CAMKII REGULATION OF ASTROCYTIC GLUTAMATE UPTAKE

Aarti R. Chawla

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Andy Hudmon, Ph.D. - Chair

____

Theodore Cummins, Ph.D.

Doctoral Committee

____

Gerry S. Oxford, Ph.D.

May 19, 2016

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Jinhui Chen, M.D., Ph.D.

____

Quyen Hoang, Ph.D

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Aarti R. Chawla

DEDICATION

This dissertation is dedicated to my parents Lalita and Rajeev Chawla. Thank you for always believing in me, especially when I struggled to do so. Your unconditional love and support are integral to this body of work.

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Aarti R. Chawla

CAMKII REGULATION OF ASTROCYTIC GLUTAMATE UPTAKE

Glutamate clearance by astrocytes is an essential part of physiological excitatory neurotransmission. Failure to adapt or maintain low levels of glutamate in the central nervous system is associated with multiple acute and chronic neurodegenerative diseases. The primary excitatory amino acid transporters (EAATs) in human astrocytes are EAAT1 and EAAT2 (GLAST and GLT-1 respectively in rodents). While the inhibition of a ubiquitously-expressed serine/threonine protein kinase, the calcium/calmodulin-dependent kinase (CaMKII) results in diminished glutamate uptake in cultured primary rodent astrocytes, the molecular mechanism underlying this regulation is unknown. In order to delineate this mechanism, we use a heterologous expression model to explore CaMKII regulation of EAAT1 and EAAT2. In transiently transfected HEK293T cells, pharmacological inhibition of CaMKII and overexpression of a dominant-negative version of CaMKII (Asp136Asn) reduces [^{3}H]-glutamate uptake by EAAT1, without altering EAAT2 mediated glutamate uptake. Surprisingly, overexpression of a constitutively active autophosphorylation mutant (Thr287Asp) to increase autonomous CaMKII activity and a mutant incapable of autophosphorylation (Thr287Val) had no effect on either EAAT1 or EAAT2 mediated glutamate uptake. Pulldown of FLAG-tagged glutamate transporters suggests CaMKII does not interact with EAAT1 or EAAT2. SPOTS peptide arrays and recombinant GST-fusion proteins of the intracellular N- and C-termini of EAAT1 identified two potential phosphorylation sites at residues Thr26 and Thr37 in the N-terminus. Introducing an Ala (a non-phospho mimetic) but not

an Asp (phosphomimetic) at Thr37 diminished EAAT1-mediated glutamate uptake, suggesting that the phosphorylation state of this residue is important for constitutive EAAT1 function. In sum, this is the first report of a glutamate transporter being identified as a direct CaMKII substrate. These findings indicate that CaMKII signaling is a critical driver of homeostatic glutamate uptake by EAAT1. Aberrations in basal CaMKII activity disrupt glutamate uptake, which can perpetuate glutamate-mediated excitotoxicity and result in cellular death.

Andy Hudmon, Ph.D. - Chair

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LIST OF ABBREVIATIONS

AC-2	Autocamtide-2
ADP	Adenosine diphosphate
Ala	Alanine
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of Variance
ASIC1	Acid-sensing cation channel 1
Asn	Asparagine
Asp	Aspartic Acid
AP-1	Activator protein 1
ATP	Adeonosine triphosphate
BBB	Blood brain barrier
CA1	*Cornus ammonis* (region 1 of hippocampus proper)
cAMP	Cyclic adenosine monophosphate
CaM	Calmodulin
CaMKII	Calcium/calmodulin-dependent protein kinase II
cDNA	Complementary deoxyribonucleic acid
ClC-3	Chloride channel-3
CN21	CaMKII inhibitory peptide (21 residues)
CN21Ala	CaMKII inhibitory peptide control (21 residues)
CNS	Central nervous system
CREB	Cyclic AMP response-element binding protein
DAPI	4',6-diamidino-2-phenylindole

D-Asp	D-Aspartate
DC	Detergent compatible
DLG1	Discs large homolog 1
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EAAC1	Excitatory amino acid carrier 1
EAAT	Excitatory amino acid transporter
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
EPSP	Excitatory post-synaptic potential
FITC	Fluorescein isothiocyanate
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate Aspartate Transporter
Gln	Glutamine
GLT-1	Glial glutamate transporter 1
Glu	Glutamate
GSK3β	Glycogen synthase kinase 3 β
GST	Glutathione S-transferases
GTRAP	Glutamate transporter associated protein
ICC	Immunocytochemistry

IP3	Inositol triphosphate
HDAC	histone deacetylase
HeLa	Henrietta Lacks cell line
HEK	Human embryonic kidney cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KN-92	2-[N-(4-Methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine, monohydrochloride
KN-93	N-[2-[[[3-(4-Chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4 -methoxybenzenesulphonamide
MAP2	Microtubule-associated protein II
mGluRs	metabotropic glutamate receptors
MMP-9	Matrix metalloproteinase-9 expression
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor-κB
NFAT	Nuclear factor of activated cells
NLS	Nuclear localization sequence
NMDA-R	N-methyl-D-aspartate receptor
OGD/R	Oxygen-glucose deprivation/reperfusion
PAC1	Pituitary adenylate cyclase-activating polypeptide -type 1 receptor
PACAP	Pituitary adenylate cyclase-activating polypeptide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDL	Poly-D-Lysine

PDZ	Post-synaptic density 95 protein, Drosophila disc large tumor suppressor 1 protein, zonula occludens-1 protein
PEI	Polyethylenimine
PKC	Protein kinase C
PSD	Post-synaptic density
PSL	Photostimulated luminescence
PTM	Post translational modification
RFP	Red fluorescent protein
SAP97	Synapse-associated protein 97a
shRNA	Short hairpin ribonucleic acid
Sp1	stimulating protein 1
Tat	HIV-1 transactivating protein
TBI	Traumatic brain injury
TBOA	DL-threo-β-benzyoxyaspartate
TCA	Tricarboxylic acid
TF	Transcription factor
TGF- α	Transforming growth factor-α
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
TRP	Transient receptor potential
Thr	Threonine
Val	Valine
SD	Standard deviation
SEM	Standard error of the mean

SRF	Serum response factor
UCPH-101	2-Amino-5,6,7,8-tetrahydro-4-(4-methoxyphenyl)-7-(naphthalen-1-yl)-5-oxo-4*H*-chromene-3-carbonitrile
USF1	Upstream stimulating factor 1
YFP	Yellow fluorescent protein
YY1	Yin Yang 1

INTRODUCTION

The non-essential amino acid, L-glutamate, is the primary excitatory neurotransmitter in the mammalian central nervous system (CNS). It is essential for physiological processes such as early brain development, synaptic plasticity during learning and memory, and cognition. However, glutamate also functions as a neurotoxin when synaptic levels are not tightly controlled. A pathological state known as glutamate-induced excitotoxicity ensues, wherein excessive activation of glutamatergic receptors ultimately results in neuronal dysfunction and death. Neurodegenerative states such as ischemia, Alzheimer's and amyotrophic lateral sclerosis are closely associated with glutamate-induced excitotoxicity (Rothstein, Martin et al. 1992, Rao, Baskaya et al. 1998, Cassano, Serviddio et al. 2012). Collectively, these disease states account for a tremendous emotional and financial burden on patients, family, health care providers, and the government. It is essential to better our understanding of the common mechanisms underlying these diseases states in order to eventually devise successful therapeutics.

A family of sodium-dependent excitatory amino acid transporters (EAATs) is responsible for glutamate clearance during neurotransmission. Astrocytes express EAAT1 and EAAT2, proteins considered to be the molecular mainstays of glutamate uptake. Interestingly, alterations in a calcium-regulated multifunctional serine/threonine protein kinase, the calcium/calmodulin-dependent (CaMKII), have been shown to disrupt glutamate uptake via the astrocytic glutamate transporters (Ashpole, Chawla et al. 2013). CaMKII is rapidly activated as a result of glutamate-induced excitotoxic calcium signaling, followed by subsequent aggregation and inactivation (Aronowski, Grotta et al. 1992). In fact, CaMKII inactivation leading to a loss of CaMKII signaling has been

shown to directly correlate with the extent of neuronal damage, a hallmark feature of neurodegenerative diseases. Not surprisingly, astrocytic EAAT dysregulation and altered intracellular calcium signaling have both been demonstrated in neurodegeneration. In keeping with these findings, the question arises: what is the mechanism of CaMKII regulation of the astrocytic glutamate transporters?

The background of this dissertation will focus on the mechanisms involved in glutamatergic neurotransmission, and the structure, function, and regulation of both glutamate transporters and CaMKII. By providing insight into these mechanisms, I hope to provide a rationale underlying the biological importance of CaMKII regulation of EAATs.

Glutamatergic neurotransmission

The earliest studies examining the role of the acidic amino acid L-glutamate were related to its involvement in the Krebs cycle (Krebs 1935, Krebs 1935) and the detoxification of ammonia (Weil-Malherbe 1950, Weil-Malherbe 1950), suggesting a crucial metabolic role for this molecule. In fact, studies demonstrating that glutamate served as a precursor for gamma-aminobutyric acid (GABA), the key inhibitory neurotransmitter in the brain (Roberts and Frankel 1950), pre-dated reports suggesting its involvement in direct chemical signaling in the brain. It is perhaps not surprising that glutamate's role as a key excitatory neurotransmitter itself temporally surface after these findings; a non-essential amino acid such as glutamate is an atypical choice for a key neurotransmitter.

In 1959 Curtis et al. (Curtis, Phillis et al. 1959) demonstrated that L-glutamate caused depolarization and excitation in feline spinal neurons, providing one of the first reports implicating glutamate as a neurotransmitter. Since these studies, glutamate has been shown to be an important player in numerous aspects of normal brain function including cognition, fast synaptic transmission, and synaptic plasticity during learning and memory (for reviews see (Fonnum 1984, Danbolt 2001, Mayer and Armstrong 2004)). Furthermore, the role of glutamate as the primary excitatory amino acid neurotransmitter in the mammalian central nervous system has been firmly established (Karlsen and Fonnum 1978, Fonnum, Soreide et al. 1981, Fonnum, Storm-Mathisen et al. 1981). Interestingly, prior to Curtis' work in the late 1950s, Newhouse and Lucas demonstrated neurotoxicity associated with injecting glutamate into the immature murine retina, thus highlighting a second role of L-glutamate- one of a neurotoxin (Lucas and Newhouse 1957, Lucas, Newhouse et al. 1957). For the next few decades, numerous studies were performed in parallel that confirmed glutamate's role as both a key neurotransmitter and a neurotoxin. To date, however, scientists still strive to establish a better understanding of how fine alterations in the concentration of glutamate in the brain switch its function from that of a neurotransmitter to a neurotoxin.

During physiological chemical synaptic transmission, the arrival of an action potential at the pre-synaptic nerve terminal results in cellular depolarization (Hodgkin and Huxley 1952, Hodgkin, Huxley et al. 1952). One of the consequences of the membrane potential becoming more positive as a result of depolarization is calcium influx through voltage gated calcium channels. Glutamate, packaged in synaptic vesicles, is released via a calcium-dependent process from the pre-synaptic neuron into the

synaptic cleft (Katz and Miledi 1968, Katz and Miledi 1968, Haydon and Carmignoto 2006). 'Resting' glutamate concentrations in the extracellular fluid and cerebrospinal fluid have been estimated to range between 3-10µM (Hamberger and Nystrom 1984), which is estimated to comprise a very small percentage of total glutamate in the entire brain which ranges from 5-15mmol per kg wet weight (Choi 1990). However, following pre-synaptic vesicular release the concentration of synaptic glutamate rises rapidly to ~3mM (Diamond and Jahr 1997). Synaptic glutamate binds to and activates glutamate receptors located on the membranes of the pre-synaptic neuron, post-synaptic neuron and glial cells to mediate depolarization and activate intracellular signaling pathways, although the receptors located on post-synaptic neurons are thought to be the most important for synaptic neurotransmission. Post-synaptic glutamate receptors are of two different types: ionotropic and metabotropic. Glutamate receptors require low micromolar (1-10µM) concentrations of glutamate to induce activation (Choi 1987). In brief, glutamate activates both ionotropic and metabotropic receptors, resulting in depolarization due to cationic influx and the activation of second messenger systems. Synaptic glutamate concentrations during neurotransmission ensure efficient excitatory neurotransmission by maintaining the synaptic concentration below those required for tonic activation of the glutamate receptors, while providing enough extracellular glutamate to saturate the receptors (Shen and Meyer 1999, Danbolt 2001, Ferraguti and Shigemoto 2006).

Ionotropic receptors are of three kinds, named so on the basis of their respective exogenous, subtype-selective pharmacological ligands: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate receptors.

Also referred to as ligand-gated ion channels, ionotropic receptors are permeable, with varying degrees of selectivity, to Na^+, Ca^{2+} and K^+ ions (for reviews see (Hollmann and Heinemann 1994, Wollmuth and Sobolevsky 2004)). In particular, NMDA receptors (NMDARs) have a higher affinity for glutamate than either AMPA or kainate receptors (Hollmann and Heinemann 1994). NMDARs require binding of glutamate as well as simultaneous cellular depolarization to occur in order to relieve the Mg^{2+} block to allow ionic flux to occur through the receptor. Once activated by glutamate, ionotropic receptors allow for the entry of both Na^+ and Ca^{2+} ions. The NMDAR is considered the molecular mainstay in controlling synaptic plasticity and memory function in the brain (Collingridge, Kehl et al. 1983, Herron, Lester et al. 1986). Generally, NMDARs have been implicated in the late or slower phase of the excitatory post-synaptic potential (EPSP) time course, while it is thought that non-NMDAR ionotropic receptors control the amplitude and early phase of the EPSP time course. Ionotropic receptors mediate and process information in the brain that can account for alterations in synaptic efficiency involved in physiological and pathological processes such as learning and memory, development, the maintenance of cellular connections and pain perception (Bliss and Collingridge 1993, Woolf and Salter 2000).

The activity of metabotropic receptors (mGluRs), on the other hand, is associated with heterotrimeric guanine nucleotide binding proteins (G proteins). Signaling mechanisms for mGluRs, based on the G-proteins they couple to, involve phosphoinositide-dependent processes, protein kinase C or cyclic AMP, which can be either inhibitory or excitatory (Ferraguti and Shigemoto 2006, Niswender and Conn 2010). mGluRs can be found on both neurons and glial cells, based on the receptor or

splice variants in question, and are typically associated with slow synaptic function and neuronal excitability. This type of glutamate receptor is thought to be necessary for synaptic development and plasticity, as well as motor control, autonomic function, cognition and the receipt of sensory information. Classically, there are three families of mGluRs: group I mGluRs, which couple to Gq $/G_{11}$ and lead to activation of PKC and the mobilization of calcium, and group II and III mGluRs, which couple to $G_{i/o}$ proteins and are thought to inhibit adenylyl cyclase. However, numerous other signaling mechanisms are also thought to couple to the activity of the mGluRs (Niswender and Conn 2010).

Excessive stimulation of ionotropic receptors is associated with a condition termed glutamate-induced excitotoxicity, ultimately resulting in neuronal death (Olney and Sharpe 1969, Choi 1992, Rothman and Olney 1995). Early studies suggested that cell death associated with glutamate-induced excitotoxicity was of a necrotic nature, based on acidophilic neurons and mitochondrial flocculent densities observed in electron microscopic images (Olney 1969, Olney 1971, Ingvar, Morgan et al. 1988). However, the observation of a calcium-dependent delayed neuronal cell death following exposure to glutamate (Choi 1987, Randall and Thayer 1992) spurred interest on the nature of glutamate-induced cell death. Studies now provide compelling evidence that apoptotic phenotypes such as intranucleosomal DNA cleavage and the involvement of caspases are a result of neuronal glutamate exposure (Kure, Tominaga et al. 1991, Ankarcrona, Dypbukt et al. 1995, Choi 1996). Thus, cell death elicited by glutamate is now attributed to both apoptosis and necrosis.

NMDAR receptor activation leads to an increase in the ion fluxes of Na^+, Ca^{2+} and K^+ ions while ion influx through the AMPA receptor is dependent on the subunit

composition of the receptor, namely the presence of the GluR2 subunit which makes it impermeable to calcium (Choi 1987, Liu and Zukin 2007). Ca^{2+} activation and overload (Coultrap, Vest et al. 2011), and Na^{+} influx through these glutamate receptors cannot be overlooked as they lead to aberrant activation of voltage-gated ion channels such as the L-type calcium channel. Prolonged neuronal depolarization, ionic imbalances and ATP depletion are secondary effects associated with glutamate-induced excitotoxicity (Yi and Hazell 2006). Possibly the most pertinent protein to the pathological state of excitotoxicity is the NMDAR, highly permeable to calcium ions and often referred to as the 'death receptor' since overstimulation leads to phenotypes of calcium overload, aberrant cellular signaling and neuronal death associated with excitotoxicity (Tu, Xu et al. 2010, Coultrap and Bayer 2012). The contribution of mGluRs to the process of excitotoxicity remains ambiguous, although the activation of Group I mGluRs has been described to contribute to neuronal cell death (Hilton, Nunez et al. 2006). Taken together, it is evident that a system must exist to effectively clear excess extracellular glutamate in order to prevent aberrant signaling and subsequent neuronal damage and ultimately, death.

Glutamate metabolism in the CNS

Glutamate is unable to enter the brain by crossing the blood brain barrier (BBB), which comprises of capillary endothelial cells lining cerebral microvasculature and astrocyte end-feet processes, perivascular neurons and pericytes that insulate the brain from plasma (Hawkins, O'Kane et al. 2006, Hawkins 2009). However, glutamate is capable of exiting the BBB via EAAT1-3 that are present on the abluminal (facing the

brain) membrane of the BBB (Hutchison, Eisenberg et al. 1985, O'Kane, Martinez-Lopez et al. 1999). Thus, in the brain, the neurotransmitter glutamate needs to be synthesized. This process can occur in a few different ways: 1) *de novo* synthesis from glucose 2) the glutamate-glutamine cycle and 3) neuronal synthesis from astrocytic lactate (reviewed in (Danbolt 2001, Krzyzanowska, Pomierny et al. 2014)). Briefly, the different mechanisms that are involved in glutamate synthesis are discussed below.

De novo synthesis of glutamate from glucose

The mammalian brain is the highest consumer of glucose in comparison to other tissues in the body, and utilizes this substrate as its primary source of energy (Erbsloh, Bernsmeier et al. 1958). Glucose can easily traverse the BBB via the GLUT1 transporter present on endothelial cells (Mueckler, Caruso et al. 1985, Hemmila and Drewes 1993), making it a readily available substrate for the *de novo* synthesis of glutamate. In fact, it is estimated that almost all the glucose that enters the brain is eventually converted to glutamate (Shen, Petersen et al. 1999). Once glucose enters an astrocyte via astrocytic end feet that are tightly coupled to the neurovascular unit and gap junctions, it is broken down into pyruvic acid via glycolysis and eventually converted to glutamate through the intermediate by-product of α-ketoglutarate via the tricarboxylic acid (TCA) cycle.

It is noteworthy that a nonessential amino acid such as glutamate serves as what is considered the key excitatory neurotransmitter in the brain. Firstly, glutamate is present in the brain at a higher concentration that any other amino acid, thus making it readily available to use for the process of neurotransmission. Secondly, glucose is ubiquitous in the mammalian diet, making the key substrate for glutamate synthesis one that is easily

available. Lastly, glucose can easily enter the brain where it serves a number of other functions, thus making the cost of glucose uptake into the brain energetically favorable.

The glutamate-glutamine cycle

Once glutamate is taken up from the extracellular fluid by glia, it is converted into glutamine by glutamine synthetase, an enzyme detected only in glia (Martinez-Hernandez, Bell et al. 1977), via an ATP-dependent process (commonly known as the glutamate-glutamine cycle). It is then shuttled back into neurons through the astrocytic system N transporter 1 (Chaudhry, Reimer et al. 1999) and neuronal system A transporter (Varoqui, Zhu et al. 2000). Glutamine is then converted back to glutamate through phosphate-activated glutaminase (Laake, Takumi et al. 1999).

Neuronal synthesis of glutamate from astrocytic lactate

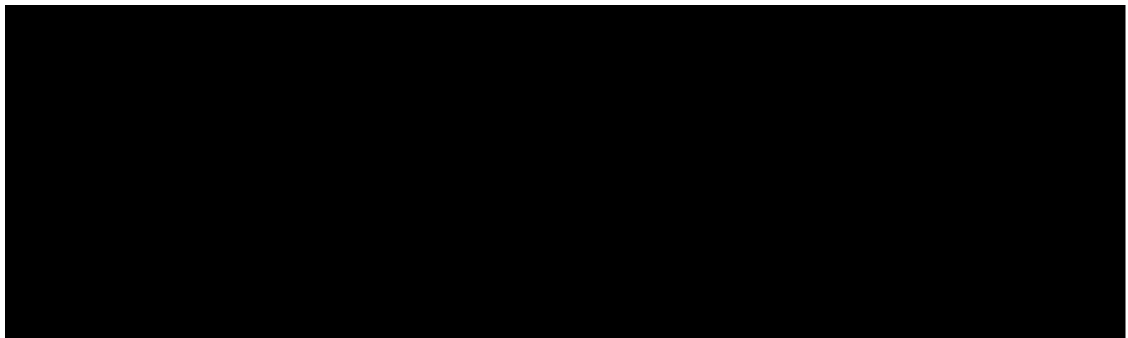
Alternately, glutamate taken up by glia is converted into α-ketoglutarate which is metabolized through the tricarboxylic acid (TCA) cycle into a number of different substrates. One fate of α-ketoglutarate is its conversion to lactate, which is then shuttled back to neurons through monocarboxylate transporters and converted to glutamate (Halestrap and Price 1999).

Regardless of what the source of the substrate that eventually results in neuronal glutamate is, once it is synthesized it is packaged into synaptic vesicles by vesicular glutamate transporters, and is ready to be exocytosed. It is this vesicular glutamate that is released by the presynaptic neuron during glutamatergic neurotransmission.

Glutamate clearance

The levels of extracellular glutamate must be tightly controlled to ensure efficient excitatory neurotransmission and to prevent aberrant receptor activation and subsequent excitotoxicity (Choi 1987, Choi 1992, Nieoullon, Canolle et al. 2006). In fact, it is thought that >99% of glutamate resides intracellularly in the brain (Danbolt 2001). Thus, a critical element of glutamatergic signaling is the clearance of excess synaptic glutamate. To date, there has been no evidence of an extracellular method of metabolizing glutamate (Danbolt 2001, Sheldon and Robinson 2007). Synaptosomal preparations measuring uptake of radiolabeled glutamate have suggested the contribution of sodium-independent transport as only a small percentage of total glutamate clearance (<5%) (Robinson 1998). Low-affinity glutamate transporters have been described, however they are poorly-characterized, appear to serve a redundant function as the high-affinity glutamate transporters mentioned below, and their existence as a separately entity is controversial (reviewed in (Danbolt 2001)). Instead, it appears that a family of sodium dependent, high affinity transporters, known as excitatory amino acid transporters (EAATs), are instrumental in removing the majority of glutamate from the synaptic cleft (Diamond and Jahr 1997, Amara and Fontana 2002, Sheldon and Robinson 2007). A schematic of glutamatergic neurotransmission encompassing the proteins and molecules described thus far is shown in **Figure 1**.

Pre-synaptic neuron
Glu
System A
Gln
Na+
Gln
System N
System x_c-
Cysteine
Na+
vesicular glutamate
EAAT1
EAAT1
Astrocyte
Astrocyte
EAAT2
EAAT2
NMDA-R
AMPA-R
Kainate-R
EAAT3
mGluR
Post-synaptic neuron



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hEAAT3     ---------------------------------------MGKPARKGCEWKRFLKNNWVL
hEAAT5     ----------------------------------------MVPHAILARGRDVCRRNGLL
hEAAT1     ---------MTKSNGEEPKMGGRMERFQQGVRKRTLLAKKKVQNITKEDVKSYLFRNAFV
hEAAT4     -MSSHGNSLFLRESGQRLGRVGWLQRLQESLQQRALRTRLRLQTMTLEHVLRFLRRNAFI
                                                                  .*  .:

hEAAT2     TLTVFGVILGAVCGGLLRLASPIHPDVVMLIAFPGDILMRMLKMLILPLIISSLITGLSG
hEAAT3     LSTVAAVVLGITTGVLVREHSNLSTLEKFYFAFPGEILMRMLKLIILPLIISSMITGVAA
hEAAT5     ILSVLSVIVGCLLGFFLRT-RRLSPQEISYFQFPGELLMRMLKMMILPLVVSSLMSGLAS
hEAAT1     LLTVTAVIVGTILGFTLRP-YRMSYREVKYFSFPGELLMRMLQMLVLPLIISSLVTGMAA
hEAAT4     LLTVSAVVIGVSLAFALRP-YQLTYRQIKYFSFPGELLMRMLQMLVLPLIVSSLVTGMAS
             :* .*::*    .   :*      :        : ***::*****:::***::**:::*::.

hEAAT2     LDAKASGRLGTRAMVYYMSTTIIAAVLGVILVLAIHPGNPKLKKQLGPGKKNDEVSSLDA
hEAAT3     LDSNVSGKIGLRAVVYYFCTTLIAVILGIVLVVSIKPGVTQKVGEIARTGSTPEVSTVDA
hEAAT5     LDAKTSSRLGVLTVAYYLWTTFMAVIVGIFMVSIIHPGSAAQK-ETTEQSGKPIMSSADA
hEAAT1     LDSKASGKMGMRAVVYYMTTTIIAVVIGIIIVIIIHPGKGTKE-NMHREGKIVRVTAADA
hEAAT4     LDNKATGRMGMRAAVYYMVTTIIAVFIGILMVTIIHPGKGSKE-GLHREGRIETIPTADA
           ** :.:.::*  : .**: **::*..:*:.:*   *:**                : : **

hEAAT2     FLDLIRNLFPENLVQACFQQIQTVTKKVLVAPPPDEEANA----------------TSAV
hEAAT3     MLDLIRNMFPENLVQACFQQYKTKREEVKPPSD---PEMN-----------------MTE
hEAAT5     LLDLIRNMFPANLVEATFKQYRTKTTPVVK-SPKVAPEEAP-PRRILIYGVQEEN-GSHV
hEAAT1     FLDLIRNMFPPNLVEACFKQFKTNYEKRSFKVPIQANETL--V-------------GAVI
hEAAT4     FMDLIRNMFPPNLVEACFKQFKTQYSTRVVTRTMVRTENGSEPGASMPPPFSVENGTSFL
           ::*****:** ***:* *:* :*

hEAAT2     VSLLNETVTEVPEE-TKMVIKKGLEFKDGMNVLGLIGFFIAFGIAMGKMGDQAKLMVDFF
hEAAT3     -ESFTAVMTTAISKNKTKEYKIVGMYSDGINVLGLIVFCLVFGLVIGKMGEKGQILVDFF
hEAAT5     -QNFALDLTPPPE----VVYKSEPGTSDGMNVLGIVFFSATMGIMLGRMGDSGAPLVSFC
hEAAT1     -NNVSEAMETLTR--ITEELVPVPGSVNGVNALGLVVFSMCFGFVIGNMKEQGQALREFF
hEAAT4     -ENVTRALGTLQEMLSFEETVPVPGSANGINALGLVVFSVAFGLVIGGMKHKGRVLRDFF
            . .    :                        :*:*.**:: *    :*: :* * ...   : .*

hEAAT2     NILNEIVMKLVIMIMWYSPLGIACLICGKIIAIKDLEVVARQLGMYMVTVIIGLIIHGGI
hEAAT3     NALSDATMKIVQIIMCYMPLGILFLIAGKIIEVEDWEIF-RKLGLYMATVLTGLAIHSIV
hEAAT5     QCLNESVMKIVAVAVWYFPFGIVFLIAGKILEMDDPRAVGKKLGFYSVTVVCGLVLHGLF
hEAAT1     DSLNEAIMRLVAVIMWYAPVGILFLIAGKIVEMEDMGVIGGQLAMYTVTVIVGLLIHAVI
hEAAT4     DSLNEAIMRLVGIIIWYAPVGILFLIAGKILEMEDMAVLGGQLGMYTLTVIVGLFLHAGI
           : *.:  *::* : : * *.**  **.***: :.*    .  :*.:*  **: ** :*. .

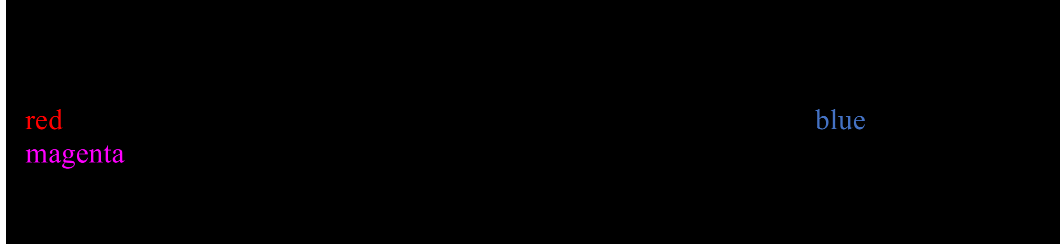
hEAAT2     FLPLIYFVVTRKNPFSFFAGIFQAWITALGTASSAGTLPVTFRCLEENLGIDKRVTRFVL
hEAAT3     ILPLIYFIVVRKNPFRFAMGMAQALLTALMISSSSATLPVTFRCAEENNQVDKRITRFVL
hEAAT5     ILPLLYFFITKKNPIVFIRGILQALLIALATSSSSATLPITFKCLLENNHIDRRIARFVL
hEAAT1     VLPLLYFLVTRKNPWVFIGGLLQALITALGTSSSSATLPITFKCLEENNGVDKRVTRFVL
hEAAT4     VLPLIYFLVTHRNPFPFIGGMLQALITAMGTSSSSATLPITFRCLEEGLGVDRRITRFVL
           .***:**.:.::**  *  *: ** : *:  :**:.***:**:*  *   :*:*::****

hEAAT2     PVGATINMDGTALYEAVAAIFIAQMNGVVLDGGQIVTVSLTATLASVGAASIPSAGLVTM
hEAAT3     PVGATINMDGTALYEAVAAVFIAQLNDLDLGIGQIITISITATSASIGAAGVPQAGLVTM
hEAAT5     PVGATINMDGTALYEAVAAIFIAQVNNYELDFGQIITISITATAASIGAAGIPQAGLVTM
hEAAT1     PVGATINMDGTALYEALAAIFIAQVNNFELNFGQIITISITATAASIGAAGIPQAGLVTM
hEAAT4     PVGATVNMDGTALYEALAAIFIAQVNNYELNLGQITTISITATAASVGAAGIPQAGLVTM
           *****:**********:**:****:*    *  *** *:*:*** **:***.:*.******

hEAAT2     LLILTAVGLPTEDISLLVAVDWLLDRMRTSVNVVGDSFGAGIVYHLSKSELDTIDSQHRV
hEAAT3     VIVLSAVGLPAEDVTLIIAVDWLLDRFRTMVNVLGDAFGTGIVEKLSKKELEQMDVSSEV
hEAAT5     VIVLTSVGLPTDDITLIIAVDWALDRFRTMINVLGDALAAGIMAHICRKDFARDTGTEKL
hEAAT1     VIVLTSVGLPTDDITLIIAVDWFLDRLRTTTNVLGDSLGAGIVEHLSRHELKNRDVEMGN
hEAAT4     VIVLTSVGLPTEDITLIIAVDWFLDRLRTMTNVLGDSIGAAVIEHLSQRELELQEAELT-
           :::*::****::*::*::**** ***:**  **:**::.:.:: ::.: ::

hEAAT2     H--EDIEMTKTQSIYDDMKNHRESNSNQCVYAAHNSVIVDECKVTLA---------ANGK
hEAAT3     N------IVNPFALESTILDNEDSD-TKKSYVNGGFAVD----KSDTISFTQTSQF----
hEAAT5     ----LPCETKPVSLQEIVAAQQNGC-VKSVAEASELTLGPTCPHHVPVQVEQDEELPAAS
hEAAT1     SVIEENEMKKPYQ---LIAQDNETE--KPI-DS-ETKM----------------------
hEAAT4     ----LPSLGKPYK---SLMAQEKGA-SRGRGGN-ESAM----------------------
                     :        :  ...     :          :

hEAAT2     SADCSVEEEPWKREK
hEAAT3     ---------------
hEAAT5     LNHCTIQISELETNV
hEAAT1     ---------------
hEAAT4     ---------------
```

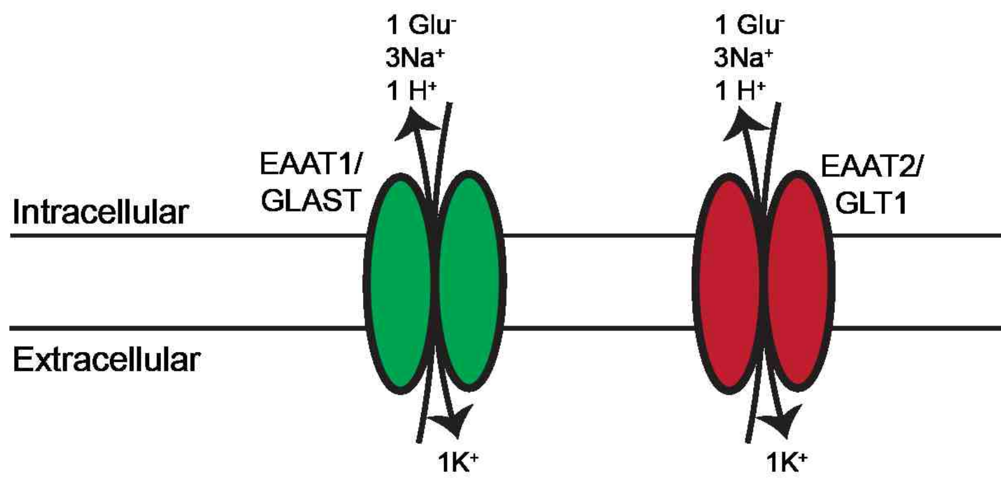


EAAT structure and function

EAATs belong to the Solute Carrier (SLC1) gene family of transporters, a diverse group of membrane transport proteins. GLAST (Storck, Schulte et al. 1992), GLT-1 (Pines, Danbolt et al. 1992), EAAC1 (Kanai and Hediger 1992), EAAT4 (Fairman, Vandenberg et al. 1995), and EAAT5 (Arriza, Eliasof et al. 1997) were identified through the 1990s, and have since been reported to share 50-60% sequence homology (**Figure 2**). The human homologs for GLAST, GLT-1 and EAAC1 are named EAAT1, EAAT2 and EAAT3 respectively (reviewed in (Danbolt 2001). Structurally, glutamate transporters have been suggested to exist as homomeric trimers, with eight transmembrane domains based on cysteine scanning mutagenesis of GLT-1 and comparison to the bacterial glutamate transporter homolog crystallized from *Pyrococcus horikoshii* (Yernool, Boudker et al. 2004, Peacey, Miller et al. 2009).

Uptake by the EAATs relies on the driving force of the thermodynamically favorable Na^+ electrochemical gradient: 1 glutamate$^-$, 3 Na^+ molecules and 1 H^+ are taken up by the cell, while a K^+ ion is counter-transported (**Figure 2**) (reviewed in (Danbolt 2001, Amara and Fontana 2002)). Initiation of this process is thought to involve the recruitment of 1 glutamate$^-$, 3 Na^+ molecules and 1 H^+ from the extracellular space, while the transporter is hypothesized to be in an outward-facing conformation. Once these substrates bind to the surface of the transporter, a conformational change occurs and the EAATs face inward, releasing these substrates into the intracellular space. At this point, a K^+ ion is recruited to the transporter surface, after which the conformation switches to one that is outward-facing again and the bound ion is released extracellularly (reviewed

in (Billups, Rossi et al. 1998, Robinson 1998)). In addition to these ionic fluxes, currents elicited by EAAT4 and EAAT5 comprise of an inward Cl^- flux.


1 Glu-
3Na+
1 H+
EAAT1/
GLAST
1 Glu-
3Na+
1 H+
EAAT2/
GLT1
Intracellular
Extracellular
1K+
1K+

EAAT expression

GLAST/EAAT1 and GLT-1/EAAT2 predominantly localize to astrocytes and to a lesser extent on other glial cells and neurons (Rothstein, Dykes-Hoberg et al. 1996, Danbolt 2001), whereas EAAC1, EAAT4 and EAAT5 are considered neuronal (Amara and Fontana 2002). GLAST/EAAT1 and GLT-1/EAAT2 will be described in greater detail in the next section, while I will focus on the neuronal transporters for the next few paragraphs. Transporter localization and nomenclature is summarized in **Table 1**.

EAAC1/EAAT3 is the primary neuronal glutamate transporter. Its expression has been described in pyramidal cells in both the cortex and hippocampus, as well as in GABAergic neurons, oligodendrocytes and even in astrocytes at very low levels (Rothstein, Martin et al. 1994, Domercq, Sanchez-Gomez et al. 1999, Kugler and Schmitt 1999). Outside of the central nervous system, EAAC1 expression is observed in the kidney and small intestine. EAAC1 is highly expressed early in development and thought to account for the majority of glutamate clearance before GLT-1 expression increases (reviewed in (Sims and Robinson 1999)). Knockdown of EAAC1 with antisense oligonucleotides resulted in a seizure phenotype but did not elevate striatal extracellular glutamate and produced only a mild excitotoxic phenotype (Rothstein, Dykes-Hoberg et al. 1996). The first EAAC1 knockout mouse model did not exhibit any overt neurological phenotype (Peghini, Janzen et al. 1997), however progressive age-dependent neurodegeneration associated with decreased glutathione levels in the brain was described in a more recent EAAC1 knockout mouse (Aoyama, Suh et al. 2006).

EAAT4 is selectively expressed on GABAergic Purkinje neurons in the cerebellum with much lower expression in the forebrain (Furuta, Rothstein et al. 1997),

and has been shown to transport glutamate with a slower turnover (Mim, Balani et al. 2005). EAAT4 expression appears to be concentrated adjacent to excitatory synapses, close to glial cell contacts. EAAT3 and EAAT4 are located peri-synaptically and/or in spines (reviewed in (Danbolt 2001)). It has been hypothesized, based on their location, that EAAT3 and EAAT4 are positioned to regulate the amount of glutamate escaping the synapse.

EAAT5 is located on presynaptic neurons in rod photoreceptors retinal bipolar cells (Arriza, Eliasof et al. 1997). A major feature of EAAT5 is its ability to conduct Cl^- ions gated by the binding of Na^+ and $Glutamate^-$, but thermodynamically uncoupled to $Glutamate^-$ flux (Wersinger, Schwab et al. 2006). This conductance, in turn, causes hyperpolarization of the presynaptic nerve terminal. EAAT5 has thus been hypothesized to play a larger role in transmitter release rather than glutamate clearance in the retina (Veruki, Morkve et al. 2006). As GLAST/EAAT1 and GLT-1/EAAT2 are the predominant glutamate transporters on astrocytes, I focus on these two transporters for the remainder of my work.

Astrocytic EAAT transporters

The glial glutamate transporters, GLAST/EAAT1 and GLT-1/EAAT2 are considered to be the primary molecular mainstays of glutamate clearance at the synaptic cleft. This idea is supported by a number of reasons. Firstly, astrocytic sodium-dependent glutamate transporters are present in extremely high densities in certain regions of the brain. Specifically, astrocytes in the hippocampal CA1 stratum radiatum and the cerebellar molecular layer have been reported to contain 15000 and 21000 glial glutamate

transporter molecules respectively per μm^3 tissue as compared to 2000 neuronal transporter (EAAT4, specifically) molecules (Dehnes, Chaudhry et al. 1998, Lehre and Danbolt 1998). Secondly, utilizing chronic antisense oligonucleotides to inhibit glutamate transporter synthesis (either GLAST or GLT-1) resulted in neurodegenerative excitotoxic characteristics, elevated extracellular glutamate levels, and progressive paralysis *in vivo* (Rothstein, Dykes-Hoberg et al. 1996). Mouse knockout models further substantiate these findings. A GLAST knockout mouse exhibits cerebellar ataxia, mild to moderate neuronal death as a result of an excitotoxic insult and inability to perform challenging tests involving coordination (Watase, Hashimoto et al. 1998). In an independent study, GLAST mice were also described to be more susceptible to seizures (Ueda, Doi et al. 2002). A homozygous GLT-1 knockout mouse exhibited severe neuronal death as a result of a cortical ischemic insult and had a higher incidence of developing lethal spontaneous epileptic seizures (Tanaka, Watase et al. 1997). Knockout models of neuronal glutamate transporters did not produce overt neurological phenotypes.

High levels of GLAST expression have been reported in the retina and cerebellum. GLAST deficiency has a much greater impact on retinal damage following ischemia in mice compared to depleting GLT-1, thus underscoring the importance of targeting glutamate uptake through GLAST during conditions of blindness and degeneration as a result of ischemic retinal disease (Harada, Harada et al. 1998). GLAST is also localized to Bergmann glia, accounting for the majority of cerebellar glutamate uptake (Rothstein, Martin et al. 1994, Rothstein, Dykes-Hoberg et al. 1996). A GLAST knockout mouse exhibits cerebellar ataxia and deficits in coordination whereas a GLT-1 knockout mouse does not have any cerebellar effects. Lastly, a study utilizing chronic

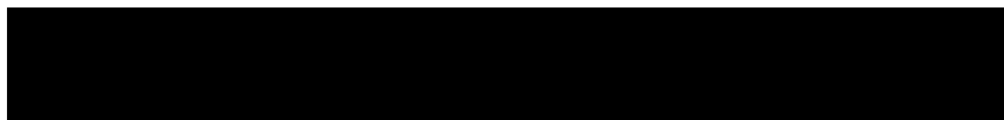
antisense oligonucleotides against GLAST and GLT-1 individually demonstrated comparable levels of neuronal injury between the two knockdowns as a result of an ischemia insult, and elevated extracellular glutamate.

GLT-1 is highly expressed throughout the neuroaxis, but is particularly prevalent in cortical and hippocampal astrocytes (Rothstein, Martin et al. 1994, Lehre, Levy et al. 1995, Lehre and Danbolt 1998). Its expression has been described in developing neurons, cultured neurons and oligodendrocytes (Rothstein, Martin et al. 1994, Domercq, Sanchez-Gomez et al. 1999) Electron microscopy studies have identified GLT-1 expression on astrocytic processes ensheathing the synapse (Lehre, Levy et al. 1995). Although GLT-1/EAAT2 has been reported to account for more than 90% of total glutamate transport activity in the forebrain, studies involving knockdown of GLAST and GLT-1 demonstrate a comparable neurotoxic phenotype, suggesting the importance of both these transporters in maintaining tight control of extracellular glutamate levels in the brain (Rothstein, Dykes-Hoberg et al. 1996).

Given these data, much of the literature on glutamate transporter activity has focused on GLT-1/EAAT2 activity. However, it is now evident that based on the region of the brain and cell type being studied, GLT-1 and GLAST expression differs and consequently account for different percentages of glutamate uptake based on these varied expression patterns. Both GLAST and GLT-1 have been shown to be crucial for astrocytic glutamate uptake, supported by both knockout models and antisense oligonucleotide studies described previously. In the next few sections, I will address mechanisms of both acute and chronic regulation of these transporters, providing

examples substantiating how alterations in both transporters result in functional changes in the brain.

Human transporter nomenclature	Rodent homolog nomenclature	Gene name	Cellular localization	Tissue localization	References
EAAT1	GLAST	SLC1A3	Astrocytes, oligodendrocytes	Highest in retina and cerebellum, also expressed in cortex, hippocampus, and basal ganglia	(Rothstein, Dykes-Hoberg et al. 1996), (Danbolt 2001)
EAAT2	GLT-1	SLC1A2	Astrocytes, oligodendrocytes, neurons	Ubiquitously expressed in the CNS	(Rothstein, Dykes-Hoberg et al. 1996), (Danbolt 2001)
EAAT3	EAAC1	SLC1A1	Neurons (pyramidal cortical neurons, both glutamatergic and GABAergic), oligodendrocytes, astrocytes at very low levels	Hippocampus, cortex	(Rothstein, Martin et al. 1994, Domercq, Sanchez-Gomez et al. 1999, Kugler and Schmitt 1999)
EAAT4	EAAT4	SLC1A6	Neurons (primarily GABAergic Purkinje cells), astrocytes	Cerebellum	(Fairman, Vandenberg et al. 1995, Furuta, Rothstein et al. 1997)
EAAT5	EAAT5	SLC1A7	Neurons (rod photoreceptors and bipolar cells)	Retina	(Arriza, Eliasof et al. 1997)



Regulation of EAATs

Regulation of the EAATs has been shown to occur at virtually any step of gene expression, from transcription, to translation, as well as post-translational modifications of the transporter protein. This section focuses on the regulation of the astrocytic transporters GLAST/EAAT1 and EAAT2/GLT1. Based on prior work, chronic versus acute alterations in transporter activity have been attributed to different mechanisms. Chronic or long-term changes in transporter activity are thought to be dependent on transcription or translation, assembly of the multimeric structure, partial glycosylation in the endoplasmic reticulum followed by terminal glycosylation, and insertion in the plasma membrane. In total, these changes occur as a result of *de novo* synthesis and a change in the total number of transporters (reviewed in (Robinson 2006, Fontana 2015)). For example, the most extensively studied of the glutamate transporters EAAC1/EAAT3, has a degradation half-life in the order of a few hours (Yang and Kilberg 2002). Conversely, rapid alterations in transporter activity that occur within minutes are typically thought to occur independently of these mechanisms. Rapid changes are thought to occur as a result of re-distribution of transporters to the plasma membrane, without changing the total number of cellular transporters available (reviewed in (Robinson 2002, Robinson 2006)). We will first discuss chronic mechanisms/examples followed by acute mechanisms/examples underlying alterations in glutamate transporter activity.

Transcription

Transcription is a key mechanism implicated in the chronic regulation of glutamate transporters. Although this dissertation focuses on acute regulatory

mechanisms of glutamate uptake, a brief discussion on a ‘long-term’ mechanism such as transcription may help us establish a temporal association between extracellular events and cellular regulatory mechanisms governing glutamate uptake.

Glutamate-induced alterations in the regulation of GLAST/EAAT1 and related genes

Although it was known that neurotransmitters resulted in the activation of transmitter receptors on astrocytes (Bowman and Kimelberg 1984), it took another decade for scientists to report that the neurotransmitter glutamate was instrumental in mobilizing calcium in astrocytes via ionotropic and metabotropic receptors on these cells (Cornell-Bell, Finkbeiner et al. 1990, Nedergaard 1994). As the glutamate transporters were cloned within that time frame, subsequent studies began to shed light on the relationship between the neurotransmitter and ligand for the glutamate transporters, glutamate, and cellular signaling pathways in astrocytes.

The application of glutamate decreased GLAST mRNA expression in cerebellar chicken Bergmann glia cells, leading to compromised glutamate uptake (Lopez-Bayghen, Espinoza-Rojo et al. 2003, Rosas, Vargas et al. 2007). Transcriptional regulation of GLAST/EAAT1 resulting in alterations in GLAST/EAAT1 expression and activity occurs through both calcium-dependent and independent mechanisms. Calcium influx through the AMPA and kainate receptors, the activation of the kinase PKC, and the availability of the transcription factor C-Jun were implicated as key players in the glutamate-mediated decrease in GLAST transcriptional regulation (Gonzalez and Ortega 1997, Lopez-Bayghen, Espinoza-Rojo et al. 2003, Lopez-Bayghen and Ortega 2004). The GLAST/EAAT1 promoter has been shown to contain a canonical sequence for the PKC-

inducible activator protein (AP-1) transcription factor (Espinoza-Rojo, Lopez-Bayghen et al. 2000). Application of glutamate and the glutamate analogue, D-aspartate (D-Asp) increases AP-1 binding directly to the GLAST/EAAT1 promoter thus decreasing its expression (Ramirez-Sotelo, Lopez-Bayghen et al. 2007, Martinez-Lozada, Hernandez-Kelly et al. 2011, Maria Lopez-Colome, Martinez-Lozada et al. 2012). Furthermore, D-Asp induced calcium influx and phosphorylation of the mammalian target of rapamycin (mTOR) in a PI3K/PKB/Akt-dependent manner in Müller radial glia, leading to increased AP-1 DNA binding (Maria Lopez-Colome, Martinez-Lozada et al. 2012). In fact, calcium influx in Bergmann glia cells has also been shown to activate CaMKII and downstream tyrosine kinases such as PI3K and MAPK, transiently phosphorylate the TF CREB (cAMP response-element binding protein) driving c-fos expression (Aguirre, Lopez-Bayghen et al. 2002, Lopez-Bayghen, Aguirre et al. 2003). CaMKII in particular, has been shown to regulate the activity of both AP-1 and C-Jun, transcription factors implicated in the regulation of GLAST/EAAT1 promoter activity (Mishra, Mishra et al. 2005). Although this finding was in human monocyte cells, we can extrapolate that the transcriptional regulation exerted by CaMKII may occur in a similar manner in astrocytes.

The EAAT1 promoter is thought to contain multiple consensus sites for Yin Yang 1 (YY1), a ubiquitously distributed TF largely known for its ability to modify histones (Karki, Kim et al. 2015, Karki, Smith et al. 2015). Glutamate has been shown to increase binding of YY1 to the GLAST/EAAT1 promoter, resulting in the negative regulation of GLAST/EAAT1 promoter activity in a time and dose-dependent manner (Karki, Kim et al. 2015). YY1 overexpression led to decreases in GLAST/EAAT1 mRNA and protein

levels (Rosas, Vargas et al. 2007, Aguirre, Rosas et al. 2008). Calcium-activated calpain proteases have been described to cleave and decrease the availability of YY1 in human vascular smooth muscle cells (Stoeckius, Erat et al. 2012), which would suggest calcium influx would increase GLAST/EAAT1 expression in astrocytes if this regulation were translatable. However, direct action of CaMKII on YY1 has not been described to date.

It is noteworthy that an independent study observed an increase in glutamate uptake and increased GLAST expression in rodent cortical astroglial cultures under similar conditions (Gegelashvili, Civenni et al. 1996) in contrast to a larger body of data that suggests GLAST expression is downregulated upon glutamate application. These differences observed may be due to a culture that is enriched in GLAST, namely Bergmann glia, versus a culture that expresses predominantly GLAST, but GLT-1 induction can occur in. Interestingly, this study observed an increase in only GLAST protein but not mRNA, suggesting this regulation is a result of translational, and not transcriptional, alterations in GLAST/EAAT1. Thus, it appears that glutamate can control the expression of GLAST/EAAT1 via the involvement of a number of transcription factors such as AP-1, CREB, YY1, and C-Jun. At least three of these (AP-1, CREB, C-Jun) are regulated by CaMKII, which may provide a basis for a long-term regulatory effect and associated gene changes in GLAST/EAAT1 as a result of CaMKII alterations.

YY1-associated regulation of GLAST/EAAT1 expression through effectors other than glutamate

We previously described that glutamate induces a reduction in GLAST/EAAT1 promoter activity through the transcription factor YY1 (Rosas, Vargas et al. 2007).

Valproate, a fatty acid used as a therapeutic for epilepsy and manic-depression, has been shown to elicit a dual regulatory effect on GLAST/EAAT1 promoter activity through YY1 via the induction of histone deacetylases (HDACs), which repress YY1 (Aguirre, Rosas et al. 2008). Recently manganese, a trace element that accumulates and acts as a neurotoxin in a number of neurological diseases, has been implicated in the reduction of GLAST/EAAT1 expression via YY1 binding to the GLAST/EAAT1 promoter. Thus, regardless of the mode of activation, YY1 appears to be key in negatively regulating GLAST/EAAT1 activity. Interestingly, YY1 represses GLT-1/EAAT2 expression; thus suggesting YY1 regulation of the two astrocytic glutamate transporters confers a similar effect (Karki, Kim et al. 2015, Karki, Smith et al. 2015).

The nuclear factor-κB (NF-κB) is thought to be a positive regulator of GLAST/EAAT1 expression by its ability to bind the GLAST/EAAT1 promoter. The EAAT1 promoter contains two consensus sites for NF-κB. In particular, mutation of NF-κB binding sites or inhibition of NF-κB decreased EAAT1 promoter activity while activation of NF-κB increased EAAT1 promoter activity, mRNA and protein levels (Karki, Kim et al. 2015). In fact, activation of intracellular Ca^{2+} and CaMKII have been shown to mediate NF-κB at multiple levels in neurons, T cells, and macrophages (Hughes, Edin et al. 2001, Ishiguro, Green et al. 2006, Liu, Yao et al. 2008). Although our studies did not examine long-term effects of CaMKII inhibition or activation on GLAST/EAAT1 levels, the transcription factor NF-κB appears to be a key player in this mechanism. In fact, long-term treatment (7 days) of mixed astroglial cultures with glutamate has been shown to increase GLAST expression and glutamate uptake (Gegelashvili, Civenni et al. 1996), contrary to the GLAST decrease elicited by a shorter

exposure to glutamate. It is conceivable that the opposing action of multiple transcription factors at play may lead to differential phenotypes when it comes to GLAST/EAAT1 expression, and furthermore, this may be dependent on cell type and duration of glutamate treatment.

Involvement of other transcription factors in GLAST/EAAT1 expression

Using gel shift and supershift analyses of a portion of the cloned GLAST/EAAT1 promoter from human fetal astrocytes, Kim et al (Kim, Choi et al. 2003) observed that stimulating protein (Sp) 1 and 3 bound to the GC-box in the GLAST/EAAT1 promoter. Sp proteins have been shown to play a role in transcriptional activation or repression of promoters lacking TATA or CAAT consensus sequences. Furthermore, upstream stimulating factor (USF1), a transcription factor that serves as a transcriptional activator, bound to the Enhancer (E) box in the GLAST/EAAT1 promoter. Although a number of other sites such as an X-box for protein RFX1, sites for the gut-enriched Krueppel-like factors, the serum response factor (SRF) and AREB6 were predicted, functional data suggested that these regulatory elements did not represent functional DNA-protein binding sites. These factors appear to be important for basal GLAST/EAAT1 activity.

A number of studies observed that application of a cAMP analogue, EGF and TGF-α resulted in increased GLAST/EAAT1 mRNA, protein and subsequent glutamate uptake in astrocyte cultures, while demonstrating TNF-α downregulated endogenous GLAST/EAAT1 mRNA and protein, suggesting transcriptional regulation of the GLAST/EAAT1 promoter by these factors (Gegelashvili, Civenni et al. 1996, Swanson, Liu et al. 1997, Zelenaia and Robinson 2000, Kim, Choi et al. 2003). The role of CREB

in the involvement of EGF-mediated GLAST/EAAT1 changes was tested, but the authors suggest this phenomenon occurs independent of CREB-involvement (Schluter, Figiel et al. 2002). A recent study demonstrated the involvement of NF-κB in GLAST/EAAT1 upregulated by EGF, suggesting it serves as a positive regulation of EAAT/1GLAST expression (Karki, Kim et al. 2015). However, for the most part, the transcriptional machinery that underlies the regulation of EAAT/1GLAST by growth factors and cAMP has not been elucidated.

PACAP regulation of GLAST and GLT-1 expression

The neuronally synthesized protein pituitary adenylate cyclase-activating polypeptide (PACAP) has been implicated in neuroprotection and shown to upregulate both GLAST and GLT-1 protein expression and subsequently increase maximum velocity of glutamate uptake in cortical glial cultures. Furthermore, PKA activation was necessary during a PACAP-mediated increase in GLAST expression, whereas a PACAP-mediated increased in GLT-1 expression required both PKC and PKA activation (Figiel and Engele 2000, Goursaud, Maloteaux et al. 2008). In particular, utilization of a PACAP analogue suggested an increase in GLAST surface expression (Goursaud, Maloteaux et al. 2008). In a PACAP-type 1 receptor (PAC1) knockout mouse, a reduction in GLAST mRNA was observed in the dentate gyrus region of the hippocampus but no significant GLAST changes were observed in the cortex whereas no changes were observed in GLT-1 in any brain regions, thus suggesting differential regulation of astrocytic glutamate transporters by PACAP (Zink, Schmitt et al. 2004). Thus far, further signaling pathways related to PACAP regulation of GLAST and GLT-1 have not been elucidated; however it

is clear that GLAST is clearly regulated by PACAP whereas GLT-1 regulation may be dependent on the model system being studied.

GLT-1/EAAT2 promoter regulation by neuronal factors

Although GLT-1/EAAT2 is expressed at high levels in brain tissue, it is expressed at low levels in pure astrocyte cultures (Gegelashvili, Danbolt et al. 1997, Swanson, Liu et al. 1997, Schlag, Vondrasek et al. 1998). Co-culturing astrocytes with neurons induced expression of GLT-1/EAAT2 (Gegelashvili, Danbolt et al. 1997, Swanson, Liu et al. 1997, Schlag, Vondrasek et al. 1998). Concurrently, the astrocytes developed a stellate morphology, suggesting differentiation *in vitro* (Swanson, Liu et al. 1997, Schlag, Vondrasek et al. 1998). Independent studies observe decreases in GLT-1/EAAT2 expression following rodent glutamatergic denervation and de-afferentiation, strengthening the idea that neurons contribute to the expression of the glutamate transporters (Ginsberg, Martin et al. 1995, Levy, Lehre et al. 1995). Astroglial cultures supplemented with conditioned media from neurons or separation of the glial cultures from neurons by a semi-permeable membrane produced a similar increase in GLT-1/EAAT2 expression, without altering GLAST/EAAT1 levels (Gegelashvili, Danbolt et al. 1997). Collectively these data suggest neurons and/or neuronal factors regulate the expression of GLT-1/EAAT2.

A number of soluble factors, primarily cytokines and growth factors, have been implicated in the induction of GLT-1/EAAT2 expression. Epidermal growth factor (EGF), the transforming growth factor-α (TGF-α), and cyclic adenosine monophosphate (cAMP) analogs induced GLT-1/EAAT2 steady-state mRNA and promoter activity,

while TNF-α decreased expression, and the platelet-derived growth factor had no effect (Swanson, Liu et al. 1997, Zelenaia, Schlag et al. 2000, Su, Leszczyniecka et al. 2003, Sitcheran, Gupta et al. 2005). No effects were observed when using insulin, basic fibroblast growth factor, and nerve growth factor (Eng, Lee et al. 1997, Schlag, Vondrasek et al. 1998, Zelenaia, Schlag et al. 2000). The GLT-1/EAAT2 promoter contains a number of putative transcription factor binding sites, including NF-κB, N-myc, and NFAT (nuclear factor of activated cells) (Zelenaia, Schlag et al. 2000, Su, Leszczyniecka et al. 2003, Yamamoto and Gaynor 2004). Deletion of the site where NF-κB binds to the GLT-1/EAAT2 promoter reduced basal GLT-1/EAAT2 promoter activity as well as abolished elevations due to EGF, TGF-α, and cAMP analogs (Su, Leszczyniecka et al. 2003, Sitcheran, Gupta et al. 2005). N-myc recruitment to the GLT-1/EAAT2 promoter has been implicated as a key event in the TNF-α associated decrease in GLT-1/EAAT2 expression (Sitcheran, Gupta et al. 2005).

A number of signaling transduction pathways have been implicated or confirmed related to the alterations in GLT-1/EAAT2 expression by neuronal factors. The activation of p42/44 MAP kinases have been shown to be important for the upregulation of GLT-1/EAAT2, with neuronal conditional media resulting in the phosphorylation of transcription factors CREM-1 and ATF-1 (Swanson, Liu et al. 1997, Gegelashvili, Dehnes et al. 2000). Inhibitors of PI3K, NF-κB, and tyrosine kinase blocked an increase in GLT-1/EAAT2 mediated by neuronal conditioned media (Swanson, Liu et al. 1997, Zelenaia, Schlag et al. 2000). These data suggest numerous independent signaling pathways mediate the induction of GLT-1/EAAT2. Given the commonality in

transcription factors implicated in this mechanism, it is possible that these pathways may converge at some point along the signaling cascade.

GLT-1/EAAT2 promoter regulation by ceftriaxone

Ceftriaxone, a third-generation cephalosporin antibiotic traditionally used as an antimicrobial, was found to be a potent neurotherapeutic by enhancing GLT-1/EAAT2 expression (Rothstein, Patel et al. 2005). Although ceftriaxone recently failed to demonstrate efficacy in a stage 3 randomized, double-blind trial to treat amyotrophic lateral sclerosis (Berry, Shefner et al. 2013, Cudkowicz, Titus et al. 2014), it has been a recent promising therapeutic targeting glutamate transporter expression. Since its initial fortuitous discovery as a neuroprotective agent, numerous studies have been performed demonstrating the ability of ceftriaxone to diminish neuronal loss associated with a variety of pathological states. These include (but aren't restricted to) ceftriaxone's ability to: delay muscle loss and neuronal death in a murine model of ALS (Melzer, Meuth et al. 2008), reduce neurodegeneration and associated motor deficits in animal models of stroke (Lipski, Wan et al. 2007, Thone-Reineke, Neumann et al. 2008), alleviate chronic neuropathic pain in rodents (Hu, Li et al. 2010) and decrease post-traumatic TBI-associated seizures. Mechanistically, ceftriaxone upregulates GLT-1/EAAT2 mRNA and protein expression (Rothstein, Patel et al. 2005, Chu, Lee et al. 2007). In primary human fetal astrocytes, this increase was mediated through the NF-κB transcription factor signaling pathway. Specifically, this study found that nuclear translocation of p65 and binding of NF-κB to the -272 position of the EAAT2 promoter were necessary for EAAT2 induction mediated by ceftriaxone (Lee, Su et al. 2008). Further interest in the

transcriptional machinery regulating ceftriaxones role in effecting GLT-1/EAAT2 expression may dwindle owing to failure of late-stage clinical trials in an ALS model, however it is clearly that at least NF-κB is a key transcription factor involved in this mechanism.

GLT-1/EAAT2 promoter regulation by estrogen receptors

Exposure of primary astrocytes to estrogen resulted in significant increase in GLAST and GLT-1 mRNA and protein levels, and increased glutamate uptake (Pawlak, Brito et al. 2005). Recently, the specific mechanisms underlying these phenotypes have been elucidated. Tamoxifen and raloxifene, selective estrogen receptor modulators, and estradiol, an endogenous type of estrogen, were found to upregulate GLT-1 expression and function via TGF-α (Karki, Webb et al. 2013, Lee, Sidoryk-Wegrzynowicz et al. 2013, Karki, Smith et al. 2014, Karki, Webb et al. 2014). Specifically, tamoxifen induced the activation of PKA, which in turn phosphorylates CREB to recruit the transcription factor to the GLT-1 promoter. Inhibition of either PKA or NF-κB abolished a tamoxifen-mediated increase in GLT-1 promoter activity. The effect of tamoxifen on GLT-1 expression was completely blocked when both CREB and NF-κB binding sites were inhibited at the same time (Karki, Webb et al. 2013). Raloxifene has been shown to upregulate GLT-1 mRNA and protein via multiple signaling pathways: ERK, EGFR, and CREB. Furthermore, raloxifene induces an increase in NF-κB reporter activity and leads to direct binding of the transcription factor to the GLT-1 promoter (Karki, Webb et al. 2014). Thus, based on the ligand, it appears that a number of different pathways are important in mediating GLT-1 expression. As most of the studies delineating signaling

pathways are recent, emerging literature may shed more light on the convergence, divergence or overlap in these pathways.

Translation

Unlike EAAT1, a number of studies report alterations in EAAT2 expression in pathological states where mRNA is not disrupted, suggesting disturbances may occur at the post-transcriptional or translational level (Rothstein, Van Kammen et al. 1995, Bristol and Rothstein 1996, Masliah, Alford et al. 1996). Protein non-coding mRNA regions (UTRs) have been shown to play important roles in regulation of gene expression, and these alterations are thought to occur post-transcriptionally. In fact, UTRs regulate mRNA stability, subcellular localization, nuclear export to the cytoplasm, alternate splicing of gene products, and translational efficiency (reviewed in (Grewer, Gameiro et al. 2014)). EAAT1ex9skip, named so since it lacks the entirety of exon 9, has thus far been the only splice variant described for EAAT1 (Vallejo-Illarramendi, Domercq et al. 2005). Multiple GLT-1/EAAT2 transcripts have been identified based on differences in untranslated UTRs that result in differential splice variants of this transporter (Pines, Danbolt et al. 1992, Arriza, Fairman et al. 1994, Utsunomiya-Tate, Endou et al. 1997, Chen, Aoki et al. 2002, Schmitt, Asan et al. 2002, Rauen, Wiessner et al. 2004, Tian, Lai et al. 2007). Extracellular factors, such as corticosterone, retinoic acid and β-lactam antibiotics, have been shown to induce the translation of some of these EAAT2 transcripts, which may not occur in a constitutive manner (Tian, Lai et al. 2007). Thus, a number of splice variants exist that are differentially regulated.

Translational effects on the glutamate transporters are now serving as the basis for devising neurotherapeutics to increase EAAT2 expression and thus reduce aberrant glutamatergic neurotransmission. For example, primary astrocyte-based enzyme-linked immunosorbent assay was devised to identify compounds that serve as translational activators of EAAT2 expression (Colton, Kong et al. 2010). This study identified 61 compounds that demonstrated a dose-dependent increase in EAAT2 protein levels. Studies involving translational enhancement of transporter may serve useful in the future as increasing glutamate uptake can serve to minimize detrimental effects during pathological states.

Post-translation modifications

Phosphorylation

Phosphorylation refers to the addition of a phosphoryl group to an amino acid (typically a serine/threonine/tyrosine as they have a free hydroxyl group) onto a protein by a kinase. Functional changes in a membrane transporter may occur due to direct phosphorylation of the protein, or indirect phosphorylation of an interacting scaffolding protein or a downstream protein or kinase that may directly regulate the transporter by another mechanism. Phosphorylation of transcription factors that leads to their recruitment to the glutamate transporter promoter has been addressed in a previous section. Interestingly, the activation of PKC in Bergmann glia cells, which predominantly express GLAST, results in a reduction in GLAST-mediated glutamate uptake. Studies reported either a reduction or no alteration in membrane trafficking of GLAST upon PKC activation (Conradt and Stoffel 1997, Wang, Li et al. 2003). Mutation of the putative

PKC sites on GLAST did not reverse this decrease in transporter activity, which the authors attributed to phosphorylation of a non-consensus PKC site. However, it is also possible that PKC may be acting through another protein that regulates GLAST directly (Conradt and Stoffel 1997). Interestingly, an increase in GLT-1 mediated glutamate uptake has been observed upon application of TPA, a potent phorbol ester that activates PKC (Casado, Zafra et al. 1991). This group further demonstrated this increase in glutamate uptake was mediated via phosphorylation of a specific residue, Ser116 in an intracellular loop on GLT-1 in virally infected HeLa cells, thus upregulating glutamate uptake. Mutation of this residue resulted in a reduction in GLT-1 mediated glutamate uptake by ~50%, suggesting its importance in this process (Casado, Bendahan et al. 1993).

Glycosylation

Glycosylation refers to the enzymatic addition of a glycan to a residue on the extracellular domains of a protein. This PTM is added during maturation of nascent polypeptides in the Golgi apparatus and the endoplasmic reticulum, and can influence protein folding, endocytosis, molecular trafficking, and signal transduction (Raunser, Haase et al. 2005, Sattler and Rothstein 2006). Clinical data demonstrate decreased glycosylation for both GLAST/EAAT1 and EAAT2 proteins in the prefrontal cortex of patients with schizophrenia compared to health controls (Bauer, Haroutunian et al. 2010). Interestingly, increased mRNA expression and decreased protein expression of EAAT1 have been described in the prefrontal cortex of schizophrenic patients (Bauer, Gupta et al. 2008), suggesting reductions in glutamate uptake can contribute to disease pathology.

Since glycosylation is important for the localization and targeting of proteins, alterations may affect transporter function during schizophrenia.

In *Xenopus laevis* oocytes, GLAST was found to be glycosylated at two residues located in the extracellular loop between transmembrane helices 3 and 4 (Conradt, Storck et al. 1995). Mutation of these residues appeared to have no effect on transporter activity, measured using voltage clamp, in oocytes suggesting glycosylation at these sites in particular is not crucial for GLAST function. However, another study found that a highly glycosylated form of GLAST resulted in the re-distribution of the transporter into raft functional membrane microdomains, which improves the ability of a cell to buffer glutamate (Escartin, Brouillet et al. 2006). Thus, while it appears that particular glycosylation residues may not contribute to GLAST activity, the overall glycosylation state of the transporter may be crucial in order to uptake glutamate. Further studies are needed in order to clarify these findings.

The contribution of glycosylation in EAAT2 function paints a conflicting picture. Studies by Kalandadze et al. (Kalandadze, Wu et al. 2002) suggest a 43-amino acid region including the last trans-membrane domain and part of the C-terminus region (475-517) is important for functionality of the GLT-1 transporter in astrocytes. Furthermore, they observed that this motif contains a leucine-rich region that suppresses a downstream-arginine based motif that serves as an ER retention motif. Mutation of the leucine-rich region causes retention of the GLT-1 transporter in the ER, leading to immature unglycosylated transporter that ultimately results in aberrant trafficking and confers a dominant-negative effect over wild-type transporter (Kalandadze, Wu et al. 2004). These data suggest increases in ER retention may account for decreased membrane expression

and uptake by transporter in GLT-1 lacking glycosylation (Trotti, Aoki et al. 2001). However another study reports that glycosylation of GLT-1 did not result in differences in liposomal reconstitution, trafficking or transporter activity compared to a non-glycosylated form of the same protein, thus making it hard to reconcile a singular functional effect associated with GLT-1 glycosylation. Interestingly, in this study, GLT-1 glycosylation appeared to enhance protein stability over time *in vitro* but did not affect its ability to form heteromers, suggesting glycosylation may play a more long-term role in cellular GLT-1 expression (Raunser, Haase et al. 2005).

Ubiquitination

Ubiquitin is a 76 amino acid long post-translational modifier that is associated with protein internalization, endosomal sorting, and proteosomal or lysosomal degradation (reviewed in (Kim and Rao 2006)). Prolonged activation of PKC using phorbol esters resulted in decreased surface expression of GLT-1 (Sheldon, Gonzalez et al. 2008, Garcia-Tardon, Gonzalez-Gonzalez et al. 2012). This phenotype appears to be mediated by internalization and ubiquitination of the GLT-1 protein (Sheldon, Gonzalez et al. 2008, Martinez-Villarreal, Garcia Tardon et al. 2012). Although both the N- and C-termini of GLT-1 contain ubiquitinated lysine residues, only the C-terminus lysine residues are required for GLT-1 endocytosis (Gonzalez-Gonzalez, Garcia-Tardon et al. 2008, Sheldon, Gonzalez et al. 2008). Although reports examining the contribution of ubiquitin as a post-translational modifier of glutamate transporter activity are limited, these findings suggest it may play an important role in activity-dependent alterations in glutamate uptake.

Sumoylation

SUMOylation is a PTM wherein a Small Ubiqutin-like Modifier (SUMO) is covalently attached or detached to lysine residues in target proteins, resulting in cellular changes, ranging from protein stability to transcriptional regulation and cell cycle progression. Initial findings in a mouse model of ALS suggested that SUMO1-conjugated C-terminal fragments of EAAT2 were associated with motor neuron impairment. Furthermore, SUMO-conjugated proteolytic fragment of EAAT2 was found to accumulate in the nucleus of spinal cord astrocytes (Foran, Bogush et al. 2011). However, a recent study has suggested that sumoylation of EAAT2 govern its intracellular compartmentalization and are not driven by pathological mechanisms. This idea is further strengthened by the presence of non-sumoylated EAAT2 on the plasma membrane (Foran, Rosenblum et al. 2014), lending credence to the idea that sumoylation of EAAT2 drives it to traffick intracellularly.

Binding

Glutamate transporter activity can be affected by glutamate transporter associated proteins, or GTRAPs. In fact, GTRAP41 and GTRAP48 increase the V_{max} of glutamate uptake via EAAT4 (Jackson, Song et al. 2001). Furthermore, EAAC1 has been shown to interact with GTRAP3-18 (Lin, Orlov et al. 2001), decreasing the affinity of EAAC1 for glutamate (from 9μM to 40μM) and reducing EAAC1 mediated glutamate transporter activity. GTRAP3-18, a binding partner of EAAT3, is a regulator of ER trafficking and thus plays a role in transporter maturation (Ruggiero, Liu et al. 2008).

Recently, Underhill et al. observed that calcium/calmodulin-dependent protein kinase phosphorylation of Discs large homolog 1 (DLG1), a PDZ protein, decreased surface expression of a splice variant of EAAT2 in both Madin-Darby canine kidney cells and cultured astrocytes. The authors propose a mechanism whereby DLG1 phosphorylation disrupts its PDZ-mediated interaction and its function as a scaffold, thus preventing stabilization of EAAT2 at the cell surface (Underhill, Wheeler et al. 2015). Thus, it appears that signaling changes associated with proteins that interact with or stabilize the glutamate transporters can disrupt either localization and/or activity of glutamate transporters.

Trafficking

The rate of membrane protein recycling is dependent on the rate of transporter delivery and re-internalization to and from the plasma membrane. Thus, at steady-state, if the rate of delivery to the plasma membrane is greater than the rate of internalization, then there will be an increase in the number of transporters at the plasma membrane. Conversely, if the rate of internalization is greater than the rate of delivery, then there will be a decrease in the number of transporters at the plasma membrane. Thus, rapid alterations in glutamate transporter activity are dependent on an intracellular pool of transporters.

The most extensively studied glutamate transporter with regards to trafficking is the neuronal transporter EAAC1/EAAT3, which has been shown to reside primarily in intracellular pools (Kalandadze, Wu et al. 2002). However, for the purposes of this dissertation I will continue focusing on GLAST/EAAT1 and GLT-1/EAAT2, the

predominant glutamate transporter in astrocytes. The primary literature is, however, not in agreement about the basal localization of astrocytic glutamate transporters. Some studies suggest 60-80% of total transporter immunoreactivity (both GLAST/EAAT1 and GLT-1/EAAT2) is localized to the cell surface in both primary astrocytes and mixed astroglial/neuronal cultures (Sclag 1998, Kalandadze 2002, Susarla 2004). However, other reports suggest that both these transporters do not exhibit intracellular immunoreactivity rodent brain slices, providing evidence that the transporter resides exclusively at the cell surface (Chaudhry, Lehre et al. 1995, Lehre, Levy et al. 1995). Yet other studies observe that GLAST and GLT-1 are found intracellularly (Duan, Anderson et al. 1999, Guillet, Velly et al. 2005). One possibility that arises from these findings is that the distinction between intracellular (or located in a cellular compartment) compared to in the plasma membrane may not be quite as straightforward as one would imagine- the transporters may residue just under the surface of the plasma membrane, making them rapidly accessible at the membrane when needed but not detectable in an intracellular compartment. Furthermore, it is conceivable that the localization of GLAST/EAAT1 and GLT-1/EAAT2 may depend on the model system, cell type, and even the culture or treatment conditions. While complex mechanisms may underlie these observations, what is clear is that numerous signaling pathways regulate trafficking of these transporters to and from the plasma membrane, rapidly altering glutamate transporter activity.

Trafficking of GLAST/EAAT1

GLAST/EAAT1 has been reported to rapidly traffick into and out of the membrane. Although thus far we have focused on the transcriptional regulation of

GLAST expression by glutamate, these changes occur in the order of a few hours. A number of independent studies report rapid increases (>30 mins) in GLAST trafficking to the membrane as the mechanism underlying augmented glutamate uptake as a result of glutamate addition. In particular, glutamate appears to increase the cell-surface expression of GLAST in primary mouse astrocyte cultures (Duan, Anderson et al. 1999). This time and dose-dependent increase in glutamate-induced glutamate uptake was attributed to actin polymerization, as inhibitors of actin polymerization reversed this effect. An additional study reported that the presence of neurons affects the glutamate uptake capacity of GLAST and GLT-1 (Poitry-Yamate, Vutskits et al. 2002). Specifically, this study demonstrates that the release of neuronal glutamate results in an increase clustering of GLAST in astrocyte cultures as measuring by immunofluorescence. Importantly, within a matter of a few minutes, GLAST trafficks to the membrane and results in increased glutamate uptake, providing evidence for an activity-dependent and substrate-induced regulation of transporter activity. Transcriptional data suggests that GLAST is not retained at the membrane, and over time decreased GLAST expression may be modulated by transcription factors.

Furthermore, signaling molecules have been implicated in alterations in GLAST/EAAT1 surface expression. For example, PKC activation has been shown to increase glutamate uptake in cortical astrocytic cultures from the rat forebrain (Casado, Zafra et al. 1991, Guillet, Velly et al. 2005) a model system where glutamate uptake is primarily mediated by GLAST/EAAT1 unless GLT-1/EAAT2 expression is induced (Gegelashvili, Danbolt et al. 1997, Gegelashvili and Schousboe 1997). However, this particular study did not examine surface expression of the glutamate transporters, as at

the time the molecular identity underlying astrocytic glutamate transport was unknown. Subsequent studies examined the phenotype underlying PKC regulation of glutamate transporters. A PKC-induced increase in glutamate uptake was attributed to increased cell surface expression of GLAST/EAAT1 in a neuron-enriched astrocyte culture (Guillet, Velly et al. 2005). However, linking surface expression of the glutamate transporter to alterations in glutamate uptake were not always straightforward. In primary astrocyte cultures derived from the forebrain, although a similar increase in glutamate uptake was observed upon PKC activation, an unexpected decrease in both total lysate and surface fraction of GLAST/EAAT1 was reported, measured by biotinylation experiments (Susarla, Seal et al. 2004). Although the authors attribute these findings to the masking of GLAST/EAAT1 epitopes as a result of PKC activation, it is possible that GLAST/EAAT1 may be rapidly re-distributed into intracellular compartments that are inaccessible by the antibodies used in the study. In addition, it appears that in different culture systems, PKC activation may have varied effects on GLAST-mediated glutamate uptake. For example, in primary cultures of retinal Muller cells, which express primarily GLAST/EAAT1, a decrease in glutamate uptake and GLAST surface expression was observed upon PKC activation (Wang, Li et al. 2003). It is clear that PKC regulation of glutamate transport is thus a complex phenomenon. One possible explanation of these data is that in cultures where GLAST/EAAT1 is the primary glutamate transporter, PKC may result in a decrease and not an increase in glutamate uptake, as is observed in mixed cultures that express both GLAST/EAAT1 and GLT-1/EAAT2. Thus, the type of cell used and supporting culture conditions are important when considering the effect of a signaling pathway on glutamate uptake. Regardless of the interpretation underlying

differential phenotypes related to GLAST/EAAT1 trafficking, numerous studies suggest that the protein can be rapidly inserted into and removed from the membrane.

Trafficking of GLT-1/EAAT2

PKC activation of GLT-1/EAAT2 appears to consistently decrease cell surface expression and activity of the transporter, whether it is in a mixed neuron/astrocyte culture system or in transfected glioma cells (Kalandadze, Wu et al. 2002, Zhou and Sutherland 2004, Guillet, Velly et al. 2005). In particular, a 43-amino acid region in the C-terminus of GLT-1/EAAT2 was found to be important for PKC-mediated re-distribution of the transporter (Kalandadze, Wu et al. 2002). Mutation of serine 486 in this domain, however, only partially prevented transporter internalization and a reduction in phosphorylation of the transporter was not detected in the mutant transporter. These data suggest that while phosphorylation may play a role in the rapid trafficking of GLT-1/EAAT2 from the membrane, other potential phosphorylation sites or an additional method of regulation not identified by this study are important for the PKC-dependent re-distribution of GLT-1/EAAT2.

A recent study demonstrates that a splice variant of EAAT2 referred to as EAAT2b can be rapidly trafficked to the membrane as a result of glutamate-induced calcium signaling (Underhill, Wheeler et al. 2015). This study implicated the stabilization of EAAT2 by a scaffolding protein DLG1, which upon phosphorylation by CaMKII can rapidly internalize the transporter from the membrane. In support of this finding, the neuronal release of glutamate leads to the formation of GLT-1 clusters that localize near neurons (Poitry-Yamate, Vutskits et al. 2002). A recent report suggests rapid alterations

in synaptic activity affect the trafficking of GLT-1/EAAT2 to the membrane and shape fast synaptic transmission, which may provide a basis for the ability of GLT-1/EAAT2 appears to rapidly re-distribute in an activity-dependent manner (Murphy-Royal, Dupuis et al. 2015).

It is evident that both the astrocytic glutamate transporters can thus be regulated in various ways, by the addition of extracellular and modifying intracellular signaling molecules or transcription factors, in order to affect glutamate uptake. Thus far, the majority of this dissertation has dealt with the EAATs in a physiological context. In order to fully understand their role in the CNS, we must next address transporter function in a pathological context.

Pathological dysregulation of the astrocytic glutamate transporters

Ischemia

Thermodynamically favorable ionic flux associated with the astrocytic glutamate transporters results in the influx of 1 glutamate$^-$, 3 Na^+ molecules and 1 H^+, while a K^+ ion is counter-ported. However, during conditions of ischemia glutamate transporter reversal has been described, leading to elevations in extracellular glutamate levels due to the release of glutamate instead of uptake by astrocytes (Phillis, Ren et al. 2000). This phenotype contributes to an increase in the concentration of extracellular glutamate, and increased excitotoxicity via the aberrant activation of glutamate receptors.

In addition, a number of studies have reported decreased expression of GLT-1 without observing changes in GLAST after cerebral ischemia in rodents, both at the level

of mRNA as well as protein (Bruhn, Levy et al. 2000, Yeh, Hwang et al. 2005, Ketheeswaranathan, Turner et al. 2011). Furthermore, decreases in GLT-1 protein have been reported following an hour of oxygen-glucose deprivation in hippocampal astrocyte cultures (Ouyang, Voloboueva et al. 2007). Combinatorial changes such as these likely contribute to diminished glutamate clearance by astrocytes during ischemic conditions.

Traumatic brain injury

In traumatic brain injury (TBI), increases in extracellular glutamate in the brain and spinal cord have been reported in both animal models and humans. Not surprisingly, a reduction in both GLAST and GLT-1 protein levels has been reported following controlled cortical impact in the hippocampus of rodents, concomitant with a decrease in radiolabeled aspartate uptake (Rao, Baskaya et al. 1998), suggesting the function of both astrocytic proteins is dysregulated during TBI. Additionally, knockdown of GLT-1 using antisense oligodeoxynucleotides has been shown to exacerbate neuronal death associated with a controlled cortical impact model of injury (Rao, Dogan et al. 2001). In a study involving TBI patients, a significant reduction in both astrocytic EAAT1 and EAAT2 levels was observed, which was attributed to astrocyte degeneration and downregulated protein in surviving astrocytes (van Landeghem, Weiss et al. 2006). Thus, the effects of TBI thus far appear to be chronically regulated as they primarily ascribe a reduction in transporter function to reductions in protein levels. Glutamate transporter reversal, a key mechanism of glutamate extrusion from astrocytes during pathological conditions, has not been described during TBI to date (Yi and Hazell 2006).

Alzheimer's disease

Alzheimer's disease, a chronic neurodegenerative condition marked by the formation of neurofibrillary plaques and tangles, is associated with excitotoxic neuronal death, decreased glutamate transporter expression and a reduction in glutamate uptake (Mookherjee, Green et al. 2011). A reduction in glutamate transporter uptake was correlated with cognitive decline (Masliah, Alford et al. 1996), and a restoration of a glial glutamate transporter in a mouse model increased synaptic integrity and benefited cognitive function (Takahashi, Kong et al. 2015). Reduced expression of GLAST and GLT-1 have been reported in brain tissue from at least two separate murine models of Alzheimer's disease, resulting in alterations in glutamatergic neurotransmission (Schallier, Smolders et al. 2011, Cassano, Serviddio et al. 2012).

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a chronic neurodegenerative disease characterized by upper and lower motor neuron degeneration (Rothstein, Tsai et al. 1990). A 100-200% increase in extracellular glutamate concentrations has been reported in the cerebrospinal fluid of patients with ALS (Rothstein, Tsai et al. 1990). Specifically, high-affinity, sodium-dependent transport was decreased in synaptosomal preparations from the spinal cord, motor, and somatosensory cortices in patients with ALS (Rothstein, Martin et al. 1992). Selective loss of the astrocytic glutamate transporter GLT-1 has been implicated as the primary molecular defect accounting for dysregulated glutamate clearance and increased extracellular glutamate in the ALS pathology (Rothstein, Van Kammen et al. 1995, Howland, Liu et al. 2002).

Thus, dysregulated glutamatergic neurotransmission and in particular, glutamate uptake, have been described in numerous neurodegenerative conditions. While it is currently unknown what mechanisms underlie these disease states, a common theme in all of these pathological states is aberrant calcium signaling. Next I explore the role of astrocytic calcium to identify potential mechanism that may contribute to the regulation of astrocytic glutamate uptake.

Calcium signaling in astrocytes

The ability of the neurotransmitter glutamate to induce astrocytic calcium oscillations and calcium waves was first observed within and among cultured astrocytes (Cornell-Bell, Finkbeiner et al. 1990, Charles, Merrill et al. 1991). Astrocytes are complex cells, and thus the sources of calcium oscillations and waves are numerous and are still being explored. Briefly, both ligand- and voltage-gated Ca^{2+} ion channels have been implicated in direct calcium influx into astrocytes. Furthermore, channels such as the TRP and Orai channels have been suggested to allow calcium entry as well (Shigetomi, Patel et al. 2016). Activation of astrocytic class I metabotropic receptors resulting in the opening of IP3-sensitive internal stores and 'spiked' calcium release, resulting in rapid propagation of a calcium response intercellularly via gap junctions (Kim, Rioult et al. 1994, Kawabata, Tsutsumi et al. 1996). Furthermore, this metabotropic response can also result in calcium oscillations of varied frequencies and amplitudes that are dependent on extracellular calcium (Kim, Rioult et al. 1994). Additionally, intracellular organelles and the mitochondria have been implicated in intracellular calcium release (Shigetomi, Patel et al. 2016). A number of signaling

molecules have since been shown to elevate astrocytic calcium in glia in brain slices: norepinephrine, GABA, acetylcholine, adenosine, ATP, and histamine (reviewed in (Haydon and Carmignoto 2006)). It is thus clear that there are numerous signaling methods that can result in the rise of intracellular calcium.

CaMKII Structure

The activation of intracellular calcium in an astrocyte using both glutamate and ionomycin has resulted in the activation of the calcium/calmodulin dependent kinase (CaMKII), a key calcium-dependent protein kinase (Yano, Fukunaga et al. 1994, Oguri, Inoko et al. 2006). CaMKII is a multifunctional serine/threonine protein kinase that comprises an N-terminal catalytic domain, an autoregulatory domain, a linker region, and a C-terminal association/hub domain. The catalytic domain of CaMKII contains an ATP binding pocket, the catalytic cleft, and the substrate targeting groove. In a basal state, the autoregulatory domain lies across the catalytic cleft, serving to inhibit CaMKII activity in the absence of calcium/calmodulin (Colbran, Schworer et al. 1989). Binding of the calcium-bound calmodulin protein to the C-terminal region of the autoregulatory domain displaces the autoregulatory domain from the catalytic cleft thus allowing ATP and substrate binding to occur (Colbran, Smith et al. 1989).

The association or hub domains within each CaMKII subunit interact to form the hexagonal ring-like structure from which arm-like structures, or the catalytic heads of each subunit, radiate (Woodgett, Davison et al. 1983, Kanaseki, Ikeuchi et al. 1991). Resolving the crystal structure for human αCaMKII provided stronger evidence for this unique dodecameric organization of the kinase, with two hexameric rings stacked on top

of one another (Chao, Stratton et al. 2011). Eight to twelve individual subunits, each ~54 to 72 kDa in size, come together to form the CaMKII holoenzyme, a protein ~600kDa in size (Kuret and Schulman 1984).

A number of different ideas have been proposed that could underlie the multimeric nature of the enzyme. Different isoforms (addressed in greater detail in the next section) assembling into the same complex produce a composite holoenzyme that can display various combinations of intermediate calcium sensitivities and calcium spike frequency dependence. Furthermore, cellular localization of CaMKII could require domains created by association domain assembly. Additionally, co-assembly of multiple isoforms or splice variants can produce distinct localization based on different targeting sequences.

CaMKII Expression

Four isoforms α, β, γ, and δ CaMKII encoded by closely related genes have thus far been described for CaMKII. 30 splice variants of mammalian CaMKII exist. The molecular weight of the isoforms ranges from 54 to 72 kDa. Alignment of the 4 CaMKII isoforms results in >60% homology, with the catalytic domains in particular being highly conserved (>95%). Most unique among the isoforms and different associated splice variants is the variable domain region, part of the hub domain. These variable regions contain different substrate binding and cellular localization motifs (reviewed in (Soderling, Chang et al. 2001, Bradshaw, Hudmon et al. 2002, Hudmon and Schulman 2002, Colbran and Brown 2004)).

CaMKII isoforms are expressed in a cellular, tissue, and sub-cellular specific manner (Hudmon and Schulman 2002). While αCaMKII and βCaMKII are primarily localized in brain tissue (Erondu and Kennedy 1985, Lucchesi, Mizuno et al. 2011), expression of αCaMKII has been reported in skeletal muscle, while βCaMKII is found in immune cells. δCaMKII and γCaMKII are present in most tissues, however δCaMKII predominates in cardiomyocytes, and γCaMKII is found in the lungs (Tobimatsu and Fujisawa 1989). In the central nervous system, αCaMKII and βCaMKII have been reported to predominate in neurons whereas δCaMKII and γCaMKII, while present in neurons albeit at much lower levels, have been reported to predominate in astrocytes (Ouimet, McGuinness et al. 1984, Bayer, Lohler et al. 1999). In the brain, αCaMKII has been reported to predominate cortically whereas βCaMKII is expressed at higher levels in the cerebellum (McGuinness, Lai et al. 1985). In addition, based on different cellular localization motifs, splice variants of the same CaMKII isoform can target to distinctive cellular compartments. For example, splice variants of some CaMKII isoforms can contain a nuclear localization sequence (NLS), as in the case of δ_B CaMKII (Zhang, Johnson et al. 2002). An NLS allows for translocation of CaMKII to the nucleus, where CaMKII is hypothesized to play a role in calcium-mediated gene transcription.

Activation of CaMKII by Ca^{2+}/CaM

As mentioned previously, the autoinhibitory domain of CaMKII is tightly bound to the catalytic region and serves as a form of autoinhibition when calcium/calmodulin are not present. Thus, basal kinase activity levels remain at <1000 fold below their maximal calcium/calmodulin-stimulated activity. At a biochemical level, it has been

observed that the autoinhibitory domain of CaMKII contains residues that mimic a protein substrate interaction with the catalytic face of the kinase, thus deeming this a pseudosubstrate interaction. In this conformation, neither the ATP-binding pocket nor the substrate-binding pocket is available for their respective binding partners. The autoinhibitory region is N-terminal and contiguous to the calmodulin-binding sequence on CaMKII. Calcium/calmodulin binding to the autoregulatory domain exposes the catalytic cleft of the kinase, as suggested by the crystal structure of one active catalytic/autoregulatory domain. While no crystal structure exists for an activated form of the CaMKII holoenzyme, the crystal structure of the inactive holoenzyme suggests a drastic structural rearrangement needs to occur in order for calmodulin to bind with its binding site on CaMKII, buried deep within the CaMKII 3D structure (Hoffman, Stein et al. 2011). CaM binding occurs in an antiparallel and temporally distinct fashion, with the N-terminal lobe of CaM first forming a contact with the autoregulatory domain (C-terminus) of CaMKII. If nucleotides are present, the C-terminal lobe will flex and bind to the N-terminal portion of CaMKII (Evans and Shea 2009, Jama, Gabriel et al. 2011). Interestingly, the N-lobe of CaM has been shown to partially activate the kinase, whereas the C-lobe of CaM is not required for CaMKII activation. Interestingly, in the presence of ATP, once 'activated', CaMKII's affinity for bound Ca^{2+}/CaM is enhanced by 1000-fold, from ~20nM to ~20pM (Meyer, Hanson et al. 1992, Forest, Swulius et al. 2008).

CaMKII Autophosphorylation

The autoregulatory domain of CaMKII contains a classical CaMKII consensus sequence (R-X-X-S/T), with Thr286 (in the α isoform, 287 in β, γ, and δ isoforms) being

the potential phosphorylation residue. Following CaMKII activation, CaMKII phosphorylates this residue (Lou and Schulman 1989). In fact, the unique dodecameric structure of CaMKII allows for each subunit of a holoenzyme to be in close proximity with another, and thus this phosphorylation event has been described to be an 'intersubunit autophosphorylation' event (Mukherji and Soderling 1994, Bradshaw, Hudmon et al. 2002). Once CaMKII is autophosphorylated at Thr286, its activity becomes Ca^{2+}/CaM-independent (Miller and Kennedy 1986, Lou and Schulman 1989). Thus, even when intracellular calcium returns to basal levels, CaMKII can function free from its normal stimulus in an autonomous state. Secondly, the affinity of CaMKII for CaM increases by 1000-fold. While the functional consequence of this CaM 'trapping' state has not been elucidated, it is possible that CaM-related cellular signaling might be altered due to its sequestration by CaMKII. Furthermore, once Thr286 is phosphorylated and Ca2+/CaM dissociate, the kinase is capable of autophosphorylating itself at Thr305/306 in the CaM-binding domain which can prevent further Ca^{2+}/CaM activation of the kinase.

The extent of a cells' autonomous activity is important as 'active' CaMKII serves as a molecular memory device for calcium signals. Ca^{2+}/CaM-independent activity as a function of Ca^{2+}/CaM-dependent activity is experimentally measured to determine % autonomy (Saitoh and Schwartz 1985). CaMKII autophosphorylation is important in a cellular context, an idea strengthened by data provided by CaMKII mutations. Thr286Ala, a mutation that prevents autonomous activity (Fong, Taylor et al. 1989), disrupts the ability of CaMKII to trap CaM (Meyer, Hanson et al. 1992). Interestingly, this mutant is capable of substrate phosphorylation due to Ca^{2+}/CaM activation (Fong,

Taylor et al. 1989) and can target to cellular compartments comparable to wild-type kinase (Shen, Teruel et al. 2000). However, Thr286Ala targets substrates in a weaker and more transient manner compared to wild-type kinase (Shen, Teruel et al. 2000, Leonard, Bayer et al. 2002). It appears that while autophosphorylation is a critical regulator of CaMKII activity, it is not necessary for substrate phosphorylation.

CaMKII Inactivation

Thr286 autophosphorylation, which is a determinant of CaMKII activity in the cell, can be reversed by a few ways. Firstly, high ADP can dephosphorylate CaMKII thus reducing autonomous activity (Kim, Hudmon et al. 2001). Second, a number of protein phosphatases in the cell, such as Ca^{2+}/CaM protein phosphatase, protein phosphatase 1, 2a and 2c, have been shown to dephosphorylate Thr286 (Fukunaga, Kobayashi et al. 1993, Strack, Barban et al. 1997).

As discussed previously, phosphorylation at Thr305/306 prevents $Ca2^{+}$/CaM binding to CaMKII thus serving as a mechanism to inactivate CaMKII. Furthermore, the activation of CaMKII under conditions of limited ATP and low pH induces inactivation of the kinase (Colbran 1993, Hudmon, Aronowski et al. 1996), a finding that is exacerbated under conditions of elevated temperatures (Hudmon, Aronowski et al. 1996). Lastly, CaMKII self-association, an activity-dependent form of aggregation involving the association and aggregation of CaMKII holoenzymes with one another, results in CaMKII inactivation. Thus, a number of different cellular events can lead to the inactivation of CaMKII.

CaMKII signaling in astrocytes

Unlike neurons, a better understanding of the functional role of astrocytic CaMKII is limited by our knowledge of substrates in this type of glial cell. CaMKII has been implicated in a migratory phenotype in both astrocytes and glioma cells. In glioma cells in particular, a migratory phenotype is associated with individual glioma cells migrating into and invading surrounding cells. Migratory gliomas are challenging to treat- even following resection, as they often reconstitute a tumor rapidly (Aubert, Badoual et al. 2008). CaMKII interaction and phosphorylation of ClC-3, a chloride channel and regulator of cell volume, results in ClC-3 activation and an increase in the migratory phenotype in glioma cells (Cuddapah and Sontheimer 2010, Cuddapah, Turner et al. 2013). This finding was confirmed using inhibitors and shRNA for both CaMKII and ClC-3, and observing a subsequent reduction in cell migration. Furthermore, CaMKII has also been implicated in the regulation of glioma cell migration by direct interaction with ASIC1, an acid sensing cation channel (Sun, Zhao et al. 2013). In this case, binding has been suggested as a potential mechanism whereby CaMKII regulates ASIC1 currents, thus leading to migration (Sun, Zhao et al. 2013). In rat brain astrocytes, CaMKII has been implicated in the expression of matrix mettaloproteinase-9 expression (MMP-9), proteinases that are upregulated during pathological conditions of inflammation and disease. Furthermore, this CaMKII-dependent upregulation of MMP-9 has been shown to correlate with an increased migratory phenotype (Wang, Hsieh et al. 2010, Lin, Lee et al. 2013). Thus, the action of CaMKII on numerous channels or proteins suggests it potentiates migration in both astrocytes and glioma cells, which may be beneficial during

the formation of a glial scar during pathological states or detrimental during an increasingly cancerous phenotype, as observed in glioblastoma.

CaMKII has also been implicated in glutamate uptake by astrocytes. Glutamate application to astrocytes has been shown to result in increased calcium signaling and CaMKII activity (Cornell-Bell, Finkbeiner et al. 1990, Kim, Rioult et al. 1994). Our lab previously reported that pharmacological inhibition of CaMKII results in a reduction in glutamate uptake in cultured rodent cortical astrocytes (Ashpole, Chawla et al. 2013). Underhill et al. report that CaMKII phosphorylates DLG1, a PDZ-protein, in turn de-stabilizing the interaction between DLG1 and EAAT2b, a splice variant of the GLT-1/EAAT2 glutamate transporter. The functional consequence of this phosphorylation is a reduction in EAAT2b cell surface expression, although glutamate uptake was not reported (Underhill, Wheeler et al. 2015). Although advances in CaMKII regulation of astrocytic glutamate transporters are recent, this field is important as astrocytic glutamate uptake is the primary mechanism of extracellular glutamate clearance in the brain.

Connexins are gap junctions that serve as regulators of intracellular ionic concentrations between cells. These hemichannels are particular important in astrocytes, as they connect astrocytes and allow calcium ions to pass between cells, resulting in a wave. CaMKII has been shown to phosphorylate connexin43, a family of connexin channels present on astrocytes, resulting in a decrease in the open channel probability of these gap junctions (Dermietzel, Traub et al. 1989, Huang, Laing et al. 2011, Xu, Kopp et al. 2012). Recently, astrocytic oxygen-glucose deprivation/reperfusion (OGD/R) was shown to decrease neurite outgrowth (Wu, Yu et al. 2015). Increased calcium signaling, subsequent CaMKII/CREB signaling, increased ephrin-A4 and decreased connexin43

expression in astrocytes as a result of OGD-R were associated with this phenotype. These data suggest that CaMKII regulates Connexin43 to maintain calcium flux from one cell to another. A loss of CaMKII activity may result in aberrant calcium signaling, as increased calcium dispersion may occur between unstimulated astrocytes.

The cellular cytoskeleton can be linked to a multitude of functions, such as cell shape, resistance, migration, and the support of numerous other functions such as organelle and protein trafficking, and neurotransmitter release. These proteins can assemble and disassemble based on various stimuli and cellular signals. CaMKII has been shown to regulate a number of cytoskeletal proteins in both neurons and astrocytes (Fink, Bayer et al. 2003, Tsui and Malenka 2006). In astrocytes, CaMKII has been shown to phosphorylate the cytoskeletal protein, vimentin and result in the disassembly of vimentin filaments (Inagaki, Nishizawa et al. 2000, Oguri, Inoko et al. 2006), which can impact cytoskeletal functions such as cell structure and migration. Furthermore, CaMKII has been implicated in the phosphorylation of GFAP, a cytoskeletal protein marker of mature and reactive astrocytes (Leal, Goncalves et al. 1997, Frizzo, Tramontina et al. 2004), although the functional relevance of this phosphorylation is not fully understood. As described previously, astrocytic CaMKII phosphorylation of a scaffolding protein DLG1 resulted in altered membrane trafficking of a splice variant of the glutamate transporter EAAT2, thus decreasing glutamate uptake (Underhill, Wheeler et al. 2015). Thus, scaffolding proteins are capable of regulating a number of functions, and CaMKII regulation of these proteins can be essential to maintain physiological function.

In sum, it appears that calcium signaling is crucial for a variety of functions in the astrocyte. Our understanding of the molecular mechanisms underlying glutamate uptake,

a key role performed by astrocytes, is limited. It is known that EAAT1 and EAAT2 are the primary molecular players governing an astrocytes' ability to clear extracellular glutamate. We chose to focus on astrocytic glutamate transporter regulation by a key calcium-regulated enzyme, CaMKII. Our studies suggest the involvement of a calcium-dependent mechanism in maintaining control of extracellular glutamate in the central nervous system.

RESEARCH GOALS

Ultimately, a better understanding of the regulation underlying astrocytic glutamate uptake improves our grasp of a key functional component of astrocyte physiology. I had previously established a link between a CaMKII activity and glutamate uptake in an astrocyte culture model. The purpose of this dissertation was to focus on which isoform of glutamate transporter was regulated by CaMKII, and addressing whether phosphorylation served as a potential mechanism of regulation.

In order to address this, I will express EAAT1 and EAAT2 in a heterologous system in order to delineate isoform-specific regulation. Furthermore, I will test the role of different CaMKII states in this regulation. Lastly, I will test the role of direct phosphorylation being associated with functional changes observed upon altering CaMKII activity. These experiments will elucidate a regulatory mechanism whereby CaMKII controls glutamate uptake via either one of both astrocytic glutamate transporters, and expand our understanding of CaMKII substrates and function in astrocytes.

MATERIALS AND METHODS

Materials

Glutamic acid, glycine, polyethylenimine (PEI) and DL-threo-β-benzyoxyaspartate (TBOA) were purchased from Sigma. 2-Amino-5,6,7,8-tetrahydro-4-(4-methoxyphenyl)-7-(naphthalen-1-yl)-5-oxo-4*H*-chromene-3-carbonitrile (UCPH-101) was obtained from Abcam. Autocamtide-2 (KKALRRQETVDAL), Tat-CN21 (YGRKKRRQRRRKRPPKLGQIGRSKRVVIEDDR) and tat-CN21Ala (YGRKKRRQRRRKAPAKAAQAAASKRVVIEDDR) were synthesized by Biopeptide Co. Inc (San Diego, CA). Lipofectamine-2000 was purchased from Invitrogen. Protease inhibitors, KN-92 and KN-93 were purchased from EMD Millipore. Radiolabeled [^{3}H]-glutamate was obtained from American Radiolabeled Chemicals and [γ-^{32}]-ATP was obtained from Perkin-Elmer.

cDNA constructs

cDNA for human EAAT1 and EAAT2 (obtained from Addgene in a pCMV5 backbone; a generous gift from Susan Amara at the National Institute of Mental Health) were subcloned into Creator-based acceptor vectors (generous gift from Dr Clark Wells at Indiana University) for mammalian expression with the N-terminal tag mCherry and a gene for ampicillin resistance as previously described (Adler, Johnson et al. 2013). For GST-fusion protein experiments, N- and C-termini of EAAT1 and EAAT2 were sub-cloned into PGEX vectors containing a GST tag. The cDNA for human δCaMKII was described previously (Ashpole, Herren et al. 2012). It was subcloned into a Creator-based vector with a YFP tag.

Site-directed mutagenesis

Site-directed mutagenesis (Agilent Technologies) was used to introduce point mutations into the cDNA for CaMKII or EAAT1. Briefly, PCR reaction mix was made according to the manufacturer's instructions. An overnight PCR reaction was performed using the following settings: Initial denaturation (95°C, 2 mins), 18 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min), and extension (68°C, 2.5 min/Kb), followed by a 7 minute elongation step at 68°C. Dpn1 was added to the PCR product for 2 hours at 37°C to digest methylated DNA. DNA was then transformed into XL-10 Gold Ultracompetent cells. Primers used for site-directed mutagenesis are annotated in Table 2. Double mutations were carried out sequentially. All mutations were verified by gene sequencing (Eurofins MWG Operon).

Cell lines

A172s, a glioblastoma cell line, and human embryonic kidney (HEK293Ts) were obtained from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5g/L glucose, 29.2 µg/mL L-glutamine, 10% heat inactivated fetal bovine serum, penicillin (10 units/mL), and streptomycin (10 µg/mL) at 37°C in 5% CO_2 as previously described (Hudmon, Lebel et al. 2005, Ashpole, Herren et al. 2012). Cells were washed at confluency with phosphate-buffered saline at room temperature, and passaged using 0.05% trypsin (Invitrogen).

cDNA	Mutation	Primer Sequence (5' to 3')
δCaMKII	Thr287Asp	atttcttcaagcagtctacatcctcctgtctgtgcatcatgg
δCaMKII	Thr287Asp	ccatgatgcacagacaggaggatgtagactgcttgaagaaat
δCaMKII	Thr287Val	atttcttcaagcagtctacaacctcctgtctgtgcatcatgg
δCaMKII	Thr287Val	ccatgatgcacagacaggaggttgtagactgcttgaagaaat
δCaMKII	Asp136Asn	attctcaggcttcaggttcctgtgaactatgccat
δCaMKII	Asp136Asn	atggcatagttcacaggaacctgaagcctgagaat
EAAT1	Thr26Ala	ttcttggccaaaagtgcgcgtttacggactccc
EAAT1	Thr26Ala	gggagtccgtaaacgcgcacttttggccaagaa
EAAT1	Thr26Asp	cactttcttcttggccaaaagatcgcgtttacggactccctgctg
EAAT1	Thr26Asp	cagcagggagtccgtaaacgcgatcttttggccaagaagaaagtg
EAAT1	Thr37Ala	gtaacttttaacatcctcctttgcaatgttctgcactttcttcttgg
EAAT1	Thr37Ala	ccaagaagaaagtgcagaacattgcaaaggaggatgttaaaagttac
EAAT1	Thr37Asp	cacacttttggccaagaagaaagtgcagaacattgataaggaggatgttaaaagtta
EAAT1	Thr37Asp	taacttttaacatcctccttatcaatgttctgcactttcttcttggccaaaagtgtg

Astrocyte cultures

Cortical astrocyte cultures were derived from postnatal day 1-2 Sprague-Dawley rat pups as per approved IACUC guidelines using methods previously established (McCarthy and de Vellis 1980, Ashpole, Chawla et al. 2013). Briefly, rodent cortices were removed from the cranium following rapid decapitation, and the meninges and blood vessels were cleaned off. Tissue was digested using papain, triturated, and re-suspended in growth media (DMEM containing 2% NuSerum, 4.5g/L glucose, 29.2 µg/mL L-glutamine, NS-21 supplement, 10 units/mL penicillin, and 10 µg/mL streptomycin) at a density of 2.5 million cells/mL and seeded on poly-D-lysine (PDL, 50 µg/mL) coated 10 cm dishes. Half the media was replaced every 3-4 days with fresh growth media. Cells were split at confluency (~7-8 days *in vitro*) using 0.1% trypsin, shaking the dishes to remove oligodendrocytes, and seeded on PDL-coated 12 mm coverslips for immunocytochemistry or on PDL-coated 35 mm dishes for [^{3}H]-glutamate uptake assays. Cultures were maintained at 37°C in 5% CO_2.

Transient transfections

A172s and HEK293Ts were transfected at 50-60% confluency with 2.5 µg cDNA per 35 mm dish. Transfection reagents Lipofectamine-2000 (10uL per 35 mm dish) and PEI (2mg/mL) were utilized as the transfection reagents for A172s and HEK293Ts respectively, in serum-free Opti Modified Eagle's Medium (OptiMEM). cDNA was mixed with transfection reagents in 100 µl OptiMEM, and incubated at room temperature for 10 (PEI) or 30 (Lipofectamine-2000) minutes, and then directly added to cells in DMEM. Assays were performed 16-24h later, when cells were 80-100% confluent.

Immunocytochemistry of astrocyte cultures

Immunocytochemistry was performed as described briefly (Ashpole and Hudmon 2011, Ashpole, Herren et al. 2012). Astrocytes seeded onto 12 mm coverslips were fixed for 15 minutes using 3-4% paraformaldehyde, permeabilized for 15 minutes using 0.25% Triton X-100 (TX-100), and blocked using blocking buffer (5% goat serum, 0.25% TX-100, 0.3M glycine) for 2 hours at room temperature. Coverslips were washed twice with PBS between each step. Next, the cultures were incubated in polyclonal rabbit anti-GFAP (1:1000) overnight at 4°C, followed by incubation in goat anti-rabbit $Alexa_{680}$ (1:5000) for an hour at room temperature. The coverslips were then washed in PBS and mounted in Prolong Gold Antifade mounting media containing DAPI (Molecular Probes), dried overnight, and imaged the next day.

Immunocytochemistry of fetal astrocyte cultures

Immunocytochemistry was performed on fetal astrocytes seeded onto collagen-coated 12 mm coverslips as described above. Specifically, the cultures were incubated in chicken anti-GFAP (1:500), mouse anti-MAP2 (1:1000), mouse anti-OX42 (1:1000), and rabbit anti-Olig2 (1:1000) primary antibodies overnight at 4°C, followed by incubation in goat anti-mouse $Alexa_{680}$ (1:5000) or goat anti-rabbit Texas red for an hour at room temperature. The coverslips were then washed in PBS and mounted in Prolong Gold Antifade mounting media containing DAPI (Molecular Probes), dried overnight, and imaged the next day.

Fluorescence Imaging

A Zeiss fluorescent microscope was utilized to monitor fluorescence intensity. Cells were imaged using the Texas red, FITC, or DAPI filters using Zeiss Axio Observer Z1 and processed with Axiovision 4 (10 or 20x magnification). All images were taken after cells had been transfected for 16-24h, in order to represent transfection efficiency as close to [^{3}H] uptake measurements as possible.

CaMKII [γ-^{32}]-ATP Activity Assays

HEK293T cultures were washed in cold PBS twice, and lysed in 20 mM Tris pH 7.4, 200 mM NaCl, 0.1 mM EDTA, using a homemade protease inhibitor cocktail (1 mM AEBSF, 300 nM Aprotinin, 2 μM E-64, 2 μM Leupeptin) as described previously (Ashpole, Chawla et al. 2013). The lysate was sonicated briefly, vortexed, and incubated with 0.5% Triton-X 100 for 5-10 minutes on ice. Total CaMKII activity was measured by incubating the lysate with 20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 100 μM ATP, 2 mM $CaCl_2$, 5 μM CaM, 50 μM AC-2 (KKALRRQETVDAL) and [γ-^{32}]ATP (3 μCi per reaction) for 5 minutes at 30°C. Autonomous CaMKII activity (Ca^{2+}/CaM-independent activity) was measured by incubating the lysate under similar conditions except 5 mM EGTA replaced Ca^{2+}/CaM. The radiolabeled reaction was spotted onto Whatman grade P81 ion exchange cellulose filter paper, and washed three times in 75 mM phosphoric acid to remove unbound [γ-^{32}]-ATP. To control for non-specific AC-2 phosphorylation in the lysate, reactions were incubated in the absence of AC-2. The linear range of the phosphorylation reaction extends >10 mins. A DC protein assay kit (Bio-Rad) was used to measure protein levels in the lysate. In brief, serial dilutions of the standard bovine

serum albumin were made in order to generate a standard curve. Samples were then diluted in buffers from the kit as per manufacturer's instructions, and concentrations were calculated based on the known concentrations of the standard. CaMKII activity was normalized to total protein to account for variability in cell density or lysis.

[^{3}H]-Glutamate Uptake Assays

HEK293Ts were transfected as described above with either vector control mCherry only, EAAT1 or EAAT2. Cultures were pre-treated with pharmacological antagonists 10 μM UCPH-101 (EAAT1 inhibitor) and 10 μM TBOA (EAAT2 inhibitor) for 20 min at 37°C to confirm isoform-specific glutamate transporter measurements. For CaMKII inhibition experiments, cultures were pre-treated with 5 μM KN-93 and tat-CN21 (CaMKII inhibitors), or 5 μM KN-92 and 5 μM tat-CN21Ala (CaMKII inhibitor controls) for 20 minutes at 37°C. Rat physiological saline (138 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2, 1.06 mM MgCl2, 12.4 mM HEPES, pH 7.4, 5.6 mM glucose; final pH adjusted to 7.3) containing 0.1 μCi/ml [^{3}H]-glutamate and 100 μM unlabeled glutamate was then applied to HEK293T cells for 20 min at 37°C. If an inhibitor or inhibitor control was previously applied to the cultures, it was also added during glutamate treatment, keeping the concentration consistent. The cultures were then washed in cold PBS twice, lysed in lysis buffer (above) containing 1%Triton X-100, vortexed briefly and incubated for 5 min on ice. Next, lysates were diluted in 8 ml RadSafe liquid scintillation cocktail (Beckman Coulter) and [^{3}H] was quantified using a scintillation counter. The linear range of the glutamate uptake reaction extends ≤1 hour. A DC protein assay was performed as described above, and uptake was normalized to total protein.

In Vitro SPOTs Phosphorylation Assay

15-amino acid long peptides spanning the intracellular regions of EAAT1 and EAAT2 (1-15, 2-16, 3-17 etc.) were synthesized onto β-alanine derivatized cellulose membranes using the SPOTS synthesis method as described previously (Adler, Johnson et al. 2013). Following synthesis, the membrane was de-protected twice with a solution of trifluoroacetic acid containing 5% phenol and 2% triisopropylsilane. Next, the membrane was blocked with 5% BSA in 50 mM HEPES pH 7.4, 100 mM NaCl, 1 mM EDTA, and 0.02% NP-40 for 30 minutes followed by three washes in 100 mM Tris-HCl pH 7.4. A pre-reaction was performed in the presence of 20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 μM CaM, 500 μM cold ATP and 500 nM recombinant purified human δCaMKII for 10 minutes on ice to autophosphorylate δCaMKII at T287 as described previously (Ashpole, Herren et al. 2012). Membranes were subjected to a kinase phosphorylation assay in the presence of 50 mM HEPES pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 μM CaM, 0.2 mg/ml BSA, 1 mM DTT, 100 μM cold ATP, 120 μCi/ml [γ-^{32}]-ATP, and 5-10 nM δCaMKII. The reactions were incubated at room temperature for 4 minutes, terminated with three washes (100mM sodium phosphate pH 7.0, 1 M NaCl, 10 mM EDTA) and dried as described previously (Ashpole, Herren et al. 2012). The extent of radioactive phosphate incorporation was quantified using a Fujifilm phosphoimager and expressed as photostimulated luminescence (PSL/mm^2) for a 1.5 mm x 1.5 mm circle centered on each spot. V3.1 of the MultiGauge software was used to detect and quantify phosphorylation of each peptide spot.

Ionomycin activation of CaMKII

HEK293T cells were transfected with δCaMKII cDNA at ~60-70% confluency. After 16-24 hours, δCaMKII was activated using 10μM ionomycin for 1 min and 20 mins, using methanol as a vehicle control. Cells were then lysed in lysis buffer (described above). [γ-^{32}] assays were performed to measure phosphate incorporation into the CaMKII substrate AC-2. [^{3}H]-glutamate uptake assays were carried out to measure the effect of increasing calcium signaling on glutamate uptake.

Co-immunoprecipitation

3x FLAG-tagged versions of EAAT1 and EAAT2 were co-transfected into HEK293T cells with δCaMKII at ~60-70% confluency. After 16-24 hours, δCaMKII was activated using 10μM ionomycin for 20 mins, following which cells were lysed using lysis buffer (described above), spun down at 200 X g for 10 minutes and the pellet was homogenized using lysis buffer containing 1% TX-100, protease and phosphatase inhibitors. 20μL of each sample was collected and labeled 'lysate' to serve as a positive control for transfection. Lysate was then exposed to either mouse IgG or mouse FLAG antibody for 2 hours at 4°C. After this incubation, washed Protein G agarose beads were added to each sample for 1 hour at 4°C. Samples were then rinsed three times using lysis buffer, and a Western blot was run to detect the FLAG-tagged protein and CaMKII.

GST-fusion protein phosphorylation

GST-tagged intracellular N- and C- termini of human EAAT1 and EAAT2, EAAT1 N terminii mutants, GluR1 and a GST vector control were expressed in BL-21 *Escherichia*

coli cells overnight at 16°C using 0.2 mM isopropyl 1-thio-β-D-galactopyranoside to induce protein expression. Cells were centrifuged at 200 X g for 10 minutes, re-suspended in lysis buffer (described above) and passed through a microfluidizer three times to lyse cells. A supernatant was collected after centrifugation (8,000 X g for 10 mins) and snap-frozen for single use aliquots. GST-fusion proteins were bound to glutathione agarose in binding buffer (20 mM Tris pH 7.4, 200 mM NaCl, 1 mM EDTA, 0.1% Tween-20 containing protease inhibitor) tumbling for 1.5 hours at 4°C. The beads were then washed three times in binding buffer to remove any unbound lysate. A pre-reaction was performed as described above to activate and autophosphorylate human δCaMKII. GST-bound fusion proteins were then phosphorylated on the glutathione beads with 100 nM δCaMKII in reaction mix containing 20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 0.25 mM $CaCl_2$, 5 μM CaM, 10 μM cold ATP, and 60 μCi/ml [γ-^{32}P]-ATP for 30 mins at room temperature. The reaction was quenched with three 500 mM EDTA washes, followed by addition of LDS sample loading buffer and β-mercaptoethanol to denature proteins at 80°C. Overnight Coomassie staining of the gel allowed for visualization of GST-fusions and protein quantification using Image J densitometry (NIH). CaMKII phosphorylation of GST fusion proteins was detected upon transfer of radioactive signal [γ-^{32}P] onto a screen, using a phosphoimager (Fujifilm) for visualization and quantified using V3.1 of the MultiGauge software as described above.

Statistical Analysis

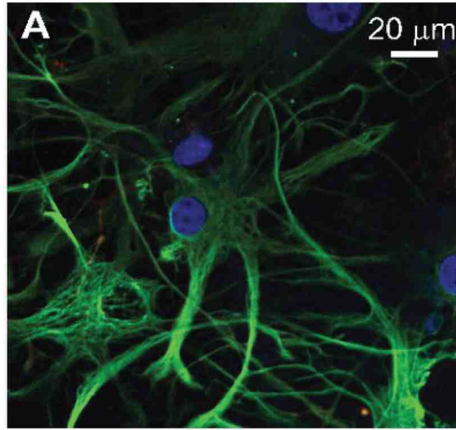
SigmaPlot version 12.5 was used to perform statistical analysis. A one-way ANOVA followed by a *post hoc* Dunnett's test was used to compare differences between the means of each group. A p value of <0.05 was considered statistically significant.

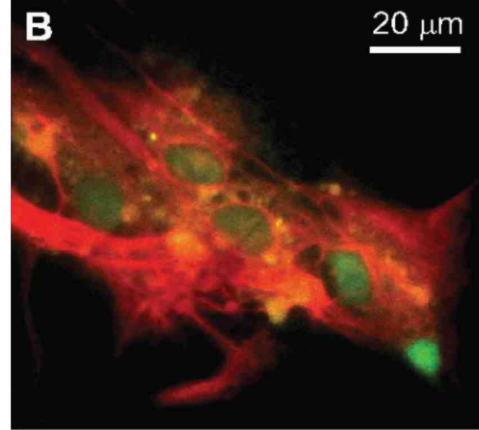
RESULTS

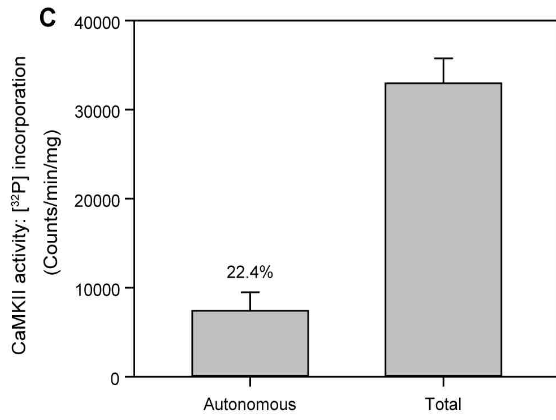
Immunocytochemical characterization of rodent cultured cortical astrocytes

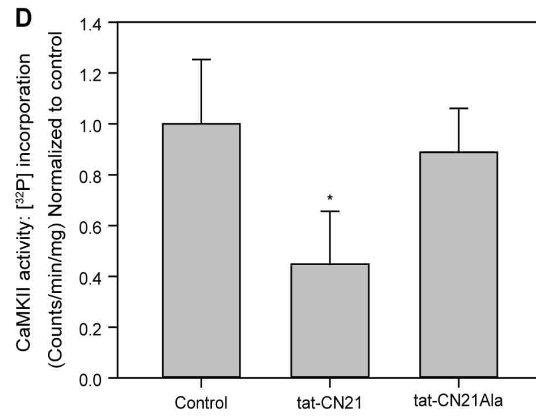
In order to assess CaMKII regulation of glutamate uptake, we generated cortical astrocyte cultures from postnatal rodents using a well-established protocol (McCarthy and de Vellis 1980). Briefly, we harvested the brain from postnatal rat pups (P1-P3) and plated the cells following mechanical and enzymatic processing of the tissue. Once the cells reached confluence (8-10 days), we mechanically dislodged contaminating cells (primarily oligodendrocytes and neurons) using forceful shaking and split the cultures to enrich for astrocytes. Although this protocol to harvest and culture astrocytes is well accepted in the field, we verified cellular content by immunostaining for specific cellular markers to identify neurons (MAP2), microglia (OX42), and astrocytes (GFAP and vimentin). We used the nuclear stain Hoechst, a dye that binds double-stranded DNA, in order to confirm nuclear integrity and to determine total cell numbers for characterizing astrocyte contamination by other glia and neurons. We observed that 93.9 +/- 10.8 % (n = 6) of these cells were GFAP positive (**Figure 4A**) and 78.9 +/- 21.2% (n = 6) were vimentin positive (**Figure 4B**), both intermediate filament proteins expressed in reactive cultured astrocytes. Furthermore, we observed that only 2.3 +/- 3.9% (n = 6) were OX42 positive, suggesting that there is little to no contamination by microglial cells. The microtubule associated neuronal protein, MAP-2, was not detected in these cultures. Overall, these findings suggest our cultures are predominantly astrocytic (~80-94% based on the protein marker being used), with minimal if any contamination from microglia and neurons. This characterization was published in the “Journal of Biological Chemistry. Ashpole N, Chawla A, Martin M, Brustovetsky T, Brustovetsky N, Hudmon

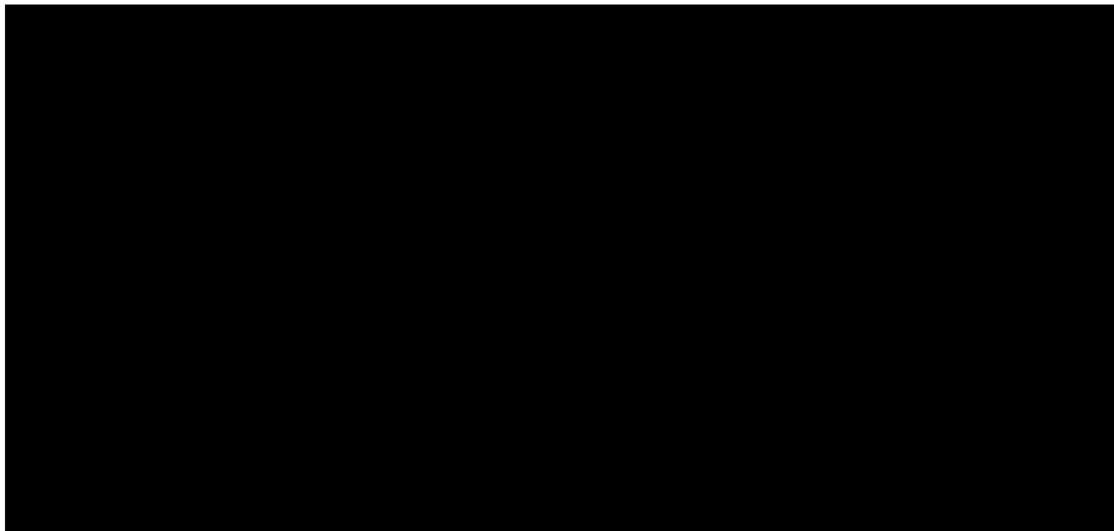
A. Loss of Calcium/Calmodulin-dependent protein kinase II activity in cortical astrocytes decreases glutamate uptake and induces neurotoxic release of ATP. Journal of Biological Chemistry. 2013; Vol 288:14599-14611. © the American Society for Biochemistry and Molecular Biology".


A
20 μm


B
20 μm


C
CaMKII activity: [32P] incorporation
(Counts/min/mg)
40000
30000
20000
10000
0
22.4%
Autonomous
Total


D
CaMKII activity: [32P] incorporation
(Counts/min/mg) Normalized to control
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
tat-CN21
tat-CN21Ala



Characterization of CaMKII in rodent cultured cortical astrocytes

Next, we tested our cultures for the presence/activity of CaMKII using a number of different methodologies. Immunocytochemical analyses detect 93.0 +/- 9.8% of our cultured astrocytes stained positive for CaMKII using a pan-CaMKII antibody, suggesting CaMKII is present in almost all the cells in our culture system (**Figure 4B**). Furthermore, the CaMKII protein appears to localize in both a nuclear and cytoplasmic context. It is important to note that CaMKII does not appear to be sequestered in intracellular compartments that may be hard to access using our pharmacological tools, based on these data. Next, we measured CaMKII catalytic activity in our astrocyte cultures. Our purpose for doing so was two-fold: one, we wanted to confirm our immunocytochemical findings that detected CaMKII expression in our astrocytic cultures, and two, we wanted to determine the extent of activated or autonomous kinase present in our cultures. In order to quantify this, we measured Ca^{2+}/CaM-dependent (total activity) and Ca^{2+}/CaM-independent activity (autonomous activity) as a function of [γ-^{32}P] incorporation into AC-2, a highly selective peptide substrate of CaMKII derived from the Thr-286 autophosphorylation site of the alpha subunit of CaMKII. Lysate activity was measured in the absence of AC-2 to control for non-specific substrate phosphorylation (<5% of total activity). We observed that 22.4 ± 5.28% of CaMKII activity is autonomous as a percentage of total CaMKII activity (**Figure 4C**). This autonomous kinase activity represents the pool of activated CaMKII or 'basal' CaMKII present in the astrocyte, which likely reflects prior CaMKII activation by canonical $Ca^{2+/}$CaM, however, this activity can also be generated by other post-translational modifications (Mattiazzi, Bassani et al. 2015), including glycosylation (Erickson, Pereira

et al. 2013), nitrosylation (Coultrap and Bayer 2014) and oxidation (Erickson, Joiner et al. 2008, Erickson, He et al. 2011). The function of this autonomous CaMKII activity is believed to generate a basal level of CaMKII activity relevant for constitutive physiological CaMKII signaling. Next, we tested the effect of the high affinity peptide inhibitor, tat-CN21 in our astrocytes cultures. CN21 is a 21 amino acid long peptide inhibitor of CaMKII generated from the naturally occurring inhibitor protein, CaMKIIN (Vest, O'Leary et al. 2010, Ashpole and Hudmon 2011). For these experiments, it was conjugated to the cell-permeable sequence, tat (YGRKKRRQRR). We did this in order to confirm the specificity of our assay (although carrying out the experiment in the absence of AC-2 tests the same) and that we could, in fact, reduce CaMKII activity. We observed a 56 ± 20% reduction in total CaMKII activity with tat-CN21 application that was significant, while the inactive control, tat-CN21Ala reduced total CaMKII activity by 12 ± 17% that was non-significant (**Figure 4D**). Overall, our findings suggest rodent cultured cortical astrocytes express CaMKII, a fraction of which exists in the activated or autonomous form.

Pharmacological inhibition of CaMKII in rodent cultured cortical astrocytes diminishes [^{3}H]-glutamate uptake

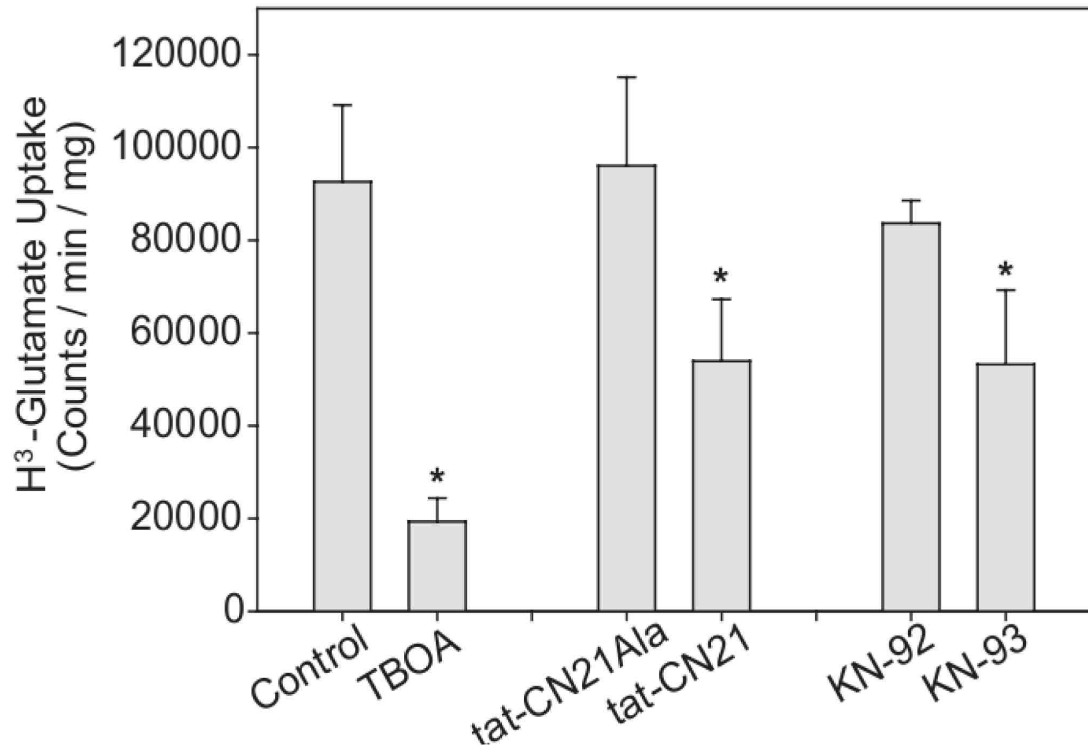
Given the correlation between CaMKII inactivation and neuronal death, and increased extracellular glutamate/dysregulation in glutamate uptake with an increase in neuronal death, we asked the question: Does CaMKII regulate glutamate uptake? We first confirmed the efficacy of our experimental paradigm. We measured [^{3}H]-glutamate uptake in the presence of a non-selective glutamate transporter inhibitor, TBOA, for 20

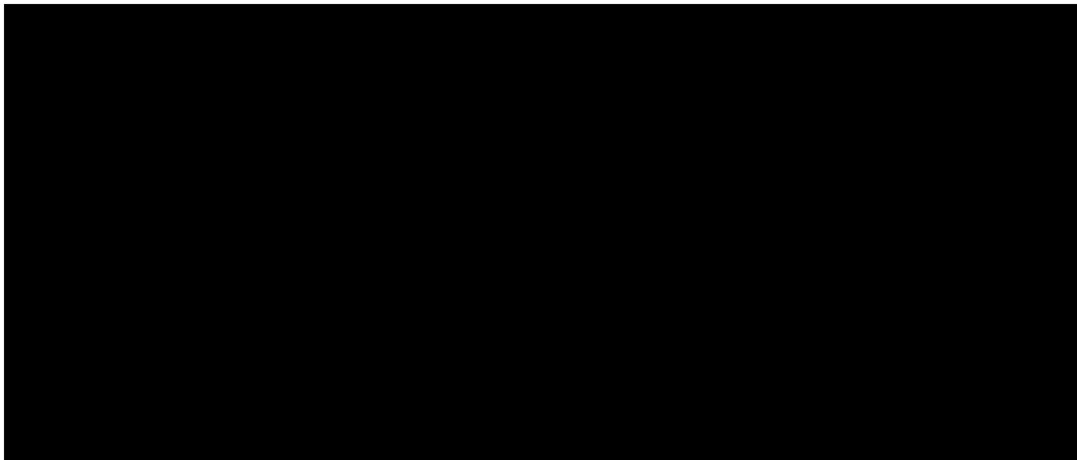
minutes at a concentration that targets all tested EAATs (100μM). Uptake was measured using a liquid scintillation counter, and counts were normalized to protein concentration in order to reduce variability between samples. We found that >85% of observable [^{3}H]-glutamate uptake is inhibited by 100uM TBOA, confirming that our [^{3}H] measurement is primarily mediated via excitatory amino acid transporters (**Figure 5**). We employed two well-established CaMKII inhibitors: KN-93, a small molecule inhibitor that competes with Ca^{2+}/CaM but not ATP for CaMKII activation (Sumi, Kiuchi et al. 1991, Pellicena and Schulman 2014), and tat-CN21, a membrane permeable CaMKII inhibitor that inhibits both stimulated and autonomous CaMKII activity (Buard, Coultrap et al. 2010). Inactive versions/controls for the respective inhibitors (KN-92 and tat-CN21Ala) were used to verify the specificity of the active compounds and control for off-target effects. We find that application of both KN-93 and tat-CN21 produce a significant reduction in uptake (45 ± 4% and 46 ± 5% respectively), while their respective inactive controls do not alter [^{3}H]-glutamate uptake (Figure 5). These data suggest CaMKII regulates astrocytic glutamate transport. Cortical astrocytes, however, express the glutamate transporters GLAST/EAAT1 and GLT-1/EAAT2 (Kondo, Hashimoto et al. 1995, Rothstein, Dykes-Hoberg et al. 1996) and thus do not represent a good model system to parse out differences in transporter regulation. In order to explore the identity and mechanisms underlying glutamate transporter regulation by CaMKII in a tightly controlled manner, we moved to a heterologous expression system previously used to study glutamate transporter function. The focus of my dissertation is to address the specificity and mechanism of CaMKII regulation of the astrocytic glutamate transporters.

Thus, the specific aims of my study were:

1. To determine the contribution of EAAT1 and/or EAAT2 in the regulation of astrocytic glutamate uptake by CaMKII
2. To determine the role of CaMKII phosphorylation in astrocytic glutamate transporter(s) activity

I hypothesize that CaMKII modulation of either EAAT1 and/or EAAT2 contributes to the phenotype of diminished glutamate uptake upon loss of CaMKII signaling in astrocytes. Furthermore, as CaMKII is a serine/threonine kinase, we hypothesized that CaMKII modulated the activity of the glutamate transporters via phosphorylation.


H3-Glutamate Uptake
(Counts / min / mg)
120000
100000
80000
60000
40000
20000
0
*
*
*
Control
TBOA
tat-CN21Ala
tat-CN21
KN-92
KN-93

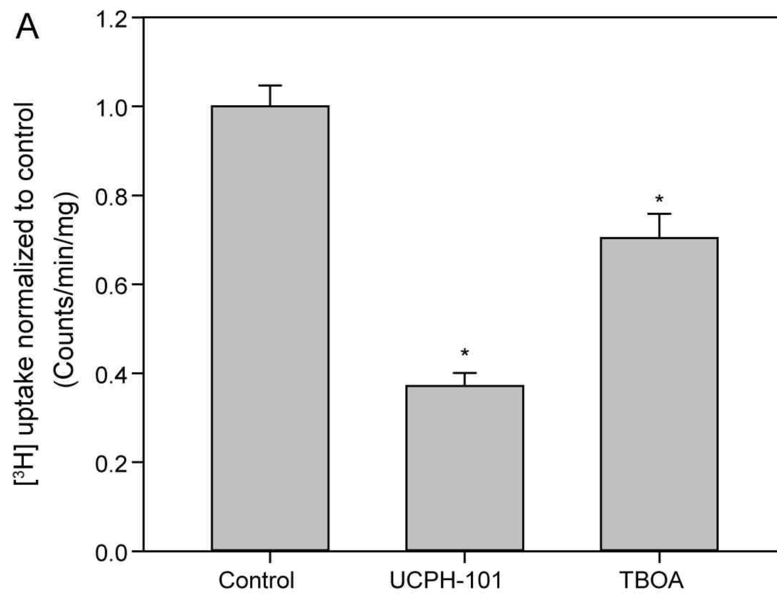


Characterization of HEK293Ts as a model to study CaMKII regulation of glutamate uptake by astrocytic excitatory amino acid transporters

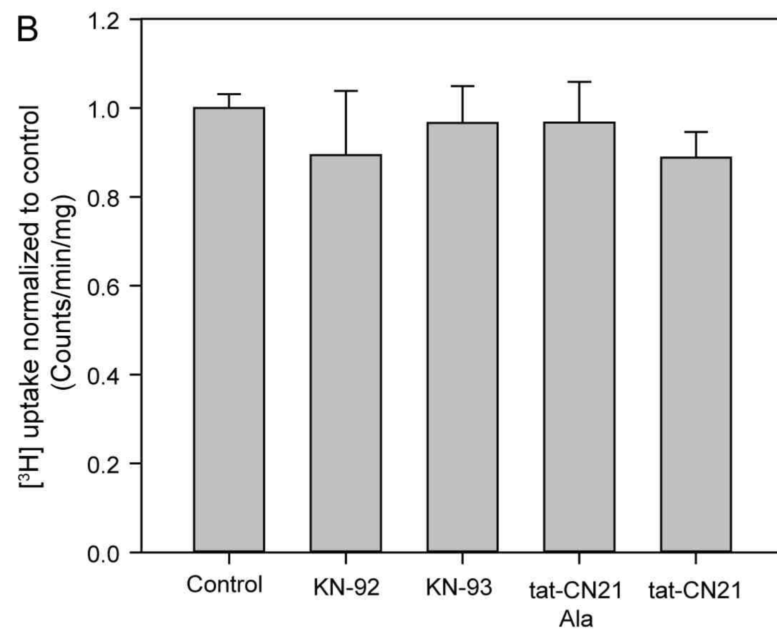
Human-embryonic-kidney cell lines (HEK293T) have been previously used as a model system to study glutamate uptake (Dunlop, Lou et al. 1999, Berry, Hayes et al. 2005, Peacey, Miller et al. 2009). However, before studying our transfected transporters we wanted to first characterize endogenous glutamate transporter activity in the HEK293T cell background. Because we observed a measurable level of glutamate uptake under our experimental conditions, we attempted to discern whether this uptake had a classical pharmacological phenotype using the EAAT1 inhibitor, UCPH-101 (Erichsen, Huynh et al. 2010) and EAAT2 inhibitor, TBOA. While TBOA is not considered a selective EAAT inhibitor, it is relatively specific for EAAT2 at low micromolar concentrations (Shimamoto, Sakai et al. 2004). We observed that 63±3% glutamate uptake is reduced by the addition of 10 μM UCPH-101 and a 30±1% reduction in glutamate uptake occurs upon the addition of 10 μM TBOA in untransfected HEK293T cells (**Figure 6A**). While these findings suggest that a portion of endogenous [^{3}H]-glutamate uptake in naïve HEK293T cells may be due to EAAT1 and EAAT2 activity, it is likely this glutamate uptake is due to EAAT3 (Toki, Namikawa et al. 1998), a subtype of glutamate transporter predominantly localized to neurons and kidney cells (Kanai and Hediger 1992). Additional studies are required to address this finding in further detail. For the purpose of our studies, however, both UCPH-101 and TBOA serve as positive controls.

We next wanted to study the consequence of pharmacological inhibition of CaMKII on glutamate uptake by untansfected HEK293T cells. Application of the

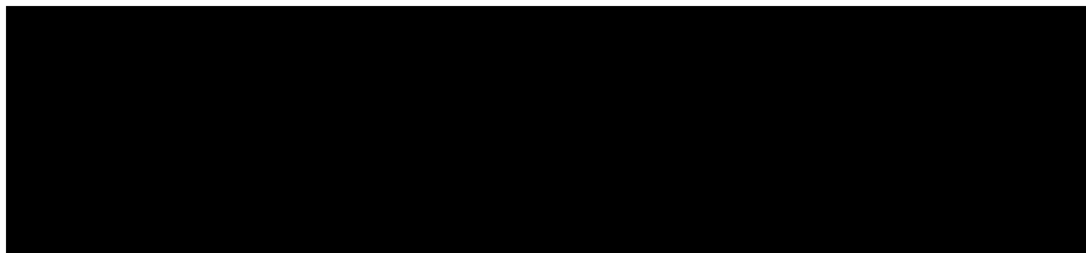
CaMKII inhibitors and inhibitor controls previously described (KN-92, KN-93, tat-CN21Ala, and tat-CN21) to untransfected HEK293T cells did not produce a significant reduction in [^{3}H]-glutamate uptake (**Figure 6B**). The findings suggest that neither the inhibitors nor the inhibitor controls have an effect on endogenous glutamate transporters in this cell line, allowing us to utilize these pharmacological agents for our assays. It is important to note that the overall [^{3}H]-glutamate uptake generated by HEK293T cells is 'low' compared to heterologous expression of EAAT1 and EAAT2 (depicted in **Figure 7D**). Furthermore, as the majority of endogenous [^{3}H]-glutamate uptake has been attributed to the glutamate transporter EAAT3, heterologous expression of EAAT1 and EAAT2 can allow us to specifically isolate these transporters in our model system.



A
[3H] uptake normalized to control
(Counts/min/mg)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
UCPH-101
TBOA



B
[3H] uptake normalized to control
(Counts/min/mg)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
KN-92
KN-93
tat-CN21
Ala
tat-CN21



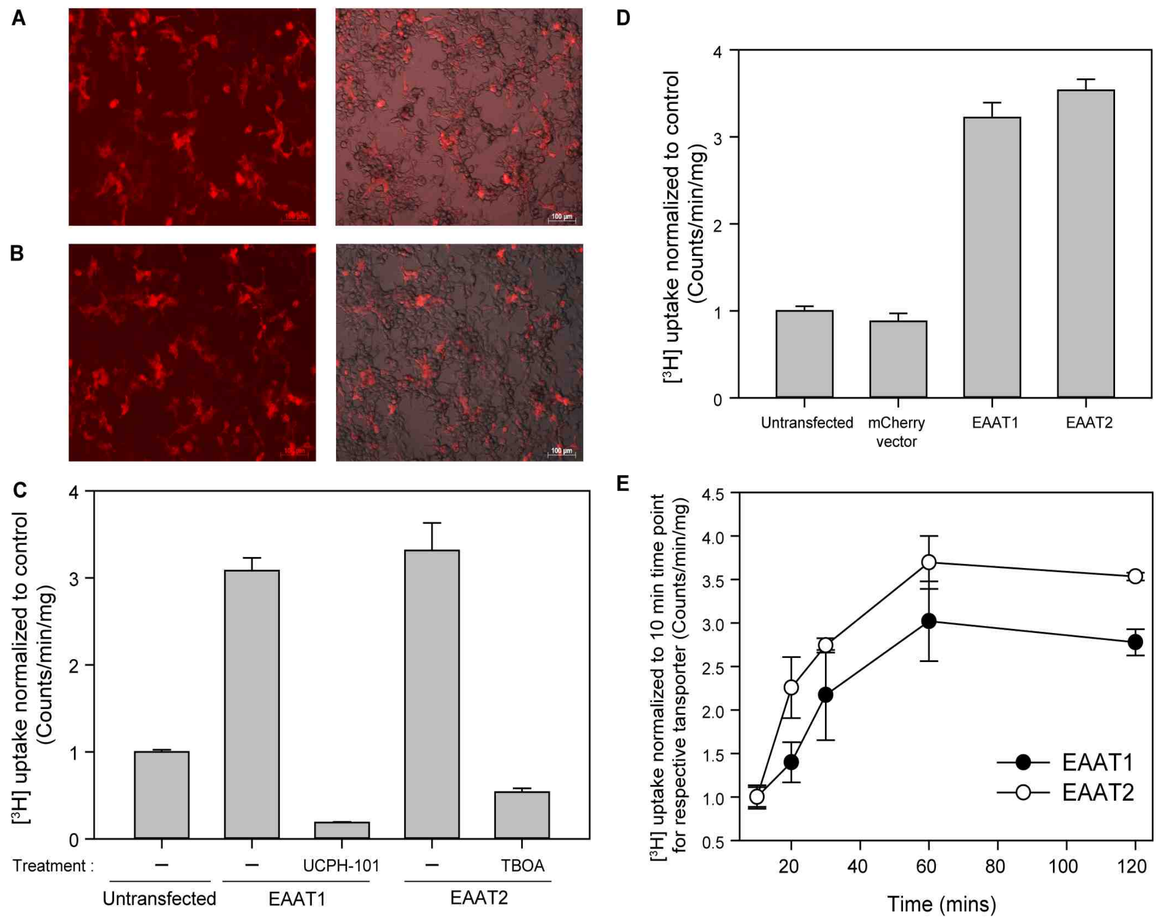
Characterization of EAAT1/2 transfected HEK293Ts as a model to study CaMKII regulation of glutamate uptake

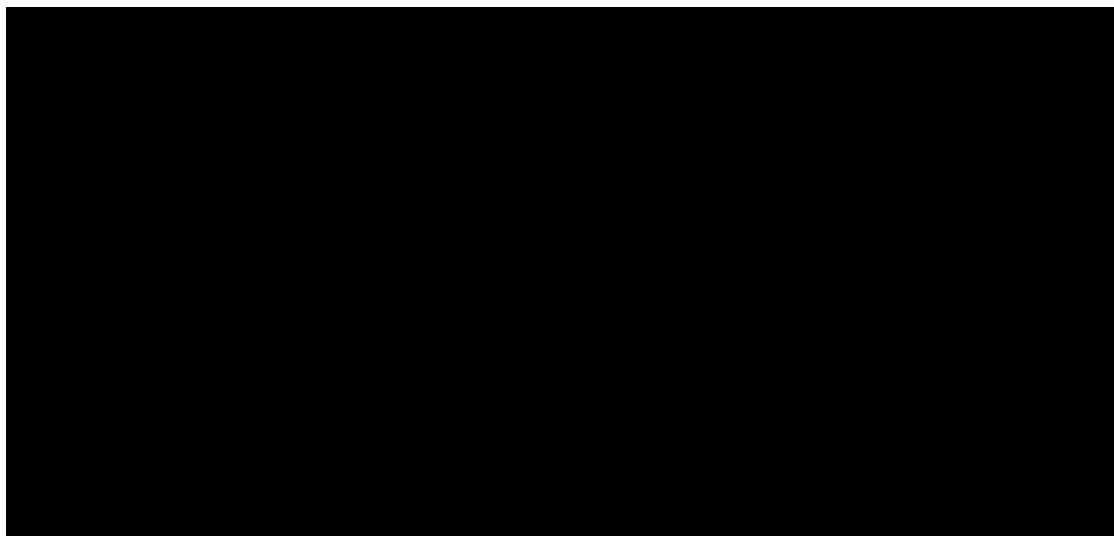
We next expressed EAAT1 and EAAT2 individually using an expression vector containing a mCherry-tag. The fluorescent reporter is fused N-terminus of the transporter where it functions as a surrogate of transporter expression. Using this heterologous expression system, we first quantified the percentage of cells expressing EAAT1 or EAAT2 by measuring mCherry fluorescence. As a function of mCherry fluorescence, EAAT1 and EAAT2 were expressed in 46% ± 7 and 43% ± 9 cells respectively, comparable to the mCherry vector control (50% ± 5, **Figures 7A and 7B**). Functionally, the transfected transporters displayed similar levels of activity 24 hours post-transfection. Heterologous expression of EAAT1 and EAAT2 produced a 3.08 ± 0.14 fold and 3.31 ± 0.32 fold increase in [^{3}H]-glutamate uptake respectively compared to untransfected HEK293T cells (**Figure 7C**). These findings were consistent with a previous study reporting a 3-4 fold increase in glutamate uptake using similar concentrations of radiolabeled and unlabeled glutamate (Dunlop, Lou et al. 1999). Using an EAAT1 inhibitor, UCPH-101 (Erichsen, Huynh et al. 2010) and EAAT2 inhibitor, TBOA (Shimamoto, Sakai et al. 2004), we were able to knock down glutamate uptake generated by transfected EAAT1 and 2 respectively (**Figure 7C**), suggesting that the ^{3}H measurements are likely via EAAT activity. However, a possible interpretation of these data is that transfection alone may result in alterations (in particular an increase) in [^{3}H]-glutamate uptake. Thus, we tested whether the control vector for EAAT1 and EAAT2 altered [^{3}H]-glutamate uptake. We find that the mCherry tag-containing control vector (1 ± 0.14 fold increase) did not alter [^{3}H]-glutamate uptake when compared to control

conditions (**Figure 7D**). These data confirm that EAAT1 and EAAT2 transfection enhances [^{3}H]-glutamate uptake in HEK293T cells as expected.

In experiments described thus far (**Figures 6, 7C and D**), we measured [^{3}H]-glutamate uptake at a time point of 20 mins based on glutamate uptake data generated in astrocytes (**Figure 5**). However, in order to determine linearity of the glutamate uptake reaction in HEK293T cells, we carried out a time course. For this assay, each dish was transfected with EAAT1 or EAAT2, [^{3}H]-labeled and unlabeled glutamate were added to HEK293T cells for varying amounts of time (ranging between 10 mins and 2 hours). We find that our [^{3}H]-glutamate uptake measurements were linear for up to 1 hour, after which uptake started plateauing (**Figure 7E**). A number of interpretations can be made from these data. First, as the reaction is linear from time points 10 mins up to1 hour, it is likely that glutamate is not used up during this duration. Second, the conditions of the uptake assay become saturating between 1-2 hours, observed by glutamate uptake values remaining consistent and even slightly decreasing in both EAAT1 and EAAT2 transfected cells from 1-2 hours. The decrease and/or saturation in uptake could be due to increased levels of intracellular glutamate building up that ultimately cause the transporters to reverse, leading to extrusion of glutamate that has been taken up. Keeping this in mind, for the rest of our assays, we chose to study [^{3}H]-glutamate uptake at a time point of 20 minutes. Our data suggests that the dynamic range for [^{3}H]-glutamate uptake generated by EAAT2 versus EAAT1 is slightly higher although we normalized the time points to their respective transporter glutamate measurements at 10 minutes. While this is suggestive of faster turnover by the EAAT2 transporter compared to EAAT1, the reason behind this phenomenon is unclear and we chose not to explore this in further detail. In

summary, HEK293T cells represent a viable heterologous expression system to study CaMKII regulation of glutamate uptake mediated by EAAT1 and EAAT2.


A
B
C
D
E
[3H] uptake normalized to control (Counts/min/mg)
Treatment :
UCPH-101
TBOA
Untransfected
EAAT1
EAAT2
mCherry vector
[3H] uptake normalized to 10 min time point for respective tansporter (Counts/min/mg)
Time (mins)

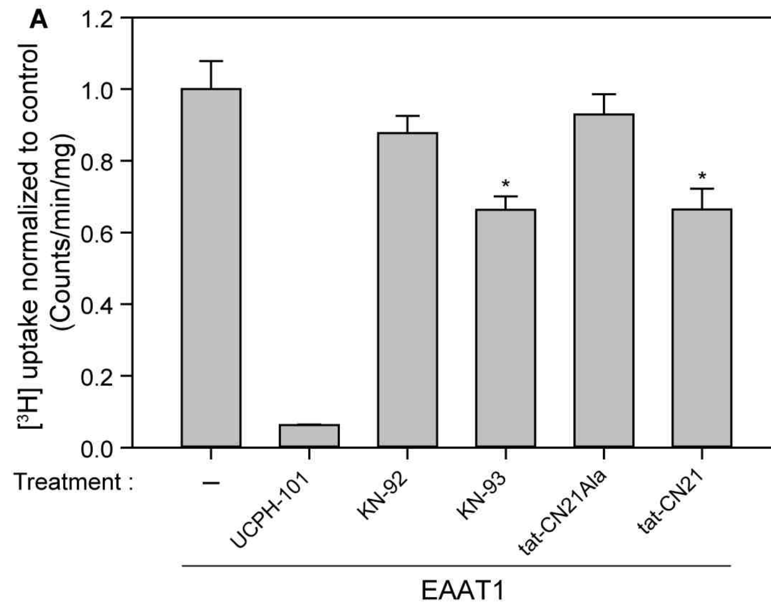


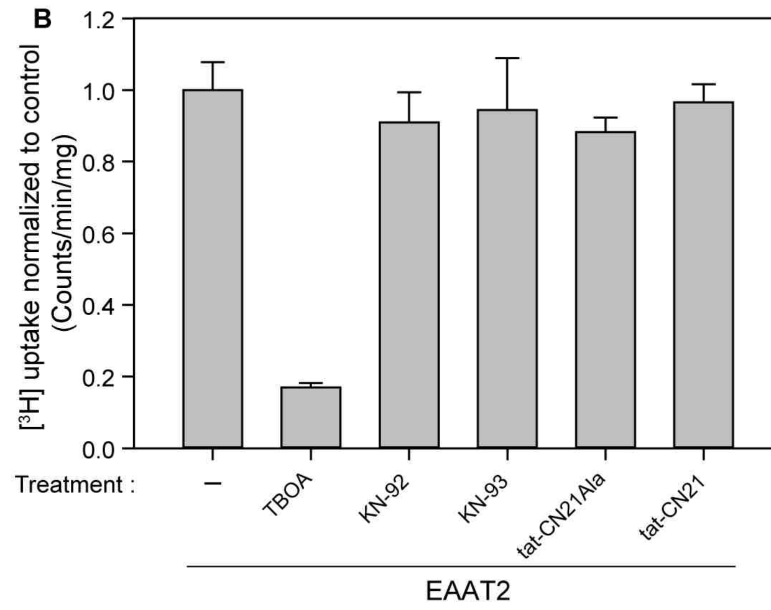
CaMKII inhibition results in a reduction in EAAT1 but not EAAT2-mediated [^{3}H]-glutamate uptake

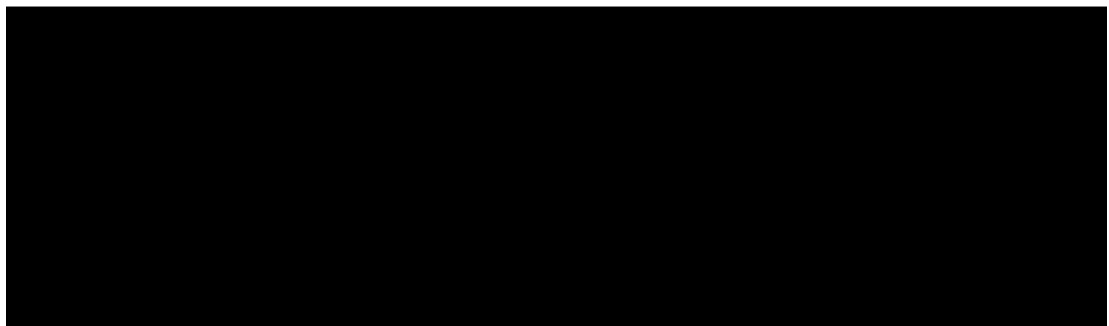
We have previously shown that pharmacological inhibition of CaMKII has no effect on naïve/untransfected HEK cells (**Figure 6B**). These data suggest that any changes observed under our experimental paradigm will not be a result of altered endogenous transporter activity, but rather the transfected transporter. Thus, while ¼ of the uptake signal is sensitive to CaMKII modulation, our signal to noise is well above background uptake levels. In addition, the linearity of the uptake signal is well within our measurement range and we can theoretically detect either inhibition or upregulation of transfected transporter activity. We thus asked the question: *Does the phenotypic regulation of astrocytic transporters by CaMKII result from regulation of EAAT1, EAAT2, or both transporters?*

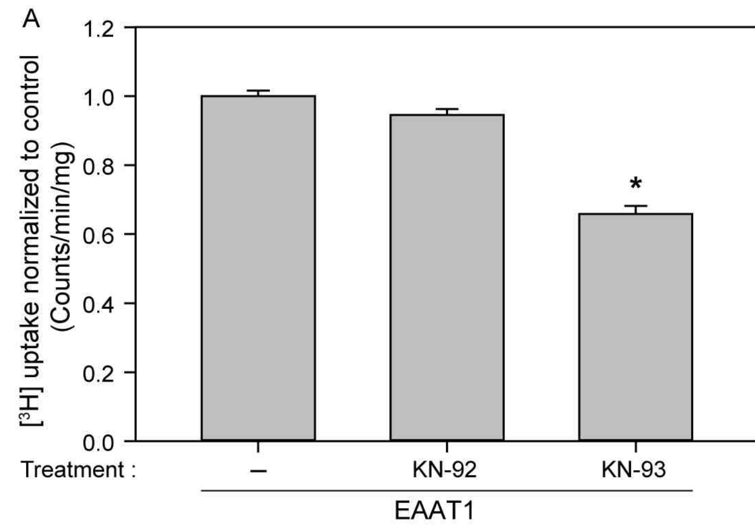
In order to answer this question, we applied CaMKII inhibitors (KN-93 and tat-CN21) and inhibitor controls (KN-92 and tat-CN21Ala) to EAAT1 and EAAT2 transfected HEK293Ts and measured [^{3}H]-glutamate uptake as described previously from our work in astrocytes (Ashpole, Chawla et al. 2013). We observed a significant reduction of 34% in [^{3}H]-glutamate uptake associated with KN-93 and tat-CN21 in EAAT1 transfected HEK293T cells compared to inactive controls KN-92 and tat-CN21Ala (**Figure 8A**). Interestingly, EAAT2 mediated glutamate uptake was not altered by CaMKII inhibition or the inhibitor controls (**Figure 8B**). These data suggest that CaMKII inhibition using pharmacological agents diminishes EAAT1 but not EAAT2 mediated glutamate uptake, appearing to have divergent effect on the two predominant astrocytic transporters for glutamate. This phenotype was further substantiated in a model

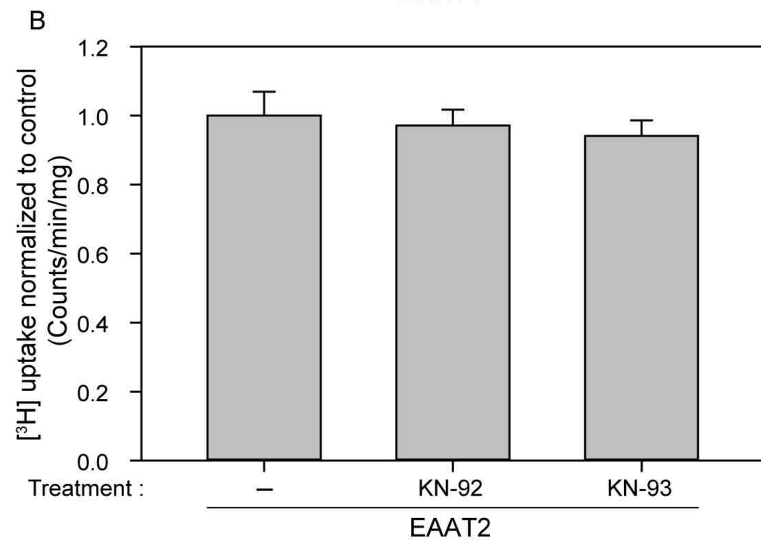
of transfected A172s (a glioblastoma cell line), wherein CaMKII inhibition reduced [^{3}H]-glutamate uptake by 34% in EAAT1 but not EAAT2 transfected A172s, substantiating our findings in HEK293Ts (**Figures 9A and 9B**). Thus, in two very different backgrounds, we observed that pharmacological CaMKII inhibition consistently targets glutamate uptake occurring via EAAT1, while leaving EAAT2-mediated glutamate unaffected. While this observation is suggestive of minimal off-target activities of our CaMKII inhibitors, our confidence in these findings is further supported by the inactive controls for our pharmacological agents not producing alterations in glutamate uptake for both EAAT1 and EAAT2. However, we wanted to use an alternative approach to corroborate our pharmacological findings in order to strengthen our conclusion.

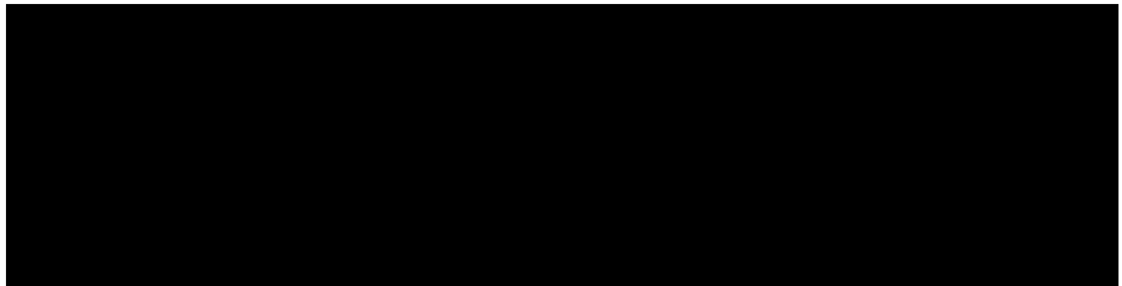

A
[3H] uptake normalized to control
(Counts/min/mg)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Treatment :
—
UCPH-101
KN-92
KN-93
tat-CN21Ala
tat-CN21
*
*
EAAT1


B
[3H] uptake normalized to control
(Counts/min/mg)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Treatment :
—
TBOA
KN-92
KN-93
tat-CN21Ala
tat-CN21
EAAT2




A
[3H] uptake normalized to control
(Counts/min/mg)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Treatment :
–
KN-92
KN-93
EAAT1


B
[3H] uptake normalized to control
(Counts/min/mg)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Treatment :
–
KN-92
KN-93
EAAT2



A dominant-negative version of CaMKII (Asp136Asn) produces a reduction in EAAT1 but not EAAT2-mediated [^{3}H]-glutamate uptake

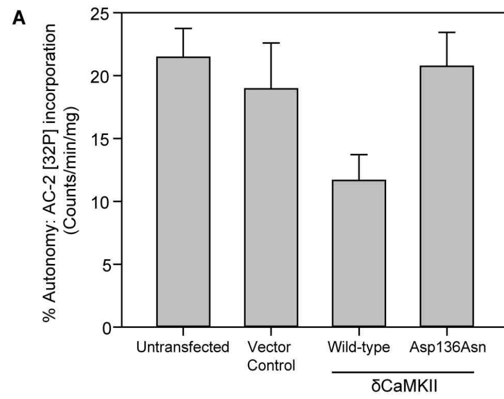
As an orthogonal approach to our pharmacological data that implicate CaMKII regulation of EAAT1 but not EAAT2, we wanted to genetically inhibit CaMKII. In rodent cultured cortical astrocytes, γ and δ CaMKII have been reported to be the predominant isoforms (Takeuchi, Yamamoto et al. 2000, Vallano, Beaman-Hall et al. 2000). This is in contrast with neuronal cells, where α and β CaMKII are expressed in much higher levels. Using pharmacology, we presumably targeted the action of both γ and δ CaMKII in astrocytes, as the inhibitors do not possess isoform specificity. Because δ CaMKII is believed to be the predominant CaMKII isoform in astrocytes (Takeuchi, Yamamoto et al. 2000), we generated a dead mutant (Asp136Asn) in δCaMKII by replacing a critical aspartate residue in the substrate binding site with an asparagine (Chao, Stratton et al. 2011). Both Asp136Asn and wild-type δCaMKII were sub-cloned into vectors expressing an N-terminal YFP tag and co-transfected into HEK293T cells with EAAT1 or EAAT2. We first characterized the autonomous activity of this mutant using [γ-^{32}P] incorporation assays into the CaMKII substrate, AC-2. Surprisingly, we did not detect a reduction in [γ-^{32}P] incorporation in cells transfected with Asp136Asn δCaMKII compared to untransfected and vector control (YFP-tagged vector); in fact we observed a slight increase in [γ-^{32}P] incorporation compared to wild-type δCaMKII (**Figure 10A**). It is possible that endogenous kinase may be overriding the inhibitory effects of Asp136Asn δCaMKII in our [γ-^{32}P] assays; however, our [^{3}H]-glutamate uptake data in **Figure 10D** and **Figure 10E** suggest that we may be failing to detect an alteration in CaMKII activity. Furthermore, in our experiments, % autonomy upon

transfection of δCaMKII wild-type consistently decreases (12±2%) compared to untransfected (20±2%) and vector control conditions (19±4%); a similar trend is observed in **Figure 11**. This may be accounted for by a 3-4 fold increase in dependent CaMKII activity.

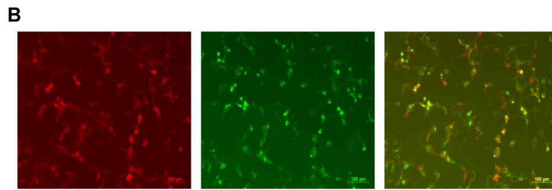
We co-transfected YFP Asp136Asn δCaMKII into HEK293T cells with either mCherry EAAT1 or mCherry EAAT2. We find that transfection efficiencies in all conditions studied are not significantly different (**Figures 10B and C**). YFP Asp136Asn δCaMKII/ mCherry EAAT1 and YFP Asp136Asn δCaMKII/ mCherry EAAT2 appear to be expressed in a similar manner intracellularly, based on a fluorometric readout. Although our [γ-^{32}P] incorporation data were puzzling, as the cellular distribution of YFP Asp136Asn δCaMKII did not appear to be different from wild-type δCaMKII and thus we decided to perform glutamate uptake assays.

Surprisingly, overexpression of Asp136Asn δCaMKII produced a 50% ± 2.9 reduction in EAAT1, but not EAAT2, mediated [^{3}H]-glutamate uptake in HEK293T cells (**Figures 10D and E**) compared to EAAT1/2 only transfected cells. We independently tested the effect of wild-type δCaMKII on [^{3}H]-glutamate uptake and did not find a significant difference compared to untreated transfected cells (depicted in **Figure 11F**), so chose not to include this control in this particular experiment. The most likely explanation for this result is that the dead CaMKII mutant protein produces a dominant negative phenotype as described previously for another kinase dead variant of CaMKII (Kabakov and Lisman 2015).Given that we observe an alteration in EAAT1- but not EAAT2-mediated glutamate uptake suggests the effect associated with Asp136Asn δCaMKII transfection is isoform specific to EAAT1. Although our [γ-^{32}P] assays (**Figure**

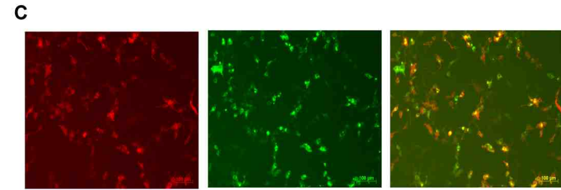
10A) do not support this idea, our functional glutamate uptake measurements suggest subunits of Asp136Asn δCaMKII combine with δCaMKII wild-type and potentially override basal signaling associated with wild-type CaMKII. In sum, this genetic approach is revealing in both being consistent with pharmacological inhibition of CaMKII regulating EAAT1, but also in the fact that along with pharmacological inhibitors, expression of the dominant negative CaMKII did not appear to alter glutamate uptake mediated by EAAT2.

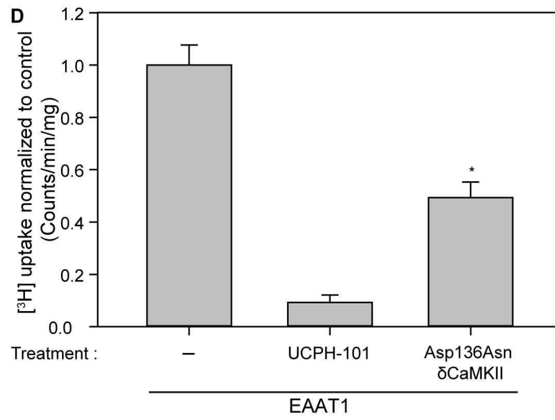


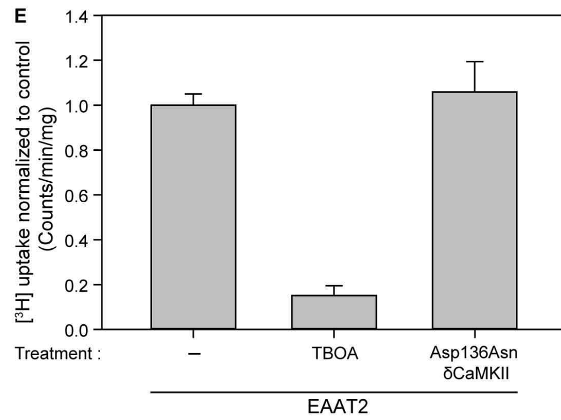
B

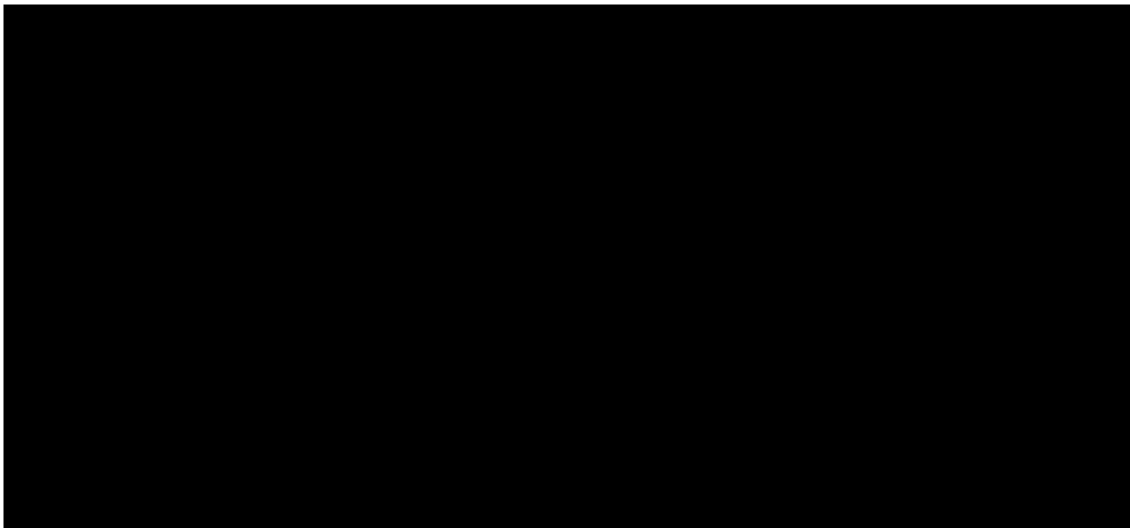


C









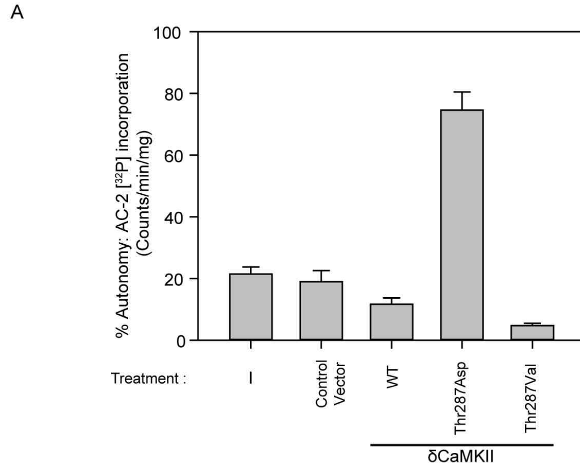
CaMKII autophosphorylation mutants at Thr287 do not alter glutamate uptake via either EAAT1 or EAAT2

Autophosphorylation of δCaMKII at Thr287 renders the kinase autonomously active, enhancing its ability to impact downstream cellular functions (Lai, Nairn et al. 1987, Miller, Patton et al. 1988, Hudmon and Schulman 2002). While there is autonomously active CaMKII in our naïve cultured HEK293T cells (21.64% ± 2.3, **Figure 11A**), we generated phosphorylation-site Thr287 mutants to examine whether the level of autonomous kinase activity alters EAAT1 or EAAT2 transporter function. First, we generated a Thr287Asp mutant that replaces threonine with a negatively charged aspartate residue to mimic phosphorylation, theoretically augmenting the level of autonomous activity. Second, we generated a Thr287Val mutant that replaces the threonine residue with a valine, a non-phosphorylatable amino acid that hypothetically reduces the level of autonomous activity. We ran [γ-^{32}P] incorporation assays to test whether our mutants did, in fact, produce the alterations we hypothesized in autonomous CaMKII activity. As described previously, we observe that transfection of wild-type δCaMKII marginally decreases % autonomous kinase activity from 21±2% to 11±2% (**Figure 11A**). Thr287Asp, a phospho-mimetic, has 74±6% autonomous activity whereas Thr287Val, the non-phosphorylatable mutant, is 4±1% autonomous as a function of AC-2 phosphorylation, as compared to untransfected HEK293T cells that exhibit 21±2% autonomous activity.

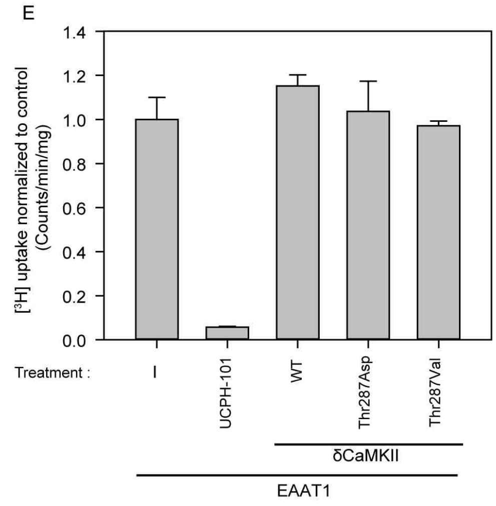
We transfected YFP-tagged versions of δCaMKII wild-type, Thr287Asp and Thr287Val into HEK293T cells. We additionally co-transfected mCherry-tagged EAAT1 into HE293Ts to observe cellular localization and distribution. δCaMKII wild-type,

Thr287Asp and Thr287Val all localized in a uniform cellular distribution in EAAT1 transfected HEK293Ts (**Figures 11 B-D**). Next, we measured the consequence of δCaMKII Thr287 autophosphorylation mutants on [^{3}H]-glutamate uptake by EAAT1 or EAAT2. Surprisingly, we find that neither the Thr287Asp nor the Thr287Val mutant alters either EAAT1 mediated (**Figure 11E**) or EAAT2 mediated glutamate uptake (**Figure 11F**). UCPH-101, an EAAT1 inhibitor, and TBOA, an EAAT2 inhibitor, produced reductions in glutamate uptake as previously shown, suggesting that our experimental paradigm is sound. Overall, these data suggest that reducing or enhancing CaMKII activity below or above basal cellular levels respectively in HEK293T cells does not further enhance glutamate transporter-mediated glutamate uptake.

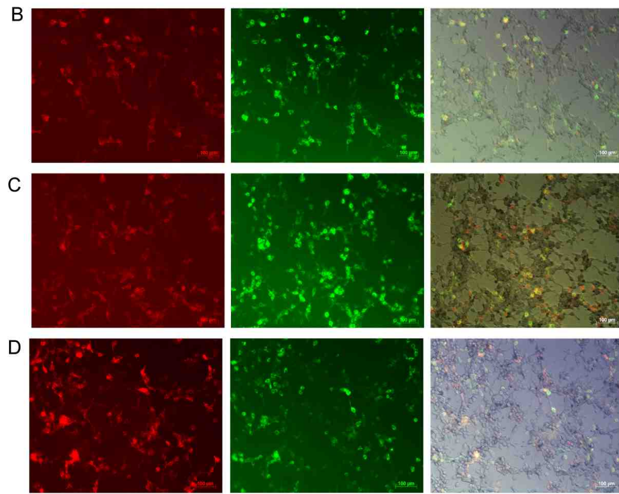
A



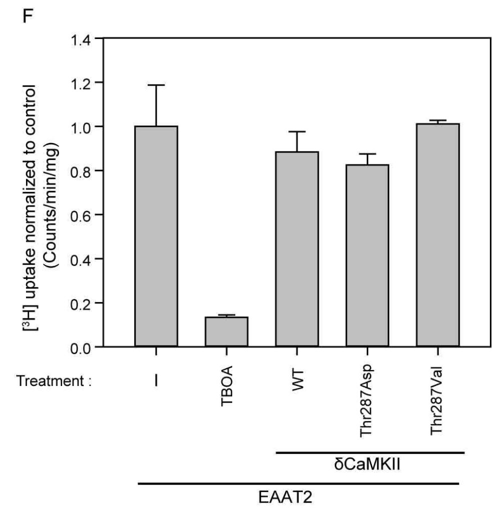
E



B
C
D



F

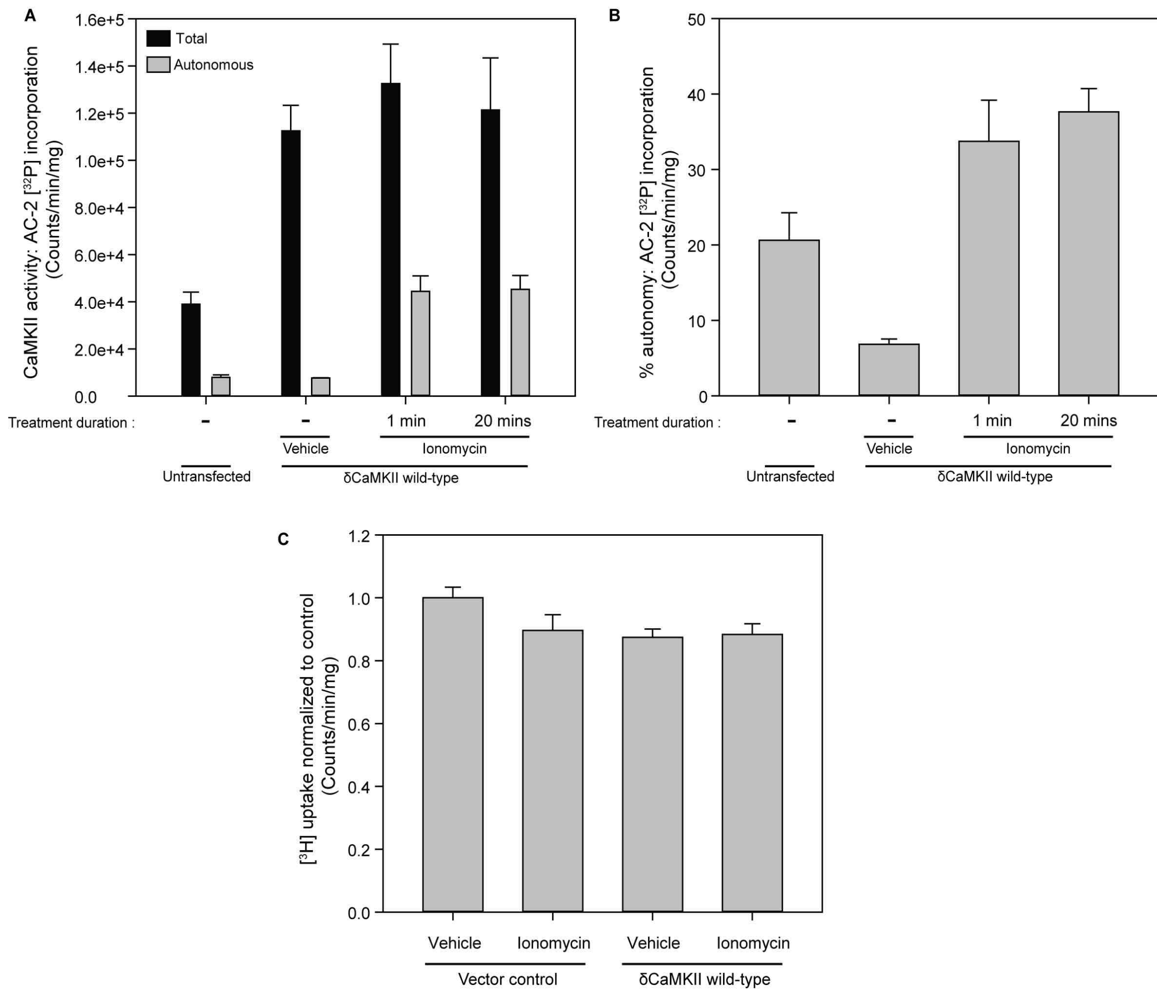


CaMKII activation by ionomycin does not alter glutamate uptake via EAAT1

We were surprised that neither Thr287Asp nor Thr287Val produced alterations in [^{3}H]-glutamate uptake. Thus, we decided to utilize a different methodology to enhance CaMKII signaling by using ionomycin, a known calcium ionophore. Although ionomycin has been reported to increase CaMKII activity, we wanted to test it in our system. In order to maximize the possible effect we would see, we overexpressed δCaMKII in HEK293T cells increasing Ca^{2+}/CaM-dependent activity by ~3-3.5 fold compared to untransfected cells (**Figure 12A**). We tested an acute (1 minute) and a chronic (20 minutes) time point for ionomycin treatment, using ionomycin at 10μM, previously described to be effective in transfected HEK cells (Hudmon, Lebel et al. 2005). We find that ionomycin increases [γ-^{32}P] incorporation into AC-2 at both the 1 and 20 minute time points, suggesting that CaMKII activation is both rapid and sustained following ionomycin treatment (**Figures 11 A and B**). Compared to % autonomy observed in δCaMKII-transfected cells, ionomycin treatment increases % autonomy significantly whereas vehicle (MeOH) treatment has no effect (**Figure 11B**). Given these findings, we decided to test the effect of ionomycin on [^{3}H]-glutamate uptake. As we did not previously observe any alterations in EAAT2 mediated glutamate uptake, we decided to focus on EAAT1 mediated glutamate uptake. We transfected HEK293T cells with vector control (YFP-tagged vector) or YFP δCaMKII, subsequently treating cells with either MeOH or ionomycin for 20 minutes. We found that ionomycin does not alter glutamate uptake in δCaMKII-transfected HEK293T cells (**Figure 12C**), corroborating our data in **Figure 11E** depicting no change in glutamate uptake when a δCaMKII Thr287Asp mutation is introduced. In total, neither site-directed mutagenesis

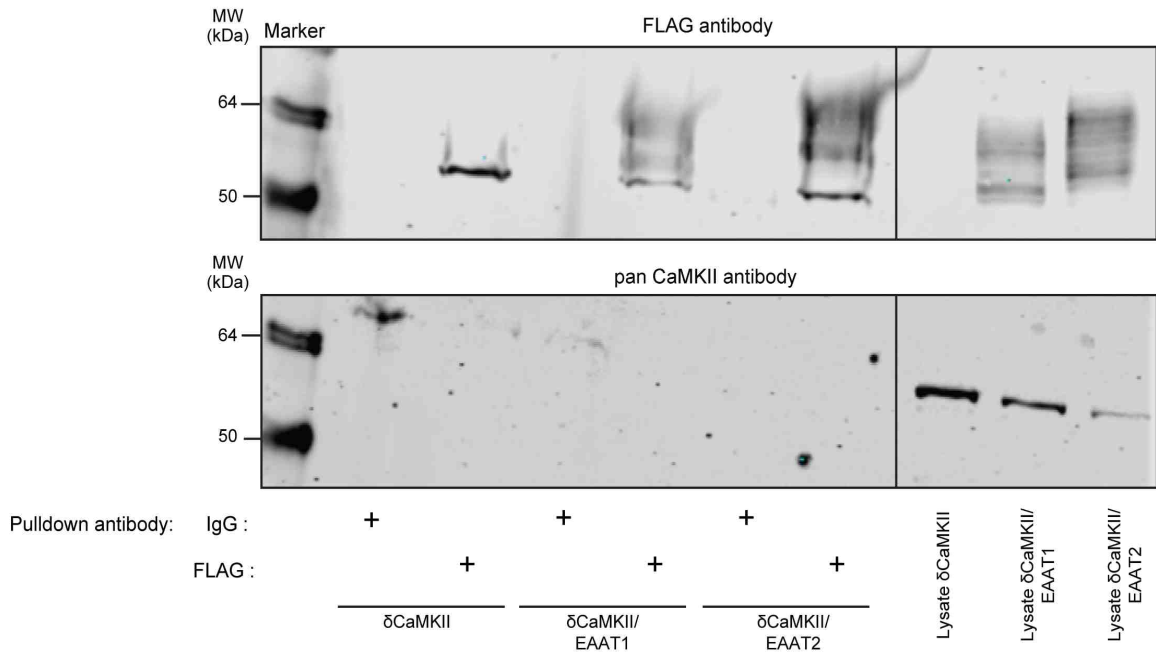
(phosphorylation-site mutants of the autophosphorylatable residue Thr287 in δCaMKII) nor enhancing intracellular calcium signaling (application of ionomycin) alter glutamate uptake in HEK293T cells expressing EAAT1 and EAAT2.

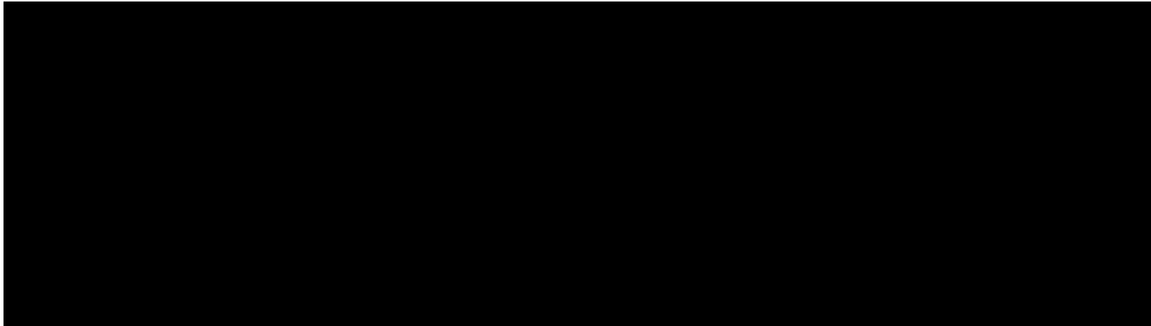
Data thus far suggest EAAT1 regulation by CaMKII. To date, none of the glutamate transporters have been described as CaMKII substrates, however one of the questions that arises from our experiments thus far is whether EAAT1 does, in fact, interact with CAMKII and serve as a CaMKII substrate? The next part of my dissertation addresses this question.


A
1.6e+5
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
Total
Autonomous
CaMKII activity: AC-2 [32P] incorporation (Counts/min/mg)
Treatment duration :
1 min
20 mins
Vehicle
Ionomycin
Untransfected
δCaMKII wild-type
B
50
40
30
20
10
0
% autonomy: AC-2 [32P] incorporation (Counts/min/mg)
Treatment duration :
1 min
20 mins
Vehicle
Ionomycin
Untransfected
δCaMKII wild-type
C
1.2
1.0
0.8
0.6
0.4
0.2
0.0
[3H] uptake normalized to control (Counts/min/mg)
Vehicle
Ionomycin
Vehicle
Ionomycin
Vector control
δCaMKII wild-type

Activated δCaMKII does not appear to bind either EAAT1 or EAAT2

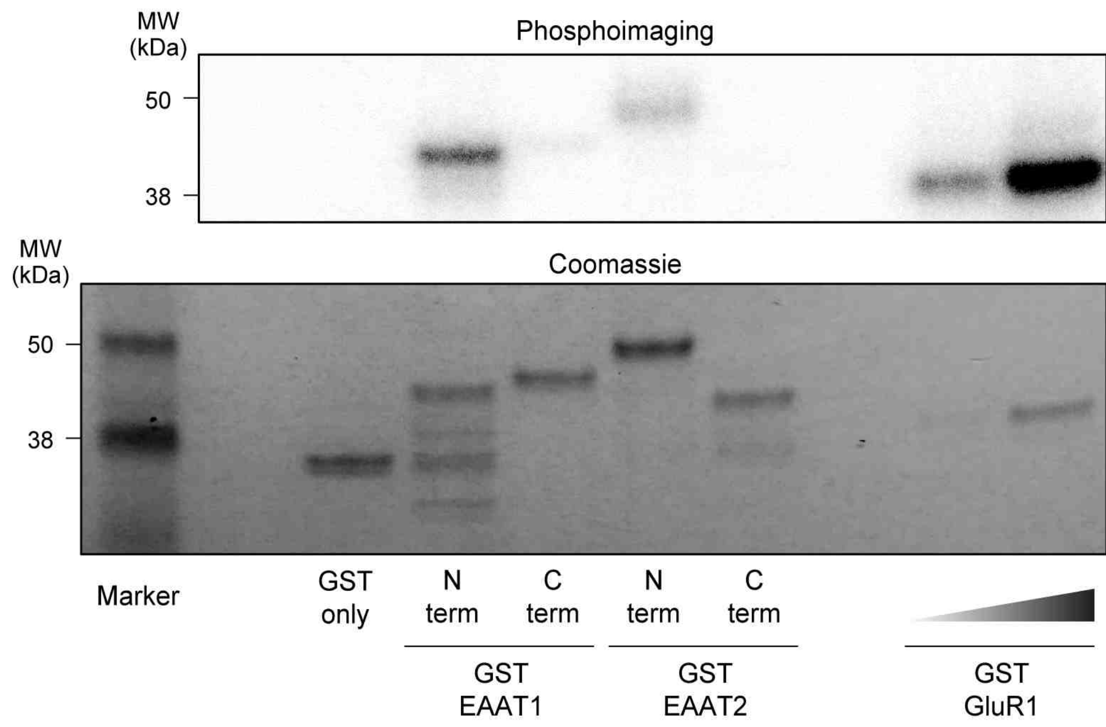
In order to test whether CaMKII interacts with glutamate transporters, we co-transfected δCaMKII and 3x FLAG-tagged EAAT1 and EAAT2 in HEK293T cells. We subsequently activated δCaMKII with 20 mins of ionomycin treatment (increase in % autonomy depicted in **Figures 12 A and B**), and tested for interaction between CaMKII and the glutamate transporters. We pulled down the transporters using a FLAG-antibody, and ran a Western blot testing our pull-down and δCaMKII interaction using a pan-CAMKII antibody. For controls, we ran lysates from our experimental transfection conditions: δCaMKII only, δCaMKII/3x FLAG EAAT1, and δCaMKII/3x FLAG 2. First, we find that we can detect substantial pull-down for both 3x FLAG-tagged EAAT1 and EAAT2 (in both the pulldown represented on the left side of the blot and lysate represented on the right side of the blot, **Figure 13**). The bottom bond observed at ~55kDa appears to be an artifact generated when the FLAG antibody is used (as it is observed when only δCaMKII is transfected and the FLAG antibody is used to pulldown protein). δCaMKII does not appear to interact with either EAAT1 or EAAT2, as evidenced by lack of detection on the left side of the blot when the pan CaMKII antibody is used for Western blot detection. However, it is clear that our pan CaMKII antibody does detect δCaMKII as it is observed in all three of our experimental conditions on the right side of the blot. In conclusion, ionomycin-activated δCaMKII does not appear to bind either glutamate transporter, EAAT1 or EAAT2.

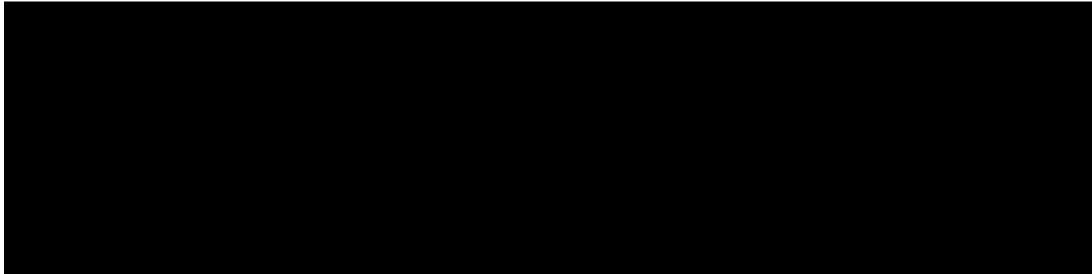

MW (kDa)
Marker
FLAG antibody
64
50
MW (kDa)
pan CaMKII antibody
64
50
Pulldown antibody:
IgG :
FLAG :
+
+
+
+
+
+
δCaMKII
δCaMKII/ EAAT1
δCaMKII/ EAAT2
Lysate δCaMKII
Lysate δCaMKII/ EAAT1
Lysate δCaMKII/ EAAT2



CaMKII phosphorylates a GST-fusion protein expressing the N terminus of EAAT1

Although CaMKII does not appear to bind either glutamate transporter, we wanted to test whether either transporter is a CaMKII substrate, as our data thus far our data suggests CaMKII regulates EAAT1 function. To address this, we generated GST-fusion proteins of the intracellular N- and C-termini of both EAAT1 and EAAT2. GST EAAT1 and -2 N- and C- termini were captured by glutathione resin, and then phosphorylated by purified human activated δCaMKII. Phosphorylation was measured by incorporation of radiolabeled phosphate [γ-^{32}P] into the substrates (upper image, **Figure 14**), and exposed onto an imaging screen for visualization. Measurements were normalized to Coomassie staining (lower image, **Figure 14**), a stain that binds to protein, to control for variance in total protein used in each treatment. A GST only lane served as a negative control (lane 1) while GluR1, a subunit of the AMPA receptor, served as a positive control (lanes 6 and 7, **Figure 14**). We observed comparable loading (lower image) among the four experimental GST fusion proteins as well as the GST control. GluR1 is a known substrate of CaMKII; we loaded variable concentrations as we expected the phosphorylation signal to be high. Qualitatively, we observe the strongest phosphorylation signal at the N terminus of EAAT1 that corresponds with the largest band of GST EAAT1 that likely represents unproteolysed fusion protein. We also observed a weak phosphorylation signal for the N terminus of EAAT2, however since the signal was barely detectable and we loaded a greater amount for the GST EAAT2 N terminus compared to the other fusion proteins we did not think this phosphorylation was of a significant amount (**Figure 14**).


MW
(kDa)
Phosphoimaging
50
38
MW
(kDa)
Coomassie
50
38
Marker
GST
only
N
term
C
term
N
term
C
term
GST
EAAT1
GST
EAAT2
GST
GluR1

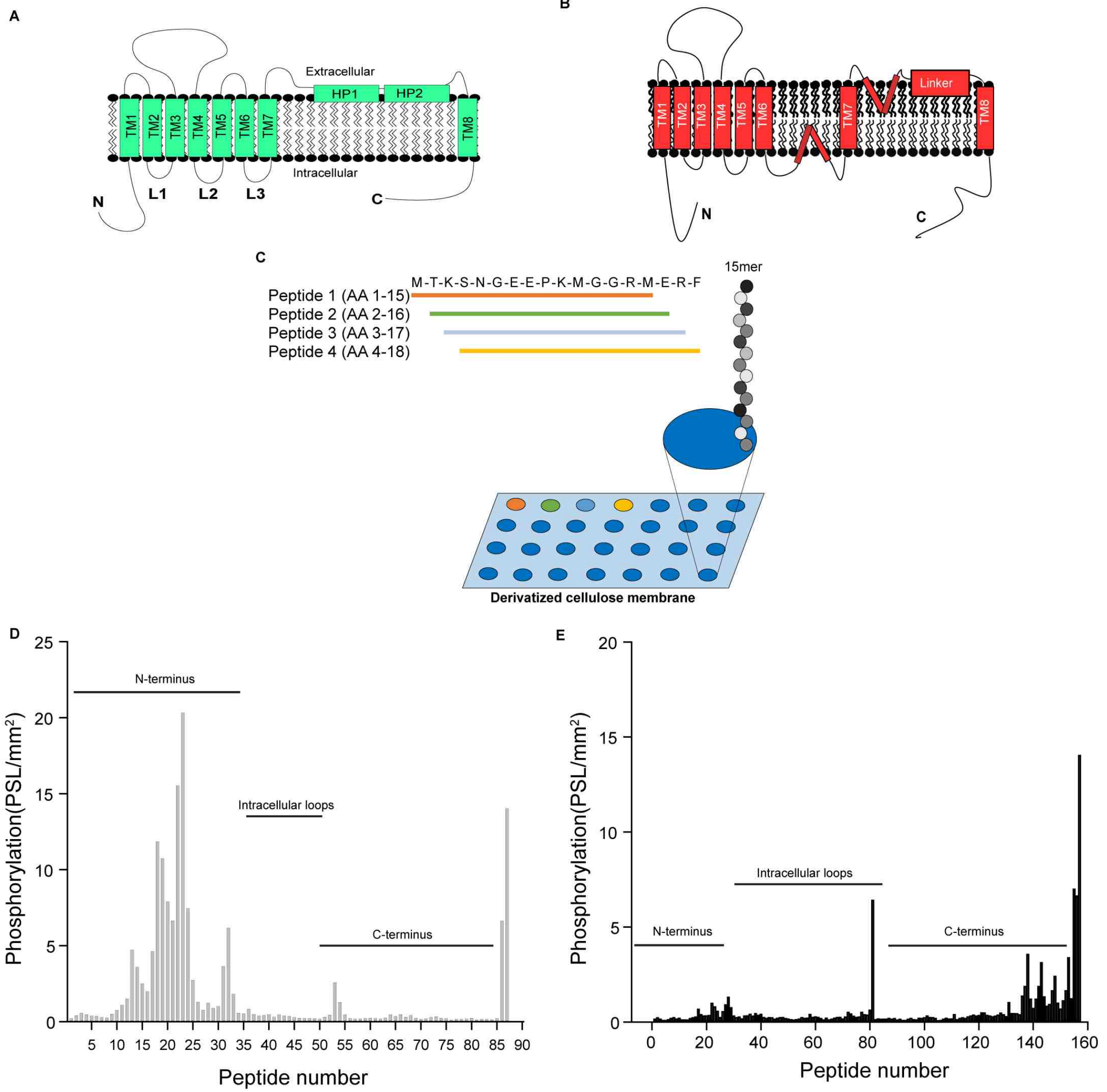


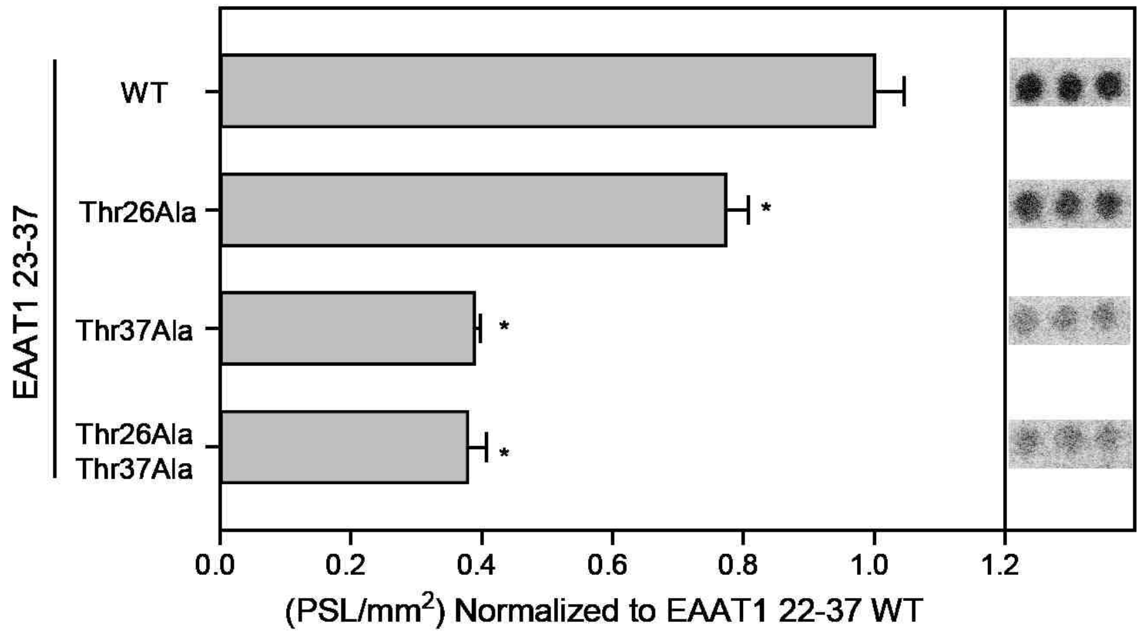
Utilization of the SPOTS blot strategy to identify potentially phosphorylatable residues in EAAT1 and EAAT2 by CaMKII

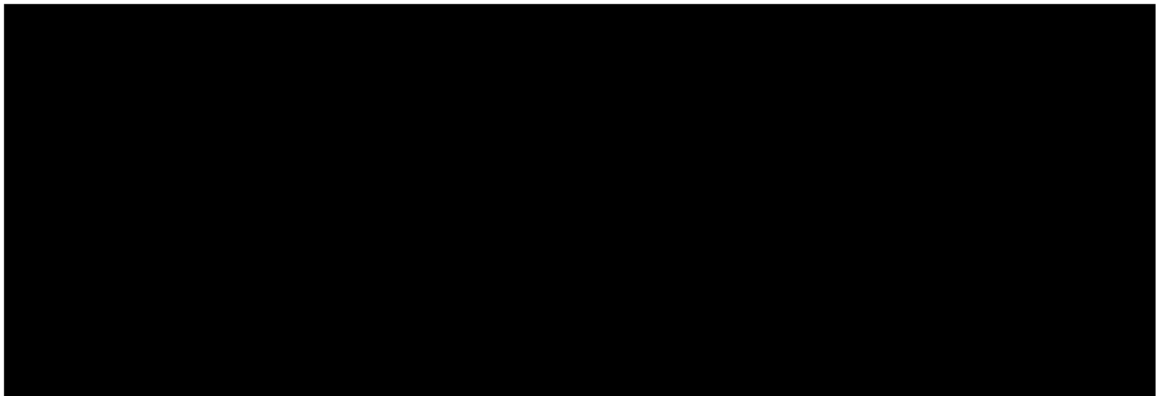
To identify specific residues phosphorylated by δCaMKII, intracellular regions of both EAAT1 and EAAT2 were tiled using immobilized peptide synthesis using the SPOTS approach as recently described (Ashpole, Herren et al. 2012). As a frame of reference, the predicted structure of EAAT1 is represented in **Figure 15A**, with 8 trans-membrane domains, intracellular N- and C- termini and 3 short intracellular loops (L1-L3). The predicted structure for EAAT2 is represented in **Figure 15B**. Similar to the predicted structure for EAAT1, EAAT2 has intracellular N- and C-termini. Furthermore, EAAT2 has 4 intracellular loops. Each 15-amino acid long peptide generated by the SPOTS approach differs by 1 amino acid, so for example as depicted in **Figure 15C** peptide 1 would span residues 1-15 of a given protein, peptide 2 would span residues 2-16, and so on. A blot was generated with the entirety of the predicted intracellular protein sequences of EAAT1 and EAAT2, as represented in **Figure 15C**. A blot containing all the testable peptides is thus treated at the same time, minimizing variability that may occur owing to differences during kinase activation and application onto membrane. Known CaMKII substrates GluR1 (described previously) and vimentin, an intermediate filament protein, were included on the tiled array as positive controls (two bars to the far right, **Figures 15D and 15E**). I*n vitro* phosphorylation of EAAT1 by CaMKII generated a number of potentially phosphorylated peptides (~peptides 18-23) in the middle of the N terminus of EAAT1, with a small spike observed at the very end of the N terminus (~peptides 32-33). Two potential residues identified from this peptide tiling analysis in **Figure 15D** were Thr26 and Thr37. Lack of [γ-^{32}P] incorporation in the predicted

intracellular loops (L, L2, and L3) and the C terminus suggest that these regions do not contain CaMKII phosphorylation sites. I*n vitro* phosphorylation of EAAT2 peptides by CaMKII generated a number of potentially phosphorylated peptides at the end of the C terminus (~peptides 137-157) as well as one peptide in intracellular loop 3 (**Figure 15E**). However, as we did not observe functional changes in glutamate uptake in EAAT2 upon altering CaMKII activity, we focused on EAAT1.

We decided to continue using the SPOTS blot strategy to replace potentially phosphorylated residues with an alanine, a non-phosphorylatable residue. First, we examined the two EAAT1 residues- Thr26 and Thr37. We synthesized the best peptides containing these residues as both wild-type and alanine mutations (as well as a double mutant) to test if specific threonine residues were phosphor-acceptors for CaMKII. Wild-type EAAT1 comprised of residues Arg23-Thr37, to be consistent with the 15mers used in **Figure 15D**. Ala mutants of both Thr26 and Thr37 as well as the double Ala mutant produced a significant reduction in CaMKII phosphorylation compared to wild-type (**Figure 16**). Specifically, Thr26Ala produced a reduction of 23±3%, while Thr37Ala and Thr26AlaThr37Ala produced a reduction of 62±1% and 63±3% respectively compared to wild-type.


A
Extracellular
HP1
HP2
TM1
TM2
TM3
TM4
TM5
TM6
TM7
TM8
Intracellular
N
L1
L2
L3
C
B
Linker
TM1
TM2
TM3
TM4
TM5
TM6
TM7
TM8
N
C
C
M-T-K-S-N-G-E-E-P-K-M-G-G-R-M-E-R-F
15mer
Peptide 1 (AA 1-15)
Peptide 2 (AA 2-16)
Peptide 3 (AA 3-17)
Peptide 4 (AA 4-18)
Derivatized cellulose membrane
D
N-terminus
Intracellular loops
C-terminus
Phosphorylation(PSL/mm²)
Peptide number
E
N-terminus
Intracellular loops
C-terminus
Phosphorylation(PSL/mm²)
Peptide number

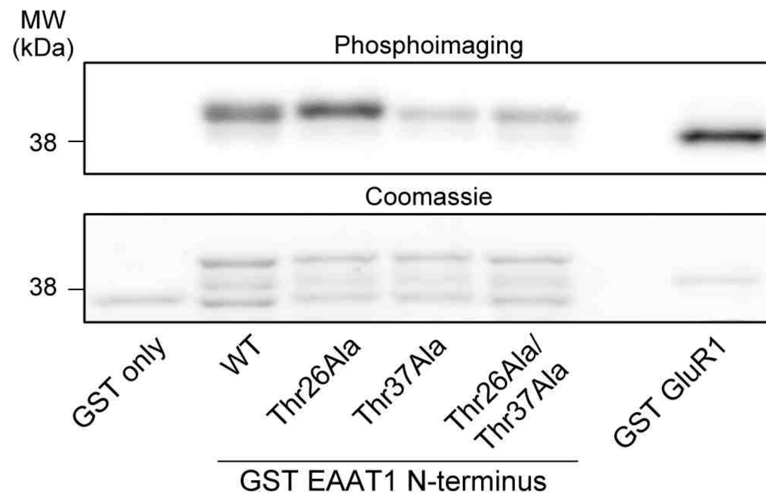

EAAT1 23-37
WT
Thr26Ala
Thr37Ala
Thr26Ala
Thr37Ala
*
*
*
0.0
0.2
0.4
0.6
0.8
1.0
1.2
(PSL/mm2) Normalized to EAAT1 22-37 WT

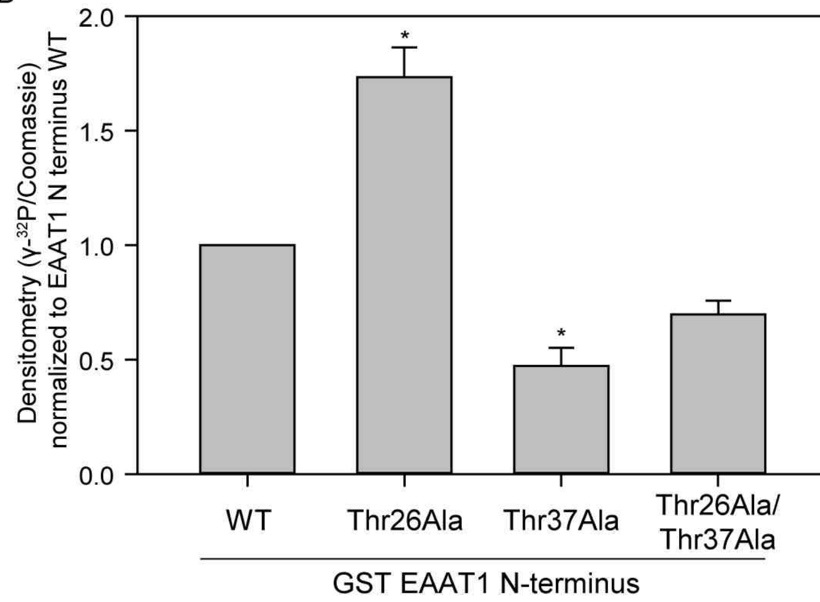


GST fusion proteins suggest Thr37 in EAAT1 is phosphorylated by CaMKII

Although potentially phosphorylated residues were identified in both EAAT1 and EAAT2, we decided to pursue only EAAT1 from hereon due to two reasons. One, we did not identify any functional contribution related to CaMKII signaling as a regulator of EAAT2 function (glutamate uptake, **Figures 8 and 9**). Second, although SPOTS blot analysis of the intracellular portions of EAAT2 suggested residues phosphorylated by CaMKII, alanine mutagenesis using the same methodology did not further suggest this is the case (data not shown). Thus, we focused our work on EAAT1 from hereon. We used GST-fusion proteins as previously described (**Figure 14**), of the EAAT1 N-terminus to parse out phosphorylation of residues Thr26 and Thr37 by CaMKII. Again, Ala substitutions were generated for Thr26 and Thr37, as well as a double Ala mutant, and we performed an *in vitro* phosphorylation reaction by δCaMKII. Thr37Ala produced a significant reduction in [γ-^{32}P] signal compared to wild-type (53±1%), while the double mutant produced a reduction that was not significant (**Figure 17B, representative image in Figure 17A**). These data confirmed our findings from **Figure 16**. However, Thr26Ala produced an increase in phosphorylation which was inconsistent with the SPOTS blot data generated in **Figure 16**. It is possible that the Thr26Ala mutation results in a mis-folded GST fusion protein that may cause [γ-^{32}P] to bind more strongly to the protein. Furthermore, this protein mis-folding may make another serine or threonine residue increasingly accessible to [γ-^{32}P], causing an increase in radioactive signal detected. Even with proper folding, the possibility of increased phosphorylation of another residue is likely. The non-significant reduction in [γ-^{32}P] incorporation observed in the Thr26Ala/Thr37Ala mutant (compared to wild-type) is in agreement with this data- if one

Ala mutation increases [γ-^{32}P] incorporation whereas the other one decreases it, the double mutant will not provide a significant alteration in phosphorylation, which appears to be the case. Although we are unsure of the exact reason underlying this discrepancy, we next decided to measure the effect of mutations at both these residues on [^{3}H]-glutamate uptake.


A
MW
(kDa)
Phosphoimaging
38
Coomassie
38
GST only
WT
Thr26Ala
Thr37Ala
Thr26Ala/
Thr37Ala
GST GluR1
GST EAAT1 N-terminus

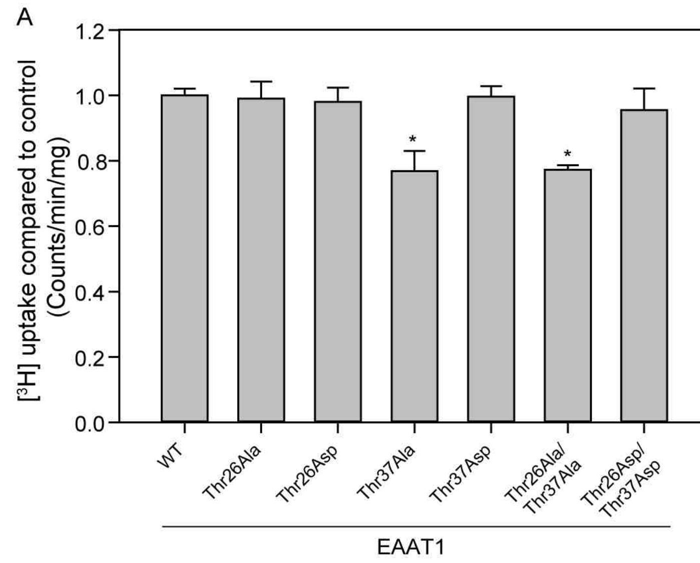

B
Densitometry (γ-32P/Coomassie)
normalized to EAAT1 N terminus WT
2.0
1.5
1.0
0.5
0.0
*
*
WT
Thr26Ala
Thr37Ala
Thr26Ala/
Thr37Ala
GST EAAT1 N-terminus

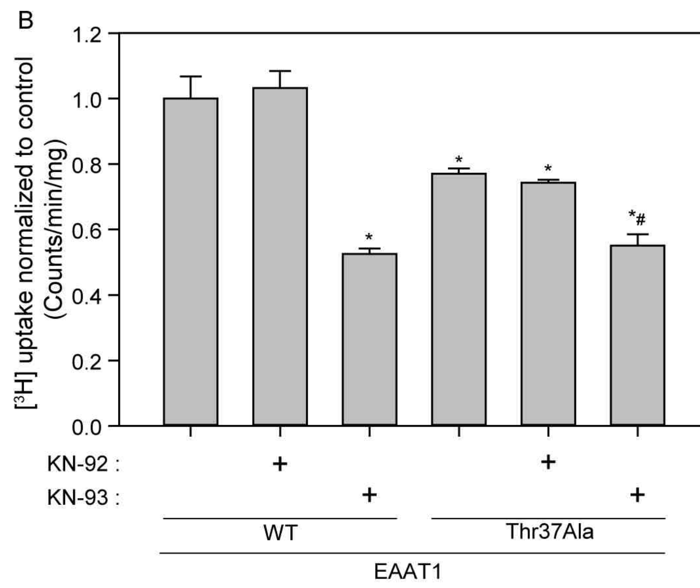
A phosphorylation event at EAAT1 Thr37 by CaMKII mediates alterations in glutamate uptake

To identify the functional significance of the Thr26 and Thr37 phosphorylation sites on EAAT1 mediated glutamate uptake, we mutated Thr26 and Thr37 into Ala residues in order to block phosphorylation. Furthermore, we generated double Ala mutants to determine the contribution of both sites. Only the Thr37Ala and Thr26Ala/Thr37Ala produced a significant reduction in [^{3}H]-glutamate uptake (**Figure 18A**). Thr26 does not appear to regulate alterations in [^{3}H]-glutamate uptake mediated by EAAT1 as the Thr26Ala mutant does not produce any change in glutamate uptake. We next mutated Thr26 and Thr37 into Asp residues, to mimic the negative charge as a consequence of phosphorylation (thereby generating phosphomimetics). Additionally, we generated a double Asp mutant (Thr26Asp/Thr37Asp) to determine whether there was an additive contribution by both sites. Surprisingly, neither Thr37Asp nor Thr26AspThr37Asp mutants increased EAAT1 mediated glutamate uptake, suggesting that CaMKII regulation of this process may occur to maintain homeostatic function.

Next, to test the specificity of Thr37 and the KN93 drug in regulating EAAT1, we performed an occlusion experiment to determine if the presence of both the CaMKII inhibitor and the Ala substitution were synergistic or additive. Corroborating our observation in **Figure 18A**, EAAT1 in the Thr37Ala background produced a significant reduction in [^{3}H]-glutamate uptake compared to EAAT1 wild-type (**Figure 18B**). When we applied CaMKII inhibitor, KN-93, onto EAAT1 Thr37Ala, we observed that the Thr37 mutation partially occluded the KN-93 effect. [^{3}H]-glutamate uptake was significantly further reduced compared to KN-92 and control conditions (**Figure 18B**),

suggesting CaMKII may have direct (phosphorylation) effects on a previously unidentified site or possibly indirect effects on EAAT1 mediated glutamate uptake via regulating EAAT1's function via its interaction with another factor/protein.


A
[3H] uptake compared to control (Counts/min/mg)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
WT
Thr26Ala
Thr26Asp
Thr37Ala
Thr37Asp
Thr26Ala/ Thr37Ala
Thr26Asp/ Thr37Asp
EAAT1


B
[3H] uptake normalized to control (Counts/min/mg)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*#
KN-92 :
KN-93 :
+
+
+
+
WT
Thr37Ala
EAAT1

DISCUSSION

General Conclusions

The differential ability of cellular glutamate uptake to diminish extracellular glutamate concentrations in order to prevent glutamate-induced excitotoxicity was discovered almost four decades ago (Hertz, Schousboe et al. 1978, Drejer, Larsson et al. 1982, Choi 1992). However, parsing out underlying mechanisms for a complex process such as glutamate uptake was complicated owing to the contribution by numerous cell types and multiple proteins. The molecular identity of the five known glutamate transporter subtypes was discovered through the 1990s (Kanai and Hediger 1992, Pines, Danbolt et al. 1992, Storck, Schulte et al. 1992, Fairman, Vandenberg et al. 1995, Arriza, Eliasof et al. 1997). These findings were pivotal as they allowed researchers to examine the contribution and regulation of each glutamate transporter subtype individually, particularly in heterologous expression systems. It is now known that the physiological pathways involving different cellular signaling proteins can result in differential alterations in surface expression, total expression, and activity of the glutamate transporters. Furthermore, subsequent pathological changes can alter glutamate transporter regulation. The findings in this dissertation uncover a novel regulation of the astrocytic glutamate transporter subtype EAAT1 by the ubiquitously expressed serine/threonine protein kinase CaMKII. Interestingly, our data suggests that it is the autonomously active form of CaMKII that is necessary for the homeostatic regulation astrocytic glutamate uptake. Since intracellular Ca^{2+} levels can change rapidly, it is thought that CaMKII autonomy serves as a key form of molecular memory for cellular Ca^{2+} signals even when the Ca^{2+} transient subsides. These findings support the idea that

CaMKII previously activated in the cell by Ca^{2+}/CaM does, in fact, serve as an important form of a molecular memory device and regulates EAAT1 via phosphorylation.

The majority of our understanding of CaMKII function comes from studies performed in neurons. CaMKII has been implicated in a number of cellular processes such as calcium homeostasis, excitability, neurotransmitter synthesis/release and neuronal plasticity (reviewed in (Hudmon and Schulman 2002, Robison 2014)). Numerous reports suggest that a loss of both neuronal and astrocytic CaMKII signaling may be an important driver of excitotoxicity (Hajimohammadreza, Probert et al. 1995, Laabich and Cooper 2000, Goebel 2009, Vest, O'Leary et al. 2010, Ashpole, Song et al. 2012, Ashpole, Chawla et al. 2013). Especially pertinent to our studies, pharmacological inhibition of astrocytic CaMKII resulted in dysregulated calcium homeostasis, altered mitochondrial membrane potential, excessive release of the gliotransmitter ATP and decreased glutamate uptake; functional changes that are associated with compromised neuronal survival (Ashpole, Chawla et al. 2013).

In this dissertation, data are presented that provide evidence suggesting CaMKII regulates astrocytic glutamate uptake via the novel substrate and glutamate transporter EAAT1, but not EAAT2. This regulation appears to be two-pronged: it is partly dependent on CaMKII phosphorylation of the Thr37 residue on EAAT1, and partly appears to occur via an unidentified mechanism that is independent of phosphorylation at this particular site. The remainder of the regulatory effect could be accounted for by phosphorylation at another site on EAAT1 that our studies may have failed to identify, or by the action of CaMKII on another protein. Although there had been no studies describing glutamate uptake regulation by CaMKII when I had undertaken my

dissertation work, a recent study by Underhill et al describes the latter form of regulation (Underhill, Wheeler et al. 2015). This study reported CaMKII phosphorylation of DLG1, a scaffolding protein, which in turn regulated surface expression of a splice variant of EAAT2 termed EAAT2b that differs at the C-terminus (Underhill, Wheeler et al. 2015). Utilizing MDCK epithelial cells and astrocyte cultures as model systems, this report also found that CaMKII did not alter surface expression of the splice variant EAAT2a (the splice variant I studied), a finding that is consistent with our observation that alterations in CaMKII activity do not affect glutamate activity through EAAT2a. In sum, CaMKII appears to differentially regulate glutamate uptake via the astrocytic glutamate transporters and their splice variants.

Cultured cortical astrocytes as a model system

Astrocytes are involved in a wide array of functions in the brain. These include but aren't restricted to buffering potassium ions, controlling extracellular pH, controlling cerebral blood flow, and taking up glutamate and GABA (Tschirgi and Taylor 1958, Orkand, Nicholls et al. 1966, Martinez-Hernandez, Bell et al. 1977, Kimelberg 1987). In order to examine astrocytic function outside of the intact brain, astrocytes were cultured to study specific cellular functions. In particular, cultured astrocytes were first utilized to study glutamate uptake almost four decades ago (Hertz, Schousboe et al. 1978, Drejer, Larsson et al. 1982). Since then, they have been extensively utilized as a model system for a number of reasons. First, dissociated postnatal astrocyte cultures represent a mature and differentiated cell line that can be used for several weeks in culture once harvested. Additionally, it is known that numerous cell types and brain tissue express a variety of

glutamate transporter isoforms (discussed in Background). Pure cortical astrocytes cultures thus provide a system that has minimal other confounding factors from other cells or transporter subtypes. Specifically, astrocytes primarily express the EAAT1 and EAAT2 (Rothstein, Dykes-Hoberg et al. 1996, Tanaka, Watase et al. 1997, Watase, Hashimoto et al. 1998) while EAAT3 has been observed to express in a neuronal background (Kanai and Hediger 1992). However, both glial and neuronal cells can express glutamate transporters in addition to EAAT1, EAAT2 and EAAT3 as a function of tissue localization (refer to **Table 1**). For example, EAAT4 is exclusively expressed in the cerebellum (Fairman, Vandenberg et al. 1995), while EAAT5 is solely expressed in retinal glial cells (Arriza, Eliasof et al. 1997). Thus, careful consideration needed to be put into generating and maintaining our dissociated astrocyte cultures. During our astrocyte culture generation protocol, we carefully excised the cortex away from the intact brain tissue, and ensured we did not support either the growth of neurons or oligodendrocytes by mechanical elimination (look at Materials and Methods). Upon characterization of our cultures using immunocytochemical analyses, we found that our cultures were primarily astrocytic (**Figure 4**), with minimal contamination by microglia and neurons. Thus, cortical astrocyte cultures represent a strong model system to study the regulation of astrocytic glutamate transporters by CaMKII.

Astrocytes are non-excitable cells that are found in close proximity to and interact with various other cells types, such as neurons, microglia, oligodendrocytes, and vascular cells, in the intact brain. While having an isolated culture enables us to study glutamate uptake occurring only via astrocytes, it is important to recognize that various other cells may provide supporting functions for those performed by astrocytes, much

like an astrocyte provides for a neuron. It is clear that astrocytes function differently in culture versus brain slices versus in an intact brain. For example, the astrocytic protein GFAP which serves as a marker for reactive changes and astrogliosis (Eng and Ghirnikar 1994) is not highly expressed in an intact brain or in brain slices, but is expressed at high levels in astrocyte cultures (Kimelberg, Cai et al. 2000). Furthermore, although GFAP is commonly used as a protein marker for astrocytes and all cells that express GFAP are considered to be astrocytes, all astrocytes do not express GFAP. Another example is that of the developmental protein and a marker of immature glial and neuronal cells, nestin. The nestin protein is expressed by most astrocytes in primary dissociated culture (Gallo and Armstrong 1995), a perplexing observation suggesting that the cells obtain a more primitive phenotype. Lastly, astrocyte cultures have also been described to express proteins typically associated with pathological states such as a proteoglycan form of the Alzheimer's protein β-amyloid precursor protein (APP), which is expressed in injured brain but not detectable in normal brain tissue (Oohira, Kushima et al. 1995). It is clear that astrocytes are highly plastic cells that alter rapidly in response to their environment. Numerous reports thus suggest cultured astrocytes may exhibit a more primitive and reactive astrocytic morphology and protein expression compared to intact astrocytes, which is an important limitation to consider when using dissociated glial cells as a model system.

Early studies indicated low levels of glutamate uptake in the rodent brain at birth, which increased to adult levels within the first few weeks (Schousboe, Lisy et al. 1976, Schmidt and Wolf 1988, Cohen and Nadler 1997), although at this time the molecular determinants underlying this functional consequence were unknown. With the increased

utilization of antibodies, immunofluorescence and immunoblotting studies reported that GLAST and GLT proteins specifically were present at early stages of development, but at very low concentrations (Ullensvang, Lehre et al. 1997). In fact, GLT-1 was barely detectable in the newborn rat while GLAST levels were more easily detectable. The expression of both these proteins increased with age and reached maximal expression at 5 weeks of age (Furuta, Rothstein et al. 1997, Ullensvang, Lehre et al. 1997). Interestingly, the expression pattern that occurs in embryonic rats is similar to the pattern observed in dissociated rodent cultures where GLAST is the primary astrocytic glutamate transporter with GLT-1 being expressed at much lower levels (Gegelashvili, Danbolt et al. 1997, Swanson, Liu et al. 1997). These findings suggest that removing astrocytes from their native environment causes the cells to revert to a more primitive phenotype. Low levels of GLT-1 expression could explain the lack of CaMKII regulation we observe, although we did supplement our astrocyte growth media with growth factors that may lead to increased GLT-1 expression. In order to substantiate these findings however, we use HEK293T cells as a heterologous expression system to study the regulation of the distinct astrocytic glutamate transporters in which we would have detected CaMKII regulation of EAAT2.

HEK293T cells as a model system

The HEK293T cell line affords numerous benefits as a model system for heterologous expression: one can rapidly and inexpensively reproduce and maintain them, they are easily and efficiently transfected using a number of different transfection methodologies and they have a high efficiency of transfection and protein production

(Thomas and Smart 2005). In fact, HEK cells have been used and characterized previously to study glutamate uptake (Dunlop, Lou et al. 1999, Berry, Hayes et al. 2005, Peacey, Miller et al. 2009).

Glutamate transporters exist as homomeric trimers, with the exception of inter-subunit interaction being described between only EAAT3 and EAAT4 thus far (Nothmann, Leinenweber et al. 2011). Our data suggests that we achieved a 46 ± 7% and 43 ± 9% transfection efficiency for EAAT1 and EAAT2 cDNA in HEK293T cells respectively, which resulted in a subsequent 3.08 ± 0.14 fold and 3.31 ± 0.32 fold increase in glutamate uptake (**Figure 7**). The low levels of endogenous uptake in HEK cells have been attributed to the expression of the glutamate transporter EAAC1/EAAT3 (Kanai and Hediger 1992, Shayakul, Kanai et al. 1997). Inter-subunit interaction has not been described between EAAT1, EAAT2, and EAAT3, and thus we can assume that the increase in glutamate uptake observed is mediated by homotrimers of EAAT1 and EAAT2.

We are limited in using HEK cells as a model system in that if CaMKII regulated either glutamate transporter via an indirect phosphorylation event involving a protein that was not present in the native HEK cell environment, we would be unable to parse out this regulatory event. However, even though the HEK cell line does not provide the complex intercellular milieu associated with a differentiated native brain cell such as a neuron or an astrocyte, they are a powerful tool to study protein functions related to glutamate signaling, transporter and ion channel physiology. In order to address the limitations involving the complexity of HEK cells, we also used cultured cortical astrocytes and a glioma cell line to confirm our findings. Our heterologous system responds in a similar

manner as a primary astrocyte culture and a transformed astrocyte cell line to alterations in CaMKII signaling, strengthening the conclusions we arrived at in HEK293T cells.

Isoform specificity of CaMKII regulation

CaMKII is expressed in high levels in the brain; in some regions it accounts for up to 2% of total protein (Erondu and Kennedy 1985). Four distinct genes encode for CaMKII in eukaryotes: α, β, γ, and δ (for review see (Hudmon and Schulman 2002)). The α and β isoforms of CaMKII, which predominate in neurons (Erondu and Kennedy 1985), have been found to play important roles in synaptic plasticity, cell survival, and excitability in a neuronal context. Cultured cortical astrocytes express the γ and δ isoforms of CaMKII (Takeuchi, Yamamoto et al. 2000, Vallano, Beaman-Hall et al. 2000), which are expressed in a number of other tissues such as skeletal muscle, pancreas and the heart (Ouimet, McGuinness et al. 1984, Tobimatsu and Fujisawa 1989).

In our studies, we used a combination of pharmacological and genetic manipulations to address the regulatory role of CaMKII with respect to astrocytic glutamate uptake. To elicit pharmacological inhibition, we utilized the small molecule inhibitor KN-93 and the peptide inhibitor tat-CN21. These pharmacological inhibitors have been previously utilized in tissues that collectively express all four isoforms of CaMKII, resulting in inhibition of CaMKII activity (Rokolya and Singer 2000, Vest, O'Leary et al. 2010, Westenbrink, Ling et al. 2015). Additionally, these inhibitors target the regulatory region, which is highly conserved among all the known CaMKII isoforms. We thus assume these inhibitors will target all four isoforms of CaMKII. For our genetic manipulations, we chose to manipulate the δ isoform as mRNA levels of this particular

isoform are expressed at much higher levels in astrocytes compared to the γ isoform (Takeuchi, Yamamoto et al. 2000). Our data suggests alterations in CaMKII regulate EAAT1 mediated changes in glutamate uptake, based on a reduction in [^{3}H]-glutamate uptake upon transfection with the Asp136Asn mutant of δCaMKII. A rather straightforward conclusion underlying this observation is that the Asp136Asn mutant forms homomultimers with wild-type δCaMKII, thus causing a dominant-negative effect and resulting in cellular functional alterations, in a manner similar to a recently described Lys42Met mutant of CaMKII (Kabakov and Lisman 2015). However, it is also feasible that the molecular mechanism underlying this finding is the generation of heteroisomeric multimers of CaMKII upon addition of this mutant. In fact, co-assembly of CaMKII isoforms within the twelve subunit holoenzyme has been described to occur before (Vallano 1989, Kolb, Hudmon et al. 1998, Brocke, Chiang et al. 1999), and thus provides a strong rationale for this mechanism of regulation. Regardless of if and how the monomeric subunits combine, it is clear that a reduction in CaMKII activity results in a reduction in glutamate uptake.

Furthermore, compensatory changes in CaMKII isoform expression and regulation can occur during pathological states, thus we cannot exclude the possibility of either a switch in isoform-specific regulation or dual regulation by both CaMKII isoforms on EAAT1 function. A recent study describes a rationale for the former mechanism, whereby a decrease in γCaMKII mRNA is accompanied by a concurrent increase in δCaMKII mRNA as a result of a lumbar spinal nerve injury (Bangaru, Meng et al. 2015). Additionally, alterations in the expression of specific CaMKII isoforms have been reported following injury or as a result of pathological conditions. In a Lysophosphatidic

acid (LPA) receptor knockout mouse, which mimics a schizophrenia phenotype, an increase in the level of βCaMKII has been reported in the knockout compared to the wild-type mouse (Musazzi, Di Daniel et al. 2011). This alteration in βCaMKII decreases the ratio between the two predominant CaMKII neuronal isoforms (α/β CaMKII), which has been shown to be important for neuronal maturation during development, subcellular localization, synaptic activity and strength (Thiagarajan, Piedras-Renteria et al. 2002, Fink, Bayer et al. 2003, Musazzi, Di Daniel et al. 2011). While the importance of such a ratio has not been elucidated for the γ and δCaMKII isoforms, one may hypothesize that in a manner similar to α and β CaMKII, skewing the endogenous ratio may play a role in altering both physiological and pathological processes.

The protein sequences of the four human CaMKII isoforms are ~95% conserved in the catalytic domain, with overall conservation of the entire CaMKII sequence being ~60%. However, major differences are found in the variable region, located within the hub domain, where sequence inserts can contain substrate-targeting motifs that are unique to each isoform. For example, it was originally thought that βCaMKII had an F-actin binding domain within the variable region that the other isoforms lack (Fink, Bayer et al. 2003). Now, it appears that both δ and γCaMKII may interact with F-actin and inhibit its polymerization, albeit with a lower affinity (Hoffman, Farley et al. 2013). However, even within the βCaMKII isoform, splice variants exist that differentially target F-actin (Zheng, Redmond et al. 2014). Substrate-binding of the different CaMKII isoforms is a complex phenomenon and needs to be examined in greater detail. While the functional consequences have not been elucidated, it is known that astrocytes express a single splice variant of δCaMKII known as δ_2, and two splice variants of γCaMKII γ_A and γ_B (Vallano,

Beaman-Hall et al. 2000). Other than affecting substrate specificity, differential domains in the variable region of CaMKII can also affect cellular localization that may result in functional discrepancies. It is thus clear that if compensatory changes in specific CaMKII isoforms were to occur, substrate specificity and cellular localization can be altered. Taken together, it is plausible that EAAT1 is a substrate for either one or both isoforms of CaMKII expressed in astrocytes.

Isoform specificity of astrocytic glutamate transporter regulation

In the rodent central nervous system, GLAST and GLT-1 are expressed on the same astrocytes (Lehre, Levy et al. 1995, Haugeto, Ullensvang et al. 1996). Where they differ is their level of expression based on the tissue or cell type they are studied in. For example, while GLAST expression is consistently described in astrocytes in the cortex (Lehre, Levy et al. 1995), higher expression is also reported in specialized cells such as the cerebellar Bergmann glia and retinal Müller cells (Torp, Danbolt et al. 1994, Rauen, Rothstein et al. 1996). In keeping with these findings, GLAST knockout mice exhibit cerebellar ataxia, the inability to perform complex tasks of coordination, and severe cerebellar edema in response to an ischemic insult (Watase, Hashimoto et al. 1998). GLAST knockout mice also display increased retinal damage as a result of ischemia, and altered electroretinograms and oscillatory potentials compared to wild-type mice (Harada, Harada et al. 1998). Furthermore, chronic utilization of GLAST antisense oligonucleotides results in neuronal excitotoxicity in motor neurons and striatal neurons, elevated extracellular glutamate and progressive paralysis *in vivo* in a rodent model (Rothstein, Dykes-Hoberg et al. 1996), suggesting that aberrations in EAAT1 activity

result in phenotypes throughout the brain due to their ubiquitous expression. Our model suggests a loss of CaMKII signaling decreases glutamate uptake via EAAT1, eventually leading to dysregulated glutamate uptake and neuronal death. Based on the cellular and tissue distribution of the EAAT1 transporter, it is plausible that a loss of CaMKII signaling will result in cortical deficits, and a particularly severe phenotype in regions like the cerebellum and retina, where glutamate uptake is largely governed by EAAT1.

Recent work from the Amara laboratory shows that CaMKII phosphorylation of a PDZ scaffolding protein, Discs large homolog 1 (DLG1/SAP97), decreases surface expression of a splice variant of EAAT2 known as EAAT2b and presumably transporter activity (Underhill, Wheeler et al. 2015). This effect was isoform specific as EAAT2a trafficking was not affected by CaMKII signaling. Our study confirms that EAAT2a is not altered by CaMKII signaling, and extends this work by showing that CaMKII can produce isoform specific modulation of EAAT activity. We did not test the regulation of EAAT2b by CaMKII, although the DLG1/SAP97 protein is endogenously expressed by HEK293T cells (Cai, Li et al. 2008). While Underhill et al. favor an indirect mechanism of regulation based on membrane stability of EAAT2b by a phosphorylated scaffolding protein, we think phosphorylation of the transporter itself is an additional key regulatory mechanism conferred by CaMKII. Interestingly, Thr37 is not conserved in EAAT2 (**Figure 2**), providing a basis for the lack of a CaMKII regulatory effect on EAAT2 observed in the experiments described in this study. Remarkably, Thr37 is conserved in only one other glutamate transporter- EAAT4. It is noteworthy to point out that EAAT1 and EAAT4 represent the entirety of glutamate transporter expression in the cerebellum, strengthening the notion that a loss of CaMKII signaling would be highly detrimental in

the cerebellar region. In fact, a βCaMKII knockout mouse exhibits cerebellar ataxia (Bachstetter, Webster et al. 2014), a phenotype identical to that observed in the GLAST knockout mouse (Watase, Hashimoto et al. 1998) which may be due to convergent molecular mechanisms. Future studies are needed to determine whether EAAT4, expressed almost exclusively in the cerebellum (Fairman, Vandenberg et al. 1995), is directly phosphorylated by CaMKII, and what the functional consequence of this post-translational modification may be.

The idea that a kinase can differentially regulate subtypes of transporters is not unprecedented. For example, glycogen synthase kinase 3β has been shown to differentially modulate GLAST and GLT-1, producing a down-regulation in GLAST but up-regulation in GLT-1 activity and cell-surface expression via phosphorylation of the transporters (Jimenez, Nunez et al. 2014). Collectively, glutamate transporter regulation by CaMKII appears complex in that it can have opposing outcomes on transporter function that are isoform dependent. Additional studies are required to determine how EAAT1 activity is regulated by CaMKII (i.e. gating or trafficking), in what manner basal regulation of EAAT1 by CaMKII is coupled to EAAT2b function, and whether specific states of CaMKII activation may be important for this isoform specific regulation.

CaMKII autophosphorylation mutants do not alter glutamate uptake

Canonical CaMKII autophosphorylation occurs when Ca^{2+}-bound CaM displaces the autoregulatory domain from the catalytic cleft of CaMKII, allowing an intrasubunit autophosphorylation reaction directed at the Thr286/287 residue in the autoregulatory domain to occur. CaMKII phosphorylation at this site renders the kinase autonomously

active independent of Ca^{2+}/ CaM, allowing CaMKII to phosphorylate substrates more efficiently (Lai, Nairn et al. 1987, Miller, Patton et al. 1988). While the majority of this dissertation pertains to astrocytic CaMKII, much of our current understanding of this enzyme comes from its role in neurons. For example, autophosphorylation at Thr286/287 on CaMKII was thought to be an essential event for its translocation to the PSD (Strack, Choi et al. 1997, Strack and Colbran 1998), a specialized area within the dendritic region of neurons that is packed with a large number of proteins, and contains over 30 CaMKII substrates (Yoshimura, Aoi et al. 2000, Yoshimura, Shinkawa et al. 2002). Once recruited to the PSD, these substrates become easily accessible to CaMKII and are increasingly phosphorylated. However, it now appears that autophosphorylation of CaMKII may not be necessary for its translocation, but it instead alters dwell time of the kinase at the PSD (Shen, Teruel et al. 2000). Thus, even in a cellular context where CaMKII has been studied in great detail, it is clear that the functional consequences related to CaMKII autonomy are rather complex and not fully understood. What is clear is that autonomous CaMKII serves as a form of molecular memory that is important for the kinase to retain past cellular activity even when calcium levels return to baseline.

Less is known about functional events associated with CaMKII autophosphorylation in the astrocyte or HEK293T cells in comparison to a neuron. Interestingly, the percentage of autonomous CaMKII activity in an astrocyte is one of the highest described for any cell type ranging from ~24-75% (Song, Bellail et al. 2006, Ashpole, Chawla et al. 2013), suggesting that astrocytic CaMKII may not need external provocation to activate calcium signaling machinery in order to activate downstream signaling mechanisms. CaMKII autophosphorylation mutants (Thr287Asp or Thr287Val

represented in **Figure 10**) did not result in altered [^{3}H]-glutamate uptake. Our data suggests that CaMKII regulation of [^{3}H]-glutamate uptake has a maximal effect with autonomous cellular kinase. This idea is supported by the inhibition of CaMKII reducing EAAT1 mediated glutamate uptake (**Figures 8-10**), but a lack of increased EAAT1 mediated glutamate uptake upon addition of a calcium ionophore ionomycin (**Figure 12**). It is unsurprising that Thr287Asp, a CaMKII mutant that increases autonomous activity, did not produce an increase in [^{3}H]-glutamate uptake if calcium mobilization does not appear to be instrumental in regulating glutamate uptake (**Figure 10**). However, upon experimental confirmation that Thr287Val produced a reduction in CaMKII autonomous activity as a measure of AC-2 phosphorylation, a lack of effect in [^{3}H]-glutamate uptake via EAAT1 was unexpected. This could be due to a number of reasons. The pool of CaMKII accessible for [γ-^{32}]P measurements versus regulating glutamate uptake may be different, for example the total CaMKII measurements we make pertain to all four isoforms whereas the regulatory effect observed is due to just one isoform. In keeping with this idea, differential changes in CaMKII isoforms have been reported as a result of pathological changes. Second, a portion of the regulatory effect of CaMKII on EAAT1 occurs independent of phosphorylation at Thr37. It is also possible that even with a small percentage of autonomous activity, downstream cellular signaling mechanisms may be adequate to result in alterations in EAAT1-mediated glutamate uptake. These explanations may thus explain a lack of regulatory effect by CaMKII autophosphorylation mutants on EAAT1-mediated glutamate uptake.

Linking our findings to astrocytic CaMKII signaling

Calcium signaling in astrocytes is important for sensing and integrating activity at the synapse and releasing gliotransmitters, among a number of functions (Halassa, Fellin et al. 2007). Based on reports from our laboratory and others, CaMKII appears to homeostatically regulate functions in the astrocyte such as glutamate uptake, release of the gliotransmitter ATP, maintaining a static phenotype/preventing migration from occurring, and maintaining calcium flux between cells. Thus, autonomous levels of astrocytic CaMKII appear to be integral to maintaining cell survival and function.

Our laboratory has previously characterized disruptions in astrocytic function associated with CaMKII inhibition. The application of pharmacological CaMKII inhibitor tat-CN21 to cortical astrocyte cultures resulted in the induction of calcium oscillations and mitochondrial membrane depolarization (Ashpole, Chawla et al. 2013). Additionally, an increase in extracellular ATP due to aberrant release of the gliotransmitter was detected as a consequence of CaMKII inhibition, and this finding was linked to calcium influx via the N-type calcium channels ($Ca_v2.2$). ATP is one of the most abundant gliotransmitters within cortical astrocytes (Coco, Calegari et al. 2003), and the release of ATP through purinergic receptors located on both neurons and astrocytes facilitates communication of ATP with neurons, microglia, and capillaries (Inoue, Koizumi et al. 2007). Interestingly, the release of astrocytic ATP has been shown to decrease neuronal excitability in the retina (Newman 2003) as well as decrease spontaneous neuronal calcium oscillations (Koizumi, Fujishita et al. 2003) and hippocampal excitatory post-synaptic currents (Zhang, Wang et al. 2003). These findings and other reports suggest ATP released by astrocytes plays an inhibitory role, thereby suppressing synaptic

transmission (Pascual, Casper et al. 2005). On the other hand, glutamate released by astrocytes has been shown to activate neurons via excitatory pathways. Thus, the interplay between the release of two key gliotransmitters from astrocytes, ATP and glutamate, appears to have opposing functions. We were surprised to find that the concentration of extracellular glutamate was not altered upon pharmacological inhibition of CaMKII in our studies. These findings suggest that the phenotype associated with ATP release may occur temporally before a phenotype associated with glutamate release cancels it out (inhibition vs excitation). It may thus be likely that neuronal synaptic function can initially be suppressed if ATP was the only gliotransmitter at play prior to excitotoxicity induced by the buildup of excess extracellular glutamate.

Conversely, reports also suggest that CaMKII activation in an astrocyte may be associated with detrimental outcomes. For example, increased phosphorylation of Cx43, a gap junction found on the astrocyte membranes, has been shown to result in a decrease in the open channel probability and diminished calcium flux between cells (Dermietzel, Traub et al. 1989, Huang, Laing et al. 2011, Xu, Kopp et al. 2012). These findings suggest limited intercellular communication between cells upon CaMKII activation in which calcium is a very important second messenger. Increased CaMKII signaling may also hyperphosphorylate vimentin, a cytoskeletal protein that has been implicated in decreased migration when phosphorylated. Interestingly, this regulation appears to occur in a manner different from the positive regulation of CaMKII on the ClC-3 channels which results in an increased migratory phenotype.

While alterations in phenotypes associated with calcium signaling do not always seem to occur in the same direction, it is clear that both increases and decreases in

CaMKII, a key enzyme regulated by calcium, can be detrimental to cellular function in astrocytes. In fact, an understanding of the functional role of CaMKII in astrocytes is only just starting to be achieved. In total, alterations in a multifunctional kinase that is known to regulate ion channels, scaffolding proteins, glutamate uptake, gliotransmitter release and cell-to-cell communication may likely result in global changes in the brain that can ultimately affect cell survival.

Implications towards neurodegenerative diseases

Studies utilizing both genetic and pharmacological tools to inhibit astrocytic and neuronal CaMKII results in a phenotype of disrupted calcium homeostasis, increased cellular excitability, aberrant glutamatergic signaling, and loss of neurons (Carter, Haider et al. 2006, Ashpole and Hudmon 2011, Ashpole, Song et al. 2012, Klug, Mathur et al. 2012, Ashpole, Chawla et al. 2013). It is thus unsurprising that dysregulated CaMKII activity has been implicated in neurodegenerative conditions such as Alzheimer's disease (Min, Guo et al. 2013). During conditions of ischemia, CaMKII has been shown to initially activate, followed by rapid inactivation of the kinase for a prolonged period of time (Aronowski, Grotta et al. 1992). In particular, the degree of CaMKII inactivation has been shown to correlate with the severity of neuronal death, suggesting that a loss of CaMKII signaling is associated with neuronal death in animal models (Hanson, Grotta et al. 1994, Waxham, Grotta et al. 1996, Coultrap, Vest et al. 2011).

In fact, alterations in glutamatergic neurotransmission, and in particular glutamate uptake, are observed in a number of neurodegenerative diseases such as traumatic brain injury, amyotrophic lateral sclerosis, Alzheimer's disease, and ischemia. In particular,

reduced GLAST and GLT-1 protein expression has been observed following a controlled cortical impact injury in the rodent hippocampus, accompanied by a decreased in radiolabeled D-Asp (Rao, Baskaya et al. 1998). Additionally, alterations in glutamatergic neurotransmission have been reported in at least two murine models of Alzheimer's disease, concomitant with a decrease in GLAST and GLT-1 expression (Schallier, Smolders et al. 2011, Cassano, Serviddio et al. 2012). Synthesizing these findings from the literature, it appears that a loss of CaMKII signaling and aberrant glutamate uptake have a similar phenotype, ultimately resulting in calcium dysregulation and neuronal death in the brain. Based on our findings in conjunction with the Underhill study (Underhill, Wheeler et al. 2015), it appears that reduced CaMKII signaling would lead to a decrease in EAAT1-mediated glutamate uptake, unaltered EAAT2a-mediated glutamate uptake, and increased EAAT2b-mediated glutamate uptake. A possibility underlying the differential CaMKII regulation of these glutamate transporters is to maintain a balance in the levels of astrocytic glutamate uptake during physiological and pathological conditions. Whether CaMKII regulation the glutamate transporters is one that is transient, with the transporters rapidly (within minutes) inserting in and out of the membrane, or a result of a longer-lasting phenotype remains to be seen.

In sum, our findings suggest CaMKII regulates glutamate uptake via EAAT1. This regulation may provide the basis for a phenotype involving excess extracellular glutamate, aberrant glutamate receptor activation and neuronal death that ensues as a result of a loss of CaMKII signaling in neurodegenerative conditions.

Future Directions

Our studies implicated phosphorylation of the Thr37 residue on EAAT1 as instrumental in CaMKII regulation of EAAT1-mediated glutamate uptake. We initially uncovered this finding by detecting significant [γ-^{32}]P incorporation of the EAAT1 N-terminus in SPOTS blot tiling assays of the intracellular portions and GST-fusion proteins of the N- and C- termini of EAAT1 and EAAT2. We then honed in on the actual residue using Ala mutants in both the SPOTS blot assays and GST-fusion proteins, and measured alterations in functional glutamate uptake in a transfected HEK293T cell line. However, these assays do not definitively test CaMKII directly phosphorylates the EAAT1 protein at Thr37. Thus, we tried to perform mass spectroscopy, an analytical technique that ionizes peptides in a protein, and then sorts the ions based on their mass to charge ratio. Using a co-immunoprecipitation strategy, we transfected HEK cells with 3x FLAG-tagged EAAT1 and performed a pull down assay using a FLAG or IgG antibody. We detect significant pulldown using a FLAG antibody from transfected 3x FLAG-tagged EAAT1 lysate (**representative image shown in Figure 12**) and minimal pulldown for the same conditions using an IgG antibody with a Western blot. No detectable protein was pulled down in the untransfected HEK cell lysate, with either the FLAG or IgG antibodies, suggesting that we were able to detect isolation of the FLAG-tagged EAAT1 using a Western strategy. However, using either Coommasie Stain or Silver stain, we were not able to detect an appreciable difference between the FLAG and IgG pulldown conditions, for either the transfected or untransfected lysate. Thus, we are continuing to optimize this technique in order to detect enough protein to perform mass spectroscopy on our samples. For the purposes of this dissertation, we were not able to

obtain a mass spectroscopic readout of CaMKII phosphorylation sites on the EAAT1 protein.

In addition to the use of a heterologous expression system such as HEK293Ts and glioma cells, we wanted to confirm our findings in mammalian astrocytes. For this purpose, we obtained human embryonic astrocytes (hEATs), an hTERT immortalized fetal astrocyte cell line. Unfortunately, we did not obtain this cell line till very recently and thus were not able to replicate the majority of our findings in it. In order to characterize the hEAT cell line, we performed immunocytochemistry to first test the purity and protein expression of the cell line. Our immunocytochemical findings suggest that hEATs are GFAP positive, a protein marker for astrocytes (**Figure 19A**). Markers for neurons (MAP2), microglia (OX-42) and oligodendrocytes (Olig-2) respectively were not detected in culture (**Figure 19B-D**), suggesting the lineage of this cell line is similar to that of differentiated astrocytes. We next tested the ability of hEATs to uptake glutamate to determine if they served as a useful model system. We found that [^{3}H]-glutamate counts were relatively low for naïve hEATs (compared to counts normalized to protein concentration in rodent cultured cortical astrocytes, HEK293Ts, and A172s), with no significant reduction elicited by the addition of the inhibitor 100μM TBOA (**Figure 19E**) suggesting this cell line does not uptake glutamate via the glutamate transporters. However, transient transfection of hEATs with EAAT1 and EAAT2 increased [^{3}H]-glutamate uptake by 10-12 fold, substantially greater compared to HEK293Ts. EAAT1 and EAAT2-mediated glutamate uptake were reduced by their respective inhibitors, UCPH-101 and TBOA. These data suggest fetal astrocytes may represent a heterologous expression system closer to native astrocytes to examine glutamate uptake in.

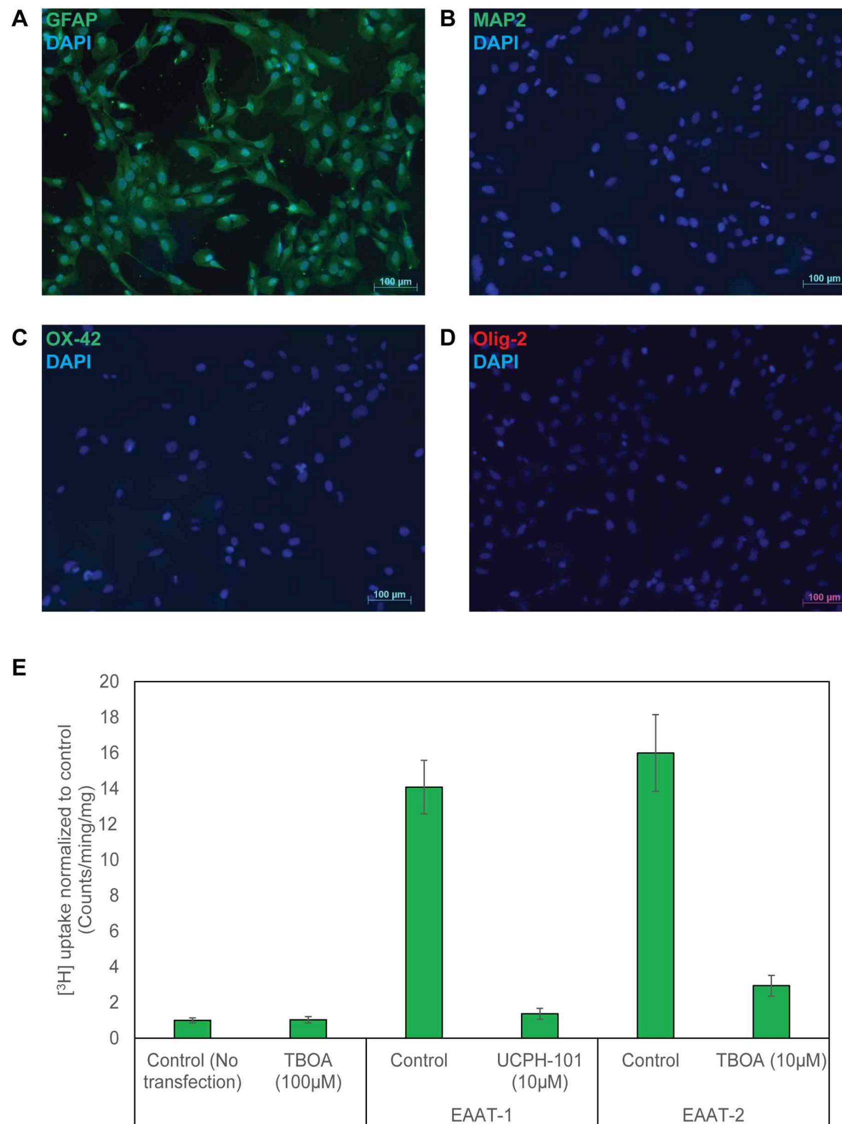


Figure 19: Characterization of human embryonic fetal astrocytes (hEATs). In A-D, shown are representative images of a field of astrocytes immunostained with DAPI (blue) and **A:** GFAP (green), **B:** MAP2 (green), **C:** OX42 (green), and **D:** Olig-2 (red). The scale bar represents 100 µM length. **E:** Average [^{3}H]-glutamate uptake in EAAT1 and EAAT2 transfected cells (n=3, ± S.D) normalized to untransfected hEAT cells. Inhibitors 10 µM UCPH-101 and 100 µM TBOA were added to EAAT1 and EAAT2 transfected cells respectively for 20 minutes prior to a 20 minute [^{3}H]-glutamate uptake measurement protocol.

CONCLUSIONS

The findings of this dissertation expand our understanding of the role of astrocytic CaMKII in the regulation of a key function, glutamate clearance via the EAAT proteins. In particular, our data identifies EAAT1 as a novel CaMKII substrate and implicates the Thr37 residue in EAAT1 as a key phosphorylation site for CaMKII that is linked to EAAT1-mediated glutamate uptake. A pathological loss of CaMKII signaling and dysregulated calcium homeostasis in astrocytes can thus diminish glutamate uptake, which may contribute to neurotoxic effects due to increased extracellular glutamate and aberrant activation of glutamate receptors.

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Zink, M., A. Schmitt, F. A. Henn and P. Gass (2004). "Differential expression of glutamate transporters EAAT1 and EAAT2 in mice deficient for PACAP-type I receptor." J Neural Transm (Vienna) **111**(12): 1537-1542.

CURRICULUM VITAE

Aarti R. Chawla

Education/Training

Institution and Location	Degree	Month/Year conferred	Field of study
Indiana University, Indianapolis, IN	Ph.D.	07/2016	Medical Neuroscience
Indiana University, Indianapolis, IN	M.S.	08/2012	Cellular and Integrative Physiology
Kelley School of Business, Indianapolis, IN	Executive Certificate	05/2012	Business of Life Sciences
Beloit College, Beloit, WI	B.S.	05/2009	Biochemistry

Peer Reviewed Publications

In preparation or under review:

Aarti R Chawla, Derrick E Johnson, Benjamin P Leeds, Ross M Nelson, Andy Hudmon. Homeostatic Regulation of the Glutamate/Aspartate Transporter EAAT1 by Calcium/Calmodulin-Dependent Protein Kinase II (Under review)

Aarti R Chawla, Andy Hudmon. Regulation of Astrocytic Glutamate Transporters (In Preparation)

Accepted:

Anne Bouchut, Aarti R Chawla, Victoria Jeffers, Andy Hudmon, William J Sullivan Jr. Proteome-wide lysine acetylation in cortical astrocytes and alterations that occur during infection with brain parasite *Toxoplasma gondii*. PloS One 2015 Mar 18;10(3):e0117966

Ashpole, NM, Chawla, AR, Martin, MP, Brustovetsky, T. Brustovetsky, N. Hudmon, A. Loss of calcium/calmodulin-dependent protein kinase II activity in cortical astrocytes decreases glutamate uptake and induces neurotoxic release of ATP. Journal of Biological Chemistry 2013 288 (20): 14599-611.

Selected Abstracts

Aarti R Chawla, Nicole M Ashpole, Andy Hudmon

Society for Neuroscience, November 2014. A peptide inhibitor derived from NR2B disrupts CaMKII-NR2B interaction and acutely neuroprotects in cultured cortical neurons exposed to excitotoxic glutamate.

Chawla, A. Johnson, D. Hudmon, A.
Biophysical Society, February 2014. Mechanisms underlying CaMKII regulation of astrocytic glutamate transporters.

Chawla, A. Spencer, S. Alloosh, M. Byrd, J. McKenney, M. Schultz, K. Mather, J. Sturek, M.
Experimental Biology, April 2012.Ossabaw miniature swine models with mutant vs non-mutant AMP kinase alleles for study of electrocardiographic properties during myocardial ischemia.

Rhee, K. Coldren, E. Chawla, A. Diez, R. O'Mealley, R. Cole, R. Sears, C.
American Gastroenterological Association, May 2010. *Bacteroides fragilis* toxin induces ectodomain cleavage of multiple membrane proteins in HT29/C1 cells

Chawla, A. Severson, K. Knight, K.
Midstates (Pew) Undergraduate Research Symposium, November 2007.Analysis of the relationship between bacterial uptake by M cells and the development of the Gut Associated Lymphoid Tissue

Awards and Honors

Medical Neuroscience Graduate Program Travel Award (2014)
Travel award, Indiana University School of Medicine (2014)
First place award for group presentation in X518: Business of Life Sciences I (2012)
Travel award, Society for General Physiologists (2012)
Travel award, Indiana University School of Medicine (2012)
Departmental honors, Biochemistry department, Beloit College (2009)
Dean's list, Beloit College (2007-2009)
Summer research grant, Chemistry department, Beloit College (2008)
Summer research grant, Schweppe Scholars Program, Beloit College (2007)

Memberships

Society for Neuroscience (2014-Present)
Indianapolis chapter of Society for Neuroscience (2013-Present)
Biophysical Society (2013-2014)
Indiana Physiological Society Member (2010-2012)
American Physiological Society Member (2010-2012)