

# Adeno-Associated Viral Vectors in Neuroscience Research

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**Adeno-associated viral vectors (AAVs) are increasingly useful preclinical tools in neuroscience research studies for interrogating cellular and neurocircuit functions and mapping brain connectivity. Clinically, AAVs are showing increasing promise as viable candidates for treating multiple neurological diseases. Here, we briefly review the utility of AAVs in mapping neurocircuits, manipulating neuronal function and gene expression, and activity labeling in preclinical research studies as well as AAV-based gene therapies for diseases of the nervous system. This review highlights the vast potential that AAVs have for transformative research and therapeutics in the neurosciences.**

## INTRODUCTION

The central and peripheral nervous system are responsible for sensing, encoding, and transmitting interoceptive and exteroceptive information in order to output intricate biological processes, such as learning, memory, and complex decision-making and the performance of both reflexes and coordinated actions. To accomplish such tasks, the primary units of the central and peripheral nervous system, neurons, are organized into a circuit-based topography that contains a diverse set of connections. In this context, neurons act as nodes in these circuits and the connections between neurons as edges. How neurons connect or “wire” together into these microscopic, mesoscopic, and macroscopic circuits, as well as the function of these assembled circuits, is of great interest to neuroscientists. Understanding the topography and function of these circuits will aid neuroscientists’ quest to elucidate where and how specific types of sensory, motor, and cognitive data are processed to produce normal and aberrant biological processes. Yet, this task is tediously complicated, and until recently, the tools needed to dependably perform such intricate investigations were lacking. The introduction of the adeno-associated viral vector (AAV) in neuroscience has helped advance both circuit identification and functional circuit analysis, and the result has dramatically expanded the capabilities of neuronal circuit characterization.<sup>1</sup> AAVs have also been important tools for deciphering the roles of specific gene products in neuronal function and animal behavior. Furthermore, AAVs are increasingly promising clinical tools for treating many neurological diseases and conditions. In this review, we aim to briefly highlight AAV-specific technological developments, comment on their implications for neural circuit identifications, manipulations, and clinical utility, and discuss future capacities for AAV-driven preclinical and clinical investigations. This review is

not intended as an exhaustive exploration of these topics, but rather as a sampling of the myriad possible uses of AAVs for neuroscience investigators.

## AAVs

AAVs are small, 4.7-kb, linear, single-stranded DNA (ssDNA) viruses in the parvovirus family that can infect multiple tissue types.<sup>2,3</sup> The AAV genome consists of two open reading frames, Cap and Rep, which are flanked by inverted terminal repeats (ITRs).<sup>4,5</sup> The ITRs are the only elements of the AAV genome that must be delivered *in cis*.<sup>6</sup> Rep is translated to produce proteins necessary for AAV replication (Rep 40, 52, 68, and 78), while Cap is translated to produce structural proteins VP1, VP2, and VP3 that form the 20- to 25-nm icosahedral capsid at a ratio of 1:1:10.<sup>4,7</sup> Cap also produces a non-structural protein, assembly-activating protein, which is involved in assembly of the capsid.<sup>7,8</sup> The ITRs allow for the formation of a hairpin structure, which permits primase-independent synthesis of the second DNA strand via host cell DNA polymerase.<sup>5</sup> Eleven natural serotypes of AAVs have been identified so far, which have different capsid structures, resulting in differing tropism.<sup>7</sup> AAV tropism can also be further altered by creating recombinant versions of multiple AAV serotypes, a process known as pseudotyping. These pseudotyped viruses can have enhanced tropism for specific cell types, as well as improved transduction efficiency in neurons.<sup>9–13</sup>

To infect a cell, the AAV attaches to the cell membrane and undergoes receptor-mediated endocytosis and endosomal trafficking.<sup>14</sup> The AAV then escapes from a late endosome or lysosome and translocates to the nucleus where the virus is uncoated. Double-stranded DNA is produced via the host cell’s polymerase.<sup>15</sup> The fate of AAV then depends on the presence of a helper virus, such as adenovirus or herpes simplex virus. If no helper virus is present, AAV does not replicate. Most AAV genomes remain in the nucleus as an episome in a latent state, although a relatively small percentage may integrate into the host’s genome assuming a latent state that does not engage the lysogenic pathway.<sup>16</sup> If a helper virus is present, AAV gene expression is

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activated, allowing the AAV to replicate. Rep and Cap genes are expressed, allowing for genome replication and progeny ssDNA particles to be synthesized. The completed virions are assembled and released from the host cell when the cell is killed through the lytic pathway by the helper virus.<sup>3,17</sup> AAV replication can also occur without the presence of a helper virus if cellular stress is induced or if the helper virus's genes needed for AAV replication are provided *in trans*.<sup>18</sup>

AAVs integrate into a specific site in the human genome, AAVS1, on chromosome 19, making AAV genomic insertions more predictable than other viral vectors.<sup>19,20</sup> Random integration into the host cell's genome does occur, but at a much lower frequency compared to other vectors.<sup>21</sup> However, the integrative capacity of AAVs can be prevented by removing Rep and Cap from the AAV genome. To construct an AAV transfer plasmid, the promoter and transgene are placed between the two ITRs and Rep and Cap are supplied *in trans* for vector production.<sup>22</sup> In order for AAV replication to occur, an adenovirus, herpes simplex virus, or a helper plasmid containing genes from adenovirus is also required to mediate AAV replication.<sup>20,22</sup>

Among types of commonly used viruses in neuroscience, AAVs can, similar to lentiviruses, infect both quiescent non-dividing cells, such as neurons, as well as dividing cells, whereas retroviruses only infect dividing cells. Additionally, AAV integration into the host genome can be prevented by removing Rep and Cap genes from plasmids.<sup>22,23</sup> When the recombinant AAV cannot integrate into AAVS1, the virus is processed into a double-stranded circular episome, which is maintained extrachromosomally and can persist in non-dividing cells for years, allowing for long-term transgene expression in non-dividing cells.<sup>23,24</sup> This property makes them a prime candidate for neuroscience research in that they are non-pathogenic, cause less immunoreactivity than other viral vectors during and after transduction, can target neurons without helper viruses or capsids, and have fewer effects on basal cell function in comparison to other viral vectors.<sup>25–27</sup> A drawback of AAVs is that they have relatively limited cloning capacity and, therefore, large genes are not suitable for use in AAVs.<sup>28</sup> When an AAV exceeds its carrying capacity, the vector genome is truncated during packaging at the 5' end. However, the use of dual or triple AAVs can theoretically increase the packaging capacity via coinfection of cells with partial sequence fragments. Partial sequence fragments are joined through specific recombination sequences, such as the bacteriophage F1 AK sequence or portions of the ABCA4 gene, “double D” format inverted terminal repeats, or by the utilization of intein-mediated splicing to reconstruct full-length sequences, thus allowing for sequences larger than 5 kb to be delivered with AAVs.<sup>24,29–34</sup> A final disadvantage of AAVs is its ssDNA genome, since the virus relies on the host cell's replication machinery to synthesize the complementary strand, which may delay transgene expression. Self-complementary AAVs have been produced to circumvent the requirement of AAV genome conversion to double-stranded DNA. Self-complementary AAV vectors contain a packaged dimeric inverted repeat, allowing the DNA to fold and base pair, forming double-stranded DNA. Self-complementary AAVs allow for rapid transductions but have half the cloning capacity of single-stranded AAVs.<sup>35–37</sup>

## Preclinical Use of AAVs in Neuroscience Research

### Circuit Identification and Classification

Neuronal circuits are currently defined by many factors, such as location, connectivity, and cell type. Within a neural circuit, neurons that originate from and terminate in the same location can be defined as a node within a larger circuit. Within these nodes, subsets of neurons that originate from and terminate to the same location may connect to different cell populations at their origin or terminal end, further differentiating the node by connectivity. Additionally, within similarly connected nodes, some neurons may release different neurotransmitters (e.g., serotonin or glutamate), which can further differentiate connected nodes by principal neuron type. Also, these nodes may have morphological and electrophysiological differences that can further specify the nodes by other cell-type attributes. A combination of any or all of these factors may characterize a circuit node while also differentiating it from a neighboring node. These differences may also be region-specific, as microcircuits can connect downstream to form larger circuits that regulate many complex cell and biological outputs.

While this complexity presents many variables for neuroscientists to grapple with during their investigations, a logical approach has been to simplify larger circuits, focusing on characterizing specific nodes, edges, and microcircuits within them. Findings from these experiments can then be fed forward to define complex, larger circuits through more multifaceted circuit manipulations and/or computational modeling. Thankfully, the unique properties of AAVs and their ability to target specific nodes in neural circuits make them an excellent choice for this paradigm.

To perform anatomical analyses of circuit nodes and comprehend these circuit topographies, a common goal is to utilize a viral vector that transduces a neuronal population of interest and expresses a fluorescent protein to visualize the target. Given that host cells infected with AAVs are unable to produce new viral particles, AAVs are therefore useful for infecting only one node of a larger circuit, as there is little concern of the virus jumping across synapses to infect connected neighbors. While low tropism and cellular transduction can be an obstacle for using AAVs in rodents, recombinant AAVs (rAAVs) have proven to be a useful solution for targeting specific neuronal cell types and increasing transduction efficiency.<sup>38,39</sup> Serotype 2 (AAV2) has a natural tropism for neurons and is the most commonly used and characterized serotype.<sup>38</sup> AAV2 vectors encoding fluorescent proteins have been instrumental in mapping connectivity between brain regions in animal models. For example, the Allen Mouse Brain Connectivity Atlas project has utilized AAV2-EGFP vectors for tracing the axonal connections between multiple brain regions in the mouse brain.<sup>40</sup> Many other laboratories also use similar techniques to map numerous cell types and neurocircuits in not only mice, but in many other species as well.

One disadvantage of AAV2 is that it is not able to be widely expressed across all neuron types.<sup>38</sup> This can prove difficult for infecting cells of interest and, as a consequence, not all cells in the node would be

**Table 1. rAAV Serotype Family Properties**

Serotype	Transport Direction		Expression by Brain Region			Cell-Type Expression		
	Anterograde	Retrograde	Striatum	Hippocampus	Cortex	Neurons	Astrocytes	Microglia
rAAV1	+	+	*	*	*	***	*	**
rAAV2	+	+	*	*	*	*		
rAAV5	+		***	***	*	**	*	
rAAV6	—	+	*	*	**	**		
rAAV7	+	+	**	**	***	**	**	**
rAAV9	+	+	**	*	***	***		

The number of \* represent relative rAAV expression levels by brain region or cell-type. \* represents the lowest level of expression, \*\* represents general expression levels, and \*\*\* represents highest expression levels in comparison to other rAAV serotypes.

This table was developed with information based on the following citations: <sup>26,161–166</sup>.

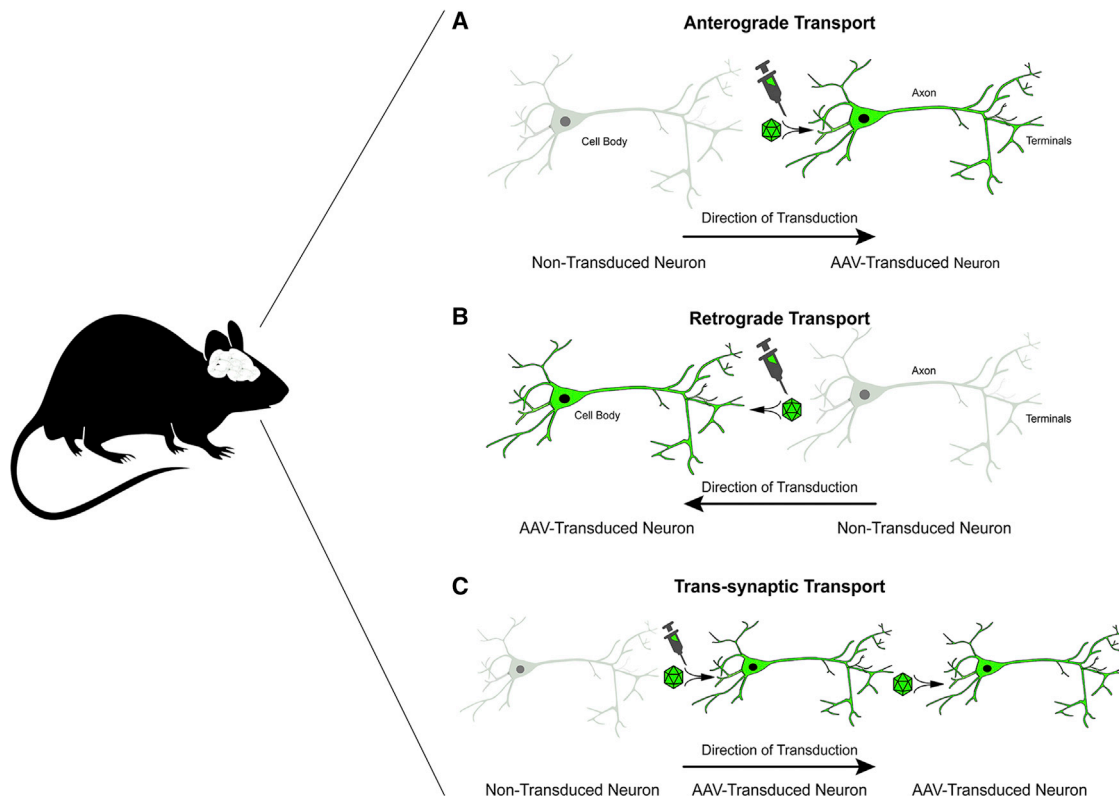
transduced when using AAV2 or one of its natural analogs. However, this can be advantageous when the goal is to transduce a small number of neurons with minimal spread in a specific brain region.<sup>41</sup> As indicated above, currently 11 natural AAV serotypes have been identified, all with different natural tropisms and transduction properties. Most AAV serotypes have the capability to transduce neurons, but that strength of that transduction varies. Some serotypes exhibit differential abilities to transduce neurons *in vivo* and *in vitro*. For example, AAVs 1, 6, and 7 show the greatest specificity for neurons over glia cells in culture, whereas *in vivo* following brain injections, AAVs 1, 2, 5, 7, 8, and 9 all show strong preference for neurons in many studies.<sup>41</sup> As previously mentioned, one way to improve the tropism of AAVs is to use pseudotyping. Pseudotyping involves engineering new viral capsids from different serotypes to create rAAVs. The most used examples are AAV2/5, which increase transduction efficiency and tropism for neurons by combining specific parts of the capsid and genome of AAV2 and AAV5.<sup>42</sup> Also, AAV-DJ, a hybrid of eight capsid types, and AAV-DJ8, a revision of AAV-DJ, have been used to increase transduction rates *in vitro* and *in vivo*, respectively.<sup>43</sup> A table for natural and recombinant AAV characteristics has been included for quick reference (Table 1).

rAAVs also aid in physical targeting and provide increased infection strategies. Most AAVs display the ability to be anterogradely transported. For example, AAVs can be placed at or near the cell body, transported into the cell, and then translocated into the nucleus for transduction as well as anterogradely down the axon to the nerve terminals (Figure 1A). Yet, some AAVs display the natural ability to be transported retrogradely and thus can be transported into the nerve terminal, transported retrogradely down the axon, and translocated into the nucleus for transduction (Figure 1B). However, most AAVs facilitate retrograde transduction poorly. The property of anterograde, retrograde, and bidirectional transport is also serotype- and concentration-dependent.<sup>44</sup> One initial solution to increase retrograde transportation was to use AAVs that utilize specific promoters, such as AAV-CAV, that recognize receptors on axon terminals to increase tropism and efficiency. New iterations of rAAVs, such as rAAV2-retro, have improved retrograde transduction efficiency and tropism for a variety of neuron types via an engineered

peptide insertion between amino acid 587 and 588 in the AAV2 BP1 capsid gene, and they allow more packaging capacity for specific promoter-driven sequences to be used for higher cell-type specificity during transduction.<sup>45,46</sup> However, some AAV serotypes (AAV1, AAV9) are capable of migrating trans-synaptically, allowing for transduction to cross synapses for circuit network labeling and manipulation (Figure 1C).<sup>47</sup> These tools aid researchers aspiring to anatomically describe circuits by providing them with a multitude of options for viral placement and provide confidence that their node of interest will be targeted and transduced by the vectors.

New technological developments in rAAVs are always ongoing. For example, a new generation of rAAVs have introduced a new delivery system for targeting AAVs to neurons in the central and peripheral nervous system. AAV-PHP.eB and AAV-PHP.S can target neurons in the CNS and PNS, respectively, when injected intravenously, bypassing the need to perform site-directed injections in the brain,<sup>23,48,49</sup> but also see Foust et al.<sup>27</sup> This approach decreases experimental complexity for research paradigms and also opens new avenues to targeting neurons for therapeutic gene delivery both in rodent and human models. Also, new AAV variants that rely on transcriptional targeting such as AAV5- glial fibrillary acidic protein (GFAP)/GfaABC<sub>1</sub>D and AAV6-scCBA, a hybrid chicken  $\beta$ -actin (hCBA) promoter fused to rAAV6, have been identified to preferentially physically target other cells in the central nervous system, such as astrocytes and microglia, respectively.<sup>50,51</sup> Not only can researchers perform anatomical circuit analysis with rAAVs, taking advantage of both anterograde and retrograde transport in neurons, but also the increasing diversity of injection strategies and the ability to directly (e.g., neurons) and indirectly (e.g., astrocytes and microglia) label circuit nodes are now accessible for experimental designs.

rAAVs display outstanding tropism and transduction efficiency for neurons, but AAVs and rAAVs also provide an improved method for infecting specific neuronal cell types using intersectional genetics approaches. rAAVs can be used to not only target specific nodes within a circuit, but also to infect cells only expressing a specific genetic marker, thus targeting specific cell populations via what transcripts those populations express. This approach allows AAVs to

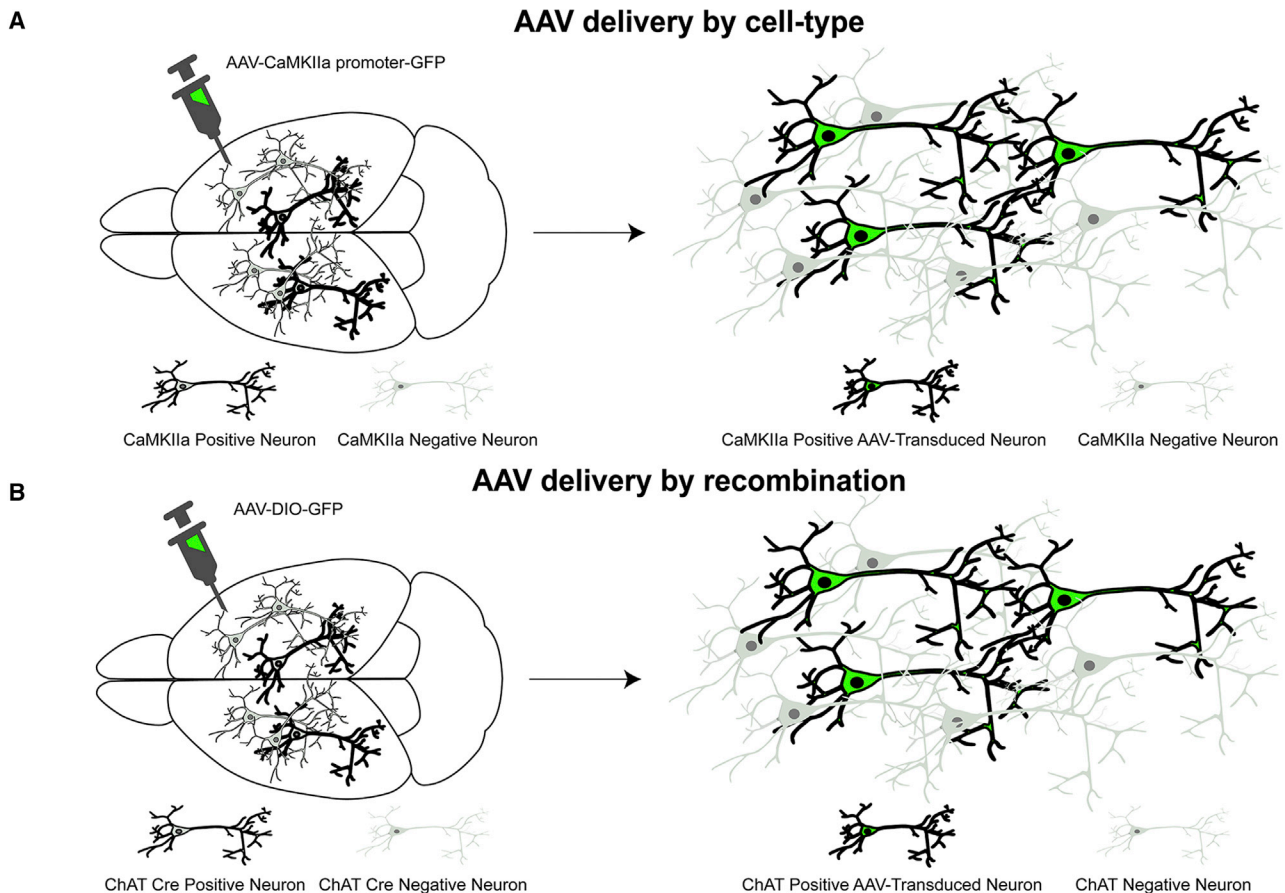


**Figure 1. Physical Targeting of Neuronal Populations**

(A) Anterograde transport methodology: AAV vector infusion results in transduction within cell bodies localized proximal to the injection target. Dependent on vector construction, subsequent cellular trafficking may result in the spread of the transgene product throughout neuronal somatodendritic compartments as well as anterograde transport along axons. This methodology may be useful for mapping neuronal projections, manipulating multiple projection targets through targeting of the cell bodies of transduced neurons at the injection site, or manipulating specific projection targets by targeting axon terminal fields. (B) Retrograde transport methodology: AAV vector infusion results in uptake within axon terminals at the injection site and retrograde transport from the terminals to the nucleus with subsequent transduction and expression of transgene. This methodology may be useful for determining neuronal connectivity as a complementary role to the anterograde methodology. (C) Trans-synaptic transport methodology: specific AAV vectors (e.g., AAV1 or AAV9) are used as described for anterograde transport methodology, but following transport to terminals they are able to migrate trans-synaptically to synaptically connected target neurons. This is especially useful for mapping of and manipulation of neurocircuits, rather than just neuronal connectivity.

only transduce cells that display specific genes of interest and thus act as a Boolean function for the virus to enter specific cell types and avoid others. To accomplish this task, AAV genomes can be modified to only be transduced in cells that express like promoters (Figure 2A) (e.g.,  $Ca^{2+}$ /calmodulin-dependent protein kinase II alpha [CamKIIa], superoxide dismutase-2 [SOD2], GFAP).<sup>52–54</sup> Furthermore, AAVs can be modified to control expression using Cre or Flp to drive recombination events, thus regulating transduction by viral recombination. By inserting a double-floxed inverse orientation (DIO) element, a molecular switch that utilizes flanking loxP recognition sites, into the AAV genome, gene expression can be driven by Cre and Flp recombinases to only express genes of interest in cells that also express Cre or Flp.<sup>55</sup> Additional information on Cre-loxP systems are reviewed elsewhere.<sup>56</sup> This allows neuroscientists to combine AAV-DIO viruses with mouse lines that express Cre in specific cell types to drive transduction in circuits across the brain. A second option is to use a second AAV virus driven by a promoter that encodes

Cre or Flp in combination with an AAV-DIO to isolate the transduction of the viral genome to highly specific cell populations within precise locations of circuits of interest (Figure 2B).<sup>55,57,58</sup> For example, Guo et al.<sup>59</sup> combined many of the above techniques to map whole-brain inputs into the dorsal striatum. By injecting two AAV-DIO vectors (AAV-DIO-EGFP-TVA and AAV-DIO-RG) under a CAG promoter into choline acetyltransferase (ChAT)-Cre, D1-Cre, and D2-Cre mice in the dorsal striatum, the group was able to transfect only neurons that project to and are within the dorsal striatum with a fluorescent reporter and two of the three necessary products for transsynaptic rabies labeling. Two weeks later, a modified rabies virus was injected that only infected cells that were previously transduced with the earlier AAV injection. This approach allows for labeling of the input neurons into the dorsal striatum across the brain by utilizing two promoter-driven AAV-DIO vectors, transsynaptic retrograde labeling techniques, as well as local anterograde labeling, and it utilizes a modified rabies virus. Thus, many of these



**Figure 2. Transcriptional Targeting of Neuronal Populations**

(A) AAV vector expression may be restricted to specific neuronal populations by using AAV vectors constructed with a cell type-specific promoter. In this example, the AAV vector encodes a GFP under the control of the  $Ca^{2+}$ /calmodulin-dependent protein kinase II alpha (CaMKIIa) promoter. Cellular transduction will only result in transgene expression in neurons expressing CaMKIIa. (B) AAV vector expression may be restricted to specific neuronal populations using transgenic mice expressing cre-recombinase within specific cell types. AAV vectors are constructed to be dependent on the activity of cre-recombinase. In this example, using a “Cre-on” strategy, an AAV vector has a GFP-encoding sequence in a double-floxed inverse orientation (DIO). It is infused into transgenic mice that express cre-recombinase within choline acetyltransferase-expressing cells (ChAT-Cre mice). Upon cre-recombinase-mediated recombination, the GFP sequence is flipped into a sense orientation allowing for expression of GFP only in cells that express ChAT. Alternatively, AAV vectors may utilize a “Cre-off” strategy under which cre-recombinase-mediated recombination flips a transgene’s sense orientation, resulting in a lack of vector expression only in cells that express cre-recombinase.

technologies can be multiplexed to answer highly specific questions in neuroscience research.

### Circuit Manipulation

Not only are AAVs an extremely flexible, efficient, and reliable tool for transducing reporters into specific nodes of circuits to understand the topography of neural circuits, but the ability to package elements into them beyond fluorescent reporters is truly where they have revolutionized neuroscience research. While the packaging capacity is relatively limited in AAVs, around 4.8 kb in size, the ability to encode a vast variety of proteins that can modulate circuit nodes has provided the ability to temporally control neuronal function with extremely tight control and accuracy in real time. Most notably, optogenetic and chemogenetic tools have revolutionized the ability to manipulate specific nodes, edges, and microcircuits. Optogenetics involves

placing the sequence for a light-inducible excitatory (e.g., Channelrhodopsin2, ChrimsonR, etc.) or inhibitory (e.g., Arch, eNpHR, etc.) ion channel within the AAV vector.<sup>60–63</sup> This allows for the insertion of these channels into the membrane after viral vector transduction that provides the investigator with the ability to alter specific neuronal properties using specific wavelengths of light with extreme temporal precision. Activation of these light-driven channels alters membrane potential dynamics and neurotransmitter release. This provides neuroscientists with the ability to use different wavelengths of light to modulate neuronal firing on microsecond timescales by either increasing or decreasing firing of neurons within nodes of interest. Alternatively, light can be used to manipulate presynaptic excitability, allowing investigators to probe inputs from one node into another. This application provides the ability to probe effects of circuit modulation in *in vitro*, *ex vivo*, and *in vivo* models. The power of *in vivo*



optogenetics for circuit modulation is vast and can be used to modulate behaviors such as altering taste perception in real time to changing positive and negative memory association.<sup>64,65</sup> Chemogenetic tools, such as DREADDs (designer receptors exclusively activated by designer drugs) can be used similarly to optogenetic tools packaged in AAVs, but these vectors encode engineered G protein-coupled receptors that bind inert chemicals such as clozapine-*n*-oxide or salvinorin B.<sup>66,67</sup> Excitatory and inhibitory DREADDs also allow for control over neuronal function; however, chemogenetics works at greater timescales in comparison to optogenetic techniques, at the minute to hour timescale. For example, Krashes et al.<sup>68</sup> targeted an excitatory DREADD to a specific set of neurons that are important in regulating feeding using an AAV-DIO and Cre-AgRP mouse line. When stimulating the DREADD receptors to activate the neurons that they were expressed on, the group was able to show that food intake increased for up to 24 h after activating the DREADD receptors. This displays the power of long-term modulatory effects that are achievable via chemogenetic techniques not only for feeding. When paired together, optogenetic and chemogenetic tools can offer interesting insights into how manipulating specific nodes within neural circuits leads to changes in neuronal function, synaptic plasticity, and ultimately complex biological behaviors.

Beyond directly manipulating nodes, packing calcium, neurotransmitter, and voltage-sensitive fluorescent reporters into AAVs has given neuroscientists the ability to sample neuronal firing in *ex vivo* and *in vivo* models. The Looger lab has developed calcium-sensitive proteins that contain a fluorescence molecule integrated with calmodulin that changes its fluorescent activity when calcium is released in the cell.<sup>69</sup> Considering that calcium is an important secondary messenger in many signal cascades and is dynamically and rapidly released during neuronal activation, these sensors act as proxies for neuronal activity that are visible using light capturing devices such as optic ferrules paired with high-powered microscopes, such as two-photon and stimulated emission depletion (STED) microscopes. This allows researchers to specifically express these sensors in neurons of interest to study node activity in freely behaving rodents and non-human primates or modulate some other input of the circuit using optogenetics or chemogenetics and image the output at some other location in the node. Studies using these approaches made possible by AAVs can decipher true measures of input/output changes within circuits. By using such techniques, investigators are able to view real-time calcium imaging data that demonstrate how dopamine neurons contribute to reward, reward prediction errors, aversion, and skill-learning tasks.<sup>70,71</sup> Also, monitoring ventral tegmental area (VTA) dopaminergic neurons has allowed for mathematical models of how different inputs, such as motor, sensory, and cognitive variables modulate dopamine release.<sup>72</sup> Beyond calcium, there are now available sensors for glutamate (iGluSnFR),  $\gamma$ -aminobutyric acid (GABA) (iGABASnFR), dopamine (dLight), and many more currently under development.<sup>73–76</sup> AAV-driven genetically-encoded fluorescent voltage sensors allow for direct visualization of membrane voltage dynamics within AAV-transduced neurons.<sup>77</sup> Not only can neuroscientists

now visualize neuronal firing changes, they can also determine the contents released from these nodes and how diversity in these signals can modulate specific circuits.

AAVs have also been useful tools for “tagging” neurons. Tagging techniques are useful for identifying recorded neurons *in vivo*, assessing neuronal network activation following completion of behavioral sequences or a specific *in vivo* treatment, or for *post hoc* isolation of activated cells for genetic or proteomic analyses.<sup>78,79</sup> For example, channelrhodopsin-2 (ChR2) can be packaged within an AAV in concert with a separate transduction of herpes simplex virus 1 (HSV1) to transduce a specific subset of neurons. The output is a grouping of neurons that are labeled with HSV1, ChR2, or both. Upon a quick pulse of light, the subpopulation of ChR2-targeted neurons can be illuminated to determine their location versus unlabeled neurons. Further functional comparisons *in vivo* can then be achieved such as functional activity assessments of ChR2-labeled subpopulations or microcircuit analysis of the connectivity between large populations of neurons.<sup>80,81</sup> Tagging activated cells that respond to complex biological behaviors, such as memory, is also achievable with AAVs. Using FosTrap, a dual-virus approach that utilizes an AAV with a molecular tag or substrate and a separate AAV-cFos, allows for the molecular tag to be transduced in neurons that were only recently functionally active. cFos, an intermediate early gene, is expressed within a cell upon neuronal activation, which then allows the second AAV carrying a reporter gene or molecular substrate to be transduced only in those activated cells. This tool has allowed researchers to tag and trace engram cells, which are cells that encode contextual memory. Such experiments have allowed for modulation of molecular substrates via optogenetic and chemogenetic techniques that underlie fear memories. In essence, researchers can effectively silence or enhance specific contextual memories at the cell level. This has in turn advanced our understanding of where and how specific memories are formed in the brain and also helped further the understanding of the molecular and circuit basis of post-traumatic stress disorder and other anxiety-related disorders.<sup>82,83</sup>

#### Other AAV Functions

Beyond manipulating neuronal firing and sampling neuronal activity, AAV vectors have provided alternative ways to alter circuit nodes. AAV-CASP3 is a cell death protein transduced by an AAV that allows specific ablations of neuronal populations of interest.<sup>84</sup> This approach iterates on classical pharmacological or mechanical lesion studies to provide advanced targeting when aiming to remove specific connections in a node while decreasing off-target effects. AAVs have been incredibly useful tools for exploring the role of specific gene products within specific cell types within specific nodes in neuronal function and behavior. This can be accomplished by AAV-driven cre-recombinase expression within defined brain regions using cell type-selective promoters in mice that are conditional knockouts for proteins of interest or in mice transduced with other cre-recombinase-dependent vectors.<sup>85–87</sup> Also, mechanistic changes downstream of neuronal activation or silencing are now achievable with AAVs that can control endosome trafficking via light, which allows for general control of



**Table 2. Summary of Disease Indications for Which AAV-Based Gene Therapies Have Been Applied in Clinical Trials Worldwide**

Inborn errors of metabolism	Neurologic
<ul style="list-style-type: none"> <li>• Lipoprotein lipase deficiency</li> <li>• Canavan disease</li> <li>• Batten disease</li> <li>• Mucopolysaccharidosis type I, II, IIIA, IIIB, and VI</li> <li>• Metachromatic leukodystrophy</li> <li>• Aromatic L-amino acid decarboxylase deficiency</li> <li>• Familial hypercholesterolemia</li> <li>• Acute intermittent porphyria</li> <li>• Crigler-Najjar syndrome</li> <li>• Tay-Sachs disease</li> <li>• Pompe disease</li> <li>• Galactosialidosis</li> <li>• Ornithine transcarbamylase deficiency</li> <li>• Glycogen Storage Disease Type I</li> </ul>	<ul style="list-style-type: none"> <li>• Parkinson's disease</li> <li>• Alzheimer's disease</li> <li>• Spinal muscular atrophy</li> <li>• Amyotrophic lateral sclerosis</li> <li>• Temporal lobe epilepsy</li> <li>• Charcot-Marie-Tooth neuropathy type 1A</li> </ul>
	Ocular
	<ul style="list-style-type: none"> <li>• Leber congenital amaurosis</li> <li>• Leber hereditary optic neuropathy</li> <li>• Choroideremia</li> <li>• Age-related macular degeneration</li> <li>• Achromatopsia (Color Blindness)</li> <li>• Retinitis pigmentosa</li> <li>• X-linked retinoschisis</li> </ul>
	Cancer related
<ul style="list-style-type: none"> <li>• Rheumatoid arthritis</li> <li>• Osteoarthritis</li> <li>• Muscular dystrophies (Duchenne, Becker, and Limb-Girdle)</li> <li>• Digital flexor tendon injury</li> </ul>	<ul style="list-style-type: none"> <li>• Gastric cancer</li> <li>• Prostate cancer</li> <li>• Nasopharyngeal carcinoma</li> <li>• Multiple myeloma</li> <li>• Malignant melanoma</li> <li>• Non-Hodgkin lymphoma and B cell acute lymphoblastic leukemia</li> <li>• Irradiation-induced parotid salivary hypofunction</li> </ul>
	Hematologic
	<ul style="list-style-type: none"> <li>• Hemophilia A and B</li> </ul>
	Multisystem
	<ul style="list-style-type: none"> <li>• Alpha-1 antitrypsin deficiency</li> <li>• Prader-Willi syndrome</li> </ul>
	Musculoskeletal
<ul style="list-style-type: none"> <li>• Cystic fibrosis</li> </ul>	
	Cardiovascular
<ul style="list-style-type: none"> <li>• Chronic heart failure</li> </ul>	
	Infectious
<ul style="list-style-type: none"> <li>• HIV</li> <li>• Hepatitis C</li> </ul>	

This table was developed based on the information provided by Gene Therapy Clinical Trials Worldwide, *The Journal of Gene Medicine* (<http://www.abedia.com/wiley/index.html>).

removal or reinsertion of proteins such as membrane signaling or adhesion molecules at specific sites.<sup>88</sup> Also, recently the machinery required for CRISPR, the directed gene editing technique, have been packaged into AAVs to perform site-specific gene editing in neurons of interest to perform gain- or loss-of-function mutations as well as gene editing in real time to determine the effects of genetic alteration on circuit function.<sup>89–91</sup>

### Clinical Use of AAVs

The search for the perfect gene-carrying vector has been underway from the moment gene therapy was first hypothesized to be a potential treatment for disease. An ideal gene-carrying vector must have a high rate of gene transfer, produce long-term gene expression, and possess low immunogenicity and pathogenicity to be a successful candidate for therapy.<sup>92,93</sup> Replication-defective retroviruses were the original vectors used during the earlier stages of gene therapy; however, the potential genotoxicity resulting from integration into the host's genome raised concerns that impeded advancements.<sup>94</sup> However, the second wave of gene therapy in the early 2000s was marked by the rising use of AAVs.<sup>94</sup> AAVs largely meet all character-

istics required of a successful gene vector. Although complex immune responses directed against AAV vector-based gene therapies are known to occur, overall, AAVs maintain an impressive safety and tolerability profile.<sup>93,95</sup> Additionally, AAVs efficiently transduce both non-dividing and dividing cell types for a clinically relevant length of time in many cases.<sup>96</sup> Although AAV2 is the most widely used variant because it was the first serotype identified, numerous other serotypes with varying tropism exist, giving AAVs wide clinical applicability.<sup>92,93,97</sup> When combined, these characteristics have established AAVs as a leading vector choice for gene therapy studies with one of the highest margins of success.<sup>98</sup>

Although AAV-based gene therapies have grown rapidly, the use of this vector is still associated with some challenges. For systemic administration of AAV-based gene therapies, the ability to target specific cell types and pass through barriers (e.g., the blood-brain barrier) to those tissues is particularly challenging.<sup>99</sup> Additionally, most of the population has been naturally exposed to many AAV serotypes, resulting in neutralizing anti-capsid antibodies.<sup>99–102</sup> Both challenges can significantly reduce the transduction efficiency of AAVs. However, some innovative strategies, such as rationally designed AAV variants through site-directed mutagenesis and directed evolution, are being employed to aid in vector improvement and circumvent these barriers to therapeutic success.<sup>23,99,103–105</sup> The packaging capacity of AAVs can also be a challenge for certain gene therapies.<sup>93,101</sup> Coding sequences larger than 5 kb are unlikely to be effectively packaged into the vector or successfully transduced.<sup>93,97</sup> While this size limitation is rarely a concern for human therapeutic applications, overlapping dual or triple AAV vectors are a possible solution for expressing genes that exceed the packaging capacity of AAVs.<sup>33,97</sup> For example, a dual AAV approach was successfully used to restore expression of otoferlin, a protein whose coding sequence exceeds the packaging capacity of a single AAV, in the cochlea of otoferlin null mice, a mouse model of genetic human deafness.<sup>106</sup> A triple AAV approach improved the capacity to 14 kb and allowed investigators to express the ciliary/centrosomal protein, ALMS1, which has a cDNA length of 12.5 kb, in mouse and pig retina.<sup>33</sup> Transduction efficiency in mice was very low, but achieved 40% transduction in pigs. The low expression in mice may be explained by species differences, but this will require additional study. Studies such as these provide promise for more flexible gene therapies in the future. As advancements in AAV virology and engineering continue, a growing repertoire of AAV variants with high tissue specificity and transduction efficiency will continue to become available for clinical studies.

AAVs have been utilized in more than 230 active or completed clinical trials worldwide.<sup>101,107</sup> See Table 2 for many of the clinical indications where AAVs were used as vectors in clinical studies. Additionally, the viral vector was among the first ever approved gene therapy in the form of Glybera, approved by the European Medicines Agency in 2012 for the treatment of lipoprotein lipase deficiency (LPLD), and also Luxturna, approved in 2017 by the US Food and Drug Administration (FDA) for a rare type of retinal dystrophy.<sup>97,99,108</sup> Although a full description of all clinical studies is not



possible here, a brief summary of some successful and promising clinical applications for AAVs is included below.

#### **Approved Applications of AAV-Based Gene Therapies**

LPLD is a rare genetic disease resulting in chylomicronemia and hypertriglyceridemia, which can put individuals at risk for pancreatitis and pancreatic insufficiency.<sup>109</sup> After promising preclinical data, the first human trial for an AAV vector to deliver a gain-of-function lipoprotein lipase gene variant began in 2005 in Europe.<sup>110</sup> In addition to being well tolerated, half of the subjects had a  $\geq 40\%$  reduction in triglycerides 12 weeks following vector administration.<sup>110</sup> Two additional clinical trials using the same AAV vector, now referred to as alipogene tiparvec (Glybera), similarly demonstrated that the gene therapy product decreased plasma triglycerides transiently, positively altered chylomicron metabolism, and led to persistent expression of the functional lipoprotein lipase enzyme.<sup>111,112</sup> After a final retrospective analysis demonstrating that the product was able to reduce the risk of pancreatitis in LPLD, Glybera became the first gene therapy approved in Western world.<sup>113</sup> Unfortunately, 5 years after this approval, the maker of Glybera announced it would withdraw the therapy due to the exceptionally high cost of treatment.<sup>114</sup>

Due to the immune privilege of the eye, the localized anatomy within a closed physical space, and post-mitotic state of retinal cells, AAV-based gene therapies for inherited retinal diseases have been extensively tested.<sup>94,115</sup> Luxturna (voretigene neparvec) delivers a functional *RPE65* gene that encodes an enzyme crucial to the retinoid cycle in patients with Leber congenital amaurosis, a rare, blinding retinal dystrophy.<sup>94,101,116</sup> In clinical trials, this AAV2-mediated therapy improved light sensitivity, navigational abilities, and visual acuity in those with the condition.<sup>116,117</sup>

Spinal muscular atrophy (SMA), the most common genetic cause of death in infants, results from a loss or dysfunction in the survival motor neuron (*SMN1*) gene.<sup>118</sup> The disease results in progressive loss of lower motor neurons and muscle atrophy, often leading to respiratory failure.<sup>118</sup> An intravenous AAV9-SMN approach was able to rescue motor neurons, leading to a significant prolongation of lifespan in a mouse model of SMA.<sup>119</sup> This same method was replicated in a small clinical study of 15 infants with SMA beginning in 2014. All patients who received either the low or high dose of the systemically administered AAV9-SMN vector were alive at 20 months of age compared to an 8% survival rate at this time in a historical cohort.<sup>118</sup> Many of these infants were able to achieve clinically meaningful motor developmental milestones such as feeding, sitting, and talking, which is typically not present in historical cohorts.<sup>118</sup> While a larger global clinical trial is still ongoing (ClinicalTrials.gov: NCT03505099), in May of 2019, the FDA approved Zolgensma (onasemnogene abeparvec-xioi; an AAV9-SMN vector) for the treatment of SMA in children less than 2 years of age.

#### **Potential Central Nervous System Applications of AAV-Based Gene Therapies**

As the blood-brain barrier prevents many systemically administered drugs from reaching the central nervous system and direct intrapar-

enchymal delivery of therapeutics would require frequent re-administration, gene therapy for neurological diseases is an attractive treatment modality.<sup>120,121</sup> To date, nearly all gene therapy clinical trials of neurological diseases have utilized AAVs as vectors for delivery.<sup>121,122</sup> Much of the promising results in these gene therapy trials have been observed in studies of choroideremia, Parkinson's disease (PD), and Alzheimer's disease (AD).

Another AAV-based gene therapy is projected to be approved for the treatment of choroideremia, a progressive X-linked recessive blinding condition due to mutations in the *CHM* gene.<sup>101,123,124</sup> Using an AAV2 vector, a functional *CHM* gene delivered into the retina of one eye significantly improved visual acuity compared to the control eye, which did not receive the functional gene. Impressively, this improvement in visual acuity remained for 3.5 years post-treatment in two out of the six patients despite progressive degeneration in the control eye that did not receive intervention.<sup>124,125</sup>

In PD trials, AAV-transduced genes are frequently neurotrophic factors to potentially prevent cell death or enzymes related to neurotransmitter synthesis that may modulate neuronal activity.<sup>104</sup> One frequently studied method involves AAV-mediated delivery of aromatic L-amino acid decarboxylase (AADC), the final enzymatic step that converts levodopa into dopamine, into the striatum. Levodopa therapy often fails to alleviate motor symptoms, as the PD progresses even with increasing doses, but AAV-AADC gene therapy replacement may offer a strategy to maintain steady dopamine concentrations in patients taking levodopa.<sup>121</sup> Early-phase studies demonstrated successful gene expression and moderate improvements in motor symptoms when an AAV-AADC was delivered to the putamen bilaterally, but larger controlled trials are necessary to accurately assess the efficacy of the treatment on disease symptomology over time.<sup>126,127</sup> Two additional phase I trials are underway using a real-time MRI-guided approach to monitor delivery of an AAV2-AADC with the goal to more accurately target the putamen (ClinicalTrials.gov: NCT01973543 and NCT03065192). An alternative GABAergic approach delivers the glutamic acid decarboxylase gene (the rate-limiting enzyme in GABA synthesis) to the subthalamic nuclei of patients. Phase I and II studies in patients with PD reported that the vector was well tolerated and mildly improved motor symptoms up to 6 months following gene transfer, suggesting it may be a promising treatment modality for the disease.<sup>128,129</sup> Intracranial administration of AAV vectors also has the potential to persistently express genes that target the progressive accumulation of abnormal proteins that characterizes many neurodegenerative diseases, including PD.<sup>121</sup> Another AAV-based gene therapy approach involves the expression trophic factors such as glial-derived neurotrophic factor (GDNF) or neurturin, a structural and functional analog of GDNF.<sup>121</sup> The delivery of GDNF or neurturin into the putamen have shown promise as neuroprotective agents in rodent and nonhuman primate phase I clinical studies of PD.<sup>121,126,127,130-133</sup> However, two larger, double-blinded randomized trials failed to show that an AAV2-neurturin gene therapy injected bilaterally into the putamen and the putamen and substantia nigra were superior





to sham surgery.<sup>130,134</sup> Although an additional phase I, open-label trial using AAV2-GDNF delivery to the putamen is underway (ClinicalTrials.gov: NCT01621581), the previous larger, double-blinded trials certainly cast doubts on the future of trophic factor gene therapy in PD; however, larger viral infusion volumes and more accurate delivery guided by real-time MRI may offer some hope.<sup>121,122,135</sup>

Neurotrophic factors have also been explored in the treatment of AD. Nerve growth factor (NGF) is one such neurotrophic factor that has been shown to promote neurorestoration and neurorepair of cholinergic neurons of the basal forebrain.<sup>136,137</sup> Dysfunction of this neuronal population is thought to underlie the clinical symptomatology of AD, and cholinesterase inhibitors, a mainstay of disease treatment, are known to exert their therapeutic effects on the basal forebrain cholinergic neurons.<sup>138,139</sup> While an initial gene therapy trial of NGF utilized an *ex vivo* approach, subsequent human studies employed an *in vivo* AAV2-based strategy to deliver the NGF gene.<sup>139–142</sup> AAV2-NGF delivered to the *nucleus basalis* of patients with AD is well tolerated and leads to persistent expression of NGF up to 4 years after treatment.<sup>142</sup> Upon autopsy in two patients, the neurons of the nucleus basalis displayed signs of a trophic response (e.g., axonal sprouting toward NGF and increased cellular signaling molecules).<sup>140</sup> Unfortunately, in a sham surgery-controlled trial of 49 patients with AD, AAV2-NGF had no benefit over placebo on cognitive measures 24 months post-injection.<sup>139</sup> As with other neurological gene therapy trials, the authors suggested that vector mistargeting could underlie the lack of successful findings for NGF in the treatment of AD. Other gene-based strategies for treating the disease include the transduction of APOE, a regulator of lipid metabolism in the brain that has been shown to alter the handling of  $\beta$ -amyloid. The APOE2 allele has been shown to be protective in AD, likely by decreasing plaque burden.<sup>143</sup> In pursuit of this protective effect, an ongoing phase I trial is investigating the effects of AAV-APOE2 transduction in patients who are homozygote for APOE4, the allelic form of the lipid regulator associated with AD (ClinicalTrials.gov: NCT03634007).

AAV-based gene therapies are quickly becoming a popular investigative treatment strategy for many other neurological conditions in animal models. Promising preclinical studies employing AAVs have also been completed in models of Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), neuropathic pain, and epilepsy.

RNA interference (RNAi) mechanisms have been harnessed to target the protein transcript and prevent the accumulation of mutant huntingtin protein (HTT) that is the hallmark of HD.<sup>144</sup> AAV delivery of therapeutic microRNAs have been demonstrated to reduce basal ganglia HTT expression in healthy rhesus macaques and selectively knock down certain mutant HTT alleles in a mouse and minipig model of HD.<sup>145–147</sup> Another novel therapeutic mechanism utilizing AAVs that has shown remarkable promise involves delivery of CRISPR-Cas9 gene-editing systems to selectively suppress mutant HTT in the striatum.<sup>148,149</sup> In these mouse models of HD, CRISPR-Cas9-mediated disruption of the mutant HTT gene reduced neuronal

inclusions, improved motor deficits, and increased lifespan. Others have used AAV vector-induced expression of zinc finger proteins to repress mutant HTT expression.<sup>150,151</sup>

Similar to HD, RNAi mechanisms and gene-editing systems using AAV vectors have also been a popular therapeutic strategy for addressing ALS. A form of familial ALS caused by an intron repeat expansion in the chromosome 9 open reading frame 72 (C9orf72) gene, which leads to the accumulation of toxic RNA foci and protein in affected cells, has been extensively studied.<sup>152,153</sup> The feasibility for a therapeutic microRNA delivered via an AAV5 vector was recently demonstrated by its ability to reduce C9orf72 expression in both HEK293T cells and induced pluripotent stem cell-derived neurons as well as *in vivo* using a mouse model of ALS.<sup>152</sup> Mutations in superoxide dismutase-1 (SOD1), which lead to toxic aggregates of misfolded protein, are another common cause of familial ALS, and an alternative to RNAi may be to deliver an AAV that expresses a protein that prevents this toxic SOD1 misfolding.<sup>153</sup> Recently, a study demonstrated that an AAV delivering macrophage migration inhibitory factor (a protein shown to inhibit mutant SOD1 misfolding) reduced spinal cord levels of misfolded SOD1 and significantly delayed disease onset, prolonging overall survival in a mouse model of overexpressing mutant SOD1.<sup>154</sup> Another potential mechanism for targeting mutant SOD1 was recently demonstrated with a targeted disruption of mutant SOD1 expression in the spinal cord using the CRISPR-Cas9 gene-editing system delivered via an AAV vector.<sup>155</sup> This reduction in mutant SOD1 was associated with significantly greater motor neuron survival (~50%) and markedly delayed disease onset in the ALS model.

The mechanisms for AAV-based therapies for neuropathic pain and epilepsy have been more diverse, likely reflecting the complex, heterogeneous underlying mechanisms that contribute to both disease states. Several studies have investigated the ability of neuropeptide Y, delivered to the brain via AAVs, to reduce seizure activity in various rodent models of epilepsy.<sup>156–158</sup> A study recently demonstrated that an AAV1 therapeutic vector could effectively transduce human neuronal cells to express neuropeptide Y, supporting further preclinical investigation.<sup>159</sup> Preclinical studies for neuropathic pain frequently include using AAVs to deliver genes that will alter the excitability of peripheral neurons, thereby reducing hyperexcitability, which is thought to underlie the hyperalgesia and allodynia often present in patients with the disease. For instance, calcium channel-binding domain 3 (CBD3) blocks N-type voltage-gated calcium channel activity in the spinal cord and dorsal root ganglion. A recent study demonstrated that AAV-mediated expression of CBD3 was able to decrease pain behavior in a rat model of neuropathic pain.<sup>160</sup> Although more investigation is needed, these preclinical studies of neurological diseases demonstrate feasibility and support the potential application of AAV-based gene therapies in the clinic one day soon.

While considerable progress in AAV-based gene therapies has occurred in the last two decades, there remains room for improvement. AAVs have been established as safe, well-tolerated viral vectors,



but barriers remain concerning inducing persistent gene expression, reducing the production of neutralizing antibodies, and targeting specific cell populations. In addition to these biological barriers, the financial burden of gene therapies on manufactures, healthcare systems, and individual patients will continue to stifle their clinical application unless alternative payment strategies are developed and/or large-scale gene therapy manufacturing methods are significantly advanced. These biological and financial obstacles must be overcome if these promising AAV-based gene therapies are expected to become practical therapeutic strategies in the everyday treatment of disease.

### Conclusion

AAVs have been truly transformative for basic neuroscience research, allowing for great advances in our understanding of brain connectivity, cellular function (including neurons and glia), and the roles of specific molecules and cell types in complex animal behaviors. As pre-clinical studies continue to provide more powerful AAV vectors for transducing specific cell types in the nervous system and more advanced methodologies for easier delivery of AAVs to the CNS, these findings from basic research have increasing translational potential for the development of novel therapeutics. Indeed, as presented herein, there is already accumulating evidence that AAV-based therapies are likely to be successful agents for treating a variety of neurological diseases. The speed at which advances in AAV-based neuroscience research tools are progressing suggests that the coming years will be further filled with exciting new discoveries and the development of new treatments that will substantially improve many people's lives.

### AUTHOR CONTRIBUTIONS

D.L.H., G.G.G., K.C.R., and B.A. contributed to the writing of the manuscript.

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