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Adeno-Associated Virus Vectors and Neurological Gene Therapy

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Abstract

Gene therapy has strong potential for treating a variety of genetic disorders, as demonstrated in recent clinical trials. There is unfortunately no scarcity of disease targets, and the grand challenge in this field has instead been the development of safe and efficient gene delivery platforms. To date, approximately two thirds of the 1800 gene therapy clinical trials completed worldwide have used viral vectors. Among these, adeno-associated virus (AAV) has emerged as particularly promising because of its impressive safety profile and efficiency in transducing a wide range of cell types. Gene delivery to the CNS involves both considerable promise and unique challenges, and better AAV vectors are thus needed to translate CNS gene therapy approaches to the clinic. This review discusses strategies for vector design, potential routes of administration, immune responses, and clinical applications of AAV in the CNS.

Keywords

gene therapy, directed evolution, adeno-associated virus (AAV), central nervous system, viral vectors

Introduction

Adeno-Associated Virus Biology

Adeno-associated viruses (AAVs) are non-enveloped, single-stranded DNA viruses that replicate only in the presence of a helper virus, primarily adenovirus (Schaffer and others 2008). The AAV viral genome is 4.7 kilobases and contains two inverted terminal repeats that flank the viral genes *rep* and *cap* (Schaffer and others 2008). Multiple proteins are produced from each viral gene through the use of alternative splicing and start codons (Fig. 1). The *rep* open reading frame (ORF) encodes four proteins necessary for AAV replication, site-specific integration, transcriptional regulation of viral promoters, and viral assembly. The *cap* ORF serves as a template for the production of three structural proteins, VP1-3, that assemble to form a 60-mer viral capsid approximately 25 nm in diameter (Fig. 2) (Schaffer and others 2008). Finally, an alternative ORF nested in *cap* encodes assembly-activating protein (AAP), which interacts with the capsid proteins VP1-3 and is necessary for capsid assembly (Sonntag and others 2010).

Vectors based on AAV are particularly promising gene delivery vehicles in large part because they exhibit low immunogenicity, can mediate long-term gene expression in both dividing and non-dividing cells, and have a low risk of insertional mutagenesis (Kaeppel and others 2013). To generate a recombinant AAV for gene delivery, the viral genome is removed and replaced with the therapeutic transgene and a promoter. The viral inverted terminal repeats are the only *cis* elements needed to ensure packaging of the genetic payload into particles, and *rep* and *cap* can be supplied separately during virus production (Kwon and Schaffer 2008). Infection with recombinant AAV vectors results in primarily episomal transgene expression that is persistent in non-dividing cells for at least 10 years (Mingozzi and High 2013). AAV has been used in an increasing number of clinical trials, including recent successes for hemophilia B and the retinal degenerative disorder Leber's congenital amaurosis type 2 (Bainbridge and others 2008; Cideciyan and others 2009; Nathwani and others 2011; Testa and others 2013), as well as efforts that led to the first approval of a gene therapy product (for lipoprotein lipase deficiency) in Western nations in late 2012 (Miller 2012).

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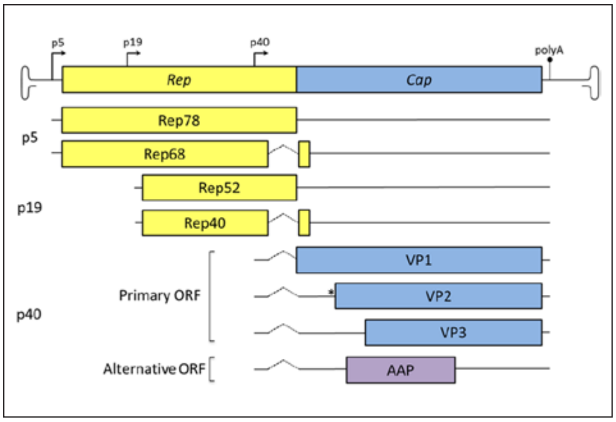

Figure 1. Key features of the AAV genome. Through alternative splicing, *rep* encodes four proteins—Rep78, Rep68, Rep52, and Rep40—that are involved in viral genome replication. The *cap* open reading frame (ORF) encodes three capsid proteins, VP1-3, that assemble to form a 60-mer viral capsid. There are two splice forms of the *cap* mRNA, where the longer form encodes VP1. In the shorter splice form the start codon for VP1 is removed, and translation initiates either from the VP3 start codon or an alternative ACG* start codon for VP2. The protein AAP, which assists in viral capsid assembly, is encoded by an alternative open reading frame with a nonconventional CUG start codon. Gene expression is driven by the p5, p19, or p40 promoter as indicated. AAV = adeno-associated virus; VP1 = viral capsid protein 1; VP2 = viral capsid protein 2; VP3 = viral capsid protein 3; AAP = assembly-activating protein.

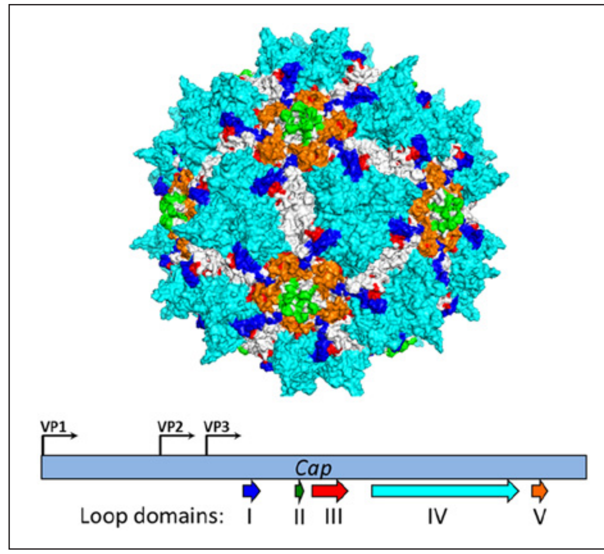

Figure 2. Adeno-associated virus (AAV) capsid structure. The locations of interstrand loop domains are indicated by the colored arrows on the *cap* gene and mapped to the three-dimensional AAV capsid structure, and these loop domains are highly diversified regions of *cap* that play a role in many virus–host interactions. The right-angle arrows indicate the translation start sites for viral proteins 1-3. This image was generated in PyMOL (The PyMOL Molecular Graphics System, Version 1.3r1, 2010, Schrödinger, LLC, Cambridge, MA) based on the crystal structure coordinates of AAV2 (Protein data bank accession number 1LP3).

Targeting the CNS

Because recombinant AAV vectors are well suited for gene therapy in the central nervous system, where most cells are post-mitotic and many chronic neurological diseases necessitate long-term transgene expression, AAV has entered into numerous CNS clinical studies (Table 1) (Asokan and others 2012). These trials have highlighted clinical challenges to successful gene therapy in the CNS. Biological transport barriers limit viral access to the brain parenchyma. For example, the blood-brain barrier (BBB) prevents global transduction of the CNS following intravascular vector administration, and clinical trials to date have thus employed intracranial injections that result primarily in localized transduction due to limited vector dispersion in the brain parenchyma. In addition, the ability to target specific cell types and regions in the CNS that are implicated in disease is important for safe and effective therapies for several indications; however, it is difficult to control AAV tropism and transgene expression. Finally, AAV vectors have an excellent safety record, though the implementation of new routes of administration and serotypes could increase the risk of an immune response to the vector and/or transgene. These challenges motivate the careful selection of natural AAV serotypes—and engineering of novel AAV variants—to yield transduction profiles that are optimally suited to specific therapeutic needs.

Adeno-Associated Virus Vector Design

Natural Serotypes in the CNS

There are 11 naturally occurring AAV serotypes and more than 100 variants of AAV (Wu and others 2006), which were isolated from human and non-human primate tissue, and which mediate a range of different cellular transduction and vector spread profiles in the central nervous system following direct injection into brain parenchyma. Most serotypes studied to date preferentially transduce neurons after intraparenchymal injection, and AAV2, for example, has particularly strong neuronal tropism (Bartlett and others 1998). However, while this vector has been favored in clinical trials because of its established safety record and historical use in the early AAV clinical studies (Ginn and others 2013), research has shown AAV1 and AAV5 to be more efficient in transducing neurons—and to a lesser extent some glia—in a number of rat and non-human primate brain regions (Mandel and

Table 1. Summary of AAV Clinical Trials in the CNS.

Disease	Clinical Trial	Serotype	Promoter	Transgene Product	ClinicalTrials.gov identifier	Refs
Alzheimer's	Phase 2	AAV2	CAG	Nerve growth factor (NGF)	NCT00876863	Mandel and others, 2010
Canavan	Phase 1	AAV2	NSE	Aspartoacylase (ASPA)	NA	McPhee and others, 2006
Late infantile neuronal ceroidlipofuscinosis	Phase 1	AAV2	CAG	Human CLN2	NCT00151216	Worgall and others, 2008
Late infantile neuronal ceroidlipofuscinosis	Phase 1/2 recruiting	AAVrh.10	CAG	Human CLN2	NCT01414985	
Parkinson's	Phase 1/2	AAV2	CAG	Neurturin (NTN)	NCT00400634 NCT00985517	Bartus and others, 2013
Parkinson's	Phase 1 recruiting	AAV2	NA	Glial cell–derived neurotrophic factor (GDNF)	NCT01621581	
Parkinson's	Phase 2	AAV2	CAG	Glutamate decarboxylase 1 (GAD)	NCT00643890	LeWitt and others, 2011
Sanfilippo syndrome (MPSIIIB)	Phase 1/2 recruiting	AAV5	NA	Human α-*N*-acetylglucosaminidase	ISRCTN[a] 19853672	Ellinwood and others, 2011

Abbreviations: AAV = adeno-associated virus; CAG = chicken β-actin promoter with CMV enhancer; CLN2 = also known as tripeptidyl peptidase 1; CMV = cytomegalovirus; NA = not available; NSE = neuron-specific enolase.
[a]International Standard Randomized Controlled Trial Number.

Burger 2004). By comparison, AAV4, one of the more phylogenetically distant AAV serotypes, preferentially transduces ependymal cells, even after intrastriatal injection (Davidson and others 2000; Markakis and others 2010). Finally, among more recently isolated AAVs, intracerebral injection of AAV7, 8, 9, and rh.10 results in primarily neuronal transduction, and vector spread is greatest with AAV9 and rh.10 (Cearley and Wolfe 2006).

In addition to neurons, glia represent potential targets. These cells play important supporting roles for neurons, including functions that could be enhanced via gene delivery. They are also involved in disorders such as amyotrophic lateral sclerosis and are thus potential direct disease targets (Ilieva and others 2009). Recently discovered serotypes hu.32, hu.11, pi.2, hu.48R3, and rh.8 are able to transduce both astrocytes and oligodendrocytes with varying efficiencies in the adult mouse brain (Cearley and others 2008).

In addition to cellular transduction, vector spread through tissue is a significant challenge, particularly for disorders that afflict large regions of the CNS such as lysosomal storage diseases. Vector can undergo transport either extracellularly or intracellularly. Intracellularly, axonal transport of AAV can occur in the retrograde (Kaspar and others 2002) or the anterograde direction, and AAV serotypes differ in their potential for such transport (Kaspar and others 2003). AAV1, 9, and rh.10 can all be disseminated along axonal projections in both the retrograde and anterograde directions after injection into the ventral tegmental area, a region dense in efferent and afferent projections (Cearley and Wolfe 2007). Axonal transport has been proposed as a mechanism to enhance therapeutic efficacy of AAV by protecting potentially both presynaptic neurons and their projective fields (Kaspar and others 2002). Such axonal transport can also be used to trace and manipulate neural circuits in the CNS (Betley and Sternson 2011).

The discovery that AAV9 can cross the BBB in both neonatal and adult mice (Foust and others 2009) has raised the exciting possibility that intravascular (IV) AAV administration could mediate widespread CNS gene expression. IV delivery leads to both neuronal and astrocytic transduction in neonates, though expression was primarily restricted to astrocytes in adults. Since intracranial injection results in mixed astrocytic and neuronal transduction, this astrocytic tropism may be due to astrocytic endfeet interfacing with endothelial cells within the BBB. Follow-up studies have demonstrated that rAAVrh.10, rAAVrh.39, rAAVrh.43 are also capable of parenchymal cell transduction after intravascular infusion in neonatal mice (Zhang and others 2011). One challenge associated with IV administration, however, is that the majority of the human population harbors neutralizing antibodies against one or more AAV serotypes because of natural exposure, which can severely limit IV-mediated AAV delivery (Bartel and others 2011). Finally, intracerebroventricular (ICV) administration (see section Therapeutic Routes of Administration) may also enable AAV to gain access to large volumes of the CNS, though ICV administration of AAV2, 4, or 5 shifts tro-

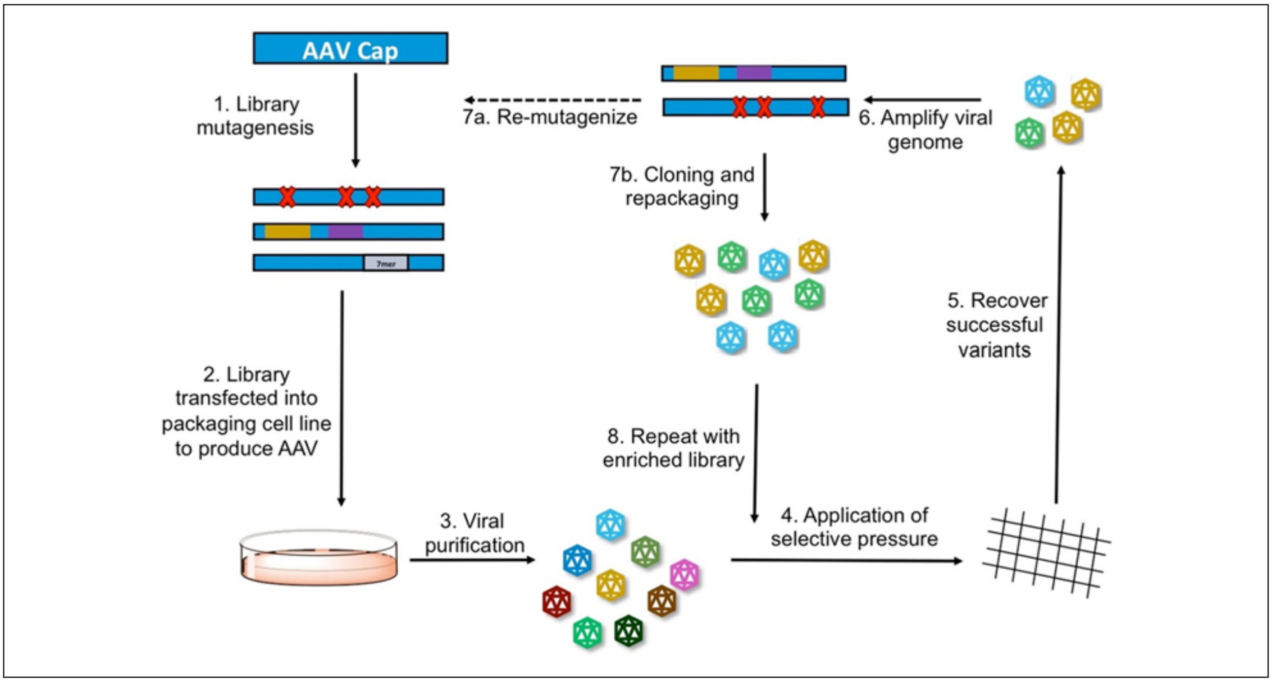

Figure 3. Adeno-associated virus (AAV)-directed evolution algorithm. (1) The AAV *cap* gene is mutagenized by techniques such as error-prone PCR, DNA shuffling, or the staggered extension process. (2) The mutagenized library is transfected into a packaging cell line (HEK293T) to produce viral particles. (3) Viral libraries are harvested and purified. (4) A selective pressure is applied. (5) Successful variants are recovered. (6) *Cap* genes are amplified by PCR. (7a) Additional mutagenesis can be conducted to increase library diversity. (7b) The enriched library is repackaged into viral particles. (8) The process is iterated to increase viral fitness.

pism primarily to ependymal cells with sparse transduction in the parenchyma (Davidson and others 2000).

Directed Evolution

Adeno-associated virus is thus a promising gene therapy vehicle; however, human therapeutic needs demand delivery properties that at best probably conferred AAVs with no selective advantages during natural evolution (i.e., CNS infection is not a prominent feature of AAV's life cycle) and at worst may be at odds with natural selection (e.g., a primary viral receptor that is promiscuously expressed at high levels can limit broad viral dispersal within tissue (Nguyen and others 2001)). As a result, there have been considerable efforts to engineer viral capsid proteins to meet biomedical needs (Schaffer and others 2008). However, the complexity of structure–function relationships in multimeric, 4 MDa viral particles renders rational design efforts difficult. An alternative approach, directed evolution, emulates how viruses naturally evolve—iterative rounds of genetic diversification and selection for improved function—but with selective pressures that can be designed to result in therapeutically useful viruses. Directed evolution has been applied to generate new viral variants with altered gene delivery specificities and enhanced evasion of neutralizing antibodies (Asuri and others 2012; Dalkara and others 2013; Excoffon and others 2009; Jang and others 2011; Koerber and others 2006; Koerber and others 2008; Koerber and others 2009; Maheshri and others 2006).

Directed evolution of AAV (Fig. 3) first involves the generation of large libraries of mutated *cap* genes, using techniques such as error-prone PCR, DNA shuffling, or the staggered extension process (Schaffer and others 2008). These genetic libraries are then converted into viral particles, where each particle contains a viral genome that encodes its capsid shell, thereby linking the virus' genotype and phenotype. After applying a selective pressure for a specific improved function, *cap* genes can be recovered, amplified by PCR, and used for additional rounds of selection. Iteration drives library convergence toward top performers, which are then benchmarked to the best available natural serotypes. In addition to providing novel and useful vehicles, the resulting mutations can be reverse engineered to elucidate new basic structure–function relationship information. Moreover, beneficial mutations identified in different studies may also be combined on a single vector to address multiple therapeutic needs.

Numerous in vitro selections to target cell types of the central nervous system have been conducted.

As discussed above, most AAV vectors predominantly transduce neurons in the central nervous system after intraparenchymal injection. Koerber and others (2009) evolved AAV variants to infect human and rat astrocytes in vitro up to 15-fold more efficiently than their corresponding parent serotypes. In addition, several evolved variants transduced astrocytes up to 5.5-fold more efficiently than parental serotypes in the rat striatum, despite the in vitro nature of the original selection. Directed evolution has also been applied to target neural stem cells (NSCs), which natural AAV serotypes do not efficiently infect. After seven rounds of selection on rat hippocampal NSCs, the variant AAV r3.45 was recovered (Jang and others 2011). This AAV, which included a peptide insertion on the capsid surface, was 15- to 50-fold more infectious on rat NSCs relative to AAV2 and AAV5. In addition, though it was selected on rat NSCs, AAV r3.45 also supported efficient gene delivery to both murine and human NSCs. Furthermore, the vector was harnessed to mediate gene correction by homologous recombination, an advantageous feature of AAV gene delivery (Khan and others 2011). Finally, this variant is selective for NSCs within the adult rodent brain (data not shown).

Glioblastoma multiforme is the most common brain cancer and has a very poor prognosis. Maguire and others (2010) conducted seven rounds of selection using a shuffled AAV library selected on glioblastoma U87 cells. A resulting, chimeric capsid with elements from AAV1, 2, rh.8, rh.10, and several point mutations transduced 97% of glioblastoma U87 cells at a dose of 10^4 genome copies/cell and also outperformed the AAV2 parental serotype on multiple glioma cell lines. Future in vivo analysis may explore the promise of such variants for gene therapy in solid tumors.

While in vitro selections have been the primary focus of work to date, numerous cellular and tissue complexities of the CNS cannot be emulated in culture. Consequently, several studies have shifted toward animal models that better represent the transport barriers that gene therapy vectors must overcome. For example, Gray and others (2010) employed directed evolution to select variants that crossed a seizure-compromised BBB. A shuffled library of AAV serotypes 1-6, 8, and 9 was injected via tail vein into rats 24 hours after kainic acid-induced limbic seizure, and AAV variants were recovered from seizure-sensitive brain sites. After three rounds of selection, two clones primarily composed of AAV1, 8, and 9 were found to selectively transduce regions in the ventral hippocampus and piriform cortex where seizures had compromised the BBB, but they did not cross the intact BBB. The evolved vectors displayed a transduction profile similar to AAV8, infecting mostly neurons and oligodendrocytes with few astrocytes or microglia transduced. Finally, the biodistribution of evolved clones was detargeted from peripheral organs when compared with the parent serotypes AAV 1, 8, and 9.

The retina is also part of the CNS, and the retinal degenerative disorder Leber's congenital amaurosis type 2 has been successfully treated in gene therapy clinical trials (Bainbridge and others 2008; Cideciyan and others 2009). The majority of monogenic retinal diseases involve mutations in genes expressed in photoreceptors and the retinal pigment epithelium, which lie several hundred microns deep within the retina, and transducing these targets with existing AAV vehicles requires an injection into subretinal space between photoreceptors and retinal pigment epithelium. The resulting transient retinal detachment can damage retinas already undergoing degeneration, and subretinal injections only transduce cells that come into contact with the "bleb" of injected liquid. Dalkara and others (2013) applied directed evolution to engineer an AAV variant that can reach the outer retina after injection into the readily accessible vitreous humor. Six rounds of selection in adult mice led to a dominant variant containing a seven-amino acid sequence inserted into loop 4 of the capsid. The evolved variant (7m8) mediated widespread transduction of the outer retina and was able to rescue disease phenotypes in two mouse models of eye diseases, X-linked retinoschisis and Leber's congenital amaurosis. Finally, the vector also showed promising clinical potential in its ability to transduce photoreceptors from the vitreous in non-human primate.

Rational Design

In some cases where specific capsid structure–function relationships are known, rational design can be effective. For example, tyrosine residues on the capsid surface are subject to phosphorylation by tyrosine kinases, leading to capsid ubiquitination and proteasomal degradation (Zhong and others 2008). Work by Srivastava and colleagues showing that mutation of tyrosines to phenylalanines can overcome this problem and enable more efficient gene delivery (Qing and others 1997), and Dalkara and others (2012) built on this work by introducing Y to F substitutions at two highly conserved, surface-exposed residues on the AAV9 capsid. The resulting tyrosine mutant AAV9-scCAG-GFP vector was administered by tail vein injection in neonatal mice, and transduced CNS cells included both neurons and astrocytes in the hippocampus, hypothalamus, cortex, and cerebellum—a pattern similar to but more efficient than wild type AAV9. Tyrosine mutant AAV9 vectors have also been demonstrated to significantly enhance gene delivery to the CNS after intracardiac injections in adult mice (Iida and others 2013). Tyrosine mutations may not substantially shift the natural tropism of parent serotypes;

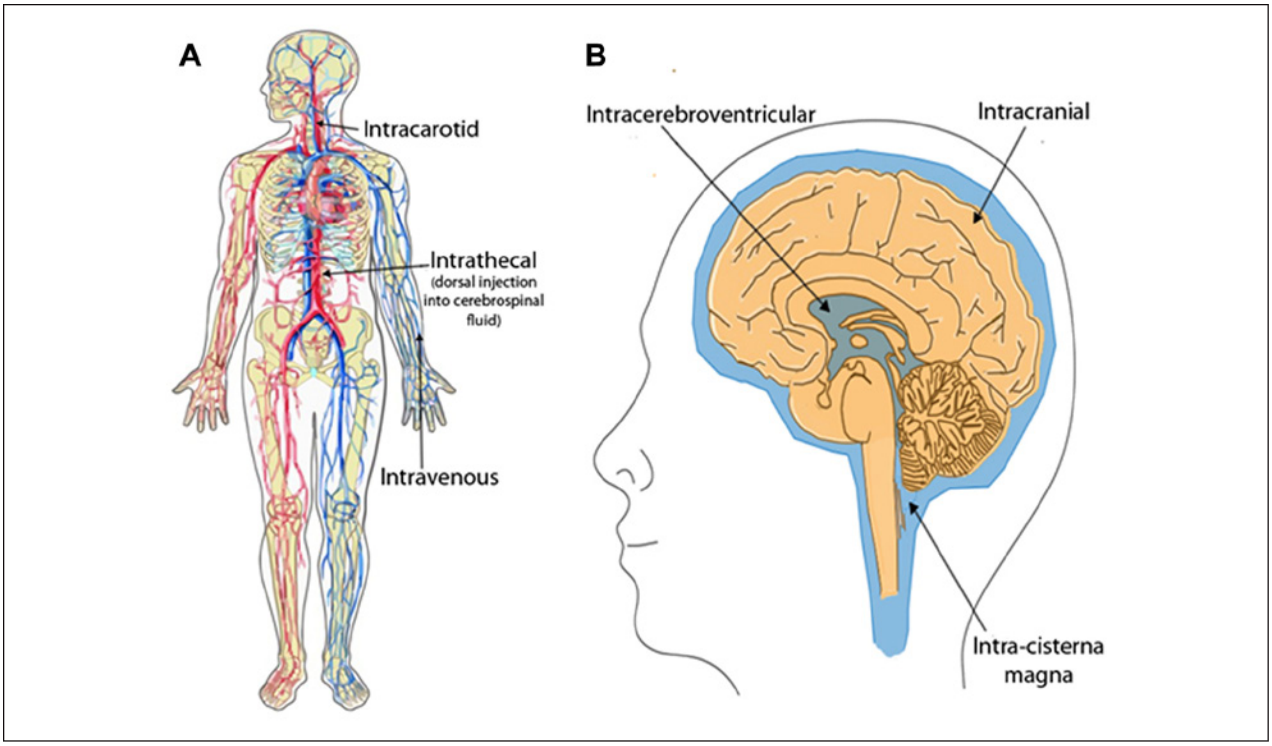

Figure 4. Routes of administration. (A) Illustration of intracarotid and intravenous injections into the bloodstream. The intrathecal route is a dorsal injection into the cerebrospinal fluid. (B) The more invasive intracranial, intracisterna magna, and intracerebroventricular injections into the CSF (blue) or brain tissue. Illustrations adapted from ChemBioDraw (Version 13.0.2.3021, Cambridge Software, Waltham, MA).

however, they may enable a reduction in vector dose and thereby lower the risk of an immune response.

Therapeutic Routes of Administration

Intracranial Administration

To date, intracranial administration of AAV—which involves insertion of flexible fused-silica infusion catheters through burr holes into the brain parenchyma, followed by slow infusion of vector (Lowery and Majewska 2010)—has been the most commonly employed route for gene delivery to the brain parenchyma (Bartus and others 2013; Ellinwood and others 2011; LeWitt and others 2011; Mandel 2010; McPhee and others 2006; Worgall and others 2008). This approach circumvents the biological transport barriers that render other administration routes challenging (Fig. 4). In addition, this route of administration does reduce the risk of vector neutralization by circulating antibodies, though anti-AAV neutralizing antibody titers in the brain parenchyma can reach 1% of levels found in systemic circulation (Treleaven and others 2012).

Intracranial injections do have significant drawbacks. As described above (see section Adeno-Associated Virus Vector Design), poor vector spread limits transgene expression to the vicinity of the injection site, a major shortcoming for diseases that affect large CNS regions such as Parkinson's or Alzheimer's diseases, or the entire CNS such as lysosomal storage disorders. It is estimated that complete transduction of the entire human infant brain for treatment of lysosomal storage disorders would require 50 to 350 injection tracts based on the limited diffusion distance (~1-3 mm) of AAV vectors (Cunningham and others 2008; Vite and others 2003). Each injection presents a risk of hemorrhaging, edema, and bacterial contamination. The spread of AAV vector throughout the brain parenchyma can be improved by convection-enhanced delivery (Cunningham and others 2008; Kells and others 2009), which increases vector transport through the interstitial fluid by inducing convective flow in addition to diffusion. Since bulk flow depends only on the pressure gradient, the injection pressure is maintained at a sufficient level to overcome the hydrostatic pressure of the interstitial fluid and thereby distribute the vector throughout the brain, though flow rates should be conservative to avoid uncontrolled vector spread along paths of least resistance such as white matter tracts (Linninger and others 2008). Convection-enhanced delivery has been used safely in a clinical trial for Parkinson's disease (Eberling and others 2008), and the development of new cannula designs to prevent reflux (Krauze and others 2005) and sophisticated MRI guidance systems to ensure accurate cannula placement (San Sebastian and others 2012; van der Bom and others 2013) will further improve safety and efficacy.

Intravascular Administration

Intravascular administration in principle offers the potential for noninvasive transduction of the entire brain with a single vector infusion, given the high density of CNS capillaries (Pardridge 2005). However, AAV transcytosis to the brain parenchyma is obviously limited by the BBB, whose tight junctions between endothelial cells preclude paracellular transport of AAV. Serotypes such as AAV9 that do cross the BBB are thought to undergo receptor-mediated transcytosis in endothelial cells (Shen and others 2011).

There are, however, several disadvantages of IV delivery: The vector circulates throughout the entire body where it is exposed to circulating antibodies and can transduce peripheral organs in addition to the CNS. The latter two represent a loss of vector, can lead to off-target side effects, and increases the risk of an immune response. In addition, due to the inefficiency in crossing the BBB, only a small fraction of injected virus reaches the brain and spinal cord, necessitating in principle high vector doses on the order of 10^{15} viral genomes to treat an adult human (Samaranch and others 2012). Moreover, transduction is primarily limited to astrocytes in adult organisms as previously discussed (see section Adeno-Associated Virus Vector Design), though vector engineering may broaden tropism.

Intra-CSF

Delivery to the CSF places vector near the CNS parenchyma, has potential to reduce peripheral off-target transduction, and limits exposure to serum neutralizing antibodies. That said, analogous to the BBB, tight junctions between ependymal cells limit the efficiency of vector penetration into the brain parenchyma.

Samaranch and others (2012) demonstrated that AAV9 infusion into the cisterna magna of non-human primates promotes significantly stronger transgene expression throughout the cortex and cerebellum compared to intravascular delivery. Transgene expression was observed primarily in astrocytes, scattered pyramidal neurons, and almost no microglia or oligodendrocytes. Moderate serum titers of preexisting anti-AAV antibodies (1:200) did prevent brain transduction, indicating that delivery to the CSF did not offer complete immunological protection.

Gray and others (2013) compared the transduction profiles of AAV2.5 (Bowles and others 2012) and AAV9 after injection into the cisterna magna and lumbar intrathecal space in non-human primates. Both vectors achieved widespread transduction of neurons and astrocytes in the brain and spinal cord, and intrathecal injections were more effective than intracisternal injections in promoting gene delivery to the dorsal root ganglia. Cross-reactivity of neutralizing antibodies between AAV2.5 and AAV9 was not observed in two of four monkeys tested, supporting the possibility of serotype switching for a second administration of vector. Furthermore, circulating neutralizing antibody titers up to 1:128, which prevent gene delivery after IV administration (Gray and others 2011), had no inhibitory effect on CNS gene transfer.

Retrograde Transport

Non-invasive gene delivery to the CNS can also be accomplished via vector administration to peripheral muscle tissue and retrograde transport along motor neuron projections to cell bodies residing in the CNS (Kaspar and others 2003). This approach is particularly relevant for diseases such as amyotrophic lateral sclerosis and spinal muscular atrophy that primarily afflict motor neurons. Hollis and others (2008) investigated the efficiency of retrograde transport of AAV serotypes 1-6 after peripheral injection into either extensor carpi muscle or sciatic nerve. AAV1 performed best in retrograde infection of lower motor neurons (1% to 4.1% of all motor neurons were transduced) after both intramuscular and intranerve injection, and the latter was more efficient.

AAV8 is also capable of retrograde transport in both neonatal and adult mice (Foust and others 2008; Zheng and others 2010). In adult mice, AAV8 does not cross the blood-nerve barrier, limiting systemic dissemination to peripheral organs (Zheng and others 2010). In another study, AAV9 was shown to undergo retrograde transport after injection into the gastrocnemius muscle of adult mice, transducing both neurons and astrocytes equally well with up to 43% of total motor neurons transduced (Benkhelifa-Ziyyat and others 2013). Spread of vector from injected muscle to the CNS and peripheral organs was also observed, likely due to the ability of AAV9 to cross the blood-nerve barrier.

Immune Responses to AAV in the CNS

Recombinant AAV vectors do not encode viral genes, but immune responses can be mounted against the viral capsid and/or the transgene product. For example, preexisting neutralizing antibodies generated from a prior exposure to AAV can opsonize and inactivate the vector. The extent of vector neutralization depends on the route of administration. IV routes that expose the vector to circulating neutralizing antibodies are sensitive to low antibody titers (Gray and others 2011), whereas relatively high titers of circulating anti-AAV neutralizing antibodies do not appear to significantly affect transduction after intracranial AAV administration in immune-primed mice (Treleaven and others 2012). Phase I clinical trials employing intracranial delivery of recombinant AAV have confirmed preclinical results, showing a minimal humoral response and limited adaptive immune response (Kaplitt and others 2007; McPhee and others 2006).

After viral transduction and the onset of transgene expression, immune responses to the transgene product can lead to clearing of transduced cells with subsequent loss of gene expression and inflammation in the CNS. Most CNS gene therapy trials have employed AAV2, a vector with neuronal tropism, but newer vectors with broader tropism such as AAV9 may increase the likelihood of an immune response by infecting antigen-presenting cells. Ciesielska and others (2013) administered AAV9 encoding human aromatic L-amino acid decarboxylase (hAADC) or green fluorescent protein into rat striatum or thalamus. Both vectors provoked a full immune response, with upregulation of MHC II in glia, lymphocytic infiltration, and inflammation leading to significant loss of neurons and generation of antibodies against the transgenes. A significant fraction of the MHC II positive cells were identified as microglia 1 week and 3 weeks after injection, and a smaller population of MHC II positive astrocytes was also observed at the 3-week time point. In contrast to AAV9, AAV2-mediated delivery of hAADC has elicited no safety concerns in human or animal studies (Christine and others 2009; Eberling and others 2008). The authors speculated that transduction of antigen-presenting cells and subsequent presentation of transgene antigen led to a full adaptive immune response. Cell-mediated responses have also been observed after intracerebral infusion of an AAV1-GFP vector in nonhuman primate (Hadaczek and others 2009) and intracranial injection of AAV5-α-L-iduronidase in a dog model of Hurler syndrome (Ciron and others 2006). Therefore, immune responses to non-self proteins expressed from AAV vectors may limit therapeutic options for patients that lack immune tolerance due to null mutations in the endogenous gene. In light of these findings, engineered vectors that both overcome CNS transport barriers and do not infect antigen-presenting cells are needed.

Strategies for Limiting Off-Target Transduction

Some natural serotypes are selective for specific cell types, and directed evolution may be used to fundamentally reengineer cell tropism (Pulicherla and others 2011). In addition, a cell type specific promoter can also be employed to restrict expression to specific transduced cells. One challenge is that such a promoter must be sufficiently small for it and the transgene to fit within the limited carrying capacity of the virus, roughly 5 kb ssDNA or 2.5 kb self-complementary dsDNA (Wang and others 2003). Neuron selective promoters that have been explored include synapsin-1, neuron-specific enolase, and human platelet-derived growth factor (Shevtsova and others 2005). In addition, the 2.5 kb rat tyrosine hydroxylase promoter has been used to drive transgene expression in midbrain dopamine neurons (Oh and others 2009). Likewise, expression can be restricted to astrocytes and oligodendrocytes using glial fibrillary acidic protein or myelin basic protein promoters, respectively (von Jonquieres and others 2013). Ideally, a matching pair of cell type specific promoter and viral capsid would be developed for each disease target.

An alternate approach to reduce transgene expression in off-target tissues is the use of microRNA (miRNA) elements. MicroRNAs are non-coding regulatory RNAs that contribute to post-transcriptional gene silencing. miRNA target sequences matching miRNAs expressed only in peripheral organs can be introduced into AAV expression cassettes to reduce transgene mRNA levels in off-target tissues. For example, Qiao and others (2011) demonstrated that five copies of liver-specific miR-122 in the 3′ untranslated region of AAV9 vectors reduced expression of the reporter genes luciferase and β-galactosidase by 50- and 70-fold, respectively, in liver tissue. Expression was not reduced in cardiac and muscle tissues, demonstrating the specificity of silencing. An analogous study (Geisler and others 2011) incorporated three copies of the same miR-122 element in AAV9 and provided additional evidence of reduced transgene expression in both hepatic cell lines and mouse liver. The discovery and cataloging of new miRNA elements (Kozomara and Griffiths-Jones 2011) will further expand applications of this technology.

Gene Therapy for Neurological Disorders

Lysosomal Storage Disorders

Lysosomal storage disorders are a family of inherited diseases involving deficiencies in enzymes that metabolize lipids, glycoproteins, or mucopolysaccharides. These deficiencies lead to the accumulation of undigested macromolecules in lysosomes, resulting in disruption of cellular function and clinical manifestations. More than 50 lysosomal storage disorders (LSDs) have been identified and collectively affect approximately 1 in 7700 births (Table 2) (Fuller and others 2006). Enzyme replacement therapies have been approved for the treatment of LSDs

Table 2. Several Lysosomal Storage Disorders Affecting the CNS.

Disease	Mutated Gene	Size of Coding Region (bp)[a]	Disease Prevalence[b]
MPSI-Hurler	Iduronidase, α-L (IDUA)	1962	1:100,000 (severe); 1:500,000 (attenuated)
MPSII-Hunter	Iduronate 2-sulfatase (IDS)	1032	1:100,000 males
MPSIIIB-Sanfilippo type B	*N*-acetylglucosaminidase, α (NAGLU)	2231	1:200,000
MPSVII-Sly	Glucuronidase, β (GUSB)	1956	1:250,000
Neuronal ceroid lipofuscinosis (Batten)[a]	Ceroid lipofuscinosis neuronal 2 gene (CLN2)	1669	1:25,000
Tay-Sachs	Hexosaminidase A, α polypeptide (HEXA)	1590	1:3500 (Ashkenazi Jewish population); 1:320,000 (general population)

[a]PubMed nucleotide.
[b]Estimates from the U.S. National Library of Medicine genetics home reference.

(Ohashi 2012) but are ineffective in the CNS since LSD enzymes do not cross the BBB. Given the short half-life of LSD enzymes, repeat intracranial infusions of enzyme would be necessary to achieve a therapeutic effect. In contrast, AAV vectors can provide sustained expression of LSD enzymes with a single vector dose. Moreover, cross-correction of non-transduced cells is possible since many LSD enzymes can be secreted and internalized by neighboring cells.

Late infantile neuronal ceroid lipofuscinosis (LINCL, also known as Batten disease) is caused by mutations in the ceroid lipofuscinosis neuronal 2 gene (CLN2). A phase 1 clinical trial for LINCL, involving AAV2 vector delivery of the CLN2 gene to 10 children via intracranial injection, has been completed (Worgall and others 2008). Gene delivery yielded a statistically significant slowing of disease progression as measured by a clinical rating scale. A secondary variable, neuroimaging results based on quantitative MRI parameters, was suggestive of improvement but did not yield a statistically significant change relative to the control group. The most common serious adverse events included seizures and the loss of one subject with severe LINCL 49 days after administration following development of status epilepticus. However, none of these adverse events were unequivocally attributed to the vector.

Alternative administration routes and vectors with increased spread (AAVrh.10; Sondhi and others 2012) are being explored to achieve the robust CNS transduction needed for correction of LSDs. Haurigot and others (2013) studied the impact of intra-CSF delivery of AAV9 vectors encoding sulfamidase in a mouse model of MPS IIIA (Sanfilippo syndrome type A). A high dose (5×10^{10} µg per adult mouse) mediated sulfamidase activity in all brain regions with up to 11% to 36% of normal expression levels in males and 7% to 39% of expression levels in females. Gene delivery resulted in reduced glycosaminoglycan accumulation in most brain regions, correction of behavioral responses, and extended survival. Intracisternal administration of the same vector in dogs resulted in primarily neuronal with some scattered astrocytic transduction, though approximately 3% to 5% of hepatocytes were also transduced, indicating that the vector exited the CSF. Expression of canine sulfamidase was sustained for weeks, but delivery of human sulfamidase caused expression to peak after 3 weeks and then decline, implicating an immune response to non-self protein in the dog. As intracisternal injections are not favored in pediatric patients, authors also evaluated intracerebroventricular administration, which resulted in widespread AAV9 vector distribution and transgene expression comparable to the intracisternal route. Serum antibody titers rose rapidly after vector exposure but remained low in the CSF (<1:10) in the absence of severe CNS inflammation. Treatment of dogs with preexisting immunity against AAV resulted in moderate gene expression in the CNS and severely reduced transduction of peripheral tissues, likely because CSF antibody titers (1:1 to 1:3.1) were much lower than in the periphery (1:1000).

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by the death of motor neurons. Of familial ALS cases (which collectively account for 10% of ALS), the most common inheritance pattern is autosomal dominant, and several genes have been implicated. Approximately 20% of these familial cases are traced to mutations in superoxide dismutase 1 (SOD1) (Nizzardo and others 2012). The mechanism of SOD1 toxicity is controversial, but it is thought to involve misfolded SOD1 aggregates.

Foust and others (2013) intravascularly injected AAV9 encoding SOD1 short hairpin RNA (shRNA) at P1 and P21 in a mouse model of ALS. Transgene expression was robust in astrocytes (P1, 34% ± 2%; P21, 54%

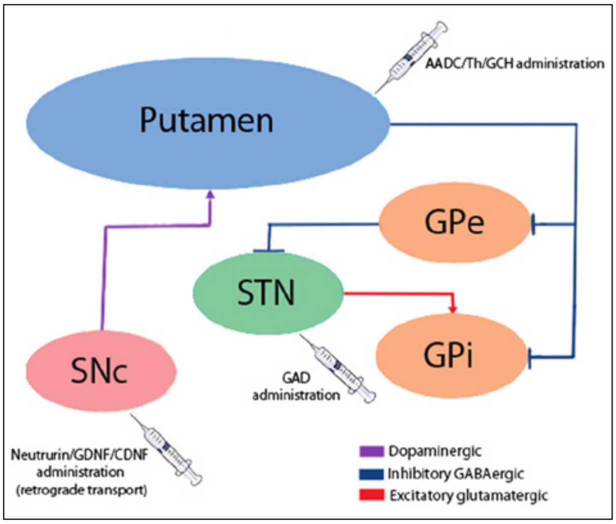

Figure 5. Therapeutic strategies for Parkinson's disease. Delivery of neurotrophic factors to the substantia nigra compacta (SNc) for retrograde delivery to the putamen. Gene therapy using glutamic acid decarboxylase (GAD) to quiet neurons in the subthalamic nucleus (STN). Delivery of neurotransmitter synthetic enzymes involved in dopamine production to the putamen. AADC = aromatic L-amino acid decarboxylase; Th = tyrosine hydroxylase; GCH = guanosine triphosphate cyclohydrolase; GDNF = glial-derived neurotrophic factor; CDNF = cerebral-derived neurotrophic factor, GPe = globus pallidus external; GPi = globus pallidus internal. Illustrations adapted from ChemBioDraw (Version 13.0.2.3021, Cambridge Software, Waltham, MA).

± 3%) and motor neurons (P1, 62% ± 1%; P21, 8% ± 1%) and persisted throughout the life span of the mice. One shRNA mediated 60% and 45% reductions in mutant SOD1 protein in P1 and P21 injected mice, and it extended survival times by 39% when treatment was initiated at birth, one of the longest extensions of survival reported in this mouse model. In addition, vector administration was impressively able to slow disease progression even after disease onset (injection at P85). The same vector administered via intrathecal injection in cynomolgus macaques led to widespread gene expression in both neurons and astrocytes in the grey and white matter of the spinal cord. The percentage of ChAT+ motor neurons expressing the transgene was 50% in the cervical region, 65% in the thoracic region, and 80% in the lumbar region. SOD1 knockdown matched this pattern, with a 60% decrease in SOD1 mRNA in the cervical region, 70% decrease in thoracic region, and 88% decrease in lumbar region. These promising results provide support for human clinical trials. In another approach, Kaspar and others (2003) treated a mouse model of ALS with an intramuscular injection of an AAV vector encoding insulin-like growth factor 1. Retrograde gene delivery improved motor neuron survival, delayed deterioration of motor function, and extended overall survival (Kaspar and others 2003).

Neuropathic Pain

AAV-mediated delivery of shRNAs has also recently been applied to treat injury-induced neuropathic pain. $Na_v1.3$ channels are up-regulated in DRG sensory neurons after injuries to the nervous system (Hains and others 2003), and are therefore potential targets for treatment of chronic pain. Samad and others (2013) injected an AAV2/5 vector encoding shRNA against $Na_v1.3$ into the lumbar dorsal root ganglion of adult rats with spared nerve injury. Two independent AAV-shRNA vectors were able to transduce ~45% of neurons and achieve ~50% knockdown of $Na_v1.3$ compared with a scrambled shRNA control. Importantly, off-target effects against other sodium channels were not observed. Moreover, $Na_v1.3$ down-regulation resulted in a significant, partial attenuation of mechanical allodynia, with up to a sixfold recovery of pain threshold; a promising proof of principle for gene therapy approaches to treat chronic neuropathic pain.

Parkinson's Disease

Parkinson's disease is a neurodegenerative disorder characterized by loss of motor function accompanying the death of dopaminergic neurons in the substantia nigra. In contrast to monogenic disorders, the mechanisms of pathogenesis in Parkinson's disease involve multiple genes and environmental factors (Shadrina and others 2010), and consequently gene therapy is not as straightforward as supplying a functional copy of a defective gene. Instead, clinical trials have utilized neurotrophic factors that prevent neuronal cell death, or alternatively neurotransmitter synthetic enzymes to modulate neuronal activity (Fig. 5). Translation to the clinic has demonstrated a strong safety profile and is progressing toward therapy efficacy.

In a clinical trial conducted by Ceregene, delivery of CERE-120—an AAV2 vector encoding the neurotrophic factor neurturin under a constitutive CAG promoter—to the putamen resulted in an excellent safety profile in a phase 1 clinical trial (Bartus and others 2013). A multicenter, double-blind, sham surgery controlled phase 2 trial strengthened claims of vector safety but failed to yield a statistically significant benefit on the primary endpoint, the motor-off component of the Unified Parkinson's Disease Rating Scale (UPDRS) evaluated after 12 months. Some clinical benefits were evident: 19 of 25 efficacy endpoints were favorable compared with the sham control. Autopsy analysis of two subjects (who died due to unrelated causes) confirmed neurturin expression in the putamen with co-localized tyrosine hydroxylase. Interestingly, neurturin staining was sparse in the substantia nigra, indicating that retrograde transport of the AAV vector and neurturin was inefficient. While unexpected based on preclinical studies in animal models, this result may indicate that Parkinson's disease in humans is characterized by accelerated degeneration of axonal transport capabilities well before the death of the neuron (Burke and O'Malley 2013).

Based on these results Ceregene initiated a phase 1/2b study incorporating direct injection of the substantia nigra and a fourfold increased dosage to the putamen. Given the strong placebo effect observed in the first trial, the time point for evaluation of the primary endpoint was increased to 15 months. Phase 1/2b results indicated no significant safety issues with gene delivery to the substantia nigra. Despite these modifications, initial phase 2b efficacy results did not demonstrate a statistically significant benefit on the primary endpoint (Ceregene 2013a). Similar to the first trial, a number of secondary endpoints produced statistically significant benefits, and a strong placebo effect was observed in the sham surgery control group. Additional analysis identified a better response on the primary endpoint in patients diagnosed 5 years prior to treatment compared with those diagnosed 10 years prior (Ceregene 2013b). Although the efficacy results were disappointing, the field should be encouraged by the exceptional safety profile and progress in vector delivery.

Other neurotrophic factors are being considered to treat Parkinson's disease. While a phase 1/2 double-blind trial with direct daily infusion of glial derived neurotrophic factor (GDNF) protein into the putamen did not produce a statistically significant benefit (Lang and others 2006), an AAV-GDNF vector may perform better than repeated GDNF infusions. A phase 1 clinical trial employing an AAV2-GDNF vector is currently recruiting (National Institute of Neurological Disorders and Stroke [NINDS]. AAV2-GDNF for Advanced Parkinson's Disease). In addition, multiple groups have shown that administration of cerebral dopamine neurotrophic factor (CDNF) to the striatum can prevent deterioration of midbrain dopamine neurons in a rat model of Parkinson's disease (Back and others 2013; Ren and others 2013), indicating the promise of this molecule.

A different therapeutic strategy is to directly target the enzymatic pathway for dopamine production. L-dopa administration is currently the most effective treatment for relieving symptoms of Parkinson's disease; however, most patients experience a decrease in therapeutic efficacy after taking the medication for several years. One possible reason for such diminishing returns is reduced levels of aromatic L-amino acid decarboxylase (AADC), the enzyme that converts L-dopa to dopamine, and gene delivery of AADC could thus improve the response to L-dopa. Furthermore, the degree of therapy can be controlled by modifying the dosing regimen of L-dopa. Preclinical studies in parkinsonian monkeys demonstrated increased L-dopa conversion lasting for more than 7 years after convection-enhanced delivery of AAV2-AADC into the striatum (Hadaczek and others 2006). Furthermore, an open label phase 1 clinical trial using an AAV2 vector for AADC delivery to the putamen improved the mean UPDRS rating scale score by 30% in the on and off states (Christine and others 2009; Eberling and others 2008). Given the strong placebo effect observed in the CERE-120 trials, double-blinded sham surgery controls will likely be included in future trials.

Additional enzymes involved in dopamine production include tyrosine hydroxylase (TH), the rate-limiting enzyme for conversion of L-tyrosine to L-dopa, and guanosine triphosphate cyclohydrolase I (GCH), the rate-limiting enzyme for production of the TH co-factor tetrahydrobiopterine. These genes have not yet been delivered in a clinical setting with AAV, but preclinical results in rat models are promising (Bjorklund and others 2010; Carlsson and others 2005). Most studies have delivered each gene in a separate virus, but a new vector designed to co-express both TH and GCH1 from a single virus (Cederfjall and others 2013) provided a dose-dependent functional recovery based on enhanced dopamine production in 6-OHDA lesioned rats. Unexpectedly, the same vector administered to parkinsonian monkeys resulted in transgene expression of GCH but not TH for unknown reasons.

Another approach involves delivery of the gene glutamic acid decarboxylase (GAD)—the rate-limiting enzyme for GABA production—into the subthalamic nucleus (STN). Increases in GABA production reduce output from the STN, an overactive region in Parkinson's disease. In a completed double-blind sham surgery controlled clinical trial, administration of AAV2-GAD vector to the STN via intracranial injection was well tolerated and resulted in improvements in the UPDRS motor score of 23.1% in patients receiving the vector compared to 12.7% in the sham control group after 6 months, the first statistically significant difference in the primary endpoint of a double-blind phase 2 trial employing AAV to treat Parkinson's disease (LeWitt and others 2011). Despite benefit in the primary endpoint, most quality-of-life measures did not improve, and as a result the trial sponsor (Neurologix) was unable to proceed to a phase 3 trial.

Conclusion

Promising clinical trials and the first gene therapy product approval underscore the exciting potential of gene delivery using AAV. Recombinant AAV vectors can provide long-lasting gene expression to treat chronic neurological disorders and have demonstrated a strong safety profile, making them promising for both monogenic and idiopathic CNS disease. That said, clinical translation in the CNS has been hindered by biological transport barriers, immune responses, and infection of off-target cells. However, these challenges have motivated the development of engineered vectors to further and fully realize the potential of gene therapy in the CNS.

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The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: DVS is an inventor on patents involving AAV directed evolution.

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