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Recent advances in the pharmacology of neurological gene therapy

Pedro Ricardo Lowenstein^{*} and **Maria Graciela Castro**

Gene Therapeutics Research Institute, Cedars-Sinai Medical Center and Department of Medicine, University of California, Los Angeles, USA

Abstract

The choice of vectors, transgenes, regulatory elements, delivery approaches and the capacity to transduce the appropriate target cell type all influence the effectiveness of gene therapy for neurological diseases. Furthermore, even if many strategies are sound and effective in experimental animals, issues relating to side effects of gene therapy, longevity of therapeutic transgene expression and diffusion throughout the brain can limit the clinical potential of gene therapy. During the past 12–18 months, there have been significant advances in the following areas: the capacity to target vectors to predetermined cells types; the development of gene therapy approaches for the treatment of dominant inherited and neurodegenerative diseases; the capacity to achieve systemic delivery of viral vectors to the brain; and the development of viral vectors to model neurological diseases.

Introduction

In this review, highlights of advances made in the use of viral vectors for gene transfer into the central nervous system (CNS) during the past 12–24 months have been selected for discussion. These include improvements in vector targeting to brain cells, the development of vectors capable of transducing large transgenic sequences, novel therapeutic approaches for recessive and even dominant inherited neurological disorders, novel systemic administration of viral vectors to target the brain, the use of viral vectors to create genetic models of neurodegenerative diseases, and the status of our knowledge on the immune responses to viral vectors injected into the brain.

Advances in viral vector technology: vectors, targeting and transduction

Lentiviral vectors were thought to specifically transduce neurons but have now been shown to achieve cell-type-specific expression in astrocytes in the rodent brain [1]. This was thought to be a function of the lentiviral envelope pseudotyped by VSV-G protein. Accordingly, alternative envelope proteins were used to pseudotype lentiviral envelopes, in some cases achieving a higher transduction of glial cells. Recent results demonstrate that the use of the astrocyte-specific glial fibrillary acidic protein promoter produces almost exclusive transgene expression in glial cells [1]. This has two major implications. Firstly, these results indicate that lentiviruses effectively infect glial cells and that, even in the absence of detectable transgene expression, glial cells were effectively transduced. Thus, any potential side effects of astrocyte transduction will need to be assessed. Secondly, lentiviruses can now be used to specifically transduce glial cells, which highlights their potential use to treat glial tumors of the brain (e.g. glioblastoma multiforme).

^{*}lowensteinp@cshs.org.

Transduction of microglial cells has recently been achieved through the use of specific transcriptional targeting. Using adenovirus-associated virus (AAV)2 and AAV5 and a variety of promoter sequences (derived from hCMV-MIE, CD11b, CD68 and F4/80), it was shown that the F4/80 promoter sequences were the most specific, both from AAV2 and AAV5 [2]. Microglial cells play important roles in brain inflammation and immune responses, and their direct and specific transduction will be of great importance for their *in vivo* engineering in models of autoimmunity, such as experimental allergic encephalomyelitis.

Viral vectors devoid of most of their coding sequences have been developed over recent years. The maximum amount of transgenic sequences in either helper-dependent adenoviral vectors or herpes simplex virus type 1 (HSV-1) amplicons has been increased to close to their absolute maximum capacity; that is, 25.4 kbp (of a potential maximum of ~ 35 kbp) for high-capacity adenoviral vectors [3], and 115 kbp (of a potential maximum of ~ 150 kbp) [4] for HSV-1 amplicon vectors. This truly opens up the possibility of encoding large genomic fragments compared with the relatively smaller transgenic sequences that have been delivered so far.

A further improvement has been achieved by the transduction of cells with adeno- and retroviral vectors. Pre-incubation of vectors with 0.05–5 mM of cell-permeable peptides derived from the HIV-1 protein tat and Antennapedia increased transduction with both vectors *in vitro* and *in vivo* [5]. These data indicate that viral transduction and uptake of viral particles is stimulated by tat and Antennapedia peptides. Although the authors did not test this in brain tissue, it is expected that the same increase in transduction would be seen in the brain *in vivo*.

Gene therapy of inherited neurological disorders

Gene transfer techniques have been very powerful in allowing overexpression of transgenes. However, inhibiting gene expression using gene therapy approaches has been more difficult. Antisense RNA and ribozymes were assayed in several different implementations. Overall, these methods to reduce mRNA levels were somewhat unpredictable. Furthermore, the specificity of antisense RNA was not sufficient to selectively inhibit mutant alleles in dominant inherited diseases (e.g. Huntington's disease) while leaving expression from the wild-type allele intact. Given the uncertainty about the function of wild-type alleles for many of these genes, selective inhibition of mutated alleles is a crucial step in the development of gene therapies for dominant diseases.

Cellular pathways that regulate the stability of RNA and lead to the degradation of specific RNA molecules (known as RNA interference) have been recently described in plants and animals. More recently, techniques have been developed by which the expression of small interfering RNAs (siRNAs) can inhibit specific mRNAs. Importantly, proof-of-concept experiments have been performed and have demonstrated that transfer of siRNAs can downregulate the expression of target sequences delivered simultaneously, both *in vitro* and *in vivo* [6,7].

This technology has now been successfully used to achieve allele-specific inhibition of dominant mutated genes in *in vitro* models of Machado-Joseph disease/spinocerebellar ataxia type 3 (SCA3), Alzheimer's disease (V337MM missense Tau mutation) and dystonia (torsinA mutations) [8,9]. Interestingly, to inhibit expression of the polyglutamine repeat responsible for SCA3, the authors had to target a single nucleotide polymorphism rather than the region encoding the glutamine repeat. Also, in these studies, the authors could inhibit mutated RNAs using either *in vitro*-synthesized duplexes, plasmid DNA or adenoviral vector-mediated expression of short hairpin RNAs. The authors optimized the use of siRNAs to specifically inhibit expression of mutated alleles, especially when it was recognized that the area coding for the poly-Q could not be used as a potential target sequence of the genes mutated in SCA3, a polyglutamine repeat disorder. So far, patients' cell lines have not been used to demonstrate

allele-specific targeting of mutated alleles using siRNA. Undoubtedly, this will be the next step in the optimization of the first credible approach to specifically inhibit the expression of mutated alleles that cause dominant neurological diseases.

Inroads have been made in the treatment of a transgenic model of amyotrophic lateral sclerosis (ALS). AAV vectors were injected intramuscularly to deliver insulin growth factor 1 (IGF1) to the spinal cord by retrograde transport of the viral vectors in a transgenic mouse model of ALS that overexpresses superoxide dismutase-1. These experiments demonstrated delayed onset of motor symptoms, extended survival of spinal cord motoneurons, delayed astrogliosis and extended survival of treated transgenic animals. Injection of a lentiviral vector that is not transported retrogradely to the spinal cord but expresses IGF1 in the muscle did not improve any measure of the disease. Thus, expression of IGF1 directly in motoneurons had a beneficial effect in an animal model of ALS, and could pave the way to development of gene therapy for this devastating disease [10••].

Systemic delivery of viral vectors

Could vectors, transgenes or transgenic proteins access the brain from the circulation? Delivery of viral vectors into the brain through a systemic route would be of great importance, but has so far neither been achieved nor explored in detail. Clearly, this would be attractive because of the large areas of the brain that are affected in human neurodegenerative diseases (e.g. Parkinson's and Alzheimer's disease). Consequently, there have been several studies that have attempted to deliver viral vectors to the adult brain through intravascular, intrahepatic, intraventricular and intravitreal routes, as well as by direct intrafetal injections. Different studies have detected transfer of viral vectors in some instances, and apparent trans-synaptic transfer of transgenic proteins in others.

Intrahepatic delivery of 8.6×10^{10} infectious units AAV2 into fetal rhesus monkeys between 30 and 120 days of gestation demonstrated low but detectable expression of transgenic enhanced green fluorescent protein (eGFP) in the brain. This was detected by PCR amplification of the CMV-eGFP region, Southern blotting using an eGFP probe, and real-time PCR for the eGFP gene. Quantitative detection of the eGFP gene showed the presence of 30–80 copies of the eGFP gene per 10^4 copies of β -actin. This was 500–2 500 times lower than in the liver, which contained the highest concentration of vector copies. Despite low levels of vector in the brain, eGFP immunohistochemistry was localized to glial fibrillary acidic protein-labeled astrocytes, demonstrating transduction of brain cells following systemic delivery into the non-human primate fetal liver [11].

Intrafetal injection of lentiviral vectors has also been attempted. Equine infectious anemia virus (EIAV) vectors (CMV- β gal; 2×10^6 transducing units, total) were injected directly into peripheral yolk blood vessels of pregnant female MF1 mice at 16 days of gestation. Staining for the transgene β -galactosidase was observed in the brain at 7 and 79 days, and was also detected by PCR for the 5' cppt region of the SMART2Z vector. However, no expression was detected in adults injected with vector into the portal circulation, suggesting that permeability to EIAV vectors may be increased in the mouse fetus [12].

Early postnatal systemic injection using E1/E3-deleted recombinant adenovirus (1×10^7 infectious units) expressing human β -glucuronidase (GUSB) under the control of the β -actin/hCMV promoter was performed in MPSVII mice within the first 24 hours of life. This maintained brain GUSB activity for up to 20 weeks at 20% of controls, while expression in peripheral organs such as liver, spleen, kidney, lung and heart were up to 10 times higher than normal. Expression of GUSB in the brain maintained the structure, facial morphology and bone morphology of normal animals. GUSB was detected by PCR, enzyme activity and histochemistry in various organs and, in the brain, was detected by PCR. Whether brain cells

expressing GUSB activity were transduced, or had taken up GUSB from the circulation, was not determined [13•].

Although neonatal gene transfer can go as far as protecting from the development of structural and functional deficits, adult intrastriatal injection of feline immunodeficiency virus vectors expressing GUSB have also been shown to reverse deficits in spatial learning [14•]. Because the corresponding developmental time points at which to administer a given therapy will be difficult to determine in humans, combined approaches should provide various potential treatment strategies.

In a model using direct injections of 9×10^9 genome equivalents of AAV1, 2 and 5 into the lateral ventricle of C3H/HeOuJ or MPS VII neonates, AAV1 transduction was much greater than that of AAV2 or AAV5. High levels of expression were maintained for up to one year post-injection [15]. Neurons, but not astrocytes or oligodendrocytes, were transduced throughout the neocortex, entorhinal cortex, striatum and hippocampus, as well as in other brain areas (ependyma and choroid plexus). This indicates that intraventricular injection using AAV vectors can achieve long-term transduction when the injection is performed in neonatal mice. It will be important to compare these data with those achieved after injection of vectors into adult ventricles, and to determine whether any kind of immune responses are generated against the vectors and/or the transgenic proteins.

Another study used E1/E3-deleted recombinant adenovirus (CMV-GFP, 2×10^8 pfu total) and injected these into the tail vein of 8–12 week old female BALB/c mice. Animals were then perfused at 3, 6, 9, 14, 21 and 28 days. Peak expression in the brain was obtained after approximately 6–14 days and was almost eliminated by 28 days. Expression was found in the hippocampus, inferior colliculus and granular and Purkinje cell layers of the cerebellum; labeled cells appeared to represent Purkinje cells in the cerebellum and astrocytes in the hippocampus [16]. Although this has not previously been reported, the fact that several groups using various types of viral vectors are able to detect transduced cells in the brain will probably force researchers to re-evaluate this issue in much detail in the near future.

Non-viral plasmid vectors were also injected into adults and appeared to provide transgene expression in the brain. A tyrosine hydroxylase (TH) expression plasmid encapsulated inside a PEGylated immunoliposome targeted with the OX26 antibody to the rat transferrin receptor was injected intravenously into adult rats, resulting in the normalization of TH activity in the striatum of animals whose dopaminergic TH⁺ fibers had been eliminated. The animals demonstrated behavioral recovery, with TH immunocytochemistry also being detected in the previously denervated striatum. However, the cellular substrate expressing TH was not identified in these studies [17].

Direct intramuscular injection of AAV vectors was performed in experimental models of Fabry disease, caused by mutations in α -galactosidase A [18,19]. In one study, injections were given to C57Bl/6 $\times$ 129/svJ hybrid adult mice either via the tail vein or through an intramuscular route [18]; no enzyme activity was detected in the brain. However, another report detected significant transgene expression and correction of metabolic abnormalities in the brain of adult knockout mice following injection of AAV vectors selectively into the quadriceps muscle [19]; vector diffusion outside the injected muscle was not detected. The discrepancies between these two studies remain to be resolved.

Could injection of vectors into an organ such as the eye lead to transfer of transgene products all the way into the brain following a trans-synaptic pathway? Intravitreal injection of 1.5×10^7 infectious particles of AAV-expressing human GUSB under the β -actin/hCMV promoter into MPSVII mice led to GUSB activity within the thalamus and tectum and, interestingly, a reduction in lysosomal storage that extended to surrounding areas such as the hippocampus

and visual cortex. This suggests that both diffusion and trans-synaptic transfer of a lysosomal enzyme may contribute to the diffusion of enzyme activity throughout the CNS [20]. Importantly, by using PCR to detect viral genomes, the authors were able to confirm that the AAV vectors had remained within the injected eye.

Taking these results together, it appears that it may be possible to target the adult brain following systemic vector injections. However, it will be important to perform systematic studies across species at various developmental stages, as well as in adults, to determine the best conditions in which to target brain tissue, in particular neurons and glial cells. Although lysosomal enzymes appear to be taken up from the systemic circulation, transgene expression is much higher and diffuse throughout the brain if vectors have been injected directly into the brain ventricles or the brain parenchyma itself [21]. Intriguingly, in some cases, individual neuronal populations (e.g. Purkinje neurons) appear to be transduced following systemic vector administration. Establishing the conditions needed to achieve CNS transduction following systemic vector delivery remains an important challenge.

Using gene transfer to create novel genetic models of Parkinson's and Huntington's diseases

Lentiviral and AAV vectors allow long-term, relatively stable gene expression in the brain. Because mutations in several genes have been shown to cause familial forms of Parkinson's disease, Alzheimer's disease and other neurological diseases, lentiviruses expressing these mutated proteins have been constructed. Injection of these vectors into normal animals has now been used to overexpress these mutated proteins in an attempt to establish novel animal models of neurological diseases.

AAV vectors encoding 13 or 97 glutamine repeats fused to GFP were constructed. Expression of 97Q-GFP led to the formation of aggregates and loss of cells at 35 days post-transduction. Lentiviral vectors expressing either wild-type (19Q) or 44-66-82Q proteins were also used. In these experiments, polyglutamine repeats were linked to 171, 853 or 1 520 amino acid sequences under the control of the phosphoglycerate kinase promoter; the results showed that pathological effects depended upon both protein and polyQ length. The largest effects were seen when the shortest protein sequence was combined with the longest polyQ repeat. Pathology (inclusions) was detected one month after vector administration, and cell loss two months later. Both these studies have been performed using rats, and there is progress towards implementing these models in primates [22•].

Lentivirus and AAV vectors have also been used to create animal models of Parkinson's disease. AAV2 expressing human wild-type and A53T-mutated α -synuclein were used to infect the rat substantia nigra [22•]. Transgene expression was detected in almost all nigral dopamine neurons at one week, and reached peak levels at three weeks. The progression of pathology showed an intriguing time course; incipient inclusion of α -synuclein aggregates was detected from three weeks and was numerous at 10 weeks; the loss of nigral dopamine cells varied from 30–80% in individual animals, TH⁺ fibers were reduced by 50%; and functional deficits were detected in 25% of animals. Surprisingly, at six months, α -synuclein-positive inclusions had disappeared and TH⁺ fibres in the striatum recovered to 80% of controls, while dopamine content remained at 50%; animals displayed minor remaining functional deficits. In addition, pathology was similar in animals that had been injected with either AAV wild-type α -synuclein or the A53T mutant. Similar results were encountered in animals injected with AAV expressing the A30P mutant of α -synuclein. These data show that AAV vectors can be used to model synuclein overexpression, which has recently been shown to cause Parkinson's disease in humans [23]. This contrasts with transgenic animal models in which the overexpression of even mutated forms of α -synuclein led only to marginal anatomical or functional deficits.

When lentiviruses expressed A30P or A53T mutated forms of α -synuclein driven by the phosphoglycerate kinase promoter, transduction was achieved in 40–50% of nigral neurons. Such expression induced a loss of TH⁺ nigral neurons of 25–35%; that is, a loss of approximately half of all transduced neurons. Furthermore, only lenti-viral vectors expressing human α -synuclein were toxic. This approach is now being used in marmosets to create a non-human primate model of α -synuclein-induced neurodegeneration. The inclusions detected were not ubiquitinated, raising the question of whether α -synuclein is processed in a different manner when expressed from a viral vector [24•].

An important issue is that for none of these studies had the vectors been fully optimized for development of models of neurological diseases. Thus, further optimization of the promoters, levels of expression, cell type-specific expression or regulated expression is likely to provide further flexibility to these experimental models, and better establish how these mutated genes cause neurological disease. In view of the differences detected between these studies and the transgenic mouse models, natural progression of the human disease or neuropathological analysis of patients' brains, it will be necessary to study these models in further detail. Finally, in these studies, the presence of systemic or local immune responses to either the vectors or transgenes was not investigated [25,26••]. It will be important to determine whether this is a relevant factor in disease progression in these animal models.

Immune challenges to gene transfer into the brain

Advances have been made in the capacity to transduce individual neural cell types, achieve high levels of transgene expression that can be regulated within individual predetermined cell types, transfer large genomic sequences through the use of vectors deleted of all viral coding sequences, and achieve wide distribution of viral vectors throughout large areas of the brain (reviewed in [25]). However, important limitations of the successful implementation of clinical neurological gene therapy are the inflammatory and immune side effects of injecting viral vectors into the brain [26••,27].

Recent work has shown that two main types of immune responses to adenoviral vectors occur during the delivery of vectors into the brain. Initially, injection of vectors causes acute inflammation, accompanied by the release of cytokines, activation of microglial cells, influx of macrophages, upregulation of major histocompatibility complex (MHC)-I and MHC-II and an infiltration with CD4⁺ and CD8⁺ T-lymphocytes. This inflammatory response is transient; even after this inflammatory response is resolved, transgene expression continues, demonstrating that the acute inflammation does not abrogate transgene expression. Systemic immunization against adenovirus, moreover, leads to a longer lived brain infiltration with T-lymphocytes, microglial activation, and MHC-I and -II upregulation, together with a complete elimination of transgene expression [25,28–36].

Conversely, high-capacity helper-dependent adenoviral vector-mediated transgene expression in the brain has been shown to remain relatively stable in the presence of anti-adenoviral immunization given either before or after injection of these vectors into the brain [25,30–34, 36].

The extent of inflammatory and immune responses to other viral vectors injected into the brain remains to be fully elucidated. Some studies have shown that HSV-1 replication-defective vectors can cause brain inflammation [37]. In other cases, however, longer term transgene expression has been observed. Nevertheless, the sensitivity of HSV-1-mediated transgene expression to immune challenges following the delivery of vectors to the peripheral nervous system or CNS remains to be explored. Interestingly, HSV-1 virus enters latency in dorsal root ganglion neurons during its normal replicative cycle. The question of whether HSV-1-derived

vectors can enter latency, and the effect of immune challenges on expression of transgenes from latent vectors, are currently attracting much experimental efforts.

AAV vectors and lentiviral vectors have always been considered not to elicit adverse inflammatory or immune reactions. Recent important data, moreover, suggest that multiple injections of AAV vectors could actually lead to brain inflammation, and that repeated administration of AAV into the brain can lead to systemic immune responses, which can be detrimental to AAV-mediated transgene expression [38••]. To what extent viral vector-induced adverse immune reactions are caused by the viral capsid, transgene expression levels, total viral dose, cell types transduced, or the capacity of vectors to interact with inflammatory brain cells, such as brain microglia, remains to be determined.

Finally, genetic paradigms are now being applied to understand the molecular basis of immune responses to adenoviral vectors. Using >20 recombinant inbred BXD mouse strains, it has been shown recently that the longevity of transgene expression correlates with levels of cytotoxic T lymphocyte activity, rather than with the levels of interferon- γ , tumour necrosis factor- α or interleukin-6. This information was then used to identify quantitative trait loci that affect the longevity of trans-gene expression [39••]. The further use of recombinant inbred strains to discover genetic loci that determine immune responses which, in turn, affect transgene expression will allow the identification of causal factors regulating longevity of transgene expression. Understanding the cellular and molecular bases through which the immune system regulates transgene expression will allow us to optimize gene delivery.

Conclusions

Over the past 12–18 months, there have been many advances in the construction of viral vectors encoding large transgenic constructs, the targeting of viral vectors to sub-populations of brain cells, the establishment of strategies to achieve allele-specific silencing in dominant inherited neurological disorders, the systemic route to target viral vectors and/or their transgenic proteins to the brain, and our understanding of the cellular and molecular bases of immune responses to viral vectors in the brain. Importantly, there have been advances in the efficacy of gene therapy treatments in animal models of neurological diseases. Furthermore, viral vectors have now been used to develop animal models of neurological diseases. Progress over this time has not been limited to the topics discussed, but has also encompassed novel approaches to the treatment of obesity through the delivery of leptin receptors or pro-opiomelanocortin to the hypothalamus [40,41], the use of a variety of antiapoptotic growth factor approaches to the treatment of ischemia [5,42–51], the delivery of neprilysin, an enzyme that degrades amyloid- β in the brain, in animal models of Alzheimer's disease [52], and the use of neuropeptides for the treatment of seizures [53] or glaucoma-induced neurodegeneration [54]. The field of neurological gene therapy has thus continued to grow and expand towards the implementation of clinical trials of gene therapy for brain diseases.

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Abbreviations

AAV	adenovirus-associated virus
ALS	amyotrophic lateral sclerosis
CNS	central nervous system
eGFP	enhanced green fluorescent protein
EIAV	equine infectious anemia virus
GUSB	β -glucuronidase
HSV-1	herpes simplex virus type-1
IGF-1	insulin growth factor 1
MHC	major histocompatibility complex
SCA3	spinocerebellar ataxia type 3
siRNA	small interfering RNA
TH	tyrosine hydroxylase

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