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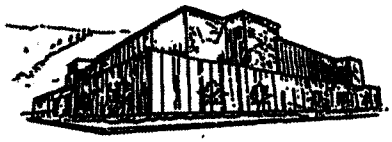

THE ROLE OF EXCITATORY AMINO ACID TRANSPORTERS IN NEUROPROTECTION AND NEUROPATHOLOGY

by

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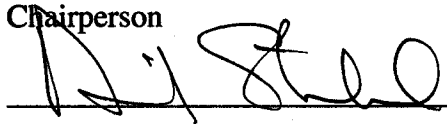

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The role of excitatory amino acid transporters in neuroprotection and neuropathology.

Chairperson: David J. Poulsen, Ph.D.

Glutamate is the most abundant excitatory neurotransmitter found within the CNS. Glutamate has been shown to play a prominent role in development, plasticity, learning and memory. Sodium dependent glutamate transporters (EAAT1-5) facilitate termination of glutamate signaling. Altered expression profiles and function of these transporters have been shown to promote neurodegradation and have been implicated in multiple disease states, including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, stroke, and epilepsy. The two studies detailed in this dissertation investigate the role of EAAT2 and EAAC1 (EAAT3) in neuroprotection and neuropathology.

Excitatory amino acid transporter 2 (EAAT2) is responsible for up to 90% of all glutamate uptake and is primarily localized on astrocytes. Many studies have attempted to elevate EAAT2 expression to compensate for loss of function and expression in disease. The aim of this study was to isolate and characterize the contribution of increased astrocytic EAAT2 expression towards neuroprotection. A novel recombinant adeno-associated virus, rAAV1-GFAP-EAAT2, was designed and used to selectively increase astrocytic EAAT2 expression. Under conditions of oxygen glucose deprivation, elevated astrocytic EAAT2 expression was shown to offer neuroprotection. This approach offers the first evidence supporting sole regulation of EAAT2 expression in astrocytes and the specific role of this transporter in neuroprotection.

Excitatory amino acid carrier 1 (EAAC1) is present throughout the CNS with a small percentage being uniquely expressed on inhibitory presynaptic terminals and provides a primary source of glutamate for GABA synthesis. Alterations to this or other pathways leading to GABA synthesis, may result in abnormal inhibitory synaptic transmission, neuronal hyperexcitability and epileptiform activity. Prior studies have shown GABA synthesis and signaling were reduced and seizure-like behaviors were heightened under global pharmacological inhibition or antisense knockdown of EAAC1. We hypothesized that increased GABAergic EAAC1 expression would enhance GABA synthesis, shift seizure susceptibility, and promote neuroprotection. Using recombinant adeno-associated virus vectors, we were able to specifically target transgene delivery to GABAergic neurons and direct over-expression and knockdown of EAAC1 in vivo. Modulation of GABAergic EAAC1 expression resulted in alterations in GABA synthesis, seizure susceptibility and neuroprotection.

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Chapter One

Introduction

The two studies outlined in this dissertation "Astrocyte specific over-expression of EAAT2 is neuroprotective under oxygen glucose deprivation conditions" and "EAAC1 glutamate transporter activity on inhibitory presynaptic terminals modulates GABA synthesis and neuroprotection" address the impact of altered glutamate transporter expression on neuroprotection and neuropathology under seizure and stroke-like conditions. Prior studies have attempted to evaluate the role of EAAT2 and EAAC1 through development of transgenic animal models, global oligonucleotide antisense knockdown, or general pharmacological inhibition but were limited by their inability to alter EAAT2 and EAAC1 expression within the hippocampus in a cell type specific manner. Additionally, prior studies have been primarily limited to inhibition of glutamate transporter function and lacked the ability to evaluate the impact of enhanced expression of selected glutamate transporters in a cell type specific manner. The studies outlined in the following two chapters utilized a novel approach in evaluating the role of these transporters through the use of recombinant adeno-associated viruses (rAAV). The rAAV1-GFAP and rAAV1/2-GAD65 viral constructs developed and characterized for these studies enabled direct cell type specific modulation of astrocytic EAAT2 and GABAergic EAAC1 expression, respectively.

The scope of the research presented in this dissertation were designed to address the following aims:

1. Use of rAAV to deliver genes for selected glutamate transporters. Sense and antisense gene sequences will be used to facilitate over-expression and knockdown of transporter expression, respectively.
2. Use selected promoters to facilitate cell type specific knockdown and over-expression of selected glutamate

transporters. The GFAP promoter and the GAD65 promoters were chosen to drive astrocyte specific EAAT2 expression and EAAC1 expression specific to inhibitory presynaptic neuron terminals, respectively.

3. Evaluate the influence of selected glutamate transporters on neuroprotection and neuropathology. The neuroprotective role astrocytic EAAT2 over-expression will be evaluated in a stroke model. The impact of EAAC1 modulation in GABAergic neurons will be evaluated using a pilocarpine induced seizure model.

Together these studies offer insight into the potential impact of altered glutamate transporter expression in disease processes and associated neurodegradation. The novel viruses used in these studies enabled efficient cell type specific modulation of glutamate transporters within select subpopulations of cells in the hippocampus.

Chapter Two

Astrocyte specific over-expression of EAAT2 is neuroprotective under oxygen glucose deprivation conditions.

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Abstract

Glutamate is the most abundant excitatory amino acid found within the CNS. This neurotransmitter has been shown to play a prominent role in development, plasticity, learning and memory. Sodium dependent glutamate transporters (EAAT1-5) facilitate termination of glutamate signaling. Excitatory amino acid transporter 2 (EAAT2) is responsible for up to 90% of all glutamate uptake and is primarily localized on astrocytes. Multiple disease states have been associated with alterations in EAAT2 expression level and function. Many studies have attempted to elevate EAAT2 expression to compensate for loss of function and expression in disease. Elevated EAAT2 expression has been noted during ischemic preconditioning (IPC) but is just one of many components altered under IPC. The aim of this study was to isolate and characterize the contribution of increased astrocytic EAAT2 expression towards neuroprotection. A novel recombinant adeno-associated virus, rAAV1-GFAP-EAAT2, was designed and used to selectively increase astrocytic EAAT2 expression. Under conditions of oxygen glucose deprivation, elevated astrocytic EAAT2 expression was shown to offer neuroprotection. This approach offers the first evidence supporting sole regulation of EAAT2 expression in astrocytes and the specific role of this transporter in neuroprotection.

Introduction

Glutamate is the most abundant excitatory neurotransmitter in the central nervous system (CNS). This neurotransmitter directs activation of ionotropic and metabotropic glutamate receptors and mediates secondary pathways involved in development, plasticity, learning and memory (Contestabile, 2000; Riedel, 2003). Excessive or prolonged levels of glutamate in the synaptic cleft may lead to excitotoxic damage and eventual cell death (Danbolt, 2001; Noraberg, 2005). Termination of glutamatergic signaling is mediated by the removal of glutamate from the extracellular space by excitatory amino acid transporters (EAATs). To date, five EAATs have been identified and exhibit distinct expression patterns throughout the CNS. Of these transporters, the high affinity, sodium dependent EAAT2 is the most abundant and responsible for more than 90% of all glutamate uptake (Danbolt, 1992). EAAT2 is primarily localized to astrocytes, with modest expression in neurons (Chen, 2004; Maragakis, 2004).

Multiple neurodegenerative diseases have been linked with glutamate –mediated excitotoxicity and altered EAAT2 expression. Amyotrophic lateral sclerosis (ALS) affected tissues have decreased EAAT2 expression focused in the motor cortex and spinal cord attributed to aberrant RNA processing and activated caspase-3 cleavage of EAAT2. (Guo, 2002; Boston-Howes, 2006). Similarly, Alzheimer's disease (AD) affected tissues show a significant loss of EAAT2 expression and funcion (Cross, 1987; Li, 1997). Neurodamage mediated by ischemia has been show to be exacerbated by decreased astrocytic EAAT2 expression and function (Rothstein, 1996; Rao, 2001). Though modifications in EAAT2 expression are a shared feature of these neurodegenerative diseases, altered EAAT2 expression is not the cause of these diseases (Howland 2002).

Increased astrocytic EAAT2 expression offers neuroprotection under excitotoxic conditions. Treatment with β-lactam antibiotics renders increased EAAT2 expression potentially through mechanisms similar to ischemic preconditioning (IPC) (Rothstein, 2005). IPC is characterized by an acute non-lethal excitotoxic event that results in altered genomic and proteomic response to a secondary excitatory stimulus. β-lactam antibiotics are antagonists of the GABA-A receptor and are capable of simulating a mild excitotoxic even through inhibition of GABA signaling (Fujimoto, 1995). Individual mediators of IPC, such as TNFα convertase, have been shown to indirectly upregulate EAAT2 expression (Romera, 2004).

Astrocytes provide a window of neuroprotection under acute excitotoxic events. Ischemia mediated energy depletion causes reversal of EAATs on neurons and may promote further excitotoxic damage (Li 1999). Astrocytes maintain extended function and ability to continue energy production through anaerobic glycolysis, supply of nutrients and antioxidants to neurons, maintenance of extracellular pH, and glutamate uptake (Benarroch, 2005). An extended time frame of neuroprotection may be afforded by the initial resilience of astrocytes under excitotoxic stress.

Studies reporting neuroprotection in the presence of elevated EAAT2 expression have used methods that alter genomic and protein expression profiles of numerous factors beyond EAAT2. These prior studies have lacked the ability to evaluate the specific neuroprotective role of EAAT2. The aim of this study was to isolate and study the contribution of astrocytic EAAT2 towards neuroprotection under stroke-like conditions. We found that cell type specific modulation of EAAT2

alone can have a profound effect on neuronal survival following excitotoxic insult.

Methods

Virus Preparation

The rAAV1-GFAP-hrGRP and rAAV1-GFAP-EAAT2 viruses were packaged in HEK293T cells cultures grown in standard growth media (DMEM, 10% heat inactivated FBS, 0.05% penicillin/streptomycin (5000 U/ml), 0.1 mM MEM nonessential amino acids, 1 mM MEM sodium pyruvate, and gentamicin (25 mg/ml)). Cells were transfected with three plasmids using Polyfect Transfection Reagent (Qiagen, Valencia, CA). The three plasmids used in the transfection were 1) adeno helper plasmid (pFΔ6), AAV helper (H21) and the AAV packaging vector containing the glial fibrillary acidic protein (GFAP) promoter followed by either humanized Renilla green fluorescent protein (hrGFP) gene (Stratagene, La Jolla, CA) or the EAAT2 gene sequence (obtained from J. Rothstein), flanked by AAV2 inverted terminal repeats. The GFAP-hrGFP construct contained the 3' Woodchuck hepatitis virus post-transcriptional regulation element (WPRE). The WPRE construct has been shown to enhance trans-gene expression and viral packaging (Loeb, 1999; Paterna, 2000; Hlavaty, 2005). Virus was isolated from HEK293T cells through repeated freeze-thaw cycles, incubation for 30 minutes at 37°C with 50U benzonase (Novagen, Madison, WI) and 0.5% sodium deoxycholate, briefly sonicated and further purified by iodixonol density gradient centrifugation as previously reported (Zolotukhin, 1999). The titer (genomic particles/ml (gp/ml)) of final virus isolate was determined by quantitative real time-polymerase chain reaction (RT-PCR) using an ABI Prism 7700 with primer and probe sets specific for the EAAT2 sequence or the WPRE sequence.

Rat hippocampal slice cultures (RHSC)

The RHSC were prepared using a modified method of Noraberg (1999). Hippocampal tissue was isolated from 7-day-old Sprague-Dawley rats. Tissue was cut into 350 μm slices (McIlwain Vibratome 800, Mickle Laboratory Engineering Co., Gomshall, Surrey, UK) and transferred to ice-cold dissection media (Hanks balanced salt solution, 20mM HEPES, 25mM D-glucose, pH 7.3, filter sterilized) and incubated for 30 minutes on ice. Slices presenting clear hippocampal architecture were transferred to dissection media alone or media containing 1 x 10^{11} gp/ml rAAV1-GFAP-EAAT2 or rAAV1-GFAP-hrGFP, oxygenated for 30 minutes and placed on Millipore organotypic inserts in 6-well plates containing 1 ml of primary RHSC media (50% Optimem (Invitrogen, Carlsbad, CA), 25% HBSS, 25% heat inactivated horse serum, 25mM D-Glucose, 2.7 μM amphotercin B, +/- 100μM penicillin G, pH 7.3, filter sterilized). On day three, media was changed to a secondary culture media (Neurobasal -A media, B-27 supplement, 1mM Gluta-max (Invitrogen, Carlsbad, CA), 25mM D-glucose, 2.7 μM amphotercin B, +/- 100μM penicillin G, pH 7.3, filter sterilized). Secondary media was changed every 3 days. Cultures were maintained at 37°C, 5% CO_2 for 10 days.

Oxygen and glucose deprivation (OGD)

OGD studies were performed using a modified method of Bonde, et al. ((Bonde, 2003). Propidium iodide (PI) is a polar compound that gains entry into dead and dying neurons and binds to nucleic acid. Binding of PI to DNA results in a red fluorescence emission (630nm) upon excitation (495nm). At least 6 hours prior to stroke, PI (Molecular Probes, Eugene, OR) was added to the media at a concentration of 2μM (Noraberg, 1999). At this concentration, staining is specific for damaged neurons. OGD was simulated by transferring inserts to deoxygenated,

glucose-free balanced salt solution (BSS) (120 mM NaCL, 5mM KCl, 1.25 mM Na2HPO4, 2mM CaCl2, 25mM NaHCO3, 20mM HEPES, 25mM Sucrose, pH 7.3, filter sterilized). Cultures were then incubating in an oxygen deprivation chamber (37°C, 5% CO_2 and 95% N_2, Biospheric, PRO-OX 110) for 1 hour. After OGD, inserts were transferred back into wells with 1ml secondary media containing 25 mM D-glucose and PI and returned to normoxic culture conditions. Fluorescent images were taken of the hippocampal slices prior to OGD and 6, 12, 18, and 24 hours post-OGD on an Olympus IMT-2 inverted microscope (Melville, NY) attached to an Olympus digital camera using the Magnafire SP software package (Optronics, Goleta, CA). Total fluorescent intensity over time was determined using Image Pro Plus software (MediaCybernetics, Silver Springs, MD).

Western Blot Analysis

Western blot analysis of rat hippocampal slice cultures were performed to evaluate EAAT2 expression levels in control slices, penicillin G treated slices and cultures transduced with rAAV1-GFAP-EAAT2. The RHSC were homogenized in lysis solution (2.5% sodium deoxycholate, 0.1% protease inhibitor cocktail set III (Calbiochem, San Diego, CA), and 0.05% benzonase (EMD Biosciences, San Diego, CA) in PBS). Protein concentrations of lysate samples were determined using the Bio-Rad DC protein assay (Hercules, CA). Aliquots of homogenized RHSC (30μg) were loaded onto a NuPAGE 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA). Proteins were transferred to Immuno-Blot PVDF membrane (Bio-Rad, Hercules, CA) and blocked in Tris buffer containing Tween-20 and 0.5% non-fat milk. Membranes were probed with antibodies to GLT-1 (1:500, ABR) and actin (1:1000, Sigma, St. Louis, MO). Proteins were visualized using ECL (Pierce, Rockford, IL) with species-specific HRP conjugated secondary antibodies. Blots

were imaged on a Kodak Image Station 440 CF. Dosimetry analysis of regions of interest were used to calculate fold change in EAAT2 protein expression corrected to actin control.

Immunohistochemistry

Cell type specific expression from the GFAP promoter was evaluated for rAAV1-GFAP-hrGFP in RHSC. Slice cultures were transduced with rAAV1-GFAP-hrGFP and fixed after 10 days in culture. Slices were fixed with 4% paraformaldehyde for 30 minutes at room temperature (RT), incubated 2 minutes in rinse in ice-cold methanol and stored at 4°C in PBS. Slices were incubated in blocking buffer (1.5% normal goat serum, 0.3% Triton-X 100) for 1 hour at room temperature (RT). After initial blocking, slices were stained with antibodies to NeuN (1:200) or GFAP (1:400) (Chemicon, Temecula, CA) for 1 hour at RT, rinsed in blocking buffer and incubated in species-specific conjugated secondary antibody (1:200, Molecular Probes, Eugene, OR). Slices were placed onto charged glass slides and coverslipped with Fluorosave (Calbiochem, San Diego, CA). Images were obtained on a Bio-Rad Radiance 2000 MP laser scanning confocal microscope (Hercules, CA).

Functional uptake assays

Uptake assays were performed using a modified method of Selkirk (Selkirk, 2005). Briefly, slices were homogenized on ice in tissue buffer (50mM Tris, 0.3M sucrose, pH 7.3) and pelleted at 14,000g for 10 minutes at 4°C. Pellet was resuspended in 250μL of either sodium containing Krebs buffer or sodium-free Krebs containing equimolar amount of choline. Hippocampal homogenates were

incubated with aspartic acid solution (200nM [^{3}H] D-aspartic acid (PerkinElmer, Boston, MA) and 2.0μM cold D-aspartic acid (Novabiochem, San Diego, CA)) in the presence and absence of 0.5 mM dihydrokainic acid (Tocris, Ellisville, MO). DHK is an EAAT2 selective uptake inhibitor and allows for assessment of non-EAAT2 mediated aspartic acid uptake. Reactions were incubated at 37°C for 4 minutes and promptly terminated by filtration through Whatman GC/F filter paper (Whatman, Brentford, Middlesex, UK) via Brandel Cell Harvester (Brandel, Gaithersburg, MD). Filtrate was incubated overnight in scintillation cocktail and counted on a Beckman LS 6500 Scintillation System. Specific EAAT2 mediated uptake of [^{3}H]-D-aspartic acid was calculated as total sodium-dependent uptake less the uptake in the presence of EAAT2 inhibitor, DHK.

Data Analysis

One-way ANOVA was performed to determine statistical significance of western blot dosimetry analysis, functional [^{3}H]D-aspartic acid uptake and level of neuroprotection offered between the control tissue and those treated with either penicillin G or transduced with rAAV-GFAP-EAAT2. Statistical analysis was performed using Prism Software (GraphPad Software, Inc., San Diego, CA).

Results

Astrocyte specific transgene expression

The GFAP promoter sequence identified by Brenner et. al. has been used in multiple studies to selectively drive protein expression in astrocytes (Brenner 1994). Feng et. al. demonstrated stable, long-term astrocyte specific expression of apolipoprotein E (ApoE) using rAAV containing the GFAP promoter (Feng,

2004). Guo et. al. utilized the GFAP promoter sequence to drive EAAT2 expression in a transgenic mouse model (2003) and noted a high degree of astrocyte specific transgene expression.

The rAAV1-GFAP-hrGFP virus was used to validate cell type specificity of the rAAV1-GFAP viral construct. Rat hippocampal slice cultures were transduced with rAAV1-GFAP-hrGFP and immunostained with anti-NeuN or anti-GFAP to identify neurons and astrocytes, respectively. Use of rAAV1-GFAP-hrGFP in RHSC revealed an astrocyte specific expression of hrGFP evident by co-localization of hrGFP expression and anti-GFAP staining. No co-localization was noted between hrGFP expression and NeuN staining (Figure 1).

Increased expression of functional EAAT2

Western blot analysis was performed to assess the level of induced EAAT2 expression in RHSC. Transduction of RHSC with rAAV1-GFAP-EAAT2 significantly increased EAAT2 expression by 129% over that of control (Figure 2A). Similarly, treatment of RHSC with 100μM penicillin rendered a statistically significant increase in EAAT2 expression of 63% over that of control. Total intensity of all three bands characteristic of EAAT2, consisting of a monomer, dimer and multimeric aggregated proteins, were used to calculate total band intensity. Additionally, each lane was calibrated to an actin control to account for variations in total protein added in each lane. Levels of EAAT1, EAAT3 and EAAT4 remained unchanged (data not shown).

To determine if the induced EAAT2 expression was functional, we compared [^{3}H]D-aspartic acid uptake levels between control, penicillin G and rAAV1-GFAP-EAAT2 transduced RHSC. Synaptosomal preparations from rAAV1-GFAp-EAAT2 and penicillin G treated slices showed a 62% and 28% increase, respectively, in

DHK sensitive uptake over controls (Figure 2B). The disparity between the level of increased EAAT2 expression to that noted in EAAT2 specific uptake may be attributed to western blot analysis quantifying total EAAT2 levels, which would include both immature intracellular transporters and mature functional transporters on the cells surface.

Increased astrocytic EAAT2 expression is neuroprotective

To evaluate the potential neuroprotective contribution of EAAT2 under excitotoxic conditions, oxygen-glucose deprivation studies were performed on control RHSC compared to penicillin G treated and rAAV1-GFAP-EAAT2 transduced slices. Elevated astrocytic EAAT2 expression, whether mediated by rAAV1-GFAP-EAAT2 or penicillin G, offers neuroprotection under OGD conditions. Propidium iodide is taken up specifically by dead and dying neurons when present in concentrations less than 2μM (Noraberg, 1999). Elevated EAAT2 levels resulted in statistically significant decrease in PI uptake at each of the 4 time points imaged post-OGD compared to control (Figure 3). No statistically significant difference in PI uptake was noted prior to OGD. The level of neuroprotection was similar between the two treatment groups at each of the observed time points compared to control.

Discussion

Prior studies have established GFAP promoter driven cell type specific protein expression in astrocytes. Feng et. al. used a similar viral construct to drive stable, long-term ApoE expression in astrocytes (Feng, 2004). Guo et. al. developed a transgenic mouse model using the GFAP promoter to drive EAAT2 expression (Guo, 2003). Over expression of EAAT2 during development in these

transgenic animals reduced life-span, litter size and overall growth. A true measure of EAAT2 mediated neuroprotection could not be conclusively defined by this study due to the potential shift in CNS development in this transgenic model. The use of rAAV to mediate changes to EAAT2 expression limits complications noted by altering essential glutamate signaling during developmental processes (Lujan, 2005). Results presented here demonstrate efficient astrocyte specific EAAT2 over-expression through the use of rAAV1-GFAP viral constructs.

Prior attempts at modifying EAAT2 function and expression in astrocytes have been limited to non-specific or indirect approaches. Pharmacological agents available for study of select glutamate transporters offer modest selectivity between the multiple glutamate transporter variants, mild potency, are unable to target transporters in a cell type specific manner and, most importantly, are limited to inhibition of transporter function. More recently, multiple agents have been identified that cause an upregulation of astrocytic EAAT2 expression. An increase in astrocytic EAAT2 expression in tissue pre-treated with β-lactam antibiotics was recently reported (Rothstein, 2005). Rothstein et. al. suggested that Ceftriaxone, the β-lactam of focus in their study, mediated neuroprotection through a mechanism similar to ischemic preconditioning (IPC). Treatment of tissue with FK506 and its non-immunosuppressive derivative, GPI-1046, both activators of the neuroimmuniphilin FK506-binding protein (FKBP12), has recently been reported to cause an increase in EAAT2 expression (Ganel, 2006; Labrande, 2006). Treatment with FK506 causes shifts in protein expression in peripheral and CNS tissue similar to that noted with IPC, such as upregulation of HSP70 and downregulation multiple of pro-apoptotic pathways (Yang, 2003; Furuichi, 2004). Upregulation of EAAT2 is just one of numerous pathways altered in IPC (Stenzel-Poore, 2003; Carmel, 2004; Romera, 2004). Previously identified components altered in the IPC response

have been shown to invoke neuroprotection when introduced individually, including erythropoietin, ceramide, TNFα and TNFα-convertase (Liu, 2000; Ruscher, 2002; Dirnagl, 2003; Romera, 2004). These single components of IPC initiate a cascade of changes similar to IPC established through an acute non-lethal excitatory stimulus. This limits the ability of these prior studies to specifically evaluate the direct impact of EAAT2 over-expression in neuroprotective pathways altered under IPC and neuroimmunophilin activation (Dirnagl, 2003; Stenzel-Poore, 2003; Yang, 2003; Furuichi, 2004). The use of rAAV1-GFAP-EAAT2 enables direct over-expression of astrocytic EAAT2 and assessment of the direct role this glutamate transporter plays in neuroprotection under oxygen-glucose deprivation.

Specific over-expression of EAAT2 in astrocytes is neuroprotective under oxygen glucose deprivation. Prior studies have shown upregulation of EAAT2 correlates with neuroprotection but were unable to conclusively state the direct impact of EAAT2 on neuroprotection due to the simultaneous activation of other neuroprotective pathways (Rothstein, 2005; Ganel, 2006; Labrande, 2006). The aim of this study was to isolate one component altered under IPC, namely the upregulation of EAAT2, and evaluate the role of this glutamate transporter in neuroprotection. Our results demonstrate the neuroprotective potential of astrocytic EAAT2 through direct and exclusive over-expression of this glutamate transporter in astrocytes.

Neuroprotection mediated by penicillin G treatment and transduction with rAAV1-GFAP-EAAT2 most likely occurs through different mechanisms. Presumably, the two approaches may invoke similar genomic and proteomic changes that occur as a result of increased EAAT2 expression and through the heightened removal of glutamate from the synaptic cleft under excitotoxic conditions. Pathways leading up to and causing increase on EAAT2 expression

in astrocytes may differ considerably between antibiotic treated and rAAV1-GFAP-EAAT2 transduced slices. Ischemic preconditioning, the proposed mechanism behind neuroprotection noted with β-lactam antibiotic treatment, is a response to an acute, sub-lethal excitotoxic event. Penicillin G is an antagonist at the GABA-A receptor (Fujimoto, 1995). Inhibition of this receptor is thought to invoke a dose dependent excitotoxic event through continued glutamate release. Penicillin G has been shown to cause a spike in glutamate release and is commonly used to induce seizure at higher doses (Shen and Lai, 2002). Moderate dosing of penicillin G may elicit the required acute, sub-lethal excitatory event needed to initiate IPC. This initial excitotoxic event could cause numerous changes to both genomic and proteomic expression (Dirnagl, 2003; Stenzel-Poore, 2003; Carmel, 2004; Romera, 2004). Upregulation of GABA-A receptor, hypoxia inducible factor-1 (HIF-1), glucose transporters, and tumor necrosis factor alpha (TNFα), and downregulation of glutamate receptors, transcriptional activators, and vesicular docking proteins are just a few of the proteins altered in IPC (Liu, 2000; Ruscher, 2002; Sommer, 2002; Dirnagl, 2003; Stenzel-Poore, 2003; Carmel, 2004; Romera, 2004; Dave, 2005; Lu, 2005). The direct over-expression of EAAT2 through the use of rAAV1-GFAP-EAAT2 bypasses activation of the additional pathways associated with IPC. The direct mechanisms mediating neuroprotection in rAAV1-GFAP-EAAT2 transduced tissue has yet to be defined. Full-scale proteomic and genomic analyses are required to definitively identify deviations and commonalities between IPC and rAAV1-GFAP-EAAT2 mediated neuroprotection.

Three mechanisms may collectively contribute to the observed neuroprotection offered through over-expression of astrocytic EAAT2 with rAAV1-GFAP-EAAT2. First, increased EAAT2 membrane expression limits excitotoxicity by removal of glutamate and termination of post-synaptic excitatory signaling

(Danbolt, 2001). Increasing the ability of astrocytes to remove glutamate from the synaptic cleft through increased EAAT2 expression may produce a refined or hypoactive glutamate signaling environment. Under low glutamate signaling conditions mediated by low glutamate synaptic concentrations or through use of glutamate receptor antagonists, glutamate receptors have been shown to shift in subunit composition and signaling potential (Molnar and Isaac, 2002). In extreme conditions, hypofunctional glutamate signaling has been observed to cause disruption in learning and memory and induce schizophrenia like behavior (Farber, 2003). While over-expression of EAAT2 may offer neuroprotection under excitotoxic events, the potential for hypofunctional glutamate signaling and altered glutamate receptor sensitivity has yet to be studied. Second, increased supply of glutamate to astrocytes may facilitate an increased production of nutrients and antioxidants to neurons (Benarroch, 2005). Astrocytes possess a cystine-glutamate exchanger that facilitates accumulation of cystine within the astrocyte. Inside the astrocyte cystine is converted to cysteine and used in the astrocytic production of glutathione precursor for use by neurons. The glutathione precursor, L-cysteinyl-glycine is converted to glutathione in the neuron by interaction with the γ-glutamyl transpeptidase coenzyme. Additionally, astrocytes are able to regenerate ascorbic acid to ascorbate for reuse by neurons (Kim, 2005). Ascorbic acid recycling is positively coupled to increased glutamate uptake, glucose utilization and glutathione production. Neurons are incapable of producing these potent antioxidants alone. Increased glutamate uptake capacity of astrocytes could enhance the supply of precursors and energy production required to synthesize these potent antioxidants essential for neuronal health. Lastly, a shift in energy production and utilization may alter the neuro-metabolic coupling between astrocytes and neurons. Glutamate uptake is coupled to a concomitant sodium influx. The Na+/K+ ATPases maintain

an intracellular sodium concentration (10-20 mM) (Kimelberg and Goderie, 1993). Increases in intracellular sodium concentrations results in the activation of energy consuming Na+/K+ ATPase and stimulates glucose uptake and glycolytic activity. Accordingly, activation of these pathways results in an increase in astrocytic lactate and pyruvate production and release. Low level increase in supply of lactate to neurons has been shown to upregulate pathways involved in lactate utilization and promotes enhanced ability to utilize lactate under successive anaerobic conditions (Dienel and Hertz, 2005). Together, increase in glutamate uptake initiates these cascades resulting in increased energy supply to neurons in the form of lactate and to a lesser extent pyruvate. This promotes a neuroprotective environment, accumulation of energy reserve and enhanced lactate utilization capacity to be used during subsequent oxygen glucose deprivation. These three proposed mechanisms of EAAT2 mediated neuroprotection together promote overall health of tissue through increased production of anti-oxidants, energy supplies, balanced extracellular environment and induce a general energy depletion tolerance.

Together, this study has identified a novel tool, rAAV1-GFAP-EAAT2, that enables cell type specific modulation of select glutamate transporter expression. While prior studies have shown capacity to increase EAAT2 expression, they have done so through indirect means or in a fashion that may cause deleterious influences on development. The novel rAAV construct used in this study offers the first direct approach to study the role of astrocytic EAAT2 expression in neuroprotection and neuropathology of disease. The mechanism in which direct over expression of EAAT2 mediates neuroprotection has yet to be fully defined and is the focus of ongoing studies.

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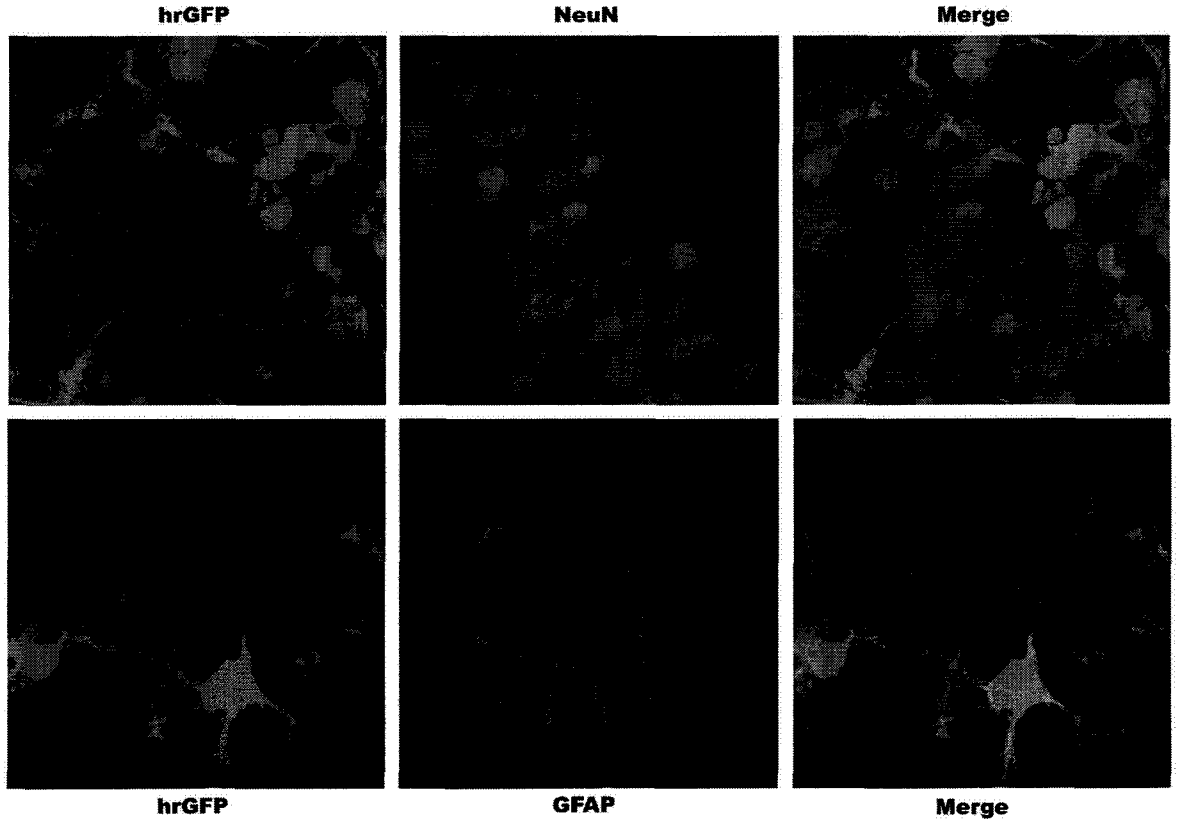

Figure 1. Rat hippocampal slice cultures (RHSC) transduced with rAAV1-GFAP-hrGFP and immunostained with anti-NeuN or anti-GFAP. NeuN is localized to neuronal nuclei and GFAP is localized to astrocytic processes. Merge panel shows co-localization of anti-GFAP staining and hrGFP. Original magnification 40x.

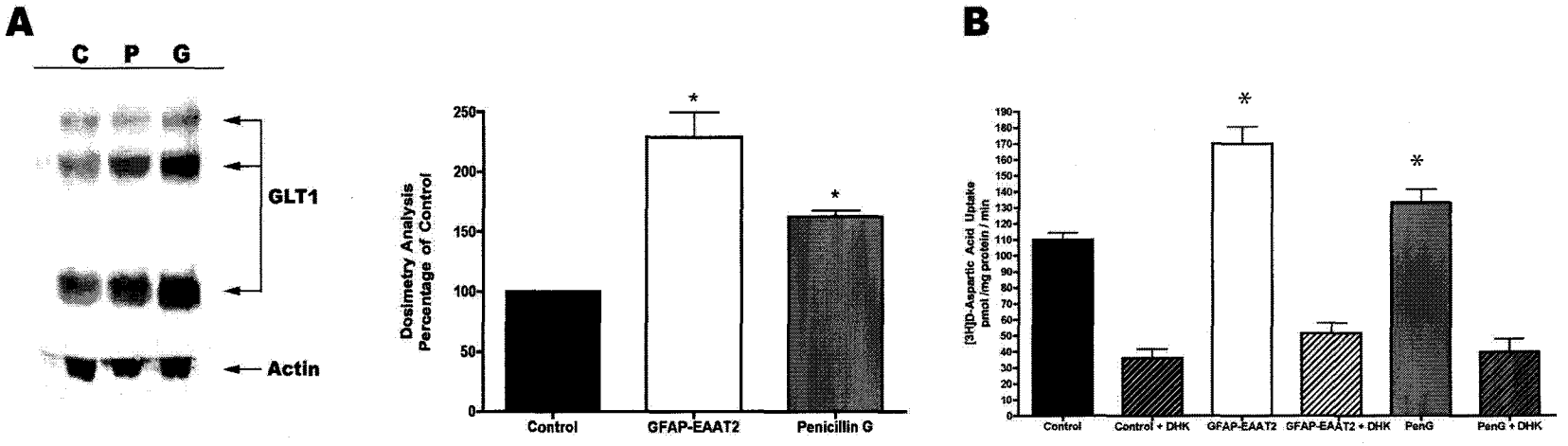

Figure 2. Increased expression of functional EAAT2. **A)** Western blot analysis of RHSC probed with anti-GLT-1. Increases in EAAT 2 expression of 129% and 63% over control were noted with rAAV1-GFAP-EAAT2 and penicillin G treatments, respectively. One-way ANOVA analysis, * = $p < 0.05$, n=3. **B)**Total EAAT2 mediated uptake of [^{3}H]-Aspartic acid in RHSC in the presence and absence of an EAAT2 specific uptake inhibitor (DHK). Increases in EAAT2 mediated uptake of 62% and 28% over control were noted with rAAV1-GFAP-EAAT2 and penicillin G treatments, respectively. Both treatment groups resulted in a statistically significant increased in EAAT2 mediated uptake when compared to control tissue. One-way ANOVA analysis, * = $p < 0.01$, n=6-9.

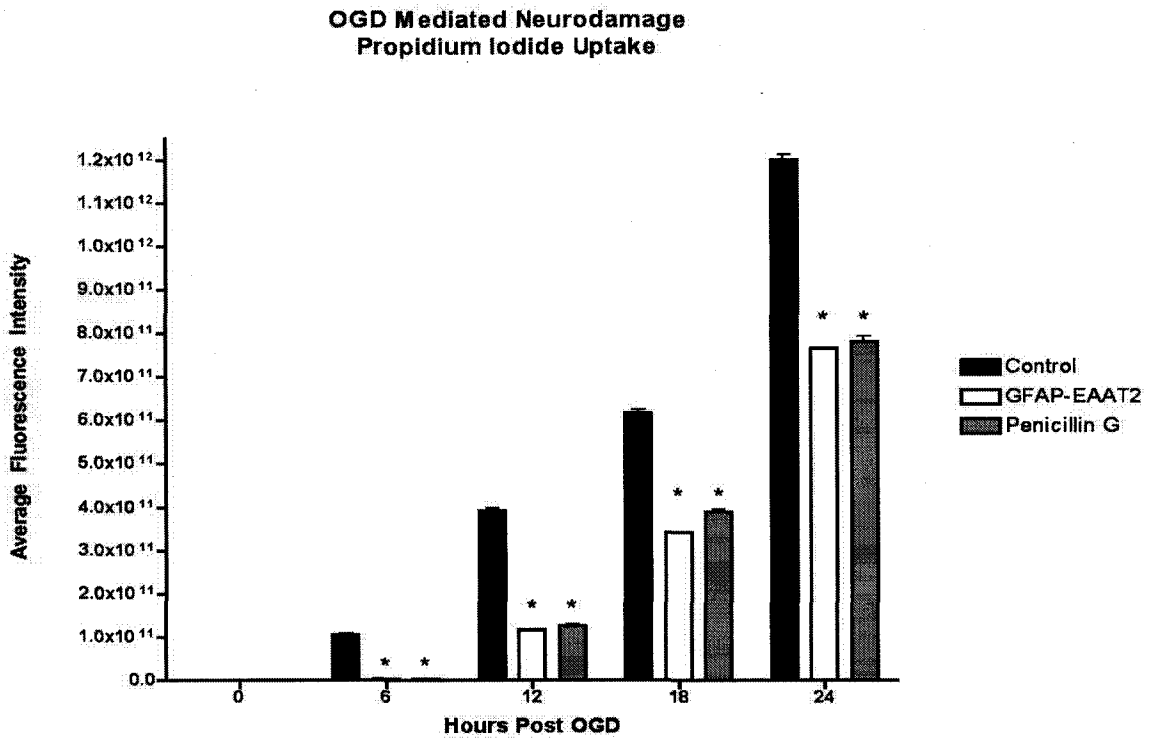

Figure 3. Neurodamage mediated by oxygen glucose deprivation (OGD) as measured by propidium iodide (PI) uptake in control, rAAV1-GFAP-EAAT2 transduced and penicillin G treated rat hippocampal slice cultures (RHSC). Total average fluorescence was measured at each time point. One-way ANOVA analysis, * = $p < 0.01$, Control (n=26), rAAV1-GFAP-EAAT2 (n=14), Penicillin G treated tissue (n=24).

Chapter Three

EAAC1 glutamate transporter activity on inhibitory presynaptic terminals modulates GABA synthesis and neuroprotection.

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Abstract

As the principle inhibitory amino acid, γ-animobutyric acid (GABA) functions to counter excitatory signaling in the CNS. Excitatory amino acid carrier 1 (EAAC1) is present throughout the CNS with a small percentage being uniquely expressed on inhibitory presynaptic terminals and provides a primary source of glutamate for GABA synthesis. Alterations to this or other pathways leading to GABA synthesis, may result in abnormal inhibitory synaptic transmission, neuronal hyperexcitability and epileptiform activity. Prior studies have shown GABA synthesis and quantal vesicular release were reduced and seizure-like behaviors were heightened under pharmacological inhibition or antisense knockdown of EAAC1. The approaches taken in these prior studies inhibited EAAC1 expression and function on a global scale and lacked the ability to regulate EAAC1 in a cell type specific manner. We hypothesized that increased GABAergic EAAC1 expression would enhance GABA synthesis and promote neuroprotection. Results presented here demonstrate that increasing EAAC1 expression causes an increase in GABA synthesis. Using recombinant adeno-associated virus vectors, we were able to specifically target transgene delivery to GABAergic neurons and direct over-expression and knockdown of EAAC1 in vivo. To evaluate the impact of altered GABA synthesis on seizure susceptibility, the pilocarpine induced seizure model was used to study the influence of altered EAAC1 expression on the rate of progression of seizure-like behavior as measure by a modified Racine scale and the development of neuropathology after tonic-clonic seizures. Modulation of GABAergic EAAC1 expression resulted in alterations in both seizure susceptibility and neuroprotection.

Introduction

The principle inhibitory neurotransmitter, γ-aminobutyric acid (GABA) functions to maintain a fine balance between excitatory and inhibitory signaling in the CNS. Disruption to this balance commonly results in a shift favoring excitotoxicity resulting in acute neuropathology and chronic neurodegradation as noted in Alzheimer's disease, Huntington's Disease, amyotryophic lateral sclerosis (ALS) and epilepsy (Enterzari-Taher, 1997; Marczynski, 1998; Kleppner and Tobin, 2001; Ben-Ari and Holmes, 2005; Cossart, 2005). The key to managing the balance between excitatory and inhibitory signaling lies in the ability to control GABA synthesis and response to excitotoxic events early in the disease process in affected regions.

GABAergic neurons comprise only 10%-20% of the total neuronal population in the hippocampus (Ben-Ari and Holmes, 2005). On GABAergic neurons, EAAC1 is uniquely localized to presynaptic terminals and provide a primary source of glutamate precursor for GABA synthesis (Rothstein, 1994; Sepkuty, 2002). Glutamate interacts with the glutamate decarboxylase (GAD) enzyme to form GABA (Martin and Rimvall, 1993) in inhibitory neurons. GAD65, one of two isoforms found in GABAergic neurons, is membrane associated and found in inhibitory presynaptic terminals co-localized with EAAC1 (Conti, 1998).

Prior studies have identified the potential impact of EAAC1 expression and function on GABA synthesis and susceptibility to excitotoxic stimulus. General antisense knockdown of EAAC1 results in a decrease in GABA synthesis, and an increase in seizure-like behaviors and susceptibility to glutamate mediated neurodamage (Sepkuty, 2002; Brustovetsky, 2004). Quantal vesicular release of GABA is reduced in the presence of non-specific glutamate transport inhibitors as observed through attenuation of mIPSC recordings (Mathews and Diamond

2003). In contrast, increased GABA synthesis and vesicular loading results from elevated synaptic glutamate levels and functional glutamate uptake. These results collectively suggest a protective role of EAAC1 that enables an increase in GABA synthesis in response to high synaptic glutamate levels. While these prior studies identified the potential role of altered EAAC1 expression and function in GABA synthesis, the approaches used to modulate EAAC1 lacked the ability to specifically isolate and study the small percentage of EAAC1 uniquely expressed on GABAergic neurons. A means to exclusively alter the expression and function of EAAC1 localized to inhibitory presynaptic terminals is needed to definitely study the impact of this transporter on GABA synthesis and inhibitory signaling.

The aim of this study was to evaluate the influence of cell type specific modulation of EAAC1 expression on GABA synthesis and the impact of altered inhibitory response on seizure susceptibility and neuroprotection. Through HPLC analysis, increased EAAT3 expression in a GABAergic neuronal cell line was shown to effectively alter the intracellular concentrations of glutamate and GABA. A recombinant adeno-associated virus (rAAV) was developed using the GAD65 promoter sequence to drive cell type specific EAAC1 expression in GABAergic neurons in vivo. This novel viral construct was used to selectively increase and knockdown EAAC1 expression in inhibitory presynaptic terminals and evaluate the impact of GABAergic EAAC1 expression on seizure susceptibility and neuropathology.

Methods

Plasmid Design and Virus Preparation

Rat cortical tissue was used to produce cDNA using the Cells-to cDNA II kit (Ambion, Austin, TX). Primers were designed for amplification of the rat GAD65 promoter sequence (Skak and Michelsen, 1999). Upstream primer contained a KpnI restriction site: 5'-GGTACCGGCGCTCCGCAG-3' and a downstream primer containing a Bcl1 restriction site: 5'-TGATCAGGGTTCTGCTAGTCTGG-3'. Amplified products were cloned into the TOPO Blunt PCR cloning plasmid (Invitrogen, Carlsbad, CA) and fragments confirmed by double stranded sequence analysis. The promoter sequence was subcloned into the KpnI and BamHI sites of the pAM-hrGFP-WPRE vector. Three additional GAD65 plasmids were designed containing an EAAC1 tagged with a flag epitope (DYKDDDK) on the 5' end (flag-EAAC1), an EAAC1 in the antisense orientation (EAAC1 AS), or an empty expression cassette (Null). Rat cDNA was used to PCR amplify the flag-EAAC1 sequence with upstream primer containing an XhoI restriction site and the flag epitope: 5'-CTCGAGATGGATTATAAAGATGACGATGACAAATGTATGG GGAAGCCCACGAG-3' and downstream primer containing a HindIII restriction site: 5'-AAGCTTAGGCATCTAAGGCCAGGC-3'. The constructs contained the 3' Woodchuck hepatitis virus post-transcriptional regulation element (WPRE). The WPRE construct has been shown to enhance trans-gene expression (Loeb, 1999; Paterna, 2000; Hlavaty, 2005). The GAD65 promoter and expression cassettes are flanked by AAV serotype 2 (AAV2) inverted terminal repeats. Prior studies have shown chimeric rAAV at select serotype ratios increases overall viral infectivity (Rabinowitz, 2004). For this study, we used a 3:1 ratio of serotype 1 to serotype 2.

The four pAM-GAD65 plasmids, containing the Null, EAAC1 AS and flag-EAAC1 expression cassettes, and one pAM-CAG-EAAT3 plasmid (Esslinger, 2005)

were used in the production of rAAV. HEK293T cells cultures were transfected with the pAM-GAD65 and pAM-CAG constructs using Polyfect Transfection Reagent (Qiagen, Valencia, CA) (Selkirk, 2005). For the production of the rAAV1-CAG-EAAT3-WPRE virus, the three plasmids used in the transfection were 1) pFΔ6 (adenoviral helper plasmid), 2) pH21 (cap gene for AAV serotype 1 and rep gene for serotype 2) and 3) the AAV plasmid containing the CAG promoter upstream from the EAAT3-WPRE sequence. For the production of the chimeric AAV1/2-GAD65 viruses, the four plasmids were used in the transfection were 1) pFΔ6 (adenoviral helper plasmid), 2) pH21 (cap gene for AAV serotype 1 and rep gene for serotype 2), 3) pRVI (cap and rep genes for AAV serotype 2), and 4) the AAV plasmid containing the GAD65 promoter upstream from the flag-EAAC1, EAAC1 AS, Null or hrGFP gene sequence, flanked by AAV2 inverted terminal repeats.

Virus was isolated from HEK293T cells through repeated freeze-thaw cycles, incubation for 30 minutes at 37°C with 50U benzonase (Novagen, Madison, WI) and 0.5% sodium deoxycholate, briefly sonicated and further purified by iodixonol density gradient centrifugation as previously reported (Zolotukhin, 1999). The titer (genomic particles/ml (gp/ml)) of the final virus isolate was determined by quantitative real time-polymerase chain reaction (RT-PCR) using an ABI Prism 7700 with primer and probe sets specific for the cis-acting enhancer WPRE sequence.

HC2S2 Cell Line

HC2S2 cells are an immortalized rat GABAergic cell line (kindly provided by Dr. Fred H. Gage, Salk Institute, La Jolla, CA) and were cultured as previously reported (Hoshimaru, 1996; Asahi, 1998). Briefly, cells were maintained in growth media (DMEM/F-12 (50:50) media containing glutamine, N2 supplement (Invitrogen, Carlsbad, CA), bFGF at 20ng/ml, and amphotercin B in flasks coated

with poly-l-ornithine and mouse laminin (Invitrogen, Carlsbad, CA). To initiate differentiation, cells were plated on coated 12 well plates at 1.0 x 10^4 cells/well in differentiation media (DMEM/F-12 (50:50) media containing glutamine, N2 supplement (Invitrogen, Carlsbad, CA), bFGF at 2ng/ml, amphotercin B and 20 μg/ml tetracyline). Tetracycline was added daily to cell culture media at 20μg/ml. After 3 days, cells stopped dividing and began to send out neuronal processes. HC2S2 cells were transduced with rAAV1-CAG-EAAT3 on day 3 in culture with 5.0 x 10^5 gp/cell. Cells were maintained in culture media which was changed every other day for 10 days.

Functional Uptake Analysis

[^{3}H]D-Aspartic-acid (PerkinElmer, Boston, MA) uptake was used to assess functional EAAT3 mediated activity. Seven days post transduction with rAAV1-CAG-EAAT3, differentiation media was removed and HC2S2 cells were gently rinsed with pre-warmed sodium-containing HEPES buffer (20mM HEPES, 120 mM NaCl, 5mM KCl, 1mM KH_2PO_4, 2mM $CaCl_2$, 10mM D-glucose, pH 7.4) or a sodium-free HEPES Buffer (20mM HEPES, 120 mM choline, 5mM KCl, 1mM KH_2PO_4, 2mM $CaCl_2$, 10mM D-glucose, pH 7.4). Media was replaced with HEPES +/- sodium containing 100nM [^{3}H]D-aspartic acid and incubated for 4 minutes at 37°C. Uptake was terminated by the removal of [^{3}H]D-aspartic acid containing media, rinsed twice in ice cold sodium-free HEPES and cells were lysed in lysis solution (0.1N NAOH, 0.01% SDS). Aliquots of the lysate were collected, incubated in liquid scintillation cocktail and counted for measure of [^{3}H]D-aspartic acid on a Beckman LS 6500 Scintillation System. Specific EAAT3 mediated uptake of [^{3}H]-D-aspartic acid was calculated as total sodium-dependent uptake less the uptake in the absence of sodium and reported as pmol/mg protein/min. Protein concentration was determined on sister wells using the Bio-Rad DC protein assay

(Hercules, CA).

Western Blot Analysis

Western blot analysis of the HC2S2 cell cultures were performed to evaluate EAAT3 expression levels in control cells and cells transduced with rAAV1-CAG-EAAT3. Cells were removed from 12 wells plates and homogenized in lysis solution (2.5% sodium deoxycholate, 0.1% protease inhibitor cocktail set III (Calbiochem, San Diego, CA), and 0.05% benzonase (EMD Biosciences, San Diego, CA) in PBS). Protein concentrations of lysate samples were determined using the Bio-Rad DC protein assay (Hercules, CA). Aliquots of homogenized HC2S2 cells (30μg) were loaded onto a NuPAGE 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA). Proteins were transferred to Immuno-Blot PVDF membrane (Bio-Rad, Hercules, CA) and blocked in Tris buffer containing Tween-20 and 0.5% non-fat milk. Membranes were probed with antibodies to EAAT3 (1:1000, ADI, San Antonio, TX) and actin (1:1000, Sigma, St. Louis, MO). Proteins were visualized using ECL (Pierce, Rockford, IL) with species-specific HRP conjugated secondary antibodies. Blot was imaged on a Kodak Image Station 440 CF. Dosimetry analysis of all three bands, representing different multimers of EAAT3, were used to calculate fold change in EAAT3 protein expression corrected to actin control.

High-Performance Liquid Chromotography (HPLC) analysis

HPLC analysis was performed to evaluate the total intracellular concentrations of specific amino acids in control or rAAV1-CAG-EAAT3 transduced HC2S2 cells. Cells were lysed in 0.1 N perchloric acid solution, centrifuged at 14,000g for 10 minutes at 4°C and supernatant was retained. Protein concentrations were determined using the Bio-Rad DC microplate protein assay (Hercules, CA). Samples (10μL) were derivatized with an *o*-phtaldialdehyde (OPA) solution (90μL)

and 40μl of the derivatized sample was loaded for HPLC analysis.

Detection of amino acid content was performed by HPLC using an Amersham Bioscience AKTA purifier system (GE Healthcare, Piscataway, NJ)., equipped with a Spherisord 5μM ODS1 reverse phase column (4.6x250mm, Waters Corporation, Tokyo Japan). Amino acid levels were quantified by fluorescence detection at 440 nm (emission) and 330nm (excitation). Mobile phase A was composed of 0.03M sodium acetate and 1% tetrahydrofuran. Mobile phase B was compose of 0.02M sodium acetate in 80% acetonitrile. Amino acids were separated using a linear increase in concentration of mobile phase B from 0% to 40% over 40 minutes at a flow rate of 0.8 ml/min. Retention time for GABA, glutamate and glutamine (Sigma, St. Louis, MO) were 29, 8, 23 minutes respectively as confirmed by amino acid standards. Specific concentrations were determined through analysis of amino acid standards over an applicable range of concentrations.

Animal Stereotaxic Injections

Eight-week old male Sprague-Dawley rats (200-250g) were anesthetized by brief isoflurane inhalation (Abbott Laboratories, North Chicago, IL) and intramuscular injection of ketamine (100mg/kg of body weight, Vetus Animal Health, Westbury, NY) then placed into a stereotaxic frame (Stoelting, Wood Dale, IL) and were maintained on 1.0 LPM of oxygen and 2% isoflurane. Injections were performed from the following coordinates beginning at midline of the bregma: -3.3mm dorsal from midline of the bregma, +/- 1.8mm lateral and -3.0mm depth from surface of the brain. The four rAAV1/2-GAD65p vectors (hrGFP, Null, EAAC1 AS, and flag-EAAC1, 1 x 10^{13} gp/ml) were diluted 1:1 with 25% mannitol (American Regent Inc., Shirley, NY) and 8 μl was delivered to each hippocampus at a rate of 0.5 μl/min.

Seizure Study

Four weeks post rAAV bilateral stereotaxic injections, animals were evaluated for seizure susceptibility. The pilocarpine solution (100mg/ml, BioChemika, Buchs, Switzerland) and atropine (100mg/ml, Sigma, St. Louis, MO) solution were prepared in a 0.9% saline solution, pH adjusted to 7.4 and filter sterilized. Thirty minutes prior to pilocarpine treatment, each animal received 100mg/kg atropine intraparitoneal (IP) to limit peripheral effects of pilocarpine. Pilocarpine solution was delivered I.P. at a concentration of 300mg/kg body weight. Animals were monitored and seizure-like behavior determined based on a modified Racine scale (Racine, 1972b; Sperk, 1994). The behaviors were broken down into the following stages: 1) chewing, salivation, walking backwards, 2) head bobbing, tremors, wet-dog shakes, 3) forearm clonus, rearing/falling, 4) 1 class 5 seizure 5) 3+ class 5 seizures and 6) general tonic-clonic behavior, status epilepticus. Time to reach each stage was recorded for each animal and times were averaged between animals within the same treatment group. Sixty minutes post-onset of SE animals received 10mg/kg body weight of Diazepam (Abbott Laboratories, North Chicago, IL).

Immunohistochemistry

Brain tissues were harvested from non-seizure animals at 4 weeks post surgery and from animals that progressed to SE in the seizure study 48 hours after onset of SE. Animals were anesthetized with isoflurane and perfused with 4% paraformaldehyde, decapitated and brain tissue removed. Tissue was incubated post harvest for 24 hours in 4% PFA, placed in 30% sucrose solution and stored at 4°C until cryosectioning. Tissue was sectioned on a cryostat (Thermo Shandon, Pittsburg, Pa) into 40μm slices, placed on charged slides, briefly dehydrated in 100% ethanol and stored at 4°C until use.

Cell type specific expression was evaluated for rAAV1/2-GAD65p-hrGFP and rAAV1/2-GAD65p-flag-EAAC1 in cryosectioned brain slices of non-seizure animals. Slices were incubated in blocking buffer (1.5% normal goat serum, 0.3% Triton-X 100) for 1 hour at room temperature (RT). After initial blocking, slices were stained with antibodies to GAD65 (1:300) or flag epitope (DYKDDDK) (1: 1000) (Chemicon, Temecula, CA) for 1 hour at RT, rinsed in blocking buffer and incubated in species-specific conjugated secondary antibody (1:200, Molecular Probes, Eugene, OR). Slices were coverslipped with Fluorosave (Calbiochem, San Diego, CA). Images were obtained on a Bio-Rad Radiance 2000 MP laser scanning confocal microscope (Hercules, CA).

A marker for neurodegradation, Fluoro-Jade B (Histo-Chem, Inc., Jefferson, AR), was used to evaluate the level of seizure mediated damage. Tissue was processed as previously published (Wang, 2000). Briefly, cryosectioned tissue was incubated in 1% sodium hydroxide and 80% ethanol solution for 5 minutes, rinsed in 70% ethanol for 2 minutes, and rinsed in distilled water for an additional 2 minutes. Tissue was then incubated with gentle agitation in 0.06% potassium permanganate for 10 minutes, rinsed with distilled water and incubated in 0.0004% FluoroJade B working solution for 20 minutes. Tissue was rinsed with distilled water and allowed to dry in a 37°C incubator over-night. Slides were immersed in xylene for 1 minute and coverslipped with DPX. Tissue was imaged using an Olympus IMT-2 inverted fluorescent microscope attached to an Olympus digital camera (Olympus, Melville, NY) and captured using Magnafire SP imaging software package (Optronics, Goleta, CA). Total fluorescent intensity per region of interest was determined using Image Pro Plus software (MediaCybernetics, Silver Springs, MD).

Data Analysis

One-way ANOVA was performed to determine statistical significance of western blot dosimetry analysis, functional [^{3}H]D-aspartic acid uptake and level of Fluoro-Jade B staining between the control tissue and transduced tissue. Statistical analysis was performed using Prism Software (GraphPad Software, Inc., San Diego, CA).

Results

EAAT3 expression regulates intracellular GABA, glutamate and glutamine levels

Prior studies have demonstrated the influence of global EAAC1 knockdown or pharmacological inhibition on GABA synthesis and signaling (Sepkuty, 2002; Mathews and Diamond, 2003; Brustovetsky, 2004). No prior studies have effectively analyzed the potential for increased GABA synthesis associated with increase in cell type specific EAAC1 expression. Therefore, to investigate the impact of elevated EAAC1 (EAAT3) expression on GABA synthesis, we used rAAV1-CAG-EAAT3 to increase functional EAAT3 expression in a GABAergic cell line and measured neurotransmitter levels through HPLC analysis. Western blot analysis and functional uptake assays were performed to assess the level of increased EAAT3 expression and functionality of the recombinant transporter. Western blot analysis indicated a 6-fold increase in EAAT3 expression (Figure 1A) in HC2S2 cells transduced with rAAV1-CAG-EAAT3 over that of control cultures. Functional [3H]D-aspartic acid uptake increased 4-fold in HC2S2 cells transduced with rAAAV1-CAG-EAAT3 over that of control, non-transduced HC2S2 cells (Figure 1B). Discrepancy between the total EAAT3 expression level and increase in uptake may be attributed to partial sub-cellular localization characteristic of EAAT3 (Krizman-Genda, 2005). Together, these initial studies validated functionality of

rAAV1-CAG mediated EAAT3 over-expression.

To further test the hypothesis that EAAT3 expression regulates GABA synthesis, intracellular amino acid content of HC2S2 cells transduced with rAAV1-CAG-EAAT3 were measured by HPLC analysis. GABA levels, as determined by HPLC, increased 136% over that measured in non-transduced HC2S2 cells (Figure 2A). Additionally, elevated EAAC1 levels resulted in a 56% increase in glutamate (Figure 2B) and 146% increase in glutamine (Figure 2C) intracellular levels when compared to non-transduced HC2S2 cells. Increased intracellular glutamate levels would be anticipated with increased functional EAAT3 expression and enhanced capacity for uptake of glutamate from the extracellular space (Figure 1A and 1B). To support further studies, a virus needed to be developed to enable selective modulation of EAAC1 expression in inhibitory presynaptic terminals.

In vivo cell type specific transgene expression

In addition to prior studies (Sepkuty, 2002; Mathews and Diamond, 2003), our in vitro results strongly suggested that in vivo modulation of EAAC1 expression in GABAergic neurons could alter GABA synthesis. To test this hypothesis, we generated an rAAV vector that carried an expression clone in which the GAD65 promoter was used to alter EAAC1 expression exclusively in inhibitory presynaptic terminals. Initial studies were performed with rAAV1/2-GAD65-hrGFP to determine cell type specificity of the viral construct. Adult Sprague-Dawley rats were sterotaxically injected with rAAV1/2-GAD65-hrGFP bilaterally into each hippocampus. Four weeks post injection, tissue was harvested and cryosections were stained with an anti-GAD65 antibody to confirm cell type specificity of transgene expression (Figure 3A). GAD65 is localized to GABAergic terminals and staining for this protein renders a punctate staining pattern. In the dentate gyrus of the hippocampus, GABAergic terminals are concentrated on pyramidal neurons.

The dentate gyrus was chosen for imaging due to the high level of GABAergic neurons as compared to other regions in the hippocampus. The hrGFP expression was co-localized with GAD65 in GABAergic neuron terminals (Figure 3A).

Utilizing the GAD65 promoter sequence, we designed rAAV vectors that facilitated over-expression and knockdown of EAAC1 in inhibitory neurons. EAAC1 is uniquely expressed on presynaptic terminals and co-localizes with GAD65 (Conti, 1998) (Figure 3B). Adult Sprague-Dawley rats received either the null, EAAC1 AS or flag-EAAC1 virus through stereotaxic injections in each hippocampus. Four weeks post injection, tissue was harvested and cryosectioned. Tissue transduced with rAAV1/2-GAD65-Flag-EAAC1 was dual stained with antibodies against GAD65 and the flag epitope. Figure 3C indicates that the flag-EAAC1 protein co-localized with GAD65. Images taken of tissue transduced with rAAV1/2-GAD65-EAAC1 AS did not reveal detectable differences in EAAC1 expression (data not shown). Due to the limited sensitivity of standard immunohistochemical techniques, knockdown of this protein could not be efficiently detected. We would predict that the antisense virus would knockdown, but not completely knockout, EAAC1 expression within inhibitory neurons. In addition, EAAC1 present in postsynaptic terminals of glutamatergic pyramidal neurons would also be detected in immunohistochemical staining. These immunohistochemical studies further support cell type specific expression patterns mediated by rAAV1/2-GAD65 viral constructs and indicates that recombinant EAAC1 proteins are trafficked to the proper subcellular location.

Modulation of EAAC1 expression on inhibitory presynaptic terminals influences seizure sensitivity and neuroprotection.

We hypothesized that over-expression of EAAC1 in inhibitory presynaptic terminals would result in increased GABA production, leading to reduced sensitivity to pilocarpine induced seizures and neuropathology. In contrast, we hypothesized

that knockdown of GABAergic EAAC1 expression would lead to reduced GABA production, increased sensitivity to pilocarpine-induced seizures and increased neuropathology. To test these hypotheses, we used the pilocarpine induced seizure model to further assess the impact of GABAergic EAAC1 expression on seizure susceptibility. Age-matched adult male Sprague-Dawley rats, transduced with rAAV1/2-GAD65-Null (Null), rAAV1/2-GAD65-EAAC1 AS (EAAC1 AS), or rAAV1/2-GAD65-flag-EAAC1 (Flag-EAAC1) and non-transduced controls, received IP injections of pilocarpine (300mg/kg) and were monitored for progression through distinct behavioral stages leading to status epilepticus (SE) (Racine, 1972a; Racine, 1972b; Sperk, 1994).

Overall, 40% of control, 23% of Null, 100% of EAAC1 AS and 50% of Flag-EAAC1 treatment groups progressed to SE. The number of animals reaching SE were similar between the control, Null and flag-EAAC1 treated groups, whereas 100% of animals in the EAAC1 AS treatment group progressed to SE. The control and the null treatment group progressed to SE at similar rates and reached SE at 16.97 +/- 0.91 and 23.68 +/- 0.88 minutes, respectively (Figure 4). In contrast, the rate at which EAAC1 AS treated animals progressed to SE was reduced by almost one half (11.28 +/- 0.65 minutes). Significant differences in the rate of progression were noted at stage 4 and stage 6. Commonly, control and null treated animals experienced 3+ seizures prior to entering SE. On average, the EAAC1 AS treatment group progressed to stage 4 (1 class 5 seizure) and directly progressed to tonic-clonic seizure. In contrast, the flag-EAAC1 treatment group advanced through the stages leading to SE at a significantly slower rate (42.02 +/- 2.42 minutes) compared to all other treatment groups. Requiring almost twice as long to reach SE compared to both the control and null treatment groups and almost four times slower than EAAC1 AS treated animals. Statistical significant differences were noted between the Flag-EAAC1 treatment group and control at

stages 4, 5 and 6.

A similar pattern of altered neuropathology was observed in the brains of animals that had progressed to status epilepticus. A stain specific for neurodamage, Fluoro-Jade B, was used to quantify degree of seizure mediated neuronal cell damage. Brain tissue was harvested 48 hours after the onset of SE from representative animals from each of the treatment groups. The relative level of Fluoro-Jade B staining within the hippocampus and cortex was determined from each group of animals. Observations were made from similar regions of the hippocampus and cortex. Seizure-mediated neuropathology was similar between controls and animals transduced with the Null virus (Figure 5A, 5B). However, tissue transduced with rAAV1/2-GAD65-flag-EAAC1 revealed significantly less neurodamage compared to control tissue. In contrast, knockdown of EAAC1 with rAAV1/2-GAD65-EAAC1 AS increased overall measured neuronal damage compared to control tissue. The differences in neuroprotection were most striking within the CA1 region of the hippocampus. It is worth noting that all four groups showed similar levels of Fluoro-Jade B staining within the cortex (Figure 6).

Discussion

Prior studies have attempted to study the role of EAAC1 expression and function on GABA signaling. Sepkuty et. al. used an oligonucleotide antisense method to knockdown EAAC1 expression globally. A severe 50% decline in GABA synthesis was observed in antisense treated animals. This associated loss of EAAC1 expression and diminished GABA synthesis resulted in increased epileptiform activity evident from EEG recordings. This study went on to report visual observation of seizure-like behaviors (staring episodes, forearm clonus) that correlated with observed EEG changes. Electrophysiology of antisense treated tissue showed an increase in hyperexcitability characterized by spontaneous burst in spike activity. From these results, Sepkuty et. al. suggested the potential impact of restricted EAAC1 expression in epilepsy. Additional studies by Mathews et. al. used a pharmacological approach to further elucidate the parameters of EAAC1 in GABA synthesis. Miniature inhibitory postsynaptic currents (mIPSC) were diminished in the presence of general glutamate uptake inhibitors but not in the presence of GLT-1 specific blockers. Similarly, quantal load of vesicular GABA release was reduced in the presence of these inhibitors. In contrast, in the presence of elevated synaptic glutamate concentrations the strength of GABA inhibition form a single vesicular release was increased. These studies suggested cytosolic GABA content may influence extent of vesicular loading and quantal release of GABA. While these prior studies provided insight into EAAC1 regulation of GABA synthesis, the inability to selectively target EAAC1 localized to GABAergic neurons was a primary drawback. From these prior studies, the potential for increased GABA synthesis and subsequent increase in tonic inhibition through over-expression of EAAC1 on inhibitory presynaptic terminals was suggested.

Prior attempts to alter EAAC1 expression have been limited to global inhibition of function and expression (Sepkuty, 2002; Mathews and Diamond,

2003). Pharmacological inhibition lack the ability to isolate and inhibit EAAC1 mediated glutamate uptake from other glutamate transporter variants much less a select subpopulation of EAAC1 localized to GABAergic neurons. Similarly, studies utilizing antisense oligonucleotide knockdown of EAAC1 have lacked cell type specificity of the ability to selectively increase expression of GABAergic EAAC1 (Sepkuty, 2002; Brustovetsky, 2004). These previous studies were essential in identifying the potential influence of EAAC1 expressed on inhibitory presynaptic terminals in GABA synthesis and the impact of EAAC1 activity on seizure susceptibility, but were limited in their ability to make conclusive statements about GABAergic EAAC1 specifically. To effectively study the role of EAAC1 localized to presynaptic inhibitory neurons, a methodology enabling cell type specific targeting and direct modulation of EAAC1 expression was required.

The novel design of the rAAV1/2-GAD65 viral constructs offers cell type specific targeting of transgene expression to GABAergic neurons The use of this viral construct to direct the over-expression and knockdown of EAAC1 located on inhibitory presynaptic terminals. Difficulty was noted in observing or measuring extent of EAAC1 AS mediated knockdown due to high level of EAAC1 background expression on non-GABAergic neurons and the sensitivity of available immunohistochemical techniques. The best means of evaluating the role of restricted EAAC1 expression was through an in vivo approach. These viral constructs offer the ability to selectively target and modulate protein expression in this small subset of hippocampal neurons. To our knowledge, these results illustrate the first successful attempt to regulate EAAC1 expression specific to GABAergic neurons.

We hypothesized that over-expression of EAAT3 (EAAC1) may increase the supply of glutamate precursor and result in increased GABA synthesis. To test this hypothesis, we evaluated the role of this glutamate transporter in GABA

synthesis in a GABAergic HC2S2 neuronal cell line. Prior studies have noted the limited cell-surface expression of EAAC1 (Fournier, 2004). Therefore, we wanted to determine whether increased expression of EAAC1 would result in increased cell surface expression. The increase in EAAC1 expression resulted in increased [^{3}H]D-aspartic acid uptake. This data strongly suggest that functional recombinant EAAT3 (EAAC1) is localized in the plasma membrane and is functional. HPLC analysis of intracellular amino acid content in HC2S2 cells transduced with rAAV1-CAG-EAAT2 revealed significant increased in GABA, glutamate and glutamine compared to control cultures. The shift in glutamate levels can be directly attributed to increased EAAT3 (EAAC1) mediated glutamate uptake capacity as evident from increased uptake of [^{3}H]D-aspartic acid. Increased glutamine levels may be caused by inhibition of mitochondrial uptake in the presence of elevated glutamate levels. Glutamate has been shown to competitively inhibit mitochondrial uptake of glutamine and offers an alternative mechanism for negative feedback in the production of glutamate in neurons (Roberg, 1999). Together, these data suggest that increased EAAC1 membrane surface expression on GABAergic neurons increases intracellular levels of glutamate promote enhanced GABA synthesis. This increase in intracellular GABA may increase vesicular loading and quantal release of GABA as suggested by Mathews and Diamond (2003). Increased quantal release of GABA may enhance the strength of the inhibitory response to pilocarpine-induced excitotoxicity.

Neuronal connectivity between the entrohinal cortex and the hippocampus provides the initiation point of excitatory signaling in the disinhibition leading to epileptiform activity (Ure and Perassolo, 2000). Axons from the cortex lead into the outer layer of the dentate gyrus (DG) which contains the highest concentration of GABAergic neurons within the hippocampus (da Silva, 2005). Excitatory signal passes through the layers of the DG and flow into the pyramidal neurons of the CA3.

The CA3 neurons feed the excitatory signal to the CA1 pyramidal neurons. Exiting of the excitatory signal from the CA1 to the entrohinal cortex completes this cyclic path. Disinhibition of this circuit lends to formation and propagation of epileptic discharges. Altered response to pilocarpine administration would be observed by shifting the ability of GABAergic inhibition to stop or hinder this cyclic epileptic discharge. We observed that expression of EAAC1 changes the threshold of seizure susceptibility and rate of progression to SE. Animals transduced with flag-EAAC1 progressed to SE at a significantly slower rate and exhibited significantly less neuropathology. This may be attributed to increased capacity to withstand pilocarpine mediated excitatory stimulus due to elevated GABA supply and increased inhibitory postsynaptic strength. Increased GABA synthesis may enable extended inhibitory signaling during cyclic epileptic discharge. In contrast, animals transduced with EAAC1 AS progressed to SE at an accelerated rate. Inability of the GABAergic neurons within the DG to counter incoming excitatory signalling following pilocarpine administration would increase excitatory signaling through the CA3 and CA1 regions of the hippocampus. These data suggest the ability to modulate susceptibility to seizure through the regulation of EAAC1 expression in inhibitory presynaptic terminals.

An alternative pathway which has been shown to amplify GABA release under excitotoxic conditions has been identified. Reversal of neuronal GABA transporters (GAT-1) under excitotoxic stimulation has been observed (Haugstad, 1997; Allen, 2004). Increased intracellular levels of GABA may additionally exit inhibitory terminals through this mechanism and further potentiate inhibitory signaling. Animals that received EAAC1 AS progressed to status epilepticus at a significantly faster rate. The inability of these animals to withstand the cyclic epileptic discharge resulting in seizure may be due to diminished capacity for GABA synthesis that may result in restricted vesicular and GAT-1 mediated GABA

release. Likewise, upregulation of EAAC1 expression would enhance these same factors mediating level of GABA release. Together, these results strongly support the essential role of EAAC1 in GABA synthesis and altered expression of this glutamate transporter in seizure susceptibility.

Patterns of neurodamage noted in each of the treatment groups follow the excitatory signaling pathways in the hippocampus (Ure and Perassolo, 2000). Overall neurodamage within the hippocampus, as identified through Fluoro-Jade B staining, was significantly decreased in the flag-EAAC1 transduced tissue. Increased capacity for GABA synthesis through the upregulation of EAAC1 expression may contribute to neuroprotection under pilocarpine induced seizures. In contrast, measure of total neurodamage in the hippocampus of tissue transduced with EAAC1 AS revealed a significant increase in seizure-mediated neurodegradation. Following the signaling pathways in the hippocampus, altered inhibitory capacity of the DG and CA3 would shift the excitatory signaling entering the hippocampus to inflict the highest degree of damage on the CA1 region. The CA1 region has been shown to have the lowest number of GABAergic neurons and GAD65 positive neuron processes and appears to be the most susceptible to seizure mediated damage in the EAAC1 AS treated animals. Together, our results show the over-expression of Flag-EAAC1 offers neuroprotection under pilocarpine-induced seizures. Likewise, the knockdown of EAAC1 results in heightened level of neurodamage under pilocarpine-mediated seizure activity. These results suggest the significant impact of altered EAAC1 expression and function in epileptogenesis and resulting seizure mediated damage.

The aim of this study was to evaluate the impact of GABAergic EAAC1 expression on GABA synthesis and seizure susceptibility. We showed elevated levels of EAAC1 expression results in increased GABA synthesis and altered intracellular concentrations of glutamate and glutamine. We developed a tool

enabling cell type specific targeting and manipulation of GABAergic neuron protein expression through the over-expression and knockdown of EAAC1 in vivo. This tool presents the only currently available mechanism for targeting this small subset of hippocampal neuron populations. The over-expression of EAAC1 offers significant neuroprotection under pilocarpine-mediated seizure activity. This neuroprotection is thought to be the result of increased GABA synthesis capacity and may result in altered vesicular loading and release of GABA. The knockdown of EAAC1 resulted in a significant increase in seizure-mediated neurodamage and is thought to be a result of restricted GABA synthesis conditions. Together, this study presents the first data suggesting the direct correlation between EAAC1 expression in inhibitory presynaptic terminals, GABA synthesis capacity and altered response to pilocarpine-induced seizure susceptibility.

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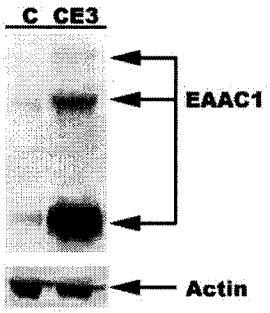

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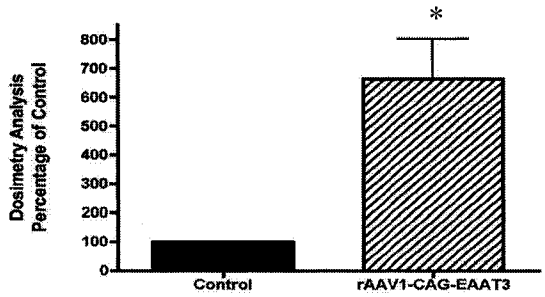

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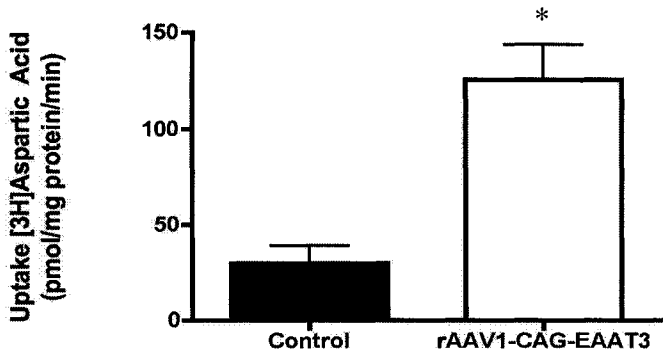

Figure 1. Increased functional expression of EAAT3 in HC2S2 cells transduced with rAAV1-CAG-EAAT3. **A)** Western blot analysis of non-transduced control HC2S2 cells (C) and cells transduced with rAAV1-CAG-EAAT3 (CE3) probed with antibody to EAAC1. **B)** Dosimetry analysis of western blot of EAAT3 in HC2S2 cells transduced with rAAV1-CAG-EAAT3 compared to control and corrected actin. **C)** Functional EAAT3 mediated uptake of [^{3}H]D-aspartic acid in non-transduced HC2S2 cells or cells transduced with rAAV1-CAG-EAAT3. Specific sodium-dependent glutamate transporter uptake of [^{3}H]D-aspartic acid was determined as the difference in uptake in the presence and absence of sodium. * = $p < 0.01$, n=3.

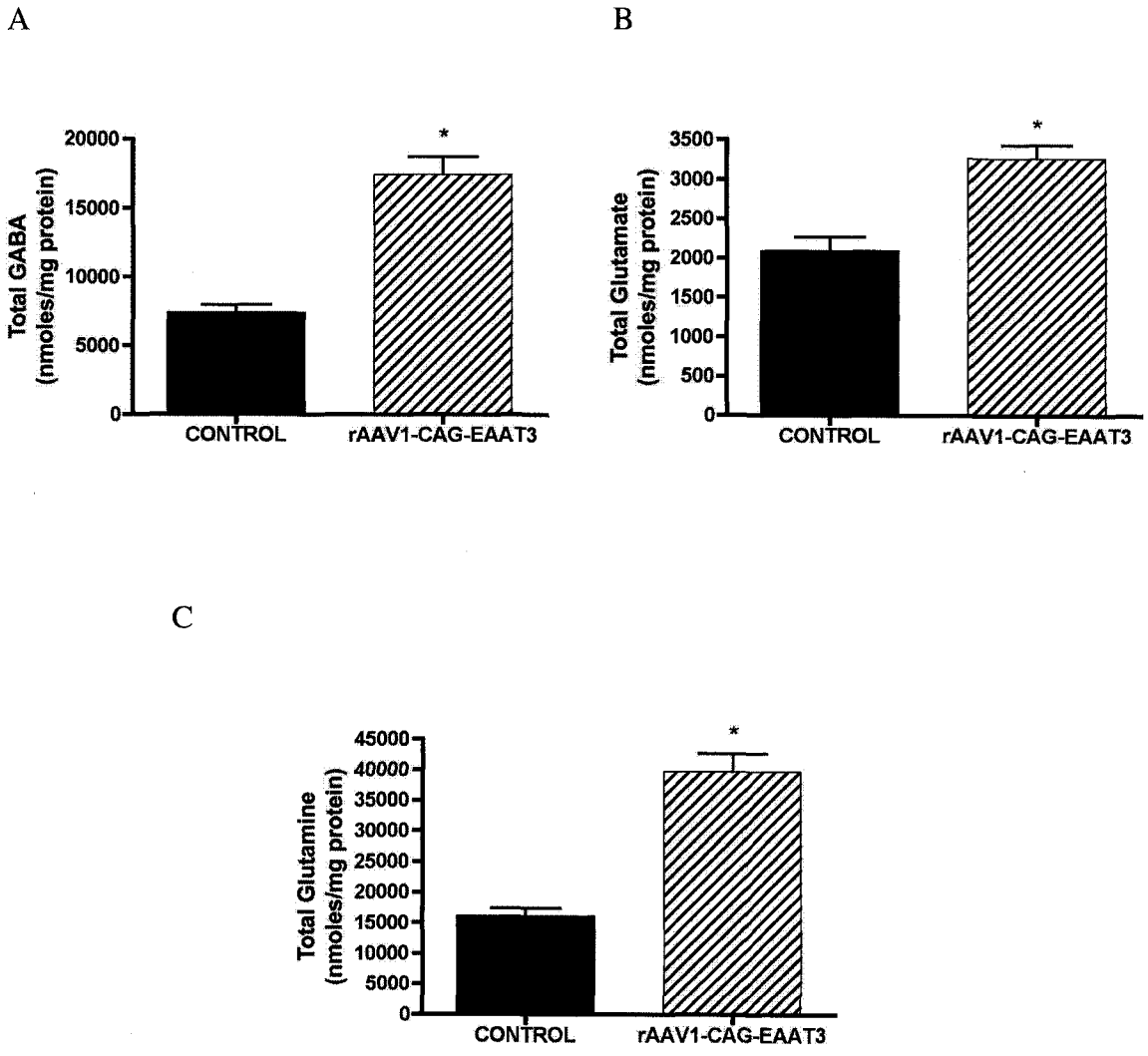

Figure 2. HPLC analysis of control and rAAV1-CAG-EAAT3 transduced HC2S2 cultures revealed a significant alteration in A) GABA, B) glutamate and C) glutamine. * = $p < 0.01$, n=6

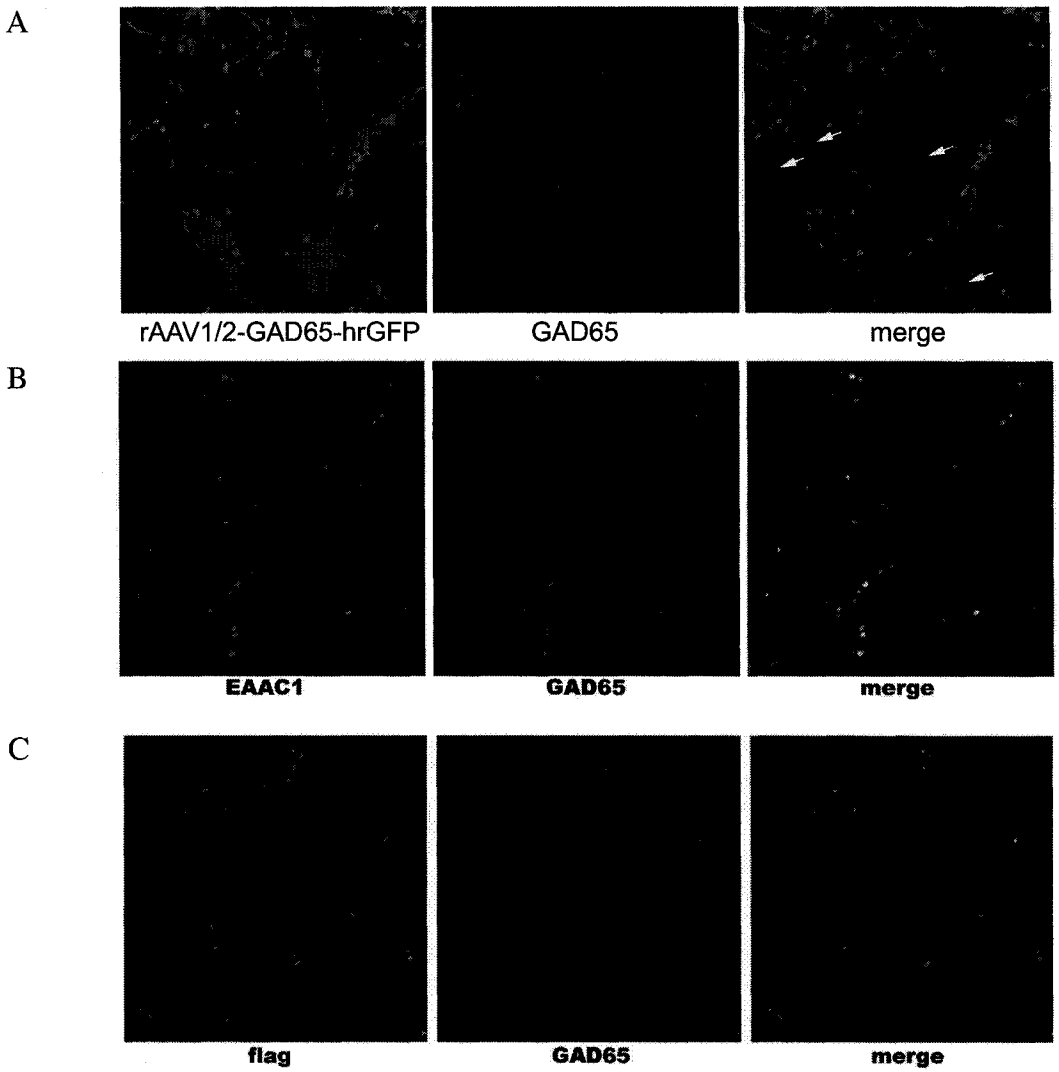

Figure 3. Cell type specific expression mediated by rAAV1/2-GAD65 viral constructs. **A)** Tissue transduced with rAAV1/2-GAD65-hrGFP and immunostained with an antibody against GAD65. The hrGFP expression co-localizes wth GAD65 in GABAErgic neuron terminals. The arrows indicate co-localization of hrGFP expression and GAD65 staining in the inhibitory presynaptic terminals (40x). **B)** Control tissue stained to demonstrate normal EAAC1 co-localization with GAD65. **C)** Tissue transduced with rAAV1/2-GAD65-flag-EAAC1 was stained with an antibody against the flag epitope located on the 3' terminus of the recombinant EAAC1 transporter and anti-GAD65. Within the dentate gyrus, flag staining co-localized with GAD65 staining. Images B & C were obtained at 100x.

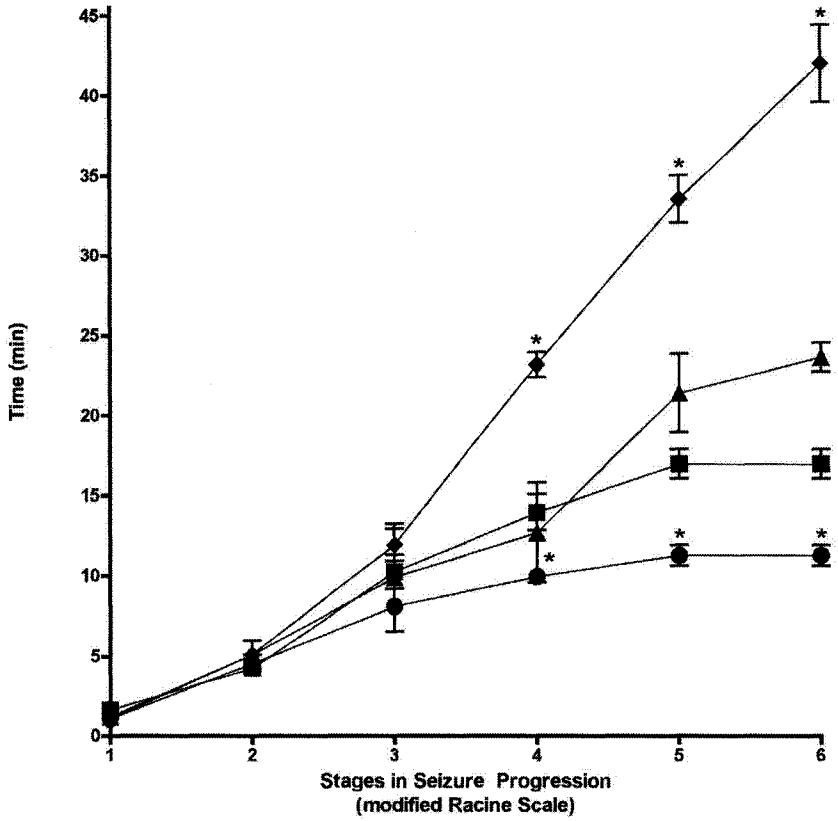

Figure 4. EAAC1 expression altered seizure susceptibility and rate of progression to SE. The pilocarpine induced seizure model was used to evaluate altered state of seizure susceptibility as a function of EAAC1 expression on inhibitory neuron terminals. Control (■), Null (▲), EAAC1 AS (●), and flag-EAAC1 (◆) animals were monitored for progression through distinct behavioral stages based on a modified Racine scale. Time to reach each of the stages was noted, averaged for each treatment group for animals that reached SE. * = $p<0.05$, One way ANOVA, n=2-7.

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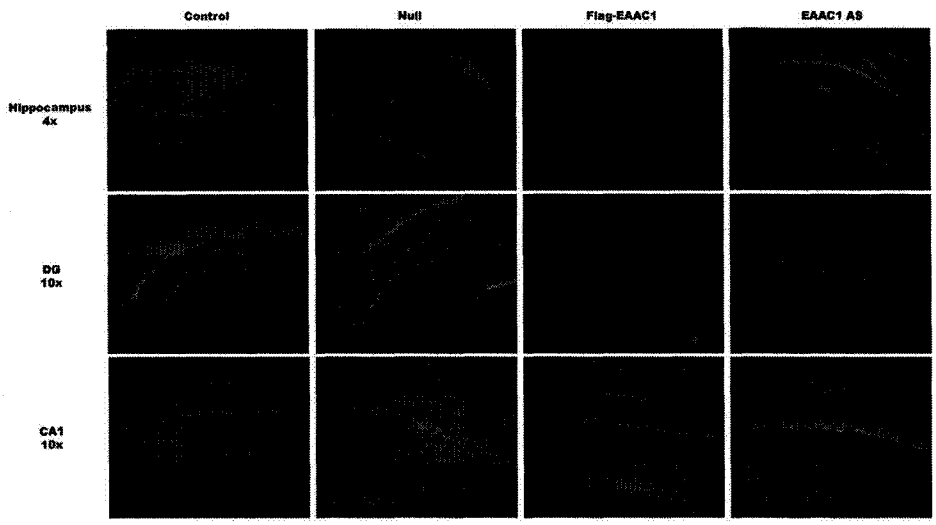

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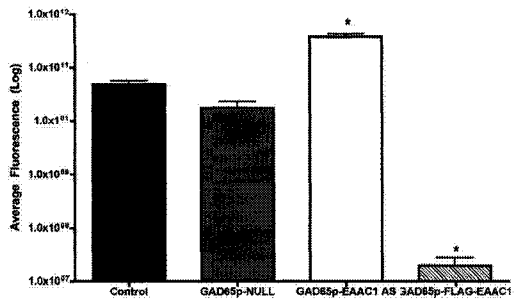

Figure 5. Modulation of EAAC1 expression on inhibitory presynaptic terminals influences neuroprotection. **A)** Tissue was harvested 48 hours post onset of status epilepticus. Tissue from each treatment group, control, null, EAAC1 AS and flag-EAAC1, were stained with Fluoro-Jade B and imaged to identify the level of seizure-mediated neurodamage. **B)** Total fluorescence intensity of the entire hippocampus was calculated. One-way ANOVA * = $p<0.05$, n=2-7.

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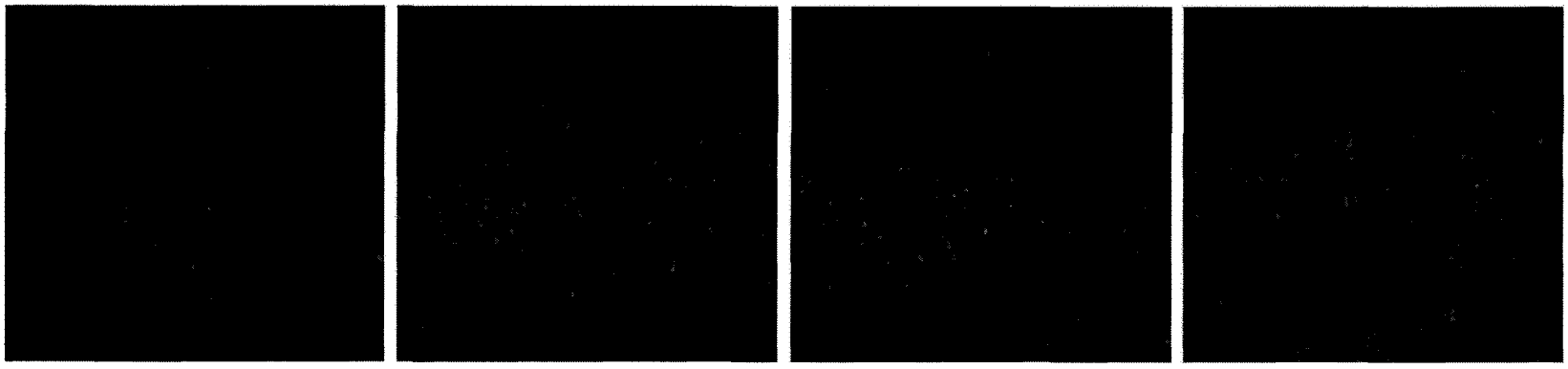

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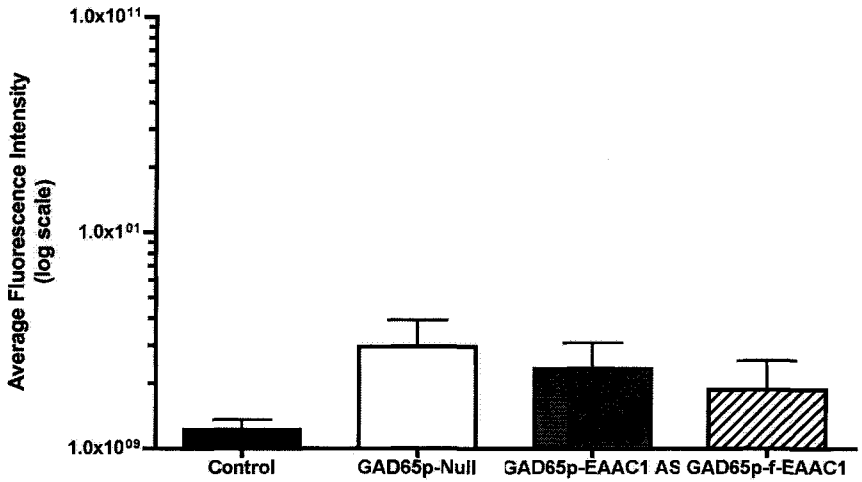

Figure 6. No difference in neuropathology was observed in cortex following pilocarpine-induced seizures. **A)** Fluoro-Jade B staining of tissue from each of the treatment groups (control, null, EAAC1 AS, and flag-EAAC1) was performed and images were obtained from similar regions within the cortex. **B)** Total fluorescence intensity from each of the sampled regions of the cortex revealed no statistical difference between each of the treatment groups.

Chapter Four

Conclusion

The data presented here offers only a glimpse at the potential of rAAV as a tool in the study of glutamate transporters. Recent advances in rAAV vector design and the identification of new serotypes offer additional regulatory features in the study of glutamate transporters (McCown, 2005; Shevtsova, 2005; Broekman, 2006). The use of chimeric rAAV1/2 may enable enhanced delivery and heightened EAAT2 expression beyond that currently achieved with the rAAV serotype 1 (Rabinowitz, 2004). Neonatal delivery of rAAV8-GFAP-EAAT2 may facilitate global upregulation of EAAT2 and allow for evaluation of wide-spread astrocytic EAAT2 upregulation (Broekman, 2006), though upregulation of EAAT2 during development may alter essential developmental processes as evident in a transgenic model over-expressing EAAT2 (Guo, 2003).

Components involved in glutamate processing in astrocytes may influence overall glutamate uptake capacity. Glutamine synthetase, the enzyme in astrocytes responsible for conversion of glutamate to glutamine, may become saturated under heightened intracellular glutamate levels. Likewise, uptake of glutamate via EAAT2 and conversion to glutamine are energy requiring processes. Components involved in energy production or homeostatic maintenance, such as α-ketoglutarate, cystine-glutamate transporter, additional enzymes involved in the TCA cycle or glucose uptake and utilization may be altered under elevated intracellular levels of glutamate.

A use of alternative GABAergic promoters, such as parvalbumin or GAD67, may provide further control of EAAC1 expression levels. Maximum EAAC1 expression levels may not be optimum in the overall viability of GABAergic neurons. Uptake of glutamate by EAAC1 localized to inhibitory presynaptic neurons is an energy dependent process and may result in rapid energy depletion under extended excitotoxic stimulus. By inducing graded expression levels of EAAC1 on GABAergic neurons, one may be able to regulate or post-

pone energy depletion and maximize the inhibitory potential of GABAergic neurons. Further study is needed to evaluate the impact of each of these factors on sustained glutamate uptake and cell viability under excitotoxic conditions.

Multiple factors impacted by altered intracellular glutamate levels may influence the level of functional glutamate uptake. Modulation of other factors involved in GABA synthesis may provide insight into the limiting steps of the synthesis pathway. We demonstrated that the upregulation of EAAC1 on inhibitory presynaptic terminals resulted in elevated intracellular GABA, glutamate and glutamine levels. The elevated level of GABA precursor, glutamate, may saturate GAD65 synthesis capacity. This could be further evaluated through the simultaneous over-expression of GAD65 in GABAergic neurons or through the addition of the GAD65 co-factor, pyridoxal-5-phosphate (vitamin B6). Other components may influence GABA synthesis capacity in the presence of elevated EAAC1 expression, such as glutaminase, components of the TCA cycle or sodium/potassium ATPases.

Lastly, the rAAV1/2-GAD65 viral constructs may prove beneficial in evaluating the role of EAAC1 in the development and progression of epilepsy. The EAAC1 is upregulated in status epilepticus (Crino, 2002) and has shown elevated expression in regions of seizure-mediated damage (Voutsinos-Porche, 2006). It has yet to be determined if increased GABAergic EAAC1 expression is a protective mechanism under epileptic conditions or is a contributing factor in the ongoing damage. Using the viral constructs and methodologies detailed in this dissertation, one could evaluate the impact of long term elevated EAAC1 expression in GABAergic neurons to further elucidate the function of EAAC1 in epileptigenesis.

Together, these studies revealed the neuroprotective nature of glutamate transporters when over-expressed in a cell type specific manner. The rAAV1-GFAP and rAAV1/2-GAD65 viral constructs address prior limitations in the

study of glutamate transporters (Sepkuty, 2002; Mathews and Diamond, 2003; Rothstein, 2005) and offer a means to further evaluate the role of glutamate transporter expression in neuroprotection and neurodegenerative disease states.

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