

Progress and challenges in viral vector-mediated gene transfer to the brain

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Gene transfer into the brain allows the manipulation of transgene expression in both time and space. Recently developed gene transfer technologies allow transgenes to be expressed in any anatomically, biochemically or functionally distinct group of brain cells. Gene transfer has been used to alter the expression of neurotransmitter receptors, ion channels, signaling proteins, neuronal growth, differentiation and survival factors, and thus to modify brain anatomy, neuron physiology, behavior and pathology. However, challenges remain in making gene therapy a more widespread tool for the treatment of neurological disease. We have identified the following as areas needing development: access and delivery of viral vectors to the brain; diffusion of viral vectors and transgenes throughout large areas of brain tissue; viral vector side effects and toxicity; inflammatory and immune responses to vectors; long-term stable transgene expression; cell type-specific expression of transgenes; and the ability of the experimenter or physician to switch transgene expression 'on' and 'off' at will. In the last year, neuro-gene therapy has shown that brain defects in experimental disease models can be prevented and corrected, and that viral vectors and encoded transgenes can be made to diffuse over larger brain areas. In addition, the cause of vector-induced inflammation and immune responses have begun to be elucidated, so that rational approaches can be developed to avoid these complications. Further improvements in viral vectors will facilitate clinical trials in the near future.

Keywords Immune responses, inflammation, longevity of expression, viral vectors

'I have no doubt that in reality the future will be vastly more surprising than anything I can imagine.'

JBS Haldane, 'Possible Worlds and Other Papers', 1927.

Introduction

Research into gene transfer to the brain has mushroomed during the last 12 years [1]. From only 14 papers published in 1990 to 294 papers published in 2001, the field has grown to produce a staggering total of over 2000 papers in the last decade. However, the number of clinical trials in the gene therapy of brain diseases is much smaller. What are the challenges that remain to be overcome in order for gene therapy to develop into an even more powerful research tool, and an easily accessible clinical resource? Two main challenges facing gene transfer to the brain, and gene therapy of brain diseases, are the delivery of viral vectors to the brain and their access to the target brain cells, and the side effects of using viral vectors as therapeutic agents, namely inflammation, immune responses and cytotoxicity.

These challenges and the progress made in these areas over the last two years will be reviewed herein.

Long-term expression in the brain in the absence of integration into host chromosomes may be a property shared by vectors based on herpes simplex virus-1 (HSV-1), adeno-associated virus (AAV) and high-capacity, helper-dependent adenovirus (HC-Adv). This feature confers an additional safety measure, since it will be less likely to lead to irreversible chromosomal alterations [2-7]. Additionally, the large cloning capacity of HC-Adv- and HSV-1-derived vectors will allow for the incorporation of regulatory sequences, which will confer further safety features by allowing transgene expression to be selectively turned 'on' or 'off' [2-5,8,9,10]. Powerful vectors will also play an important role in the examination of the molecular basis of brain function. Although transgenic animals can be constructed to overexpress ('knock-in'), or abolish ('knock-out'), individual genes throughout the life of an animal, it remains difficult to selectively manipulate the expression of individual genes in adult experimental animals. New technologies being developed ostensibly for gene therapy will undoubtedly be of use in manipulating the molecular basis of neural function in adult, non-genetically manipulated, experimental animals.

Viral vector access to target brain cells

Entering the brain

Direct physical access to brain cells is limited. Isolated from the outside world, the brain is protected by the cranium and the meninges, and it is separated from the internal milieu by the blood-brain barrier (BBB), and the brain-cerebrospinal fluid (CSF) barrier (BCB). Access to any given region of the brain from the outside requires crossing either the bone/meningeal barriers, while accessing the brain from within the vasculature requires crossing the BBB or BCB.

Direct access to neurons in the spinal cord, autonomic ganglia and the brain can be gained through nerve, such as those from motor neurons, the autonomic nervous system or olfactory neurons that have terminals located outside the central nervous system (CNS). Retrograde transport from these nerves to the brain allows direct access to the CNS, and is the entry pathway used by a number of neurotropic viruses, such as HSV-1, rabies and polioviruses. However, due to the incapacity of defective vectors to move trans-synaptically between neurons, this pathway has not been much exploited to achieve distributed delivery of transgenes throughout the brain. Powerful neuroanatomical tracing techniques using pseudo-rabies viruses suggest that circuit-specific gene delivery may one day become possible [11,12,13]. For restricted delivery, however, retrograde transport from muscles back to the cell bodies of motoneurons in the anterior horn of the spinal cord provides local secretion of growth factors. Since secreted growth factors become available to more cells than those that became infected by a viral vector, this could constitute a reasonable approach to gene therapy. Expression of survival factors acting on intracellular targets would be efficient, but

would only allow for survival of the transduced cells. Similar delivery approaches are being applied to sensory nerves for the treatment of pain, and could be adapted to other sensory neurons that have nerve terminals of relatively easy access to external viral vector applications, such as olfactory neurons through the nose, retino-thalamic neurons through the eye, and the vestibular and acoustic neurons through the ear.

Crossing the BBB

If vectors could be targeted to cross the BBB directly and specifically, then systemic administration of vectors to the brain would become possible. Current methods used to allow viral vectors, or even chemotherapeutic agents, to cross the BBB include osmotic opening, pharmacological opening and the use of targeting vectors to facilitate crossing of the BBB by binding to specific receptors that mediate transcytosis through the endothelium, eg, the transferrin receptor. Although manitol-induced osmotic shock, bradykinin-induced BBB opening and transferrin-mediated transport have all been used, these methods mostly allow only a low level and anatomically diffuse delivery of vectors to the brain. However, a number of reports have indicated that in the case of brain tumors, more specific delivery has been achieved due to the blood-tumor barrier being 'weaker' than the BBB [1]. The passage of cells and proteins through the BBB in inflammatory brain diseases, such as multiple sclerosis, is also compromised, a fact that could aid the systemic delivery of vectors selectively to the affected brain regions in such patients. Whether molecules present on the intact BBB could be used to target specific and restricted neuroanatomically defined regions, such as the substantia nigra, through intravascular systemic delivery, remains to be demonstrated.

Intravascular delivery presents a number of further challenges. There is the potential for systemic toxicity through interactions of the viral vector particles with elements of the innate immune system, such as complement and the macrophages, and the effect these interactions have on reducing the bioavailable viral vector in blood. Chiocca and collaborators [14-16] have shown that complement depletion significantly increases the amount of HSV-1 viral vector that reaches the brain. The precise role of perivascular macrophages in the trans-endothelial delivery of viral vectors needs to be examined in detail.

Diffusion throughout the brain

The distribution of transgene expression throughout the CNS

Once a vector is injected into the brain, it will diffuse until interacting with specific receptors on the surface of target cells that allow its internalization, or binding sites in the extracellular matrix that can sequester the virions. Only relatively small volumes are usually injected into the brain parenchyma proper, preferably below 3 μ l into a rodent brain. However, neuroanatomists will usually limit their injections to only a few nanoliters. These smaller volumes can also be employed when using replication-competent viruses. However, to transduce reasonable amounts of brain tissue with a non-replicating vector, at least several microliters need to be injected, independently of the method of injection.

Bankiewicz *et al* have recently utilized convection-enhanced delivery directly into the brains of rodents and non-human primates [17,18,19]. Convection allows greater CNS diffusion and an increased volume of fluid to be delivered to the brain in a non-traumatic manner. This method resulted in a significant increase in the area of diffusion of AAV-2-derived viral vectors throughout the brain, and a corresponding increase in the distribution of intracellular proteins (eg, L-amino acid decarboxylase and HSV-1-tk) and secreted proteins (eg, glial-derived neurotrophic factor (GDNF) and neurturin). While regular pressure injection is limited to 2 to 3 μ l of virus vector solution, convection allows the injection of 20 μ l (to diffuse over an entire rat striatum) to 100 μ l (to diffuse over an entire rat hemisphere) of vector solution. In primates, 100 μ l will mediate viral vector-mediated transduction of the entire putamen. Convection-mediated delivery enables the injection of large volumes of vector-containing solution to transduce large brain areas without causing any damage to the brain, which would ensue if such large volumes were to be injected using conventional methods [17,18,19].

In an ingenious experiment, Nguyen *et al* [18] and Hamilton *et al* [19] coated viral vectors non-covalently with heparin to block the interaction of the AAV viral capsid with tissue heparan sulfate proteoglycan. The prediction was that by reducing the interactions between the viral particles and the tissue, the vectors would be able to diffuse further away from the injection site. This occurred as predicted, and suggests that an important factor limiting the diffusion of viral vectors throughout the brain is their interactions with cellular receptors and extracellular matrix molecules, which effectively trap the vector. These data are further supported by work using adenovirus type 5 (Ad-5)-derived vectors. Thomas *et al* used adenovirus to which poly-lysine had been covalently added to the virions, or vectors carrying mutations in the genes encoding fiber and penton base that abolished their capacity to interact with the receptors CAR and integrins [20]. While the capsids containing poly-lysine remained very close to the injection needle track into the brain, virions unable to bind CAR and integrins appeared to diffuse further than wild-type virions. These results suggest that receptor interactions determine the diffusion of virions from the injection site. Poly-lysine presumably binds to ubiquitous heparan sulfate proteoglycan, and thus blocks the virions from diffusing throughout the brain, while virions unable to bind to any receptors remain relatively free to diffuse over larger distances. Importantly, this doubly ablated vector did not transduce any brain cell type, while still causing an acute brain inflammatory response.

The diffusion of viral vector virions, genomes and transgenes throughout the brain: How far can they get?

Although viral vector diffusion is limited, the extent of these limits remains to be determined. So far, different authors have determined the degree of viral vector diffusion using different methodologies. For example, a straightforward method is to label the viral capsid itself and determine its diffusion throughout the brain. However, these experimental approaches have yet to be applied in detail to the currently used viral vector systems.

Viral vectors injected into the brain diffuse until encountering receptors that can bind their capsid proteins. If those receptors

are located on a cell, viral capsids may be internalized; if those binding sites are part of the extracellular matrix, they will retain the virions therein and thus limit diffusion. Upon infection, the genome of DNA viral vectors is delivered to the nucleus, while the capsid is either disassembled or degraded during the infection process. While this process has been studied in detail in epithelial cells, it remains to be characterized in neurons. For example, which neuronal surface do different viral vectors use to infect/enter neurons? Are neurons infected by virus attached to their cell bodies or dendrites, or are viral vectors preferentially taken up by axonal terminals? Do viral vectors move anterogradely (from cell body to axon terminals), retrogradely (from axonal terminals to cell body), or both, upon injection of vectors into the nervous system? Do all viral capsids use common intracellular transport mechanisms, or will this process be shown to be specific to each viral vector? This is an important issue, because mechanisms of virus uptake into brain cells may determine the best route of infection. For example, if uptake by axon terminals would be the vector's primary mechanism of entry, and transduction of subcortically projecting neurons distributed throughout large areas of the cortex is the aim, then an injection into the white matter of the corpus callosum would be the ideal administration site. At present, the mechanisms of virus uptake into brain cell subtypes are not known.

Vector diffusion will also be modified by the nature of the brain cells targeted by each vector. Information gathered so far indicates that lentiviruses and AAV preferentially infect neurons, both *in vivo* and *in vitro*, while adenoviruses infect both astrocytes and neurons [10,21••,22•]. Furthermore, different virus serotypes, in the case of AAV, diffuse over different brain volumes, with AAV-5 transducing more cells and covering a larger area than AAV-2 [22•]. However, it has not been determined how viral vectors enter into neurons. A number of gene transfer vectors (eg, adenovirus, lentivirus, HSV-1 and plasmid DNA) injected into muscle allow transgene expression in neuronal cell bodies in the spinal cord. This demonstrates that the axons of motoneurons in the spinal cord can take up viral vectors and transport them retrogradely to the anterior horn cell bodies. There is further evidence that injection of adenoviruses into the CNS can lead to a very wide distribution of transgene throughout the brain, presumably also by retrograde intraneuronal distribution of viral vectors taken up by axonal terminals at the site of injection [23•]. Similar data have also been reported using AAV and HSV-1 vectors. Interestingly, Zermansky *et al* [23•] reported the presence of the transgene-encoded protein and evidence for the presence of the viral vector genome. This suggests that viruses could diffuse throughout larger areas than hitherto suspected, a phenomenon also detected for AAV virions and AAV genomes [JM Heard, personal communication]. Widespread distribution thus depends on a number of physical variables, such as injection method (eg, convection versus direct injection), modifications used to make the virion less 'sticky' (eg, by adding heparin to block binding of virions to tissue receptors like proteoglycans), the site of injection allowing greatest uptake (eg, muscle and corpus callosum injection maximizing uptake into axonal terminals), and the expression cassette itself (eg, promoter and transgene), as well as the sensitivity of the detection system.

Virally encoded transgene diffusion: Is what you see what you get?

Intracellular gene products

Viral vector diffusion is most commonly assessed through transgene expression, ie, using the histochemical or immunohistochemical distribution of the bacterial transgene product β -galactosidase from *Escherichia coli*. A shortcoming of this method is that virion diffusion measured in this way represents the end result of a complex series of processes, namely the actual diffusion of the virion throughout the brain's extracellular space, efficiency of infection of brain cells and efficiency of transgene expression. Thus, if transgene expression is suboptimal, it will be read as reduced virion diffusion. A complete description of the efficiency of this process, ie, the efficiency of physical diffusion throughout the brain tissue, viral vector uptake into cells, genome nuclear targeting and transgene expression, remains to be determined for any viral vector.

In 1999, Dewey *et al* [24] indicated that the protein thymidine kinase (tk), encoded by a gene derived from HSV-1, within adenoviral vectors, could be detected over much larger regions of the forebrain than if the transgene used encoded β -galactosidase. Furthermore, recent experiments by Zermansky *et al* [23•] demonstrated that this increased distribution was not related to the concurrent administration of ganciclovir, nor was it dependent on the injection of the HSV-1-tk-expressing vector into a brain tumor. Thus, when compared side by side, the distribution of HSV-1-tk was much more widespread than the distribution of β -galactosidase. The vector genomes, which contained either genes encoding tk or β -galactosidase (first-generation Ad-5), as well as the promoter driving their expression (major immediate early murine cytomegalovirus; MIEhCMV), were identical.

The logical conclusion of these experiments is that the HSV-1-tk gene must contain genetic elements that allow higher expression levels that are consequently detected as more widespread distribution when compared with the expression of β -galactosidase. It is unlikely that the inclusion of the HSV-1-tk gene would increase the intracerebral diffusion of the adenoviral vector. Furthermore, similar results have been shown when HSV-1-tk is encoded by AAV vectors [18•]. Since vector genome sequences could be detected throughout the brain (most likely distributed through retrograde axonal transport), adenovirus genomes reach much larger brain regions than is usually recognized. Such enhanced expression in distant sites also raises new safety issues. In addition, it forces us to consider whether β -galactosidase is a reliable reporter transgene, or whether it underestimates the true extent of expression from viral vectors throughout the brain? This is an important issue that needs to be urgently addressed.

It will be important to quantitatively analyze each of the processes that contribute to the diffusion of viral vectors and their transgenes. To state it briefly, viral vector diffusion could be studied using fluorescently labeled virions; viral vector infectivity by using *in situ* quantitative PCR for vector genomes at short times after virus infection; quantitative RT-PCR to quantify the levels of mRNA; and transgenes could be quantified using various quantitative anatomical or

biochemical methods. A systematic application of these methods will determine the extent and limitation of all the steps involved in the diffusion of viral vectors, and those involved in determining the area of brain sustaining transgene expression, from the injection of the viral vector into the brain, to the expression of the desired transgene. Identification of limiting steps will facilitate the development of more powerful vectors.

Extracellular gene products

In principle, secreted transgene products, such as the dopamine neuron growth factor GDNF, will distribute over a larger area of the brain than either β -galactosidase or HSV-1-tk, due to the fact that each transduced cell actually secretes the protein into the extracellular space, from where it can diffuse to affect the function of other cells. Indeed, this has been observed independently of the viral vector used. Recent elegant work using lentiviral vectors expressing GDNF has shown powerful neural protection of substantia nigra neurons against MPTP-induced neurodegeneration, as well as in age-related decrease of dopaminergic striatal innervation in non-human primates [25••]. While secreting a protein into the extracellular space will improve the overall biological efficiency of a growth factor acting on plasma membrane receptors, secretion cannot improve the efficiency of a protein that needs to act intracellularly, and cannot be taken up from the extracellular space.

This challenge has been approached using essentially three methods. Lysosomal enzymes are absent or defective in a number of metabolic diseases, many of which have debilitating CNS symptomatology. Importantly, lysosomal enzymes are targeted to lysosomes through an intracellular targeting pathway, the mannose-6-phosphate (M6P) pathway. Lysosomal enzymes are thus recognized by the M6P receptor (M6P-R), which targets lysosomal enzymes to the lysosomes. This pathway also allows lysosomal enzymes to be released into the extracellular space, from where they can again be taken up by M6P-Rs present on the plasma membrane of neighboring cells. Such M6P-R bound to lysosomal enzymes will target these to the lysosomes. Thus, affected cells can take up lysosomal enzymes from the extracellular space, and target these enzymes to the correct intracellular compartment where they are needed. While in some cases the enzymes can be administered systemically to improve liver function, brain disease is never modified by systemic administration of lysosomal enzymes, due to the poor penetration of the BBB by these enzymes. In some patients, bone marrow transplantation has been shown to ameliorate brain disease, supporting the hypothesis that cells migrating to the brain can release lysosomal enzymes, which can be taken up by brain cells and thus improve the functioning of surrounding (non-transduced) cells missing such enzymes. An important advance in this direction was presented in a recent publication that demonstrated that treatment of already affected animal models of mucopolysaccharidosis type VII (MPS VII) or metachromatic leukodystrophy (MLD) with lentiviral vectors expressing β -glucuronidase (for MPS VII) or arylsulfatase A (for MLD) either protected animals from the disease and/or reversed its neurological symptomatology. This demonstrates that lysosomal enzymes, even if expressed from more restricted anatomical distributions, will diffuse over larger areas of the brain, and thus provide sufficient enzyme to produce therapeutically relevant anatomical and behavioral changes [21••,26••].

Lysosomal enzymes thus contain within their structure all the signals to ensure their release and re-uptake into deficient cells. To improve the intracerebral distribution of proteins that are normally neither secreted nor taken up as part of their normal life cycle requires new approaches. The HSV-1-encoded VP22 protein is released from infected cells, and taken up by neighboring, non-infected cells. Although the mechanism by which VP22 is released and taken up is not understood at the molecular level, various groups have now shown that fairly large proteins can be synthesized as chimeric proteins and that these chimeras, even though of higher molecular weight, follow the normal distribution of wild-type VP22, ie, they are released into the surrounding extracellular space, and are taken up by otherwise non-transduced cells [27,28].

Recent studies have shown that relatively short peptides derived from the protein transduction domain of the HIV Tat protein enable various types of proteins to be taken up into cells. Again, as with VP22, the underlying mechanism of action is not completely understood, but if the transduction domain is linked to a marker protein such as β -galactosidase or β -glucuronidase, entry into cells and diffusion throughout the brain was markedly increased [29]. Although an earlier report suggested that systemic administration of a chimeric Tat- β -galactosidase protein was able to cross the BBB when partially denatured [30], it could not when expressed from an AAV vector [29]. This may depend on whether the chimeric protein is partially unfolded or other experimental factors. This intriguing result will need to be explored in further detail, since at this time there is no reagent available that allows reliable crossing of the BBB in the absence of active disruption. Interestingly, the protein transduction domain of Tat is able to increase the diffusion of a protein normally secreted in the brain. Furthermore, although Xia *et al* [29] were unable to see the chimeric peptide crossing the BBB, injections into the brain's lateral ventricle led to diffusion of the chimeric protein into the brain tissue surrounding the ventricles. Thus, these authors have shown that Tat- β -glucuronidase expressed from AAV vectors could cross the brain-ventricular barrier. It remains to be determined whether a highly efficient infection of the brain endothelium will likewise produce enough protein to be delivered directly to the brain, from the systemic vasculature. Both the ventricular ependymal layer and the brain microvascular endothelium are polarized cells. If vectors can be engineered to selectively infect these cells, and further engineered to take advantage of the macro-pinocytosis and transcytosis targeting mechanisms of these cells (already employed by HIV to enter the brain), these methods will provide some very interesting tools for generalized and diffuse gene delivery to the brain [31].

How do viral vectors affect the brain?

Thresholds: Toxicity, innate inflammation and adaptive immune responses

Viral vectors can induce side effects upon injection into the brain. This toxicity can be detected *in vitro* or *in vivo* following the injection of vectors directly into the brain, and depends on a number of factors [20•,24,32,33,34•,35•,36•], such as the viral capsid itself, viral proteins packaged into the virions and the vector's genome. For first-generation adenoviral vectors, or non-defective herpes virus vectors, toxicity can also depend on the expression of viral proteins

encoded by the viral genome. In addition, transgene toxicity could be due to either the protein itself or its uncontrolled overexpression. The effects that high-level and prolonged constitutive transgene expression may have on neuronal function have not yet been evaluated in sufficient detail.

Although experiments have shown that adenoviral-mediated gene therapy can have potent pharmacological effects in experimental models of brain disease, the vectors utilized do have shortcomings. First-generation recombinant Ad (RAd) vectors injected into the brain parenchyma cause acute cellular- and cytokine-mediated inflammatory responses, and do not stably transduce in the presence of anti-adenoviral immune responses. However, adenovirus-induced cytotoxicity is only seen at high vector doses used to transduce cells, and the consequent high-level expression of viral genes from the genome of first-generation RAd vectors. Additionally, direct vector-toxicity, acute inflammation and/or the anti-adenoviral T-cell responses limit long-term transgene expression from first-generation adenoviral vectors.

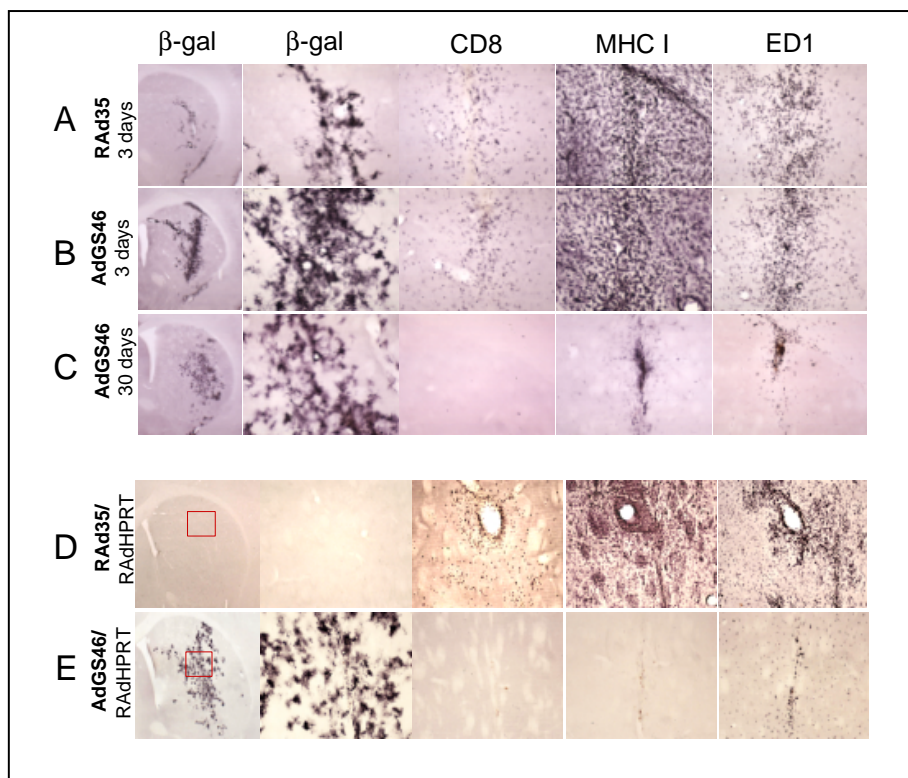
Our experiments have confirmed that injections of RAd or HC-Adv into the brain do not prime a systemic anti-Adv

immune response. Priming of immune responses occurs in lymph nodes. Thus, to prime an anti-adenoviral immune response, Adv needs to be injected peripherally, outside of the brain, eg, intradermally in our experiments.

Toxicity

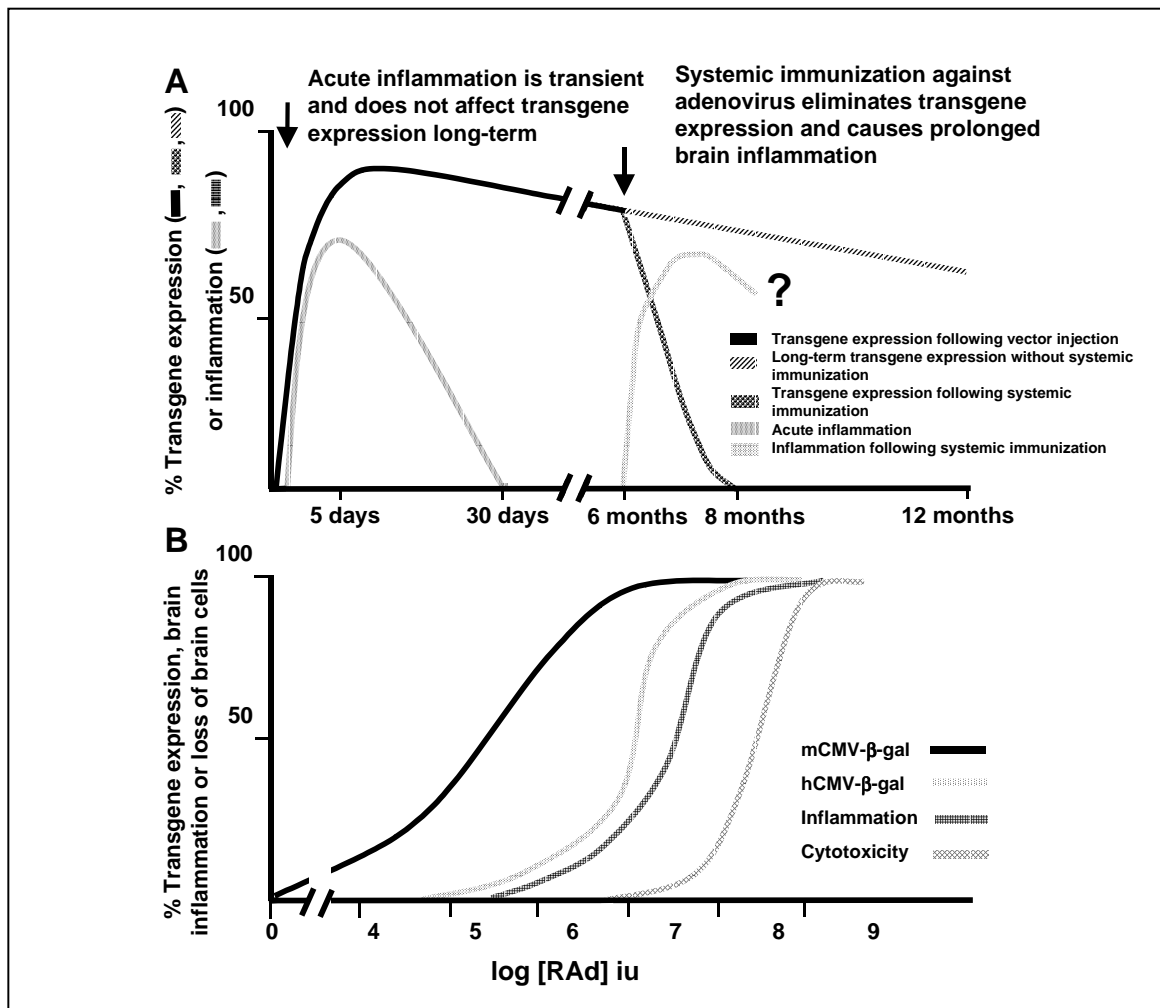
Adenoviruses are efficient vectors to transfer genes into the brain. However, they have been associated with acute dose-dependent inflammatory and cytotoxic reactions. On the dose-response curve to the injection of first generation adenoviral vectors into the brain, inflammation (measured as influx of macrophages, activation of astrocytes and upregulation of MHC class I and II, as described in [35•]), is first detected between 1×10^6 to 1×10^7 iu (see Figures 1 and 2, and described in detail below), individual inflammatory markers displaying slightly different dose-response curves [36•]. No cytotoxicity (measured as decrease in the brain area occupied by neurons or astrocytes [36•]) is seen at these doses of viral vector. The severity of the inflammation peaks at 1×10^8 iu. Importantly, however, no cytotoxicity is detected following the injection of up to 1×10^8 iu (injected in a total volume of $3 \mu\text{l}$), ie, no decrease in neuronal and glial cell markers (Figure 2A and 2B).

Figure 1. Transgene expression (β -galactosidase) and inflammation in the brains of animals injected with either the E1/E3-deleted vector RAd35 or the HC-Adv AdGS46.



Rows **A** and **B** show β -galactosidase expression (columns 1 and 2), CD8+ cell infiltration (column 3), MHC class I upregulation (column 4) and microglial cell activation (ED1, column 5), 3 days after injection of 1×10^7 iu of RAd35 (row **A**) or AdGS46 (row **B**) into the brains of naïve animals. Levels of transgene expression and inflammation mediated by the E1/E3-deleted, or the HC-Adv vector, were indistinguishable in naïve animals. Transgene expression from 1×10^7 iu of both RAd35 and AdGS46 is stable for at least 30 days in naïve animals, and inflammation resolves within this time frame (shown in row **C** only for AdGS46). However, transgene expression from E1/E3-deleted vectors in the CNS is rapidly eliminated and accompanied by severe brain inflammation if animals receive a subsequent peripheral infection with adenovirus (row **D**; RAd35 injected in the brain at day 0, RAdHPRT [56] injected in the skin at day 60). In contrast, transgene expression from HC-Adv remains stable, and no brain inflammation is elicited when animals are subsequently injected with RAdHPRT in the skin (row **E**; AdGS46 injected in the brain at day 0, RAdHPRT injected in the skin at day 60). For further details see [41•].

Figure 2. Time course of inflammation and dose-response curves of gene expression.



(A) Time course of acute brain inflammation in response to adenoviral vector injected into the brain, and inflammation in response to a peripheral immunization with adenovirus in animals previously injected into the brain. (B) Dose-response curves of expression of β -galactosidase expression from different promoters, intensity of acute inflammation and cytotoxicity in the striatum in response to the injection of first-generation adenoviral vectors. (A) Illustrates that acute inflammation in response to adenovirus injection into the brain is transient, and decreases to negligible levels 30 days post-injection, while transgene expression can be detected for up to 12 months. Both the acute inflammation and the longevity of transgene expression are comparable for first-generation and HC-Adv vectors (illustrated in Figure 1). However, upon systemic immunization, eg, skin, with a first-generation adenovirus containing a different transgene from that being expressed in the brain, a very strong immune response ensues that can be detected in the brain and completely eliminates transgene expression. The question mark at the end of the curve representing brain inflammation following skin immunization indicates that although it is known that the brain inflammation persists for much longer than the acute inflammation, its extent has not yet been established. (B) Shows curves comparing transgene expression from either the murine cytomegalovirus (mCMV) or the human cytomegalovirus (hCMV) promoter, acute inflammation and acute cytotoxicity. This schematic figure illustrates that the dose-response curve is different for each parameter measured. Note that using the mCMV promoter, one obtains maximal expression in the absence of inflammation or cytotoxicity, while when using the hCMV promoter to drive transgene expression, much higher viral doses are needed, and thus high-level expression will be compromised by concomitant inflammation and/or cytotoxicity. For further experimental details, please consult the original publications [36•,39••,41•,42•]. The cell types responsible for recognizing the adenovirus particles and brain cell infection, and mediating the acute innate or adaptive immune inflammation in the brain, are currently being established.

During our experiments on brain immune responses to adenoviral vectors, we uncovered an important threshold following the injection of 1×10^8 iu of first generation adenoviral vectors into the brain. Doses above 1×10^8 iu cause acute cytotoxicity, with a consequent loss of neurons and glial cells, as well as an acute and long-term reduction in levels of transgene expression. Over time, this high-dose injection leads to loss of brain substance. This pattern of cytotoxicity is only observed at doses above 1×10^8 iu. These results explain the commonly reported finding that at such

higher, but not at lower, doses of adenovirus long-term transgene expression is curtailed. Acute cytotoxicity, which we believe is due to the high concentration of virions injected into the brain, indicates that the highest dose of adenoviral vectors that can be safely injected into any single site within the brain should not exceed the dose of 1×10^8 iu. Although these experiments were performed using first-generation viral vectors, the fact that this toxicity is not transgene related, but can be eliminated by heat-inactivation of the adenoviruses, strongly suggests that acute toxicity is

indeed virion-dependent. Therefore, we would expect very similar acute toxicity profiles from injection of high-capacity helper-dependent adenoviral vectors. Thus, our experiments establish 1×10^8 iu as the maximum tolerated dose per direct injection site into the brain. Since there is no priming of the systemic adaptive immune response, this upper dose limit would potentially hold for each completely independent injection site into the brain; higher total doses could still be safely delivered to the brain (Figure 2B).

Aside from the capsid, long-term presence of a transgenic protein or even neuronal infection with a viral vector per se could lead to toxicity in the medium to long term. To examine this possibility, Kaspar *et al* used transgenic mice carrying a *GFP* gene that is not expressed due to a transcriptional 'stop' signal situated in front of it [37••]. The transcriptional 'stop' sign was flanked by *loxP* elements. Kaspar *et al* subsequently injected AAV vectors expressing *Cre* recombinase into various areas of the brain, and observed that *Cre* expression was able to remove the 'stop' signal and allow GFP expression. Furthermore, they showed that GFP expression was stable for up to 6 months. This important experiment demonstrates that long-term infection of neurons, and presumably long-term non-regulated expression of *Cre*, is non-toxic. It is now crucial to repeat this experiment with different viral vectors, different transgenes and in different brain cell types.

Finally, transgenes themselves could be neurotoxic. This applies to therapeutic transgenes and also to the marker transgenes that are used to set up the experimental designs and monitor the efficiency of transgene expression from the viral vector. Very recently, Detrait *et al* have examined the neuronal toxicity of three different reporter transgenes delivered by two different types of HSV-1-derived vectors [38••], and determined that both enhanced green fluorescent protein (eGFP), nuclear-targeted β -galactosidase and cytoplasmic β -galactosidase were cytotoxic. Cytotoxicity was marker-dependent, with the highest toxicity attributable to eGFP. These toxicity data are of crucial importance, and should now also be examined for other viral vectors. How the vector context influences transgene expression and toxicity will have to be studied in each case.

Inflammation and innate immune responses in the CNS

Early brain inflammation following the injection of both E1/E3-deleted, and high-capacity helper-dependent adenovirus vectors into the brain is transitory and does not reduce β -galactosidase expression

Detailed dose-response curves showed that brain inflammation (measured as influx of macrophages, and activation of astrocytes, among other markers), is first detected between 1×10^6 and 1×10^7 iu; below these doses, no sign of inflammatory cell influx or upregulation of expression of MHC molecules is present. Cytotoxicity, however, occurs only above the threshold of 1×10^8 iu injected into a single brain site (Figure 2A (time course), and 2B (dose-response curve)). Taken together, data supporting that both inflammation and cytotoxicity are not transgene-dependent [36•,39••,40], and that cytokine release and fever induction could be blocked by heat inactivation of adenovirus virions [40], suggest that the adenoviral capsid itself may be

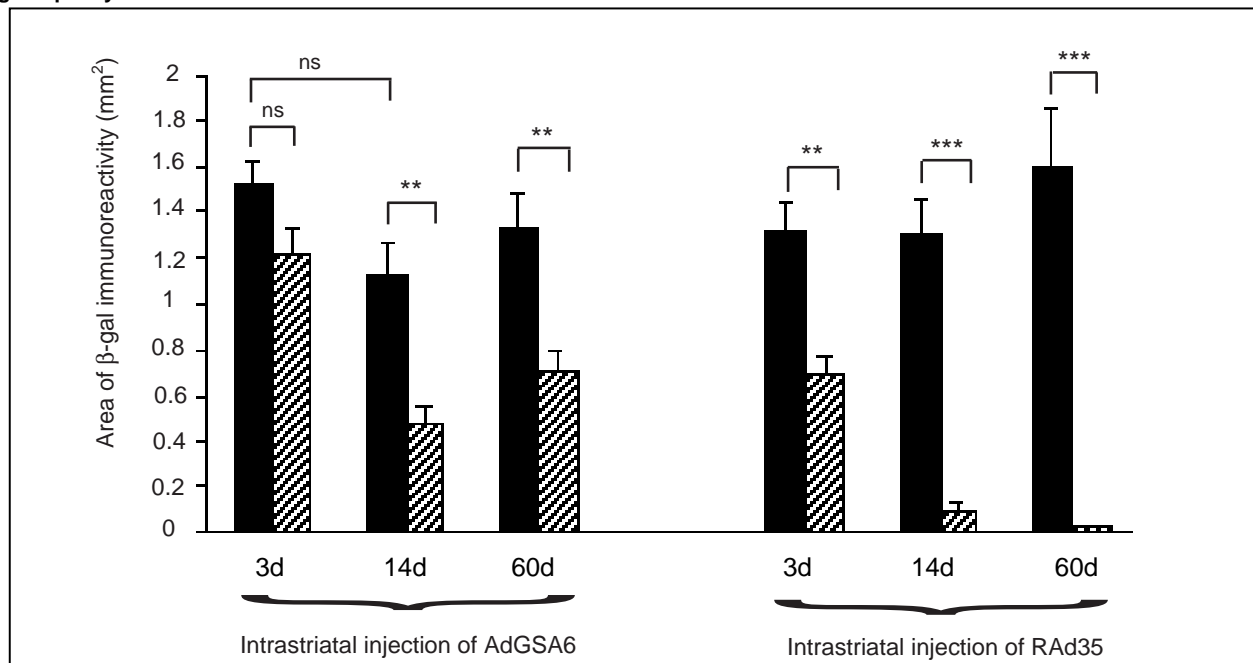
responsible for the inflammation and cell death observed at very high doses. However, the available data do not enable the determination of whether inflammation and cytotoxicity would be contingent on the presence of the adenoviral capsid alone, or the actual adenoviral vector genome.

To unravel the mechanisms underlying adenovirus-induced inflammation, we compared the inflammatory response to the 1×10^7 iu of either a first-generation adenoviral vector, or the novel high-capacity helper-dependent adenoviral vectors. These two vectors share exactly the same capsid, but the genome of the high-capacity adenoviral vector only contains the inverted terminal repeats (ITRs), which are needed for viral and vector DNA replication, and the packaging signal from the wild-type adenovirus genome. In contrast, the genome of first-generation vectors contains most of the wild-type genome, with the exception of the E1 and E3 regions. The question to be addressed was whether the capsid was responsible for the inflammatory response, in which case both vectors would cause identical inflammation, or whether the vector genome played a major role, in which case the inflammation caused by either vector would differ substantially, either quantitatively or qualitatively [41•].

Injection of either vector directly into the striatum demonstrated the following: injection of 1×10^7 iu of either E1/E3-deleted vectors or HC-Adv into the brain caused comparable acute, transitory inflammation, with both cellular (eg, activation of microglia) and molecular components (eg, upregulation of MHC class I and II). Importantly, and in confirmation of data already published previously, the acute inflammation caused by either vector did not affect long-term transgene expression [24,41•] (Figures 1 to 3); expression for both first-generation and HC-Adv persisted for up to 6 months. Transgene expression was further detected at 12 months, although the expression of a marker transgene from first-generation vectors appeared to decrease, when compared to the expression of the high-capacity vector [41•, PR Lowenstein, unpublished data].

The experiments described above provided strong support for the hypothesis that the adenovirus capsid was indeed responsible for inducing inflammation. Thus, we explored whether the interaction of adenovirus with its receptors for binding to the target cells and cell entry were necessary to induce inflammation. Thus, the capacity to bind to CAR and integrins is necessary to transduce brain cells, but binding to these receptors is not necessary for adenovirus virions to induce inflammation. Vectors unable to bind CAR or integrins did not support any transgene expression in the brain, but still caused brain inflammation. An interesting observation was that the brain area over which these double-deleted vectors caused inflammation appeared to be larger than that caused by vectors using the wild-type receptors. Thus, the capacity of vectors to bind to various cellular receptors determines both the diffusion of vectors throughout the brain, and their capacity to cause acute inflammation. These data demonstrate that adenovirus-induced inflammation is independent of binding and signaling through CAR and integrins, but dependent on the structural integrity of the capsid, which if dissociated is unable to cause inflammation [20•] (Figure 4).

Figure 3. Pre-immunization against adenovirus rapidly eliminates transgene expression from E1/E3-deleted vectors, but not from high-capacity vectors.



Quantification of the area within 40 μm thick brain sections occupied by β -galactosidase. Error bars show the SEM value from the five animals in each experimental group. Student's t-test was used to calculate the degree of significance of differences between levels of transgene expression and inflammation in the brains of non-immunized animals (black bars) and immunized animals (hatched bars) after intrastriatal injection of RAD35 or AdGSA6 [34•,40]. Note that in the absence of pre-immunization, transgene expression from either viral vector is stable. In the presence of pre-immunization, most transgene expression from a first-generation vector is eliminated by 14 days, while that from an HC-Adv remains at 50%. The lack of a decrease of expression from the HC-Adv at 3 days suggests that humoral immunity is not responsible for the decrease seen for the first-generation vector, since anti-adenovirus antibodies cannot distinguish the capsids of either vector. That the inhibition of expression from the HC-Adv is transient suggests that this vector allows the transient presentation of an antigenic epitope to the activated T-cells. We suggest that transient presentation of capsid epitopes is responsible for this decrease. In the case of the first-generation vector, continued production of capsid epitopes allows T-cells to eventually eliminate transgene expression completely. Modified from [42•].

Adaptive immune responses

Expression from high-capacity adenovirus vectors is stable in the presence of systemic anti-Ad-5 immune responses

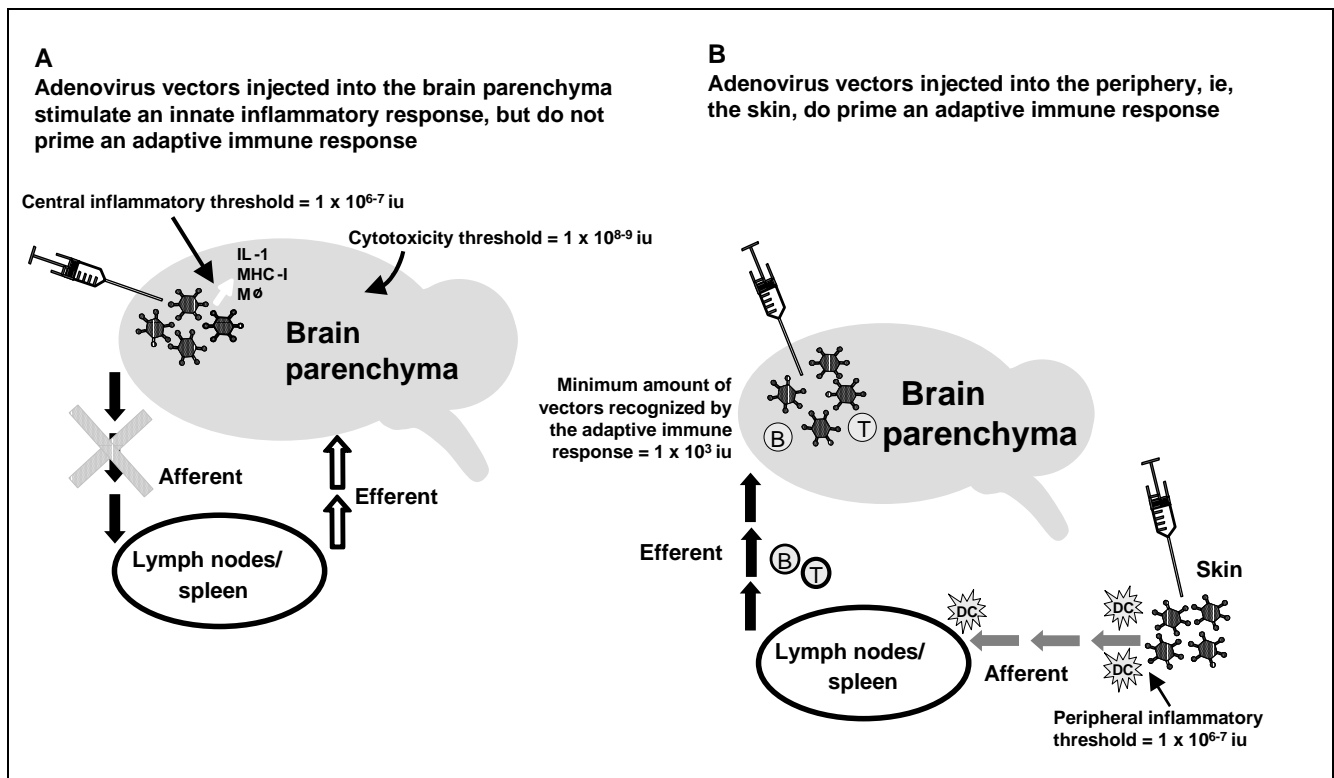
To evaluate the responses of brain transgene expression in response to systemic immunization against adenovirus, inflammatory responses and transgene expression were studied following the intrastriatal injection of Ad-5 E1/E3-deleted (first-generation) or HC-Adv vectors [41•,42•]: (i) long-term, in naïve animals, followed by a peripheral immunization against Ad-5 (CNS first, skin second) [41•]; and (ii) medium-term, in animals immunized against Ad-5 preceding intracranial vector injection (skin first, CNS second) [42•]. These studies demonstrated that following peripheral immunization against Ad-5, transgene expression from an E1/E3-deleted adenovirus vector is completely abolished within 45 days, while expression from an HC-Adv remains unaffected (Figure 3). The mechanism responsible for downregulating transgene expression has not yet been determined. Either the immune system can eliminate transduced cells [43•,44], or it downregulates transgene expression through a non-cytolytic mechanism [45•,46•] (Figure 1). In the pre-immunization model that mimics gene transfer into patients pre-exposed to adenovirus, expression from E1/E3-deleted adenovirus in the brains of animals previously immunized against Ad-5 is almost completely

eliminated by 14 days post-injection, while expression from HC-Adv is only reduced to 50%, and remains stable for at least 2 months [42•]. Longer expression is currently being examined. These experiments demonstrate the advantages provided by HC-Adv vectors.

Promoters, transgenes, viral vector distribution and transduction efficiency: Have we got the right standards?

We thus reasoned that an absolute reduction in the dose of vectors, and an increase in transgene expression per vector genome, is necessary in order to eliminate acute cytotoxicity and chronic inflammation. In search of higher levels of transgene expression, we explored the activity of various other promoter systems, which included the human cytomegalovirus (hCMV) promoter, neuron-specific enolase promoter, the glial fibrillary acidic promoter and the prolactin promoter, as well as the inducible tetracycline regulatory element driven by constitutive or cell-type-specific promoters [10,39••,47-51]. Other promoters, including the synapsin 1, tubulin α 1, and myelin basic promoters, have also been utilized [52-54]. The best results were obtained with the MIEmCMV promoter, which was 1000-times more potent than the hCMV promoter in the brain [39••], and thus allows us to reduce the dose of vector needed accordingly (Figure 4). Injection of very low

Figure 4. Schematic of immune responses in the brain.



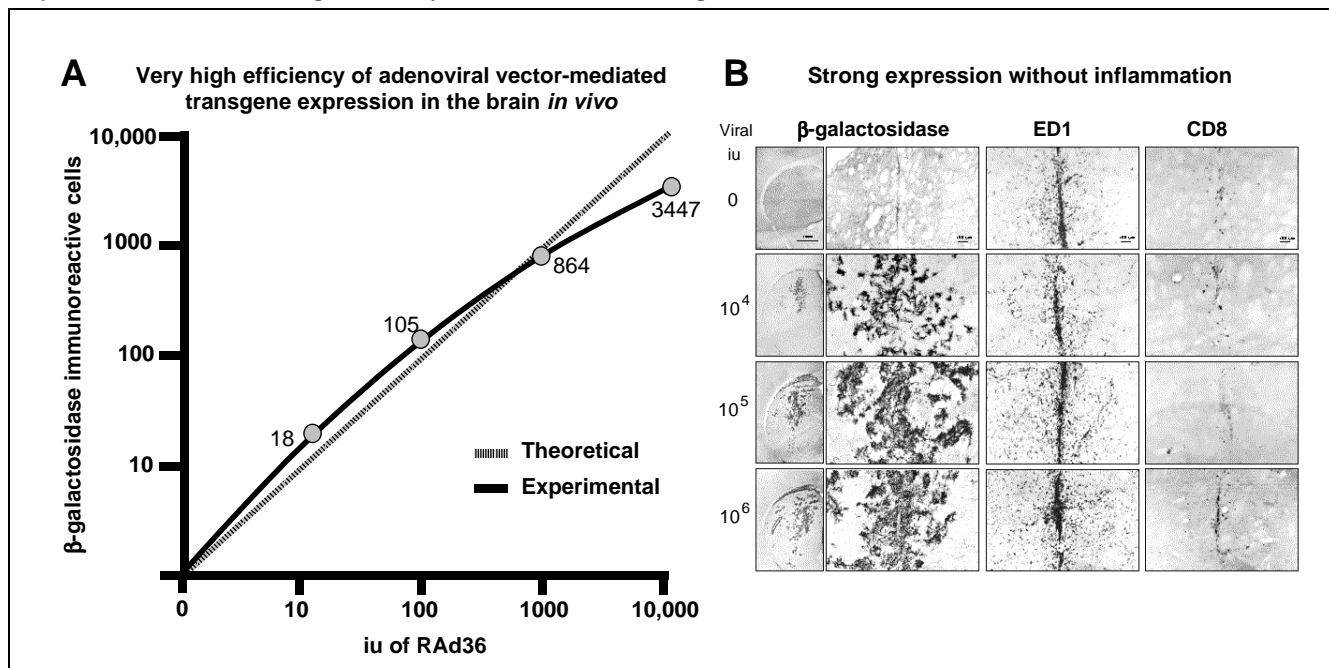
This figure summarizes the inflammatory and immune responses to adenovirus vectors either injected into a naïve animal (A), or when systemic anti-adenoviral immunity is primed. (A) Illustrates the injection of adenovirus into the brain of a naïve animal, where it will cause the release of inflammatory cytokines and activation of glial cells and macrophages (M ϕ) and microglial cells. The threshold of adenovirus needed to activate the inflammatory response or the threshold to cause cytotoxicity are indicated in the figure. Notice that the cytotoxic threshold is approximately one log higher than the inflammatory threshold. Expression at viral doses below 1×10^6 iu, which can be obtained using strong promoters (eg, MIEhCMV), result in high-level expression in the absence of side effects. Because of the lack of an afferent arm of the immune response in the brain (possibly due to a lack of dendritic cells from the naïve brain parenchyma), no priming of the immune system will occur following the careful delivery of adenoviral vectors into the brain parenchyma [56,57]. An injection into the brain ventricular system, however, will lead to a different result due to the presence of dendritic cells in this compartment. (B) Illustrates the situation in which an animal previously injected with adenoviral vectors into the brain is immunized against adenovirus in a peripheral site such as the skin. Injection of adenovirus into the skin causes an immune response that eliminates transgene expression in the brain. The mechanisms of the immune-mediated elimination of transgene expression are currently being investigated. The peripheral inflammatory threshold (defined as the dose of adenovirus vector needed in the skin to prime an anti-adenoviral immune response) has been determined to be $1 \times 10^{6-7}$ iu. This threshold is the same dose of virus needed to cause acute brain inflammation (central inflammatory threshold in (A)). Also notice the high efficiency of the adaptive immune response. Detailed dose-response curves [CA Gerdes *et al*, unpublished data] indicate that the adaptive immune system can eliminate transgene expression from as little as 1000 infected brain cells. However, it cannot do so when there are only 100 cells expressing the transgene in the brain. In summary, this figure illustrates that the acute inflammatory response, while inducing the release of inflammatory mediators and recruiting inflammatory cells, is unable to abolish transgene expression per se, and does not lead to the priming of a systemic anti-adenoviral immune response. Peripheral immunization, however, leads to a potent anti-adenoviral immune response that completely eliminates transgene expression from as little as 1000 transduced cells. Elucidating the mechanisms by which the adaptive immune response can eliminate transgene expression is an important issue in gene therapy for brain disorders that will need to be elucidated in order to develop safer and more stable neurological gene therapy.

doses (1×10^1 to 1×10^4 iu) of a first generation adenovirus expressing β -galactosidase under the control of the MIEhCMV promoter demonstrated a linear relationship with the number of detected transduced cells (Figure 2 and 5). This indicates that a single infectious adenoviral vector particle is able to transduce one cell, which can be detected by sensitive immunohistochemical detection methods. The linear dose-response relationship was lost at 1×10^4 iu, possibly indicating that due to diffusional constraints, double infection events will occur from this viral dose onwards. Importantly, this is the lowest dose at which we detect any expression from the hCMV promoter, a promoter for which we previously established that

multiple infection is necessary to allow expression in brain cells [55]. This contributes to understanding adenoviral vector toxicity, since if promoters display weak activity, this will result in under-representation of the total number of infected cells in the brain.

Widespread distribution of the protein product encoded by the HSV-1-*tk* gene throughout large areas of the nervous system was observed as an unexpected 'side effect' in a trial of gene therapy for glioblastoma [24]. A follow-up to this study demonstrated that the widespread distribution was not a function of either the co-administration of the vector with a glioblastoma (known to downregulate immune responses), nor

Figure 5. The powerful mCMV promoter allows high level of transgene expression in the absence of inflammation: A linear dose response demonstrates the high efficiency of adenovirus-mediated gene transfer in the brain.



(A) Dose response of β -galactosidase expression versus increasing doses of RAD36 (MIEmCMV- β -gal). The hatched line indicates the number of transduced cells expected if each particle were infectious, and if each particle's infectious event could be detected. The black line indicates the values obtained experimentally using Rad36 ($n = 3$). Note that linearity is lost at 1×10^4 iu, indicating that double infection occurred, possibly due to a limitation in vector diffusion through the brain tissue, thus lowering the total number of transduced cells obtained, compared with theoretical values. The linearity obtained between 1×10^1 and 1×10^3 iu strongly suggests that each infectious event is being detected. Interestingly, 1×10^5 iu is the dose at which we begin to see significant expression from the human CMV promoter, a promoter that needs multiple infectious events in order to activate transcription within an adenoviral vector [52]. (B) The mCMV promoter allows high-level expression at low doses of vector without inflammation. Increasing doses of RAD36 (mCMV- β -gal) (1×10^4 to 1×10^6) were injected into the striatum of adult Sprague-Dawley rats in a total volume of $3 \mu\text{l}$, and animals were perfused 5 days later. Brains were processed for immunohistochemistry, to detect β -galactosidase (transgene expression; first two left-hand side columns), ED1 (macrophages-microglial cells) and CD8 (infiltrating lymphocytes and NK cells). Low power view of the striatum is shown in the left-hand side column, and a higher power view is shown in the column next to it. Scale bar, shown in the upper left = 1 mm; all others = 100 μm . Note that RAD36 allows good transduction of the striatum in the absence of recruitment of inflammatory cells, compared with animals injected with saline. Also note that we have previously shown quantitatively [39••] that transduction measured as expression of β -galactosidase at the dose of 1×10^4 to 1×10^5 iu is comparable to that seen with Rad35 (hCMV- β -gal) at a dose of 1×10^7 iu, which induces much higher inflammation.

the administration of ganciclovir (known to have an immune-suppressant effect) [23•]. This argues in favor of the expression of HSV-1-*tk* being very high, or the protein or its mRNA being very stable, in the brain. Although it is possible that HSV-1-*tk* could diffuse and be taken up by neurons, this is unlikely since HSV-1-*tk* is not a protein that is normally released. Thus, the choice of transgene can influence levels of expression, distribution and detection throughout the brain.

Conclusions

Optimizing gene therapy: From the right promoter to the correct and safe therapy

Gene therapy has made many advances over recent years. Optimization has been the target of gene transfer into the brain, and important milestones have been achieved. We know that viral vectors are very efficient at transferring genes into brain cells, and recent improvements in vector design are bringing clinical trials ever closer. Adenoviral, HSV-1, AAV and lentiviral vectors have all been improved step by step, from transgenic constructs through to vector capsids. Progress has also been achieved in the design of better transgenic

constructs, more powerful cell-type-specific and inducible promoters, and vector capsids that allow re-targeting to predetermined cell types. The MIEmCMV promoter, for example, can increase expression in the brain 1000-fold, and is more powerful than the hCMV promoter in all species tested. Additionally, it is glial-specific, and thus probably ideal for expressing genes that encode proteins to be secreted into the brain's extracellular space.

In addition, great care needs to be exercised in the selection of marker transgenes. β -galactosidase and eGFP may be the easiest to use, but they appear to be toxic to neurons, and will thus curtail long-term gene expression [38••]. HSV-1-*tk* is an ideal replacement to be used as a reporter gene in the brain. It is expressed at high levels, and can be detected by immunocytochemistry in many areas of the brain where, by contrast, β -galactosidase was not detected [23•]. HSV-1-*tk* also demonstrates sustained long-term presence in the CNS. In regard to the potential toxicity of HSV-1-*tk*, although HSV-1 remains latent for many years in dorsal root and trigeminal ganglia neurons where it may express *tk* at different stages of its replicative cycle, no toxicity has been detected in these

cells. Importantly, as far as we know, no other genes encoding for intracellular proteins are expressed at comparable levels in the CNS. Thus, whereas β -galactosidase expression may underestimate the potential of vector-mediated expression, HSV-1-*tk* may overestimate it. Either way, the transgene itself has the potential to influence the level of expression detected, an important fact that needs to be taken into account in the design and testing of viral vectors. Many advances have been made towards elucidating the potential neurotoxicity and/or immunogenicity of viral vectors and their encoded transgenes in the brain, as well as the mechanisms underlying innate and adaptive immune responses in the brain (Figure 4), and these will bring clinical trials closer to fruition.

During the last 18 months, experimental models of inherited metabolic brain diseases have demonstrated that gene therapy cannot only prevent [21••,25••], but also truly restores, brain function [26••]. Future improvements in vector targeting, transgene expression and safety profiles make the implementation of further clinical trials for the treatment of chronic neurodegenerative diseases, and brain cancer, a likely topic for discussion in these pages. After a long journey through a dark tunnel, neurological gene therapy is finally beginning to see glimmers of therapeutic light.

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 • Excellent review on the mechanisms of brain immune responses presenting a powerful case for the lack of dendritic cells from the naïve brain determining the quality of brain immune responses.