Vector Design and Considerations for CNS Applications

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Introduction

A decade ago, strategies for gene delivery to the brain were limited mostly to stereotaxic injection of viral vectors to the brain. Any measure of widespread gene delivery was achieved by the use of multiple injections to create pockets of transgene expression throughout the brain. More recently, advancements in vector design and the exploration of alternative routes of administration have made global CNS gene delivery a possibility. This chapter will explore these advancements and provide an overview of the capabilities and limitations of existing gene delivery technology for CNS disorders. For a detailed review of these topics, see Gray et al. (2010a).

The most prominent CNS gene delivery vector is currently adeno-associated virus (AAV). Although AAV naturally infects humans, it is nonpathogenic and is classified as a dependovirus because it is unable to execute a lytic infection without coinfection with a helper virus such as adenovirus or herpesvirus (Goncalves, 2005). Important for CNS gene therapy applications, AAV can transduce nondividing cells and has the ability to confer long-term stable gene expression without causing associated inflammation or toxicity (Goncalves, 2005). Recombinant AAV packages any DNA cassette within its size constraints (~4.8 kb) as long as the DNA is flanked by ~145 bp inverted terminal repeats (ITRs).

Lentivirus-based vectors are also playing an increasingly significant role in CNS-directed gene therapy, and they have the advantage of a larger packaging capacity (~8 kb of foreign DNA as opposed to ~4.5 kb for AAV). However, this chapter will focus on AAV vectors and applications.

Vector and Gene Expression Needs

Gene delivery and expression needs can vary considerably depending on the specific disease paradigm. Generally, if a disease can be treated with a factor that is expressed from a transduced cell and provides a benefit to neighboring cells, the gene therapy approach is easier. In this scenario, the efficiency of delivery does not necessarily need to be high, and any cell type is potentially a viable target. Each transduced cell will essentially become a factory for producing the therapeutic factor. If enough cells are transduced to provide the secreted therapeutic factor to the entire CNS (or the affected portions of the CNS), the treatment can be efficacious.

The simplest type of disease target (at least from a delivery standpoint) is a disease in which a relatively small area of the brain would need to be treated. In the example of Parkinson's disease (PD), there is a loss of dopaminergic neurons in the substantia nigra (SN), so gene therapies for PD aim to compensate for this loss of neurons in the SN rather than targeting the entire brain. Using optimized injection parameters, such as MRI-guided and convection-enhanced delivery (Varenika et al., 2009), a focal area of the brain can be transduced with high efficiency and accuracy.

Most diseases broadly affect the brain and/or spinal cord, and in such cases, the challenge is to utilize a vector and delivery approach to broadly deliver the therapeutic reagent to enough of the CNS to impact the course of the disease. Lysosomal storage diseases (LSDs) are caused by the loss of an essential enzyme that results in the toxic accumulation of that enzyme's substrate. In many cases, the missing enzyme is secreted from the expressing cell and can be taken up by neighboring cells via the mannose-6-phosphate pathway (Sands and Davidson, 2006). For LSDs, hypothetically, the ideal approach would be to broadly transduce cells throughout the CNS in order to secrete enough therapeutic enzyme in a spatially appropriate manner to reduce the toxic substrate in the entire CNS. The same principal can be applied to therapeutic approaches utilizing neurotropic factors for other diseases. The spatial pattern of gene delivery does not necessarily need to target all the affected cells or areas as long as the secreted factor does. One may intuit that a higher degree of gene expression per cell could compensate for a lower efficiency of gene delivery, as long as the factor is not toxic to the expressing cell.

The most challenging indications for CNS-directed gene therapy are those that are widespread and have cell-autonomous effects; that is, only the cells that directly receive the therapeutic transgene would benefit. Included in this group of CNS disease therapies are gene replacement strategies for fragile X syndrome, spinal muscular atrophy (SMA), Rett syndrome, and Huntington's disease (HD), to name a few. These are diseases that, from a hypothetical genetic standpoint, could be treated by replacing the defective gene or, in the case of HD, by knocking down or otherwise removing toxic aggregates formed by the mutant protein. The biggest challenge in treating these disorders lies in delivery, since the affected cells pervade large areas of the CNS, sometimes severely affecting the entire brain and/or spinal cord. Another potential complication is illustrated by Rett syndrome, in which overexpression of the missing gene (*MeCP2*) by as little as twice the endogenous levels can lead to separate progressive neurological effects distinct from

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classical Rett syndrome and early mortality in mice (Collins et al., 2004). Therefore, the regulation of transgene expression can be a critical component to the success of these gene therapy strategies.

Optimizing Gene Expression

Gene expression can be optimized in several ways, depending on the needs of the gene transfer application. Starting with the vector itself, the use of self-complementary (sc) AAV genomes provides faster gene expression and a 10- to 100-fold increase in transduction efficiency compared with traditional single-stranded (ss) AAV (McCarty et al., 2003). This increase in efficiency comes at the cost of packaging capacity: sc AAV can package only ~2.1 kb of foreign DNA compared with ~4.5 kb for ss AAV. The restricted packaging capacity of AAV (especially sc AAV) has made the development of minimal promoters, 5' and 3' untranslated regions (UTRs), and polyadenylation signals an important component of vector development, especially for larger transgenes.

Most often, gene expression is regulated by changing the promoter to provide cell-specific or ubiquitous expression and to control the overall amount of transcript produced. However, the choice of 5' UTR, 3' UTR, enhancer, and polyadenylation signal can have a strong effect on promoter strength (see Table 1 for a list of expression control elements). Commonly used strong and "ubiquitous" promoters include the cytomegalovirus (CMV) promoter and the truncated chicken beta actin (CBA) promoter, each of which is ~800 bp (including the CMV enhancer and 5' UTR sequence) (Gray et al., 2011b). Compared with CBA, the CMV promoter is stronger but is prone to silencing over time in the CNS. By utilizing a hybrid CBA and MVM intron with the CBA promoter, this hybrid CBA promoter (CBh) can provide long-term, ubiquitous gene expression at high levels (Gray et al., 2011b). The CMV promoter can be further strengthened and silencing avoided by incorporating the woodchuck hepatitis virus posttranscriptional response element (WPRE) in the vector, but this addition comes at the cost of 600 bp of packaging size (Hermening et al., 2006). The beta glucuronidase (GUSB) or ubiquitin C (UBC) promoters can provide ubiquitous gene expression with a smaller size of 378 bp and 403 bp, respectively, but they are considerably weaker than the CMV or CBA promoter (Husain et al., 2009; Qin et al., 2010).

To achieve cell-specific expression, neuron- or astrocyte-specific promoters can be employed. To restrict expression to neurons, the neuron-specific enolase (NSE), synapsin, or MeCP2 promoter can be utilized with sizes of 2.2 kb, 470 bp, and 229 bp, respectively (Peel et al., 1997; Kugler et al., 2001; Gray et al., 2011b). A truncated 681 bp glial fibrillary acidic protein (GFAP) promoter can be used to restrict expression to astrocytes (Lee et al., 2008).

Modifying Vectors

Identifying the optimal serotype

When contemplating a particular target in the CNS, it is important to review the potential routes of administration and identify the optimal serotype to use. The simplest modification of AAV to modulate its tropism is to package the genome within capsids from different serotypes. More than 100 different AAV variants have been identified, each with potentially different cell tropism, providing a broad toolkit of vectors for optimized delivery to the target cells (Wu et al., 2006).

The AAV serotypes most commonly used for CNS applications include AAV1, AAV2, AAV4, AAV5, AAV6, AAV8, and AAV9. AAV2 was the most

Table 1. Promoters.

Enh	Promoter	5' UTR/intron	Strength	Size	Specificity
CMV	CMV	SV40	High	800 bp	Ubiquitous
CMV	CBA	SV40	High	800 bp	Ubiquitous
CMV	CBA	CBA-MVM	High	800 bp	Ubiquitous
None	UBC	None	Weak	430 bp	Ubiquitous
None	GUSB	None	Weak	378 bp	Ubiquitous
None	NSE	None	Strong	2.2 kb	Neuron
None	Synapsin	None	Medium	470 bp	Neuron
None	MeCP2	None	Weak	229 bp	Neuron
None	GFAP	None	Medium	681 bp	Astrocyte

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studied serotype during the early development of AAV vectors and has been used in clinical studies, but in most regards for CNS applications, it does not perform as well as more recently characterized serotypes. Using direct injection into the brain parenchyma, AAV1, AAV5, and AAV9 provide the best vector spread and highest efficiency of transduction. AAV1 and AAV9 provide almost exclusively neuronal tropism, while AAV5 provides a mix of neurons and glia, and AAV4 targets mostly astrocytes (Davidson et al., 2000; Burger et al., 2004; Cearley and Wolfe, 2006). AAV1 and AAV6 have superior retrograde axonal transport capabilities following peripheral injection (Hollis et al., 2008), while AAV9 undergoes efficient axonal transport within the brain (Cearley and Wolfe, 2006). AAV6, AAV8, and AAV9 have demonstrated efficient delivery to the spinal cord and dorsal root ganglia following intrathecal administration, targeting different subsets of cells depending on the specific serotype (Storek et al., 2008; Towne et al., 2009; Snyder et al., 2011). Intracerebral ventricular injection of AAV4 efficiently transduces ependymal cells (Liu et al., 2005a). Interestingly, AAV9 (and to a lesser extent AAV8) can cross the blood–brain barrier (BBB) following intravenous administration to transduce neurons and glia within the brain and spinal cord (Gray et al., 2011a).

Engineering the virus coat

The virus coat is an obvious molecular target to engineer the virus toward a specific therapeutic application. This can be done by rational mutagenesis, incorporation of peptide ligands on the virion surface, and directed evolution to produce new AAV variants. In one example of capsid mutagenesis, Pulicherla et al. (2011) introduced point mutations into AAV9 to knock down its liver tropism, potentially creating a safer version of AAV9 to deliver intravenously to the CNS. To make an AAV2-based vector for enhanced retrograde transport and neuron targeting, peptides derived from an NMDA receptor agonist and a dynein binding motif were incorporated into the capsid (Xu et al., 2005). These peptides synergistically enhanced retrograde transport of AAV2 10- to 100-fold and allowed retrograde delivery to the CNS from peripheral injection *in vivo*; in contrast, unmodified AAV2 delivery to the CNS was undetectable. This strategy can be taken one step further to bypass any knowledge of the target cell and utilize a phage-display library to generate a peptide with tropism for the given tissue. Chen et al. (2009) cycled a phage-display library of random peptides in normal mice and mice modeling LINCL (late infantile neuronal ceroid lipofuscinosis) or mucopolysaccharidosis VII (MPS VII), injecting the library into the tail vein and recovering it from the brain. The dominant recovered peptide from each selection was incorporated into an AAV2 capsid, then tested *in vivo*. The peptide specifically targeted AAV2 to the cerebral vascular endothelial cells after intravenous injection in mice. The engineered vectors had a striking preference for the CNS vasculature of the LINCL and MPS VII disease mouse models where the phage-display selection was performed, so much so that it did not work in a wild-type mouse and vice versa.

DNA shuffling and directed evolution

DNA shuffling and directed evolution make up another method used to generate novel "mixtures" of AAV capsid genes and then exert selective pressure to identify new AAV variants with desired characteristics. The generation of random AAV capsid libraries, termed "directed evolution," was pioneered by Schaffer and Maheshri (Maheshri et al., 2006). Multiple variations of the AAV directed-evolution process have since been utilized, but the overall strategy is similar. First, the capsid genes are randomly mutagenized, or mixed, to form a library of pooled capsid variants in the context of a replication-competent backbone. Next, this library is subjected to multiple rounds of selective pressure. At the end, the recovered library clone(s) should be enhanced for whatever characteristic was selected for, above that of the parent serotype(s). Described methods for producing the library include random mutagenesis of the capsid gene of a single serotype by error-prone PCR, randomly mixing capsids from multiple serotypes by DNA shuffling, or a combination of the two methods (Koerber et al., 2006; Maheshri et al., 2006).

We applied the directed evolution process directly to a CNS application, namely to specifically target a therapeutic vector to sites of seizure damage (Gray et al., 2010b). Our group took advantage of the disruption of the BBB that occurs at sites of seizures and selected for AAV capsids that could enter the brain only at these sites of seizure damage. These clones had the additional benefit of a near-complete loss of liver, heart, and muscle tropism, giving them a favorable safety and biodistribution profile.

Global CNS Delivery

Emerging AAV vector technologies are allowing global delivery of a gene-based therapy to the entire CNS. As discussed in Vector and Gene Expression Needs, above, ideal global therapeutic approaches utilize factors that are expressed and secreted. In this scenario, the biodistribution of the expressed factor can be more pervasive than the vector

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biodistribution, possibly leading to disease correction even in the event of suboptimal vector delivery. Early and ongoing strategies based on this principle utilized one of two methods:

(1) Multiple intraparenchymal brain injections to provide pockets of gene expression throughout the brain or
(2) Transduction of the ependymal cells lining the ventricles as a means of expressing the factor into the CSF, where it would then be distributed throughout the brain.

Especially for direct intracranial injections, it is important to note that the degree of vector distribution (and resulting phenotypic correction) seen in rodent models will be much less in larger animal models and humans, owing to the difference in brain volume. This reality has halted the human translation of many very encouraging rodent efficacy studies. The rest of this section will focus on approaches that utilize a fluid volume for gene delivery, which should make clinical translation more feasible.

Multiple groups have now reported in detail the ability of AAV9 vectors to cross the BBB and transduce neurons and astrocytes following intravenous injection in neonatal mice, adult mice, cats, and nonhuman primates (Duque et al., 2009; Foust et al., 2009; Gray et al., 2011a). This process is at least ten times more efficient with sc AAV vectors than with ss AAV vectors (Gray et al., 2011a). Using doses of sc AAV ranging from 5×10^{12} to 2×10^{14} vg/kg, strategies employing intravenous delivery of AAV9 vectors have successfully treated SMA (Foust et al., 2010) and MPS IIIB (Fu et al., 2011) in mice. This is especially interesting for SMA, where the delivery efficiency is apparently high enough to achieve efficacy even though the transferred gene (*SMN1*) should exert only a cell-autonomous effect. Although this approach utilizes a fluid volume that should be amenable for direct-dose scaling between rodents and humans, the translation of this approach is questionable because of the reduced delivery efficiency in nonhuman primates, the high amounts of vector required, and the high biodistribution of the vector to peripheral tissues (Gray et al., 2011a). A 10-fold lower dose can be efficacious for MPS IIIB compared with SMA, likely owing to the secretion of the expressed enzyme for MPS IIIB, and this lower dosing threshold may increase the translational feasibility of this approach.

LSDs in general lend themselves well to a gene therapy approach (Sands and Davidson, 2006). In a gene therapy approach, delivery efficiency and biodistribution of the vector can be suboptimal as long as the secretion and biodistribution of the therapeutic enzyme are sufficient to treat the entire CNS. The efficacy of bone marrow stem cell (BMSC) replacement (either by heterologous donor or by autologous transduction via retroviral vectors) as a treatment for Krabbe's disease and adrenoleukodystrophy (ALD) attests to the potentially low threshold of delivery that may be therapeutic. In these cases, it is hypothesized that the CNS treatment efficacy results from the migration of enzyme-expressing microglial cells derived from the BMSCs. These microglia make up a very small percentage of the cells in the brain but would be widely distributed, suggesting that a similar approach using gene delivery vectors could strongly impact these diseases. In this regard, preclinical studies in large animal models for glycogen storage disease type 1a (GSD-1a), MPS type I, MPS type VI, and MPS type VII have been strikingly successful. In these studies, gene therapy permanently (and sometimes completely) alleviated the disease phenotype long after untreated controls had died from the disease (Sleeper et al., 2004; Traas et al., 2007; Koeberl et al., 2008; Tessitore et al., 2008). It should be noted that some of these approaches utilized retroviral vectors, and others utilized AAV vectors. Overall, however, these studies indicate the great potential of using gene therapy to significantly impact these diseases if a translatable mode of gene delivery can be utilized.

Conceptually, the best strategy for treating LSDs would be to deliver the therapeutic gene vector such that the expressed enzyme biodistribution would reach all cells in the CNS. Four major strategies have been employed to accomplish this:

- Direct intraparenchymal injection of the vector has limited vector distribution, especially in large animals. The spread of vector and enzyme can be enhanced by utilizing networks of axonal transport within the brain (Varenika et al., 2009), but many regions are still left untreated;
- Intravenous delivery, as discussed above, is an attractive approach that is minimally invasive but would achieve even vector distribution across the entire CNS. It remains to be determined whether the existing barriers for human translation (e.g., dose, preexisting neutralizing antibodies, reduced transduction, and peripheral organ tropism) (Gray et al., 2011a) can be successfully overcome to

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attain a therapeutic option for human translation;

- Delivering AAV vectors to the CSF via intracerebroventricular (ICV) injection to target ependymal cells is a third option. These cells form the interface between the CSF and brain parenchyma, so they have the potential to secrete the therapeutic enzyme into the CSF, where it can be circulated to the entire CNS. Using AAV4 vectors to efficiently target the ependymal cells, this strategy was successfully employed to treat mice with MPS VII (Liu et al., 2005b). Given that AAV genomes do not persist in dividing cell populations, the long-term efficacy of this approach is questionable and remains to be tested, since the ependyma has a turnover rate of approximately 130 d (Chauhan and Lewis, 1979);
- Utilizing intra-CSF delivery to target neurons and/or glia within the brain and spinal cord parenchyma is a fourth approach. Systemic administration of mannitol at the time of intraventricular AAV2 injection can allow penetration of the vector into the brain parenchyma (Ghodsi et al., 1999; Fu et al., 2007). Intracisternal injection of AAV1 vectors also showed diffuse global transduction of ependymal cells and Purkinje neurons, mostly localized along areas of the brain in proximity to the CSF, but also efficient transduction of cervical dorsal root ganglia (Iwamoto et al., 2009); and
- Lumbar intrathecal injection of AAV vectors provides a possibility for widespread gene delivery with a routine and low-risk clinical procedure. Intrathecal administration of AAV2 within the thoracic region of the spinal cord led to transduction of neurons distributed throughout the entire brain, albeit at very low efficiency (Watson et al., 2006). This study demonstrated the possibility of vector transport through the CSF into the brain from an intrathecal injection.

Comparing several routes of delivery (intravenous, intramuscular, intranerve, and intrathecal), Towne et al. (2009) found that intrathecal injection of AAV6 vectors led to the most efficient and widespread transduction of cervical and lumbar dorsal root ganglia. AAV9 vectors have also recently been shown to efficiently target spinal cord motor neurons and dorsal root ganglia following intrathecal injection (Gray et al., 2011b; Snyder et al., 2011). With the exception of the direct brain injection, these approaches all utilize a fluid volume that should be amenable to direct scaling of doses: from rodents to large animals to humans.

Conclusions

AAV vector technology allows focal or widespread transgene delivery to the CNS, resulting in long-term stable gene expression in nondividing cells. Naturally occurring serotypes provide a broad toolkit of effective vectors, while next-generation engineered vectors offer more efficient and specific delivery of the therapeutic transgene, potentially tailored to specific disease applications. Transgene expression can be ubiquitous or restricted to specific cell populations through vector choice, route of administration, and/or promoter control. LSDs represent a promising and immediate family of diseases that could benefit from gene therapy. The main obstacle in the translation of LSD gene therapies has been the availability of a global gene delivery system applicable to large animals; however, promising technological developments utilizing vasculature or intra-CSF vector delivery are beginning to meet that need. Increasing the efficiency of these delivery strategies should make treatments for cell-autonomous diseases (e.g., Huntington's disease, SMA, Rett syndrome) a more realistic possibility.

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