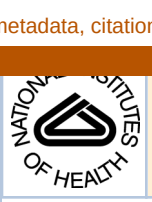

Published in final edited form as:

Neurobiol Dis. 2012 November ; 48(2): 179–188. doi:10.1016/j.nbd.2011.09.014.

Viral Vectors for Gene Delivery to the Central Nervous System

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Abstract

The potential benefits of gene therapy for neurological diseases such as Parkinson's, Amyotrophic Lateral Sclerosis (ALS), Epilepsy, and Alzheimer's are enormous. Even a delay in the onset of severe symptoms would be invaluable to patients suffering from these and other diseases. Significant effort has been placed in developing vectors capable of delivering therapeutic genes to the CNS in order to treat neurological disorders. At the forefront of potential vectors, viral systems have evolved to efficiently deliver their genetic material to a cell. The biology of different viruses offers unique solutions to the challenges of gene therapy, such as cell targeting, transgene expression and vector production. It is important to consider the natural biology of a vector when deciding whether it will be the most effective for a specific therapeutic function. In this review, we outline desired features of the ideal vector for gene delivery to the CNS and discuss how well available viral vectors compare to this model. Adeno-associated virus, retrovirus, adenovirus and herpesvirus vectors are covered. Focus is placed on features of the natural biology that have made these viruses effective tools for gene delivery with emphasis on their application in the CNS. Our goal is to provide insight into features of the optimal vector and which viral vectors can provide these features.

Keywords

Viral vectors; Central Nervous System; Gene Therapy; Adeno-associated virus; Retrovirus; Adenovirus; Herpesvirus

Introduction: Viruses as Vectors for Therapeutic Gene Delivery

The idea to treat human disease by delivering a therapeutic gene was first conceived as early as the 1970s (Friedmann and Roblin, 1972). The potential of viruses as vectors for gene delivery was recognized even then. Viruses are among the simplest biological agents capable of delivering genetic information to a cell and have evolved properties that make them useful as vectors for gene delivery (Shen and Post, 2007). Structural virions carry and protect viral genetic information (DNA and RNA), providing stability for transit throughout the human body. Determinants on the surface of the virion specify which cells the virus will enter while minimizing immunostimulatory potential within the host. Signals in the genetic material control gene expression from the virus in the infected cell. Finally, many viruses have developed mechanisms of replication that allow them to be propagated in cell culture. Though these innate properties have been advantageous in developing vectors for gene

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delivery, using viruses comes with potential risks as well. Many viruses cause disease and vectors derived from them have the potential to be pathogenic. The goal in designing vectors is to minimize this potential risk by using only components of the virus necessary for transduction of the target cell and expression of the transgene, while also preserving the efficiency of production. In this review, features of the ideal vector for delivering genes to the CNS are outlined. We then discuss the molecular biology of several viruses currently being developed as vectors and how they compare with our model of the ideal vector. Emphasis is placed on the application of these vectors in the CNS.

Model of the Ideal Vector for Gene Delivery to the CNS

Many viral systems have been developed as vectors for gene delivery and each offers unique solutions to the challenges of gene therapy. Determining which features a vector must possess to effectively treat a disease is the first step toward evaluating the particular strengths and weaknesses of different viral systems. To discuss the potential of different viral vectors for use in the CNS, we have developed a model of the ideal vector. One feature of the ideal vector is specific tropism for and highly efficient transduction of the target tissue and minimal transduction of ‘off-target’ cells, tissues or organs. Neurological diseases are often the result of loss or malfunction of a specific tissue within the CNS. For example, Parkinson’s is associated with the death of dopaminergic neurons in the substantia nigra. Treatment of diseases such as Parkinson’s requires that delivery of the therapeutic transgene be targeted to the affected tissue. Second, the ideal vector will express the transgene for a length of time and at a level as to have maximal therapeutic impact. It is important that these two parameters be empirically determined for the specific disease being treated. If the duration is too short or the level is too low, expression of the transgene may not produce a therapeutic effect. If too high, expression could have a cytotoxic effect on cells. High levels of expression may also be prone to elicit a host immune response against the therapeutic gene. A third feature of the ideal vector, and perhaps the most important, is that side effects such as vector related pathologies and/or host immune response be minimal. Some viruses (e.g. herpesviruses) lyse cells as a part of their normal replication cycle. For these viruses, the potential to generate replication competent particles during vector production is a significant concern. Other viruses (e.g. adenovirus) can be highly immunogenic. A strong host immune response can cause inflammation and/or mediate clearance of the transduced cells, resulting in lack of therapeutic effect and possibly consequences for patients’ health. Finally, methods of manufacture that are scalable and meet guidelines for therapeutic application must be available for the ideal vector. This is necessary to produce vector in high enough quantities and purity as to be practical for delivery to patients.

Comparison of the available viral vectors against the ideal vector reveals that the natural biology of the virus plays a significant role in whether a vector is suitable for use in the CNS. The packaging capacity of a virus can limit the size of transgene it is able to deliver. The viral proteins exposed on the surface of the virion can determine tissue tropism and immunogenicity. Even the form of the viral genome and how long it persists in a cell can govern the level and duration of transgene expression. However, innovations in the molecular biology of a vector can overcome the limitations imposed by the biology of the virus. Tissue tropism can be expanded and the specificity enhanced using methods of transencapsidation or pseudotyping to change receptor binding properties of a vector. Manipulation of the genetics, such as removal or introduction of functional sequences, can promote formation of more stable forms of a vector and thus increase the duration of transgene expression. Thus, it is important to consider the biology of the virus alongside the vectorology.

Adeno-Associated Virus Vectors

Adeno-associated virus (AAV) is a member of the family *Parvoviridae* in the genus *Dependovirus* (Berns and Parrish, 2007). Dependoviruses are non-autonomous, requiring co-infection with a helper virus (e.g. adenovirus or herpesvirus) in order to replicate, and non-pathogenic (Atchison et al., 1965; NIH, 2011). These aspects of its natural biology greatly reduce the risks of side effects associated with use of AAV for gene delivery. The virion is small, ~20 nm, consisting of a protein capsid that surrounds the viral genome (Cassinotti et al., 1988; Rose et al., 1971; Xie et al., 2002). The capsid is made up of viral capsid (Cap) proteins VP1, VP2, and VP3. These proteins facilitate binding of the virion to the cell surface receptor, which varies between serotypes. AAV2, the predominant serotype capsid of use in clinical applications, uses heparin sulfate as its primary receptor (Summerford and Samulski, 1998). Other serotypes have been shown to use sialic acid (Serotypes 1, 4, 5 and 6 (Kaludov et al., 2001; Wu et al., 2006b)), Laminin receptor (serotype 8 (Akache et al., 2006)), and N-linked galactose (serotype 9 (Shen et al., 2011)). The target tissue of natural infection has not been determined, but tropism can vary considerably between capsids of different serotypes (Chao et al., 2000; Rabinowitz et al., 2002; Wu et al., 2006a; Xiao et al., 1999). The genome of AAV is a single-stranded (ss) DNA of ~4.7 kb flanked on 5′ and 3′ ends by an inverted terminal repeat (ITR). The ITR is predicted to form a “T” shaped stem loop structure due to complementarity within its sequence, and functions as an origin for genome replication, which proceeds through a rolling hairpin mechanism (Berns and Parrish, 2007; Lusby et al., 1980; Nash et al., 2008; Straus et al., 1976; Tattersall and Ward, 1976). Second-strand synthesis occurs within the transduced cell and requires several cellular enzymes (Nash et al., 2008). Expression of the viral replication (Rep) proteins, 78, 68, 52, 40 (name reflects molecular weight), is also necessary for replication and packaging of the genome (Im and Muzyczka, 1990; Im and Muzyczka, 1992; Snyder et al., 1990). In the absence of helper virus the viral genome persists episomally in a latent state as monomers and multimers in linear and circular forms (Duan et al., 1998; Schnepp et al., 2005). Integration of the genome is observed at a relatively low frequency and has preference for a specific site on chromosome 19 (McCarty et al., 2004). However, integration is greatly reduced and loses site specificity in the absence of Rep 78/68 (as is the case with vectors derived from AAV).

Several different types of vectors have been derived from AAV, each using a different strategy to facilitate transgene expression. One requirement of all AAV vectors is that the transgene cassette be flanked by the ITR sequence. This is necessary for packaging the transgene into the viral capsid. AAV serotype 2 was the first serotype to be used extensively and, as a result, the AAV2 ITR is the most widely used. Another requirement is that the vector not exceed ~4.7 kb in size, the packaging capacity of the viral capsid (Dong et al., 2010; Dong et al., 1996; Hirsch et al., 2010; Lai et al., 2010; Wu et al., 2010). This packaging capacity is rather stringent, as vectors that exceed this size are packaged in truncated forms. Single-stranded AAV (ssAAV) vectors were the first to be developed and consist of a transgene expression cassette flanked on 5′ and 3′ ends by the ITR sequence (Samulski et al., 1989). In these vectors the transgene cassette simply replaces the coding region of wild-type (WT) AAV and is packaged as single-stranded DNA, as the name indicates. No viral genes are encoded by the vector, reducing the potential for host immune recognition of transduced cells. One limitation of these vectors is that transgene expression depends on second-strand synthesis, which is a rate limiting step in cell transduction (Ferrari et al., 1996). Self-complementary (scAAV) vectors were developed to overcome the dependence on second-strand synthesis (McCarty et al., 2001). scAAV vectors contain a mutated ITR positioned centrally between two copies of the transgene sequence, one inverted and complementary to the other. This allows the genome to self-anneal to form a double-stranded molecule. These vectors express the transgene earlier and to greater levels

than ssAAV vectors, but limit the size of the transgene cassette to ~2.2 kb. However, limited coding capacity has prevented the use of scAAV and even ssAAV vectors for applications requiring large transgene cassettes, >5 kb. To circumvent this, dual vector approaches have been developed that can increase transgene capacity up to ~8 kb (Duan et al., 2001; Hirsch et al., 2010; Hirsch et al., 2009). In these strategies, the transgene cassette is separated over two vectors and assembly of the full-length transgene relies on co-infection of a single cell and subsequent recombination. Split (trans-splicing) vectors are designed with a splice donor site on the vector encoding the 5′ half of the transgene and a splice acceptor site on the vector encoding the 3′ half of the transgene such that recombination between the two occurs within an intron (Duan et al., 2000; Ghosh et al., 2011; Nakai et al., 2000; Sun et al., 2000; Yan et al., 2000). This approach enables expression of larger transgene cassettes, but the low efficiency of co-transduction of a single cell and vector recombination significantly reduces the level of transgene expression relative to ssAAV and scAAV vectors.

Several features of AAV vectors stack up well against our model of the ideal vector for gene delivery to the CNS. Though not thought to be a natural site of infection, AAV vectors transduce many different cell types of the CNS, depending on which capsid is used. This includes neurons, which most serotypes transduce with high efficiency, as well as astrocytes, glial and ependymal cells, which are selectively transduced well by a subset of capsids (Alisky et al., 2000; Broekman et al., 2006; Burger et al., 2004; Davidson et al., 2000; Kaplitt et al., 1994; Lawlor et al., 2009; Van der Perren et al., 2011; Van Vliet et al., 2008; Wang et al., 2003). The capsid of serotype 9 has even been shown to cross the blood brain barrier (Duque et al., 2009; Foust and Kaspar, 2009; Foust et al., 2009; Gray et al., 2011). AAV vectors can also engender long-term, stable transgene expression in the CNS, persisting in the murine brain for >6 months (Klein et al., 1999). Other reports have demonstrated persistence in other tissues for >6 years in primates and >8 years in dogs (Niemeyer et al., 2009; Rivera et al., 2005; Stieger et al., 2009). No significant side effects have been found associated with delivery of AAV vectors (Bessis et al., 2004). The possibility that AAV vectors may be correlated with an increased risk of tumorogenesis has been presented, but a study covering over 600 animals found no association (Bell et al., 2006; Bell et al., 2005; Kay, 2007). Finally, scalable systems for vector production have been developed and are currently in use (Grieger et al., 2006; Grieger, personal communication; Lock et al., 2010; Vandenberghe et al., 2010).

While AAV vectors are being tested for clinical use in the CNS, the field is also moving quickly to improve upon these vectors. Vectors derived from AAV, particularly serotype 2, are the predominant viral vectors of use to treat neurological diseases, accounting for 76% of therapy trials in the CNS (Wiley, 2011). Trials targeting Parkinson's, Amyotrophic Lateral Sclerosis (ALS), Alzheimer's, Batten Disease, and Epilepsy are ongoing. Strategies are being developed that may expand this list to include Huntington's Disease, Mucopolysaccharidoses (MPS) and Spinocerebellar Ataxia (McCown, 2011). One focus is to expand the list of possible tissue targets by synthesizing vectors with novel tropism and increased efficiency/specificity of transduction (Michelfelder and Trepel, 2009; Muzyczka and Warrington, 2005; Van Vliet et al., 2008). Historically, AAV2 has been used more widely than other serotypes and early vectors employed the ITR of serotype 2 which could only be packaged in the corresponding capsid. Development of a cross-packaging system allows a single vector carrying the serotype 2 ITR to be trans-encapsidated with the capsids of most AAV serotypes (Rabinowitz et al., 2002). This system provides a way to customize the capsid of a vector for tissue specificity for the CNS and can overcome the possible complication associated with pre-existing immunity against AAV2 (Taymans et al., 2007; Van der Perren et al., 2011; Vandenberghe et al., 2006). Random diversification and selection of capsids in a process termed molecular evolution has further increased the potential for customization. This method has yielded capsids with improved transduction of

astrocytes *in vitro*, ~15-fold, and *in vivo*, ~6-fold, when injected into the rat striatum (Koerber et al., 2009). Similar strategies have been employed to customize vectors for specific therapeutic applications or disease states. For example, shuffling the Cap protein coding sequence of different serotypes has produced novel capsids capable of crossing the seizure-compromised blood brain barrier in a rat model for epilepsy (Gray et al., 2010a). Additionally, Chen *et al.* cycled a phage display library of random peptides in normal, LINCL, or MPS VII mice, injecting the library into the tail vein and recovering it from the brain (Chen et al., 2009b). The recovered peptides were then incorporated into an AAV2 capsid, which specifically targeted to the cerebral vascular endothelial cells after i.v. injection in mice. The peptides identified were highly specific for the disease state they were selected in, and were not cross-compatible with WT mice. Another focus of vector engineering is to reduce the potential risk of vector mobilization. Evidence that vectors may be replicated in the presence of WT AAV and helper-virus infection has been produced from experiments in cell culture and *in vivo* (Afione et al., 1996; Cheung et al., 1980). To address this, novel ITRs have been developed that are not replicated by Rep proteins of WT AAV and thus, cannot be mobilized (Hewitt et al., 2009; Hewitt and Samulski, 2010).

In summary, AAV vectors are currently the preferred gene delivery vehicle for CNS applications. They provide efficient gene transfer, long-term transgene expression, minimal pathogenicity, low immunogenicity, and scalable manufacture for clinical applications. However, AAV vectors are hindered by their 4.7-kb packaging limit. For a detailed review of the many applications of AAV in the CNS, see references (Gray et al., 2010b; McCown, 2011).

Retrovirus vectors

Retroviridae is a family of single-stranded (ss) RNA viruses that reverse transcribe their genetic material into DNA and integrate into the host genome as part of their life cycle (Goff, 2007). Genera of this family can be categorized as *simple* (alpharetroviruses, betaretroviruses, gammaretroviruses, and epsilonretroviruses) or *complex* (deltaretroviruses, lentiviruses and spumaviruses) retroviruses depending on the proteins they encode. Both simple and complex retroviruses have Gag, Pol and Env genes (Leis et al., 1988). In addition, complex retroviruses encode several small regulatory proteins that play a role in virus-host interaction. Retroviral virions average ~100 nm in size and are composed of a phospholipid envelope surrounding a capsid, within which two copies of the ssRNA genome, both positive sense, and several viral proteins are organized into a nucleocapsid (Chen et al., 2009a; Goff, 2007; Liu et al., 2010). The envelope is acquired upon budding from the host cell plasma membrane and is comprised of a phospholipid bilayer containing several viral glycoproteins, encoded by the Env gene (Forster et al., 2005; Johnson, 2011). Envelope glycoprotein SU (surface) interacts with the cell surface receptor and TM (transmembrane) appears to play a role in membrane fusion. A variety of cell surface receptors are used by different retroviruses, and target predominantly T cells and macrophages (Miller, 1996; Sommerfelt, 1999; Weiss and Tailor, 1995). Inside the envelope the Gag poly-protein is the major structural component, interacting with both the interior surface of the envelope membrane as well as the RNA genome (Freed, 1998; Scarlata and Carter, 2003). When the virus buds from a cell, Gag is cleaved by the viral protease, encoded by the Pol gene, into matrix (MA), capsid (CA), and nucleocapsid (NC) subunits. A structural rearrangement of the virion, termed maturation, then takes place, forming the conical capsid and nucleocapsid, which are referred to collectively as the viral core (Briggs et al., 2003; de Marco et al., 2010). Reverse transcription of the ssRNA by the viral reverse transcriptase, encoded by the Pol gene, takes place within the core, during or shortly before entering a cell. This process results in the synthesis of a linear double-stranded (ds) DNA genome and formation of a pre-integration complex (Telesnitsky and Goff, 1997). This

process is primed by tRNA at the primer binding site (PBS) and requires several template switches, which are facilitated by long terminal repeat (LTR) sequences present on the termini of the genome. The pre-integration complex then enters the nucleus either through active transport (complex retroviruses) or upon cell division (simple retroviruses) depending on the virus (Bukrinsky et al., 1993; Lewis and Emerman, 1994). Once in the nucleus, the viral integrase, encoded by the Pol gene, facilitates integration of the linear dsDNA into the genome of the cell, forming a provirus which serves as the template for viral gene expression (Brown et al., 1989).

Vectors for gene delivery have been generated from simple (e.g. Moloney murine leukemia virus; gammaretrovirus) as well as complex (e.g. HIV-1; lentivirus) retroviruses (Escors and Breckpot, 2010; Maier et al., 2010). The predominant difference between vectors derived from these two types of retroviruses is in their reliance on cell division for transduction. Vectors derived from simple retroviruses require passage through the cell cycle for transduction while vectors derived from complex retroviruses are capable of transducing non-dividing cells (Lewis and Emerman, 1994). This represents a distinct advantage of complex over simple retroviral vectors for gene delivery to the quiescent cells of the CNS (Escors and Breckpot, 2010; Jakobsson and Lundberg, 2006; Wong et al., 2006). Though simple retroviruses have been employed to study aspects of neurogenesis, lentiviral vectors have become the predominant retroviral vector for gene delivery to the CNS.

Vectors derived from lentivirus have a majority of the viral genes deleted and retain only the *cis*-acting sequence elements necessary for nuclear export of the RNA, RNA dimerization, packaging, and reverse transcription (Dull et al., 1998; Zufferey et al., 1997). This yields vectors with an available packaging capacity of ~8 kb. Comprehensive analysis of lentiviral vector packaging capacity revealed that this is not an absolute limit, but rather that there is a near-linear negative correlation between increasing vector size and virus production (Kumar et al., 2001). Packaging cell lines have been developed for simple as well as complex retroviral systems that provide the viral genes necessary for vector production in *trans* (Cockrell et al., 2006; Danos and Mulligan, 1988; Kafri et al., 1999; Klages et al., 2000; Markowitz et al., 1988). Modern vectors have been modified in an effort to eliminate integration into the host genome. Initially seen as an advantage, integration comes with a significant risk of insertional mutagenesis, as was realized with the incidence of leukemia in 4 of 20 patients treated for X-linked SCID (Bokhoven et al., 2009; Hacein-Bey-Abina et al., 2003; Pike-Overzet et al., 2007). It is not clear if the risk of insertional mutagenesis in the *ex vivo* cell therapy approach used in this X-linked SCID trial reflects that of *in vivo* treatment of non-dividing cells, such as neurons of the CNS, but this risk has hindered the progress of these vectors. One strategy to overcome this challenge has been to try directing integration to ‘safe’, heterochromatin regions of the genome, which may minimize the risk of gene activation (Gijsbers et al., 2010). Alternatively, introduction of self-inactivating (SIN) mutations, which knock out promoter activity of the LTR, has greatly reduced the risk of insertional gene activation (Miyoshi et al., 1998; Zufferey et al., 1998). Another major advancement was the development of non-integrating lentiviral (NIL) vectors, which carry either mutant integrase or mutations in their LTRs that inhibit integrase binding (Apolonia et al., 2007; Philippe et al., 2006; Sarkis et al., 2008). Though still observed at ~0.35 – 2.3 %, integration is greatly reduced with NIL vectors (Cornu and Cathomen, 2007). Instead of integrating the vector genome persists episomally in linear and circular forms (Cara and Reitz, 1997; Philpott and Thrasher, 2007). Efficiency of transduction of target cells, including neurons, is not significantly affected by these mutations and is similar to that of integrating vectors (Cornu and Cathomen, 2007; Rahim et al., 2009; Sarkis et al., 2008). Recently, removal of a sequence element involved in plus-strand DNA synthesis was shown to further reduce integration and increase the efficiency of formation of circular episomes (Kantor et al., 2011).

Many features of lentiviral vectors compare well with our model of the ideal vector. Though these vectors do not naturally infect cells of the CNS, there is significant potential for modifying tissue tropism for specific therapeutic applications. The mechanism for envelopment of lentiviruses allows vectors to be pseudotyped with envelope proteins from other viruses (e.g. vesicular stomatitis virus G) (Bischof and Cornetta, 2010; Chen et al., 1996; Naldini et al., 1996). Taking advantage of this feature of the biology of the virus has generated vectors capable of transducing neurons and astroglial cells (Colin et al., 2009; Desmaris et al., 2001; Greenberg et al., 2007; Jakobsson et al., 2006; Rahim et al., 2009). Also, innovations in the genetics of the vector, leading to the creation of SIN and NIL vectors, have reduced the risk of integration and vector-related pathologies. Finally, systems of vector production have been developed that can be scaled up for manufacture of clinical reagents (Kafri et al., 1999; Klages et al., 2000). However, one shortcoming of NIL vectors is that duration and level of transgene expression are not well characterized. Transgene expression has been shown to persist up to 3 months in the rat brain and as long as 9 months in other tissues, but more extensive studies are required to determine the limit of expression (Bayer et al., 2008; Yanez-Munoz et al., 2006).

Retroviral vectors are currently the second most commonly used viral vector for gene delivery to the CNS. *Ex vivo* transduction of cells with a Moloney leukemia virus vector expressing nerve growth factor (NGF) and introduction to the brain has been used in treatment of Alzheimer's disease with moderate success (Tuszynski et al., 2005). A trial using a lentiviral vector delivering three genes involved in dopamine biosynthesis, ProSavin, to treat Parkinson's disease is also underway (Oxford_Biomedica, 2011; Wiley, 2011). Preliminary studies in animal models suggest these vectors might also be used in treating Huntington and lysosomal storage diseases (Jakobsson and Lundberg, 2006; Lundberg et al., 2008; Wong et al., 2006).

NIL vectors are at the forefront of retroviral vector development and hold promise as tools for gene delivery to the CNS. Further reduction in the rate of integration and characterization of the longevity of transgene expression are necessary to meet our requirements for the ideal vector. For a review of the application of retroviral and/or lentiviral vectors in the CNS, see references (Jakobsson and Lundberg, 2006; Lundberg et al., 2008; Wong et al., 2006).

Adenovirus vectors

Adenoviruses belong to the family *Adenoviridae* and range in size from ~70 – 100 nm (Berk, 2007). The virion consists of an icosahedral protein capsid, composed of subunits hexon, penton and fiber, surrounding a core, which contains the genomic material of the virus (Reddy et al., 2010). Hexon is the predominant subunit of the capsid, making up the surface of the structure, while penton and fiber subunits reside only at the vertices, the latter protruding from the surface in a characteristic spike. Virus attachment to cells is mediated via interaction of the fiber protein with a specific cell surface receptor, the coxsackievirus and adenovirus receptor (CAR) (Bergelson et al., 1997; Tomko et al., 1997). This receptor is present on the surface of a number of tissues, including cells of the CNS. The adenoviral genome is a linear, dsDNA molecule of ~36 kb (Davison et al., 2003). Inverted terminal repeat (ITR) sequences flank the genome and serve as the origin for replication. Additionally, a *cis* element has been identified near the end of the genome that is required for efficient genome packaging (Hearing et al., 1987). Adenoviruses do not integrate into the host genome as part of their life-cycle and the frequency of integration events is very low. Instead, the genome is maintained as a linear episome in the nucleus of a cell (Hillgenberg et al., 2001).

Adenoviral vectors have gone through several generational steps of development. First generation vectors contain deletions of the E1 and/or E3 regions of the genome, the former encodes proteins necessary for early gene expression the latter is dispensable for replication and packaging (Bett et al., 1994). This yielded a transgene capacity of up to ~8.3 kb, but this generation of vectors retains a majority of the viral genes and cytoxicity related to expression of the viral genes has limited their utility. Second generation vectors have more extensive deletions of the adenoviral sequence, removing the E2 or E4 regions along with E1/3 (Amalfitano et al., 1998; Armentano et al., 1995). These vectors are incapable of replicating on their own and require helper vectors or cell lines to complement the function of these two regions. Removal of these additional regions has helped reduce inflammation and host immune response to the vectors, but it remains a problem. More recently, helper-dependent ('gutted') vectors were created that are devoid of most of the viral genes (Parks et al., 1996; Volpers and Kochanek, 2004). Helper-dependent vectors contain the minimal sequence elements necessary for production, the ITRs and the packaging signal, and have a capacity of ~36 kb (Parks, 2000). Similar to WT adenovirus, adenoviral vectors are maintained episomally as a linear molecule in the nucleus of a cell (Jager and Ehrhardt, 2009; Rauschhuber et al., 2011). Circular molecules can also be produced, but the appearance of these forms may depend on the sequence contained within the vector and/or the presence of adenovirus proteins (Kreppel and Kochanek, 2004). Integration of vector into the host genome is observed only minimally, even when extensive homology exists between the recombinant vector and the host genome (Stephen et al., 2010; Stephen et al., 2008).

Adenoviral vectors exhibit some of the features desired in our model of the ideal vector, but still have to overcome the risk of host immune response and the lack of helper-free production methods before they can be used extensively in the CNS. Helper-dependent vectors have been shown to transduce of neuronal, astroglial, and human glioma cells, demonstrating that targeting of neuronal tissues is possible with these vectors (Candolfi et al., 2006; Candolfi et al., 2007). Additionally, methods of modifying vectors to broaden tropism and reduce off-target transduction have been developed (Everts and Curiel, 2004; Glasgow et al., 2006). Transductional targeting is perhaps the most promising of these methods and employs bi-specific molecules that bind to the fiber protein, concealing the ligand for CAR, and expose a new receptor binding ligand. Specificity for various tissues has been accomplished in this manner. Duration of transgene expression also shows promise for therapeutic applications. Expression from helper-dependent vectors persists significantly longer than earlier generation vectors, lasting >2 years in the liver of baboons (Morral et al., 1999; Morsy et al., 1998). The foremost challenge confronting adenovirus-derived vectors has been to eliminate host immune response to the vector (Bessis et al., 2004). Early generation vectors cause significant toxicity in the CNS as well as other tissues, resulting in clearance of transduced cells (Lozier et al., 2002; Thomas et al., 2001; Thomas et al., 2002). This has led to successful application of these vectors to treat cancers of the CNS, but has limited progress forward in treating other diseases (Germano et al., 2003; Immonen et al., 2004). Host immune response against helper-dependent vectors is reduced compared with that of earlier generation vectors, but remains a problem (Chirmule et al., 1999; Morral et al., 1999; Morsy et al., 1998). Development of scalable methods of manufacturing helper-free virus has not yet been achieved and is another issue that must be overcome. Production of adenoviral vectors require that viral genes, deleted in the vector sequence, be provided in *trans* from a complementing plasmid or cell line. Recombination between the complementing sequence and the vector sequence can produce vector that carries viral genes. One future direction of the field has been to develop systems of production that eliminate this risk. In a recent advance, Suzuki et al. have taken advantage of the natural packaging limit of adenoviral capsid to ensure that if helper virus is produced by

recombination between vector and helper genomes it will not be packaged (Suzuki et al., 2011).

Adenovirus vectors have not yet achieved the success of AAV- and lentivirus-derived vectors for gene delivery to the CNS. Transgene coding capacity of ~36 kb and the ability to transduce cells types of the CNS are promising attributes. However, toxicity and immunogenicity currently impede the advancement of these vectors to the clinic.

Herpesvirus vectors

Herpesviridae is a broad family of viruses grouped by common biology and virion morphology (Pellett and Roizman, 2007). Vectors derived from herpes simplex virus (HSV) have come to dominate the field, but several members of this family have been considered for gene delivery (e.g. Epstein-Barr virus and Herpesvirus saimiri) (Griffiths et al., 2006; Robertson et al., 1996). For a detailed description of HSV and its life cycle, see reference (Roizman et al., 2007). The virion of HSV is large relative to other viruses, ~ 186 nm, and consists of a lipid envelope surrounding a capsid and a pleiomorphic layer called the tegument (Grunewald et al., 2003). The envelope is a phospholipid bilayer embedded with several viral glycoproteins and is acquired upon budding into membranous compartments in the cytoplasm of a cell. The tegument, found just beneath the envelope, is largely unstructured and contains at least 20 viral proteins and possibly viral and cellular RNA. The capsid has a regular icosahedral structure and is composed of capsomer subunits. Within the capsid, the linear dsDNA genome, ~152 kb, is associated with several viral proteins to make up the viral core. The genome can be described as having two distinct coding regions, unique long (U_L) and unique short (U_S) coding regions, both flanked by terminal repeat sequences (Wadsworth et al., 1975). HSV encodes ~90 genes, the functions of which are numerous. However, many of these genes are dispensable and can be removed without inhibiting genome replication or packaging of the virus.

Infection with WT HSV can be either lytic or latent. Glycoproteins on the exterior of the envelope mediate entry of a cell by first binding glycosaminoglycans (i.e. heparan sulfate proteoglycan) on the cell surface (Shieh et al., 1992). Subsequent internalization steps involve additional interaction with one of several possible secondary receptors. Though the primary receptor can be found on many different cell types throughout the body, infection with HSV typically occurs at the cutaneous or mucosal epithelium where replication of the virus is initially lytic. The virus can also invade axons of sensory neurons in the affected area and undergo retrograde transport, up the axon, to the dorsal root ganglia (Bearer et al., 2000). Proteins in the tegument have specific roles upon infection of a cell, including involvement in travel up the axon. In fact, it has become apparent that one function of this layer of the virion is simply to transport these proteins to the infected cell, pre-assembled. Once the virus has reached the nucleus of a cell, the linear genome circularizes and is maintained as an episome with minimal integration (Garber et al., 1993; Strang and Stow, 2005). The genome can be maintained in this state for the life of the infected individual. After circularization, genome replication proceeds through one of two mechanisms, initially following a theta mode and later switching to a rolling-circle mode of synthesis (Boehmer and Lehman, 1997).

Several different types of vectors have been derived from HSV (Marconi et al., 2009; Shen and Post, 2007). Attenuated (‘replication-conditional’) vectors are deficient in expression of viral genes essential for replication in non-dividing, but not dividing cells (e.g. thymidine kinase and ribonucleotide reductase) (Wu et al., 1996). Because replication of these vectors is highly toxic to infected cells, they can be used to target proliferating cells for killing. Though not desirable for delivering therapeutic genes, these vectors have been used in the

CNS, primarily for treatment of cancers such as glioblastoma multiforme (Andreansky et al., 1997; Lou, 2003; Markert et al., 2009; Mineta et al., 1995). More extensive removal of viral genes led to the creation of replication-defective vectors, which lack the ability to replicate autonomously and require complementing helper function for propagation (Krisky et al., 1998a; Marconi et al., 1996; Samaniego et al., 1998). Removal of immediate-early genes, necessary for expression of early and late genes, from these vectors greatly reduced vector toxicity. This eliminated cytotoxic vector replication within the cell and reduced host immune response to viral gene products. As a result, these vectors can deliver and facilitate expression of a transgene, which can persist for as long as ~3 weeks in cell culture and ~1 month *in vivo* (Krisky et al., 1998b; Lilley et al., 2001). Identification of the minimal cis-acting sequences necessary for virus replication, the cleavage/packaging signal and the viral origin, provided another significant advancement in the design of HSV vectors (Spaete and Frenkel, 1982). Amplicon vectors contain only these elements and have a packaging capacity of ~150 kb (Epstein, 2009; Marconi et al., 2009). These vectors have several advantages over attenuated or replication-defective vectors, including lack of viral genes that might cause cytotoxicity and maximal transgene coding capacity. Additionally, in instances in which the vector is significantly smaller than the packaging capacity of the virion, head-to-tail concatemers of the vector can be packaged (Saeki et al., 2003). The rolling circle mechanism of genome replication produces head-to-tail concatemers of the genome that are normally cleaved to liberate monomers for packaging. If these concatemers do not exceed capacity they can be packaged without cleavage. Delivering multiple copies of the transgene in each vector may produce increased levels of transgene expression in the transduced cells, another potential advantage. Transgene expression with these vectors has been demonstrated to persist for up to 7 months in the rat brain, but may not be stable over this period of time (Sun et al., 2003; Zhang et al., 2000). Due to the natural biology of the virus, all vectors derived from HSV are neurotropic and can efficiently transduce neurons of the CNS (Pakzaban et al., 1994; Wolfe et al., 1992). The envelope can be pseudotyped with the envelope proteins of other viruses to alter tissue tropism and reduce off-target transduction as well, as has been demonstrated with VSV-G protein (Anderson et al., 2000).

Possibly the greatest hurdle to the progress of HSV vectors has been the lack of a method of production that eliminates the potential for contaminating helper virus and yields high-titer vector. Production of HSV vectors requires complementation with helper vectors or cell lines that provide the necessary viral proteins in *trans*. Early versions of helper vectors could be packaged and were present at high levels in vector preparations. An improvement in production was the separation of helper vector sequence onto different cosmids, none of which carry the viral packaging signal (Fraefel et al., 1996). This system was able to produce vector with reduced levels of contaminating helper virus, but cytotoxic helper virus replication was not eliminated. A more recent method of production uses bacterial artificial chromosome (BAC) to express the complementing viral genes (Saeki et al., 2003). Working with a single BAC has technical advantages over working with multiple different cosmids. However, contamination with cytotoxic helper virus remains a problem. Further advancement in this area is needed in order to identify scalable and clinically relevant methods of vector manufacture.

HSV-derived vectors have several positive attributes but do not stand up well against our model of the ideal vector for gene delivery to the CNS. These vectors are able to be targeted for cells of the CNS and thus, possess one feature of our model for the ideal vector. It is also worth noting, that these vectors have the largest packaging capacity of all of the vectors we have discussed in this review. Though not a requirement of the ideal vector, this may serve as an advantage over other vector systems in certain applications. The shortcomings of these vectors are in the duration of transgene expression, the potential for harmful side effects

associated with co-production of helper virus, and the lack of a scalable system for vector production.

HSV vectors have not yet been used in the clinic for therapeutic gene delivery to the CNS, but progress has been made in developing vectors for the treatment of neurological diseases. Amplicon vectors expressing the anti-apoptotic peptide Bcl-2, neurotrophic factors GDNF and BDNF, and enzymes of the dopamine biosynthesis pathway have shown protection against neuron degeneration in a rat model of Parkinson's disease (Sun et al., 2005; Sun et al., 2003; Yamada et al., 1999). Also, replication-conditional vectors have been researched extensively for their oncolytic properties and applied in the clinic to treat glioblastoma multiforme and other cancers (Marconi et al., 2009; Markert et al., 2009; Markert et al., 2000; Shen and Post, 2007).

In summary, herpesvirus-derived vectors hold promise as tools for gene delivery to the CNS, but are still in their infancy. Further progress in the duration of transgene expression and scalable methods of safe vector production is required to compete with other available vector systems.

Conclusion

Several viral systems have been developed as vectors for gene delivery. We have described features of the ideal vector for clinical application in the CNS. Of the viral systems available, AAV vectors compare well against the ideal vector. AAV is minimally toxic and does not elicit a strong host immune response. Transduction can be achieved in several tissues of the CNS and therapeutic levels and duration of transgene expression have already been demonstrated. Additionally, scalable methods of production are readily available. Retroviral vectors have several promising attributes as well, including a larger packaging size, capacity for cell targeting and scalable production. However, the potential for vector integration and insertional mutagenesis has limited the use of these vectors. Continued improvement of non-integrating derivatives may eventually eliminate this risk and produce a vector with a stronger safety profile. Other viral systems, adenovirus and herpesvirus, still have several hurdles to overcome in meeting the standards of an ideal vector. Both are associated with potential pathologies related to strong host immune response (adenovirus) or cytotoxicity (herpesvirus). Factors such as lack of scalable production (for herpesvirus) and duration and level of transgene expression have also impeded the progress of these vectors. For these reasons adenovirus and herpesvirus vectors are currently not considered for most CNS applications. Thus, in most circumstances AAV is currently the most appropriate vector for therapeutic gene delivery to the CNS.

Acknowledgments

The authors would like to thank Adam Cockrell for critical review of the manuscript.

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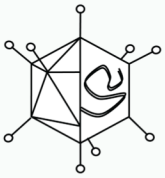
Vector	Specifications	Application in the CNS
Adeno-Associated Virus (AAV)  ~20 nm	Genome: ssDNA Capacity: ~4.7 kb (~ 2.2 kb with scAAV, ~8 kb with dual vectors) Forms circular and linear episomes; integrates with very low frequency Shown to infect neurons, astrocytes, glial and ependymal cells	Used extensively in clinical trials, including Parkinson's, Alzheimer's, Batten, and Canavan diseases. Preliminary studies suggest AAV vectors could also be used to treat Mucopolysaccharidoses (MPS), Spinocerebellar Ataxia, Amyotrophic Lateral Sclerosis (ALS), Epilepsy, and Huntington's disease.
Retrovirus: Human Immunodeficiency Virus (HIV)  ~100 nm	Genome: ssRNA Capacity: ~ 8 kb NIL vectors form linear and circular episomes; integration is low. Other HIV vectors integrate with high efficiency Shown to infect neurons and astroglial cells	Used in clinical trials for treatment of Parkinson's and Alzheimer's diseases. Vectors are being developed for use with Huntington's and lysosomal storage diseases.
Adenovirus  ~70 - 100 nm	Genome: dsDNA Capacity: ~36 kb Maintained as linear episomes; integration is minimal even with extensive homology to genome Shown to infect neural, astroglial and human glioma cells	Not in clinical use for gene therapy in the CNS due primarily to vector toxicity. Has been used for oncolytic potential as an anti-cancer agent.
Herpesvirus: Herpes Simplex Virus - 1 (HSV-1)  ~186 nm	Genome: dsDNA Capacity: ~150 kb Genome circularizes upon entering nucleus and is maintained episomally; integration is minimal Shown to infect neurons	Not in clinical use for gene therapy in the CNS due to problems with vector toxicity and production. Vectors are being developed for use with Parkinson's disease. Has also been developed for anti-cancer therapy.

Figure 1.
Viral Vectors for Gene Delivery to the CNS

Table 1

Comparison of viral vectors to the model of the ideal vector for gene delivery to the CNS.

Features of Ideal Vector →	Transduction of target tissue	Duration of transgene expression	Minimal Pathogenesis	Scalable production
AAV vectors	+++ (neurons, astrocytes, glial and ependymal cells)	+++ (6 months in brain, 6 years in other tissues of primates)	+++ (no associated pathologies)	+++ (large scale production of highly pure vector)
Retrovirus Vectors (NIL/Integrating)	++ (neurons and astroglial cells)	+/+++ (3 months in brain, 9 months in other tissues of murines)	+++/+(potential pathologies associated with integration)	++ (moderately scalable production of highly pure vector)
Adenovirus vectors	++ (neural, astroglial, and glioma cells)	++ (2 yrs in non-brain tissues of primates)	− (immune response to vector and helper-virus contamination present pathologies)	++ (large scale production; helper-virus contamination)
HSV vectors	+ (neurons)	+ (7 months in brain of murine, unstable)	− (helper-virus contamination presents pathologies)	− (scalable production not yet achieved)

How well each vector compares with the ideal vector on each feature is indicated with ‘+’ or ‘−’ signs.