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Engineered AAV vectors for improved central nervous system gene delivery

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Adeno-associated viruses (AAV) are non-pathogenic members of the *Parvoviridae* family that are being harnessed as delivery vehicles for both basic research and increasingly successful clinical gene therapy. To address a number of delivery shortcomings with natural AAV variants, we have developed and implemented directed evolution—a high-throughput molecular engineering approach to generate novel biomolecules with enhanced function—to create novel AAV vectors that are designed to preferentially transduce specific cell types in the central nervous system (CNS), including astrocytes, neural stem cells, and cells within the retina. These novel AAV vectors—which have enhanced infectivity *in vitro* and enhanced infectivity and selectivity *in vivo*—can enable more efficient studies to further our understanding of neurogenesis, development, aging, and disease. Furthermore, such engineered vectors may aid gene or cell replacement therapies to treat neurodegenerative disease or injury.

Keywords: adeno-associated virus, astrocytes, directed evolution, gene delivery, neural stem cells, retina, viral engineering

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Adeno-Associated Virus Background

Adeno-associated viruses (AAV) are a family of parvoviruses with a 4.7 kb single-stranded DNA genome contained inside a non-enveloped capsid.[1] The viral genome has 2 inverted terminal repeats (ITR)—which function as the viral origin of replication and packaging signal—flanking 2 primary open reading frames (ORF): *rep* (encoding proteins that function in viral replication, transcriptional regulation, site-specific integration, and virion assembly) and *cap*.[1] The *cap* ORF codes for 3 structural proteins that assemble to form a 60-mer viral capsid.[1] Many naturally occurring AAV variants and serotypes have been isolated,[2-8] and none has been associated with human disease. Additionally, recombinant versions of AAV can be used as gene delivery vectors, where a marker or therapeutic gene of interest is inserted between the ITRs in place of *rep* and *cap*.[9] These vectors have been shown to transduce both dividing and non-dividing cells *in vitro* and *in vivo* and can result in stable transgene expression for years in post-mitotic tissue.[1]

Directed Evolution of Adeno-Associated Virus for CNS Targets

Differences in the protein capsid sequences of the natural AAV serotypes are associated with different gene transfer properties. Therefore, strategic modifications to the *cap* gene have the potential to yield delivery properties that are distinct from, and potentially highly advantageous compared to, any of the natural AAV variants. While rational design has the potential to increase the efficiency of AAV vectors, incomplete knowledge of viral delivery mechanisms coupled with the vast range of possible sequence modifications argues for unbiased, high-throughput algorithms for AAV engineering. In particular, we have developed directed vector evolution as a high-throughput molecular engineering approach to successfully generate novel, "designer" AAV variants with specific sequence modifications that enable them to overcome formidable barriers to efficient gene delivery in

a broad range of applications.[10–14] Within this process, highly diverse libraries composed of $\sim 10^7$–10^8 genetic variants of the AAV *cap* gene are iteratively subjected to selective pressures, resulting in enhanced gene delivery properties such as altered receptor binding, neutralizing antibody-evasion capabilities, and novel cell tropism.[10-13]

Directed evolution has for example been applied to create novel AAV vectors designed to overcome gene delivery barriers and/or preferentially transduce specific cell types in the central nervous system.[15-18] For instance, because natural AAV serotypes preferentially transduce neurons,[19-21] research has also been undertaken to develop AAV vectors that are capable of efficient transduction of other neural cells.[16–18] For example, Koerber et al. utilized a diverse library of AAV variants generated through DNA shuffling, random mutatgenesis, and surface loop replacement to evolve vectors for the ability to infect primary human astrocytes in culture.[16] The resulting vectors transduced between 2-fold and 15-fold more primary human astrocytes compared to AAV serotype 2 (AAV2).[16] Two variants tested *in vivo* also transduced 3.3-fold and 5.5-fold more astrocytes than wild-type AAV2 within the rat striatum following intracranial injection.[16] These 2 variants display similar peptide motifs at amino acid 262 (in loop 2 of the AAV capsid), which may enhance cell binding or internalization.[16]

Using a library of shuffled *cap* genes from several wild-type serotypes, Gray et al. isolated AAV variants capable of gaining access via intravenous administration to regions of the brain in which seizure had compromised the blood–brain barrier.[15] After kainic acid-induced seizure, 2 clones composed primarily of wild-type AAV1, 8, and 9 could transduce cells in the piriform cortex and ventral hippocampus of rats upon tail vein administration of the vectors, but no transduction occurred in brain areas where the blood–brain barrier was not compromised.[15] Within these brain areas, the variants efficiently transduced oligodendrocytes and neurons, but not astrocytes or microglia.[15] Comparison of the *in vivo* biodistribution of these clones with parental serotypes AAV1, 8, and 9 revealed that the portion of AAV8 within these clones likely led to the ability to cross the seizure compromised barrier, while the AAV1 portions of the clones likely led to peripheral organ transduction pattern.[15]

In addition to efforts to target largely postmitotic cells, Jang et al. applied directed evolution to engineer an AAV variant capable of efficient neural stem cell (NSC) transduction.[17] Using a *cap* library generated by DNA shuffling, random mutagenesis, and random peptide insertion, selection for the ability to infect cultured adult hippocampal NSCs yielded AAV r3.45, an AAV2 variant with a 7 amino acid peptide insertion at position 588.[17] Compared to wild-type AAV2, AAV r3.45 mediated 50-fold increased transduction of rat NSCs and significantly increased transduction of murine NSCs, human fetal NSCs, and human embryonic stem cell (hESC) derived neural progenitor cells *in vitro*.[17] A subsequent study showed that within an hESC-derived culture containing a mixture of cells including NSCs, neurons, and astrocytes, the percentage of NSCs infected by AAV r3.45 was significantly higher compared to wild-type AAV.[18] The variants still bound heparan sulfate proteoglycans (HSPG, AAV2s primary receptor), though peptide insertion at amino acid 588 in the parental AAV2 capsid lowered AAV 3.45s HSPG affinity. In addition, the inserted peptide, which did not have homology to any known cell surface proteins, likely conferred affinity to an undetermined secondary receptor that potentially mediated viral internalization.[17] AAV r3.45 also efficiently and preferentially transduced rat and mouse NSCs following intracranial administration to the hippocampus. Approximately 65% of the cells transduced in the rat hippocampus by AAV r3.45 were Type 2a NSCs, and 9% were Type 1 NSCs.[18] Furthermore, 60% of Type 2a NSCs and 41% of the Type 1 NSCs, respectively, were transduced by AAV r3.45 in the rat brain.[18] Similarly, in the mouse brain approximately 38% of Type 2a NSCs were transduced in the hippocampus.[18]

There has also been considerable work in engineering novel AAVs for delivery to the neuroretina. The cells most commonly involved in retinal disease, photoreceptors and retinal pigment epithelium, are located in the outer retina, separated from the vitreous fluid of the eye by the inner limiting membrane and several dense cell layers. As a result, no natural AAVs are capable of transducing these cells following an intravitreal injection, a preferred, noninvasive route of administration that could conceivably enable vector to transduce the entire retinal surface area. Therefore, clinical trials conducted to date—which have been increasingly successful for Leber's congenital amaurosis type 2[22–24] and choroideremia[25]—have relied on vector administration via subretinal injection, a surgery that can damage both healthy and diseased retinas and that results in transduction in a relatively small portion of the retina.

Two vectors capable of improved outer retinal transduction upon intravitreal injection have recently been developed in rodent. First, Klimczak et al. engineered an AAV variant, ShH10, capable of highly specific (over 94% of transduced cells) and efficient infection of Müller cells, glial cells that span the thickness of the retina, when delivered intravitreally.[26] Further analysis of the individual point mutations in ShH10 revealed that its N451D mutation decreased the variant's dependence on N-linked sialic acids, and this single point mutation was sufficient to cause intravitreal Müller cell tropism. In addition, the D532N mutation appears to increase affinity to HSPG, which are not a receptor for the parental AAV6.[26] In a subsequent study, this vector mediated broad expression of glial cell-derived neurotropic factor (GDNF), which slowed retinal degeneration in a rat model of retinitis pigmentosa.[27]

In another study, using *in vivo* directed evolution in a mouse, Dalkara et al. generated the AAV variant 7m8, which was capable of transporting genetic cargo through the retina and directly infecting photoreceptors after intravitreal delivery.[14] The peptide insertion at amino acid 588 in the parental AAV2 capsid lowered 7m8s affinity for HSPG, though infectivity was still HSPG-dependent.[14] In addition, the 7 amino acid peptide insertion at

position 588 mediated substantially higher gene expression in mouse photoreceptors *in vivo* compared to wild-type AAV, and led to the functional rescue of murine models of X-linked retinoschisis and Leber's congenital amaurosis type 2.[14]

Applications of Engineered Viral Vectors to the Study of the CNS

There has been a strong and increasing interest in applying newly developed technologies—ranging from optogenetics[28] to site-specific nucleases[29,30]–to investigate basic neuroscience problems including neurodevelopment, adult neurogenesis, brain activity mapping,[31] learning and memory, CNS aging, and human neurological disease biology. As model systems for investigation in each of these areas, transgenic and mutant mice offer the capacity to study the functional effects of targeted gene modifications in an *in vivo* environment. For example, in the area of adult neurogenesis, transgenic mouse lines such as the Nestin-CreER^{T2} mouse have enabled a number of basic advances.[32-34] However, deriving new lines to study each new gene is highly time and labor intensive, taking months to years to create, and their application to study phenotypes associated with aging or neurodegenerative disease models adds considerably more time.[35] Furthermore, the mouse genetics "infrastructure" is not available in other organisms, such as large animals.

As a versatile and powerful alternative, efficient and selective gene delivery to specific cell types within the CNS offers the potential to greatly accelerate basic investigation. Compared to a months-to-years timeframe to generate (and, if relevant, age) a new mouse, studies involving targeted gene delivery with a wild type mouse can proceed from design to data in a matter of weeks, even taking into account the time needed for surgery. For example, different studies have implicated Wnt/β-catenin signaling in both the self-renewal and the lineage commitment of adult hippocampal stem cells, ostensibly contradictory results.[34,36-38] To address this question, the NSC-selective AAV r3.45 was harnessed to deliver constitutively-active β-catenin (CA β-catenin) in the mouse hippocampus.[18] The result was significant increases in the number of NSCs, neural progenitor cells, and neurons in the hippocampus, indicating that β-catenin signaling increases neurogenesis via modulating both proliferation and differentiation of NSCs. This first use of an engineered, targeted AAV to investigate neurogenesis presages future studies with, for example, astrocyte or NSC-selective vectors to study cell-extrinsic[34,39] and cell-intrinsic mechanisms that control NSC quiescence, proliferation, self-renewal, and differentiation.

In the retina, the Müller cell-tropic AAV vector ShH10 was utilized to study the role of glial cell secretion of ciliary neurotrophic factor (CNTF) in axonal regeneration. ShH10-mediated expression of CNTF was sufficient to promote long-distance regeneration of severed optic axons in a mouse optic nerve crush trauma model.[40] The efficient and selective transduction of glial cells in the retina and subsequent CNTFR secretion resulted in sustained regeneration and protection of axons in the optic nerve.[40] This study also reported previously unexplored aspects of the mechanism of CNTFR-mediated neuronal growth, including the induction of massive ectopic sprouting of axons in the retina and axonal misguidance in the absence of additional guidance signaling.[40] AAV variants ShH10 and 7m8 were also used to elucidate the mechanism by which cellular retinaldehyde-binding protein (CRALBP) supports cone photoreceptor function.[41] In particular, Xue *et al.* utilized ShH10-mediated expression of CRALBP in Müller cells and 7m8-mediated expression of CRALBP in retinal pigment epithelia in mice lacking the *Rlbp1* gene (which encodes CRALBP) to determine that rescuing CRALBP expression specifically in Müller cells but not in retinal pigment epithelia improved M-cone sensitivity and restored the retinal visual cycle in these mice.[41]

Considerations for Future Engineering of AAV for Central Nervous System Administration

Different natural and engineered AAV variants have delivery properties that vary by cell type, tissue target, and species; therefore, the variant must be carefully chosen to suit a given application. For example, although different natural AAV serotypes share some highly conserved capsid regions, there are 9 hypervariable regions[42] that result in differences in cell surface receptor binding and tissue tropism.[43] In a study comparing the biodistribution of AAV1–9 following tail vein administration to mice, differences in both transduction efficiency and specificity of the various serotypes could be observed.[43] For example, AAV2 transduction resulted in 26- to 30-fold more gene expression from the liver compared to the skeletal muscle and heart, respectively.[43] AAV6 gene expression from the heart was 2.7- to 4.7-fold higher than gene expression in the skeletal muscle or liver.[43] AAV9 broadly transduced the heart, liver, lungs, and skeletal muscle tissues evaluated.[43]

Furthermore, the transduction efficiencies and biodistributions of various AAV serotypes differ between animal species. In mice and non-human primates, the natural serotype AAV8 has a much greater liver transduction efficiency compared to AAV2.[44] However, in clinical trials for hemophilia B, AAV2 and AAV8 vectors mediated similar peak levels of Factor IX protein expression in patients.[45,46] These species differences are also seen with engineered AAV vectors. Despite strong transduction in the mouse retina using the variant 7m8, infection in the non-human primate retina was limited.[14] These proof of concept studies in mouse thus illustrate another consideration for directed evolution: gene delivery barriers change with species, and thus vectors engineered for optimized delivery in rodents may not translate to large animals or to patients. However, future engineering in large animal models may yield vectors with a strong translational path to human clinical studies.

In addition to engineering AAV variants for specificity to certain cell types or

tissues as described above, another mechanism for restricting gene expression to a cell type of interest is to engineer the payload being delivered. Endogenous, cell-type specific promoters have commonly been used to restrict expression to specific cells. For example, a rhodopsin promoter can mediate AAV-mediated gene expression specifically in rod photoreceptors.[14] Alternatively, the incorporation of short target sequences for tissue-specific microRNA into the transgene cassette can be used to detarget gene expression from specific tissues. When cells that express the corresponding microRNA are transduced, binding of the microRNA to the vector payload functions to downregulate expression of the transgene. This strategy has been employed to show that expression from AAV vectors can be reduced using liver-specific,[47–49] heart-specific,[49] and antigen presenting cell-specific[50] microRNA. The use of promoters or microRNA regulatory elements to express the transgene only from the cell type of interest will help reduce off-target gene expression or possible immune responses to the transgene resulting from antigen presentation of the therapeutic protein. The use of endogenous promoters can also serve to decrease the level of expression in cells of interest compared to stronger viral promoters, which could in some cases maintain transgene expression within a therapeutic efficacy window.

Clinical Applications of Engineered AAV in the Central Nervous System

In addition to basic studies, gene delivery can be harnessed for gene or cell replacement therapies to treat neurodegenerative disease or injury. A number of CNS clinical trials have established safety but not strong evidence for efficacy to date, and enhanced vectors may aid future clinical efforts. For example, delivery of aspartoacylase (for Canavan's disease) and CLN2 (for late infantile neuronal ceroid lipofuscinosis) transgenes using natural AAV have been established as safe.[51,52] Use of an engineered vector that achieves more efficient spread and thus broader transduction of the central nervous system could improve efficacy for these monogenic disorders. AAV-mediated delivery of transgenes such as aromatic L-amino acid decarboxylase (AADC), glutamic acid decarboxylase (GAD), and glial derived neurotrophic factor (GDNF) have been explored in clinical trials for Parkinson's disease,[53-58] though these trials have not yet achieved the efficacy necessary to become a treatment, potentially due to limited delivery efficiency. In one study, the AAV2 vectors used in the AADC clinical trial transduced only 5–6% of neurons near the injection site in the nonhuman primate brain.[59] Use of an engineered vector for higher infectivity and broader distribution could again yield higher efficacy in such studies.

Analogously, within the eye, subretinal injections of AAV2 vectors have yielded highly promising results in clinical trials for Leber's congenital amaurosis type 2 and choroideremia.[22-25] However, many additional retinal diseases that are candidates for gene therapy feature heavily damaged retinas that would benefit from non-invasive delivery via the vitreous, which can also offer broader expression across the retina compared to a subretinal injection. Engineered AAV variants are needed for outer retinal transduction from the vitreous. As a final example of future potential applications, inducible, conditional expression of the *fmr1* gene in NSCs resulted in restoration of fragile X mental retardation protein expression specifically in adult NSCs and rescued mice from learning deficits in a murine model of fragile X syndrome.[60] Use of an NSC-specific gene delivery vector could offer a path for clinical translation.

Gene delivery vectors based on adeno-associated virus (AAV) have emerged as effective research tools and promising clinical vectors. Although the central nervous system presents many barriers to delivery, novel, engineered AAV vectors capable of more efficient delivery, more specific targeting, and broader transduction are increasingly helping to surmount these challenges and further enable biomedical research in the CNS.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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