Adeno-Associated Virus Vector-Mediated Expression and Constitutive Secretion of Galanin Suppresses Limbic Seizure Activity

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Summary: Theoretically, gene therapy techniques offer an attractive alternative treatment option for intractable, focal epilepsies. Although logical gene therapy targets include excitatory and inhibitory receptors, variable viral vector tropism interjects an uncertainty as to the direction of change, seizure suppression, or seizure sensitization. To circumvent this therapeutic liability, adeno-associated virus (AAV) vectors have been constructed where the gene product is constitutively secreted from the transduced cell. Using AAV vectors, the fibronectin secretory signal sequence (FIB) was placed in front of the coding sequence for green fluorescent protein or the active portion of the neuroactive peptide galanin (GAL). Subsequent studies showed that these vectors supported expression and constitutive secretion of these gene products from transfected cells *in vitro*. More importantly, upon transduction *in vivo*, AAV-FIB-GAL vectors significantly attenuated focal seizure sensitivity, and this seizure attenu-

ation could be controlled *in vivo* by using a tetracycline-regulated promoter. The expression and constitutive secretion of green fluorescent protein, or the expression of GAL alone, exerted no effect on focal seizure sensitivity. Moreover, unilateral infusion of the AAV-FIB-GAL vectors into the hippocampus prevented kainic acid-induced hilar cell death. With regard to limbic seizures, bilateral infusion of AAV-FIB-GAL vectors into the piriform cortex prevented both behavioral and localized electrographic seizure activity after the peripheral administration of kainic acid. Also, when rats were electrically kindled to class V seizure activity, subsequent infusion of AAV-FIB-GAL proved capable of significantly elevating the seizure initiation threshold. Thus, these studies clearly demonstrate the anti-seizure effectiveness of AAV vector-mediated expression and constitutive secretion of galanin. **Key Words:** Epilepsy, seizure, galanin, gene therapy, adeno-associated virus.

INTRODUCTION

Epilepsy afflicts approximately 2.5 million people in the United States, making epilepsy one of the most prevalent neurological disorders.¹ Although current, anti-epileptic medication effectively controls the seizures in approximately 70% of this population, medications do not adequately control the seizures for the remaining 30%.² In these intractable cases, surgical resection offers a final option, but only if a circumscribed site of seizure genesis can be identified and does not impinge on the brain sites associated with critical functions, such as speech.³ Certainly epilepsy surgery has proven palliative for many patients, but a less invasive, effective therapy

would greatly benefit this treatment refractory, epileptic population.

Theoretically, gene therapy techniques offer an attractive alternative treatment option for focal epilepsies. Focal epilepsy arises from a circumscribed site of seizure genesis, so *in vivo*, viral vector gene therapy should prove capable of influencing the site that initiates seizure activity. Furthermore, seizure activity emanates from excessive synchronous discharge by large populations of neurons where the underlying basis has been attributed to a decrease in inhibitory function, an increase in excitatory function or both. Because some viral vectors, such as adeno-associated virus (AAV) vectors or lentivirus vectors, exhibit a dominant neuronal tropism,^{4,5} these viral vectors provide direct access to the cell type that mediates seizures. Thus, manipulations that decrease excitation or increase inhibition should prove capable of modulating seizure sensitivity *in vivo*.

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THE LIABILITY OF VARIABLE VIRAL VECTOR TROPISMS

Excitatory or inhibitory amino acid receptors comprise obvious targets for antiepileptic gene therapy, particularly given the fact that pharmacological increases in gamma-aminobutyric acid (GABA) inhibition or blockade of excitatory amino acid receptors attenuates seizure activity *in vivo*.⁶ N-methyl-D-aspartic acid (NMDA) receptors directly influence seizure sensitivity, but a prerequisite for NMDA receptor function is the presence of the NMDA receptor 1 protein (NMDAR1).^{7,8} Thus, removal of this subunit protein should significantly reduce NMDAR1-mediated excitation. By targeting NMDA receptors however, Haberman et al.⁹ found that variable patterns of the viral vector-mediated gene expression dramatically influenced the final outcome. The AAV vector delivery and expression of an antisense construct to the NMDAR1, indeed reduced NMDA receptor function *in vitro* and NMDAR1 protein *in vivo*. Using a focal seizure model, cytomegalovirus (CMV) promoter driven expression of the NMDAR1 antisense (NR1A) significantly decreased the seizure sensitivity, proving the principle that viral vector derived antisense could influence

FIG. 1. The effects adeno-associated virus (AAV)-CMV-NR1A, AAV-tTAK-NR1A or AAV-tTAK-eGFP on the seizure threshold sensitivity within the seizure sensitive area of the inferior collicular cortex. After virus infusion and electrode implantation, the seizure initiation threshold was determined weekly. As seen across weeks 2 to 4, the AAV-CMV-NR1A group exhibited a significant increase in the seizure threshold by week 4, indicating a reduction in seizure sensitivity. In marked contrast, infusion of the AAV-tTAK-NR1A caused a significant decrease in the seizure threshold, indicating an increase in seizure sensitivity. No significant changes were noted for the AAV-tTAK-eGFP group. Thus, a slight change in only the promoter element resulted in diametrically opposed changes in seizure sensitivity. NMDA = N-methyl-D-aspartic acid; eGFP = enhanced green fluorescent protein. (Reproduced with permission from Nature Publishing Group.⁹)

native NMDA receptor function *in vivo* (FIG. 1). However, when expression of the same antisense construct was driven by a tetracycline regulated minimal CMV promoter (tTAK),¹⁰ the seizure sensitivity actually increased, resulting in animals that were more seizure sensitive. The fact that NMDA receptor-mediated excitation drives endogenous GABA inhibition, provided a tenable explanation for these diametrically opposed results. In the case of the CMV promoter, primary seizure output neurons likely comprised the preponderance of transduced cells, thus removal of the NMDA receptor excitation blunted the seizure sensitivity. In the case of the regulated minimal CMV promoter, the preponderance of gene expression probably occurred in inhibitory GABA interneurons, so removal of the endogenous excitatory drive to these interneurons would cause an increased seizure sensitivity. The likelihood of such an occurrence received substantial support, when equal amounts of each AAV vector were administered together. Some neurons supported expression from both promoters, but significant portions exhibited transduction from only one or the other of the promoters. Thus, a slight change in only the promoter resulted in a dramatic shift in the final outcome of the gene expression. These surprising findings greatly complicate the targeting of neurotransmitter receptor proteins or ion channels for the treatment of epilepsy. Certainly, it is possible that the viral vector tropism and/or promoter tropism could differ between experimental animals and humans. Thus, without some detailed *a priori* knowledge of the transduction pattern in humans, any manipulation of neurotransmitter receptor proteins or ion channels could as easily lead to heightened seizure sensitivity instead of the desired reduction of seizure sensitivity.

A MEANS TO CIRCUMVENT TROPISM LIABILITIES

One means to circumvent the problems of cell-specific transduction would be to express an inhibitory factor and then constitutively secrete that factor from the transduced cell. If the receptors for that factor are present in the area of transduction, then one should be able to achieve seizure attenuation regardless of the transduction pattern. Certainly a number of endogenous neuroactive peptides have well established anti-seizure actions, findings that present a number of viable choices for an appropriate inhibitory factor. Furthermore, the inhibitory factor should be secreted in a nonregulated manner. For example, most neuroactive peptides are expressed initially as a prepro-peptide. This prepro-peptide is then appropriately trafficked such that the active peptide is stored in presynaptic vesicles for subsequent stimulus dependent release. Thus, to obtain a physiological effect from viral vector expression of a prepro-peptide, one

must assume first that the prepro-peptide will be trafficked appropriately within the transduced cell. Second, one must assume that the inhibitory influence will be exerted in the region of transduction, not some distal site of projection. Finally, one must assume that a vector-derived peptide will enhance stimulus-dependent release either on a per stimulus basis or over an extended period of time. In contrast, if the active peptide was constitutively secreted from the transduced cell, these assumptions would no longer apply.

THE CASE FOR GALANIN GENE THERAPY

Galanin is a 29 to 30 amino acid neuropeptide that exerts a wide range of effects within the CNS from modulating consummatory behaviors and attenuating LTP to suppressing seizure activity.^{11–14} Galanin exerts this diverse range of actions through any one of three galanin receptors (GALR1, GALR2, GALR3) that belong to the G-protein coupled receptor super family. For example, activation of GALR1 or GALR3 results in the opening of K⁺ channels and inhibition of cAMP, whereas activation of GALR2 receptors increases phospholipase C activity and mobilizes intracellular calcium.¹⁵ Electrophysiologically, the actions of galanin prove to be mostly inhibitory,¹⁶ likely attributable to the ability of galanin to significant reduce presynaptic release of acetylcholine,¹⁷ as well as presynaptic release of glutamate.¹⁸ Not only did Mazarati et al.¹³ demonstrate that local galanin application significantly suppressed local seizure activity *in vivo*, but a subsequent study by Mazarati and Wasterlain¹⁹ found that the anticonvulsant properties of galanin were more substantial and irreversible when compared with those actions of somatostatin or neuropeptide Y. Clearly, galanin exerts significant anti-seizure effects *in vivo*, but the question remains as to how the viral vector gene therapy techniques would be used to express and secrete galanin.

A NOVEL APPROACH TO NEUROPEPTIDE GENE THERAPY

As mentioned previously, one means to circumvent the problems of cell-specific transduction would be to express and constitutively secrete the active galanin peptide from the transduced cell. Normally, the laminar protein, fibronectin, is constitutively secreted from a wide range of cell types, and this constitutive secretion depends on the secretory signal sequence that precedes the fibronectin coding sequence. Utilizing this fact, Haberman et al.²⁰ constructed an AAV vector where the coding sequence for the active galanin peptide was preceded by the fibronectin secretory signal sequence (AAV-FIB-GAL). Since fibronectin is normally, constitutively secreted from the cell, it was reasoned that the secretory

signal sequence of this laminar protein would traffic the gene product into this constitutive secretion pathway. Thus, the active peptide would be secreted from the transduced cell in a nonregulated fashion. As long as the appropriate neuropeptide receptor was present in the area of transduction, the effect of the peptide would not depend on a specific pattern of neuronal transduction, as was found for excitatory receptor manipulation.⁹ Indeed, these studies showed that the fibronectin secretion signal sequence did cause constitutive secretion of the gene product from the transduced cells *in vitro*, and upon transduction *in vivo*, exerted significant attenuation of focal seizure sensitivity (FIG. 2). By using a tetracycline-regulated promoter,¹⁰ the gene expression and anti-seizure effect could be controlled *in vivo*, but unlike studies with NMDAR antisense, changing the promoter did not alter the anti-seizure effect of galanin expression and secretion. When this AAV vector was unilaterally infused into the hippocampus, subsequent peripheral kainic acid administration produced the expected limbic seizure activity, but galanin expression and secretion proved sufficient to prevent seizure-induced hilar neuronal damage. Clearly, sufficient galanin was expressed and constitutively secreted to significantly alter *in vivo* excitability. In all of these models, no effects were found for vectors that either expressed and secreted green fluorescent protein (GFP) or expressed but did not secrete galanin. A subsequent publication by Lin et al.²¹ showed that AAV-

FIG. 2. The effects of adeno-associated virus (AAV) vector microinjections on the focal seizure sensitivity in the inferior collicular cortex. After AAV-fibronectin secretory signal sequence (FIB) galanin (GAL) vector infusion (λ) the amount of stimulation current increased significantly for a 4-week period (**p* < 0.05; paired *t*-test). The oral administration of doxycycline caused a return to baseline, and with removal of the doxycycline, the seizure threshold slowly increased. In contrast, infusion of an AAV-GAL vector, which expresses but does not secrete galanin or an AAV-FIB-GFP vector, which expresses and constitutively secretes GFP, had no significant effect on seizure threshold. (Reproduced with permission from Nature Publishing Group.²⁰)

mediated expression of a human galanin cDNA significantly decreased the number seizure episodes that followed locally applied kainic acid, but this approach to vector-mediated galanin expression did not alter the seizure latency or kainic acid-induced neuronal damage.

INFLUENCES ON LIMBIC SEIZURE ACTIVITY

Although Haberman et al.²⁰ provided a basic proof of principle, it remained to be shown that this gene therapy approach could significantly attenuate limbic seizure activity. Subsequent studies by McCown²² established that AAV-mediated expression and secretion of galanin exerts a substantial influence on seizures of limbic origin. Normally, peripheral treatment with the excitatory amino acid agonist, kainic acid, results in the appearance of generalized limbic seizure behaviors and electrographic seizure activity. However, when AAV-FIB-GAL vectors were infused bilaterally into the rat piriform cortex, subsequent peripheral treatment with kainic acid did not evoke behavioral or localized, electrographic seizure activity (FIG. 3). A subsequent study addressed the clinically relevant issue of prior seizure exposure. Rats received daily electrical kindling stimulation until three

FIG. 3. The effects of adeno-associated virus (AAV)-fibronectin secretory signal sequence (FIB)-GFP and AAV-FIB-galanin (GAL) vectors on the expression of limbic seizure behaviors. Seven days after infusion of AAV-FIB-GFP ($n = 8$) into the piriform cortex, kainic acid administration (10 mg/kg, i.p.) elicited wet dog shakes, class III and class IV seizure activity with the same latency as in untreated controls ($n = 8$) ($p > 0.1$; t -test). Seven days after infusion of AAV-FIB-GAL ($n = 12$) into the piriform cortex, kainic acid administration elicited wet dog shake behaviors with the same latency as in untreated control animals. In contrast, a single, brief class III seizure was exhibited by only 1 of the 12 rats over the 240 minute post-kainic acid observation period, whereas the other 11 rats did not exhibit any limbic seizure behaviors. None of the animals exhibited any class IV seizure behaviors over the entire 240 minute post-kainic acid observation period ($p < 0.01$; t -test). (Reproduced with permission from Nature Publishing Group.²²)

class V seizures were elicited. Once this fully kindled state was achieved, AAV-FIB-GAL vectors were infused into the area of the stimulating electrode. One week later, the seizure stimulation threshold was significantly elevated, so clearly enough that galanin was expressed and secreted to significantly alter local limbic seizure sensitivity. In total, these findings firmly establish the ability of vector-derived galanin expression and constitutive secretion to suppress limbic seizure activity.

CONCLUSION

Although excitatory amino acid receptors, inhibitory amino acid receptors, and ion channels provide logical targets for antiepileptic gene therapy, variability in cellular tropism can negatively influence the eventual outcome. To circumvent this therapeutic liability, a viral vector gene therapy approach has been developed where transduced cells express and constitutively secrete galanin. Using AAV vectors, galanin expression and constitutive secretion is sufficient to significantly attenuate limbic seizure activity and prevent seizure-induced cell death. These studies provide an important foundation for the future progression of antiepileptic gene therapy.

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