Brief Communications

Enhancing $GABA_A$ Receptor α1 Subunit Levels in Hippocampal Dentate Gyrus Inhibits Epilepsy Development in an Animal Model of Temporal Lobe Epilepsy

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Differential expression of $GABA_A$ receptor (GABR) subunits has been demonstrated in hippocampus from patients and animals with temporal lobe epilepsy (TLE), but whether these changes are important for epileptogenesis remains unknown. Previous studies in the adult rat pilocarpine model of TLE found reduced expression of GABR α1 subunits and increased expression of α4 subunits in dentate gyrus (DG) of epileptic rats compared with controls. To investigate whether this altered subunit expression is a critical determinant of spontaneous seizure development, we used adeno-associated virus type 2 containing the α4 subunit gene (GABRA4) promoter to drive transgene expression in DG after status epilepticus (SE). This novel use of a condition-dependent promoter upregulated after SE successfully increased expression of GABR α1 subunit mRNA and protein in DG at 1–2 weeks after SE. Enhanced α1 expression in DG resulted in a threefold increase in mean seizure-free time after SE and a 60% decrease in the number of rats developing epilepsy (recurrent spontaneous seizures) in the first 4 weeks after SE. These findings provide the first direct evidence that altering GABR subunit expression can affect the development of epilepsy and suggest that α1 subunit levels are important determinants of inhibitory function in hippocampus.

Key words: **spontaneous seizures; epilepsy; gene therapy; rat; AAV; GABA**

Introduction

GABA is the major inhibitory neurotransmitter in the brain, and its fast inhibitory action is mediated via $GABA_A$ receptors (GABRs) that are composed of the products (α1–α6, β1–β4, γ1–γ3, δ, ε, ϕ, π) of multiple subunit genes (Macdonald and Olsen, 1994). Subunit expression and subsequent receptor composition and function vary regionally, developmentally and in certain disease states. Our previous studies in humans with temporal lobe epilepsy (TLE) and in rodent models of TLE found reduced expression of GABR α1 subunits and increased expression of GABR α4 subunits in the dentate gyrus (DG) of epileptic individuals (Brooks-Kayal et al., 1998, 1999). These changes begin within 24 h of pilocarpine-induced status epilepticus (SE) in adult rats and persist for months as these animals uniformly become epileptic (Brooks-Kayal et al., 1998). These subunit alterations are associated with marked changes in receptor function and pharmacology, including enhanced inhibition of GABA currents by zinc and diminished augmentation of GABA currents by type I benzodiazepine agonists (Buhl et al., 1996; Gibbs et al., 1997; Brooks-Kayal et al., 1998). In contrast, rats that experience pilocarpine-induced SE at postnatal day 10 have increased GABR α1 expression in DG and enhanced type I benzodiazepine augmentation of GABA currents, and none develop epilepsy later in life (Zhang et al., 2004). Together, these findings suggest that diminished α1 levels in dentate granule neurons may contribute to epileptogenesis and/or that elevated α1 levels may be protective, but they do not establish a causal link between changes in this subunit and epilepsy. To further investigate the potential importance of GABR α1 in TLE, in the present study, we test the hypothesis that enhancing α1 levels in DG after SE using viral vector gene transfer can inhibit subsequent development of epilepsy in the rat pilocarpine model of TLE.

Materials and Methods

Production of viral vectors. Adeno-associated virus (AAV) vectors were constructed from pTR–UF4 (Passini et al., 2004) and internal ribosomal entry site (IRES)–enhanced yellow fluorescent protein (eYFP) (Clon-

Received Aug. 2, 2006; revised Sept. 7, 2006; accepted Sept. 25, 2006.

This work was supported by National Institutes of Health Grants NS38595 (A.R.B.-K.), NS051710 (A.R.B.-K., S.J.R.), NS050393 (S.J.R., A.R.B.-K.), NS51943 (I.V.L.), NS38690 (J.H.W.), DK47757 (J.H.W.), and DK63973 (J.H.W.) and a grant from the American Epilepsy Society (A.R.B.K., S.J.R.). Funding for statistical support was provided by the Mental Retardation and Developmental Disability Research Center of the Children's Hospital of Philadelphia (Grant P30 HD26979). We are very appreciative for the statistical expertise of Dr. Paul Gallagher in the Division of Biostatistics, Department of Pediatrics at Children's Hospital of Philadelphia.

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DOI:10.1523/JNEUROSCI.3329-06.2006

 0270-6474/06/2611342-05$15.00/0

tech, Mountain View, CA). The human minimal GABRA4 promoter (500 bp) (Roberts et al., 2005), with or without the rat α1 subunit gene (GABRA1) cDNA, was directionally cloned into IRES–eYFP. These constructs were then cloned into the pTR–UF4 AAV vector backbone to create the AAV–α1 and AAV–eYFP constructs. The recombinant vector plasmids were packaged into virions by cotransfection of HEK 293T cells with a helper plasmid and a plasmid expressing the AAV5 serotype capsid. Transfections, purification, and titering were performed by the Penn Vector Core (University of Pennsylvania, Philadelphia, PA). The AAV–α1 vector virus had a titer of 1.9×10^{13} genome particles/μl and the AAV–eYFP vector virus had a titer of 5.3×10^{12} genome particles/μl.

Viral injection. Adult male Sprague Dawley rats aged 75–120 d (Charles River Laboratories, Wilmington, MA) were anesthetized with 100 mg/kg ketamine, placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and put under continuous halothane (Sigma, St. Louis, MO) for the duration of the procedure. Two microliters of virus at a rate of 0.25 μl/min was stereotaxically injected into dentate gyrus in three sites at two different depths/site as described previously (Roberts et al., 2005).

Status epilepticus induction. At 2 weeks after AAV or sham injections, rats received intraperitoneal injections of pilocarpine (385 mg/kg) to induce SE according to a standard protocol (Brooks-Kayal et al., 1998; Shumate et al., 1998). Rats that did not exhibit convulsive seizures within 1 h of pilocarpine injection were given a second dose of pilocarpine (192.5 mg/kg) to produce equivalence in seizures between animals. All rats included in the study developed status epilepticus. Diazepam (6 mg/kg; Hospira, Lake Forest, IL) was administered at 1 h after the onset of SE to stop seizure activity and was administered again every 2 h (3 mg/kg) until rats stopped seizing.

Behavioral and electroencephalographic monitoring for spontaneous seizures. After completing the viral injection, a subset of rats ($n = 4$ for sham controls and $n = 7$ for AAV–α1) were implanted with electrodes in frontal cortex and CA1 of hippocampus for combined intracranial electroencephalography (EEG) and video monitoring. All other rats were assessed using video monitoring only. All rats were monitored continuously starting immediately after pilocarpine injection until the day they were killed.

RNA amplification and reverse Northern blotting. Dissection of DG and RNA isolation were performed using standard procedures as described previously (Roberts et al., 2005; Raol et al., 2006). T7-$(dT)_{24}$ primer and SuperScript II RNase H− reverse transcriptase were used for cDNA synthesis, and the AmpliScribe T7 High Yield Transcription kit (Epicenter Technologies, Madison, WI) was used for RNA amplification according to published methods (Raol et al., 2006). The cRNA was purified using the RNeasy MinElute Cleanup kit protocol (Qiagen, Valencia, CA) according to the protocol of the manufacturer. [P^{32}]dCTP was incorporated into the radiolabeled cDNA probe using SuperScript II RNase H− reverse transcriptase and hybridized for 18 h against a slot blot containing GABR cDNAs (α1–α6, β1–β3, γ1–γ3, δ, ε, ϕ, π) using published protocols and conditions (Raol et al., 2006).

Reverse transcription-PCR. Reverse transcription-PCR was done using standard procedures as described previously (Roberts et al., 2005). Each sample was run in triplicate and contained the following: 1.25 μl of GABR α1 Taqman primer probe (Rn00788315_m1; Applied Biosystems, Foster City, CA) or 1.25 μl of Taqman cyclophilin probe (CCG TGT TCT TCG ACA TCA CGG CTG; Applied Biosystems) with 1.25 μl of each cyclophilin primer (cyclophilin reverse, 5′ CCC AAG GGC TCG CCA 3′; cyclophilin forward, 5′ TGC AGA CAT GGT CAA CCC C 3′; IDT Technologies, Coralville, IA), 12.5 μl of Taqman Master mix, and 10 μl of sample cDNA. All values were normalized to cyclophilin expression to control for loading amount variability.

Protein analysis. Western blotting was performed using our published protocol (Raol et al., 2006). A total of 12.5 μg of DG protein homogenates were run on 10% SDS/polyacrylamide gels and then transferred to nitrocellulose. Blots were blocked with 5% milk, incubated with 1:400 anti α1-subunit primary antibody (Upstate Biotechnology, Lake Placid, NY) overnight at 4°C, and then incubated with anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (HRP) (1:5000 dilution; Amersham Biosciences, Buckinghamshire, UK) using standard methods. Protein bands were visualized using Super Signal West Pico chemiluminescent substrate kit (Pierce, Rockford, IL) and quantified using NIH Image software (National Institutes of Health, Bethesda, MD). All of the blots were normalized to β-actin for which the blots were striped and reprobed with anti-β-actin polyclonal antibody at 1:10000 dilution (Sigma) and anti-rabbit IgG secondary antibody conjugated with HRP, and results are percentage of control values.

Statistical analysis. Because there was no statistical difference between the control groups (AAV–eYFP, saline, or no injection) killed at the 1, 2, or 4 week time points after SE in any of the parameters studied, the data from all control groups were pooled and are represented as "sham control" for statistical comparison with the AAV–α1-injected rats. Statistical analysis for the results of reverse Northern, RT-PCR, and Western blotting was done using one-way ANOVA, followed by Bonferroni's *post hoc* test comparing selected groups. Kaplan–Meier survival analysis procedures were used to examine differences between the AAV–α1 and sham control rats in time (day) to first spontaneous seizure. Kaplan–Meier survival analysis is a method for modeling time-to-event data in the presence of censored cases. The Kaplan–Meier model is based on estimating conditional probabilities at each time point when an event occurs and taking the product limit of those probabilities to estimate the survival rate at each point in time. Unpaired Student's *t* test was used to analyze the difference in SE characteristics between AAV–α1 and sham control rats. One-way ANOVA, Bonferroni's, and *t* test analysis was performed using Instat (GraphPad Software, San Diego, CA). Kaplan–Meier survival analysis was performed using SAS software, version 8 (SAS Institute, Cary, NC). A p value ≤ 0.05 was considered statistically significant. Statistical analysis was performed in consultation with the Division of Biostatistics at the Children's Hospital of Philadelphia.

Results

To directly test the hypothesis that higher α1 levels inhibit development of epilepsy after SE, we used an AAV serotype 5 gene transfer vector to express a GABR α1 cDNA in DG after SE. We chose the novel approach of using the GABRA4 promoter to express the α1 cDNA because GABRA4 promoter activity is upregulated in dentate after SE (Roberts et al., 2005). An AAV vector containing either the α1 cDNA and eYFP reporter (AAV–α1) or the eYFP reporter only (AAV–eYFP) was injected into DG of adult rats, and SE was induced 2 weeks later. SE was also induced in two additional sets of control rats, saline-injected and uninjected naive. All of the rats were then continuously monitored for spontaneous seizures and were killed 1, 2, or 4 weeks after SE.

AAV–α1 injection selectively enhanced α1 subunit levels in dentate after SE

Levels of 16 GABR subunit mRNAs (α1–α6, β1–β3, γ1–γ3, δ, ε, ϕ, π) in microdissected DG were assessed by RNA amplification and reverse Northern blotting. Rats injected with AAV–α1 showed higher levels of α1 subunit by 2 weeks after SE ($n = 5$; $p < 0.02$ by one-way ANOVA for effect of group; $p < 0.01$ for pairwise comparison by Bonferroni's *post hoc* test) (Fig. 1*A*). α1 mRNA and protein levels were similar between the control groups (AAV–eYFP injections, $n = 8$; saline injections, $n = 6$; or no injections, $n = 3$) and between the three time points at which the control groups were analyzed (1, 2, or 4 weeks after SE; $n = 8$, 3, and 6, respectively), so all data were pooled as a single sham control value (total $n = 17$). There were no significant differences in mRNA levels between the AAV–α1 and sham control groups for any GABR subunit other than α1 (data not shown). To further quantify α1 mRNA changes, RT-PCR was performed. α1 mRNA levels were higher in DG from rats that were injected with AAV–α1 at 1 week ($n = 6$) and 2 weeks ($n = 5$) but not 4 weeks ($n = 4$) after SE compared with sham controls ($p < 0.01$ by one-way ANOVA for effect of group; for pairwise comparison,

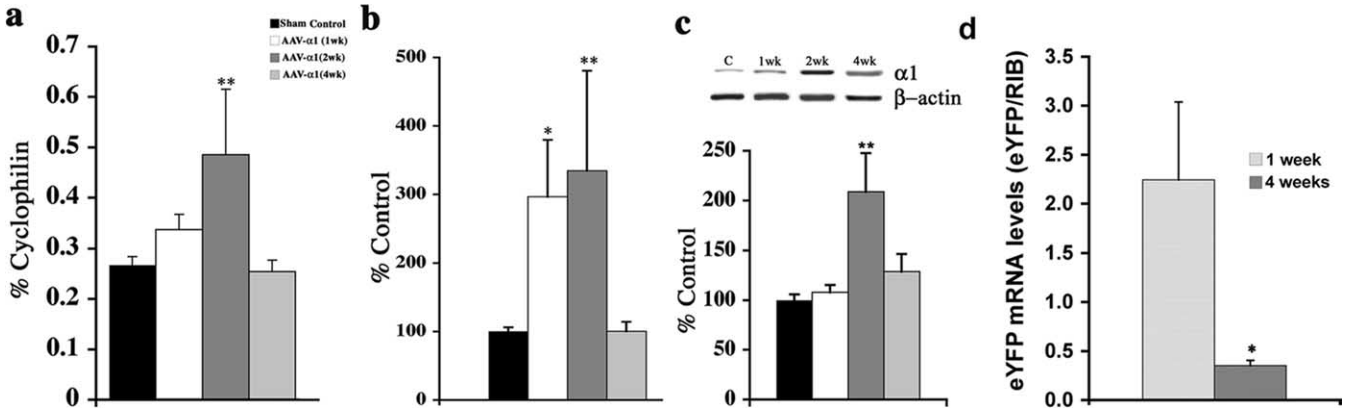

Figure 1. GABR α1 subunit levels in the dentate gyrus after SE were higher in rats injected with AAV–α1. ***a***, α1 subunit mRNA (mean ± SEM; normalized to cyclophilin) at different time points after SE measured using RNA amplification. ***b***, α1 mRNA levels measured with RT-PCR (normalized to cyclophilin). ***c***, α1 subunit protein levels. Top, Representative Western blots demonstrating α1 subunit and β-actin protein levels in DG homogenates from sham control rat (c) and rats that were injected with AAV–α1 and killed at 1, 2, and 4 weeks after SE. Bottom, Quantitation of protein levels (mean ± SEM) at different time points after SE (normalized to β-actin values and expressed as percentage of control values). ***d***, Histogram represents eYFP mRNA levels (mean ± SEM) in the dentate gyrus of the rats that were killed after either 1 or 4 weeks after SE determined using RT-PCR. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

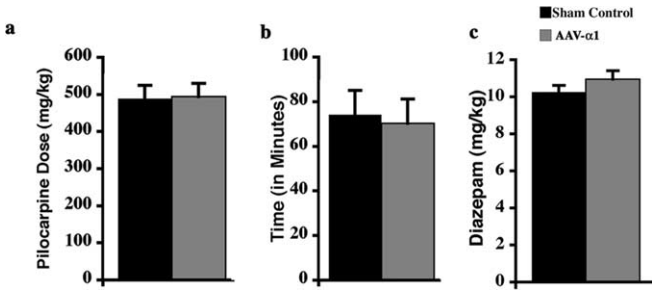

Figure 2. The SE characteristics were not different between AAV–α1 and sham control rats. ***a***, Bar graphs (mean ± SEM) shows the amount of pilocarpine required to induce SE was similar between groups. ***b***, The mean latency to onset of SE (in minutes) was not different between sham and the AAV–α1 group of rats. ***c***, The histogram (mean ± SEM) shows that the cumulative amount of diazepam required to stop seizures beginning 1 h after SE onset was the same between groups.

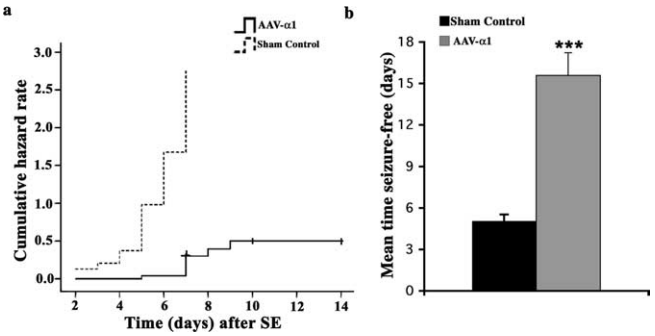

Figure 3. Viral-mediated transfer of α1 subunit into DG decreased the risk of developing spontaneous seizures after SE. ***a***, The rate of spontaneous seizure development after SE per unit time is lower in AAV–α1 rats than in sham controls (Kaplan–Meier hazard function, $p < 0.0005$). The cumulative hazard rate (*y*-axis) is the summation of the probability per time unit that a rat that has remained seizure free will develop seizures in a specific time interval. Censored cases, represented as +, are cases in which rats had not developed first seizure by the end of their follow-up period (total of 14 censored cases, all in the AAV–α1 group, with follow-up time ranging from 7 to 14 d). ***b***, Length of time (mean ± SEM) rats remained seizure free after SE was threefold longer in AAV–α1 rats compared with sham controls (***$p < 0.0005$).

$p < 0.05$ for 1 week and $p < 0.01$ for 2 week by Bonferroni's *post hoc* test) (Fig. 1*B*). α1 subunit protein levels were also higher in DG of the AAV–α1-injected rats by 2 weeks after SE ($n = 5$; $p < 0.0001$ by one-way ANOVA for effect of group; $p < 0.001$ by Bonferroni's *post hoc* test for pairwise comparison) (Fig. 1*C*) but were similar to control by 4 weeks after SE ($n = 4$). To differentiate whether the decline in α1 subunit levels by 4 weeks after SE was attributable to loss of transferred or endogenous α1, we assayed mRNA levels for eYFP (which is transcribed in tandem with the α1 cDNA) in AAV-injected rats killed at 1 or 4 weeks after SE. RT-PCR analysis demonstrated that mean eYFP mRNA levels declined over sixfold in AAV-injected rats between 1 and 4 weeks after SE (Fig. 1*D*), suggesting that the decline in α1 subunit levels by 4 weeks after SE most likely results from loss of α1 cDNA expression from AAV rather than from changes in transcription of the endogenous α1 gene or enhanced α1 turnover.

AAV–α1 injection inhibited development of spontaneous seizures after SE

All of the rats in both groups experienced SE after pilocarpine injection, and there was no difference in any of the characteristics of SE between the groups, including amount of pilocarpine required to induce SE (Fig. 2*A*), latency to SE onset (Fig. 2*B*), and amount of diazepam required to stop seizures (Fig. 2*C*). All of the rats were continuously video monitored from the time of SE until the day they were killed to determine whether they developed spontaneous behavioral seizures. AAV–α1 injection significantly increased the mean time to first spontaneous seizure after SE ($p < 0.0005$ by Kaplan–Meier survival analysis; $n = 23$) (Fig. 3*A*,*B*), and only 39% of AAV–α1-injected rats (9 of 23) were observed to develop spontaneous seizures compared with 100% of rats receiving sham injections ($n = 17$). A subgroup of AAV–α1-injected rats ($n = 7$) were monitored using intracranial EEG in addition to video monitoring to determine whether any electrographic seizure activity might be occurring in the absence of behavioral seizures. No electrographic seizures were seen in the absence of behavioral correlates, and all behavioral seizures were associated with electrographic seizure activity. In AAV–α1-injected rats that went on to develop spontaneous seizures, the severity and frequency of seizures did not differ from sham-injected rats.

In addition to the effects on seizures, elevation of α1 expression was also associated with a behavioral phenotype in a fraction of the AAV–α1-injected rats. Although most of the AAV–α1-injected rats behaved similarly to sham-injected rats, 30% were observed to have abnormal behavior, including excessive seda-

tion, anorexia, and weight loss that persisted for days to weeks after SE, and was sometimes severe enough to require supplemental feedings and hydration to ensure survival. Formal testing of memory and behavior was not performed. This observed behavioral effect was not seen after SE in any of the other groups (including the AAV–eYFP control), suggesting that the effect likely resulted from elevated α1 levels rather than the effects of AAV injection or SE. Such effects are not surprising given that α1-containing receptors are the major form of GABRs mediating sedation in the nervous system (Mohler et al., 2002).

Discussion

The current findings provide the first direct evidence that increasing the levels of a single $GABA_A$ receptor subunit, namely the α1 subunit, in dentate gyrus can inhibit the development of spontaneous seizures after SE. The current results are consistent with a working model in which α1 subunits in DG are limiting and their decrease in response to SE, in the background of increased α4, impairs inhibition in the hippocampus and contributes to the subsequent development of spontaneous seizures. Some (Brooks-Kayal et al., 1998, 1999) but not all (Schwarzer et al., 1997; Fritschy et al., 1999) previous studies of TLE have found lower expression of α1 in the DG, however, and the importance of α1 subunit changes for epileptogenesis has remained controversial. Although the present data do not prove a causal role for α1 changes in the pathogenesis of epilepsy, they strongly suggest that α1 levels in DG can play a determining role in whether epilepsy develops after SE. It must be noted that the current short-term study cannot determine whether enhanced α1 subunit expression is simply suppressing seizures (i.e., having an anticonvulsant effect) or would permanently prevent development of epilepsy in some animals (i.e., an antiepileptogenic effect). Such a determination will require a much longer-term study using a different viral construct that produces more persistent elevation of α1 subunit levels. Because SE is a known risk factor for later development of epilepsy in humans (Cendes et al., 1993), the current results suggest that such additional studies are warranted to determine whether therapeutic strategies designed to enhance GABR α1 subunit expression may be effective in reducing the risk of later epilepsy development in patients experiencing SE.

The specific cellular mechanisms by which the increased α1 is exerting its effect on seizure development remains to be determined. Several potentially interesting future lines of investigation are raised by our results. GABRs require multiple subunit proteins to be functional (typically 2α, 2β, and a γ or δ). Thus, to have an effect, the α1 expressed from the AAV vector must assemble with endogenous subunits, such as β and γ, and then be trafficked to the cell membrane. Future studies to identify subunit partners of the viral α1 gene product within the cell and at the cell surface after SE may provide insight into the mechanisms of α1 gene transfer effects as well as assembly and trafficking of α1-containing GABRs. Physiological studies will be useful to assess the effect of α1 gene transfer on inhibitory function at both a cellular and circuitry level. Finally, although in the current study, we did not observe an obvious difference in hippocampal morphology between the AAV–α1 and sham control groups using routine light microscopy, more detailed studies including stereology and quantitative cell counting will be needed to be certain that α1 gene transfer is not affecting SE-induced cell death or synaptic reorganization.

Our results demonstrate that a vector containing the GABR α1 gene downstream of the GABRA4 promoter can be used to successfully increase α1 levels in DG. To our knowledge, this is the first reported use of a condition-specific promoter upregulated in disease to drive enhanced expression of a gene that was transferred by a somatic gene transfer vector into diseased cells. Surprisingly, AAV transfer of the α1 transgene driven by the GABRA4 promoter resulted in enhancement of α1 gene expression in DG for only the first 2 weeks after SE. The reduction of α1 expression at 4 weeks suggests that the GABRA4 promoter in AAV vector is either silenced or transcriptionally downregulated. Although gene silencing has been reported in the CNS for integrated retrovirus vectors (Prasad-Alur et al., 2002; Rosenquist et al., 2005), it is less likely to alter AAV-mediated expression because AAV vector genomes are both episomal and associated with high-molecular-weight DNA in the brain, and robust gene expression has been documented from AAV using various promoters for much longer than 4 weeks (Skorupa et al., 1999; Xu et al., 2001; Passini et al., 2002). Our previous work strongly suggests that BDNF and Egr3 (early growth response 3) are major regulators of enhanced α4 expression after SE (Roberts et al., 2005, 2006), so downregulation of these transcription factors could explain our findings. However, the endogenous α4 subunit expression in AAV-injected animals did not vary at 1, 2, or 4weeks after SE, as would be expected if Egr3 or BDNF were depleted or downregulated, making this hypothesis unlikely. Additional studies will be required to define the precise mechanism responsible for the decrease in expression of the α1 cDNA from AAV over time.

Several previous studies have reported successful use of gene transfer of inhibitory neuropeptides galanin (Haberman et al., 2003; Lin et al., 2003; McCown, 2006) and neuropeptide Y (Richichi et al., 2004) into the CNS to increase the threshold to seizure induction by kainic acid or electrical kindling. However, none of those studies examined the effects of gene transfer on later development of spontaneous seizures or epilepsy after the provoked seizure events. Another study in a genetic rat model in which the aspartoacylase gene is deleted demonstrated that replacement of this gene reduced the spontaneous tonic convulsions that typically occur in this mutant but did not improve their survival time (Seki et al., 2004). The current study provides the first documentation of the successful use of viral gene transfer to inhibit development of an acquired epilepsy. These findings suggest that strategies designed to modify abnormal gene expression during the latent period between an initial precipitating injury known to increase risk of epilepsy, such as status epilepticus or severe head trauma, and the onset of spontaneous seizures may have therapeutic potential for the prevention of epilepsy.

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