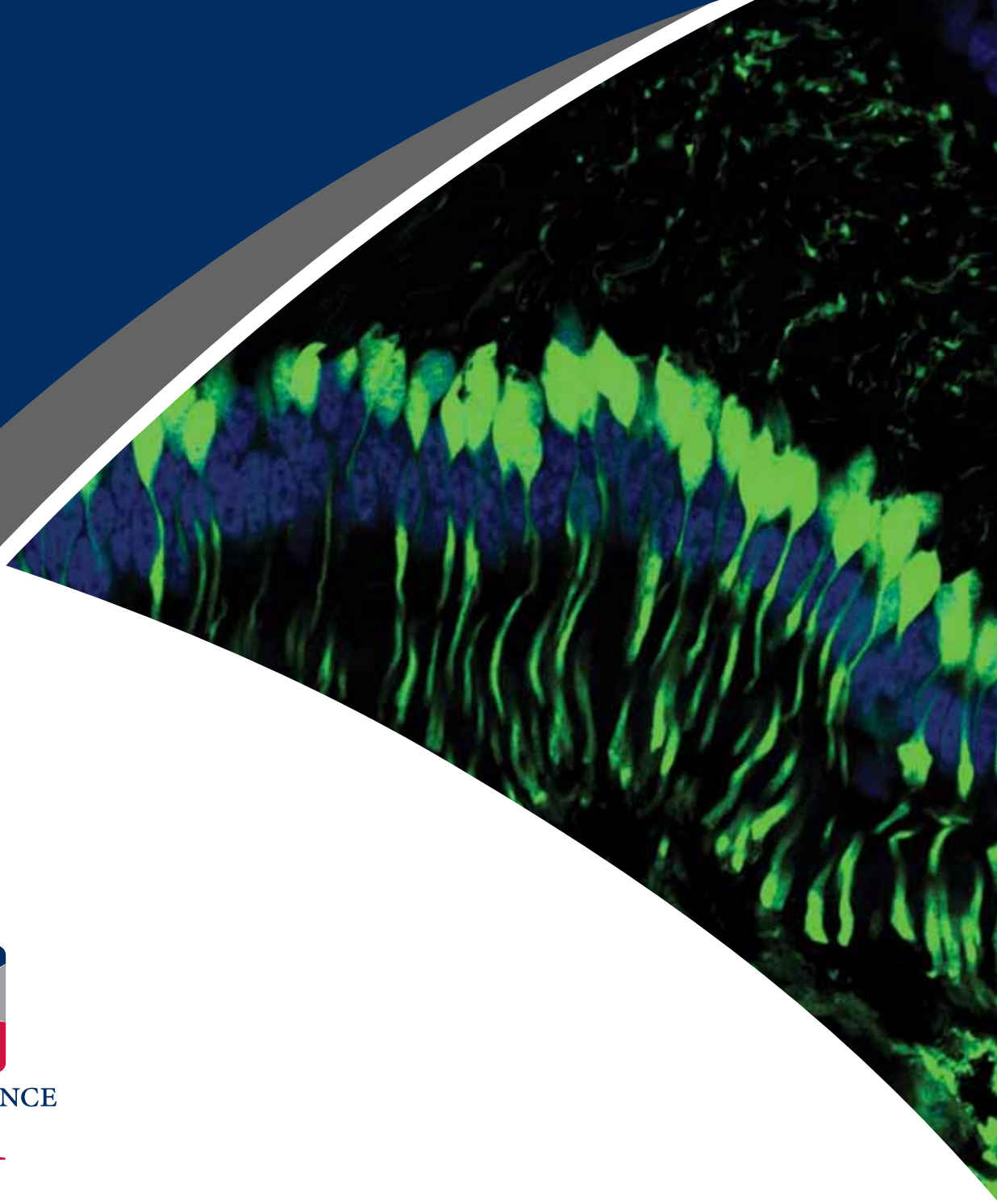

NEUROSCIENCE 2011

SHORT COURSE I

Gene Vector Design and Application to Treat Nervous System Disorders

Organized by Joseph C. Glorioso, PhD

SOCIETY FOR NEUROSCIENCE

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Table of Contents

Introduction

Vector Design and Considerations for CNS Applications
Steven J. Gray, PhD and R. Jude Samulski, PhD 7

Gene Transfer Vectors: Applications to the Treatment of Retinal Degenerations
Jean Bennett, MD, PhD and Luk Vandenberghe, PhD 17

Viral Vector Gene Delivery to the Brain to Treat the Disseminated Lesions of Neurogenetic Diseases: Focus on Lysosomal Storage Diseases
John H. Wolfe, VMD, PhD 25

Delivering Therapies to the Brain: A Brief Review of Current Strategies for Huntington's Disease
Maria Beconi, PhD; Alla Katsnelson, PhD; and Allan J. Tobin, PhD 33

Gene Therapy for Motor Neuron Disease
Nicholas M. Boulis, MD 41

Gene Transfer to the Peripheral Nervous System: Treatments for Polyneuropathy and for Pain
David J. Fink, MD 51

Gene Therapy for Epilepsy
Michele Simonato, MD 59

Gene Therapy for Malignant Brain Tumors: from Experimental to Clinical Neuro-oncology
M.G. Castro, PhD and P.R. Lowenstein, MD, PhD 69

Introduction

The neurobiology of human disease is a rapidly progressing field and encompasses a broad range of pathological processes that include abnormal development, metabolism, cognitive and emotional states, memory and sensory functions. The nervous system is also afflicted with unique forms of autoimmune processes, malignancy, infections, and trauma. These disease processes can affect peripheral and central nervous system functions either independently or through their connected activities and related structures. The nervous system is by far the most complex and difficult to study of the organ systems and most of our most elusive and pervasive disease processes arise in the nervous system. The nervous system detects our environment, interprets our world, and directs our activities.

Many of the nervous system's pathological states are coming to light, although tremendous gaps in our understanding of disease mechanisms remain. Indeed, fundamental brain processes such as memory storage and recall, consciousness, and integrative functions are nearly completely mysterious. Most serious diseases that involve degenerative processes such as Huntington's, Parkinson's, and Alzheimer's disease are without lasting or even palliative treatments. Therapies that involve the use of pharmaceutical approaches often have nonspecific and global unwanted effects and many drugs in use to treat brain-related diseases are used for off-target applications. Our best insights into disease processes center on single gene defects and metabolic disturbances. However, even in these instances, interventions to correct disease processes are disappointingly limited. Moreover, the protective blood-brain barrier (BBB) can inhibit treatment approaches even though the BBB is crucial to protecting the brain. The application of genetic approaches to develop nervous system therapies may offer an important approach to correction of nervous system diseases, and clearly there is a great deal of interest in disease prevention, progression, and nervous system repair through the delivery of functional genes to affected nerve tissues. These interventions generally fall under the purview of gene therapy.

The goal of this workshop is to provide information on the latest gene therapy vehicles and delivery methods in which treatment of animal models of nervous system disease has proven effective and early human clinical trials suggest that applied gene therapy can be translated to humans. While we are still in the early phases of the development of this technology, gene therapy will very likely become standard medical practice for many nervous system-related diseases where well-accepted treatment practices simply may not work. This is largely because nervous system disease often involves multiple cellular systems where drug-related therapy will not be sufficient to tackle problems inherent in complex systems.

We have brought together experts in the gene therapy field that include those in vector biology and design, in the application of gene therapy to clinical problems, and gene therapy pioneers who can provide insight into the potential future of gene therapy applications to human disease. The goal of this workshop is to open discussion of the utility of gene therapy for nervous system disease conditions, the future of clinical research, and prospects for first successes.

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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

NOTES

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

NOTES

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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Gene Transfer Vectors: Applications to the Treatment of Retinal Degenerations

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Introduction

The first two genes implicated in blindness were identified and cloned in 1990: the choroideremia gene (*CHM*) and the rhodopsin (*RHO*) gene (Cremers et al., 1990; Dryja et al., 1990; Farrar et al., 1990). Two decades later, 172 different genes have been identified which, when mutated, can cause retinal degeneration (http://www.sph.uth.tmc.edu/RetNet). This number is likely 80% of the total number of retinal disease-causing genes because loci have been identified for at least 32 additional blinding conditions. The progress in genetics has led to the identification of disease-causing genes in spontaneous mutant animal models and, in turn, to the development of additional animal models of blindness and an improved understanding of disease pathogenesis. With the simultaneous development of reagents and approaches with which to carry out retinal gene transfer, it has become possible to harness the pathogenetic data to develop rational gene-based treatments.

The mammalian eye, because of its ease of access, benign immunologic response to gene transfer, and ability to perform noninvasive functional and structural studies, has been at the forefront of therapeutic trials based on gene transfer. Gene transfer strategies have been used in both small and large animal models to demonstrate proof-of-concept. These preclinical studies have allowed the field to reach the point where gene therapy to treat a form of inherited blindness has been tested in clinical trials.

Background

Vectors and retinal gene transfer: nonviral gene delivery

A number of physicochemical methods have been evaluated for their ability to deliver nucleic acids to retinal cells. These methods include the use of physicochemical agents to compact the DNA and/or transport it across the membrane lipid bilayer (Table 1). Retinal gene transfer has also been achieved through electroporation or iontophoresis. Nonviral gene transfer is attractive because it can be used to deliver DNA of unlimited size and is less likely than viral gene transfer to incur a detrimental immune response. Several studies have demonstrated proof-of-concept for retinal gene therapy using nonviral DNA delivery, and additional studies are expected to reveal the long-term safety, stability, and efficacy of this approach.

Vectors and retinal gene transfer: viral vector–mediated gene delivery

A large number of recombinant viruses have been tested for their ability to target the retina. Different viruses have different attributes and challenges, including cargo capacity, ease of purification, cellular specificity, and immune response (Table 1). Many of these have been used to demonstrate efficacy in animal models of retinal degeneration.

Adenovirus type 5 (Ad5) vectors, deleted of the adenoviral *E1, E3* genes, were the first to be evaluated for retinal gene transfer in the differentiated retina (Bennett et al., 1994; Li et al., 1994). Adenovirus vectors result in high levels of gene expression within 24 to 48 h. When injected subretinally, they target retinal pigment epithelium (RPE) cells efficiently in the adult eye and also Müller cells. When injected intravitreally, they target Müller cells and many cells in the anterior segment (including cells in the cornea, lens, iris, and outflow tract). Because the early generations of vectors carry viral open-reading frames, these vectors can elicit an immune response that limits the duration of transgene expression. Efforts have been made to generate adenovirus vectors lacking any viral open-reading frames, the so-called gutted or helper-dependent vectors, thereby reducing immune clearance and allowing stable transgene expression (Kumar-Singh and Chamberlain, 1996). Such vectors result in more stable transgene expression than did the first-generation vectors and, further, have a much greater cargo capacity than the original adenoviral vectors (Table 1). They are more difficult to manufacture, however.

Lentivirus vectors, unlike recombinant adenovirus (rAd) and recombinant adeno-associated virus (rAAV) vectors, have RNA genomes that are reverse-transcribed by virally encoded reverse transcriptase. These vectors integrate into the host genome and thereby can result in stable gene transfer. Vectors developed from a variety of different wild-type viruses, including human, simian, and feline immunodeficiency virus and equine infectious anemia virus, have been generated. Lentiviral vectors target RPE cells efficiently after subretinal injection and, in undifferentiated retina, target neural progenitor cells. Lentiviral vectors can carry a cargo of ~7.5 kb (Table 1).

Recombinant AAV vectors do not carry any viral open-reading frames and therfore are generally more favorable from an immunologic standpoint than adenovirus vectors (Table 1). Also, an abundant amount of safety data is available on AAV

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Table 1. Vectors tested *in vivo* in retinal gene therapy proof-of-concept studies[a].

Vector	Cargo limits	Integration	Stability (in large animal models)	Easy to purify for animal studies	Retinal cell targets	Risk of toxic immune response	Used in human ocular studies
Electroporation	Unlimited	No	Unknown (unlikely)	Yes	RPE, PRs; BPs	Low	No
Compact nanoparticles; POD	Unlimited	No	Unknown	Yes	PRs, RPE; GCs, IRs	Low	No
Adenovirus	7.5 kb	No	No	Yes/No	RPE, Müller	High	Yes
Helper independent ("gutted") adenovirus	34 kb	No	Unknown	No	RPE, PRs	Unknown	No
Adeno-associated virus	4.8 kb	No	Stable	Yes	RPE, Müller, PRs, GCs	Low	Yes
Lentivirus	7.5 kb	Yes	Stable	Yes	RPE, PRs	Low	Yes

[a]Although a number of retinal cell targets are listed, the exact targets depend on the route of administration, dose, species, and modifications to the vector. BP, bipolar cell; GC, ganglion cell; IR, inner retinal cell; Müller, Müller cell; POD, peptide for ocular delivery; PR, photoreceptor cell; RPE, retinal pigment epithelium.

administration in animals and in humans, both systemically and intraocularly. Recombinant AAV vectors have the added benefit that they target a more diverse set of cell types than do adenoviral (or other) vectors. AAV vectors do not integrate, or do so only rarely (Table 1). However, AAV-mediated transgene expression in the retina is stable since the transgene persists in episomal fashion in postmitotic differentiated cells. Expression persists for the life of small animals (e.g., mice and rats) and at least for many years in large animals and humans. rAAV vectors are useful for delivering genes efficiently to many types of retinal cells. A disadvantage of these vectors is their relatively limited cargo capacity (a maximum of 4.8 kb) (Table 1).

The retinal gene delivery properties (e.g., cellular specificity, onset of expression) of rAAV vectors can be modified by swapping the capsid from one AAV serotype with another (i.e., creating cross-packaged AAVs) or by altering amino acids in the capsid. This iformation is important for selecting vectors for particular applications. For example, an AAV that targets photoreceptor cells efficiently (AAV8) will be more useful in treating a photoreceptor disease than an AAV that predominantly targets retinal pigment epithelium cells (AAV2) (Vandenberghe et al., 2011).

Preclinical studies: proof-of-concept

Gene augmentation strategies, whereby a wild-type copy of a gene is delivered, have been tested successfully in animal models of approximately 12 different diseases. The animals model conditions such as autosomal recessive retinitis pigmentosa (ARRP), autosomal dominant (AD) RP, Leber congenital amaurosis (LCA), cone rod dystrophy, macular dystrophy, oculocutaneous albinism, achromatopsia, mucopolysaccharidosis VI, AR Stargardt disease, and RP found in disorders such as Bardet-Beidl syndrome and Usher syndrome. Keys to the success of retinal gene augmentation studies include selecting the appropriate vector (see above) and deciding when and where to deliver the vectors. The outcome measures used in the various studies include physiological assays such as electroretinograms (ERGs), evaluations of pupillary light reflexes and optokinetic responses, and tests of visual behavior (e.g., ability to swim through a water maze or to select light or dark areas).

Some toxic gain-of-function mutations have also been treated successfully using gene transfer techniques. Such strategies are necessarily more complex than gene augmentation strategies. The best studied examples of intervention in gain-of-function gene defects include rhodopsin mutations found in ADRP. Such defects result in abnormal cellular trafficking as well as altered functional properties. Deleterious effects of the endogenous mutant genes can be minimized by either a knock-down or a combined knock-down and gene augmentation strategy. The mutant mRNA can be specifically targeted, leaving the wild-type mRNA (either endogenous or delivered via gene augmentation) intact. Knock-down has been achieved successfully by using ribozymes, RNA interference (RNAi), delivery of micro RNAs, and zinc finger nucleases.

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"Generic" gene therapy strategies have been devised that are not specific to a particular disease-causing gene and potentially could be applied to a diverse set of conditions. One approach is to use genes encoding growth or neurotrophic factors or hormones to maintain the health of the diseased photoreceptors. Another approach is to deliver light-sensitive channels, originally isolated from single-cell organisms, to either inner retinal ganglia or remnant cone photoreceptors. This so-called optogenetic therapy has been used to deliver retinal/visual behavior to animals that were previously insensitive to light (Bi et al., 2006; Tomita et al., 2007; Lagali et al., 2008; Busskamp et al., 2010; Caporale et al., 2011; Doroudchi et al., 2011).

Current Status of Gene Therapy Trials for Retinal Degeneration

LCA is a severe, congenital blindness that can be caused by mutations in any one of at least 15 different genes. LCA2, the form resulting from mutations in the *RPE65* gene, which is involved in the retinoid cycle, has been the target of three different gene augmentation therapy clinical trials, all initiated in 2008. Each of the studies used an AAV serotype 2 (AAV2) vector delivering the wild-type human *RPE65* cDNA subretinally to the RPE in one eye. However, the studies differed in terms of dose, inclusion criteria, type of promoter, location of injection, and outcome measures. Each group reported a high degree of safety, and the various groups demonstrated efficacy in the first set of subjects through increases in light sensitivity, improved visual acuity and visual fields, improved pupillary light reflex, and/or improved mobility (Bainbridge et al., 2008; Hauswirth et al., 2008; Maguire et al., 2008). A fourth clinical study, in which the investigational AAV gene therapy was provided under compassionate use only, reported encouraging results for one patient (Banin et al., 2010).

The entire set of results of the Phase 1/2 study were reported by the group at The Children's Hospital of Philadelphia. They indicated that not only was the AAV delivery safe, but each of the 12 clinical trial subjects, aged 8–45 years, showed evidence of improved retinal and visual function, as judged by both subjective and objective testing (Maguire et al., 2009). The children in the study showed particularly large improvements, now being able to read books and play sports, although the older individuals also showed evidence of gain in function.

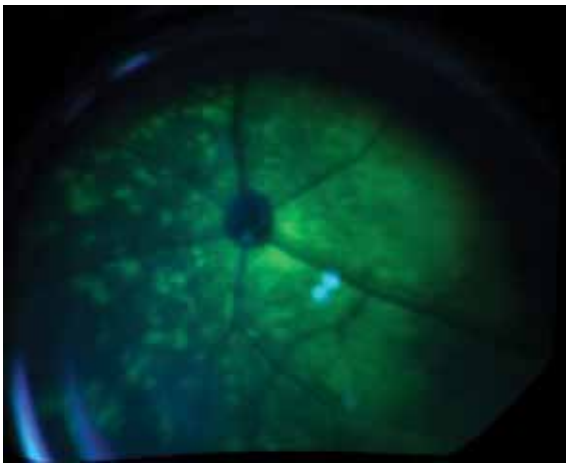

Figure 1. GFP is visible through illumination with blue light with an ophthalmoscope in the injected control eye of this mouse. The mouse had received subretinal injection of 1E11 vector genomes (vg) AAV2/8.CMV.EGFP. CMV, cytomegalovirus promoter; EGFP, enhanced green fluorescent protein.

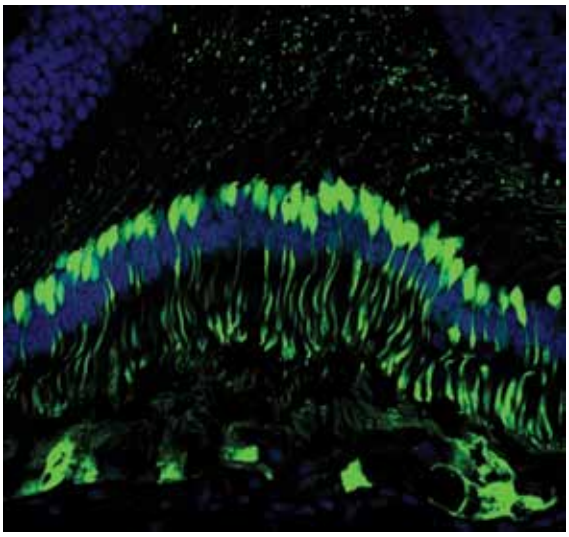

Figure 2. Histologic section from a retina of a monkey injected subretinally in the macula with 1E11 vg AAV8.CMV.EGFP. CMV, cytomegalovirus promoter; EGFP, enhanced green fluorescent protein.

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Challenges of Bringing Retinal Gene Transfer from Bench to Bedside

The successes of the first human gene augmentation therapy studies involving retinal degeneration, the LCA-RPE65 studies (Bainbridge et al., 2008; Hauswirth et al., 2008; Maguire et al., 2008, 2009; Banin et al., 2010; Simonelli et al., 2010), provide the foundation for gene therapy approaches to the treatment of other forms of inherited retinal degenerative diseases. There will be many challenges in extrapolating these approaches to treat other retinal degenerative diseases, as follows:

- Treatment of some retinal diseases will require use of a large transgene cassette — one that does not fit into the current AAV capsid or even the larger confines of lentiviral vectors;
- It will be important to continue to expand the vector toolkit in order to generate reagents that are efficient at targeting photoreceptors and other inner retinal cells;
- Although many animal models of retinal diseases have been described, many are not accessible or are imperfect; thus, additional models are needed;
- It will be important to continue to evaluate the safety of retinal gene transfer, both with respect to responses to the vector and the transgenic protein and with respect to repeat administration (in the contralateral eye); and
- Systematic genetic screening programs of wide breadth are needed to identify subjects who could participate in retinal gene therapy clinical trials.

Many physicians in the United States still tell their patients, "There is nothing that we can do." There are very few guidelines on what is an acceptable level of improvement in retinal/visual function. Additional studies will be needed to develop and adapt outcome measures in order to assess the efficacy of retinal gene therapy. The initial results from functional magnetic resonance imaging (fMRI) studies have shown that the visual cortex can become responsive to visual input after retinal gene therapy, even after prolonged (up to 35 years in the oldest patient) visual deprivation. Additional studies should evaluate the limits to restoration of retinal-cortical pathways.

Conclusions

A huge amount of progress has been made toward developing proof-of-concept of gene therapy for retinal degeneration. In addition, the results of the first few human clinical trials have shown both safety and efficacy. It will not be long before clinical trials are developed for additional gene targets. With continued improvements in vector design and progress toward understanding the genetic and pathologic bases of retinal degenerative diseases, it is likely that gene therapy successes will be reported for other blinding diseases that are currently untreatable.

Acknowledgments

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Viral Vector Gene Delivery to the Brain to Treat the Disseminated Lesions of Neurogenetic Diseases: Focus on Lysosomal Storage Diseases

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Introduction

Neurogenetic diseases have a widely distributed pathology in the brain because most defective genes are involved in metabolic pathways (the inborn errors of metabolism) and consequently affect cells throughout the CNS. Gene transfer can correct biochemical defects within a diseased cell, but delivering a gene to a whole brain is a significant challenge, especially in humans, whose brains are 2000–3000 times larger than a mouse brain.

Certain viral vectors can be transported along neural pathways to distal sites. Combining virus properties with selected neuronal pathways can thus form a strategy for delivering a therapeutic gene over a large volume of brain from localized injection sites. Several strategies have attempted to deliver genes across the blood–brain barrier, as the vascular bed is distributed uniformly throughout the brain. Some vectors have shown promise for intravascular gene delivery, which would obviate the need for surgery. In addition to vector properties, some proteins' properties allow for wider deliver of the therapy, e.g., the secretion uptake mechanism of many lysosomal enzymes, which are responsible for a large number of the neurogenetic disorders. Recent advances in animal experiments suggest it may be possible to treat a large portion of the human brain using a clinically feasible number and volume of injections.

Brain Size and Structure

The human brain is ~800 g at 1 year of age (when many human genetic-disease patients are likely to be treated) and grows to ~1200 g in adults. In contrast, the mouse brain is only ~400 mg in the adult: 2000–3000 times smaller. Although rat brains are about 5 times larger (2 g), there are very few human neurogenetic disease models in the rat. The brains of domestic mammals are much larger than rodent brains, and a large number of animals with human genetic diseases have been identified, mainly in cats and dogs, many of which involve the CNS. Their brains are approximately 100–200 times larger than a mouse brain, and they have a gyrencephalic structure (cortical folds with gyri and sulci), compared to the lyssencephalic rodent brain. The large animals model the obstacles to global gene and/or protein delivery in humans better than mice.

Animal Models of Human Neurogenetic Diseases

Testing gene therapy methods in valid animal disease models is valuable for designing successful and predictable approaches for human gene therapy. With human patients, it is not feasible to study the large numbers of individuals, at multiple time points, needed to compare various parameters of vector design, control of gene expression, and the fate of transduced cells. For these purposes, a large number of mouse models of human neurogenetic diseases have been identified or created. Many are good phenocopies of the human disorder, but they vary in fidelity from one gene to another. A large database on the genetics, biochemistry, pathology, and comparison of various treatment modalities now exists for these models, providing the basis for comparing and improving new methods of therapy.

Testing experimental therapies under actual disease conditions provides information on the responses of the affected organs that would not be apparent in normal animals. Genetic diseases typically result in lesions distributed throughout the brain, requiring global distribution of the gene vector or the therapeutic protein. The large animal brains provide a more accurate model of the conditions that are present in human diseases. The large animals also provide better models for testing noninvasive imaging modalities, such as magnetic resonance imaging or spectroscopy of disease processes, and can be performed using human clinical magnets and apparatus.

Another advantage of large animal models is that they are treated and evaluated as individual patients, so the range of success and failure can be assessed. At the same time, large statistically significant cohorts of affected animals and normal controls can be produced and evaluated. Variations in age at treatment and analysis, class of gene therapy vector, route of administration, and other experimental variables can be studied. In human genetic disease populations, by comparison, it would be virtually impossible to assemble the statistically significant cohorts or to treat and evaluate normal controls. In addition, human genetic disease populations are small but often harbor a variety of mutations, whereas genetically diseased animal breeding colonies typically contain a single mutation. These animal colonies provide a uniform genetic mutation that causes the disease, while the genetic background of large animals within a colony is relatively outbred compared with inbred strains of mice. Another

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advantage for translation to clinical trials is that the large animal species live much longer than rodents. This longevity allows studies on long-term effects of treatments, both for efficacy and adverse results.

Lysosomal Storage Diseases: Biochemical and Cell Biological Basis for Correction

The lysosomal storage diseases (LSDs) are caused by inherited deficiencies of lysosomal enzyme activity. More than 60 such human diseases have been identified. Although most are caused by mutations in the enzyme structural genes, some involve defects in modifying genes that are necessary for enzyme maturation or transport molecules. Most of the LSDs are characterized by severe, progressive degenerative syndromes with onset in early childhood that affect multiple organ systems.

Treatment strategies for these diseases are based on the observation that most lysosomal enzymes can be released from normal cells and taken up by mutant cells. The extracellular enzyme is endocytosed and is activated when the endosome fuses with the acidified lysosome. In the lysosome, it degrades the accumulated substrate to correct the metabolic deficiency (called cross-correction). Studies of corrected cell transplantation (*ex vivo* gene therapy) and direct viral gene transfer (*in vivo* gene therapy) in animal disease models have shown that sufficient levels of normal enzyme can be expressed in order to arrest or reverse some of the pathological effects of the diseases. Gene therapy for LSDs thus depends on correcting a portion of the deficient cells in the patient, which can then supply the missing enzyme to non-transduced cells. Although the effectiveness varies with the disease, treatment performed at a young age produces a better clinical outcome than in older animals. However, most patients with a LSD are not diagnosed until they begin to display significant pathology during early childhood, making it important to understand the effects of intervention after the disease is considerably advanced.

Treating the brain disease is critical in human patients with LSDs, most of which involve developmental disabilities in brain function. Although non-CNS pathology can be at least partially treated (using bone marrow transplants, enzyme replacement therapy, or gene therapy to provide soluble enzyme into the circulation), treating the CNS component is more difficult because the enzyme does not easily cross the blood–brain barrier.

Viral Vectors

Many animal models of LSDs have been treated using viral vector-mediated gene transfer strategies to deliver the normal enzyme. Similar distribution of the gene can be expected for most other genes as well, but efficacy will depend on the biochemistry and cell biology of the gene product. The most commonly used vector systems for *in vivo* transduction have all been studied in the CNS in animal models of LSD. Variable amounts and patterns of transduction can be achieved in the brain with lentivirus (LV), herpes simplex virus (HSV), adenovirus (AdV), or adeno-associated virus (AAV) vectors (Cearley and Wolfe, 2009). When animals are treated as neonates or fetuses, the progression of disease can be arrested before significant pathology occurs. However, initiating treatment in adult animals even when the pathology is advanced has shown that significant reversal of established lesions can be achieved; also, improvements in behavioral tests and neurodegeneration parameters can occur. Furthermore, studies have demonstrated that in some neural pathways, the enzyme can be transported to distal neuronal cell bodies, which can amplify the sphere of correction far beyond the injection site.

Lentivirus

Lentivirus (LV) vectors have been developed from human immunodeficiency viruses, simian immunodeficiency virus, feline immunodeficiency virus, and equine infectious anemia virus (Jakobsson and Lundberg, 2006). The major advantage of LVs for delivery to the CNS is that they can transduce post-mitotic cells such as neurons; thus, they can be injected directly into the brain. LV vectors become stably integrated into the host-cell genome and can mediate long-term expression of the transgene. To reduce the potential for activating a proto-oncogene, LV vectors are designed to be self-inactivating; in LVs, the long terminal repeat promoter and enhancer are deleted from the integrated provirus form. This design also eliminates competition with the internal promoter in the expression cassette.

LV vectors can transduce most cell types within the CNS, including neurons, astrocytes, oligodendrocytes, and stem cells. The host range can be expanded by pseudotyping LVs with surface glycoproteins from a number of other enveloped viruses, including vesicular stomatitis virus G, MuLV, Mokola, lymphocytic choriomeningitis virus, and rabies virus. Each pseudotyped vector transduces different subgroups of cells within regions of the CNS. The selectivity is mediated by variable expression

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of the cell-surface receptors to which the viral envelope protein binds. Thus, targeted transduction within the CNS can potentially be achieved using a combination of cell-tropism and cell-type-specific promoters. Some evidence also shows that LV vectors undergo retrograde transport, but this does not occur in all nervous system pathways.

Herpes simplex virus

HSV is the only naturally neurotropic virus used as a vector. HSV has a large double-stranded DNA genome with approximately 80 genes, but many of the genes are not required for replication (Mata et al., 2003; Berges et al., 2007). Thus, much of the genome can be deleted so that HSV vectors can potentially hold large inserts of foreign DNA. Human HSV-1 is the most commonly used herpes virus vector system for experiments involving the CNS, although porcine pseudo-rabies virus has been used for short-term tract-tracing studies. HSV-1 vectors establish an episomal latent infection in neurons; thus, insertional mutagenesis is not thought to occur. There are two types of HSV-1 vectors: recombinant replication-competent vectors and non-replicating amplicons that must be made in packaging cells. Both types can mediate gene delivery to CNS cells.

Recombinant HSV vectors

Recombinant HSV vectors consist of a wild-type HSV genome, selected for either loss of viral pathogenic effects or deletion of genes necessary for replication. Because HSV-1 is a naturally neurotropic

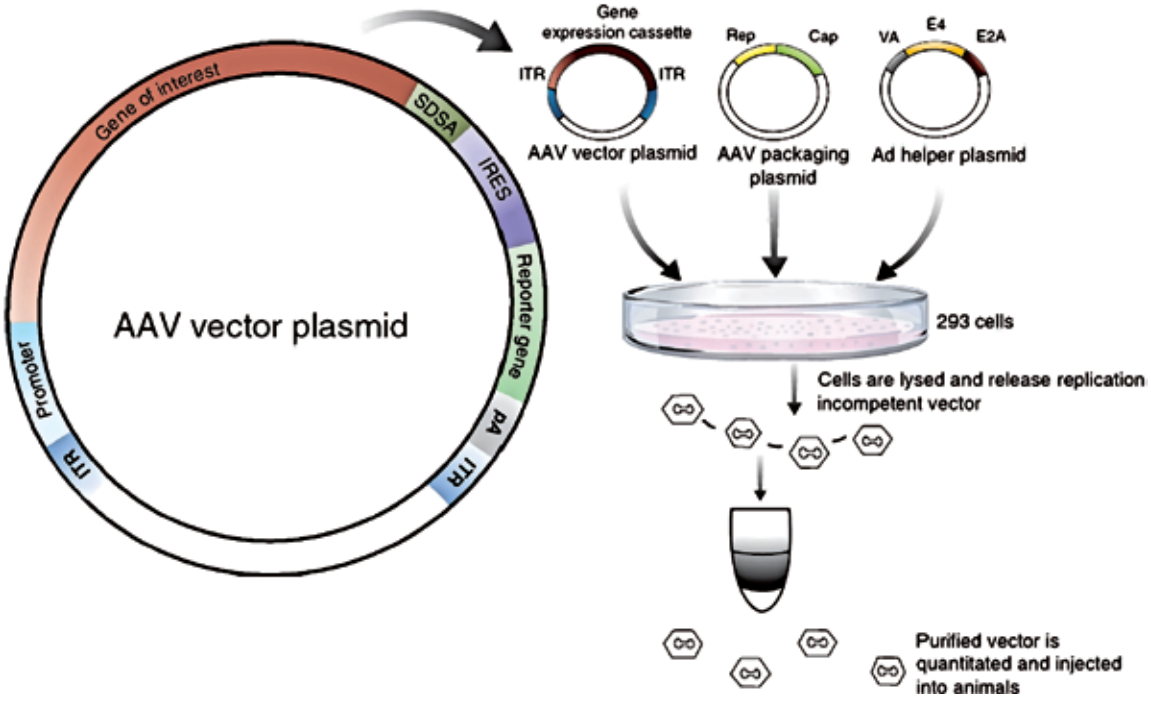

Figure 1. Example of viral vector design and production. To make an AAV vector, a 3-plasmid transfection system is used, consisting of the AAV vector plasmid, an AAV packaging plasmid, and an Ad helper plasmid. The AAV vector plasmid (enlarged view on the left) contains the gene of interest, flanked by the inverted terminal repeats (ITRs), which are the only elements from the wild-type AAV included in the plasmid. The AAV packaging plasmid contains the AAV genes necessary for genome replication (Rep) and capsid protein formation (Cap), but these are not flanked by ITRs and thus are not incorporated into the virion. AAV is a dependovirus, which requires functions supplied by a helper virus, e.g., AdV or HSV. For vector production, a helper plasmid encoding the minimum gene functions needed for replication is used. The 3 plasmids are transfected together into a packaging cell line, such as 293T cells, and normal cellular pathways are used to express the genes and proteins leading to the assembly of viral particles. The particles are released into the media after cell lysis or budding from the cell surface, depending on the type of virus. The vector particles are isolated from the lysate using cesium chloride gradient, pelleted by centrifugation, or column-purified. The vector viral particles are then quantified and can be used for experimental purposes. Production of most vector viruses is done using a similar strategy, with the functions supplied either in trans on plasmids, or by helper viruses, which vary depending on the virus type. IRES, internal ribosomal entry site; pA, poly-adenylation; SDSA, splice donor–splice acceptor.

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virus, only low titers are needed in order to spread the replicating virus in the CNS. Recombinant HSV vectors are thought to establish a persistent latent state as a nonintegrated nuclear element, similar to wild-type HSV. In natural infection, after latency is established, only one viral gene is expressed: the latency-associated transcript (LAT), which is expressed for the lifetime of animals. Thus, the promoter of the LAT sequence has been used to drive long-term expression of therapeutic genes in the CNS.

Nonreplicating HSV amplicon vectors

The HSV amplicon vector system is based on a eukaryotic expression plasmid that can be packaged (with the viral genes supplied in trans) or a helper virus-free method. HSV amplicons have a theoretical packaging capacity of up to 130 kb. Because the HSV amplicon vector can accommodate such a large insert, typical vectors are constructed with 13–15 kb foreign sequences, which are concatamerized in a single vector. This packaging results in an increased transgene dose per infected cell. A disadvantage of using the amplicon-based system is that these vectors result in relatively limited transgene expression, and expression in dividing cells is transient because vector DNA is lost during mitosis. For these reasons, hybrid vectors have been developed that combine components of the HSV-1 amplicon with genetic elements from other types of viral vectors. HSV–AAV hybrid vectors have been made that combine the benefits of both vector systems, resulting in a vector that can package very large inserts, target multiple cells types, and result in high expression levels for an extended period of time.

Adenovirus

Adenovirus (AdV) vectors are non-enveloped, with a double-stranded DNA genome. AdV vectors have both significant advantages and disadvantages for gene therapy experiments in the CNS (Davidson and Breakefield, 2003). Advantages include their simplicity to generate and ability to transduce a variety of cell types, both dividing and post-mitotic, including neurons, astrocytes, oligodendrocytes, and ependyma. Transduction using AdV vectors also results in high levels of transgene expression. Another potential advantage is that, in some cases, AdV vectors are transported along neuronal pathways.

The main disadvantage of using AdV vectors is that they are highly immunogenic, a quality that is the main contributor to loss of transgene expression after administration. AdV vectors target dendritic cells, which facilitates immune stimulation. Most humans carry circulating antibodies against adenoviruses as a result of natural infection, which is a potential barrier to clinical use. One approach to reduce this effect is to alter the capsid protein to resemble less immunogenic forms. Another is to use a "gutless vector," which has a genome deleted of all the Ad proteins, which are supplied in trans to form a virus. Gutless AdV vectors have mediated, stable, long-term expression of a transgene in the CNS and can accommodate an insert of approximately 30 kb, whereas replication-defective AdV vectors carry only approximately 8 kb.

Adeno-associated virus

AAV is a category of non-enveloped parvoviruses with a single-stranded DNA genome. AAVs are naturally replication-defective and can only replicate when co-injected with a helper virus (AdV or HSV). AAV vectors are made in a transfection cell system in which the replication and other functions are provided in trans (Mandel et al., 2006; Wu et al., 2006). This system results in the vector genome containing only about 300 nucleotides of viral sequence.

AAV vectors are one of the most widely used gene therapy vectors in CNS experiments. When directly injected into the brain, AAV vectors transduce mostly neurons, but some serotypes can also transduce other neural cell types. AAV vectors result in a delayed-onset but persistent transgene expression in the CNS. AAV vectors can be concentrated and purified to very high titers, resulting in widespread and stable transduction with low toxicity. The limitations of AAV vectors include their small cloning capacity (4.5 kb), which limits their use for large therapeutic genes or many genetic regulatory elements. AAV vectors are relatively inefficient for *in vitro* experiments compared with other vectors, but they can transduce a number of neural cell types. AAV vector production is also labor-intensive and time-consuming.

Numerous serotypes of AAV capsid genes are available (~100) and each is a sequence variant of the capsid protein (Cearley et al., 2008). Each serotype has been shown to have different transduction characteristics in the CNS. The AAV2 serotype vector has been widely applied in CNS applications, though it has relatively low transduction. The use of several other serotypes in the CNS is increasing, especially serotypes 1, 5, 9, and rh10, and many new variants have been tested. Different serotypes can produce different patterns of transduction when injected into

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specific regions of the CNS, and these characteristics can be used to selectively target certain cells. There is evidence that the AAV vector serotypes 1, 5, 9, rh10, and others are transported within the CNS along various neuronal pathways.

Combining Vector Properties with Routes of Delivery to Increase Enzyme Distribution

The properties of vectors and lysosomal enzymes have been combined in different ways to target larger volumes of brain tissue in mice. For example, AAV4 injected into the ventricles can transduce ependymal cells, and the secreted enzyme is distributed via the circulating CSF (Liu et al., 2005). When AAV1, 9, or rh10 is injected in a small volume into projection nuclei originating in the brainstem/pons region, it can be transported widely enough so the subsequent enzyme secretion from the dispersed gene expression sites is sufficient to treat the whole brain (Cearley and Wolfe, 2007). Other sites with widespread connections within the brain (including the hippocampus, striatum, and thalamus) have been used to disseminate the enzyme and/or gene (Cearley and Wolfe 2007; Baek et al., 2010). These studies have shown that when the correct vector and neuronal system are used, the volume of treated brain tissue can be significantly increased while reducing the number and volume of injections. Another promising approach is the recent finding that intravenous injection of AAV9 crosses the blood–brain barrier and can deliver therapeutic amounts of a lysosomal enzyme (Foust et al., 2009; Fu et al., 2011).

Human Trials

Phase 1 clinical trials have been performed with AAV in two neurogenetic diseases: Canavan disease and late infantile Batten disease. These trials were designed to assess safety of the reagents and procedures. Although there is little evidence that the diseases were improved, that is probably the result of the limited number of injections allowed and use of AAV2, which is the least effective AAV. Thus, the total amount of normal protein delivered was probably below the threshold needed for efficacy. This conclusion is supported by experiments using multiple injection sites spaced throughout the brain, which have been shown to be medically beneficial in large animal disease models such as cats with alpha-mannosidosis (Vite et al., 2005). Using more efficient serotypes and targeting the injections to the neural systems most likely to disseminate the gene and/or protein may allow effective scale-up to the human brain. Additional trials are planned in Batten's disease and other LSDs using AAV serotypes that have shown wider distribution in animal studies.

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Delivering Therapies to the Brain: A Brief Review of Current Strategies for Huntington's Disease

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Introduction

Most potentially neuroprotective therapies now being explored for Huntington's disease (HD) and other neurodegenerative disorders will need the assistance of a drug delivery system to breach the blood–brain barrier (BBB) and to reach their targets in the human brain. Outside of cancer therapeutics, however, no drug delivery system has been shown safe and effective for delivering chronic treatments to the brain. A successful therapy for HD will likely have to achieve three crucial milestones: (1) crossing the BBB into the brain, (2) reaching the specific cells to which it is directed, and (3) performing its ameliorative function there. Further, any therapeutic must have a safety profile that supports chronic dosing and demonstrate success in a relevant HD preclinical model.

Drug Delivery Routes

Direct delivery to the brain

In HD patients, mutant huntingtin (*HTT*) is present in every cell of the body. One key question, then, is which cells should be targeted for the best chance of eliciting a therapeutic effect. Most discussions so far have focused on the striatum, with the hope that reducing the levels of *HTT* mRNA will rescue striatal neurons and motor signs.

Isis Pharmaceuticals (Carlsbad, CA) is using intrathecally delivered antisense oligonucleotides (ASOs) to reduce *HTT* levels in the brain, while a collaboration between Alnylam Pharmaceuticals (Cambridge, MA) and Medtronic (Minneapolis, MN) is pursuing the same goal by using small interfering RNA (siRNA) delivered intraparenchymally (Smith et al., 2006; Akinc et al, 2010). A proprietary Medtronic infusion system uses an implantable, battery-powered drug-infusion pump to deliver siRNA to the striatum, using convection-enhanced delivery for a wider distribution of the siRNA (Dickinson et al., 2010; Sah and Aronin, 2011).

At this point, neither Isis's antisense oligonucleotides nor Alnylam's siRNA is specific to mutant *HTT*; in both cases, wild-type *HTT* mRNA is also reduced. Since *HTT* participates in many cellular processes, excessive loss of *HTT* may be toxic, thus raising concerns about the therapeutic window for such treatment.

Intranasal delivery

Delivering drugs via the nasal passages could provide a noninvasive way to bypass the BBB and avoid toxicity due to systemic administration (Dhuria et al., 2010). In the olfactory epithelium, primary sensory neurons regenerate every 30 days or so (a unique property among neurons), which means that tight junctions are lacking in areas of immature cells. Work in animal models and humans suggests that a variety of particles — e.g., small molecules, neurotrophins, chemotherapeutics, oligonucleotides, stem cells — could be delivered to the brain in this way.

The highest concentration of particles delivered through the nose ends up in the olfactory bulb, medulla, and brainstem (at the entry point of the trigeminal nerves); however, widespread delivery to the striatum and cortex also occurs. Leah Hanson and her colleagues have shown that cargo distribution appears similar among different types of molecules. The fraction of the total dose delivered to brain, however, is highly variable and amounts to only 2–4%.

In rodents, cargo appears in the brain within 5 to 10 min, peaking after 30 min after intranasal administration. How the drug is delivered (for example, where it is placed on the mucoepithelium, whether the animal is anaesthetized or not) can change that time course. Some molecules inhaled through the nose (like cocaine) can also first go to the blood and then cross over the BBB to the brain. Tracking the distribution with real-time imaging has been a challenge, however, because a big halo at the nose obscures the particles' trajectories.

The observed time course suggests that the particles do not travel by diffusion or active transport but by some unknown mechanism. Electron microscopy shows a lack of tight junctions between olfactory sensory neurons, suggesting particles might travel within bundles of axons through the cribiform plate and into the olfactory bulb. Within these bundles of axons, there are channels with evidence of ciliary movement. Once in the brain, a bulk flow mechanism, that is, motion created within the perivascular space by the pulsatile motion of blood flow through the brain, could explain the movement of particles so quickly.

Transient BBB opening

Proteins on the meningococcus bacterium interact with beta-2 adrenergic receptors to open the paracellular route, thus allowing *Neisseria meningitidis* to invade the meninges. Xavier Nassif and his colleagues are working to use this mechanism for paracellular transport across the BBB (Coureuil et al,

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2010). The mechanism leads to a transient opening of the BBB by making the tight junctions between cells temporarily leaky.

Targeting a drug to intracellular destinations in the brain would require an additional mechanism. Alternatively, the technique could be applied at a chosen spot in the BBB close to the intended destination. Disrupting the BBB with a pathogen would be a relatively invasive technique, having significant safety hurdles and implications. However, the use of bacterial fimbriae may serve the purpose in the absence of liver organisms.

Drug Delivery Vehicles

Adeno-associated virus

Viral vectors, especially those derived from adeno-associated virus (AAV) and herpes simplex virus (HSV), allow transgenes to access the intracellular machinery of transcription and translation. Although several virus types are known to cross the BBB, such as HIV and rabies virus, HSV and adenovirus require the targeting of a transcytosis mechanism for delivery across the BBB. AAV can cross the BBB of mice inefficiently; however, thus far, none of the AAV serotypes have been shown to traverse the BBB of primates to any useful extent.

AAV can deliver *HTT* siRNA to brain (Xia et al., 2004; Boudreau et al., 2009). Primate data on the persistence of AAV-delivered transgenes suggest that a single injection provides 10 years of expression, and AAV delivery has been extended to human trials, for example, of Parkinson's disease.

In a single injection to the striatum, AAV infects neurons at very high multiplicities, spreading to several million striatal neurons. In the absence of cell division, AAV fails to integrate into the host chromosome. AAV2 is the most widely used AAV serotype, but AAV5 or AAV9 may actually be better suited for delivery to brain neurons (Foust et al., 2009; Gray et al., 2011).

More research is needed to determine whether AAV-mediated gene therapy could be administered systemically. Systemic delivery would increase the chance of immune effects, which are less of a concern for local delivery. Up to 85% of the population has circulating antibodies to AAV2, which could compromise even initial systemic delivery.

Besides possible immunologic complications, dosing viral gene delivery will depend on the needed multiplicity of infection, available viral titers, and the strength of the promoter used. So far, virally delivered transgenes are constitutively active, so clinical studies would require careful examination of possible side effects and the development of an "exit strategy" in the event that something goes wrong. One possibility would be to use regulatable promoters, though in the past, the FDA has rejected this option out of concern that introducing extra proteins into the construct could itself compromise safety. Another issue, as in the case of direct delivery, is how to achieve a specified level of *HTT* knock-down that is not itself toxic.

Herpes simplex virus

HSV-derived vectors could provide either direct or systemic delivery. Joseph Glorioso has reported that HSV does not naturally cross the BBB, but such ability might be engineered by replacing its machinery for infecting cells with single-chain antibodies that bind to transcytosing receptors, such as transferrin. The modified virus could then be endocytosed into endothelial cells of the BBB and exocytosed to the brain's extracellular space. The choice of an appropriate transcytosing target, however, is challenging since the presence of the target's natural ligand (transferrin) would compete with viral uptake. Other options include the incorporation of other transcytosing viral glycoproteins into the HSV envelope in order to mediate delivery to the brain across the BBB. Such vectors would require detargeting the natural viral receptors to prevent infection of endothelial cells. The detargeted vectors could be supplied with new binding ligands (retargeting) that mediate viral infection of specific neuronal subpopulations in the brain.

HSV is a human virus that infects neurons efficiently and persistently, and it could allow the delivery of multiple therapeutic genes. HSV can express genes long term in neurons, and it can accommodate a DNA cargo up to 40 kb long. The virus does not integrate in the host genome but persists as an extra-chromosomal element in the nucleus. In its engineered vector form, its lytic functions are removed, and it expresses only the engineered gene.

HSV-mediated gene delivery is already in the clinic, with a Phase 2 trial for cancer pain and an early trial for brain cancer (Glorioso and Fink, 2009). In the pain trial, the treatment achieves efficacy with the delivery of a total of 10^8 virus particles. Dosing for HD would likely be different, probably requiring higher doses to breach the BBB. Once established in neurons, however, the vector is highly stable, and repeat dosing might not be needed.

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So far, neither animal experiments nor clinical trials have raised issues with immunogenicity. Most people carry antibodies for HSV, just as they do for AAV, but the dosing level used to date (10^7 to 10^9 /ml) in patients has not proven immunogenic. Nonetheless, long-term dosing will raise safety issues, including immunogenicity, long-term regulation of gene expression, and potential toxicity.

Nanoparticles

Nanoparticles have been used extensively for drug delivery, with some such therapies approved for cancer treatment. Nanoparticles have many functional groups and can conjugate many different molecules. Miqin Zhang's group is developing a 40-60 nm particle with an iron-oxide core coated with a natural polymer called chitosan, present in the exoskeleton of crustaceans (Veiseh et al., 2011). Chitosan is a transcytosing molecule that is able to cross the BBB, and the iron oxide allows the particle to be imaged. The particles are injected systemically and can cross the BBB and deliver drugs to tumors in the brain, with 6-8% of the molecules taken up by the brain. Synthetic nanoparticles might also deliver a gene or siRNA, so it would be possible to try to use the particle to silence *HTT*.

For antisense oligonucleotides and siRNAs, however, the question remains of how to move the nanoparticle cargo into the cytoplasm. One possibility is to use a cell-penetrating peptide, though that would mean limiting the amount of cargo. Another possibility is to make use of existing transporters, for example, the dopamine transporter, but some such strategies may be confounded by HD-associated decreases in transporter concentration.

An endogenous nanoparticle: high-density lipoprotein

Endogenous mechanisms can also carry molecules across the BBB. One such system depends on ApoA1, the major protein component of high-density lipoprotein (HDL). "Nascent HDL" is an ApoA1 molecule surrounding a phospholipid core ~12 nm in diameter. ApoA1 normally acts as a cholesterol acceptor, penetrating tissues and removing cholesterol from fats. In plasma, HDL already carries microRNA, so getting its core protein to carry siRNA might not require major feats of engineering. Also, the structure of ApoA1 is well known, so it's possible to modify it with a single-chain or monoclonal antibody to target it to specific cell types.

ApoA1 performs complex tasks difficult to recapitulate in a synthetic nanoparticle: produced in the liver, ApoA1 travels around the body, picks up its payload, and brings it back to the liver, taking on a variety of structures during its life cycle (Fan et al., 2009). Michael Oda's group has modified ApoA1's structure to deliver both large and small cargos through the pulmonary and transnasal pathways (Oda et al., 2006; Burgess et al., 2010). Some 90% of the cholesterol in circulation exchanges with molecules in the plasma, limiting the utility of this system for reliably delivering cargo. But a more stable form of HDL, further modified by adding polyethylene glycol (PEG), can deliver the highly toxic antifungal compound amphotericin B.

As in the case of synthetic nanoparticles, HDL-derived nanoparticles must not only traverse the BBB and get into brain cells, but also escape the endosomal compartment and deliver the ASO or siRNA to the cytoplasm. AlCana Technologies (Vancouver, British Columbia, Canada) has developed ApoE-dependent systems containing ionizable cationic lipids to deliver nucleic acids to specific cells. The flux of such complexes across the BBB is limiting, so it becomes important to choose especially potent molecules; small molecules, for example, may not be delivered in sufficient quantity to reach an effective concentration. Higher probability of success may require high-capacity transporters and receptors, to minimize potential interaction with the transport of endogenous substrates. Despite the complexity of these systems, the fact that components are naturally occurring reduces safety concerns.

Issues in Translation

CSF and drug delivery

Understanding the flow of CSF is important for predicting the distribution of therapeutic molecules in the brain. This is especially true for drugs delivered by direct administration to the CSF, either intrathecally or intracerebrovascularly. It is also important for identifying biomarkers via CSF sampling and predicting how a drug, once in the CSF, is cleared.

According to the textbooks, cells of the choroid plexus, which line the brain vesicles, secrete CSF into the vesicles; CSF then flows unidirectionally from the ventricles to the cisterna magna, with an unidentified quantity flowing down the spinal column. According to Marijan Klarica, however, this view is incorrect (Vladić et al., 2009; Bulat and Klarica, 2011). Instead, there is no net formation of CSF within the brain

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ventricles; rather, an exchange takes place between CSF and interstitial fluid. Altering the position of the cranium does not change the volume of intracranial fluid, and pressure in the cisterna magna (in head-up position) is normally about zero. Because the volume of the cranium is fixed, the enlargement of the large intracranial blood vessels during systole forces CSF from the ventricles and cortical subarachnoid space into the subarachnoid space of the spinal cord. During diastole, the flow is reversed, so there is continual craniospinal mixing of ventricular, cisternal, and spinal CSF.

Large macromolecules such as proteins are removed from the CSF quite slowly, so they distribute throughout the CSF over time. Klarica's work on distribution dynamics suggests that the concentration of such long-residence-time molecules after 24 h is highest in the lumbar region. Sampling by lumbar puncture should therefore provide a surprisingly good representation of the contents of the CSF. The active mixing of CSF also provides a rationale for intrathecal delivery of brain-directed drugs, as in the case of Isis Pharmaceuticals' ASOs.

Immunogenicity and hypersensitivity

One major concern when taking molecules from the preclinical to the clinical stage is the possibility that they can provoke an innate or acquired immune response in humans. The FDA will probably ask for extensive data to show that a molecule is not immunogenic—not just in rodents, but also in large animals such as primates or dogs.

Immune responses do occur, a concern in all the modalities discussed. A possible exception is the use of nanoparticles to deliver small molecules, though some nanoparticles do cause hypersensitivity reactions in some human research participants. One way researchers have tried to control immune responses to nanoparticles is to treat research participants with steroids and antihistamines. Other approaches are to induce immune tolerance to the carrier particle in advance of treatment and to exclude potentially hyperresponsive patients in advance. No one yet knows whether any of these approaches will eliminate the problem: it may be necessary to accept that 10% of people will not be able to receive a second dose.

Pharmacokinetics and pharmacodynamics

Many factors contribute to whether a drug and its delivery vehicle will perform well. Because the design process contains so much trial and error, some CHDI Foundation Workshop participants have suggested that characterizing a molecule's distribution should await a demonstration of some efficacy in an animal model within a reasonable therapeutic safety window. The importance of measuring drug levels (pharmacokinetics) and the engagement of the potential drug with its presumed target (pharmacodynamics) should be underscored. Without such information, no one would be able to say why a particular molecule might or might not have worked.

Overall Strategy

A drug that shows disease modification in any neurodegenerative disease would help the field, blazing the trail for others. Some have suggested a stepwise approach: start with naked siRNA delivered through a pump to see if it reduces levels of mutant *HTT* in the brain; then find a readout that indicates a desirable change; next deliver the same molecule with a viral vector; and, if successful, move to systemic delivery, perhaps in a viral vector or nanoparticle.

There is general agreement with this staged approach and with the idea that direct intracranial delivery of *HTT*-silencing siRNA offers the current best therapeutic potential, but opinions diverge on the best way to move forward. Alex Kiselyov (CHDI, Los Angeles CA) has suggested the possibility of coadministering an agent with a treatment that opens the BBB. Overall, the global strategy should be using everything that is approved for chronic use in humans.

Pieter Gaillard has noted the parallel between the development of neuroprotective therapeutics and the more mature indication of lysosomal storage diseases (LSDs) (Van Weperen and Gaillard, 2010). To date, the only true disease-modifying approach for LSDs has been obtained in patients using intrathecal infusion (as well as direct intraventricular administration, which is more invasive) of the therapeutic enzymes, and with BBB-penetrant small molecules (substrate reduction therapies). All other approaches (e.g., local or global gene delivery; functionalizing enzymes to target the brain, either specifically targeted or generally by cell-penetrating peptides; nanoparticles; and liposomes) have thus far failed to change clinical practice. Comparing lessons learned there could provide guidance for the HD field.

Many knowledge gaps stand in the way of designing an effective BBB-crossing delivery vehicle and therapeutic for HD and other neurodegenerative

disorders. Do the properties of the BBB change with disease onset and severity? Does brain metabolism, required to clear the nontherapeutic components from the body, differ in individuals with the disease? What brain region does the disease affect first? Should a treatment aim to reverse or arrest the disease after symptoms have already appeared, or is it better to treat before the disease has manifested? Will treating one area of the brain be enough to achieve a therapeutic effect? An additional and crucial issue is the current dearth of biomarkers, both of disease progression and of the engagement of HD-relevant targets.

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Gene Therapy for Motor Neuron Disease

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Introduction

Gene therapy continues to be a potential option for treating amyotrophic lateral sclerosis (ALS), a fatal adult motor neuron disease (MND) with no cure. The only U.S. Food and Drug Administration (FDA)–approved drug for ALS, riluzole, offers a modest improvement, prolonging survival by a maximum of three to five months (Shaw and Ince, 1997). A variety of gene therapy approaches are available to justify clinical intervention in this rare condition, whether preventing degeneration by protecting motor neurons (MNs) from external insults or by silencing the genetic mutations that cause some familial forms of the disease.

This chapter will inform the reader about promising therapeutic transgenes and proof-of-principle studies in transgenic rodent models of ALS. It will also discuss challenges regarding the disease targets and timing for therapeutic intervention. Finally, it will briefly review potential restorative approaches for ALS, as well as gene therapy for other MNDs.

Facts and Demographics of ALS

ALS, also known as Lou Gehrig's disease in the United States, is a fatal MND with adult onset and relatively short course, culminating in death within three to five years postdiagnosis. This neurodegenerative disease is characterized mainly by the progressive degeneration of upper and lower MNs in the spinal cord, brainstem, and motor cortex. As MNs degenerate, muscles lose strength, and voluntary movements are compromised. Death is usually caused by respiratory failure, when diaphragm and intercostal muscles become disabled (Vincent et al., 2008).

Although clinically indistinguishable, ALS can occur in one of two forms: a most common or sporadic (sALS) form, which affects approximately 90% of the patients; or a familial (fALS) form linked to specific genetic mutations, which affects approximately 10% of ALS patients.

In the United States, the prevalence of ALS is approximately 30,000, and the incidence is slightly greater (60%) in the male population. The disease generally occurs between the ages of 40 and 70 years.

Etiology and Pathogenesis of ALS

In addition to the identification of specific genetic mutations linked to the inherited familial form of ALS, complex and multifactorial processes are involved in the disease pathway.

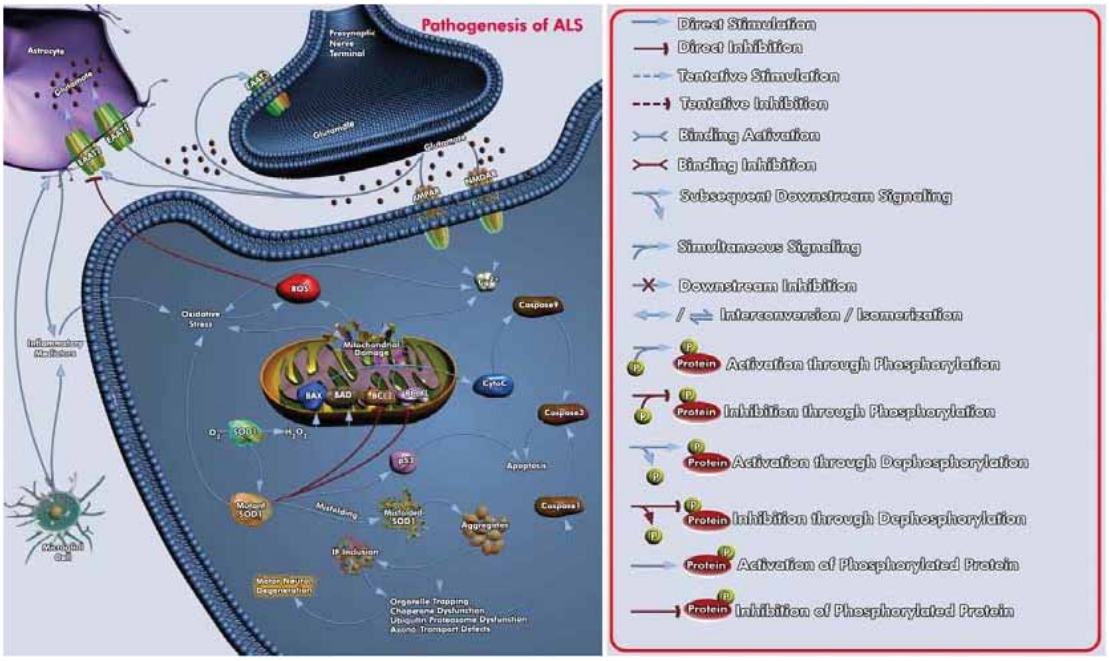

Figure 1. Pathogenesis of ALS. Multiple mechanisms are implicated in the pathogenesis of ALS, including excitotoxicity, oxidative stress, mitochondrial dysfunction, defective axonal transport, and abnormal protein aggregation. Reprinted with permission. Copyright ©2009 Qiagen. All rights reserved.

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Approximately 20% of the fALS cases are caused by an identified mutation in the Cu/Zn superoxide dismutase type-1 (*SOD1*) gene, whereas a mutation in the transactive response–DNA binding protein (*TARDBP*) gene has been recently linked to ~5% of fALS cases. More recently, mutations in other genes, including the angiogenin (*ANG*), vesicle-associated membrane protein–associated protein B (*VAPB*), and fusion in malignant liposarcoma/translocated in liposarcoma (*FUS/TLS*) genes, have been identified in patients with fALS (Millecamps et al., 2010; Traub et al., 2011).

Whether sporadic or caused by specific genetic mutations as listed above, the disease invariably has a common pathological feature: the selective death of MNs. Oxidative stress, neurofilament abnormalities, excitotocixity, apoptosis, mitochondrial dysfunction, defective axonal transport, mutations in RNA binding proteins, and inflammation are among the multiple factors playing a role in the pathogenesis of ALS (Fig. 1). We invite the reader to further explore the literature on these different pathogenic mechanisms by visiting timely reviews, such as those of Bruijn et al. (2004) and Rothstein (2009).

Possible Therapeutic Targets

In ALS, simultaneous treatment of the spinal cord (i.e., MN cell bodies and/or glial cells) and skeletal muscle (i.e., neuromuscular junctions [NMJs]) might be necessary to fully cover the pathways involved in MN degeneration.

Motor neurons

Although MNs are known predominantly as the primary cell type implicated in the disease, increasing evidence indicates that they are perhaps not the sole target for therapeutic intervention in ALS. Gene therapy strategies for ALS had once focused mainly on treating MNs. However, defining a specific therapeutic target for ALS remains a challenge. Despite the selective vulnerability of MNs in ALS, astrocytes can play a modulatory yet detrimental role in the disease process by triggering apoptotic and inflammatory mechanisms, thereby contributing to MN death (Barbeito et al., 2004). Moreover, reduced levels of glutamate transporters in astrocytes may cause impaired glutamate uptake and the consequent excitotoxicity occurring in ALS. Nonetheless, halting MN degeneration is the ultimate goal of any therapeutic strategy for ALS.

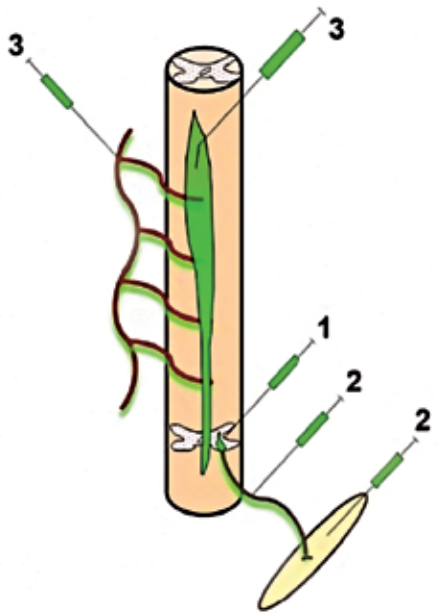

Figure 2. Methods for motor neuron gene delivery. Therapeutic transgenes can be delivered to spinal motor neurons (1) by direct injection; (2) by peripheral gene delivery, using the intramuscular or intraneural routes of administration; or (3), more recently, by systemic gene delivery via intramuscular or intrathecal administration.

Astrocytes

Downregulation of the excitatory amino acid transporter 2 (EAAT2), expressed mainly in astrocytes, has been suggested as a cause of MN excitotoxicity (Howland et al., 2002). In fact, cells engineered to overexpress EAAT2 can dramatically increase glutamate uptake and confer neuroprotection on motor neurons in coculture systems *in vitro* (Wisman et al., 2003). Increased expression of EAAT2 in *SOD1* mice can delay the loss of MNs in these double transgenic mice (Guo et al., 2003); conversely, a reduced amount of this receptor in *SOD1* mice caused them to exhibit earlier MN loss (Pardo et al., 2006). In conclusion, increasing the expression of glutamate receptors in glial cells could be beneficial for the treatment of ALS.

Neuromuscular junctions

Because end-plate denervation is one of the initial events in ALS (Fischer et al., 2004), targeting NMJs at early stages can be critical to preserving MN connections (Dupuis and Loeffler, 2009; Dupuis and Echaniz-Laguna, 2010). In newborn *SOD1* mice, intramuscular injection of an adeno-associated viral vector encoding cardiotrophin-1 delayed

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neuromuscular degeneration (Bordet et al., 2001). Similarly, in *SOD1* rats, *ex vivo* gene delivery of glial cell line–derived neurotrophic factor (GDNF) within muscles significantly increased the number of neuromuscular connections and, consequently, MN cell bodies during the midstages of the disease (Suzuki et al., 2008). On the other hand, a recent study has demonstrated that bodywide intramuscular injections of adeno-associated virus 6 (AAV6)–expressing small hairpin RNAs (shRNAs) against *SOD1* into newborn mice halted muscle atrophy but failed to stop disease progression (Towne et al., 2011).

Lessons from Transgenic Models of ALS

Rodent models carrying mutated forms of the human *SOD1* gene develop an MND that closely replicates the human disease. Such models have been widely used to help elucidating the disease mechanisms as well as to assess the efficacy of a variety of therapeutic strategies for ALS, including gene therapy (Gurney et al., 1994).

Numerous studies have reported promising results in *SOD1* rodent models, prolonging survival of the animals and preventing MN loss. Even so, the therapeutic relevance of animal models remains questionable because the vast majority of interventions occur in asymptomatic animals. In medical practice, ALS patients are diagnosed as the symptoms manifest themselves, most commonly reported as muscle weakness, which indicates distal axonal degeneration.

Gene delivery to MNs: routes of administration

When designing a therapy for ALS, the degree of success directly correlates with how adequately MN pools are targeted across the spinal cord (Fig. 2). Moreover, the biodistribution of the therapeutic transgene can determine the extent of a treatment effect. In reality, efficacious and safe gene delivery to spinal MNs remains a challenge for successful gene therapy in ALS, a disease process that ideally requires diffuse gene delivery throughout the cord.

Different routes of viral vector administration for MN gene delivery have been evaluated over the years. Noninvasive approaches via peripheral intramuscular or intraneural administration, which yielded promising results in mice (Kaspar et al., 2003), have failed scale-up validation in larger species owing to inefficient vector transport and negligible amounts of gene expression in the spinal cord (T. Federici and N. Boulis, unpublished observations). Robust gene expression following intramuscularly injected AAV6 was recently described in monkeys (Towne et al., 2009). Nonetheless, the feasibility of such an approach for treating ALS in humans remains questionable in a disease process with distal axonopathy. Alternatively, intraspinal injections have been investigated as a more direct means of achieving gene delivery in the spinal cord. Although promising, with positive outcomes in wild-type and *SOD1* animals (Azzouz et al., 2000; Franz et al., 2009; Lepore et al., 2007), such an approach has yet to be validated for clinical translation. Our group is currently performing a Phase 1 clinical trial for intraspinal cellular delivery in ALS patients, tempering the safety concerns that have hampered the translation of invasive surgery for therapeutic delivery (Lunn et al., 2011). Preclinical assessment of instraspinal gene delivery in large animals is still necessary in order to validate scalability and assess biodistribution with this type of approach (Federici et al., 2009). Intramuscular, intraneural, and intraspinal injections cannot target the entire spinal cord and are considered segmental approaches for gene delivery.

Current research in gene therapy has focused on AAV9, an AAV vector that is capable of crossing the blood–brain barrier following intravenous or intrathecal administration with age-dependent and promoter-dependent but preferential transduction of MNs (Duque et al., 2009; Foust et al., 2009; Snyder et al., 2011). Moreover, our group and others have recently demonstrated MN transduction following systemic delivery of AAV9 in large animals (Duque et al., 2009; Federici et al., 2011; Foust et al., 2011; Gray et al., 2011). These results confirm the highly translationable profile of this combination of vector and noninvasive approaches for diffuse gene delivery.

Neuroprotection

Numerous studies have demonstrated that protecting dying MNs can prolong survival in rodent models of ALS. Despite producing only a modest effect, such an approach has become the basic premise for ALS treatment. However, attempts to systemically deliver recombinant trophic factors such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), or human insulin-like growth factor-1 (IGF-1) have shown no benefit to ALS patients (Federici and Boulis, 2006; Sorenson et al., 2008). These trophic factors' inadequate or insufficient delivery and systemic inactivation/short half-life have been suggested as potential explanations for the disappointing results in humans. In contrast,

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gene-based delivery of neurotrophic factors may be a more effective alternative, as will be discussed in this section.

Of relevance to ALS, the safety and therapeutic potential of gene-based delivery of neurotrophic factors are being evaluated in clinical trials for Parkinson's and Alzheimer's diseases (Tuszynski et al., 2005; Marks et al., 2008; Snyder et al., 2010). The rationale for gene delivery of neurotrophic factors for ALS comes from animal proof-of-principle data demonstrating that secreted neurotrophic factors can support MN survival in a diseased milieu and thereby prevent progressive degeneration. Numerous successful demonstrations of MN protection have been reported following viral vector–mediated delivery of various growth factors, most notably GDNF, IGF-1, and vascular endothelial growth factor (VEGF), in transgenic rodent models of fALS. In these models, animals overexpress the human gene coding for the mutated forms of *SOD1*, developing a disease with very similar characteristics to ALS.

Neurotrophic-based gene therapy has been able to delay disease onset and slow progression of the disease in *SOD1* mice and rats more or less effectively, depending on the delivery approach. By a mechanism involving retrograde axonal transport of the transgene, intramuscular injections have been widely used to deliver neurotrophic-based viral vectors. Intramuscular delivery of AAV2-IGF-1 prolonged survival and delayed disease progression in *SOD1* mice (Kaspar et al., 2003). Similarly, intramuscular injection of an equine infectious anemia virus (EIAV)–based lentiviral vector expressing VEGF resulted in prolonged survival in the same mouse model (Azzouz et al., 2004a) (Fig. 3). Our group demonstrated segmental neuroprotection but no effect on survival following intraspinal delivery of AAV2-IGF-1 in *SOD1* rats (Franz et al., 2009).

How robust such effects need to be in order to take them to the level of preclinical development, compared with riluzole, which offers only a marginal effect (Gurney et al., 1996; Scott et al., 2008), remains questionable. The market opportunity for therapeutic development exists, and the ALS patient population urges for more effective treatments. Nonetheless, as previously discussed, devising scaling-up delivery strategies from rodents to large animals remains one of the main hurdles that limits the translation of spinal cord gene therapy. To date, MoNuDin (an EIAV-based lentiviral vector system for the delivery of VEGF) is the only gene therapy technology in preclinical development for the treatment of ALS.

As one of the mechanisms implicated in the pathogenesis of ALS, apoptosis has been targeted as a means of preventing neuronal cell death. Gene delivery of Bcl-xL and Bcl-2 — molecules with known anti-apoptotic activity — yielded MN protection *in vitro* and in *SOD1* mice (Azzouz et al., 2000; Yamashita et al., 2002; Garrity-Moses et al., 2005). However, despite the promise of some proof-of-principle studies, the state-of-the-art literature does not indicate that this strategy is advancing gene therapy.

Gene silencing

Because *SOD1* fALS arises through a toxic gain of function, RNA interference (RNAi) has been proposed as a means to knock down mutant *SOD1*. While proof-of-principle research has provided substantial evidence of successful selective silencing of the *SOD1* mutant allele, attempts to elucidate the mechanisms of ALS or to distinguish among the roles that different cell types play in disease pathogenesis by selectively knocking down mutant *SOD1* in astrocytes, MNs, or muscle cells have been somewhat disappointing. Moreover, while viral vector–mediated *SOD1* gene silencing significantly increased the lifespan of *SOD1* mice (Ralph et al., 2005; Raoul et al., 2005), systemic delivery has been proven insufficient for preventing disease progression (Towne et al., 2008). Recently, intramuscular delivery of AAV6.shRNAs.*SOD1* in newborn mice has also failed to stop disease progression (Towne et al., 2011). Nonetheless, Isis Pharmaceuticals (Carlsbad, CA) has initiated a Phase 1 study to assess the safety of ISIS-SOD1Rx, an antisense oligonucleotide–based drug that inhibits *SOD1* production (clinical trial identifier NCT01041222).

Gene therapy for spinal muscular atrophy

Spinal muscular atrophy (SMA, broadly considered the pediatric version of MND) has defined mutations in the survival motor neuron gene 1 (*SMN1*); therefore, SMA is a desirable disease target amenable to gene therapy. Even though humans have a nearly identical gene, *SMN2*, the protein is less stable and truncated owing to an alternative splicing and, therefore, does not compensate for the absence of *SMN1*. SMA is classified into three different forms, and the presence of variable levels of full-length *SMN* determines the severity of disease.

As in ALS, SMA patients have selective loss of lower MNs, and gene therapy neuroprotection strategies have been equally proposed for SMA (Lesbordes et al., 2003; Federici and Boulis, 2006). In addition, viral

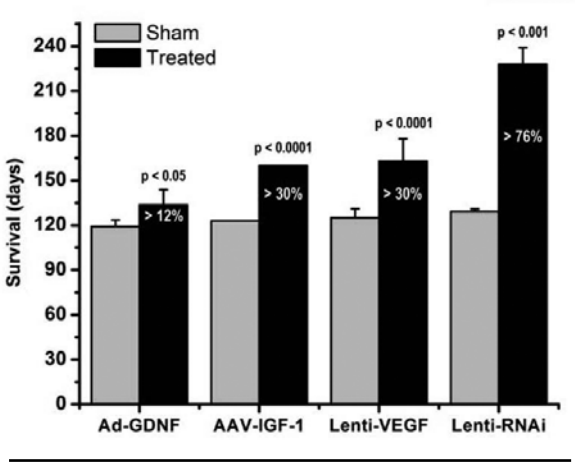

Figure 3. Survival effect of gene therapy in *SOD1* mice. Kaplan–Meier graph demonstrates increased lifespan of *SOD1* mice after intramuscular injection of adenovirus vector (Ad)–GDNF (Acsadi et al., 2002), AAV–IGF-1 (Kaspar et al., 2003), EIAV–VEGF (Azzouz et al., 2004a), or EIAV-mediating expression of RNAi targeted to the human *SOD1* gene (Ralph et al., 2005). Untreated *SOD1* mice survive an average of 135 days (black line). Federici and Boulis (2006), their figure, reprinted with permission.

vector–mediated *SMN* gene replacement has been tested in animal models of SMA, with variability of efficacy depending on time of intervention and biodistribution of the therapeutic transgene. For example, despite successfully restoring *SMN* protein levels following intramuscular delivery of a lentiviral vector expressing the human *SMN* gene, only marginal efficacy in survival was observed (Azzouz et al., 2004b). More recently, several groups have reported prolonged survival in SMA mouse models following (systemic) intravenous delivery of AAV9.*SMN* (Foust et al., 2010; Valori et al., 2010; Dominguez et al., 2011). Finally, a different approach, based on the delivery of translational read-through compounds, has been described as capable of reducing disease severity in SMA mice by producing a more stable isoform of the truncated protein (Mattis et al., 2008).

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Gene Transfer to the Peripheral Nervous System: Treatments for Polyneuropathy and for Pain

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Introduction

Gene transfer to the peripheral nervous system poses special challenges. The target cells—sensory neurons with cell bodies located in the dorsal root ganglia—are widely distributed and relatively inaccessible. In addition, the pseudo-unipolar axons projecting peripherally to the target organ and centrally to the spinal cord are large in comparison with the size of the cell body.

The two categories of disease processes for which gene transfer to the peripheral nervous system would be desirable are sensory polyneuropathy and chronic pain. Polyneuropathy refers broadly to a family of conditions in which peripheral sensory axons degenerate, often in a length-dependent fashion. With the exception of immune-mediated neuropathies that can be treated by immunomodulation, there are no available treatments to effectively prevent the progression of neuropathy resulting from systemic illness (e.g., that caused by diabetes), toxic exposure (e.g., chemotherapy-induced peripheral neuropathy), or genetic defect (e.g., Charcot-Marie-Tooth disease).

Challenges in the Development of Treatments for Pain and Polyneuropathy

Pain is a complex experience comprising sensory discriminative, cognitive, and emotional components. Acute pain is initiated by the activation of a subset of sensory afferents (nociceptors). These nociceptors transmit nociceptive information centrally through a well-characterized ascending pathway that serves to warn the individual of potentially harmful stimuli in the environment, often leading to a reflex withdrawal response. Chronic pain that results from continued activation of nociceptors, or from damage to the neural structures serving pain perception, is equally unpleasant but characteristically results in reduced activity and avoidance of contact. Although the dorsal root ganglia (DRG) are neither necessary nor sufficient for the perception of chronic pain, most of the common forms of chronic pain proceed through the same anatomical pathways as those utilized for acute pain and involve first-order synapses in the dorsal horn of the spinal cord.

For the treatment of polyneuropathy, extensive preclinical animal studies beginning in the 1990s demonstrated that neurotrophic factors delivered by intraperitoneal injection could effectively prevent the progression of polyneuropathy resulting from any of a number of causes, including diabetes, toxic exposures, or genetic defect. Subsequent work extended the range of factors from the classical neurotrophins (e.g., nerve growth factor [NGF], neurotrophin-3) to other peptides with neurotrophic effects, including insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and erythropoietin (EPO). However, despite the abundant evidence that systemically administering these factors is effective in preventing the progression of neuropathy in animal models, clinical trials have failed to demonstrate a therapeutic effect in patients. Although there are many possible explanations for these discordant results, one obvious problem is that the dose of peptide factor utilized in the animal studies (typically in the range of 5–10 mg/kg) was much higher than the doses tolerated by patients. For example, in the Phase 3 clinical trial of NGF for diabetic neuropathy, a dose of 0.1 μg/kg proved ineffective in preventing the progression of neuropathy in these patients.

A conceptually similar difficulty confronts the treatment of chronic pain. Nociceptive neurotransmission at the first synapse in the dorsal horn between the primary nociceptor and second-order neurons projecting rostrally is subject to complex modulatory influence. This influence is mediated by inhibitory neurotransmitters released from interneurons under the control of descending inputs. Pharmacological activation of these inhibitory neurotransmitter receptors (predominantly but not limited to opioid and GABA), either presynaptically on primary afferents or postsynaptically on second-order neurons, represents one effective means of modulating chronic pain. However, the same receptors are widely distributed throughout the central neuraxis and, in the case of opioids, on nonneural structures as well. Therefore, off-target effects unrelated to analgesia that are elicited by systemic administration of opiates or GABA-active drugs limit their use for pain relief. Alternatively, targeting delivery of these drugs to the spinal level by intrathecal administration allows one to increase the effective dose 10-fold.

Gene transfer offers the possibility of a highly selective, targeted release of bioactive molecules within the nervous system. This method is able to target delivery of neurotrophic factors to the primary sensory afferent for treating polyneuropathy, or of inhibitory neurotransmitters for relieving chronic pain. Through autocrine and paracrine effects, the release of neurotrophic factors from transduced primary sensory afferents could protect sensory neurons from degeneration without requiring high-

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dose systemic delivery. Similarly, the release of inhibitory neurotransmitters from primary sensory afferent terminals in the dorsal horn could provide an analgesic effect without engendering side effects by activating these receptors in other sites within the nervous system or other tissues.

Herpes Simplex Virus as a Gene Transfer Vehicle

Among the available gene transfer vectors, herpes simplex virus (HSV) is particularly well suited for the delivery of genes to the DRG. HSV possesses a natural tropism for peripheral sensory neurons of DRG, where the virus establishes a latent state in which viral genomes persist for the life of the host as intranuclear episomal elements. The lifelong persistence of latent genomes in trigeminal ganglion, without the development of sensory loss or histologic damage to the ganglion, attests to the effectiveness of these natural latency mechanisms. Wild-type virus may be reactivated from latency under the influence of a variety of stresses. However, recombinant vectors that are entirely replication-defective retain the ability to establish persistent quiescent genomes in neurons but are unable to replicate (or reactivate) in the nervous system.

Anatomy of HSV and the latent state

The HSV particle consists of a nucleocapsid surrounded by an envelope containing glycoproteins essential for virus attachment and penetration into cells. The HSV genome contains 152 kb of linear, double-stranded DNA encoding more than 80 gene products and consisting of two segments: a unique long (U_L) and unique short (U_S) segment, each of which is flanked by inverted repeats containing important immediate-early (IE) and latency genes. The viral genes are found almost entirely as contiguous transcribable units, making their genetic manipulation relatively straightforward.

In wild-type infection, the virus is transmitted by direct contact, replicating initially in epithelial cells of skin or mucous membranes. Second-generation virions are taken up by sensory nerve terminals and carried by retrograde axonal transport to the neuronal perikaryon in DRG, where viral DNA is injected through a modified capsid penton into the nucleus. In the lytic replication cycle, expression of the viral IE genes (which occurs in the absence of *de novo* protein synthesis) serves to transactivate expression of early (E) genes. Removing essential IE genes from the HSV genome results in the creation of vectors that are unable to enter the lytic cycle in noncomplementing cells but are nonetheless transported in a normal fashion to the nucleus, where they establish a persistent latent state (Krisky et al., 1998; Wolfe et al., 1999; Fink et al., 2000).

The latent state occurs naturally only in neurons. In this state, following injection of the viral genome into the nucleus, expression of the gene products characteristic of lytic infection is repressed, and the viral genome persists as an intranuclear episomal element. Latent genomes continue to transcribe only one segment of the inverted repeat sequences in UL, just downstream of, and from the strand opposite, the IE *ICP0* gene to produce a family of latency-associated transcripts (LATs). Latent genomes are partially methylated and sequestered as an episomal minichromosome-like structure bound by nucleosomes; in this state, they have no discernible effect on host-cell metabolism or phenotype. Nonreplicating vectors constructed by deleting essential IE genes are forced into a pseudolatent state.

Preclinical studies of HSV-mediated gene transfer in models of polyneuropathy

We have tested nonreplicating HSV vectors in several models of neuropathy. In selective large myelinated fiber degeneration caused by high-dose pyridoxine (PDX), subcutaneous inoculation of a nonreplicating HSV vector coding for neurotrophin-3 (NT-3) resulted in the preservation of sensory nerve amplitude, sensory nerve conduction velocity, and amplitude of the H-wave. Further, it protected large myelinated fiber proprioceptive sensory function and preserved large myelinated fibers in nerve and in the dorsal horn of spinal cord (Chattopadhyay et al., 2002). Similar results were obtained by injecting a nonreplicating HSV vector expressing NGF. In a model of toxin-induced neuropathy caused by cisplatin, subcutaneous inoculation of HSV vectors constructed to express either NGF or NT-3 took place just before a 6-week course of cisplatin. The treatment resulted in significant protection against the development of neuropathy, as assessed by electrophysiological, behavioral, and morphological outcomes (Chattopadhyay et al., 2004).

A model of type 1 diabetes in Swiss Webster mice was created by injecting the animals with streptozotocin (STZ). The subcutaneous inoculation of a nonreplicating HSV vector expressing either NGF (Goss et al., 2002a), VEGF (Chattopadhyay et al., 2005a), or EPO (Chattopadhyay et al., 2009) into both hind feet 2 weeks after inducing diabetes

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prevented the loss of sensory nerve action potential amplitude characteristic of neuropathy. Results were measured 4 and 8 weeks after the injection of STZ.

In these initial studies, we employed the human cytomegalovirus immediate early promoter (HCMV IEp) to drive transgene expression and examined the biological effect of vector-mediated transgene expression up to 2 months after inoculation. To achieve prolonged transgene expression, we employed the HSV latency-associated promoter 2 (LAP2) element (nucleotides 118866–119461 of the HSV genome). LAP2 is the sequence responsible for lifelong expression of latency-associated transcripts in neurons infected with wild-type virus. Using a vector with the LAP2 promoter driving expression of NT-3, we found that five and a half months after vector inoculation, animals inoculated with the LAP2-driven NT-3-expressing vector showed preservation of peripheral nerve function in the face of subacute PDX intoxication (Chattopadhyay et al., 2005b). Similarly, in the STZ diabetes model, mice inoculated with the NT-3 expressing vector were protected against the progression of diabetic neuropathy during the course of 6 months (Chattopadhyay et al., 2007).

Because prolonged expression of neurotrophic factors could have unwanted adverse effects, we constructed a nonreplicating HSV vector, vHrtEPO, to express EPO under the control of a tetracycline response element (TRE)–minimal CMV fusion promoter. Primary DRG neurons in culture infected with vHrtEPO express and release EPO in response to exposure to doxycycline (DOX). Animals infected with vHrtEPO by footpad inoculation demonstrated regulated expression of EPO in DRG under the control of DOX administered by gavage. Mice rendered diabetic by injection of STZ, inoculated with vHrtEPO, and treated with DOX 4 days out of 7 each week for 4 weeks were protected against the development of diabetic neuropathy, as assessed by electrophysiological and behavioral measures. These studies indicate that intermittent expression of EPO in DRG, achieved from a regulatable vector, is sufficient to protect against the progression of neuropathy in diabetic animals and provides proof-of-principle preclinical evidence for the development of such vectors for clinical trials.

Preclinical studies of HSV gene transfer for pain

The efficacy of HSV-mediated gene transfer of enkephalin has been tested in several different models of pain in rodents. Pohl and colleagues first showed that a *tk*-defective HSV recombinant, injected subcutaneously in the paw, will transduce DRG neurons that express enkephalin in DRG (Antunes Bras et al., 1998). Wilson and colleagues subsequently demonstrated that a similar tk^- HSV–based vector containing the human proenkephalin gene, and injected subcutaneously into the paw, reduces hyperalgesic C-fiber responses ipsilateral to the injection (Wilson et al., 1999). Pohl and colleagues went on to show that subcutaneous inoculation of the vector reduces pain-related behaviors in a rodent model of chronic pain related to polyarthritis induced by injection of complete Freund's adjuvant (CFA) (Braz et al., 2001). Expression of enkephalin from the vector not only reduced pain-related behaviors but also prevented cartilage and bone destruction in the inflamed joints, presumably owing to the release of enkephalin from the peripheral sensory terminals in the joint (Braz et al., 2001). These findings correlated with those demonstrating that axonal transport of the transgene product from transduced cells carries the transgene product toward the periphery (as well as toward the spinal cord), an effect that could be demonstrated by applying a ligature to the nerve (Antunes Bras et al., 2001).

Subcutaneous inoculation of an enkephalin-producing, nonreplicating vector produces an analgesic effect in the delayed phase of the formalin model of inflammatory pain (Goss et al., 2001) in two disease models: the selective spinal nerve ligation model of neuropathic pain (Hao et al., 2003) and the infraorbital nerve constriction model of craniofacial pain (Meunier et al., 2005).

In experiments designed to test the effect of the vector on visceral pain, investigators have injected the vector directly into the end organ rather than the skin. Yoshimura et al. (2001) demonstrated that injecting the nonreplicating enkephalin-expressing HSV vector into the rat bladder wall results in enkephalin expression in relevant DRG. They also demonstrated that vector-mediated enkephalin effectively attenuated capsaicin-induced bladder irritation and resultant bladder hyperactivity (Goins et al., 2001; Yoshimura et al., 2001). Similarly, Westlund and colleagues have shown, in rodent models of acute and chronic pancreatitis, that directly injecting an enkephalin-expressing HSV vector into the pancreas attenuates evoked nocisponsive behaviors (Lu et al., 2007; Yang et al., 2008). In the pancreas, enkephalin expression appeared to reduce the inflammatory response, analogous to the effect reported in polyarthritis (Braz et al., 2001). In a mouse model of bone cancer

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pain, subcutaneous inoculation of the HSV vector expressing enkephalin resulted in an attenuation of spontaneous nocisponsive behaviors (Goss et al., 2002b).

Studies of the enkephalin-expressing HSV vector have been extended to primates. Yeomans et al. (2006) demonstrated that peripheral application of the HSV vector expressing enkephalin to the dorsal surface of the foot of macaques reduced A-delta and C-fiber–mediated pain-related responses.

Taken together, these results from several different groups of investigators provide proof-of-principle that HSV vector–mediated delivery of enkephalin can provide an analgesic effect and set the stage for a human trial to treat chronic pain using HSV vector–expressed enkephalin (see below).

Our groups have examined HSV vectors constructed to express other inhibitory neurotransmitters. Endomorphin-2 (EM-2; Tyr-Pro-Phe-Phe-NH2) is an endogenous, highly selective μ-opioid receptor agonist (Zadina et al., 1997), but the gene coding for EM-2 has not yet been identified. We therefore constructed a tripartite synthetic gene cassette with the N-terminal signal sequence of human preproenkephalin (PENK), followed by a pair of EM-2 coding elements, including the addition of a C-terminal glycine residue flanked by dibasic cleavage sites. The gene product is processed by the cellular machinery that processes PENK to enkephalin (Wolfe et al., 2007), and the C-terminal glycine in the cleaved product directs amidation of the cleaved peptide by the widely distributed enzyme peptidylglycine α-amidating mono-oxygenase.

Our findings showed that subcutaneous inoculation of the endomorphin-expressing HSV vector into the footpad of rats with neuropathic pain (induced using selective L5 spinal nerve ligation) resulted in a significant reduction in both mechanical allodynia and thermal hyperalgesia that could be blocked by the highly selective μ-opioid receptor antagonist CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide) (Wolfe et al., 2007). It also resulted in a substantial reduction in nocisponsive behaviors in the delayed phase of the formalin test and in the CFA model of inflammatory pain (Hao et al., 2009).

Nocisponsive behaviors can also be attenuated by using HSV vector–mediated gene transfer to modulate the expression of μ-opioid receptors in primary sensory afferents. Consistent with a centrally mediated effect, cutaneous application of an HSV vector (defective in its expression of the viral *thymidine kinase* gene and with the human μ-opioid receptor cDNA in reverse orientation) results in decreased expression of μ-opioid receptors on the central primary sensory afferent terminals in the dorsal horn of the spinal cord and reduced potency of intrathecal [D-Ala^2,*N*-MePhe^4,Gly-ol^5] enkephalin (DAMGO) on C-fiber nociceptive responses (Jones et al., 2003). Conversely, cutaneous application of an HSV vector expressing the μ-opioid receptor gene in the sense orientation increases μ-opioid receptor immunoreactivity in primary sensory afferents and a leftward shift in the dose response to intraperitoneal lopiramide, indicating an effect at transgene-mediated μ-opioid receptors expressed on the peripheral terminals of the primary sensory neurons (Zhang et al., 2008).

We constructed a replication-incompetent HSV vector encoding the 67 kD isoform of human GAD (Liu et al., 2004). In the selective spinal nerve ligation model of neuropathic pain, inoculation of the GAD-expressing vector resulted in a substantial reduction in mechanical allodynia and thermal hyperalgesia (Hao et al., 2005). In neuropathic pain, the analgesic effect of the GAD-expressing vector is greater in magnitude than the effect produced by either the enkephalin- or endomorphin-expressing vectors. This finding is consistent with the evidence that development of chronic pain after peripheral nerve injury is accompanied by the loss of GABAergic tone in the dorsal horn of the spinal cord (Moore et al., 2002) and the clinical observation that opiate drugs are relatively ineffective in the treatment of neuropathic pain. The GAD-expressing HSV vector also reduces pain-related behaviors in a model of central neuropathic pain created by T13 spinal cord hemisection (Liu et al., 2004).

Phase 1 human trial of a preproenkephalin-expressing HSV vector

Based on the preclinical data, we proceeded to conduct a multicenter, dose-escalation Phase 1 clinical trial of NP2, a replication defective, HSV-based vector expressing human PENK, in subjects with intractable focal pain caused by cancer. NP2 was injected intradermally into the dermatome(s) corresponding to the radicular distribution of pain. The primary outcome was safety. A secondary endpoint, efficacy of pain relief, was assessed using a numeric rating scale (NRS), the Short Form McGill Pain Questionnaire (SF-MPQ), and concurrent opiate usage.

Ten subjects with moderate to severe intractable pain despite treatment with more than 200 mg/day of morphine (or equivalent) were enrolled into the study. Treatment was well tolerated, with no study agent–related serious adverse events observed at any point in the study. Subjects receiving the low dose of NP2 reported no substantive change in pain. Subjects in the middle-dose and high-dose cohorts reported pain relief, as assessed by NRS and SF-MPQ. Treatment of intractable pain with NP2 was well tolerated. There were no placebo controls in this relatively small study, but the dose-responsive analgesic effects were encouraging, and a Phase 2 placebo-controlled trial has been initiated.

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Gene Therapy for Epilepsy

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Introduction

Epilepsy is the most common serious brain disorder: it is estimated to affect 50 million people worldwide, with a prevalence of 1–2% of the population (World Health Organization, 2009). The term "epilepsy" actually includes a large group of genetic and acquired chronic neurological disorders whose single common feature is a persistent increase of neuronal excitability occasionally and unpredictably expressed as a seizure. An epileptic seizure is "the transient occurrence of signs and/or symptoms due to abnormal, excessive or synchronous neuronal activity" (Fisher et al., 2005). Seizures can be of two types: generalized, when occurring in and rapidly engaging bilaterally distributed networks, or focal, when occurring within networks limited to one hemisphere (Berg et al., 2010). Etiologically, epileptic syndromes are classified as genetic (when resulting from a known genetic defect), structural/metabolic (when resulting from a structural or metabolic lesion), and of unknown cause (Berg et al., 2010). Genetic epilepsies are most often associated with generalized seizures, whereas most structural epilepsies are associated with focal seizures that originate within or around the lesion area.

There is a significant unmet medical need in epilepsy. First, no truly antiepileptogenic therapy is currently available. None of the antiepileptic drugs in clinical use can prevent the development of epilepsy in cases in which the cause of the epileptogenic lesion is identifiable (e.g., head trauma, episode of status epilepticus [SE], stroke, brain infection). Second, pharmacological therapy is unsatisfactory: one third of the patients treated with antiepileptic drugs continue to experience seizures. Furthermore, in patients in whom seizures are well controlled, drugs may exert debilitating side effects and, in time, refractoriness to their therapeutic effects may develop. For some patients with focal seizures that are refractory or become refractory to pharmacological therapy, one final option is the surgical resection of the epileptogenic region. Third, there is a need for disease-modifying therapies. Antiepileptic drugs do not prevent the progression of the disease, and we lack therapies that can ameliorate or prevent the associated cognitive, neurological, and psychiatric comorbidities or epilepsy-related mortality.

Possible Gene Therapy Interventions

At least 30% of the epilepsies are believed to be of genetic origin. At first glance, it may seem that these diseases are good candidates for gene therapy, but this is not the case. Only rare forms of epilepsy are caused by a single mutant gene, whereas more common types result from the inheritance of two or more susceptibility genes (Berkovic et al., 2006). Moreover, the pathology in these cases often affects a large part of the brain and, thus, would require widespread gene transfer; however, currently available gene therapy methods provide only localized effects.

Researchers are attempting to develop strategies for globally delivering genes to the brain by crossing the blood–brain barrier (BBB) after administering vectors in the peripheral blood. One such strategy is to employ a pathway used by many circulating endogenous molecules, such as transferrin or insulin, to reach neurons and glia (de Boer and Gaillard, 2007; Simionescu et al., 2009). After these ligands bind on the luminal side of the capillary endothelial cell membrane, a caveolar vesicle is formed, engulfing the receptor and the bound conjugate. The caveola and its cargo are then transported across the endothelial cell cytoplasm, from the luminal to the abluminal side, via an intracellular transport mechanism known as transcytosis. For gene therapy, a vector can be conjugated to a ligand (such as a single-chain antibody against the transcytosis receptor or a peptide) that mimics the natural ligand for the receptor, e.g., transferrin or insulin. The vector–ligand conjugate remains intact and unmodified while in transit and is therefore released intact into the interstitial space. Recently, adeno-associated virus (AAV) vectors (Di Pasquale and Chiorini, 2006; Foust et al., 2009) have been shown to undergo transcytosis of the rodent BBB. However, much work remains to be done to prove that this approach can be applied to the treatment of genetic epilepsies.

Meanwhile, epileptic syndromes that are characterized by a focal lesion appear to be much better candidates for gene therapy. As described above, most of these diseases have an identifiable cause, and it is thought that these damaging insults set in motion a cascade of neurobiological events that eventually lead to epilepsy: a phenomenon termed "epileptogenesis." Thus, these forms of epilepsy offer the opportunity for intervention at different levels: preventive (antiepileptogenic), symptomatic (antiseizure), and disease-modifying (Fig. 1).

Choice of vector and route of delivery

The vector types employed thus far in epilepsy studies have been AAV (different serotypes) and herpes simplex virus (HSV) (Table 1). Typically, the route of delivery has been the stereotactic injection of the vector into the epileptogenic region (the hippocampus, in most instances). This approach

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ensures high levels of transgene expression and a limited immune response. (Note, however, that the surgical procedure may induce breakdown of the BBB and penetration of lymphocytes.)

Scientists have taken advantage of the biological properties of the different viruses to calibrate the spread of the viral particles in order to adequately cover the target area while limiting the number of injections and their volume. For example, Paradiso et al. (2009) have taken advantage of the retrograde transport of HSV to deliver therapeutic genes bilaterally after injection into one hippocampus, HSV being transported contralaterally by commissural fibers. Richichi et al. (2004) found degrees of spread around the injection site with different AAV serotypes.

Researchers have tested other routes of administration to obtain sufficiently specific accumulation of the transgene in the region of interest without facing the technical hurdles of direct intracerebral administration. In this respect, intranasal delivery is a feasible approach that has been tested using a replication-defective HSV-2 vector to deliver the anti-apoptotic gene *ACP10PK* (Laing et al., 2006).

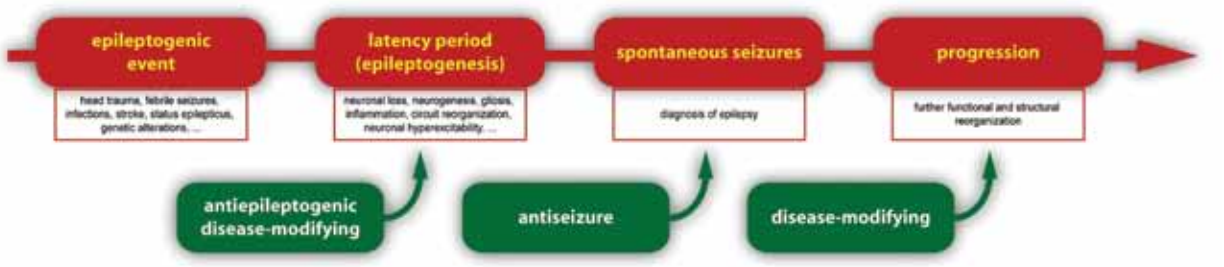

Figure 1. Natural history of acquired focal epilepsy (red) and possible therapeutic interventions (green).

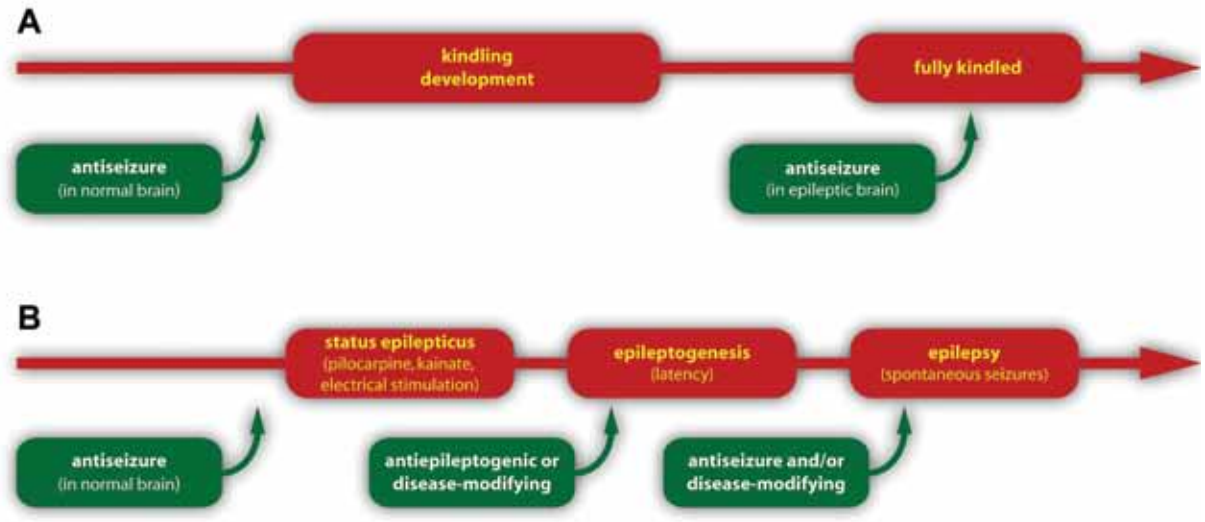

Figure 2. Murine models of acquired epilepsy employed in gene therapy studies. The time of gene transfer and its therapeutic significance are shown in green.

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Unfortunately, the transgene expression that resulted was not specific to the area of interest and, further, its level of expression was low. More recently, Gray et al. (2010) reported having identified an AAV clone capable of crossing the seizure-compromised, though not the intact, BBB. This finding opens up the possibility of creating vectors that may selectively target the brain areas involved in seizure activity after peripheral administration.

Models and endpoints employed in gene therapy studies

Research into gene therapy for epilepsy has been conducted essentially in two types of models. In the kindling model (Fig. 2*A*), the repeated administration to a discrete limbic brain area of an initially subconvulsive electrical stimulation induces seizures that progressively intensify in duration and severity, from focal to secondarily generalized. Kindling can be evoked by stimulating different areas of the brain, including the amygdala, hippocampus, and piriform cortex. Second, chemically (pilocarpine or kainate) or electrically (self-sustained SE) evoked SE (Fig. 2*B*) are models in which induction of an epileptogenic insult (SE) is followed by a latency period during which the animals are apparently well, and then by spontaneous recurrent seizures (SRSs), i.e. epilepsy. This situation closely mimics the one occurring in humans who acquire structural epilepsies.

These two models allow the exploration of the three main intervention levels identified above and shown in Figure 1: antiepileptogenic (preventing the development of epilepsy in subjects at risk after having received an epileptogenic insult), antiseizure (reducing the frequency and/or severity of seizures), and disease-modifying (altering the natural history of the disease). However, special care should be taken in choosing the model and the endpoint for evaluating treatment effectiveness in order to correctly allocate the results in terms of translation to clinical relevance. We will adopt here the conservative approach proposed by Dudek (2009). When gene transfer is performed before SE or kindling stimulation, therapeutic effects should be considered as antiseizure even when parameters relative to latency, SRSs, or kindling development are altered, because it is essentially impossible to guarantee that the treatment did not alter the initial SE or suppress each individual stimulus-evoked seizure during kindling. Accordingly, we will consider as potentially antiepileptogenic only treatments in which gene therapy was applied after the epileptogenic insult. Even in this scenario, undisputable evidence of an antiepileptogenic effect comes from prolonged observation (lasting several months) of treated animals and verifying that the effect is maintained well after termination of transgene expression or overexpression. Otherwise, it seems more appropriate to define the effect as disease-modifying. A disease-modifying effect may also be documented as either neuroprotection or arrest of disease progression during the chronic phase (when SRS frequency progressively increases).

Gene therapy: antiepileptogenic effects

Based on the above criteria, there is no undisputable evidence so far of gene therapy strategies that can actually exert antiepileptogenic effects. A series of studies, however, although not yet providing a final proof, strongly supports this notion (Bovolenta et al., 2010; Paradiso et al., 2009, 2011). In both humans and animals, epileptogenesis is associated with focal pathological abnormalities, including cell death (most prominently, a loss of neurons in the hippocampus termed "hippocampal sclerosis"); axonal and dendritic plasticity; neurogenesis; neuroinflammation; and functional alterations in ion channel and synaptic properties. The molecular mechanisms underlying these cellular alterations are still poorly understood, but impairment in neurotrophic factor (NTF) support may be a key causal element (Simonato et al., 2006).

Among the NTFs, fibroblast growth factor 2 (FGF-2) and brain-derived neurotrophic factor (BDNF) may be particularly implicated in epileptogenesis. Both protect neurons from ongoing damage and, further, FGF-2 is a potent proliferation factor for neural stem cells, while BDNF favors their differentiation into neurons (Simonato and Zucchini, 2010). Thus, Paradiso et al. (2009) reasoned that supplementing FGF-2 and BDNF in the epileptogenic hippocampus could attenuate seizure-induced damage, enhance repair, and ultimately, alleviate epileptogenesis. To test this hypothesis, they developed a replication-defective HSV-1 vector expressing these two NTFs and injected it into one hippocampus four days after pilocarpine-induced SE, i.e., during latency and after the establishment of hippocampal damage. These conditions are similar to those of a person who, following the occurrence of an epileptogenic insult, is in the latency period preceding the beginning of spontaneous seizures. The HSV vector was retrogradely transported to the contralateral hippocampus, allowing bilateral expression of the transgenes. Transgene expression was transient, lasting approximately two weeks. However, short-term expression is an advantage in these specific

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settings because NTFs can trigger plastic changes that remain detectable when they are no longer expressed, whereas their long-term expression may be detrimental for brain function (Thoenen and Sendtner, 2002). The goal of this approach was to increase the extracellular levels of FGF-2 and BDNF by generating cells capable of constitutively but transiently secreting these factors; achievement of this goal was verified by performing *in vitro* and *in vivo* analysis of both NTFs processing and release.

Administering the vector expressing FGF-2 and BDNF slightly attenuated the ongoing cell loss, indicating that, *in vivo*, its neuroprotective effect is limited or may require more prolonged or higher-level transgene expression. In contrast, the effect on neurogenesis was remarkable: the proliferation of early progenitors was favored and led to the production of cells that entered the neuronal lineage of differentiation, while aberrant aspects of SE-induced neurogenesis were reduced. One month after SE, all untreated animals displayed hippocampal sclerosis and SRSs. Treated animals, in contrast, had a highly significant reduction of cell loss in the hippocampus, and in particular, a nearly complete preservation of somatostatin interneurons.

To verify that these beneficial effects were sufficient to ameliorate the outcome of the disease, animals were video-EEG monitored for 20 days, and the occurrence, severity, and duration of SRSs were recorded. As expected, all non–vector-injected pilocarpine rats exhibited SRSs. In contrast, rats treated with the vector displayed a highly significant improvement: a subset of animals never developed SRSs in the time frame of observation, and the average number of seizures per day and their severity were significantly reduced. Finally, the authors controlled the possible effect of FGF-2 and BDNF therapy on ictogenesis (generation of spontaneous seizures) in a separate group of animals. They found that the effect was negligible in this respect, arguing that the treatment interferes selectively with epileptogenesis (Paradiso et al., 2009).

Gene therapy: antiseizure effects

A primary logical target for the gene therapy of seizures in drug-resistant individuals consists of modulating excitability by either increasing the strength of inhibitory signals or reducing the strength of excitatory signals. One study focused on $GABA_A$ receptors. In the granule cells of the hippocampus of epileptic (pilocarpine) rats, the expression of $GABA_A$ alpha-1 subunits is decreased, while expression of alpha-4 subunits is increased compared with controls (Brooks-Kayal et al., 1998). This altered expression pattern may be critical for the generation of seizures. Thus, Raol et al. (2006) designed an AAV2 vector containing the alpha-4 subunit gene promoter to drive alpha-1 expression. They injected this vector into the hippocampus two weeks before pilocarpine SE, obtaining increased alpha-1 expression in the granule cells, increased latency time, and decreased number of rats developing SRSs in the first four weeks after SE. Although these effects may be interpreted as antiepileptogenic, it cannot be ruled out that the vector attenuated SE and only secondarily protected from SRSs.

Haberman et al. (2002) tested out the idea of protecting from seizures by reducing the strength of excitatory signals. They did so by cloning in antisense an essential subunit for the functioning of NMDA receptors (NR1) in an AAV vector, under control of two different promoters. They found that, depending on the promoter, the cells expressing the transgene (those in which NMDA currents were downregulated) were either inhibitory interneurons or primary seizure output neurons; thus, the two different vectors had opposite effects on focal seizures (Haberman et al., 2002). This study underscores the importance of transducing a specific cell population anytime the transgene codes for a receptor (or a channel) that is expressed on both inhibitory and excitatory neurons.

As described for NTFs, one means of circumventing the problem of selectively targeting certain cell populations could be to express an inhibitory factor in a way that it is constitutively secreted from the transduced cells. If the receptors for that factor are present in the injected area, seizure control can be achieved without the need to target specific cells. Indeed, significant antiseizure effects have been obtained by overexpressing the NTF glial cell line–derived neurotrophic factor (GDNF) in the hippocampus (Kanter-Schlifke et al., 2007) and increasing hippocampal levels of the endogenous anticonvulsant adenosine with an AAV8 vector expressing the enzyme that catabolizes adenosine (adenosine kinase [ADK]) in antisense (Theofilas et al., 2011). However, the most promising results have been obtained with the inhibitory neuropeptides galanin (GAL) and neuropeptide Y (NPY).

Galanin

GAL is a 29-amino-acid neuropeptide released during seizures that inhibits glutamate release in the hippocampus (Lerner et al., 2008). Administration of GAL receptor agonists attenuates seizures, whereas pharmacological blocking exerts proconvulsant effects.

Table 1. Summary of the gene therapy studies in epilepsy.

Antiepileptogenic						
Gene	**Vector**	**Model**	**Site of injection**	**Timing**	**Results**	**Reference**
FGF-2 and BDNF	HSV-1	Pilocarpine	Hippocampus	Latency (4 days after SE)	DM: reduced cell loss, increased neurogenesis AE: reduced seizure frequency and severity	Paradiso et al., 2009
					DM: reduced neuroinflammation	Bovolenta et al., 2010
					DM: reduced mossy fiber sprouting	Paradiso et al., 2011
Antiseizure						
Gene	**Vector**	**Model**	**Site of injection**	**Timing**	**Results**	**Reference**
$GABA_A$ subunit alpha-1	AAV2	Pilocarpine	Dentate gyrus of the hippocampus	Before pilocarpine	AS: decreased % of animals with SRS at 4 weeks	Raol et al., 2006
NMDA subunit NR1 (antisense)		Inferior collicus stimulation	Inferior collicus	Before stimulation	AS or PC (depending on the promoter and the transduced cells)	Haberman et al., 2002
GAL	AAV2	Intrahippocampal kainate	Hilus of dentate gyrus in the hippocampus	Before kainate	AS: attenuation of seizures DM: reduced hilar cell loss	Haberman et al., 2003
		Inferior colliculus stimulation	Inferior colliculus	Before inferior collicus stimulation	AS: increased seizure threshold	
		Intrahippocampal kainate	Hippocampus	Before kainate	AS: reduction of seizure frequency and severity	Lin et al., 2003
		Intraperitoneal kainate	Piriform cortex	Before kainate	AS: reduction of seizing animals	McCown 2006
		Piriform cortex kindling	Piriform cortex	Fully kindled	AS: increased seizure threshold	
NPY	AAV2 AAV-1/2	Intrahippocampal kainate	Hippocampus	Before kainate	AS: delayed latency and reduction of seizure frequency	Richichi et al., 2004
		Rapid hippocampal kindling	Hippocampus	Before kindling	AS: retardation of kindling development	
	AAV2	Intraperitoneal kainate	Piriform cortex	Before kainate	AS: delayed latency	Foti et al., 2007
	AAV-1/2	Self-sustained SE	Hippocampus (bilateral)	In the chronic period (with spontaneous seizures)	AC: reduction of seizure frequency in a subset of rats DM: arrest in disease progression	Noè et al., 2008
	AAV-1/2	Rapid kindling	Hippocampus	Before kindling	AS: retardation of kindling development AR: no alteration in LTP	Sørensen et al., 2009
	AAV1	Intrahippocampal kainate	Hippocampus	Before kainate	AC: reduction of seizure frequency and dwuration AR: no alteration in learning and memory, anxiety, locomotor activity	Noè et al., 2010
Y2 receptor	AAV-1/2	Rapid hippocampal kindling; subcutaneous kainate	Hippocampus	Before kindling or kainate	AS: retardation of kindling development and reduction of kainate seizure frequency	Woldbye et al., 2010
NPY + Y2 receptor		Rapid hippocampal kindling	Hippocampus	Before kindling	AS: potentiation	
GDNF	AAV2	Hippocampal kindling Hilus of dentate gyrus		Before kindled	AS: no seizure generalization	Kanter-Schlifke et al., 2007
		hippocampal kindling		Fully kindled	AS: increased currents to evoke seizures	
		self-sustained SE		Before SE	AC: reduction of seizure severity and mortality	
ADK (antisense)	AAV8	ADK transgenic mice	Intra-CA3	Spontaneously seizing mice	AC: reduction of spontaneous seizures	Theofilas et al., 2011
ICP10PK (anti-apoptotic gene)	HSV-2	Intraperitoneal kainate	Intranasal	Before kainate	AC: prevention of seizures DM: prevention of neuronal loss and inflammation	Laing et al., 2006

Results are classified as antiepileptogenic (AE), antiseizure (AS), proconvulsant (PC) and disease-modifying (DM). Evaluation of possible adverse reactions (AR) of the treatment is also reported.

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Transgenic mice with functional deletion of *GAL* and *galanin type 1* receptor genes have spontaneous seizures or enhanced susceptibility to seizures, whereas transgenic mice overexpressing *GAL* in seizure pathways are resistant to epilepsy. Several synthetic agonists of galanin type 1 and type 2 receptors have been shown to inhibit experimental seizures.

In order to obtain constitutive secretion of GAL from transduced cells in the seizure-generating area, Haberman et al. (2003) constructed an AAV vector in which the GAL coding sequence was preceded by the secretory signal sequence of fibronectin (FIB), a protein that is constitutively secreted. This vector was tested in two seizure models. After injection into the hippocampus, this vector attenuated kainate seizures and prevented kainate-induced hilar cell death; after injection in the inferior colliculus, it increased the seizure threshold in this area (Haberman et al., 2003).

Congruent with these findings, other studies have reported that AAV-mediated expression of GAL in the hippocampus reduces the frequency and severity of seizures caused by intrahippocampal injection of kainate (Lin et al., 2003) and that AAV-mediated expression of GAL in the piriform cortex reduces the number of seizing animals after peripheral administration of kainate (McCown, 2006). Notably, these effects were independent of the promoter driving GAL expression. Together, these studies support the notion of an antiseizure effect in normal animals (Fig. 2). To determine whether this effect may hold true in an epileptic brain, McCown (2006) injected the AAV-FIB-GAL vector into fully kindled rats, obtaining a significant elevation of seizure threshold. Thus, vector-derived GAL expression and constitutive secretion appear to be able to suppress epileptic seizure activity.

Neuropeptide Y

NPY is a 36-amino-acid neuropeptide that is overexpressed during seizures (Noè et al., 2009). Activation of the NPY Y2 and Y5 receptors inhibits glutamate release in the hippocampus and attenuates seizures. Transgenic rats overexpressing NPY show reduced seizure susceptibility, whereas knock-out mice lacking NPY or the Y2 or Y5 receptor gene are more vulnerable to chemically or electrically induced convulsions. In hippocampal slices from epileptic patients, NPY potently inhibits perforant path-evoked excitatory responses in granule cells.

The effect of chronic overexpression of NPY in the hippocampus has been extensively studied. The NPY-coding gene has been transferred into the hippocampus using two types of vectors, based on AAV2 or AAV-1/2 (a vector consisting of a 1:1 mixture of AAV1 and AAV2 capsid proteins), eight weeks before intrahippocampal injection of kainate or rapid kindling. Researchers observed a decreased occurrence of seizures or a retardation in kindling development (Richichi et al., 2004). Similarly, bilateral piriform cortex infusions of AAV vectors that constitutively secrete NPY (AAV-FIB-NPY) increased latency to seizures after kainate administration (Foti et al., 2007). Moreover, AAV-induced overexpression in the hippocampus of the Y2 receptor has been found to exert seizure-suppressant effects per se and potentiate the effects of NPY overexpression (Woldbye et al., 2010). Together, these findings strongly support an antiseizure effect in the normal brain.

To evaluate whether the antiseizure effect was also present in the epileptic brain, Noè et al. (2008) injected the NPY-expressing AAV-1/2 vector bilaterally into the hippocampus of rats that were experiencing SRSs after electrically induced SE. They found a significant reduction in seizure frequency in 40% of the animals. Even more interestingly, they observed a remarkable decrease in the progression of seizures (in this model, the frequency of spontaneous seizures increases over time), i.e., a disease-modifying effect. More recent studies have explored the possible side effects that may be expected because of the many functions of NPY in the CNS. However, the NPY-expressing AAV-1/2 vector did not affect epilepsy-induced impairment of long-term potentiation (LTP), an indication that it will not further impair epilepsy-associated memory loss (Sørensen et al., 2009). Furthermore, an NPY-expressing AAV1 vector, while demonstrating potent anticonvulsant activity, did not cause alterations in learning and memory, anxiety, and locomotor activity behavioral tests (Noè et al., 2010). Taken together, the overall evidence supports the application of AAV-NPY gene therapy for human epilepsy.

Future Developments

Gene therapy offers a wealth of opportunities for epileptologists. Vectors can be tailored to the desired experimental needs in several respects:

- Spread from the zone of inoculation. For example, different degrees of spread for different AAV serotypes or retrograde transport for HSV. Also, new vectors may be available soon for peripheral administration with either selective localization in lesion areas (for the treatment of focal epilepsies) or widespread distribution in the brain (for the treatment of generalized epilepsies);

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- Duration of transgene expression. Relatively short-acting expression is achieved with HSV and long-lasting expression with AAV vectors; and
- Targeting specific cell populations (e.g., employing population-specific promoters).

In turn, patients with partial epilepsies selected for surgical resection of the epileptogenic area are ideal candidates for gene therapy. Their pathology is focal, optimal medical treatment has failed, and the success of surgery in leading centers (~70% seizure-free at one year) supports the hypothesis that local, sustained release of an inhibitory molecule might suffice to "silence" hyperactivity. In a way, tissue resection represents the most extreme form of cellular "silencing," so gene therapy may provide a realistic alternative. Gene transfer of inhibitory factors such as GAL or NPY into the epileptogenic area in patients selected for surgery does not require ad hoc stereotaxical intervention, because these patients undergo implantation of depth electrodes for diagnostic purposes before surgery. Also, gene transfer therapy has a built-in rescue procedure because, should it fail to produce any advantage, patients would simply undergo surgery as originally planned.

No doubt, accurately verifying gene therapy's safety and efficacy in nonhuman primates is needed before beginning studies in humans. However, clinical experience of gene therapy in humans with other diseases is encouraging. Once these last hurdles are overcome, the GAL and NPY gene therapy strategies for treating epilepsy will likely progress to Phase 1 clinical trials.

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Gene Therapy for Malignant Brain Tumors: from Experimental to Clinical Neuro-oncology

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The Clinical Challenge

Glioblastoma multiforme (GBM) grade IV is the most common malignant brain tumor in humans. These tumors are most common in patients over the age of 60 but also appear in younger patients. The presumed diagnosis of GBM is based on magnetic resonance imaging appearance but ultimately depends on tumor neuropathology. Histological features diagnostic of GBM are pseudopalisades, microvascular proliferation, and necrosis. Current standard of care includes surgical resection, radiotherapy, and temozolomide. Progression is usually fast, following the first line of treatment. Upon progression, patients are treated with second-line chemotherapies and antagonists of angiogenesis, e.g., bevacizumab, an antibody to vascular endothelial growth factor (VEGF). Treatment of tumor recurrence is not effective. Median survival in academic, high-complexity medical centers is 18–21 months. Few patients survive up to five years postdiagnosis, and longer-term survival is uncommon (Grossman et al., 2010).

Patient survival has improved only marginally during recent decades, prompting the development of novel therapies ranging from inhibitors of angiogenesis to chemotherapy, inhibitors of signaling molecules, vaccination (e.g., against tumors, tumor antigens, mutated epidermal growth factor receptor [EGFR]), and most recently, gene therapy (Candolfi et al., 2009).

The Genetic Mutations

Genetic alterations found in GBM are complex (Furnari et al., 2007). Most cases are sporadic, and a small number of familial gliomas are associated with germline mutations, i.e., neurofibromatosis I and II, tuberous sclerosis complex, von Hippel-Lindau disease, Cowden disease, Li-Fraumeni cancer syndrome, Turcot syndrome, and Gorlin's syndrome. Genes mutated in GBM include EGFR, *p53*, $p16^{INK4a}$/$p14^{ARF}$, *PTEN*, and *IDH-1*. Gliomas also display a mutator phenotype that leads to chromosomal abnormalities, most commonly on chromosomes 1p, 7, 8q, 9p, 10, 12q, 13q, 19q, 20, and 22q, which are also linked to altered signaling pathways. The identification of altered signaling pathways allows for the development of novel specific inhibitors. Mapping of copy number alterations and gene mutations identifies alterations in the following signaling pathways:

(i) The receptor tyrosine kinase/PI3K class 1 signaling through AKT to affect cell division (e.g., EGFR, NF1, PI3K, PTEN);
(ii) The receptor tyrosine kinase/RAS signaling through RAS/RAF to alter cell-cycle progression;
(iii) The PI3K class 2 signaling through PIP3 to affect cell migration;
(iv) *p53* signaling, altering G2/M arrest and apoptosis (i.e., CDKN2A, p53); and
(v) The retinoblastoma pathway (G1/S progression, e.g., *CDKN2B-CDK4, RB1*).

The latest attempt to relate primary molecular lesions to clinical patterns of GBM classifies GBMs as follows:

(i) *Classical*: EGFR, PTEN, *CDKN2A*;
(ii) *Proneural*: PDGF, *IDH-1*, *p53*, PTEN, *CDKN2A*;
(iii) *Neural*: EGFR, *p53*, PTEN, *CDKN2A*; and
(iv) *Mesenchymal*: NF1, *p53*, PTEN, *CDKN2A*.

Novel methods described below will test experimentally whether distinct combinations of mutations induce experimental tumors with individual morpho-functional characteristics and test their responses to novel treatments (Verhaak et al., 2010).

The Experimental Challenge: How to Model Glioma Tumors

Rodent glioma cell lines

Intracranial (adults) or intravenous (during pregnancy) injections of mutagens have been used since the 1930s to induce gliomas in rats, mice, rabbits, and gerbils. The most common cell lines used in rats are C6, 9L, T9, RG2, F98, BT4C, RT2, and CNS1. The alkylating agent methylnitrosourea was used to induce the C6, 9L, T9, and CNS1 cells. Most cell lines can be grown in syngeneic hosts. C6 cells were derived from outbred Wistar rats, a fact that curtails the possibility of using these cells to study antitumor immune responses. Syngeneic lines were derived from Fisher rats (using methylnitrosourea [e.g., 9L, T9] or ethylnitrosourea [e.g., RG2, and F98]); Lewis rats (e.g., CNS1, induced by methylnitrosourea); and BDIX rats (e.g., BT4C cells, induced by ethylnitrosourea). These cell lines are grown in culture and form reliable tumors upon implantation of 100–10,000 cells into the brain of their respective hosts. Mutations in genes that are also mutated in human tumors have been detected in these cell lines, although the whole complement of mutations induced by alkylating agents is likely to be more widespread than mutations in human tumors. Cell lines are a favorite model for experimental studies of novel treatments for brain tumors. To optimize immunotherapies, some of the

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least immunogenic cell lines, e.g., CNS-1, RG2, or F98, are ideally suited for such studies (Candolfi et al., 2007a; Barth and Kaur, 2009).

The most common cell lines used in mice are the SMA-560 astrocytoma cells, derived from VM/Dk mice, and the GL26/GL261 cells, derived from C57Bl/6 mice. SMA-560 astrocytoma cells were derived from a spontaneous astrocytoma. GL261 was derived from C57BL/6 mice implanted in the brain with 3-methylcholantrene pellets. Both have reduced immunogenicity and form tumors reliably upon implantation (Curtin et al., 2009; Maes and Van Gool, 2011). Tumors display increased vascular proliferation and invasion but do not form pseudopalisades. These tumors constitute excellent experimental models for testing the effectiveness of genetic therapies in the presence of the systemic adaptive immune system.

Human glioma cell lines

Human GBM-derived cells are of great interest but can be studied only in immune-suppressed animals. Human glioma cells are well suited for studies of experimental radiotherapy, chemotherapy, or gene therapy but not experimental immunotherapies. In the past, primary clonal cell lines derived from resected GBM were used. The extent to which human glioma cell lines are representative of the original GBM cannot be addressed. Increased interest in the study and characterization of stem cells in human GBM has led to the isolation of glioma stem cells from human GBM by growing human tumors *in vitro* as neurospheres. Growth in immune-suppressed rodents preserves characteristics of human GBM stem cells, e.g., migration throughout the CNS and tumor formation. The study of human glioma stem cells is important, given their presumed central role in the formation and recurrence of human gliomas (Rich and Eyler, 2008; Le et al., 2009; Bonavia et al., 2011).

Genetically inducible models

Germline gliomagenesis: transgenic and knock-out models

DNA sequences encoding for particular mutations known to be important in gliomagenesis can be delivered to mouse brain progenitor cells using transgenic techniques. Targeting the expression of the pathogenic genes to the brain is achieved using cell-type–specific promoters. Alternatively, particular genes can be knocked out to mimic inactivating mutations.

In many cases, transgenic expression of a mutated gene, in combination with gene knock-outs, has been necessary to induce brain tumors in mice. Overexpression of v-src in astrocytes (using the GFAP promoter) induces astrocytomas of mainly low and high grade, whereas overexpression of ^{V12}H-Ras induces low-grade astrocytomas. The GFAP promoter has also been used to express the EGFR wild type (Wt), or the EGFRvIII, which by itself did not cause gliomas unless ^{V12}H-Ras was also added. (Mostly oligodendrogliomas and oligoastrocytomas were detected in such animals.) Expression of v-erbB or SV40-1gT$_{121}$ did cause oligodendrogliomas and astrocytomas, but tumor induction and the degree of tumor aggressiveness were increased if the experiments were performed in *Ink4a/arf*$^{-/+}$, *p53*$^{-/+}$, or *PTEN*$^{-/+}$ mice. Animals with combined germline mutations in *NF1*$^{-/+}$ and *p53*$^{-/+}$ displayed low- and high-grade astrocytomas.

In spite of the advantages of transgenic and knock-out models of brain tumors, the following challenges remain: tumors are induced mainly in very young animals; the strain of mice used influences glioma penetrance; there is a variability in the genetic background because of the process used to produce transgenic animals; and tumor penetrance varies from generation to generation as transgenic lines are backcrossed to achieve homogeneous genetic background (Alcantara Llaguno et al., 2009; Le et al., 2009).

Somatic gliomagenesis: virally induced models

Replication-competent avian leukemia virus system. An alternative to germline modifications is to introduce mutations into somatic mouse cells. The first system to do so was the replication-competent avian leukemia virus (RCAS) system. Because mammalian cells are not permissive to ALV, transgenic neonatal animals expressing the viral receptor TV-A under the control of either the nestin promoter (to target progenitor cells) or the GFAP promoter (to target astrocytes) have been generated. This system has been used to express mutations in Wt animals or animals carrying germline deletions of tumor suppressors (e.g., *p16*^{INK4a}/*p19*^{ARF}, PTEN, *p53*). Various types of gliomas have been generated via RCAS-mediated expression of Akt and k-Ras, or PDGF-B in Wt animals; expression of k-Ras in PTEN$^{-/-}$; expression of Akt and k-Ras in *p16*^{INK4a}/*p19*$^{ARF\ -/-}$; expression of PDGF-B in *p16*^{INK4a}/*p19*$^{ARF\ -/-}$ and PTEN$^{-/-}$; expression of PDGF-B in *p53*$^{-/-}$; and expression of EGFRvIII in *p16*^{INK4a}/*p19*$^{ARF\ -/-}$. Tumors obtained vary from low-grade to high-grade astrocytomas and oligodendrogliomas and are now being used to test novel therapies (Huse and Holland, 2010).

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Retroviral vectors. Moloney murine leukemia virus (MMLV) vectors have been utilized to overexpress PDGF-B in rats. Tumor penetrance is 100%, and the tumors have the typical histological characteristics of high-grade gliomas seen in human patients. This model has been exploited to study glioma biology and, most recently, as a model to test novel glioma therapeutics (Assanah et al., 2006; Lopez et al., 2011).

Lentiviral vectors. Lentiviral vectors have been engineered to induce gliomas in C57Bl/6 mice (Marumoto et al., 2009). Lentiviral vectors expressing floxed Akt and H-Ras were injected into $p53^{+/-}$ mice expressing GFAP-Cre. Cre recombination in cells expressing GFAP activates expression of the encoded oncogenes. Tumors display the morphological and behavioral characteristics of high-grade glioma, and brain tumor–initiating stem cells could be isolated and used to propagate glioma cells *in vitro* and *in vivo*. High-grade glioma tumors have also been induced in Sprague Dawley rats using lentiviral vectors expressing PDGF-B, Akt, and H-Ras. Injections of lentiviral vectors expressing PDGF-B and H-Ras (but not PDGF-B and Akt) induced a rapidly progressive, high-grade glioma. PDGF-B expression on its own did not induce a highly penetrant phenotype, and Akt and H-Ras on their own induced a slowly progressive, low-grade glioma. These tumors are now being used to test the effectiveness of gene therapies (M. Wibowo, M.G. Castro, and P. Lowenstein et al., unpublished observations).

How to Treat Glioma Tumors with Gene Therapy

Vectors for experimental and clinical Neuro-oncology

Brain tumor gene therapy strategies attempt to kill tumor cells through a variety of means: conditional cytotoxicity, direct cytotoxicity, apoptosis, correction of genetic deficits, inducing inflammation, or inducing immune responses. Many different vector systems have been developed and used experimentally. Here we will discuss only those that have advanced to clinical testing.

Nonreplicating retroviral vectors

Nonreplicating retroviral vectors are single-stranded RNA vectors, with a total genome size of 3–9 kb, which provides for a packaging capacity of up to 8 kb. Expression from these vectors is obtained only following the infection of dividing cells, where they integrate into the host cell genome. Expression is expected to be long-lasting, but in some cases, inactivation of promoters curtails expression. Retroviral vectors have limited immunoreactivity and cause limited inflammation. These were the first vectors developed and used in experimental and clinical gene therapy. For the treatment of brain tumors, vectors have encoded the conditional cytotoxic gene *HSV1-TK*, cytostatic IL-4, antiangiogenic dn-VEGF-R2, and apoptosis-inducing FasL. Initial work with these vectors was encouraging, leading to rapid clinical translation.

Nonreplicating retroviral vectors were the first vectors used in clinical trials for patients suffering from malignant brain tumors. A series of initial Phase 1/2 trials was performed that gave encouragement to proceed to larger-scale trials. A large multicenter, Phase 3 clinical trial was performed but showed no benefit to patients, owing to several factors: the logistics of the trial; the low transduction of retroviral vectors; and immune responses to vector-producing cells. As of this writing, this approach is not being pursued (Klatzmann et al., 1998; Chiocca et al., 2003).

Replication-competent retroviral vectors

Given the shortcomings eventually detected when using retroviral vectors for the treatment of brain tumors, various groups developed replication-competent retroviral vectors based on amphotropic murine leukemia virus (MLV). These vectors can also be engineered for replication to become tissue-specific, express a marker protein such as GFP, and be armed with a prodrug-activating gene such as cytosine deaminase. These vectors are now being used in clinical trials for GBM. Limitations of nonreplicating retroviral vectors have given way to the hope that replication-competent ones may overcome such shortcomings. Replicative vectors have been developed relatively recently and are now being tested in early GBM clinical trials (Solly et al., 2003; Tai et al., 2010).

Nonreplicating adenoviral vectors

Adenoviral (AdV) vectors derive from nonenveloped, double-stranded (ds) DNA viruses, are nonintegrating, and have a total genome size of ~36 kb. Their packaging capacity is 8–10 kb in first-second generation AdV and up to 30 kb in high-capacity, helper-dependent AdV. AdVs grow to high titers and are made replication-deficient through deletion of the E1 region. They do not integrate into the host genome; thus, their expression is potentially transient. However, transient expression in the CNS *in vivo* is linked to inflammation and immune responses, as following careful experiments

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that minimize inflammation and immune responses allow brain expression for 6–12 months in immunocompetent animals (Dewey et al., 1999). AdV vectors have been used in a variety of GBM models, leading to various clinical trials (Eck et al., 1996, 2001; Curtin et al., 2005; Lowenstein et al., 2007; Candolfi et al., 2009):

- Conditional cytotoxic HSV1-TK or cytosine deaminase;
- HSV1-TK + immune-stimulatory Flt3L;
- *p53* or *p16/CDKN2* to correct genetic defects;
- Antiangiogenic angiostatin;
- Pro-inflammatory IL-12 and tumor necrosis factor–alpha (TNF-α);
- Na^+/I^- symporter to increase delivery of radioactive iodine; and
- Decorin or small hairpin RNA (shRNA) to block immune-suppressive TGF-β.

In early clinical trials, AdV-TK vectors were more effective than retroviral vectors encoding HSV1-TK. This success led to a double-blind, randomized, multicenter European Phase 3 trial of Adv-TK for treating GBM. No serious side effects were seen, but neither was a clear survival benefit (Immonen et al., 2004; van Putten et al., 2010), causing the European Medicines Agency not to approve this vector for treating GBM. Lack of therapeutic benefit was most likely the result of variations in patients' treatment across different clinical centers. This variability prompted investigators in the United States to continue testing AdV-TK to advance it toward an improved controlled, larger-phase trials in the future.

Clinical trials of AdV expressing *p53* and IFN-β were performed. In spite of the absence of adverse events attributed to Ad-*p53*, transduction and distribution of the vector throughout the tumor needed improvement. The IFN-β trial was stopped because some participants experienced acute inflammation. In spite of overall negative results, however, individual centers reported longer-term survival in some patients (Eck et al., 2001; Vecil and Lang, 2003; Gomez-Manzano et al., 2004).

Our group developed a combined approach using HSV1-TK and Flt3L to induce specific immune responses in the CNS. In April 2011, the FDA allowed an investigational new drug application (IND) to proceed to a Phase 1 clinical trial in patients with resectable primary GBM. This trial is expected to start by December 2011. We are currently performing an open, controlled clinical trial using helper-dependent, high-capacity AdV vectors expressing constitutive HSV1-TK and inducible Flt3L to treat GBM in dogs. Dog tumors are resected, and AdV is injected into the resection cavity, followed by induction of Flt3L expression valacyclovir to stimulate conditional cytotoxicity of HSV1-TK and temozolomide. Control vectors express nontherapeutic genes. More than a dozen dogs have been treated, and this study is ongoing (Ali et al., 2005; Candolfi et al., 2007b; King et al., 2008; Curtin et al., 2009; Larocque et al., 2010; Pluhar et al., 2010; King et al., 2011; Mineharu et al., 2011).

Replication-competent adenoviral vectors

Replication-competent, or oncolytic, AdV vectors have been produced; they contain mutations that are compensated for by factors present in cancer cells but not normal cells. D-24–type vectors have a 24 bp deletion from the pRB binding site in E1A. Altered E1A protein cannot bind Rb, which is needed to release E2F to activate the viral E2 region and viral replication. In cancer cells with inactivations in the Rb pathway, E2F remains available and induces oncolytic AdV replication. Onyx-15 (dl520) contains mutations in the E1B-55kDa protein, which normally inactivates *p53*, required for induction of S-phase and viral replication. Onyx-15 mostly replicates in cells lacking *p53*. Cell-type-specific promoters (e.g., melanoma, prostate, tumor-specific regulatory sequences) driving the expression of genes necessary for viral replication have been used to restrict replication to predetermined cell types. Oncolytic AdV vectors are being used in experimental gliomas and in clinical trials. Δ-24-RGD, a tropism-enhanced oncolytic virus targeting the *Rb* pathway, is being tested in a Phase 1 clinical trial (Geoerger et al., 2002; Vecil and Lang, 2003; Chiocca et al., 2004; Jiang et al., 2009; Fueyo et al., 2011).

HSV-1 replicative, attenuated, or conditionally replicative vectors

HSV-1 is an enveloped dsDNA virus containing 152 kb of genomic DNA. It infects dividing and noninvading cells, does not integrate into the genome of host cells, and achieves long-term persistence in neurons. The packaging capacity in replication-defective vectors is more than 30 kb; fully deleted amplicon HSV-1 vectors allow larger inserts (e.g., bacterial artificial chromosomes [BACs]). Vectors (e.g., G207, 1716) are deleted in specific viral genes to reduce neuropathogenicity. Common mutations used are those in γ*34.5*, the major neuropathogenicity gene, *ICP6*, U_L24, U_L56, and α*47*. Early clinical trials in the United States and United Kingdom showed vectors to be safe. Newer vectors (e.g., OncoVEX; BioVex, Woburn, MA)

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include therapeutic genes (e.g., immune-stimulatory granulocyte macrophage colony-stimulating factor [GM-CSF]) or are replication-competent, and are combined with chemotherapy (Markert et al., 2006; Marconi et al., 2010; Kanai et al., 2011).

Measles virus

Measles viruses are being used to treat a number of different tumors, including GBM. Attempts to retarget measles virus to glioma cells are ongoing. An early-phase trial using engineered oncolytic measles virus for GBM reported no dose-limiting toxicity with up to 10^7 tissue culture infectious dose 50 (TCID50) (Allen et al., 2006, 2008).

Newcastle disease virus, Reovirus

Two replication-competent viruses have been used to treat GBM in early-phase clinical trials. The MTH-68/H strain of Newcastle disease virus and Reovirus, serotype 3 (Dearing strain), are given via systemic administration to treat GBM. These human trials remain to be published (Freeman et al., 2006).

Future Challenges of Translational Neuroscience and Neuro-oncology

In spite of major advances made over the last 20 years, future clinical success will depend on our capacity to address the following challenges (Lowenstein and Castro, 2009):

- Defining sufficient experimental efficacy to warrant a move from the lab to clinical trials;
- Determining which criteria are necessary to make such decisions;
- Assessing carefully what can be learned from past failures in clinical trials;
- Determining a criterion for failure in clinical trials;
- Advancing our understanding of the biology of human GBM;
- Determining the relevant genetic contribution to brain tumors;
- Improving the delivery, safety, and efficacy of viral vectors; and
- Achieving GBM-specific systemic delivery.

In summary, to improve the clinical outcome of GBM, we need to accomplish several tasks. We need to develop tools to predict the likelihood of clinical success of novel therapies initially tested in experimental models. Further, the clinical significance of small improvements in patient survival needs to be carefully considered. We should establish a "failure" criterion for experimental and clinical trials (i.e., when should novel strategies not be pursued further) and improve the statistical evaluation of both experimental and clinical trials by moving away from "statistical significance" and toward "clinical significance." Finally, we need to increase the recruitment of patients into clinical trials and intensify our study and understanding of the human tumors. Median survival of patients is now 18–21 months; in 1941, it was reported to be 13 months. Seven months' increased survival after seven decades of research and clinical developments highlights the seriousness of the challenge and the desperate need for original solutions.

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