Original Article

Adenosine kinase, glutamine synthetase and EAAT2 as gene therapy targets for temporal lobe epilepsy

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Astrocytes are an attractive cell target for gene therapy, but the validation of new therapeutic candidates is needed. We determined whether adeno-associated viral (AAV) vector-mediated overexpression of glutamine synthetase (GS) or excitatory amino-acid transporter 2 (EAAT2), or expression of microRNA targeting adenosine kinase (miR-ADK) in hippocampal astrocytes in the rat brain could modulate susceptibility to kainate-induced seizures and neuronal cell loss. Transgene expression was found predominantly in astrocytes following direct injection of glial-targeting AAV9 vectors by 3 weeks postinjection. ADK expression in miR-ADK vector-injected rats was reduced by 94–96% and was associated with an ~50% reduction in the duration of kainate-induced seizures and greater protection of dentate hilar neurons but not CA3 neurons compared with miR-control vector-injected rats. In contrast, infusion of AAV-GS and EAAT2 vectors did not afford any protection against seizures or neuronal damage as the level of transcriptional activity of the glial fibrillary acidic promoter was too low to drive any significant increase in transgenic GS or EAAT2 relative to the high endogenous levels of these proteins. Our findings support ADK as a prime therapeutic target for gene therapy of temporal lobe epilepsy and suggest that alternative approaches including the use of stronger glial promoters are needed to increase transgenic GS and EAAT2 expression to levels that may be required to affect seizure induction and propagation.

INTRODUCTION

Gene therapy is an attractive prospect for treatment of neurologic diseases because it has the potential to produce long-term therapeutic benefits for diseases that are typically chronic in nature.\(^1\) Outcomes from gene therapy clinical trials including Parkinson’s disease\(^2\) and Canavan disease\(^3\) have been promising. These studies suggest that gene therapy can be conducted safely, is well tolerated and that symptomatic relief or modification of disease progression is achievable. However, further optimization of these and other existing strategies, as well as identification of new approaches, are still needed.

To date, neurons have been the main cellular target for gene therapy in the brain, with strategies primarily centered on modification of neuronal function to enable a therapeutic outcome. Glial cell types such as astrocytes and microglia have received less attention, to a large extent, because of the lack of vectors capable of efficient and selective targeting of transgenes to these cell types. This landscape has changed in the past few years with advances in viral vector technology; lentiviral\(^7\) and recombinant adeno-associated viral (AAV) vectors were based on AAV serotypes 5, 8 or 9 (AAV5, AAV8 and AAV9),\(^8\)–\(^10\) which are capable of astrocytic transduction. Combined with the clear recognition that astrocytes have a number of diverse and important roles in the healthy brain and can contribute to disease progression in neurodegenerative diseases,\(^11\) attention has now been turned to exploring astrocytes as an alternative cell target for central nervous system gene therapy. Their abundance in the face of neuronal depletion in neurodegenerative diseases makes them an attractive choice for gene therapy.

The identification of molecular targets and the development of strategies aimed at altering astrocyte function to achieve therapeutic benefit have not been fully explored. One disease amenable to an astrocyte-targeting gene therapy approach is mesial temporal lobe epilepsy (TLE), one of the most common forms of drug-resistant epilepsy that is characterized by the unpredictable and recurrent occurrence of seizures. Spontaneous seizures often originate from a spatially restricted focal region of neuronal hyperexcitability in the anteromedial temporal lobe that includes the epileptic hippocampus. Surgical resection of this brain region is highly effective in controlling seizures in about 85% of these patients, thereby validating this as a specific target for therapeutic intervention.\(^12\) The main neuropathologic hallmark of TLE is Ammon’s horn sclerosis, defined by a selective pattern of neuronal cell loss in the hippocampus, aberrant axonal sprouting and reorganization of neuronal circuitry, as well as reactive gliosis.\(^13\) Although the past two decades have largely focused on unraveling the contribution of neurons as the underlying substrate for generation of chronic recurrent seizures, in recent years, research in this field has shifted to gaining an understanding of the contribution of astrocytes to epilepsy.

Reactive gliosis, whereby astrocytes undergo structural and biochemical changes, is a prominent feature of TLE in humans and many animal seizure models, and there are several mechanisms by which reactive astrocytes could contribute to the evolution of abnormal network excitability in TLE.\(^14\)–\(^15\) Two possible contributing processes could involve the altered ability of astrocytes to regulate synaptic glutamate and adenosine levels.

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Received 12 February 2014; revised 15 July 2014; accepted 6 August 2014; published online 18 September 2014
Increased extracellular glutamate is strongly linked to the initiation of spontaneous seizures in humans, with proconvulsant effects replicated in animal models by administration of glutamate agonists. Astrocytes are largely responsible for preventing glutamate receptor overactivation by mediating glutamate clearance from the synapse via the excitatory amino-acid transporter 1 (EAAT1) and astrocyte-specific EAAT2 glutamate transporters. Astrocytic glutamate is then converted to glutamine by glutamine synthetase (GS) before being shuttled back to neurons to act as a precursor for glutamate or GABA synthesis. Alterations in EAAT2 and GS expression in sclerotic hippocampal tissue removed during epilepsy surgery, and the demonstration that elevated extracellular glutamate levels appear to trigger spontaneous seizures in human TLE has provided some impetus into establishing whether these proteins would be suitable gene therapy targets. However, whether glutamate transporters are altered in human epilepsy is controversial; no change in transporter expression in resected tissue from TLE patients was found in the two studies, whereas regionally specific changes in EAAT1–3 expression have also been reported. Differences in tissue processing or disease severity might explain these discrepancies. GS expression in astrocytes is also downregulated in resected epileptic tissue. Although the changes in glutamate transporter expression in human TLE warrants further investigation, studies in animal models provide compelling evidence that impaired glutamate uptake and processing by astrocytes has the potential to contribute to the development of seizures. Genetic deletion of either EAAT2 or GS expression alone, or pharmacologic inhibition of glutamine synthetase by methionine sulfoximine is sufficient to cause the development of spontaneous seizures in rats and mice.

An alternative target is adenosine kinase (ADK), an enzyme expressed exclusively in astrocytes and involved in regulating extracellular concentrations of adenosine. Adenosine is a potent natural anticonvulsant and protective molecule that has a pivotal role in seizure termination and contributes to postictal refractoriness to further seizures. Work led by Boisson et al. has shown ADK expression to be a key molecular link between astrogliosis and neuronal dysfunction in epilepsy. Transgenic mice engineered with increased ADK activity are prone to the development of spontaneous seizures and, conversely, boosting local adenosine concentration by transplantation of ADK-deficient cells are sufficient to prevent epileptogenesis. Pharmacologic activation of adenosine A1 receptors by adenosine analogs provides effective seizure control in various experimental seizure models including mice resistant to treatment with conventional antiepileptic drugs. Moreover, AAV8-mediated astrocytic expression of an ADK cDNA in antisense orientation prevents the occurrence of spontaneous recurrent seizures in ADK transgenic mice.

These results highlight EAAT2, GS and ADK as potential therapeutic targets for TLE, but whether they are suitable in a gene therapy context has not been fully investigated. In this study, we determine whether AAV9-mediated overexpression of EAAT2 and GS or silencing of ADK expression using an alternative RNA interference-mediated mechanism in astrocytes can mitigate against kainate-induced seizures and neuronal cell death in rats.

RESULTS

AAV8 and AAV9 vectors mediate widespread transduction of astrocytes in the rat hippocampus

Previous studies have demonstrated that systemic administration of AAV9 vectors expressing green fluorescent protein (GFP) under the control of the strong constitutive chicken β-actin/cytomegalovirus hybrid (CAQ) promoter directs GFP expression to astrocytes and neurons. In contrast, direct injection of this vector into the brain leads predominantly to neuronal transduction. We and others showed previously that use of cell-specific promoters in AAV8 vectors can direct transgene expression in glial cell types including astrocytes and oligodendrocytes following direct injection of vector into the rat brain. This prompted us to test the capacity of the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter to direct and restrict transgene expression to astrocytes following direct infusion into the rat brain. We infused titer-matched AAV9 or AAV8 vectors expressing GFP into the dorsal hippocampus of rats and analyzed the spatial distribution and cellular transduction patterns of transgene expression at 3 weeks. A highly sensitive anti-GFP antibody was used to detect GFP expression by immunohistochemistry. GFP expression was widely distributed throughout the dorsal hippocampus, spreading ~4 mm over the rostral–caudal axis with both vector serotypes, and was restricted to the injected hemisphere (Figure 1a). The total volume of the hippocampus transduced by these vectors was similar (Figure 1b); but notably, GFP-immunoreactive cells were more homogeneously distributed throughout the dorsal hippocampus, spreading up to the border with the corpus callosum and beyond the lateral border of the mossy fiber pathway in AAV9-vector-injected rats (Figure 1c). The large majority of GFP-immunoreactive cells were astrocytes based on their location away from the hippocampal dentate granule and pyramidal neuron cell body layers and cellular morphology. Immunostained cells with an intricate bushy-like morphology, characteristic of hippocampal astrocytes, were clearly visualized toward the outer edges of the transduced zone, such as the dendritic fields of the CA1 region. GFP-positive cells were found in abundance in the granule cell molecular layer and hilus adjacent to the infusion site (Figure 1c). GFP immunoreactivity was also found in a subset of dentate granule neurons that was supported by staining of the mossy fiber pathway, the axonal projections from these cells (Figure 1c). A few GFP-positive CA1 neurons were also found (Figure 1c, inset), but neuronal transduction was not found in the CA3 region with either vector serotype (Figure 1c). Immunofluorescent labeling with antibodies to cell-specific markers confirmed the identity of transduced cells. GFP-positive cells colocalized with the astrocyte marker GFAP, and with NeuN-immunoreactive neurons scattered throughout the granule cell layer but not with the microglial marker Iba1 (Figure 1d). Overall, the transduction patterns were very comparable between the two AAV serotypes, and shows that the use of an astrocyte-specific promoter in AAV9 vectors can cause a significant shift in transgene expression to astrocytes.

Transgene expression in the rat hippocampus following AAV9-mediated gene transfer

Next, we developed AAV plasmids expressing the putative therapeutic transgenes, a hemagglutinin-tagged-GS and a FLAG-tagged EAAT2 (Figure 2a). The functionality of both these epitope-tagged transgenes was confirmed using in vitro models (Supplementary Data). A luciferase construct was generated as a control. Two AAV plasmids expressing miRNA sequences targeting rat ADK mRNA or a control miRNA linked to an Emerald GFP reporter to facilitate cell tracking of miRNA sequences were also developed (Figure 2a). Because we observed marginally more widespread astrocyte transduction with the AAV9-GFP vector, we packaged these constructs into AAV9 vectors. Subgroups of rats were injected with these vectors and we confirmed expression of these transgenes 3 weeks later. The cellular expression patterns of anti-HA, anti-FLAG, anti-luciferase and anti-GFP immunoreactivity, which was used to visualize cells transduced with the AAV-miRNA vectors, were consistent with that found with the AAV9-GFP vector, with robust transgene staining in astrocytes with all vectors (Figure 2b). Highly dense transgene expression was found in the dentate granule cell molecular layer, with fewer transduced astrocytes across the CA2 and lateral CA3 regions on the injected side and no transgene expression observed in the contralateral non-injected hippocampus.
Anticonvulsant efficacy of AAV9-mediated overexpression of EAAT2 and GS and ADK knockdown in astrocytes

We then asked whether overexpression of EAAT2, GS or suppression of ADK expression in astrocytes could modulate seizure development, duration or severity in an experimental rat model of TLE. We used the intrahippocampal kainate infusion model that has been shown to elicit a characteristic pattern of recurrent and/or continuous status epilepticus that persists for ~ 2 h, but is associated with a low mortality rate.43

Subgroups of rats were generated into two cohorts: rats in the first group received a bilateral hippocampal infusion of EAAT2, GS or luciferase vectors, whereas the second group of rats received microRNA-targeting ADK (miR-ADK) and miR-control (miR-con) vectors. After 4 weeks, rats received an infusion of kainate,
miR-ADK protects against kainate-induced neurotoxicity

Intrahippocampal injection of kainate elicits epileptic seizures in the CA3 region and causes local neurodegeneration at the infuson site.\textsuperscript{41,43} Seizures that propagate to distal limbic structures can also induce neurodegeneration if they are of sufficient duration and intensity.\textsuperscript{43,44} To determine whether the vector treatments could afford neuroprotection against kainate and seizure-induced neurotoxicity, we stained sections with Fluorojade B (FJ) to label-degenerating neurons.\textsuperscript{46} No FJ-positive cells were found in the hippocampus in any vector treatment group in the absence of kainate treatment (Figure 4a). In contrast, numerous FJ-positive cells were clearly evident in the CA3 pyramidal region in the hippocampus ipsilateral to kainate injection (Figure 4a), with one or two FJ-positive cells observed in the contralateral hippocampus in some animals (results not shown). We quantified numbers of FJ-positive cells in the dentate hilus and CA3 region (Figure 4b) ipsilateral to kainate injection and found that FJ-positive cell numbers in the CA3 region were similar between all treatment groups relative to their respective controls (one-way ANOVA, F\textsubscript{2,24} = 0.38, P = 0.68 for luc, EAAT2, GS animals; unpaired t-test, t\textsubscript{15} = 0.15, P = 0.88 for miR-con versus miR-ADK). However, hilar cell loss in the miR-ADK-treated rats was significantly attenuated relative to the miR-con-injected rats, whereas the extent of hilar cell loss between EAAT2, GS and luciferase-overexpressing rats was similar (one-way ANOVA, F\textsubscript{2,24} = 0.02, P = 0.98 for luc, EAAT2, GS animals; unpaired t-test, t\textsubscript{15} = 2.5, P = 0.02 for miR-con versus miR-ADK). Immunohistochemistry with the NeuN antibody confirmed that miR-ADK expression was associated with a neuroprotective effect in the hilus, with a greater preservation of NeuN-immunoreactive hilar neurons in the miR-ADK-vector-injected rats (Figure 4c). This was confirmed by volume measurements of the hilus in NeuN-immunolabeled sections, with significant atrophy of this region in the miR-con compared with miR-ADK-vector-injected rats (Figure 4d). Reduced neuroinflammatory responses as visualized by attenuated Cd11b immunoreactivity indicative of reduced microgliosis was also found in the miR-ADK rats (Figure 4e). The differential protection between the hilar and CA3 neurons in the miR-ADK-injected animals can be attributed to the differences in the spatial distribution of transgene expression, as we observed a higher density of transduced astrocytes in the hilar/dentate granule cell region and fewer transduced astrocytes in the CA3 region.
Significant attenuation of ADK expression by miR-ADK but low-level expression of transgenic GS and EAAT2

We next determined whether the antiseizure and neuroprotective effects observed in the miR-ADK vector-injected rats, and lack of effect in the AAV-GS and EAAT2 vector-injected rats, could be explained by the overall change in protein level and/or function that was attained. The extent of knockdown of ADK expression achieved following infusion of the AAV9-miR-ADK vector was analyzed by western blot analysis of hippocampal homogenates prepared from additional rats injected with AAV9-miR-ADK and miR-con vectors or phosphate-buffered saline (PBS) vehicle. ADK protein levels were significantly reduced by 94–96% in three out of four miR-ADK animals relative to the miR-con and PBS-injected controls, and down to 56% in the remaining miR-ADK animal (Figure 5a). The smaller effect observed in the last animal was likely due to an off-target infusion. Anti-ADK immunohistochemistry showed that ADK expression was reduced in astrocytes in the regions transduced by the miR-ADK vector (Figure 5b) as identified by anti-GFP immunoreactivity in an adjacent brain section (not shown). Although we did not measure adenosine concentrations, it is highly likely that the seizure and neuroprotective effects observed in our study are attributable to increased extracellular levels of adenosine in the transduced hippocampus given that previous studies have shown an 80% reduction in ADK expression is associated with increased extracellular adenosine levels in an ex vivo paradigm.47

We then looked at the relationship between endogenous and transgenic GS and EAAT2 protein levels in the hippocampus of additional subgroups of rats unilaterally infused with AAV vectors. Anti-GS immunohistochemistry showed high levels of endogenous GS expression in astrocytes but no obvious difference in the intensity of GS immunoreactivity between non-injected and vector-injected sides, despite the strong detection of transgenic GS using an anti-HA antibody (Figure 5c). Similarly, high endogenous levels of EAAT2 were also found highly, but a low-level increase in EAAT2 immunoreactivity on the vector-injected side compared with the non-injected side was observed (Figure 5c).

To further investigate these findings, we conducted western blot analyses to determine the relative contribution of transgenic GS and EAAT2 to endogenous levels of these proteins in vector-injected hippocampal lysates. The higher molecular weight transgenic HA-tagged GS protein band was detected with both GS and hemaglutinin antibodies in AAV9-GS vector-injected rats, but band intensities suggested that transgenic GS was in low abundance relative to endogenous GS levels (Figures 5d and e). Transgenic EAAT2 was detected with the anti-FLAG and EAAT2 antibodies, but no clear distinction between endogenous and transgenic forms of EAAT2 was observed (Figure 5d). We also sought to determine whether astrocytic expression of either of these transgenes would alter expression levels of the other protein as they are functionally linked. AAV-mediated GS expression did not appear to alter EAAT2 protein levels, and...
conversely, GS protein band intensities in AAV-EAAT2 vector-injected samples appeared similar when comparing between the contralateral and ipsilateral vector-infused sides. We also investigated whether reporter gene expression might also affect GS expression, as previous studies have shown that astrocytic GFP expression can downregulate GS expression. GS protein levels across all transgene-expressing vector treatment groups were similar to that of PBS-injected hippocampal lysates and lysates prepared from rats injected with an AAV vector expressing no transgene (AAV empty); however, GFP overexpression appeared to attenuate GS expression levels consistent with previous reports. We then conducted a semiquantitative analysis of western blot band intensities in the GS vector- and EAAT2 vector-injected animals, and compared this with the AAV-luciferase group (as used in our seizure study). There were no significant differences in GS protein levels between the vector-injected and non-injected hemispheres nor between the luciferase vector compared with the GS vector-injected sides (luc ipsi: 100.0 ± 4.3% vs GS ipsi 107.7 ± 4.3%; unpaired t-test, t<sub>9</sub> = 1.18, P = 0.26) (Figure 5f). Similarly, no differences in EAAT2 levels were found in the EAAT2 versus luciferase vector-injected hippocampus (luc ipsi: 100.0 ± 4.4% versus EAAT2 ipsi 103.3 ± 5.2%, t<sub>6</sub> = 0.4886, P = 0.642). However, there was a small but significant increase in EAAT2 levels on the vector-injected side relative to the non-injected side (EAAT2 ipsi: 103.3 ± 5.2% versus EAAT2 contra: 93.45 ± 4.5%; paired t-test, t<sub>3</sub> = 4.542, P = 0.02), consistent with the slight increase in EAAT2 immunolabelling (Figures 5c and g).

As an additional approach, we also looked to see if we could detect a functional change in GS activity across all AAV vector-injected treatment groups (Figure 5h). We found no difference in...
Figure 5. Significant knockdown of ADK expression but no increase in GS or EAAT2 expression with AAV vector infusions. (a) Western blot analysis of hippocampal homogenates from individual PBS, mir-con and mir-ADK vector-injected rats, and positive control liver homogenate probed with anti-ADK antibody. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control. (b) Immunolabeling with anti-ADK antibody showed loss of ADK staining in hippocampal astrocytes in the zone transduced by AAV9-miR-ADK vector (inset shows high magnification image) compared with the same region in a non-injected brain. Note: in this miR-ADK vector-injected hippocampal section, the dentate hilar region was not transduced by the AAV-miR-ADK vector. (c) Representative images of hippocampal HA, GS and EAAT2 immunostaining in AAV9-GS- or AAV9-EAAT2-injected rats. (d) Western blot analysis of hippocampal lysates from rats injected unilaterally with PBS or AAV vectors at 4 weeks probed with antibodies to GS, HA, EAAT2, FLAG, LUC, GFP or GAPDH. (e) Western blots showing individual fluorescent signals detected by anti-HA and GS antibodies and colocalization of hemaglutinin (HA) and GS transgenic bands. (f) Semiquantitative analysis of band intensities from western blots of hippocampal lysates from luciferase (luc) or GS vector-injected rats or (g) EAAT2 vector-injected rats probed with GS or EAAT2 antibodies, respectively. (h) Hippocampal GS activity (μM mg⁻¹ protein h⁻¹) in PBS and AAV vector-injected rats. Bars represent the mean ± s.e.m., n = 4–7 per treatment, *P < 0.5, **P < 0.01 (Tukey’s post hoc analysis). Scale bar for (b) 250 μm for low-power image and 63 μm for inset (c) 250 μm.
GS activity between the GS and luciferase vector group consistent with the protein expression data. Surprisingly, there was an overall attenuation in GS activity across the transgene expression groups relative to PBS-injected animals (one-way ANOVA, F_{5,26} = 4.76, P = 0.003) and rats that received an infusion of AAV vector that does not express a transgene (AAV empty), suggesting that transgene expression per se in astrocytes causes this effect.

DISCUSSION

Astrocytes are an attractive cell target for new genetic therapies given their prevalence in the brain and potential contribution to the pathophysiology of neurodegenerative diseases. However, the identification and validation of astrocyte-specific therapeutic targets is still needed. Here, we report that miR-mediated knockdown of hippocampal astrocytic ADK expression showed antiseizure and neuroprotective efficacy in the kainate model of epilepsy in rats, whereas expression levels of transgenic GS or EAAT2 transgene were too low to exert any therapeutic effect in this model.

Astrocyte-specific targeting with AAV vectors

Advances in viral vector technology have enabled transgene expression in astrocytes, with lentiviral and AAV vectors leading the way. An expanded repertoire of AAV vectors has been developed on the back of the discovery of more than 100 novel AAV capsid variants, with much of the work to date centered on the development and characterization of vectors derived from serotypes AAV1–9. In comparison with the AAV2 vectors used in early pioneering central nervous system gene therapy studies, most of these new-generation vectors have superior transduction properties and vary in their tropism for specific cell populations in different brain regions, providing a more versatile toolkit that will enable the development of targeted and optimized therapeutic strategies. Moreover, these newer generation AAV vectors have opened up avenues for achieving cell-specific targeting through the engineering of novel capsids, or the optimization of viral-delivered vector genomes. Differential expression of transgenes in neurons, astrocytes and oligodendrocytes in the brain can be achieved using cell-specific promoters to drive transgene expression in the context of AAV5, AAV8 vectors and chimeric AAV1/2 vectors, with the promoter specificity generally over-riding the effect of capsid tropism, unlike AAV2 vectors that are highly neurotropic regardless of the promoter used.

We were intrigued by reports that intravenous delivery of AAV9 vectors expressing GFP under the control of the CAG promoter transduces astrocytes throughout the central nervous system in the adult mice and non-human primates, with neuronal transduction also observed in one study. Paradoxically, our lab and others have found that direct injection of the same vector into the brain leads predominantly to neuronal transgene expression. This raises the possibility that coupling a transgene to the GFAP promoter might lead to a vector optimized for astrocytic expression of transgenes in much the same way as we had previously observed with AAV8. Indeed, we found that this combination of promoter and AAV9 capsid led to transgene expression to astrocytes, with an almost identical transduction pattern following vector infusion into the hippocampus.

Our vector stocks were highly pure as visualized by detection of only AAV viral capsid proteins on protein gels, eliminating the possibility that cellular protein contaminants in our vector stocks influenced the cell transduction patterns observed.

A few dentate granule neurons were also transduced, with GFP noticeably filling granule cell axonal projections to the CA3 pyramidal neurons. This is in line with reports that the 2.2 kb human GFAP promoter can also direct low-level transgene expression in neurons, a feature that is more evident using sensitive anti-GFP immunohistochemical detection methods. It is unclear whether this residual neuronal expression would influence the antiseizure results we obtained. Nevertheless, one approach that could be used to increase the stringency of astrocyte-specific targeting, if required, is to take advantage of the differential expression of endogenous miRNA in specific cell types. By engineering miRNA binding sites for miR124, a neuron-specific miRNA into lentiviral vector genomes, Colin et al. demonstrated an effective reduction in the number of transduced neurons in the striatum from 18% to 6%.

Antiseizure effects found with astrocytic knockdown of ADK expression but not GS or EAAT2 overexpression

Gene delivery to the human brain still presents a significant hurdle, particularly for diseases that are associated with widespread neuropathology. Systemic delivery of a gene delivery agent is seen as a promising minimally invasive approach that could be used to achieve global gene transfer, provided that vector systems capable of passage across the blood–brain barrier are available. AAV9 vectors might fulfill some of these criteria as a systemic gene delivery agent, but there are still some barriers to overcome. Moreover, although intravenous delivery is less invasive than direct intracerebral injection, the systemic approach is likely to require much greater doses of vector, immunologic responses and risk of transduction of non-target organs. While the concept of miR-mediated detargeting can be extended to silencing of transgene expression in peripheral organs such as the liver, the presence of neutralizing antibodies to AAV capsid proteins might pose a significant limitation in the clinical translation of this approach. Gray et al. have also described a vector capable of crossing the seizure-compromised blood–brain barrier. While systemic delivery approaches have therapeutic applicability for generalized forms of epilepsy that involve large areas of the brain, targeting of therapies to defined site of seizure origin may be more desirable to minimize the possibility of perturbation of normal brain function.

Gene therapy strategies for epilepsy to date have focused on modulating neuronal excitability by overexpressing inhibitory neuropeptides such as neuropeptide Y or galanin or reducing the strength of excitatory signaling by modulating N-methyl-D-aspartate receptor function. Adenosine is a potent natural anticonvulsant, but attempts to develop adenosine-based therapies suitable for systemic administration have been thwarted because adenosinergic drugs are associated with sedative and cardiovascular side effects that limit their clinical usefulness. Strategies allowing augmentation of adenosine levels locally within a specific target site will circumvent these problems. We show here that local miR-mediated downregulation of ADK expression in hippocampal astrocytes is sufficient to limit the duration of kainate-induced seizures. At the vector dose used, a >90% reduction in ADK levels was achieved. Future studies will be required to confirm that ADK knockdown in vivo using this miR-based approach is associated with increased extracellular adenosine concentrations at baseline and/or during the course of seizures using microdialysis approaches. We speculate that this seems likely given that a lentiviral-mediated knockdown of ADK transcripts in human mesenchymal stem cells by 80% is sufficient to increase adenosine release and our results are consistent with the observation that mice that received hippocampal grafts of these ADK-modified cells showed reduced seizure durations and attenuated cell loss following kainate injection. Similarly, AAV8-mediated expression of ADK antisense sequences in astrocytes also completely abolishes spontaneous seizures in mice. Transplantation of copolymers or cell types engineered to release adenosine by manipulating ADK activity provides effective seizure control in several experimental seizure paradigms including kindling, and spontaneous seizures in the CA3 region.
We found that the greater neuroprotection afforded to neurons in the hilus compared with CA3 neurons, which was consistent with the extent of transgene spread observed, with miR-ADK sequences predominantly expressed in the medial dorsal hippocampus, while expression was weaker toward the lateral CA3 region. Further refinements of this strategy could involve determining whether stronger therapeutic efficacy could be achieved by specifically targeting transgene expression to the CA3 region in light of studies demonstrating complete protection against seizures and injury in this region in forebrain-deficient ADK transgenic mice following intramygdaloid challenge with kainate. Moreover, the long-term effects of miR overexpression on endogenous gene expression and ADK knockdown will need to be determined. Our results lend support to the concept that astrocytes are a good alternative cell target for gene therapy for epilepsy, with ADK being a prime therapeutic target.

The selection of GS and EAAT2 as potential therapeutic targets stemmed from observations that reduced astrocytic levels of GS21,25 and potentially glutamate transporters22,23 in the epileptogenic hippocampus could lead to perturbed glutamate regulation and provide a potential mechanism for elevated synaptic glutamate levels that are involved in triggering seizures.49 Therefore, increasing the astrocytic capacity for glutamate clearance and breakdown in the face of extracellular glutamate concentrations that can be up to sixfold higher than basal levels during spontaneous seizures in rats50 and in the human epileptic hippocampus51 might be sufficient to suppress excessive glutamate levels. We found that AAV-mediated expression of GS or EAAT2 had no effect on kainate-induced seizures nor did it protect against neuronal cell loss. Contrasting with the robust detection of transgenic GS and EAAT2 using antibodies to the respective epitope tags, it was surprising to observe that transgenic GS and EAAT2 did not contribute to any significant increase over endogenous levels of these proteins, particularly in light of the substantial knockdown of endogenous ADK achieved with miR-ADK. Thus, the level of transcriptional activity of the GFAP promoter, whereas sufficient to drive production of miR sequences at concentrations capable of powerfully suppressing ADK expression, was not sufficient to drive a substantial increase in GS or EAAT2 expression at levels that might be required to impact on glutamate processing and seizure protection. Astrocyte-specific EAAT221 or Aldh1l122 promoters could be tested as alternatives to see if these might produce higher levels of GS expression. Higher vector titers might possibly also be tested, although our results suggest a cautious approach should be taken. GS expression is sensitive to AAV vector-mediated GFP expression in a titer-dependent manner as observed in astrocytes in the CA1 region, with high titer (3 x 1010 genome copies per injection) almost leading to complete loss of GS expression and reactive gliosis in the absence of neuronal cell loss.48 Reactive gliosis results in deficits in neuronal inhibition, which would further perpetuate a shift toward increased hyperexcitability in hippocampal circuits,48 an unwanted effect in an epileptogenic hippocampus. Moreover, high viral loads are more likely to elicit cell-mediated immunological immune responses.49 We also showed that transgene expression in astrocytes leads to a slight attenuation in GS activity levels by some unknown mechanism, an effect not found in hippocampal lysates from rats injected with AAV vector that does not express a transgene (AAV empty). Whether this is of significance and whether there is a functional consequence on hippocampal excitability remains to be investigated.

In contrast to the GS vector-injected rats, a low-level increase in EAAT2 expression was observed in the vector-injected hemisphere but only relative to the contralateral non-injected side. A recent study found that pilocarpine-induced seizure induction and propagation parameters were not different in transgenic mice expressing 1.5–2-fold increased EAAT2 protein levels compared with control mice.74 This suggests that approaches may be needed to increase EAAT2 levels by at least twofold to potentially exert any effect on seizures. Downstream chronic epileptogenic processes including mossy fiber sprouting, seizure-induced neurogenesis and frequency of spontaneous seizures were reduced in EAAT2-overexpressing mice.74 This suggests that EAAT2 may be useful for preventing specific epileptogenic processes. An increased capacity for glutamate uptake needs to be matched with an increased capacity to metabolize glutamate. In fact, the rapid metabolism of intracellular glutamate is a prerequisite for efficient glutamate clearance from the extracellular space by glutamate transporters—thus, high intracellular concentrations of glutamate can slow net transport of glutamate into the cell75 and therefore the capacity to metabolize glutamate may be the rate-limiting step. Thus, GS may be a better therapeutic target than EAAT2 or a combination of both may be required to provide effective seizure control. Our results suggest that alternative strategies aimed at increasing GS and EAAT2 levels should be tested before any definitive conclusion on their suitability as therapeutic targets can be made.

Our studies show that prophylactic expression of miR-ADK in the naive rat brain can affect seizure durations; future studies will require evaluation of the miR-ADK knockdown approach in seizure models with existing hippocampal neuropahty and spontaneous seizures. The cellular environment into which the vectors will be introduced could influence cell targeting, as AAV5 vectors expressing GFP under CAG promoter appeared to switch from preferentially transducing neurons in the naive hippocampus to reactive astrocytes in a hippocampus showing extensive seizure-induced neuronal loss and reactive astrogliosis.10 It is unclear whether reactive astrocytes assume altered phenotypes that passively or actively allow cell uptake of viral vector, but nevertheless it suggests that viral vector tropism could be influenced by pathologic changes in expression levels of receptors on astrocytes. It will be interesting to determine whether astrocyte-specific targeting in the epileptic brain can be maintained using a GFAP promoter-driven transgene, and whether upregulation of GFAP gene activity in astrocytes that become reactive following injury can be mimicked by increased transgene expression levels.76 Finally, from a broader perspective, glutamate receptor overactivation and neuronal cell loss are overarching concepts applicable to other neurodegenerative disease, suggesting that ADK knockdown gene therapy could have therapeutic potential for other neurodegenerative disorders. Our study adds to a growing list of astrocyte-specific therapeutic strategies including utilization of these cells as reservoirs for local secretion of growth factors such as glial-derived neurotrophic factor that might enhance protection of dopamine neurons in Parkinson’s disease8 or inhibitory neurochemicals such as adenosine as shown in this study and those of others.39 Modulation of astrocytic processes that contribute to neuroinflammation could be another strategy. VIVT, a peptide that interferes with the immune/inflammatory activation could be another strategy. VIVT, a peptide that interferes with the immune/inflammatory processes could be another strategy. VIVT, a peptide that interferes with the immune/inflammatory processes including kainate, 5-HT or inhibitory neurochemicals such as adenosine as shown in this study and those of others.39 Modulation of astrocytic processes that contribute to neuroinflammation could be another strategy. VIVT, a peptide that interferes with the immune/inflammatory processes including kainate, 5-HT or inhibitory neurochemicals such as adenosine as shown in this study and those of others.39 Modulation of astrocytic processes that contribute to neuroinflammation could be another strategy. VIVT, a peptide that interferes with the immune/inflammatory processes including kainate, 5-HT or inhibitory neurochemicals such as adenosine as shown in this study and those of others.39
control of a 2.2 kb human GFAP promoter (GenBank accession number: M67446), and containing a truncated woodchuck hepatitis posttranscriptional regulatory element and short polyadenylation signal flanked by AAV2 inverted terminal repeats to generate pAM-GFP-GFP, pAM-GFAP-EAAT2, pAM-GFAP-GS and pAM-GFAP-luciferase plasmids. Three mir-targeting rat ADKs (nucleotides 241–261, 5'-UCACCGAGAUAUAUAUGAGA-3'; 458–478, 5'-ACCCUGUGGCGCCCGCAAUGUUG-3'; 844–864, 5'-CAAGGGAGAGAUGACA CUAAU-6') were designed using BLOCK-iT RNAi designer (Invitrogen) or miR-negative control (5'-GUCUcAGGccCUAGAUUUU-3') and inserted into an AAV plasmid containing the GFAP promoter, woodchuck hepatitis posttranscriptional regulatory element and short polyadenylation, as well as an Emerald GFP reporter to facilitate cell tracking of miR sequences, to posttranscriptional regulatory element and short polyadenylation, as well as an AAV9 plasmid containing the GFAP promoter, woodchuck hepatitis posttranscriptional regulatory element as described previously. Highly pure vector stocks were produced by transient transfection and genomic titers were determined by real-time PCR using primers to woodchuck hepatitis posttranscriptional regulatory element as described previously. Highly pure vector stocks were produced as only bands corresponding to the AAV viral capsid proteins VP1, -2 and -3 were visible on electrophoresis gels. The vector titers used for the expression and seizure study were (in vector genomes ml⁻¹) as follows: AAV8-GFP, 1 x 10¹²; AAV9-GFP, 1 x 10¹²; AAV9-miR-ADK, 9.48 x 10¹₁; AAV9-miR-con, 4.12 x 10¹²; AAV9-GS, 2.64 x 10¹²; AAV9-EAAT2, 1.68 x 10¹²; and AAV9-luciferase, 2.0 x 10¹². The AAV backbone plasmid consisting of the GFAP promoter, polylinker site, truncated woodchuck hepatitis posttranscriptional regulatory element and short polyadenylation was also used to generate AAV9 vector containing these regulatory elements but no transgene (AAV9-empty), vector titer, 3 x 10¹².

Vector infusions All studies were conducted under the ethical approval and guidelines from the University of Auckland Animal Ethics Committee. Male Sprague-Dawley rats (250–300 g) were anesthetized with pentobarbital (70 mg⋅kg⁻¹, intraperitoneally) and rats were positioned in a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Rats were randomly assigned to receive 3 μl of AAV vector infused unilaterally (for the expression and functional studies) or bilaterally (for the seizure analysis) into the hippocampal (coordinates: anterior–posterior – 3.6 mm, medial–lateral ±2.1 mm, dorsal–ventral – 4.3 mm from skull surface, bregma = 0) at a rate of 70 nl min⁻¹ controlled by a microinjection pump as described previously. Immunohistochemistry For analysis of transgene expression, rats were killed 3 weeks following AAV infusion by pentobarbital overdose (100 mg⋅kg⁻¹, intraperitoneally) at the end of 90 min to terminate any residual seizure activity. Rats were killed 1 week later and transcendally perfused as described above.

FJ staining Every twelfth section spanning the dorsal hippocampus (6 sections, 480 μm apart) was stained with 0.00004% FJ as described previously. An investigator blinded to the treatment groups quantified FJ-positive cells, from all sections, into two regions of the hemisphere ipsilateral to kainate infusion: the hilar region, defined as the zone between the upper and lower blades of the dentate granule cell layer, and the hippocampal CA3 pyramidal neuron region. Data were expressed as the total number of FJ-positive cells per region over six sections.

Western blotting Five weeks following infusion of AAV9-miR-con (n = 2), miR-ADK vector (n = 4) or PBS (n = 2), rats were killed, the brains removed and hippocampi dissected. Each hippocampus was homogenized by sonication in ice-cold lysis buffer (50 mm Tris-HCl, 2 mm EDTA, 0.05% Triton X-100, pH 7.5) containing protease inhibitors (Complete Protease inhibitor Cocktail; Roche Diagnostics, Mannheim, Germany). Lysates were centrifuged at 20 800 g for 15 min at 4 °C and supernatants taken for analysis as described in Supplementary Information. For western blot analysis of GS and EAAT2 levels, 10 μg of hippocampal lysate (as prepared for the G3 assay) was resolved by 10% sodium dodecyl
sulfate-polyacrylamide gel electrophoresis, transferred to Hybond-ECL membrane (GE Healthcare, Uppsala, Sweden) and probed as described in Supplementary Information.

GS assay
Additional subgroups of rats received unilateral hippocampal infusions of PBS (n = 6), AAV9-empty (n = 4), AAV9-luciferase (n = 4), AAV9-GFP (n = 7), AAV9-GS (n = 7) or AAV9-EAA2 vector (n = 4), and at 4 weeks, rats were killed and the hippocampus on the injected and non-injected sides was dissected. Individual hippocampi were homogenized in 40 μl Tris, 1 μl EDTA (pH 7.4), centrifuged at 1500 g for 10 min at 4 °C and the supernatant diluted to a protein concentration of 1 mg ml⁻¹. GS activity was assayed as described previously⁴² and in Supplementary Information.

Statistical analysis
Data are expressed as mean ± s.e.m. A one-way ANOVA with Tukey's post hoc test was used to compare different group means. Unpaired or paired Students t-tests, where appropriate, were performed using GraphPad Prism 5.02 (GraphPad Software, La Jolla, CA, USA), with significance levels set at *P < 0.05, **P < 0.01 and ***P < 0.001.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
This work was supported by funding from Lottery Health Research (NZ) and the Royal Society of New Zealand Marsden Fund to DY, PAL and MJD, HRC funding to MD, and NIH NINDS RO1 award NS44576 to MJD. We thank M Brenner for providing the 2.2 kb AAV2-GAD gene therapy for advanced Parkinson’s disease: a double-blind, placebo-controlled, intracerebral injection study. Exp Neurol 2011; 228: 41–52.

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