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Current Prospects and Challenges for Epilepsy Gene Therapy

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Abstract

This review addresses the state of gene therapy research for the treatment of epilepsy. Preclinical studies have demonstrated the anti-seizure efficacy of viral vector-based gene transfer through the use of a variety of strategies – from modulating classic neurotransmitter systems to targeting or overexpressing of neuropeptide receptors in seizure-specific brain regions. While these studies provide substantive proof of principle for viral vector gene therapy, future studies must address the challenges of vector immunity, cellular specificity and effective global delivery. As these issues are resolved, viral vector gene therapy should significantly impact the treatment of intractable epilepsy.

Introduction

In this year alone, nearly 150,000 new cases of epilepsy will be diagnosed in the United States, adding to the estimated 2.5 million Americans (Hirtz, et al., 2007) and 50 million people worldwide (Hauser, 1990) who suffer from epilepsy. With this disease also comes a tremendous financial burden; epilepsy associated medical costs, lost or reduced earnings and decreased production result in an estimated annual cost to the United States of $15.5 billion (Shafer and Begley, 2000). Although current anti-epileptic medication effectively controls seizures in approximately 70% of people receiving optimal care, these medications are inadequate for the remaining 30% of patients (Cascino, 2008, Kwan and Brodie, 2000). Fewer than 10% of patients with drug refractory epilepsy are considered for surgical resection (Engel, et al., 1992, Sander, 1993, Shafer, et al., 1988, Siegel, 2004), leaving many epilepsy sufferers with no therapeutic recourse. Limited progress has been made since the early 1990s in the development of antiepileptic drugs with improved efficacy or tolerability (Loscher and Schmidt, 2011). Moreover the number of patients with drug-resistant epilepsies has not decreased, (Loscher and Leppik, 2002) providing an impetus for development of the new, more effective anti-epileptic drug treatments.

Gene therapy has significantly advanced in both preclinical and clinical research venues for the study and treatment of a wide array of human diseases, including disorders of the central nervous system (CNS). Mounting clinical successes (Bainbridge, et al., 2008, Cideciyan, et al., 2009) and promising safety, tolerability and longevity findings in human (Kaplitt, et al.,

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2007) and non-human primate (Hadaczek, et al., 2010) CNS have solidified gene therapy as a realistic alternative to small molecule treatments. The CNS has proven quite permissive to viral vector gene transfer and expression for many of the conventional delivery vectors. Adenovirus, herpes simplex virus, lentivirus, and adeno-associated virus are the most frequently utilized viral vectors for brain and spinal cord gene delivery. While each confers unique strengths and weaknesses many of these viral vectors support long-term, non-toxic delivery of foreign genetic information to host cells. Common uses of viral vectors include replacing deleted or mutated genes, targeted knockdown of dysfunctional or pathogenic genes, cellular expression of therapeutic proteins and expression or knockdown of a particular gene for molecular genetics studies.

This review addresses the state of gene therapy research for treating epilepsy. We begin by describing the major gene transfer strategies for treatment, including strategies that mimic or bear similarity to the currently available major small molecule anticonvulsants, and also those strategies that are less conventional. Epilepsy and seizure disorders are studied using a limited number of common epilepsy models intended to mimic selective aspects of clinical disease. Currently, most epilepsy gene therapy studies involve limbic brain structures such as the hippocampus, piriform cortex or entorhinal cortex where seizure activity is induced by kainic acid, pilocarpine or electrical kindling stimulation. These animal models are intended to be reflective of human temporal lobe epilepsy. Still other studies have involved non-limbic brain areas that support focal seizure activity. The first half of this review describes the strategies and results of viral vector gene transfer preclinical epilepsy studies The second half of this review explores the major considerations and current challenges in working with CNS gene transfer.

Gene transfer based on pharmacological anti-epilepsy targets

Unlike genetic disorders such as hemophilia or Duchenne's muscular dystrophy, for which gene therapy offers promise of specific gene correction (Manno, et al., 2006, Mendell, et al., 2010), intractable epilepsy often occurs without any known genetic linkage. While there are examples of familial mutations resulting in seizure disorders (e.g. sodium channel, voltage-gated, type I, alpha subunit [SCN1A] mutations), most intractable epilepsy cases are idiopathic. Thus, the predominant therapeutic strategy has focused upon attenuating the seizures through manipulation of excitatory or inhibitory function in the CNS. Certainly, one rational focus for gene therapy has been to recapitulate or improve upon existing anti-epileptic small molecule drugs. As such, targets have included excitatory neurotransmitter receptors or inhibitory GABA receptors.

NMDA Receptors

Given the involvement of N-Methyl-D-aspartic acid (NMDA) receptors in excitatory amino acid neurotransmission, several studies focused on altering NMDA receptor (NMDAR) function to decrease glutamatergic hyperexcitability. Haberman et al. (2002) initially found that AAV-mediated delivery of an antisense RNA specific to NMDAR1 reduced NMDA receptor function in primary cortical neurons and NMDAR1 protein *in vivo*. When the expression of this antisense RNA was driven by the cytomegalovirus (CMV) constitutive promoter, the stimulation threshold for focal seizure activity was significantly elevated. However, if the antisense NMDAR1 expression was driven by a tetracycline-off (TET-off) promoter, the opposite effect was found, i.e., a significant increase in focal seizure sensitivity. In another study, AAV-mediated partial knockdown (~50%) of the NR1 subunit of the NMDAR in the dorsal hippocampus increased seizure latency, decreased the total number of seizures, and decreased the total time in EEG seizures after hippocampal kainic acid (KA) administration (Kalev-Zylinska, et al., 2009). Thus, while these studies validate the ability to influence NMDA-seizure interactions, they also emphasize the importance of

vector tropism to the final outcome (discussed further in a subsequent section). Finally, using a unique gene therapy approach to target NMDAR function, During et al. (2000) orally administered NMDAR1 cDNA-packaged AAV, which elicited a humoral autoimmune response against the NR1 subunit. These anti-NR1 antibodies persisted for at least 5 months, were able to cross the blood-brain barrier, and reduced KA-induced seizures and seizure-related cell death.

GABA and GABA Receptors

As the chief inhibitory neurotransmitter in the brain, gamma-aminobutyric acid (GABA) and GABA receptor (GABAR) modulation are common targets of current anti-epileptic drugs. Mutations of GABAR subunits have been associated with familial generalized epilepsies (Macdonald, et al., 2003), and clinical and rodent models of temporal lobe epilepsy have revealed persistent altered hippocampal GABAR function and subunit composition (Brooks-Kayal, et al., 1999, Brooks-Kayal, et al., 1998, Buhl, et al., 1996, Gibbs, et al., 1997).

Mixed effects have come from altering relative GABAR subunit expression. An early study using AAV2-mediated gene transfer successfully upregulated or knocked down the GABA-A receptor α1 subunit in the rat inferior colliculus (Xiao, et al., 1997). While GABA-A receptor α1 antisense expression increased electrical stimulation-induced seizure duration, overexpression of the receptor subunit did not increase the seizure threshold. Other studies have found greater therapeutic success. Since GABAR-α4 levels increase, while GABAR-α1 levels decrease in neurons of the epileptic dentate gyrus (Brooks-Kayal, et al., 1998), a clever strategy was devised in which Raol et al. (2006) utilized the increased α4 promoter activity after pilocarpine-induced *status epilepticus* to drive α1 expression. When AAV-α1 expression was driven by the α4 promoter, pilocarpine-treated rats exhibited a three-fold increase in α1 subunit expression versus controls, and moreover a significantly increased latency to first spontaneous seizure. Raol et al. (2006) demonstrated for the first time that increasing the GABARα1 subunit can lead to inhibition of spontaneous seizures, although the effects were transient. Unfortunately, vector-derived transgene levels were markedly reduced within just two weeks. A likely explanation for this occurrence was silencing of the α4 promoter by the transduced hippocampal cells. The phenomenon of promoter silencing in brain tissue is not unique to the α4 promoter; a similar lack of long-term promoter function has been observed even with the strong constitutive CMV promoter (Klein, et al., 1998, McCown, et al., 1996).

Another GABAergic antiepileptic strategy involves *ex vivo*-engineered GABA-producing cells. Initially, studies relied upon transplantations of largely GABAergic crude cell populations, such as those from fetal striatal eminence (Loscher, et al., 1998). With the advent of advanced gene transfer techniques GABA-producing cells can now be engineered *ex vivo* for transplantation. Initially, Gernert et al. (2002) injected GAD65-engineered GABA-producing immortalized neurons into the central piriform cortex and subsequently initiated basolateral amygdala kindling. Pretreatment with GABA-producing cells caused a 200% increase in the pre-kindling partial seizure threshold, but the post-kindling seizure threshold change did not reach significance. Subsequently, Thompson et al. (2005) targeted the GABA-producing cells to the dentate gyrus of the hippocampus where the GAD-65 activity was linked to a TET-off promoter. The GAD-65 cells significantly reduced the incidence of severe limbic motor seizures, increased the seizure threshold and shortened afterdischarge duration elicited by granule cell stimulation. Therapeutic efficacy was confirmed, because adding doxycycline in the rats drinking water, which suppresses gene expression, prevented the seizure-suppressing effects. Similarly, Castillo et al. (2006) delivered GAD67 expressing, GABA-producing cells intranigrally prior to KA administration. Following a low dose KA regiment (5mg/kg KA per hour), the GABA cell-treated rats required a greater number of KA injections to produce the first convulsion,

showed a greater latency to class V seizures, and exhibited fewer total class V seizures. Following these studies, Castillo et al. (2008) tested whether GABAergic cell transfer could reduce the number of spontaneous seizures in the weeks following acute KA administration. Applying the same repeated low-dose KA regiment (5mg/kg/hour) over four hours, the researchers subsequently delivered GAD67-producing cells intranigrally. At 4 and 12 weeks post-injection, these rats exhibited fewer class IV or V motor seizures than sham or control cell-line treated rats. Thus there is good evidence from multiple investigators that genetically engineered GABAergic cell transplantation can alter seizure induction. Still, the promise of cell transplantation is not without problems: the long-term viability of transplanted cells remains poor (Thompson and Suchomelova, 2004). Further, when GAD-67 engineered cells were transplanted into the substantia nigra of post-kindled rats, Nolte et al. (2008) observed a considerable inflammatory immune reaction, and subsequent graft rejection. Because such rejection was not found from pre-kindling cell transplantation, the authors concluded that kindling-induced glial activation facilitated an immune response against the transplanted cells. Thus, presently both cell survivability and potential hazards of a seizure milieu complicate this genetic approach for seizure control.

Neuropeptides

In recent years neuropeptides and their receptors have become a novel gene therapy target for use in the treatment of focal epilepsy. Often termed endogenous anticonvulsants, many neuropeptides and their receptors are widely distributed throughout the brain. Moreover, a growing number of these small peptide ligands have been shown to exhibit anti-seizure properties in addition to modulating a wide range of physiological functions. Furthermore, endogenous expression of several neuropeptides and their receptors are dramatically altered in epileptic tissue, whereas the absence of normal ligand/receptor levels in transgenic animals can result in a more seizure-prone state. With regard to epilepsy, neuropeptide Y (NPY), galanin, and possibly somatostatin viral vector-based expression can result in seizure attenuation or prevention. Thus gene transfer treatment strategies hinge on directed overexpression of the neuropeptides and/or receptors to achieve an anti-convulsive state.

Galanin

In rodent epilepsy models, seizures rapidly and persistently deplete galanin stores largely from septum/diagonal band complex cholinergic fibers terminating on dentate gyrus/CA3 areas and can result in transient ectopic galanin expression in hilar neurons (Mazarati, et al., 1998). Galanin receptor-expressing cells are found throughout the CNS, and particularly in the limbic system, including in the piriform, amygdala/periamygdaloid areas, entorhinal and insular cortices and subiculum area of the hippocampus (Melander, et al., 1988, Skofitsch, et al., 1986). Also, galanin receptor expression can be affected by seizure activity. For example, Lu et al. (2005) found that pilocarpine-induced seizures resulted in 30% less galanin receptor 2/3-specific radiolabeled peptide binding within one day after the first class IV seizure.

When recombinant galanin peptide is delivered directly to the brain, seizure activity is attenuated *in vivo* (Mazarati, et al., 1998). Also, galanin knockout mice have lower seizure thresholds, and galanin-overexpressing mice have higher seizure thresholds in multiple seizure models (Kokaia, et al., 2001, Mazarati, et al., 2000). Current studies suggest that galanin conveys these anti-seizure properties via presynaptic actions where it can suppress glutamate release during epileptic discharge as well as hyperpolarize neurons (Lundstrom, et al., 2005). Galanin also exerts neuroprotective properties, as mice deficient in galanin production show greater KA-induced cell death while galanin overexpressing mice suffer less KA-induced cell death (Elliott-Hunt, et al., 2004).

Several researchers have examined whether viral vector-derived galanin can serve as an anticonvulsant. Initially, Haberman et al. (2003) created an AAV vector that expressed and constitutively secreted galanin, and upon its infusion into the inferior collicular cortex the threshold for focal seizure activity was significantly elevated. In this study, expression was driven by a TET-off promoter, so when expression was suppressed by doxycycline administration, the anti-seizure effects were reversed. Furthermore, infusion of this AAV vector into the dorsal hippocampus provided significant neuroprotection from KA-induced cell death. In another study, Lin et al. (2003) found that infusion of an AAV-galanin vector into the hippocampus decreased the quantity of and time spent in seizures from intra-hippocampal KA administration, but did not protect against cell death. The disparity between these two investigations may have resulted from subtle but important differences in transgene cloning strategies. Although discussed to a greater extent in subsequent sections, Haberman et al. (2003) utilized a constitutive secretory signal sequence that ensured the secretion of galanin in the area of the transduced cells. In contrast, Lin et al. (2003) did not include a specific signaling sequence within their galanin vector, which may have reduced overall levels of secreted, active neuropeptide. Lastly, a more recent study by McCown (2006) found that AAV vector expression and constitutive secretion of galanin in the piriform cortex prevented KA-induced EEG and behavioral seizure activity, while amygdala expression significantly elevated the seizure threshold in previously kindled rats. In total, these studies firmly establish the *in vivo* anticonvulsant and neuroprotective actions of AAV vector-derived galanin.

Neuropeptide Y (NPY)

Like galanin, NPY and NPY receptor expression is altered in seizure-related tissue. In both human and rodent epileptic tissue, NPY receptor Y2 is upregulated presynaptically at Schaffer collaterals, and Y1 receptor is downregulated in the dentate gyrus molecular layer as compared with normal tissue. Further, in epileptic, but not healthy control tissue, NPY-containing interneurons innervate perforant path and mossy fiber terminals (Vezzani and Sperk, 2004). Lastly, NPY plasma levels may be inversely correlated with febrile seizures because children with recurrent or long-lasting febrile convulsions have lower plasma NPY than controls or children having typical febrile convulsions (Lin, et al., 2010).

NPY seems to have potent anti-seizure properties. Direct infusion of recombinant NPY significantly attenuates seizure activity (Woldbye et al., 1997; Mazarati and Wasterlain, 2002). Conversely, transgenic mice lacking NPY expression exhibit increased susceptibility to GABA antagonist-induced seizures (Erickson, et al., 1996) and experience greater seizure progression and mortality associated with KA treatment (Baraban, et al., 1997). These NPY-related changes in seizure susceptibility are likely mediated by presynaptic NPY–Y2 receptors inhibiting glutamate release (Colmers and Bleakman, 1994, Greber, et al., 1994).

A number of studies have firmly established the anticonvulsant potential of NPY gene transfer and expression. Richichi et al. (2004) found that AAV-mediated expression of a preproNPY sequence markedly reduced EEG seizures and delayed seizure onset from intrahippocampal kainate. However, significant results were achieved only through the use of an AAV1/AAV2 chimeric virus (recombinant virus made by co-transfecting helper plasmids for AAV1 and AAV2) but not AAV2 alone. More recently improved anticonvulsant effects were found by packaging the preproNPY cDNA into AAV1-only capsids and driven by a stronger promoter (chicken beta actin) flanked by a woodchuck post-regulatory element (WPRE) (Noe, et al., 2010). Finally, delivery of AAV-NPY to the rat hippocampus after rapid hippocampal kindling decreased both the number of rats progressing to spontaneous seizures, and the overall frequency of spontaneous seizures in a subgroup of rats, an effect that appeared to be positively correlated with NPY expression

levels in the hippocampus (Noe, et al., 2008). Clearly, vector mediated expression of preproNPY can substantially influence seizure activity *in vivo*.

An important concern for epilepsy gene therapy studies is whether the anti-seizure effects of the treatment impair normal behavioral or physiological processes. In the case of NPY, some research suggests that preproNPY expression does not lead to any apparent learning and memory, emotive, or locomotor deficits (Noe, et al., 2010). However, one study found that naive rats treated with AAV-NPY in the hippocampus showed transiently delayed hippocampal-based learning and attenuation of long-term potentiation (LTP) in CA1 (Sorensen, et al., 2008). Subsequent research by the same group showed that a kindling model of epilepsy similarly led to compromise of both short-term synaptic plasticity and LTP, and that an NPY vector did not further exacerbate the kindling-induced changes in hippocampal function (Sorensen, et al., 2009).

Several gene transfer-based studies have evaluated and taken advantage of the receptor specificity responsible for NPY's anticonvulsant properties. When an AAV-NPY vector was infused into the hippocampus of NPY-Y1 receptor knockout mice, the mice exhibited fewer seizures and no mortality after peripheral KA administration. In contrast, Y2 knockout mice showed no difference in KA-induced seizures or mortality, when pretreated with an AAV-NPY vector. These findings suggest that Y2 NPY receptors further the anticonvulsant properties of NPY in the hippocampus (Lin, et al., 2006). Foti et al. (2007) provided further validation of NPY Y2 receptor influence on seizure activity. These investigators used AAV vectors to constitutively express and secrete a carboxy-terminus peptide fragment of NPY (NPY[13–36]), which acts as an NPY receptor agonist with high affinity for the Y2 NPY receptor (Sheikh, et al., 1989). AAV-NPY[13–36] transduction of the piriform cortex significantly attenuated KA-induced limbic seizures to the same degree as full length NPY (Foti, et al., 2007). Most recently, researchers have used AAV vectors to overexpress Y2 receptors in the hippocampus using AAV vectors. Expression of NPY Y2 receptors resulted in fewer generalized limbic seizures, increased the number of stimulations to reach severe limbic seizure grades during rapid kindling and shortened afterdischarge duration. Additionally, receptor overexpression led to greater latency to first motor seizures and *status epilepticus* following subcutaneous KA administration. While receptor overexpression was effective based on endogenous ligand activation, overexpressing both the Y2 receptor and NPY produced a more pronounced seizure-suppressant effect (Woldbye, et al., 2010). Recently, similar findings were also demonstrated using NPY and Y5 receptor overexpression in a KA model (Gotzsche, et al., 2011).

Somatostatin

Some evidence supports somatostatin as a seizure-related neuropeptide. Somatostatin knockout mice do not show spontaneous seizures, but have a shorter latency to KA-induced class V seizures. Also, perforant pathway kindling in somatostatin knockout mice results in longer afterdischarge duration, but does not alter the number of stimulations required to reach generalized limbic seizures (Buckmaster, et al., 2002). Although another transgenic mouse study found that somatostatin 2 receptors do not influence seizure sensitivity (Moneta, et al., 2002), preliminary findings have shown that AAV5 mediated expression of somatostatin in the dentate gyrus and CA1 of the hippocampus may delay amgydala kindling relative to GFP-treated rats (Zafar, et al., 2010).

Neurotrophic Factors

Neurotrophic factors (NTFs) and their receptors have long been considered important molecules involved in brain injury and repair (Abe, 2000), and are thought to be key mediators of both synaptic plasticity (Schinder and Poo, 2000) and the changes associated

with neural circuit reorganization occurring after seizures and neuronal cell death. Their substantial, yet complex role in the development and/or suppression of epileptogenesis, discussed in greater detail elsewhere (Simonato, et al., 2006), warrants examination of NTFs and their receptors as antiepileptic gene transfer targets.

Strong evidence suggests that glial-derived neurotrophic factor (GDNF) gene transfer can protect brain cells (e.g. dopaminergic neurons) against damage (Choi-Lundberg, et al., 1997, Mandel, et al., 1997). Increasing evidence shows that GDNF expression also may be therapeutic against both epilepsy and epilepsy-associated cell damage. Yoo et al. (2006) overexpressed GDNF in the rat hippocampus using adenovirus (Ad) seven days prior to KA administration. Compared to controls, Ad-GDNF-treated rats showed reduced KA-induced limbic seizure activity and fewer apoptotic cells in hippocampal CA3 and the dentate hilus. While these findings unquestionably demonstrate the therapeutic function for GDNF in their model, it is uncertain whether the function of GDNF is primarily neuroprotective or secondarily associated with neuroprotection via anti-convulsive properties. However, a more recent study points to GDNF specifically as an anticonvulsant protein. Kanter-Schlifke et al. (2007) reported that transduction of dorsal and ventral hippocampus with AAV-GDNF reduced the number of generalized seizures and shortened the seizure duration evoked by ventral hippocampal kindling. Additionally, post-kindling treatment with AAV-GDNF led to an increased seizure threshold in these animals and reduced seizure frequency during rapid stimulation-induced *status epilepticus*.

Beyond GDNF, a cluster of related research studies have recently found that short-term NTF overexpression can prevent progression to epileptogenesis. Using herpes-based vectors, the researchers transiently expressed fibroblast growth factor-2 (FGF-2) and brain-derived neurotrophic factor (BDNF) to the rat dorsal hippocampus 3 days after pilocarpine-induced status epilepticus. This supplementation strategy was found to reduce seizure-related neuronal cell death (Paradiso, et al., 2009), lessen hippocampal neuroinflammation (Bovolenta, et al., 2010), and prevent mossy fiber sprouting (Paradiso, et al., 2011). Moreover, in all aforementioned studies, NTF-treated rats had fewer and less severe spontaneous seizures than control-treated animals. Although these data demonstrates the powerful anti-epileptogenic potential of FGF-2/BDNF overexpression, the researchers point out that the molecules' antiepileptogenic effects may not translate into antiseizure therapy *per se* because treating rats with the viral vectors after spontaneous seizure development did not diminish future seizure events (Paradiso, et al., 2009). This important negative result, coupled with the potential detrimental effects of long-term expression of NTFs (Thoenen and Sendtner, 2002), suggest a narrow but potentially valuable use for these transgenes in preventing epilepsy.

Adenosine (ADK)

Adenosine, like some neuropeptides, has been classified as an 'endogenous anticonvulsant' (Dragunow and Goddard, 1984), given its apparent ability to suppress seizure activity in the brain. Patients experiencing seizures have strong upregulation of dialysate adenosine levels at seizure foci, and this increased adenosine concentration is sufficient to suppress epileptiform activity *in vitro* (During and Spencer, 1992). *In vivo* the astrocytic enzyme adenosine kinase (ADK) limits extracellular adenosine levels by catabolizing adenosine into 5′-AMP upon cellular uptake (Pascual, et al., 2005, Zimmermann, 2000), and in fact several studies have linked seizures and ADK activity. For example, intra-amygdala KA delivery in mice causes spontaneous electrographic seizure activity in CA3 with a concomitant increase in hippocampal ADK activity (Li, et al., 2008). Also, transgenic mice that overexpress ADK in the hippocampus develop spontaneous CA3 electrographic seizures while below normal ADK expression attenuates the seizures (Li, et al., 2008). In an effort to translate these findings into therapy, Theofilas et al. (2011) used an AAV8 vector to express an ADK

antisense in CA3 of the hippocampus where expression was driven by as astrocyte-specific promoter. This antisense ADK mRNA successfully blocked spontaneous EEG seizure activity in ADK overexpressing mice. Future studies will most certainly test this promising anti-epileptic strategy in non-transgenic epilepsy models as well.

Important considerations for epilepsy gene therapy

Successful epilepsy gene therapy will require targeting the appropriate cells with a therapeutic level of gene expression that is persistent yet controlled and safe. As the previous sections have outlined, there are a multitude of promising anti-epileptic gene transfer approaches. Moreover, the choice of transgenes will continue to advance as basic discoveries provide additional insight into the basis for epilepsy. Inevitably, the strategies for translating such discoveries into gene therapy applications will be governed largely by the properties of the gene therapy vehicles. There are currently a number of diverse alternatives for gene delivery, including adenovirus, lentivirus, retrovirus, and AAV. All have the potential to serve as adequate gene delivery systems to the CNS, but at present AAV vectors are the leading platform for this purpose: Beyond the vast number of preclinical and basic mammalian studies involving AAV delivery to the brain and/or spinal cord, the large majority (approximately 75%) of clinical trials that have been initiated for CNS gene therapy have utilized AAV, as compared with the percentage of trials utilizing adenovirus (6%), lentivirus (12%) or retrovirus (6%) (Gray, et al., 2010). As such, AAV serves as an appropriate example for both the possibilities and current limitations of gene therapy. The following sections address many of the limitations and liabilities involved with CNS gene transfer, as well as recent progress to overcome such obstacles.

Vector packaging size

Viral vectors differ greatly in packaging capacity. Herpes simplex virus type 1 can package more than 100 kb, whereas adenovirus can package approximately 25kB, lentivirus 9kb and AAV only 4.7kb. Thus, vector-packaging limitations can limit the utility of certain vectors, such as AAV. For example, SCN1A gene mutations comprise the most common channelopathy-related epilepsies, but the SCN1A gene is 6030bp (NCBI Reference Sequence: NM_001165963.1), a size beyond the maximal capacity of AAV. However, a number of strategies have been developed that address packaging limitations. In general these strategies focus upon improving DNA recombination for delivering multiple partial constructs (Hirsch, et al., 2010). In addition development of truncated promoters allows the packaging of larger transgenes. For example, Gray et al. (2011) has described a truncated hybrid chicken beta actin promoter (~800 bp) that is substantially smaller than the full-length hybrid chicken beta actin promoter of >2000bp.

Targeting the right cells

In previous sections we cited examples in which epilepsy researchers utilized viral vector gene transfer to overexpress inhibitory receptors, receptor subunits, neuroactive peptides and even antisense mRNA to cell-specific enzymes. Each strategy ostensibly assumed that the viral vectors would transduce the intended CNS cells and cause negligible effects to other cells. However, the pattern of AAV transduction depends upon viral tropism, promoter permissiveness, route of administration, purity of the vector preparation and the state of the *in vivo* milieu. When constitutive promoters drive expression, direct CNS infusion of AAV2, AAV5 or AAV8 results in near total neuronal tropism. In contrast, when gene expression is driven by an oligodendrocyte specific promoter, AAV2 transduction is limited to oligodendrocytes (Chen et al., 1998), while astrocyte expression can be obtained by using an astrocyte-specific promoter with AAV5 or AAV8 (Lawlor, et al., 2009). Notably, the use of a cell type-specific promoter in a viral vector expression cassette does not always result in cell-specific transgene expression. Peel and Klein (2000) found that AAV2-GFAP-driven

GFP expression in the uninjured rat cervical spinal cord was localized predominantly to neurons (rather than astrocytes, as intended), whereas nerve root avulsion led to robust astrocytic expression of the transgene. Route of administration can also determine cellular tropism. When gene expression is driven by a constitutive promoter, direct CNS injection of AAV9 results in almost exclusive neuronal transduction (Klein, et al., 2008), whereas after peripheral administration, AAV9 vectors transduce both neurons and astrocytes in the CNS (Foust et al., 2009; Duque et al., 2009). Another factor affecting cell-specific expression is vector preparation purity. By controlling both serotype and promoter, Klein et al. (2008) found that AAV vectors purified by cesium chloride gradients led to higher astroglial expression than vectors purified by iodixonal. The likely source for this difference involves the activation of astrocytes by vector production-related impurities remaining in the cesium preparation. Beyond vector preparation purity, the state of the CNS milieu can also alter the pattern of vector derived gene expression. As mentioned previously, nerve injury can redirect spinal cord viral vector transgene expression from neurons to glia when the transgene is under a glial-specific promoter (Peel and Klein, 2000). Recently Weinberg et al. (2011) found that cell-specific expression can shift even using a constitutive, non-selective promoter. In KA treated rats, AAV5-CBA-GFP mediated gene expression resulted in significant astroglial expression whereas in normal rat brain, transgene expression was restricted to neurons. This effect was undoubtedly linked to neuroinflammation, gliosis, and/or neuronal cell loss observed in this epilepsy model. Finally, even within neuronal cell populations, variables such as viral titer have been shown to correspond to the extent of pyramidal versus interneuron gene expression (Nathanson, et al., 2009). Thus vector preparation purity, CNS milieu, and viral titer can all influence both the outcome and interpretation of *in vivo* gene therapy studies.

Given the inherent uncertainty in vector-based expression patterns *in vivo*, an important question is: “how important is cell-specificity to the experimental design?” In the previously mentioned NMDAR1 antisense study, NMDAR1 knockdown resulted in either a seizure sensitization or seizure suppression depending on the choice of constitutive promoter (Haberman, et al., 2002). For increased seizure sensitivity the preponderance of gene expression likely occurred in inhibitory neurons, whereas in the case of decreased seizure sensitivity, the preponderance of expression likely occurred in principle output neurons. In support of these explanation, injecting a mixture of two recombinant AAV vectors packaging different reporter genes behind separate constitutive promoters resulted in a significant number of neurons expressing only one or the other transgene (Haberman, et al., 2002). However, results from other research suggest that this concern may not apply to all therapeutic applications. For example, overexpression of GABR-alpha1 (Raol, et al., 2006), knockdown of NMDAR1 (Kalev-Zylinska, et al., 2009), and NPY Y2 receptor overexpression in the dorsal hippocampus (Woldbye, et al., 2010) all resulted in seizure suppression, despite the theoretical possibility of opposing or null effects, should the vector have transduced unintended cell types.

Other anti-epileptic gene transfer strategies may avoid the complications of vector related cell specificity. For example, Theofilas et al. (2011) used an astrocyte specific promoter to restrict expression of adenosine kinase (ADK) antisense to astrocytes. Another strategy involves directing transduced cells to constitutively secrete the therapeutic transgene by including a secretory signal sequence in the transgene coding sequence. Haberman et al., (2003) first demonstrated the utility of such an approach that was further validated with different transgenes in subsequent studies (Foti et al., 2007). Using a constitutive secretory platform, as long as the therapeutic target exists in the region of transduction, the specificity of cellular transduction should not significantly influence outcome.

Vector immunity

Persistent genetic manipulation generally represents the major goal of CNS gene transfer, but even though the CNS is considered somewhat immune privileged, a number of studies have identified immune responses against viral vectors in the CNS. Several reports have verified that neutralizing antibodies (nAb) present in the serum can prevent brain transduction by AAV (Mastakov, et al., 2002, Peden, et al., 2004). Of the currently recognized AAV serotypes, immunoglobulins against AAV2 are most prevalent in the human population (72%), whereas those against other serotypes are far less prevalent (e.g. AAV5 the least prevalent, at 3.2%) (Boutin, et al., 2010). In a recent study of prior AAV2 immunization, Weinberg et al. (2011) found that in rats, nAb levels comparable to those found in most healthy human subjects blocked AAV2, but not AAV5 transduction, and led to an influx of serum IgG into AAV2 but not AAV5-injected brain areas. In addition to humoral immunity, cytotoxic T responses against the vector capsid transgene are considered a major reason for long-term loss of peripheral therapeutic gene expression. Despite the fact that 95% of the viral genes have been removed from current AAV vectors, therapeutic transgenes and the viral capsid both have demonstrated the capacity to elicit a cell-mediated immune response. A recent clinical trial for Duchenne's muscular dystrophy demonstrated the potential for cytotoxic T-cell destruction of transduced skeletal muscle arising from transgene immunity (Mendell, et al., 2010). Another study found that capsid-specific CTLs eliminated AAV2-transduced liver cells which led to therapeutic failure in a hemophilia B clinical trial (Manno, et al., 2006). Further investigations suggested that CD8+ T-cells had targeted capsid antigens on the AAV2-transduced cells, indicating that the AAV2 capsid could induce a CTL response in humans. Evidence has revealed that CD8+ T-cells actively survey the brain and can rapidly and specifically respond to CNS peptide presentation through trafficking, clonal expansion, and extravasation into the parenchyma (Galea, et al., 2007). Because the potential CTL-mediated killing of vector transduced brain cells presents a significant concern, future studies should address the likelihood of such an event. Despite the potential for immune responses acting against successful long-term viral vector gene transfer, it is important to note that CNS gene transfer can persist long term. For instance, in a non-human primate model of Parkinson's disease AAV2 mediated gene expression has been demonstrated for at least eight years (Hadaczek, et al., 2010).

Peripherally based CNS gene delivery

A major strength of viral vector-based gene transfer is the ability to target a limited population of cells sufficient to influence focal epilepsy. For other non-focal epilepsies however, localized transduction likely would probably not prove effective. Epilepsies with a clear genetic basis, such as SCN1A gene mutations, do not exhibit a localized focus for vector targeting. Thus, direct parenchymal injection would not prove effective, even with multiple injections. In meeting the need for global CNS gene delivery, recent studies have demonstrated the means by which widespread CNS gene transfer can be obtained through peripheral vector administration. Intravenous administration of self-complementary (see (McCarty, 2008) for review) AAV9 crosses the blood-brain barrier of neonate and adult mice and transduces substantial numbers of neurons and glia (Duque, et al., 2009, Foust, et al., 2009). Translation to humans, however, requires overcoming several challenging impediments. For example, Gray et al. (2011) found that scAAV9 delivery to the CNS of nonhuman primates was significantly hampered by pre-existing nAbs, low relative neuronal to glial transduction, and high viral tropism to peripheral organs. Recent advances have been made that address some concerns over peripheral delivery of viral vectors to the CNS. One recent study demonstrated that by incorporating a liver-specific microRNA (miR-122) binding site into the 3 untranslated region (UTR) of an AAV expression cassette, normally high liver expression was dramatically reduced without effecting expression in other transduced tissues (Qiao, et al., 2011). Another study applied this approach towards CNS

gene transfer. Xie et al. (2011) demonstrated that by incorporating a binding site for miR-122 and miR-1 (a microRNA found in heart and skeletal muscle) into a peripherally administered AAV9 cassette, transgene expression in major peripheral organs was substantially reduced without interfering with CNS expression.

Another peripheral vector administration strategy has been developed with a specific epilepsy focus. The novel approach was based upon the fact that seizures cause a localized compromise in the blood-brain barrier. In their study, Gray et al. (2010) created a library of chimeric AAV capsid clones using *in vitro* capsid DNA shuffling. The library was infused intravenously after KA-induced seizure activity, a time when the blood-brain barrier was compromised. Two clones were rescued that exhibited the ability to selectively cross the seizure compromised blood-brain barrier and transduce cells after intravenous administration. Moreover, unlike AAV9, the clones did not cross the intact blood brain barrier and surprisingly exhibited little peripheral accumulation. With the advancement of these and other novel approaches, it may be possible to influence the full extent of non-focal seizures after intravenous vector administration.

Conclusion

More challenging work still lies ahead for the epilepsy gene therapy research community. Improvements in vector production and utilization will be necessary for advancing the safety, stability and efficacy of gene therapy strategy. However, the core determinant of success still hinges on the choice of genetic target. Given the growing number of promising antiepileptic gene therapy vectors under preclinical development, a key challenge will be in deciding which of these promising therapeutics have the greatest likelihood of clinical success. Because rigorously validating a single therapeutic vector in multiple epilepsy models can be extensive, few proposed antiepileptic gene therapy vectors have been studied in a chronic epilepsy model. Moreover, a clear benchmark for anti-epileptic gene therapy has not been determined. Thus, what degree of efficacy in which preclinical models warrants translation to human subjects, and what is the minimal human disease status that warrants antiepileptic viral vector consideration? Progress towards establishing these preclinical benchmarks will be essential to reaching clinical success.

In closing, although the pharmaceutical industry has introduced a number of new antiepileptic drugs over the last 20 years, the proportion of intractable, drug resistant patients has remained the same. Converging data from numerous preclinical animal studies provide substantial support for the clinical application of AAV based gene therapy to treat focal epilepsies. These advances offer significant promise, while the challenges of overcoming vector immunity, improving cellular specificity and global delivery remain challenging but resolvable obstacles. With improvements in the basic understanding of epilepsy pathologies and gene transfer methodologies, viral vector gene therapy should significantly impact therapeutic success for intractable epilepsy.

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Table 1

Epilepsy gene therapy-based preclinical studies.

Genetic target	Placement	Delivery Vector	Experimental Model	Functional Outcome	References
ADK antisense	Hippocampus	AAV-GFAP-tg	ADK-overexpressing transgenic mice (spontaneous EEG seizures)	Prevention of spontaneous EEG seizure activity	(Theofilas, et al., 2011)
ASPA	Intracerebroventricular	Ad-CBA-tg	SER rats	Transient tonic seizure reduction	(Seki, et al., 2004)
FGF-2/BDNF	Hippocampus	HSV-CMV/ICP0-tg	Pilocarpine ip	Reduced spontaneous EEG seizures, neuronal cell death, inflammation, mossy fiber sprouting	(Bovolenta, et al., 2010, Paradiso, et al., 2009, Paradiso, et al., 2011)
GABAR α1 antisense	Inferior colliculus	AAV-CMV-tg	Stimulation-induced seizure activity	Increased seizure duration	(Xiao, et al., 1997)
GABAR α1	Hippocampus	AAV-GABR α4-tg	Pilocarpine-induced status epilepticus	Transiently reduced spontaneous seizures	(Raol, et al., 2006)
Galanin (+Fib)	Collicular Cortex	AAV-TET off-Fib-tg	Focal Electrical stimulation	Increased seizure threshold	(Haberman, et al., 2003)
Galanin (+Fib)	Hippocampus	AAV-TET off-Fib-tg	Kainic acid ip	Neuroprotection	(Haberman, et al., 2003)
Galanin	Hippocampus	AAV-NSE-tg-WPRE	Kainic acid ih	Reduced number of seizures	(Lin, et al., 2003)
Galanin (+Fib)	Piriform Cortex	AAV-CBA-Fib-tg	Kainic acid ip	Seizure inhibition	(McCown, 2006)
Galanin (+Fib)	Piriform Cortex	AAV-CBA-Fib-tg	Focal electrical stimulation	Increased seizure threshold	(McCown, 2006)
GDNF	Hippocampus	AAV-CBA-tg+WPRE	Hippocampal kindling	Decreased number of generalized seizures, increased seizure threshold	(Kanter-Schlifke, et al., 2007)
GDNF	Hippocampus	Ad-CMV-tg	Kainic acid ip	Delayed seizure behaviors,	(Yoo, et al., 2006)
ICP10PK	Intranasal	HSV-ICP10-tg	Kainic acid ip	Reduced behavior seizures, neuroprotection	(Laing, et al., 2006)
NMDAR1 antisense	Collicular cortex	AAV-CMV-tg	Focal electrical stimulation	Increased seizure threshold	(Haberman, et al., 2002)
NMDAR1 antisense	Collicular cortex	AAV-TET off-tg	Focal electrical stimulation	Decreased seizure threshold	(Haberman, et al., 2002)
NMDAR1	Peroral administration	AAV-CMV-tg	Kainic acid ip	Reduced kainic acid-induced seizures and cell death	(During, et al., 2000)
NMDAR1 NR1 antisense	Hippocampus	AAV-CBA-tg-WPRE	Kainic acid ih	Increased seizure latency, decreased total number of seizures, decreased total time in EEG seizures	(Kalev-Zylinska, et al., 2009)
NPY	Hippocampus	AAV-NSE-tg-WPRE	Kainic acid ih	Delayed seizure behavior	(Richichi, et al., 2004)
NPY	Hippocampus	AAV-NSE-tg-WPRE	Hippocampal kindling	Increased seizure threshold and delayed rate of kindling	(Richichi, et al., 2004)
NPY (+Fib)	Piriform Cortex	AAV-CBA-Fib-Fib	Kainic acid ip	Reduced and delayed seizure behaviors	(Foti, et al., 2007)
NPY	Hippocampus	AAV-CBA-tg-WPRE	Rapid hippocampal kindling	Less progression to spontaneous seizures; Fewer spontaneous seizures	(Noe, et al., 2008)
NPY	Hippocampus	AAV-CBA-tg-WPRE	Kainic acid ih	Fewer EEG seizures	(Noe, et al., 2010)
NPY 13–36 (+Fib)	Piriform cortex	AAV-CBA-Fib-tg	Kainic acid ip	Reduced and delayed seizure behaviors	(Foti, et al., 2007)

Genetic target	Placement	Delivery Vector	Experimental Model	Functional Outcome	References
NPY/NPY Y2 receptor	Hippocampus	AAV-NSE-tg-WPRE	Rapid hippocampal kindling	Increased stimulations to severe limbic seizure, shorter afterdischarge duration	(Woldbye, et al., 2010)
NPY/NPY Y2 receptor	Hippocampus	AAV-NSE-tg-WPRE	Kainic acid, sc	Greater latency to first behavioral seizure and status epilepticus	(Woldbye, et al., 2010)
NPY/NPY Y5 receptor	Hippocampus	AAV-NSE-tg-WPRE	Kainic acid, sc	Greater latency to first behavioral seizure, shorter seizure duration	(Gotzsche, et al., 2011)

Abbreviations are: AAV: adeno-associated virus; Ad: adenoviral; ADK: adenosine kinase; ASPA: aspartoacylase; BDNF: brain-derived neurotrophic factor; CBA: cytomegalovirus enhancer with a chicken beta-actin promoter; CMV: cytomegalovirus; EEG: Electroencephalographic; Fib: fibronectin secretory signal peptide; FGF-2: basic fibroblast growth factor-2; GABAR: gamma-Aminobutyric acid A receptor; GDNF: glial-derived neurotrophic factor; GFAP: Glial fibrillary acidic protein; HSV: herpes simplex virus; ICP0: infected cell polypeptide 0; ICP10PK: ICP 10 protein kinase; ih: intrahippocampal; ip: intraperitoneal; NMDAR1: N- methyl-D- aspartate receptor 1; NPY: Neuropeptide Y; NSE: neuron- specific enolase; SER: spontaneously epileptic rats; SN: substantia nigra; TET off: tetracycline-off regulatable promoter; TG: transgene; WPRE: woodchuck virus post-transcriptional regulatory element.