening strategies such as those presented here can be used to optimize clinically desirable features such as tropism for certain regions of the nervous system, access to disease-relevant compartments (e.g., specific cell types and tissue microenvironments), derisking safety and tolerability issues (e.g., immunogenicity and toxicity), and improving efficacy. Several preclinical studies demonstrate the capability of AAVs [including engineered systemic vectors (**Supplemental Table 3**)] to treat CNS disorders. Wegmann et al. (2021) delivered gene-silencing zinc finger protein transcription factors, either locally into the hippocampus with AAV9 or brain-wide with AAV-PHP.B, to repress tau expression in a mouse model of Alzheimer's disease. Sinnett et al. (2021) engineered miRNA regulatory elements into the viral genome of an AAV packaging a therapeutic transgene to prevent overexpression after intra-CSF delivery to a mouse model of Rett syndrome (improving the safety

Supplemental Material >

of AAV gene transfer without compromising efficacy). Further development and implementation of AAV components that exert control over the time, place, and quantity of transgene expression will improve the safety and efficacy of AAV gene therapies for diverse pathologies.

## 5.2. Genome Editing

Coupled with advances in AAV methodology, tools for editing and manipulating the genome offer new possibilities for understanding gene function in disease and identifying potential therapeutic targets (Doudna 2020, Porteus 2019, Zhang 2019). Over the past decade, the number of clustered regularly interspaced short palindromic repeat (CRISPR)-derived tools has grown substantially, enabling researchers to manipulate the genome, epigenome, and transcriptome of a cell (Liu & Jaenisch 2019, D. Wang et al. 2020, Xu & Qi 2019, Zhang 2019). If coupled with efficient and targeted vector delivery, the broad functionality of the CRISPR toolbox promises to transform preclinical research.

The large size of many Cas proteins has limited the integration of CRISPR-based tools with AAV delivery. The well-characterized and widely used *Streptococcus pyogenes* Cas9 is 4.1 kb in length, leaving only a few hundred base pairs for all regulatory elements. Smaller Cas proteins have been characterized (Kim et al. 2017, Ran et al. 2015, Wu et al. 2021, Xu et al. 2021), however, and can be packaged into a single AAV vector alongside the guide RNA (gRNA) cassette. AAV-mediated delivery of CRISPR-based tools can also be achieved through a two-vector system: delivering the gRNA and Cas effector separately, delivering a split Cas protein in AAV genomes to be recombined after transduction, or through mRNA- or protein *trans*-splicing. CRISPR-based tools stand to benefit from broader efforts to minimize and optimize promoters, enhancers, and regulatory elements (Choi et al. 2014, Lau & Suh 2017).

Despite these difficulties, a number of studies have demonstrated the promise of AAV-delivered CRISPR tools for preclinical research. Duan et al. (2021) used a single AAV to deliver both Cas9 and gRNA, either intraparenchymally with AAV9 or brain-wide with AAV-PHP.eB, to edit a pathogenic mutation in a mouse model of familial Alzheimer's disease, improving molecular, electrical, and cognitive markers of disease progression. Taking advantage of split-intein protein *trans*-splicing, Levy et al. (2020) delivered cytosine base editors (CBEs) and adenine base editors to produce defined single–base pair conversions in multiple target tissues. Systemic administration of a split CBE with AAV-PHP.eB produced robust C·G-to-T·A in the cortex and cerebellum and, when used to correct a causative mutation, resulted in modest increases in survival in a mouse model of Niemann-Pick disease. Matharu et al. (2019) used a two-vector approach to deliver (through intraparenchymal injection) gRNA and a nuclease-deficient Cas9 fused to a transcriptional activator to rescue haploinsufficient obesity in mice through transcriptional activation of the target genes.

CRISPR-based tools can also be used to conduct large pooled genetic screens in vivo (e.g., for disease modifiers) (Przybyla & Gilbert 2021). Chow et al. (2017) used intraparenchymal AAV9 to deliver a gRNA library into Cas9 reporter mice, followed by targeted-capture sequencing of genomic target sites to identify glioblastoma driver genes. As the scale and utility of these screens is fundamentally limited by the number of cells that can be assayed, AAV capsids that broadly transduce the brain may enable larger-scale pooled screening with CRISPR-based tools.

In addition to engineering Cas proteins (Kleinstiver et al. 2016) and proper design and validation of gRNAs (Akcakaya et al. 2018), cell type–specific targeting through pairing of AAV capsids and cargo, as well as methods to hone spatial and temporal expression (Davis et al. 2015, Dow et al. 2015, Kumar et al. 2018), may reduce off-target effects and immune responses. Self-inactivating CRISPR vectors (Li et al. 2019) or anti-CRISPR proteins (Lee et al. 2019) can also be used to turn off CRISPR systems following on-target editing.

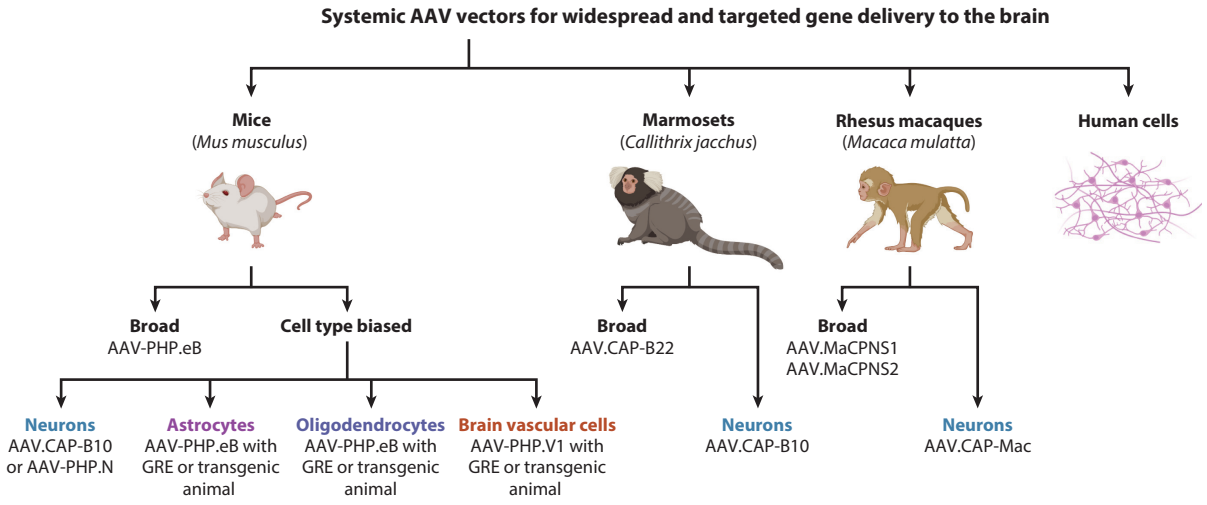



**Figure 5**

Systemic AAV vectors for widespread and targeted gene delivery to the brain. The diagram summarizes engineered systemic AAVs for use in mice, marmosets, and rhesus macaques. AAV capsid choice depends on a number of factors, including the delivery method, target cell population, and model organism under study. AAV-PHP.B and AAV-PHP.eB can transduce human-derived brain organoids (Deverman et al. 2016) and ex vivo human neocortical slices (Mich et al. 2021), respectively. However, much work remains to identify systemic AAVs for use in humans. The discovery and molecular characterization of cell types comprising the human cerebrovasculature (Garcia et al. 2022) could facilitate the development of BBB-crossing AAVs in humans. For reagents and protocols, visit **https://clover.caltech.edu**. Figure adapted from image created with BioRender.com. Abbreviations: AAV, adeno-associated virus; GRE, gene regulatory element.

## 6. CONCLUSION

Recent years have witnessed rapid progress in the development of AAV vectors [including systemic AAVs (**Figure 5**)] for neuroscience research. The modular design of AAV capsids and cargo allows easy adoption of the latest advances in actuators, sensors, and gene regulatory elements to target diverse brain cell types in rodents and NHPs. A growing armamentarium of molecular tools is now widely available through Addgene's plasmid repository (**https://www.addgene.org**) and elsewhere, and customized vectors can be made in-house (Challis et al. 2019, Gray et al. 2011a, Grieger et al. 2006). Open platforms such as Addgene's AAV Data Hub (**https://datahub.addgene.org/aav/**) can help researchers find and share AAV data to accelerate biomedical research. By expanding the availability and utility of the AAV toolkit, researchers can achieve minimally invasive, cell type-specific cargo expression across species using engineered AAVs.

## DISCLOSURE STATEMENT

The California Institute of Technology has filed and licensed patent applications for some of the work described in this manuscript, with S.R.K., X.C., and V.G. listed as inventors. V.G. is a cofounder and board member of Capsida Biotherapeutics, a fully integrated AAV engineering and gene therapy company. The remaining authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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