Exploring the treatment of epilepsy through intrahippocampal GABA modulation with an HSV-vector expressing GAD67

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Abstract

This study explores the effect of a herpes-simplex viral (HSV) vector expressing glutamic acid decarboxylase 67 (GAD67) in the rat hippocampus. GAD67 gene transfer increases GABA release in transfected neurons by 3 days and its effect diminishes over 3 weeks. Transgene expression was associated with a decrease in voltage-gated sodium channel α-subunits 1.2 (NaV1.2) and 1.6 (NaV1.6) in the hippocampus. Changes in VGSC levels, along with enhanced GABA neurotransmission, are likely responsible for the prevention of status epilepticus in rats expressing the GAD67 transgene using a pilocarpine-based model of epilepsy. Thus, gene transfer of GAD67 under a latency promoter for prolonged expression may have therapeutic value for patients with intractable epilepsy.

Introduction

Epilepsy is a serious neurological condition characterized by recurrent, unprovoked seizures.[8] An epileptic seizure is a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain.[32] It is among the most common neurological conditions[78], affecting approximately 5 out of every 1000 people in developed countries[96], and it is reported that up to 5% of people will experience seizures at some point in their life.[6] Decades of epilepsy research have identified genetic and acquired abnormalities of synaptic and neuronal network instability, and over forty subtypes of epilepsy are recognized today.[24,27] Thus, epilepsy is not a single disease but a symptom of brain dysfunction: a final common pathway of many different cerebral insults.

Epilepsies may be classified based on etiology as either primary (idiopathic) or secondary (symptomatic). Primary epilepsies are those that are not caused by another known disorder or syndrome and their etiology is presumed to be genetic. Secondary epilepsies, on the other hand, are the result of an insult to the patient's brain, such as a tumor, stroke, infection, or trauma.[42] Types of epilepsy are further divided into partial or generalized based on the clinical presentation; partial seizures remain confined to a particular brain region while generalized seizures occur bilaterally and throughout the forebrain.[27]

Despite the variety of subtypes, all epileptic seizures can be unified as abnormalities in the ongoing electrical activity of the brain, and most, if not all, epileptic events arise from some type of imbalance between excitatory and inhibitory activity within the neuronal circuitry.[71] The interconnectivity of neurons makes them particularly susceptible to seizures through "runaway" excitation. Here, activated neurons stimulate neighboring neurons in a feed-forward loop, and the result is the initiation of synchronous electrical events that are characteristic of seizures.

In the case of partial epilepsy, the region of the brain that is responsible for initiating the excitatory activity is often identifiable by the sensations, known as auras, perceived at the onset of a seizure. Electroencephalography (EEG) aids in the localization of focal epileptogenic abnormalities by detecting aberrant electrical firing through electrodes placed on the scalp, and today, functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) are often utilized for the accurate localization of abnormal brain activity.[24] These tools provide physicians with the insight necessary for diagnosis and treatment of a particular type of epilepsy.

After diagnosis, the first line of treatment utilized in epileptic patients is pharmacotherapy. A range of anti-epileptic drugs (AEDs) are currently in use, but no single drug has proven to be universally effective, and combinations of AEDs are often used in an attempt to control seizures. Most AEDs have similar mechanisms of action, either blocking voltage-gated sodium channels (carbamazepine, phenytoin, and lamotrigine) or enhancing inhibitory neurotransmission (barbiturates, vigabatrin, and tiagabine). Understanding the effects of these drugs requires knowledge of the neuronal voltage-gated sodium channels (VGSCs) and their role in generating and propagating action potentials, as well as a familiarity with GABA synaptic transmission; these topics will be briefly reviewed below.

VGSCs consist of three protein subunits: a pore-forming α-subunit and two auxiliary β-subunits, β1 and β2.[76] There are nine distinct mammalian α-subunits, and specific α-subunits are expressed differentially throughout the central and peripheral nervous system. The embryonic hippocampus predominantly expresses α-subunit 1.3 (NaV1.3), while the adult hippocampus expresses α-subunits 1.1, 1.2. and 1.6 (NaV1.1, NaV1.2, and NaV1.6, respectively).[5,29,36]

These proteins are described as voltage-gated because their sodium ion (Na^+) pore opens and closes in response to changes in cellular membrane voltage. Gating allows for rapid changes in electrical activity to be transferred down the axon, and it is essential for the propagation of the action potential. At rest, VGSCs are closed and do not allow Na^+ to flow through their pores. However, they have threshold voltages that cause them to open and allow for Na^+ flow through their pore region. Once this threshold is reached and the pore opens, the Na^+ that is accumulated on the outside of the cell

is allowed to flow into the cell, and the cell becomes depolarized. This depolarization of the membrane allows more VGSCs along the axon to reach their threshold voltage and open their pores, allowing the electrical signal to proceed down the axon.

By a similar voltage-gated mechanism, the VGSC pore closes to ion flow once the cell reaches a specific membrane voltage, and this is known as inactivation. The closing of the pore allows the cell to re-establish the baseline ion gradient and to prepare for the firing of another action potential. Thus, the timing of gating events and the membrane voltages at which they occur determine the properties of a particular neuron and are important to consider when analyzing the excitatory/inhibitory events associated with different types of neurons.

Carbamazepine, phenytoin, and lamotrigine are AEDs that alter VGSC gating events in order to decrease neuronal excitability. These drugs bind to sodium channels that have been inactivated and stabilize the inactivate conformation so that the VGSCs remain closed to ion flow. This has the effect of inhibiting action potentials in these neurons. Based on probability, neurons that fire rapid action potentials are more likely to be bound and constitutively inhibited, while neurons that fire action potentials intermittently are less likely to be inhibited. Therefore, in theory, the proper dose of these AEDs should inhibit the aberrant firing of seizure foci while leaving the firing associated with normal processes relatively unaffected.[80]

A second class of drugs that is capable of controlling seizures is effective at the level of the synapse. In the prototypical neuron, depolarizing events in the cell body trigger the action potential to be propagated down the axon and neurotransmitters to be released from the axon terminals into the synaptic cleft. Here the neurotransmitters can bind to receptors and trigger changes in the molecular or electrical environment of the neighboring cells. Barbiturates and benzodiazepines are known to be effective at the level of the synapse and were some of the first drugs utilized for epilepsy treatment. The efficacy of barbiturates and benzodiazepines lies partly within their ability to mimic the principal inhibitory neurotransmitter in the brain, γ-amino butyric acid (GABA).

GABA is produced in nerve terminals from the brain's principal excitatory neurotransmitter, glutamate, and is released into the synaptic cleft by both vesicular and non-vesicular mechanisms.[119] Once present in the synapse, GABA can bind to receptors and inhibit the firing of action potentials in neurons. This is achieved by evoking hyperpolarizing currents in cells. Hyperpolarizing currents shift the membrane potential away from the threshold required to fire an action potential, and thereby inhibit neuronal firing. $GABA_A$ receptors achieve this by allowing chloride ions (Cl^-) to flow into the cell through an ion pore, while $GABA_B$ receptors achieve this by intracellular signaling cascades that open potassium channels and allow K^+ to flow out of the cell.

In recent years, other AEDs have been developed around mechanisms of enhancing GABA-mediated neuronal inhibition. Vigabatrin and tiagabine, for example, are drugs that serve to increase the brain's endogenous levels of GABA. Vigabatrin accomplishes this by inhibiting GABA transaminase[39], the enzyme that degrades GABA in the nerve terminals. Tiagabine, on the other hand, increases GABAergic transmission by inhibiting the plasma membrane's GABA transporter (GAT).[38] GAT typically serves in the reuptake of GABA from the synaptic cleft. Therefore, inhibition of GAT serves to increase the concentration of GABA in the synapse, facilitating GABA binding to receptors and increasing inhibitory neural transmission.

Despite the development of new AEDs, approximately 20-30% of patients with epilepsy remain refractory to drug therapy[54,100], and surgical resection of the seizure focus must be considered for improving seizure control. This method was first explored as a treatment option in the 19th century, and the first successful resection was reported in a patient with temporal lobe epilepsy (TLE) in 1879.[64,24] TLE is an important subtype to consider when discussing surgical treatment options since it is the most common type of focal epilepsy, the most refractory to pharmacotherapy, and the most accessible to surgical therapy.[98,24] Unfortunately, despite extensive improvements in surgical techniques, not every epileptic patient is a good candidate for surgery and not every seizure focus is readily localizable.[7] Furthermore, nearly all patients that undergo surgery still require additional anticonvulsant therapy[117] and suffer from the cognitive and behavioral side effects of AEDs.

If surgery has been rejected, very few treatment options remain. As a result, alternative treatments are being explored using animal-based models of epilepsy, and a variety of techniques have been established that can closely mimic TLE in humans. Most commonly, electrical stimulation, electrical kindling, and excitotoxin (kainic acid or pilocarpine) administration are used for this purpose. Each of these techniques has been proven to mimic the pathophysiologies associated with human TLE, including hippocampal sclerosis and electrophysiological alterations.[83,109,110] In our studies, the pilocarpine model of seizures was used to induce status epilepticus (S.E.) and to explore the possibility of using gene transfer as a novel therapeutic approach to epilepsy.

The following studies were designed to explore the efficacy of a non-replicating herpes-simplex virus (HSV)-based vector in mediating the gene transfer of glutamic acid decarboxylase 67 (GAD67) for the treatment of epilepsy. GAD67 is an enzyme that synthesizes GABA from glutamate. Neurochemical studies suggest that a decrease in γ-aminobutyric acid (GABAergic) inhibition, particularly in the dentate gyrus of the hippocampus, contributes to epilepsy[53], and that there is potential for treatment through modulation of GABA inhibitory neurotransmission.[13]

In our laboratory, the HSV-based vector that encodes GAD67 (QHGAD) has been shown to increase GABA synthesis and release by transduced neurons.[59] The principle objective of our study was to test whether focal injection of QHGAD in the rat hippocampus would decrease abnormal electrical discharges and thus reduce seizure activity in an animal model of epilepsy.

Based on current AEDs' ability for seizure prevention (discussed above), we hypothesized that enhanced GABA

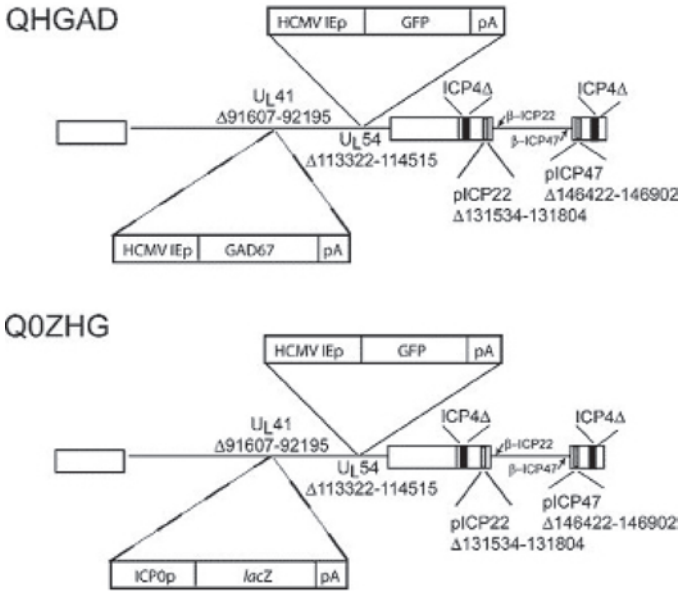
neurotransmission would reduce seizure activity in a pilocarpine-based model of epilepsy. In addition to its behavioral effects, we hypothesized that enhanced GABA release from transfected neurons acting on $GABA_A$ and/or $GABA_B$ receptors in these, or neighboring, neurons would alter neuronal expression of VGSCs. We aimed to explore this effect and to determine the mechanisms by which our vector alters these channels.

Methods

Experimental Animals. Female Sprague Dawley adult rats (210-230 g) were used. They were housed with a fixed 12 hr light/dark cycle and *ad libitum* access to food and water. Housing conditions and experimental procedures were approved by the University of Michigan Committee on Use and Care of Animals.

Vector Construction. The HSV-based vector had four immediate early genes deleted, (ICP4, ICP22, ICP27, and ICP47) to block viral gene expression and abort the HSV lytic cycle. The human GAD67 gene is under the control of the human cytomegalovirus immediate early promoter (HCMV IEp) at the UL41 locus in QHGAD. A green fluorescent protein (GFP) reporter gene is also under control of HCMV IEp at the UL54 locus. The control vector, QOZHG, is defective in the same four IE genes and contains the GFP reporter gene, but the GAD67 gene is substituted with the *Escherichia coli lacZ* reporter gene under control of the herpes promoter infected cell polypeptide 0 (ICP0), at the UL41 locus. Vector stocks were produced and stored at −80 °C until thawed for use. The QHGAD and QOZHG vectors were purified to a titer of 5 x 10^6 pfu/μL. Vector Constructs are shown in **Figure 1**.

Stereotactic injections. Two vectors were used in these experiments: Control vector, QOZHG, and experimental vector, QHGAD. Animals were injected with the appropriate vector bilaterally into the dentate gyri (nose bar, 0 mm; lateral, +/-2.8 mm from Bregma; anterior/posterior, -3.8 mm from Bregma; ventral, -3.5 below the dura) using a 10 μL Hamilton syringe. 3 μL of the vector was injected at a rate of 0.33 μL/min and all animals were fully anesthesized throughout surgery (4% chlorohydrate, 1 mL/kg, intraperitoneally (i.p.)). Damage to the dentate gyrus was avoided to our best ability, and no behavioral differences were seen in these animals post -stereotactic injection. For seizure experiments, control vector and QHGAD vector were aliquoted and labeled numerically by an outside source. The numbered vectors were injected, and behavioral testing was performed before the identity of the vector was known, eliminating researcher bias. Pilocarpine was administered three days post-stereotactic injection.



Pilocarpine administration. As **Figure 2** illustrates, each rat was pre-treated with scopolamine (1 mg/kg, i.p.) before pilocarpine administration (400 mg/kg, i.p.). The animals were closely monitored and any seizure behavior was recorded for 90 minutes. At 90 minutes, each animal, regardless of seizure severity, was given a dose of diazepam (5 mg/kg, i.p.) regardless of seizure severity. The animals were then given a dose of 4% chlorohydrate (1 mL/kg, i.p.) to ensure full anesthesia before sacrificing the animals.

Behavioral Measurements. After pilocarpine injection, animals were observed for 90 minutes for seizure-specific behaviors. The latency to mouth/facial movements and head bobbing, defined as stage 1 and stage 2 seizures, respectively, was recorded.[87] In strongly affected animals, these behaviors were followed by recurrent myoclonic convulsions, rearing, falling, and S.E.[109] S.E. was defined as persistent seizure activity that was uninterrupted by voluntary or coordinated movement. The occurrence of S.E. was recorded in all animals, and the latency to S.E. was noted when applicable.

Cell Culture. Hippocampal neurons from Sprague Dawley rat pups on post-natal day 1 were obtained, dissociated, and plated over poly-lysine coated 24-well plates. Hippocampal neurons were cultured in defined Neurobasal media supplemented with B27, Glutamax I, and Albumax II. After 3 weeks in culture, cells were incubated with the appropriate viral vector (MOI 3) for 2 hours before the media was removed and fresh media was added. The cells were left to express the vector for 48 hours, at which point approximately 95% of cells expressed the reporter gene, GFP, as observed using fluorescent microscopy.

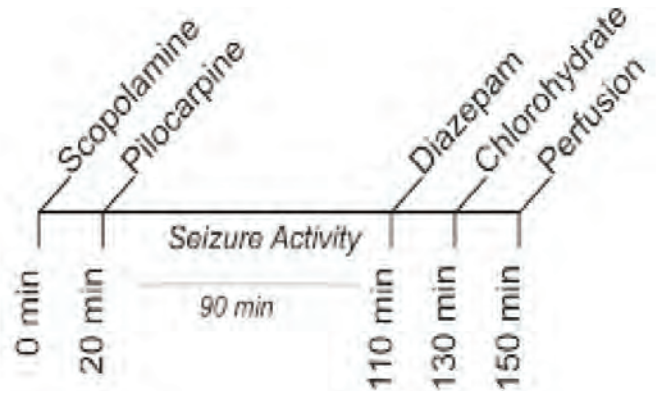


Figure 2: *A schematic representation of the seizure induction protocol.*

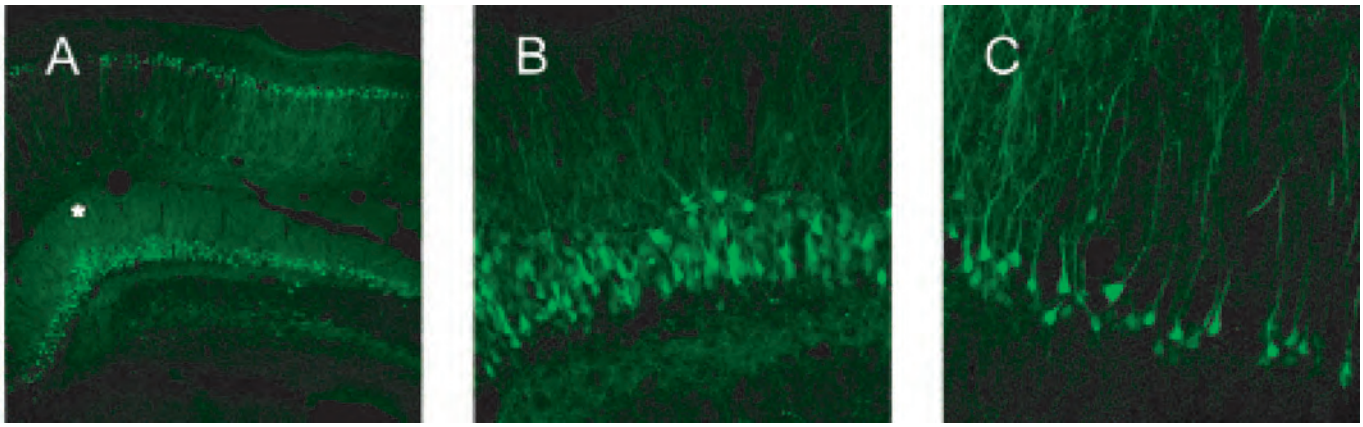


Figure 3: *GFP expression in the rat hippocampus upon QHGAD inoculation. 3 days post-injection, GFP is present throughout the hippocampus, and the injection did not seem to cause any serious tissue disruption (a). Granule cells of the dentate gyral superior blade display the strongest expression (b), and significant expression is also seen in the CA1-CA3 regions (CA1, c). The injection site is indicated with an asterisk(*).*

Western Blot Analysis. Animals were perfused transcardially with 100 mL 0.9% NaCl and the brain was collected and frozen on dry ice immediately. The hippocampus was then dissected from the surrounding cortex and the tissue was homogenized in sample buffer (2% SDS, 1% glycerol, 0.5% Tris Buffer) containing phosphatase inhibitors (Sigma, p2850 & p5726) and a protease inhibitor (Sigma, P8340). Care was taken to avoid the precipitation of SDS. The homogenate was then sonicated and subsequently centrifuged at 14,000 rpm for 5 min before the supernatant was collected. High-speed fractionation studies were performed on noted samples, which included a spin at 80,000 rpm for 60 minutes. Bio-RAD Dc protein assay was performed to determine all protein concentrations and 50 µg aliquots were prepared for analysis. All samples were run on 4-15% or 7.5% tris-HCl gels from Bio-RAD. Primary antibodies were obtained from Chemicon, Temecula, CA (GAD67, 1:1000; NaV1.2, 1:200; NaV1.6, 1:200; β2, 1:300) and Sigma-Aldrich, St. Louis, MO (β-actin, 1:2000)), and species-specific secondary antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA (1:2000). All antibodies were diluted in 5% milk solution prepared in 1x PBS. Exposure of antibody binding was performed with chemiluminescent HRP antibody detection reagent from Denville Scientific (E-2500). Densitometry analysis was performed using Quantity One software. Experiments using cell lysates were also prepared according to the methods listed above.

Immunohistochemistry. Animals were perfused transcardially with 150 mL 0.9% NaCl and subsequently with 200 mL 4% paraformaldehyde (PFA) buffered at a pH of 7.4. The brains were then dissected and post-fixed for 24 hours in PFA and dehydrated for 48 hours in a 30% sucrose solution. Forty µm thick sections were taken using a sliding microtome, and slices were kept in antifreeze solution (sucrose, ethylene glycol, phosphate buffer) at -20°C until use. Antibodies used include those listed above, as well as c-fos (1:500, Santa Cruz, SC-7202). Fluorescent, species-specific, secondary antibodies (1:2,000, Alexa Fluor) were used in immunofluorescent assays, and DAB stains were performed using reagents from Vector Laboratories, Inc.

Immunocytochemistry. Cells were directly fixed in 10% formalin solution, blocked (5% NGS, 1% BSA, 0.01% 1000x sodium azide, 0.2% Triton), and probed with the appropriate antibodies diluted in solution (5% NGS, 1% BSA, 0.01% 1000x sodium azide). Cells were then probed with fluorescent, species-specific, secondary antibodies (1:2000, Alexa Fluor)), and cells were mounted using Fluoromount-G (Electron Microscopy Sciences, 17984-25).

Imaging. Confocal microscopy was performed using the Zeiss 510 Meta Microscope from the Microscopy and Image Analysis Core Facility in the University of Michigan Biomedical Science Research Building.

Statistical Analysis. Statistical significance was obtained for the seizure animals using a Fisher Exact Test.[32] For Western blot analysis, one-tailed Student t-tests were applied to densitometry ratios when the number of samples per group was sufficient.

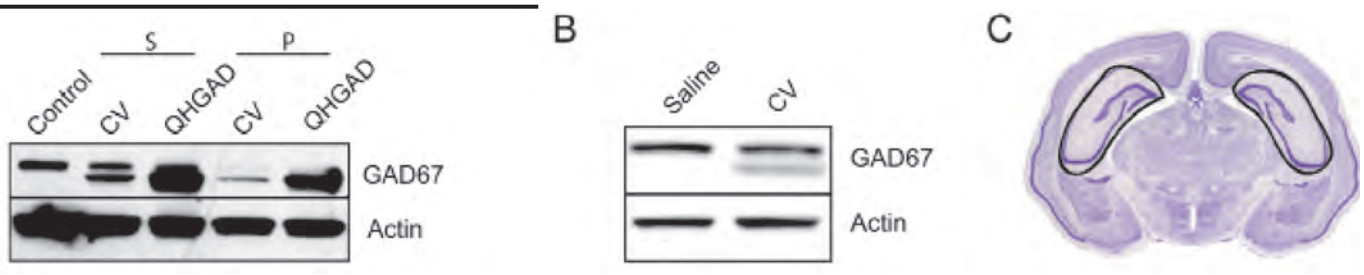
Results

GFP and GAD67 transgene expression in rat hippocampus following HSV-vector delivery

To determine the extent of neuronal infectivity and transgene expression in rat hippocampus after HSV-vector delivery to the dentate gyrus, a series of histological and biochemical studies were performed.

Injection of the HSV-vector into the rat hippocampus was adjusted to give the maximum amount of expression in the dentate gyrus region and to minimize the damage to surrounding tissue. Ultimately, 3 µL of the vector injected at a rate of 0.33 µL/minute in a region dorsal to the dentate gyral superior blade was determined to be ideal; these settings were utilized for all subsequent experiments.

The extent of vector-mediated transgene expression was visualized using a green fluorescent protein (GFP) reporter gene present in QHGAD and QOZHG vector control (**Figure 3**). Injection of the vector resulted in widespread expression in the granule cells of the dentate gyrus (3b), as well as the



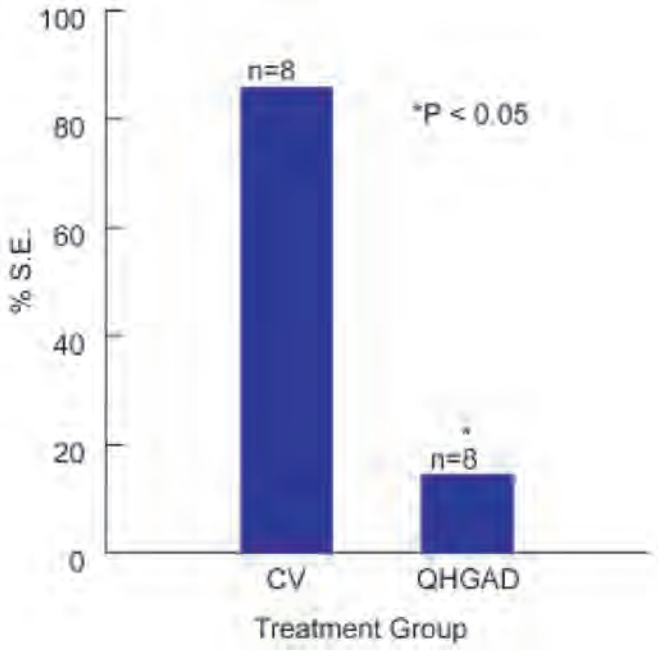


Figure 4 (Top): *GAD67 transgene protein expression from rat hippocampus. A shows the Western blot analysis of hippocampal protein three days post-injection and the strong expression of GAD67 with the QHGAD vector. CV is control vector, S is the supernatant fraction and P is the pellet fraction of homogenates. B illustrates that the lower band detected with the GAD67 antibody is not due to trauma (saline injection), but is present with injection of control vector. C depicts the regions of the hippocampi that were isolated from rat brain for Western blot analysis.*

Figure 5 (Left)*: QHGAD inoculated animals are resistant to status epilepticus (S.E.) upon pilocarpine administration. Six out of eight control vector inoculated animals reached S.E. in this seizure assay while only one out of eight QHGAD inoculated animals reached S.E. when given the same dose of pilocarpine. Statistical analysis was performed using a Fisher Exact Test and the difference observed is significant, $p < 0.05$.*

pyramidal cells of the hippocampal CA1-CA3 regions (3c) 3 days post-injection. The expression in the dentate gyrus was specific to the granule cell layer and did not show notable expression in the neurons of the dentate hilus. GFP is observed in neuronal cell bodies, as well as neurites, indicating widespread transgene expression intraneuronally. These results were maintained at the 1 week time point, but were largely diminished by 2 weeks and were absent by 3 weeks.

The level of GAD67 transgene expression was measured by Western blot of protein homogenates from animals injected in the dentate gyrus with QHGAD, control vector (CV), or saline. Results showed that QHGAD treated hippocampi expressed very high levels of GAD67 protein at 3 days post-injection as compared to sham animals and control vector injected animals (**Figure 4**). The electrophoretic mobility of the GAD67 transgene protein appears slightly faster than the endogenous band seen in sham animals. Animals injected with saline alone showed the same band as sham animals, suggesting that the change in mobility was not due to trauma. We are unclear about the exact nature of this slight increase in electrophoretic mobility of the GAD67 transgene protein, but it is possibly due to differences in glycosylation levels or phosphorylation state. In the high speed pellet fraction of these samples (100,000g), a single molecular weight band was observed instead of the doublet, indicating a possible membrane associated fraction of GAD67. Again, protein expression was robustly increased in the QHGAD sample.

These results showed that the direct delivery of a non-replicating HSV-vector to a focal brain regions leads to a high efficacy of neuronal infection and robust levels of transgene expression. Also, the time course observed was characteristic of what we have previously seen using the HCMV promoter. Thus, these vectors were determined suitable for further studies in epilepsy.

Over-expression of the GAD67 transgene provides resistance to pilocarpine-induced status epilepticus

Using the behavioral analysis described above (see Methods), seizure activity characterized by mouth/facial movements, head bobbing, convulsions, and status epilepticus was recorded. All animals were observed for 90 minutes following the administration of pilocarpine, and latencies to different seizure activities were recorded. Preliminary experiments were performed to determine an optimal dose of pilocarpine for inducing status epilepticus (S.E.) in vector injected animals, and it was concluded that a dose of 400 mg/kg, preceded by scopolamine (1 mg/kg), was the maximum dose that could be administered without risking mortality. This dose was capable of producing S.E. in 75% of QOZHG-injected animals, which is similar to the percentage reported by others for sham animals.[53]

Three days prior to pilocarpine administration, all animals were injected with 3 μL of the appropriate vector containing $5x10^6$ pfu/μL. These animals were injected bilaterally in the dentate gyri, as described previously in studies of GAD67 transgene expression.

Treatment with QHGAD was capable of reducing the percentage of animals to reach S.E. in this model of seizures, and the results were statistically significant ($p < 0.05$) (**Figure 5**).

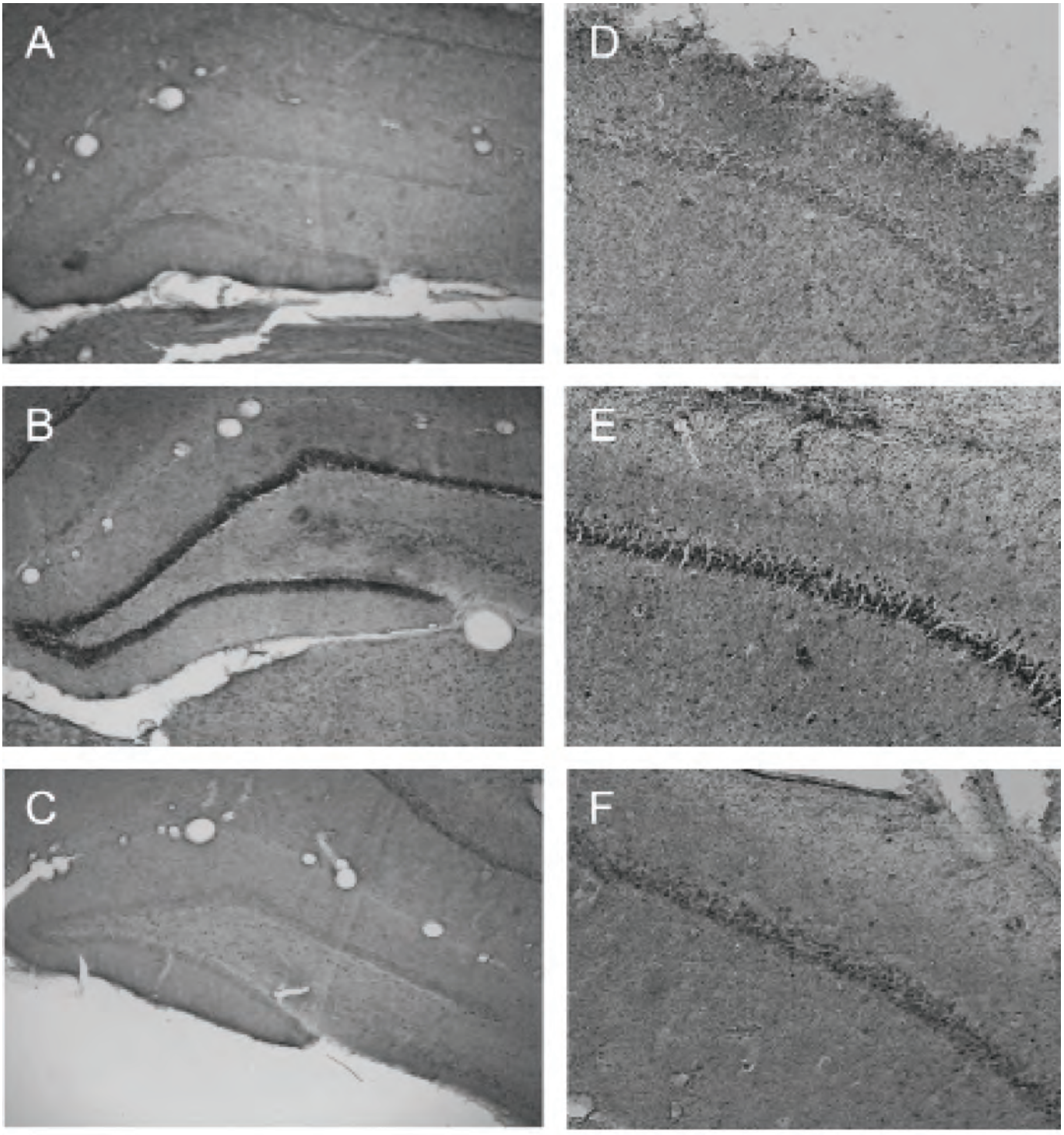
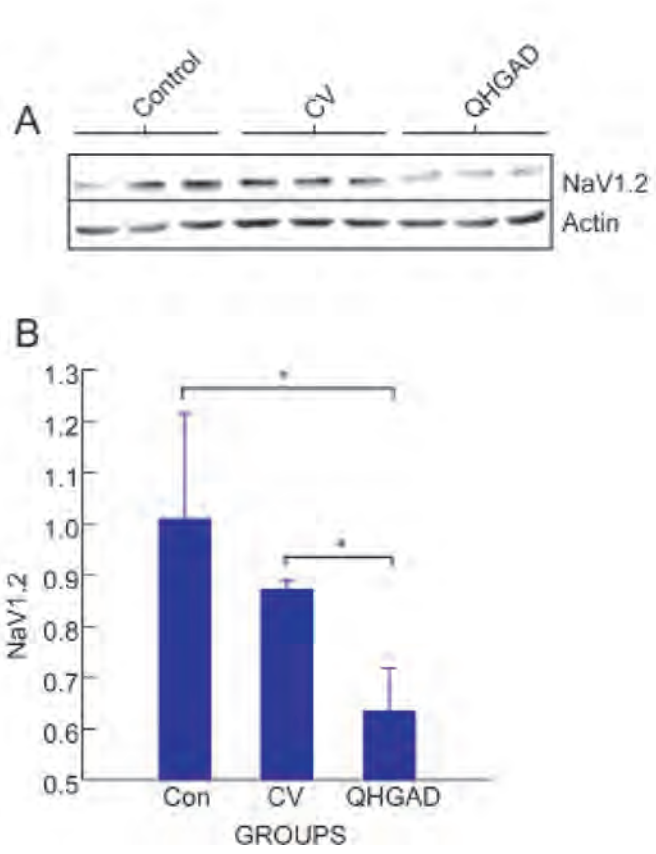


Figure 6: *Expression of c-fos in the dentate gyrus (a,b,c) and CA2 regions (d,e,f) after pilocapine administration. a and d are sections from a QHGAD injected brain that did not reach S.E. b and e are sections from a control vector injected brain that reached S.E. Strong expression of c-fos is seen in the dentate gyrus and CA2 region. c and f are sections from a QHGAD vector injected animal that reached S.E. The dentate gyrus of this animal resembles the non-S.E. dentate gyrus (a) with low c-fos expression, but the CA2 region of this brain shows expression of c-fos similar to that in the S.E.*

Six out of eight animals injected with control vector experienced S.E. while only one out of eight animals injected with QHGAD experienced S.E. The latencies to onset of stage 1/2 seizure activity and S.E. were highly variable between groups and results were not statistically significant.

In addition, we examined brain sections from these animals for c-fos expression with immunocytochemistry. Analysis of c-fos expression in the hippocampus can be correlated with seizure activity, as reported by others.[21,43,22] Animals that did not reach S.E., independent of treatment group, show low bas-



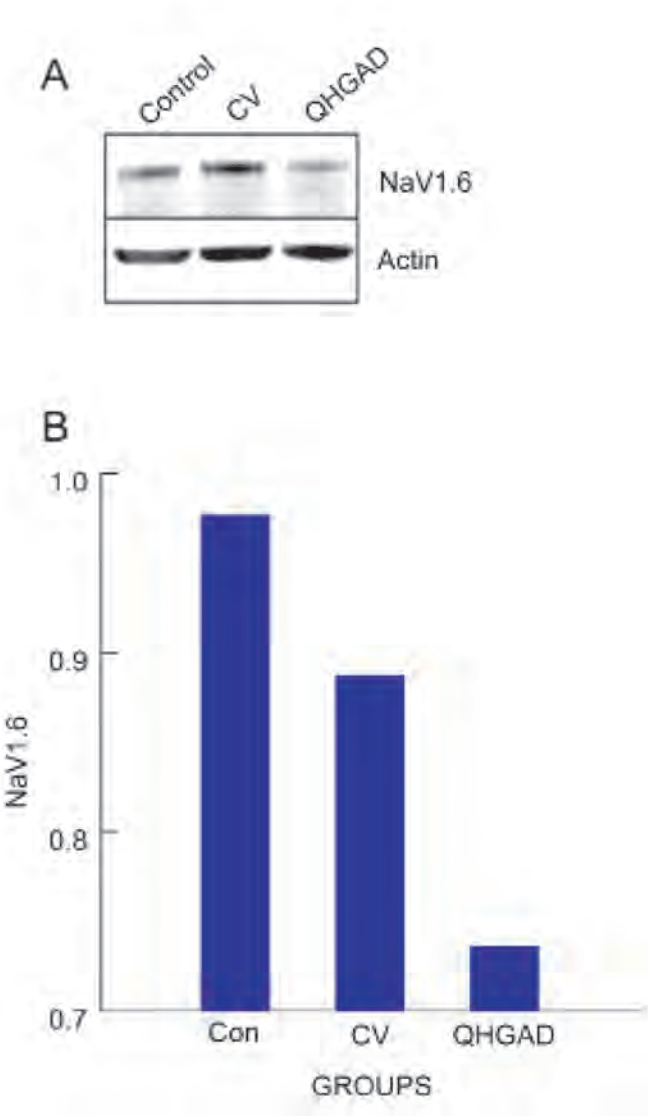


Figure 7 (Top): *NaV1.2 is down regulated in QHGAD injected animals. Western blot analysis of hippocampal tissue shows decreased levels of NaV1.2 at 3 days post-injection (A). A ratio of the mean intensity of the NaV bands to control (β-actin) is depicted in B. Bars represent standard error of the mean. Differences between the groups indicated were statistically significant, p<0.05, using a one-tailed Student's t-test.* **Figure 8 (Right)***: NaV1.6 is down regulated in QHGAD injected animals. Western blot analysis of hippocampal tissue shows decreased levels of NaV1.6 at 3 days post-injection (A). A ratio of the mean intensity of the NaV bands to control (β-actin) is depicted in B. The blot shown is representative of several trials showing NaV1.6 down regulation.*

al levels of c-fos expression in the hippocampus, and QOZHG injected animals that reached S.E. showed a strong up-regulation of c-fos in dentate granule cells and CA1-CA3 pyramidal cells (**Figure 6**). Interestingly, the single animal injected with QHGAD that reached S.E. did not show c-fos up-regulation in the dentate granule cells but did show some level of c-fos immunostaining in the hippocampal CA1-3 regions as compared to animals with no seizures, indicating that another pathway may have been responsible for initiating S.E.

The results from these experiments showed that prevention of clinical seizures may be obtained though HSV-mediated GAD67 transgene delivery to the seizure focus.

Long term enhanced synthesis and release of GABA from over-expression of GAD67 down-regulates VGSCs in treated hippocampus

Changes in VGSC expression and properties have been described in different animal models of epilepsy, and it has been reported that these changes can play a critical role in the development of seizures.[1, 23, 51, 61, 116] For this reason, we hypothesized that the enhanced release of GABA resulting from QHGAD inoculation may affect the levels of the voltage-gated sodium channels. We examined the presence of NaV1.1, 1.2, and 1.6, since these are the subunits present within the adult hippocampus. Protein levels were examined 3 days after vector injection in animals that were not treated with pilocarpine.

We observed a strong decrease in the presence of NaV1.2 and NaV1.6 in response to QHGAD compared to QOZHG and control brains (**Figure 7, Figure 8**). We then examined the time dependence of this down-regulation and concluded that NaV1.2 and NaV1.6 were down-regulated to the greatest degree when the GAD67 expression was the highest. By Western blotting, we show that these α-subunit protein levels are greatly diminished at one week but are increasing at two and three weeks when GAD67 transgene expression is diminishing (**Figure 9, Figure 10**). NaV1.1 was unable to be examined with this method due to antibody limitations. Immunofluorescent staining of α-subunit proteins was also attempted, but the resolution of this method was not sufficient to display these changes in protein levels.

We examined the levels of β2 protein in the hippocampus

since the β-subunits of VGSCs are important for the insertion and maintenance of α-subunits in the plasma membrane, but we did not observe significant change upon administration of QHGAD (**Figure 11**). These findings suggest that the regulation of the NaV1.2 and 1.6 protein levels by GAD67 transgene expression may be independent from the regulation of the β2-subunits in these cells.

The occurrence of VGSC α-subunit down-regulation upon QHGAD inoculation signifies that QHGAD prevention of S.E. may not have been strictly due to enhanced GABA release, but may have been partly due to a decrease in the VGSC α-subunits that are crucial for action potential propagation.

VGSC α-subunits are localized in hippocampal neurons

In order to examine if the regulation of VGSC α-subunits was localized in neurons or glial cells of the hippocampus, immunocytochemistry was performed. NaV1.1, 1.2, and 1.6 α-subunits were all seemingly localized to neurons, as visualized by the co-localization of VGSC proteins with the neuronal marker MAP2 (**Figure 12**). The co-localization we detected was specific to granular layer neurons of the dentate gyrus and pyramidal neurons of the CA1-CA3 regions, and strong expression of NaV1.1 and 1.2 was observed in neuronal plasma membranes. NaV1.6 was detected more uniformly throughout the cell body, but this effect may be due antibody quality.

These findings suggest that the down-regulation in VGSC α-subunits observed upon QHGAD injection in the hippocampus is specific to neurons, not glial cells, and therefore may have a direct effect on action potential propagation in these cells.

Absence of QHGAD regulation of VGSC α-subunits in vitro

Experiments using hippocampal neurons in culture were performed in an attempt to further characterize the cellular mechanisms involved in the down-regulation of VGSC α-subunits in response to QHGAD.

Hippocampal neurons were obtained from neonatal rat pups on post-natal day 1 and cultured for 3 weeks. VGSC α-subunits were examined in this cell culture by immunocytochemistry, as they were examined *in vivo*. Co-localization experiments were performed with antibodies to NaV1.1, 1.2, 1.6, and the neuronal marker MAP2. These experiments showed dominant VGSC α-subunit expression in neurons, rather than astrocytes (**Figure 13**).

It should be mentioned that low levels of VGSC staining were observed in occasional astrocytes *in vitro* when double labeled with GFAP, but this effect was not uniform, and the

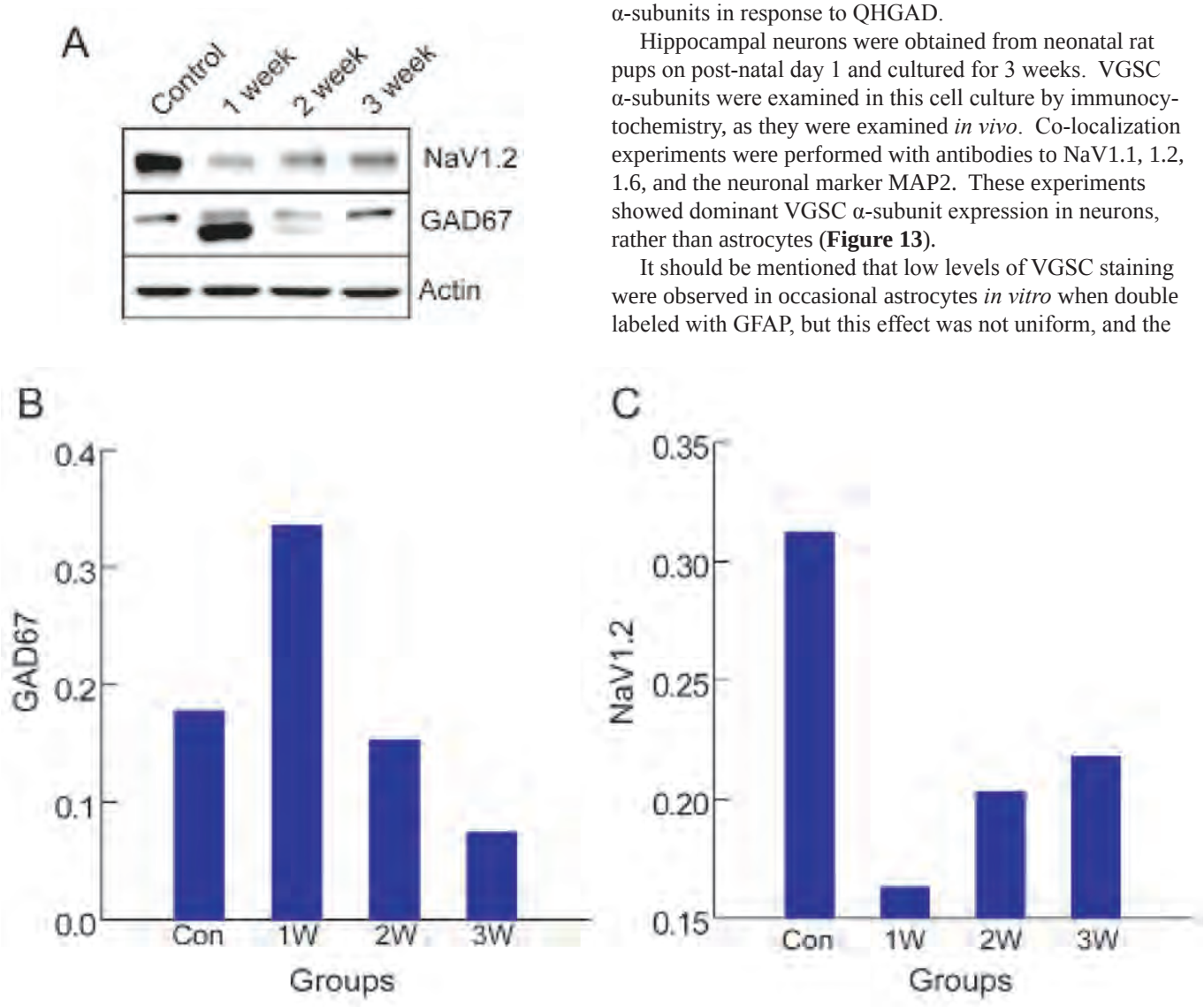


Figure 9: *Response of NaV1.2 to GAD67 transgene expression over a 3-week timecourse. NaV1.2 protein expression is inversely related to GAD67 transgene expression over this time period. B and C illustrate the ratio of the GAD67 band mean intensity or VGSC band mean intensity to control (β-actin), respectively.*

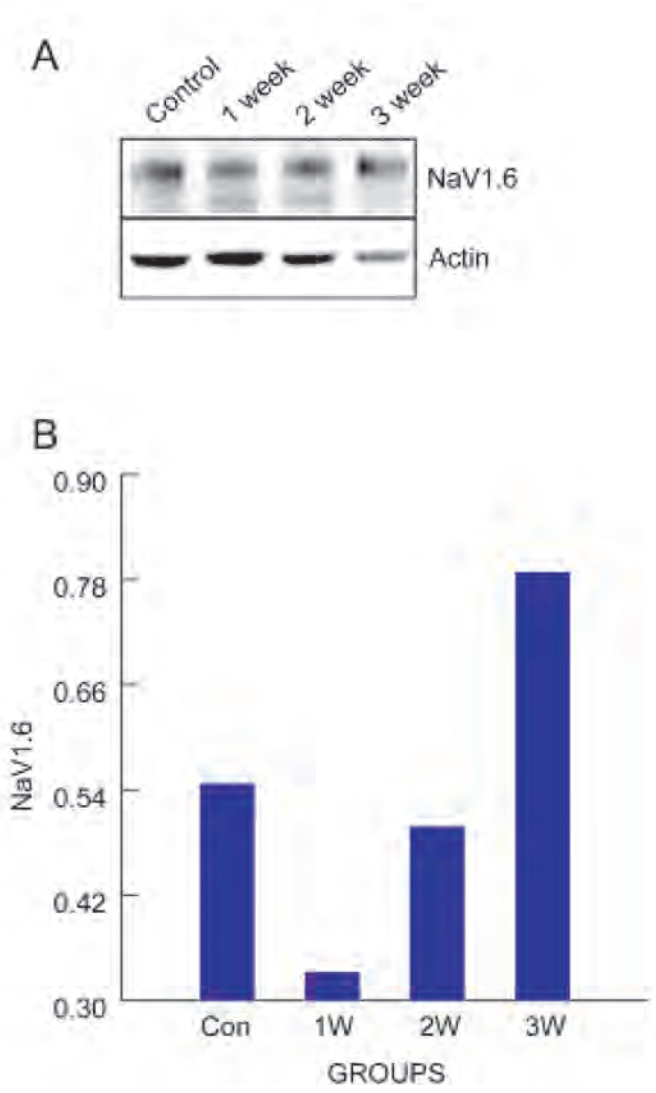


Figure 10: *Response of NaV1.6 over a three week timecourse. The NaV1.6 protein level is regulated in a similar manner to NaV1.2. It is decreased at 1 week, but increases in expression in week 2 and 3 (A). B is a ratio of the mean intensity of the VGSC bands to control (β-actin).*

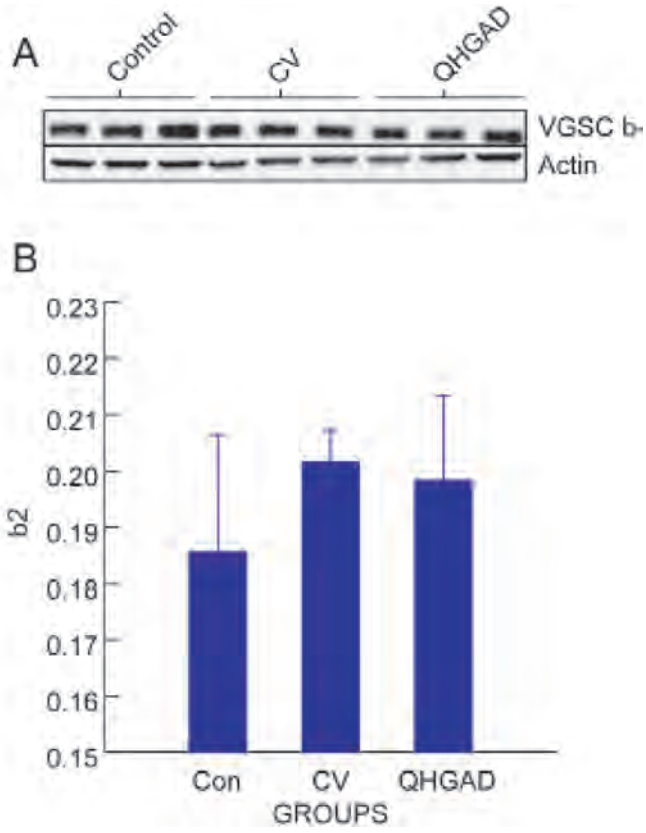


Figure 11: *VGSC β2-subunit protein does not show any alteration between control, control vector, and QHGAD after 3 days of expression, as is seen in the NaV1.2 and NaV1.6 levels. Western blot of the protein is shown in A. B shows a ratio of the mean intensity of the β2 bands to control (β-actin) bands. Bars represent standard error of the mean. Differences are not statistically significant.*

levels were substantially lower than those detected in neurons.

Upon transfection with QHGAD, cultured hippocampal neurons displayed pronounced GFP expression and displayed a large increase in GAD67 protein observed with Western blotting (**Figure 14**). However, in preliminary experiments these neurons did not show the regulation of VGSCs observed *in vivo*.

These experiments lead us to believe that the VGSC α-subunit regulation that we observe *in vivo* is occurring in neurons, but the effect is somehow specific to adult hippocampal neurons or is dependent on the intact neuronal circuitry present in an *in vivo* model.

Discussion

The high prevalence of drug-resistant epilepsy requires patients to undergo surgical resection of the seizure focus often as a last treatment option. Unfortunately, not all patients are eligible for surgery based on their medical history and/or the location of their seizure focus. The development of other treatment options for these patients is necessary, and our studies suggest that GAD67 gene transfer to the seizure focus may provide a therapeutic option for epilepsy management.

Progress in the development of non-replicating viral vectors has greatly advanced their potential use in clinical gene therapy, and HSV-based vectors have gained a great deal of attention due to its their many desirable characteristics. HSV's ability to establish a latent state in the nucleus of non-dividing cells, such as neurons, as a non-integrated episomal element makes it particularly useful. This mechanism allows the viral genome to remain latent in the host cell without disrupting the host genome.[74] Furthermore, HSV's genome can be manipulated to decrease its toxicity and to carry relatively large genes for transfer to host cells.[31] For these reasons, HSV-based vectors have been explored as clinical treatment options, and they have been shown to be well tolerated and safe when delivered to a variety of tissues, including the brain.[40, 68, 79, 85, 89] The QHGAD vector was previously shown to be effective in the transduction of dorsal root ganglion neurons and was able to greatly increase the release of GABA from these cells *in vitro* and *in vivo*.[59] In our studies, QHGAD was capable of

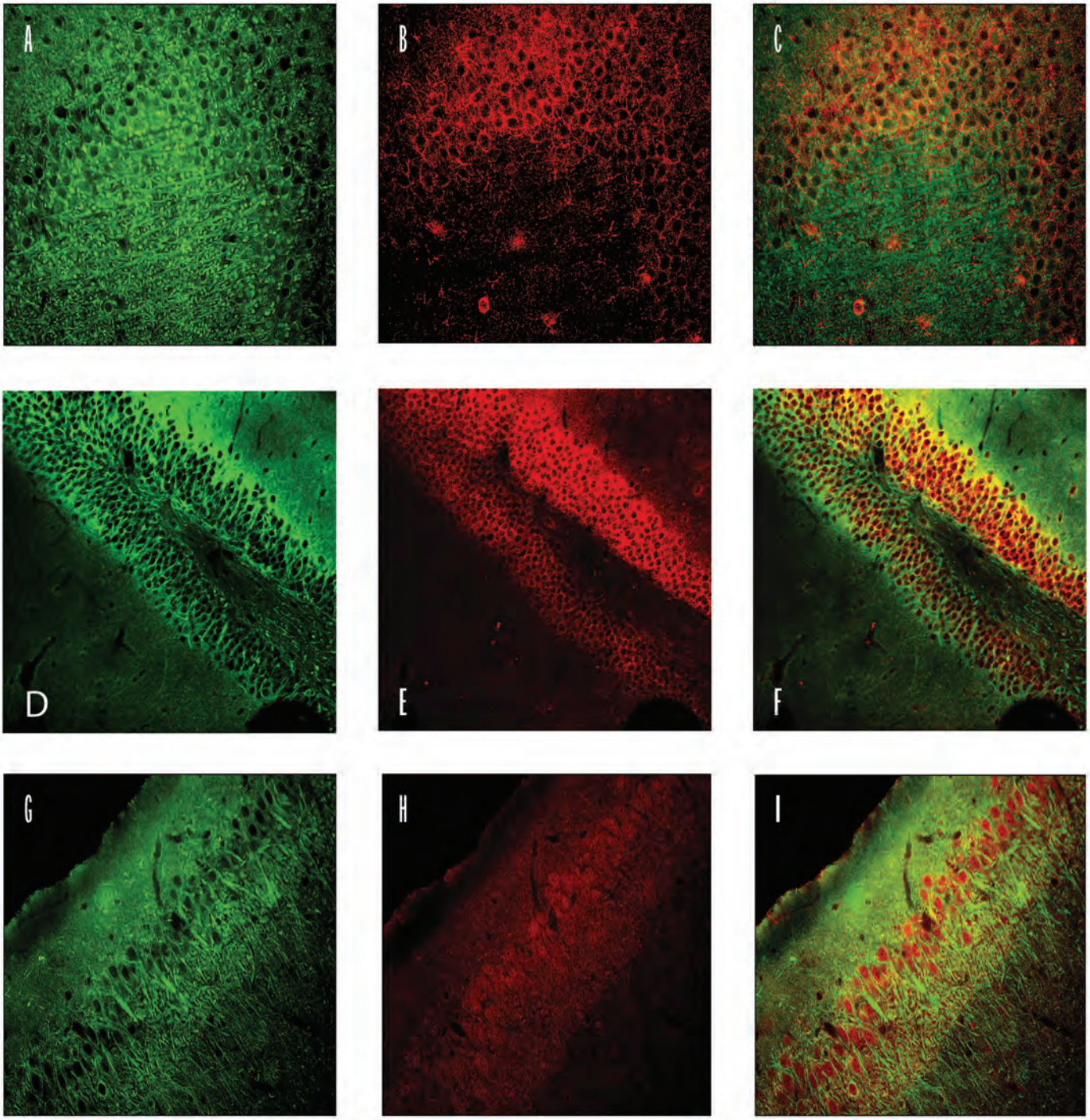


Figure 12: *Tissue sections show the possible co-localization of NaV1.1 (A,B,C), 1.2 (D,E,F), and 1.6 (G,H,I). A, D, and G are hippocampal neurons stained with MAP2, and images B, E, and H are VGSC α-subunits stained with the antibody corresponding to the specific subtype. C, F, and I are overlays of these images displaying co-localization.*

transfecting hippocampal neurons (**Figure 3**) and expressing GAD67 protein in this tissue (**Figure 4**).

Endogenous properties of GAD67 make it particularly favorable for epilepsy treatment. The two isoforms, GAD65 and GAD67, are each encoded by a single, separate gene, and they are distinct in their intracellular distributions in neurons.[11] In general, GAD65 is prevalent in axon terminals and synaptic vesicles, whereas GAD67 is located in cell bodies and uniformly distributed throughout the neuron.[56] The levels of GABA synthesized by these enzymes differ since GAD65 is bound to its cofactor, pyridoxal 5'-phosphate (PLP), less often than GAD67, making GAD67 more constitutively active in neurons.[3] Thus the transfer of the GAD67 gene rather than the GAD65 gene allows for the bypass of this regulation and the constitutive production of GABA in transfected neurons.

Sequestering of neurotransmitters in synaptic vesicles provides another level of control for neurons. Shifts in membrane potential and subsequent increases in intracellular calcium ions are able to control the trafficking of synaptic vesicles to the plasma membrane, where they release their

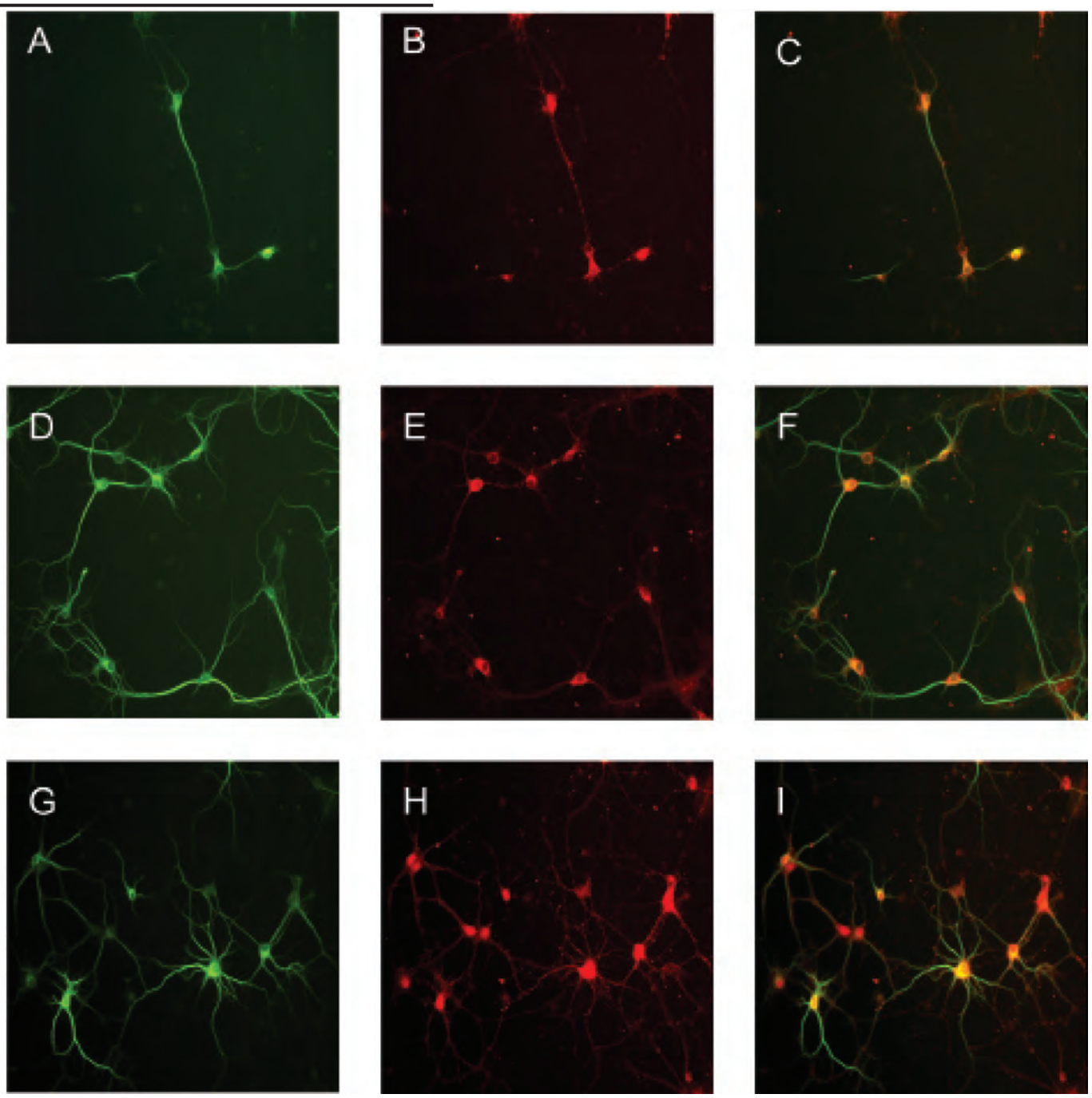


Figure 13: *Immunofluorescent staining of hippocampal neurons in vitro shows the strong co-localization of a neuronal marker (MAP2) with NaV1.1 (a,b,c), 1.2 (d,e,f), and 1.6 (g,h,i). A, C, and Fare hippocampal neurons stained with MAP2, and images B, E, and H are VGSC α-subunits stained with the antibody corresponding to the specific subtype. C, F, and I are overlays of these images displaying co-localization.*

contents into the synaptic cleft. Typically GABA is released at the synapse, binds to GABA receptors, and causes hyperpolarization. Interestingly, it has been reported that a decreased quantal release of GABA from synaptic vesicles may contribute to epileptogenic activity in experimental models of temporal lobe epilepsy.[44]

In addition to synaptic release, the reversal of the GABA transporter, GAT-1, has been observed as a non-vesicular mechanism of GABA release. GAT-1 undergoes the coupled translocation of Na^+, Cl^-, and GABA in a ratio of 2:1:1[63], and the transporter can be reversed by increased intracellular concentrations of Na^+ and GABA (119, 120, 121). QHGAD has been reported to release GABA in this fashion in transfected DRG neurons, and thus it has the potential to release GABA constitutively, independent of membrane potential and synaptic vesicle activity.[59]

Animal models of seizures and epilepsy allow for the mechanisms, as well as the treatments, of epilepsy to be extensively researched. In the 1980s, the excitotoxin pilocarpine was determined to be valuable for designing new therapeu-

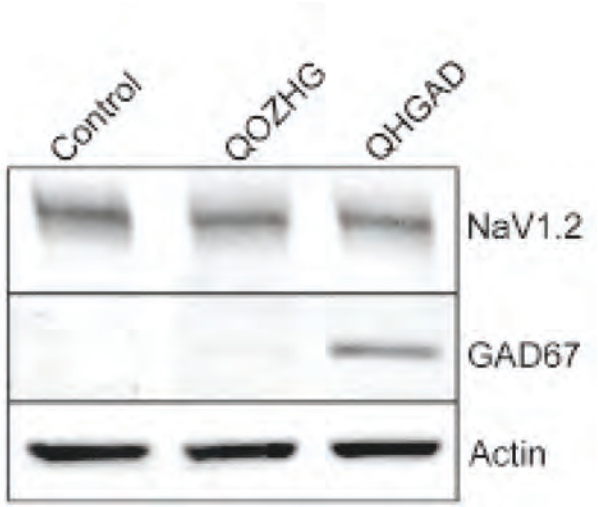


Figure 14: *Western blotting detects a strong increase in GAD67 protein upon QHGAD infection in vitro, but a decrease in NaV1.2 and/or 1.6 is not detected. NaV1.2 is shown here. The blot displayed is representative of several trials.*

tic approaches to epilepsy.[109] Pilocarpine was observed to provoke seizure-characteristic behaviors, and to produce long-term effects of epilepsy and seizure related brain damage.[83] Today, the pilocarpine model of epilepsy allows us to conduct two types of pharmacological studies: acute studies concerning our treatment's efficacy against status epilepticus; and chronic studies concerning our treatment's potential for prevention of recurrent seizures.

To date, we have only completed the first round of studies using QHGAD, but the efficacy of the vector is encouraging. We showed that QHGAD inoculation in the rat dentate gyrus was able to prevent status epilepticus in 87.5% of animals injected with pilocarpine, while only 25% with control vector inoculation were protected from S.E. upon pilocarpine administration. Results from the QOZHG group are consistent with other reports from sham animals[53], and inter-animal variability likely accounts for the percentage of these animals that did not reach S.E.[58]

It has been previously determined that seizure activity results in rapid and transient c-fos up-regulation in dentate granule cells and CA1-CA3 cells, reaching maximum levels within 30 minutes of seizure termination.[20,21,22] We used this marker as a confirmation of seizure activity in QOZHG and QHGAD injected animals in an attempt to support behavioral findings. We found that the seizures experienced by control and QOZHG inoculated animals were consistent with an up-regulation of c-fos in these regions. Furthermore, QHGAD injected animals that did not exhibit S.E. did not show an up-regulation of c-fos.

Interestingly, the single QHGAD inoculated animal that reached S.E. did not follow this trend. This animal did not show the strong up-regulation in dentate granule cells, but did show some degree of up-regulation in CA1-3 regions (**Figure 6**). We are unable to fully explain this novel finding, but we hypothesize that the high expression of GABA in the dentate granule cells and their strong inhibition remained intact, and status epilepticus was reached through another pathway in the brain. The perforant pathway connecting the entorhinal cortex (EC) to the CA1-3 regions is a possible candidate. The EC is believed to be a seizure origin for many patients with TLE[38], and it has been reported that neurons leaving the EC can stimulate the CA1-3 regions without stimulating the dentate gyrus[26]. Based on *in vitro* studies showing the contribution of the CA regions to seizure initiation and maintenance, we believe that this mechanism was the possible pathway for generalized seizure induction[2].

Many alterations in brain protein composition have been observed in animal models of epilepsy as well as human epileptic hippocampus, and compensatory mechanisms of VGSC regulation are noted to occur in sodium channels' α-subunits, as well as their β-subunits.[23,111,116] We hypothesized that the continuous release of GABA from QHGAD transfected neurons would alter the electrical environment and result in an alteration of VGSCs. We examined NaV1.1, 1.2, and 1.6 because of their marked presence in adult hippocampus[29], and the β2-subunit because of its association with these channels and its reported alterations with seizure activity.[17,76]

Analysis after 3 days of QHGAD expression revealed a strong down-regulation of NaV1.2 and 1.6 proteins (**Figure 7, Figure 8**); the decrease was statistically significant for NaV1.2, and experiments are currently underway to increase the sample size and to perform statistical analysis on NaV1.6 bands. We also observed that this down-regulation correlated with the level of vector-mediated GAD67 expression, as shown in the 3 week time course (**Figure 9, Figure 10**).

The continuous release of GABA having an effect on VGSC α-subunit protein levels is a novel finding that has not been reported by other groups. In order to gain insight to this effect, we designed experiments to determine what cell types were expressing these VGSCs and what mechanisms were responsible for their down-regulation.

Since it has been reported that hippocampal astrocytes, like neurons, exhibit VGSC electrophysiological properties[10,103], we utilized immunofluorescent staining to determine the cellular localization of NaV1.1, 1.2, and 1.6 in rat hippocampus. Immunostaining of tissue slices suggests that the proteins being detected are neuronal in origin, but due to the limitations of light microscopy, we cannot rule out that a portion of the proteins detected are in astrocytes in close association with labeled neurons. These experiments were repeated *in vitro*, and a confirmation of the prevalent neuronal localization was observed (**Figure 11**), with the exception of occasional astrocytes staining for low levels of these α-subunits.

The down-regulation of VGSCs is associated with several neurological diseases and insults, such as epilepsy and ischemia, yet little is known about the mechanisms of down-regulation. It has been proposed that neuronal VGSCs may be the targets of the ubiquitin-protein ligases of the Nedd4 family[33,95], but we have not ruled out decreased transcription of VGSC genes as a mechanism of the down-regulation resulting from QHGAD. Experiments looking at VGSC α and β-subunit mRNA levels by reverse transcription-PCR are currently underway.

In an attempt to explore the mechanism of VGSC α-subunit down-regulation, we utilized the dissociated hippocampal neurons from rat neonates and transfected these cells with

QHGAD. We aimed to determine if blocking $GABA_A$ and/or $GABA_B$ receptors would abolish the observed down-regulation. However, the down-regulation of α-subunits was not observed in this cell culture as it was observed *in vivo*, despite strong GAD67 expression (**Figure 14**). We hypothesize that this may be due to several factors including the lack of an intact neuronal network (as is present *in vivo*) or the molecular differences between neonatal and adult hippocampal neurons.

It has been reported that early in development, GABA serves as an excitatory neurotransmitter in the brain due to the decreased expression of the potassium chloride cotransporter, KCC2.[57,92] We hypothesize that this reversed effect of GABA in neonatal cells may be responsible for eliminating the mechanism of voltage-gated sodium channel regulation.

Another possible explanation for this phenomenon lies within the relative abundance of NaV1.1, 1.2, 1.3, and 1.6 in the hippocampus. Levels of these proteins are reported to change between neonatal and adult life, with 1.3 being the predominant VGSC α-subunit in neonatal hippocampi.[5,29] We hypothesize that NaV1.3 levels are high in our hippocampal neuron culture and that these proteins, rather than NaV1.2 and 1.6, may undergo down-regulation after QHGAD infection. Experiments to test the levels of NaV1.3 have yet to be conducted, but we plan to complete these experiments in the near future.

Also, experiments using an organotypic slice preparation of adult rat brains are being planned to determine if an intact neuronal network is necessary for the regulation of VGSC α-subunits and to eventually explore the electrophysiological properties of this type of VGSC regulation.

In addition to exploring VGSC α-subunit regulation, future experiments will need to assess the ability of QHGAD to be an effective treatment for chronic epilepsy. It may prove that QHGAD does not provide the long-term protection that is needed due to the short time span of GAD67 expression, and in this case, a vector should be constructed with a longer acting promoter in place of the HCMV promoter. Studies using the HSV-latency promoter LAP2 for long term expression have been previously published and show transgene expression for up to one year.[18,91] Chronic studies will allow us to determine whether GAD67 transgene expression is capable of preventing recurrent seizures over months or years. The results of this type of study will provide better insight to the clinical capabilities of gene therapy in the setting of anticonvulsant-resistant epilepsy.

It has been extensively observed that the cellular environment of the cortex and the hippocampus can give rise to seizure activity by a variety of mechanisms. These include alterations in VGSCs[23], "burst-generating" cell activity[71], shifts in EGABA due to K^+/Cl^- cotransporter regulations[86], and synchronization of cell firing due to ephaptic interactions[47]. Despite the variation, all of these epileptic mechanisms could be conducive to improvement by enhanced GABAergic activity, and GAD67 gene transfer provides a reasonable method of achieving this enhancement.

Thus, the development of novel treatments for epilepsy is ongoing, and the results from our experiments, as well as other studies in gene therapy, show promise toward management of intractable epilepsies.[49,72,91,121] As previously shown, HSV-vectors can provide a safe and effective means for transgene delivery, and hold great potential for the future of gene therapy. The findings presented above are encouraging for the possible use for GAD67 transgene expression as a treatment for epilepsy, but many critical experiments remain to be pursued.

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