THE EFFECTS OF CAMKII SIGNALING ON NEURONAL VIABILITY

Nicole M. Ashpole

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Andy Hudmon, Ph.D. - Chair

_____

Nickolay Brustovetsky, Ph.D.

Doctoral Committee

_____

Thomas D. Hurley, Ph.D.

October 29, 2012

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Weihua Lee, Ph.D.

_____

Gerry S. Oxford, Ph.D.

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DEDICATION

I would like to dedicate this dissertation my family- my parents Scott and

Suzie Ashpole, my brother Matthew Ashpole, and my husband Matt McBride.

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ABSTRACT

Nicole M. Ashpole

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Calcium/calmodulin-dependent protein kinase II (CaMKII) is a critical modulator of synaptic function, plasticity, and learning and memory. In neurons and astrocytes, CaMKII regulates cellular excitability, cytoskeletal structure, and cell metabolism. A rapid increase in CaMKII activity is observed within the first few minutes of ischemic stroke *in vivo*; this calcium-dependent process is also observed following glutamate stimulation *in vitro*. Activation of CaMKII during pathological conditions is immediately followed by inactivation and aggregation of the kinase. The extent of CaMKII inactivation is directly correlated with the extent of neuronal damage. The studies presented here show that these fluctuations in CaMKII activity are not correlated with neuronal death; rather, they play a causal role in neuronal death. Pharmacological inhibition of CaMKII in the time immediately surrounding glutamate insult protects cultured cortical neurons from excitotoxicity. Interestingly, pharmacological inhibition of CaMKII during excitotoxic insult also prevents the aggregation and prolonged inactivation of the kinase, suggesting that CaMKII activity during excitotoxic glutamate signaling is detrimental to neuronal viability because it leads to a prolonged loss of CaMKII activity, culminating in neuronal death. In support of this, CaMKII inhibition in the absence of excitotoxic insult induces cortical neuron apoptosis by dysregulating

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intracellular calcium homeostasis and increasing excitatory glutamate signaling. Blockade of the NMDA-receptors and enzymatic degradation of the extracellular glutamate signal affords neuroprotection from CaMKII inhibition-induced toxicity. Co-cultures of neurons and glutamate-buffering astrocytes also exhibit this slow-induced excitotoxicity, as CaMKII inhibitors reduce glutamate uptake within the astrocytes. CaMKII inhibition also dysregulates calcium homeostasis in astrocytes and leads to increased ATP release, which was neurotoxic when applied to naïve cortical neurons. Together, these findings indicate that during aberrant calcium signaling, the activation of CaMKII is toxic because it supports aggregation and prolonged inactivation of the kinase. Without CaMKII activity, neurons and astrocytes release stores of transmitters that further exacerbate neuronal toxicity.

<div align="right">Andy Hudmon, Ph.D.- Chair</div>

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LIST OF ABBREVIATIONS

AC-2	Autocamtide 2
ADP	Adenosine diphosphate
AIP2	Autocamtide 2-like peptide
AMPA-R	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of Variance
ATP	Adensosine triphosphate
BSA	Bovine serum albumin
Ca2+	Calcium
CaM	Calmodulin
CaMKII	Calcium/calmodulin-dependent protein kinase II
CaMKIIN	CaMKII-Inhibitory protein
CaMKIINtide	CaMKII-inhibitory peptide (27 residues)
CN21	CaMKII-inhibitory peptide (21 residues)
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPT	Glutamate pyruvate transaminase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KN-62	4-[(2S)-2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl] phenyl isoquinolinesulfonic acid ester
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
LTP	Long-term potentiation
MAP2	Microtubule-associated protein II
MK801	(5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate
Myr	Myristolated
NMDA-R	N-methyl-D-aspartate receptor
PBS	Phosphate buffered saline
PSD	Post-synaptic density
SD	Standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Tat	HIV-1 transactivating protein
TTX	Tetrodotoxin

INTRODUCTION

Our brains are aging. As they age, our probability of developing a neurodegenerative disease increases. Whether these diseases are inherited (Huntington's Disease), slowly manifesting (Alzheimer's Disease), or rapidly acquired (ischemic stroke), the devastating effects of neurodegenerative diseases can play both an emotional and financial toll on patients, family members, and society. Ischemic stroke alone is known to affect nearly 800,000 Americans annually and nationwide costs associated with stroke exceed 30 billion dollars each year (Roger et al., 2012). Medical and societal advances have led to a longer life-span; thus, the incidences and costs of neurodegenerative diseases are only expected to grow. In order to develop better therapeutics for the treatment of these diseases, a deeper understanding of the mechanisms underlying how neurons die is essential.

One common feature of neurodegenerative diseases is the dysregulation of intracellular calcium (Ca^{2+}) signaling. Under physiological conditions, intracellular Ca^{2+} is maintained at a very low concentration (~100 nM) until stimulation induces a transient increase through influx and release of Ca^{2+} from intracellular stores (Figure 1) (Maravall et al., 2000). Through a variety of downstream Ca^{2+}-effector proteins, this increase in Ca^{2+} can regulate a wide array of cellular processes including transcription, translation, excitability, and cellular metabolism (Ghosh and Greenberg, 1995, Trewavas and Malho, 1998, Graef et al., 1999, McCormack and Denton, 1999, Santella and Bolsover, 1999). Excitable cells like neurons tolerate these increases in intracellular Ca^{2+} as long

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as they are quickly buffered and the downstream Ca^{2+}-effector pathways are effectively reset. The inability to buffer the increased Ca^{2+} and the continued transduction of the Ca^{2+} signal can cause physiological signaling pathways to become pathophysiological. This dysregulation is prevalent within several neurodegenerative diseases and is subsequently associated with a number of deleterious effects such as synaptic dysfunction, metabolic impairment, gliosis, macrophage infiltration, and ultimately cellular necrosis and/or apoptosis.

This dissertation focuses on understanding the role of the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) pathway in aberrant calcium signaling. Upon activation, this kinase is known to target a variety of substrates in a variety of cellular compartments, thereby impacting a wide array of cellular processes, including gene transcription, cytoskeletal rearrangement, and protein degradation (Figure 1) (reviewed by (Colbran, 1992, Hudmon and Schulman, 2002)). Activation of CaMKII during aberrant calcium signaling could be pathological for neurons and their support cells as both increases and/or decreases in CaMKII are associated with neurodegenerative insults marked by dysregulated calcium signaling (Aronowski et al., 1992, Perlin et al., 1992, Hanson et al., 1994, Westgate et al., 1994, Churn et al., 1995, Zalewska and Domanska-Janik, 1996). Whether these fluctuations- both the transient increase and prolonged inactivation- play a causal role in the ensuing cell death is not known. By examining the influence of aberrant activation and inactivation of CaMKII in neurons and astrocytes, we will be able to identify how CaMKII signaling influences normal and pathophysiological conditions in the brain.
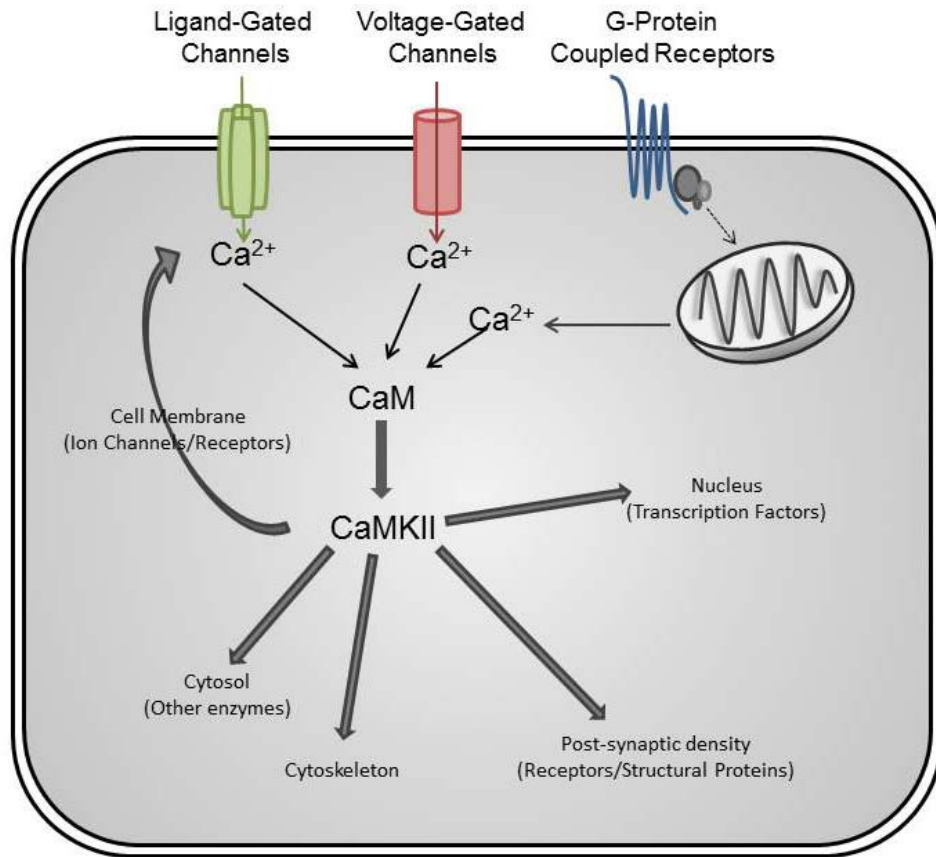
Figure 1: Multifunctional CaMKII signaling. CaMKII is activated by increases in cytosolic calcium via ligand-gated and voltage-gated calcium channels as well as release of calcium from intracellular stores. Activation of CaMKII following increases in cytosolic calcium leads to the targeting of the enzyme to a wide variety of substrates in several cellular compartments, thereby influencing numerous cellular processes.

The next few sections will provide insights into the structure, regulation, and function of CaMKII. I will also examine the influence of CaMKII signaling in other model systems. Together, this knowledge is necessary to understand how CaMKII function may be altered by, and contribute to aberrant calcium signaling within the brain.

CAMKII EXPRESSION

CaMKII is a Ca^{2+}-signaling Ser/Thr kinase ubiquitously-expressed throughout the body. There are four major isoforms of CaMKII- α β δ and γ- with several identified splice variants for each isoform. These isoforms are encoded by different genes that in humans are found on chromosome 5, 7, 4, and 10, respectively (Li et al., 1994, Epigenomics, 2012). Alignment of the entire full-length protein of all four isoforms indicates that over 60% of the sequence is conserved. As might be expected, this conservation is highest within the catalytic domain of the kinase. Sequence alignment of the catalytic domain of the four major isoforms found in humans indicates that 95% of the sequence is conserved (Figure 2). The most divergence between isoforms is found in an area termed the variable region (within the hub domain), where sequence inserts known to contain substrate targeting motifs unique to each isoform exist.

CaMKII isoform expression is regulated by tissue specific expression, cell-specific patterning, developmental patterning, and subcellular distribution (as reviewed by (Hanson and Schulman, 1992b, Hudmon and Schulman, 2002)). Robust αCaMKII expression is observed in skeletal muscle, while βCaMKII is found in immune cells, δCaMKII predominates in cardiomyocytes, and γCaMKII

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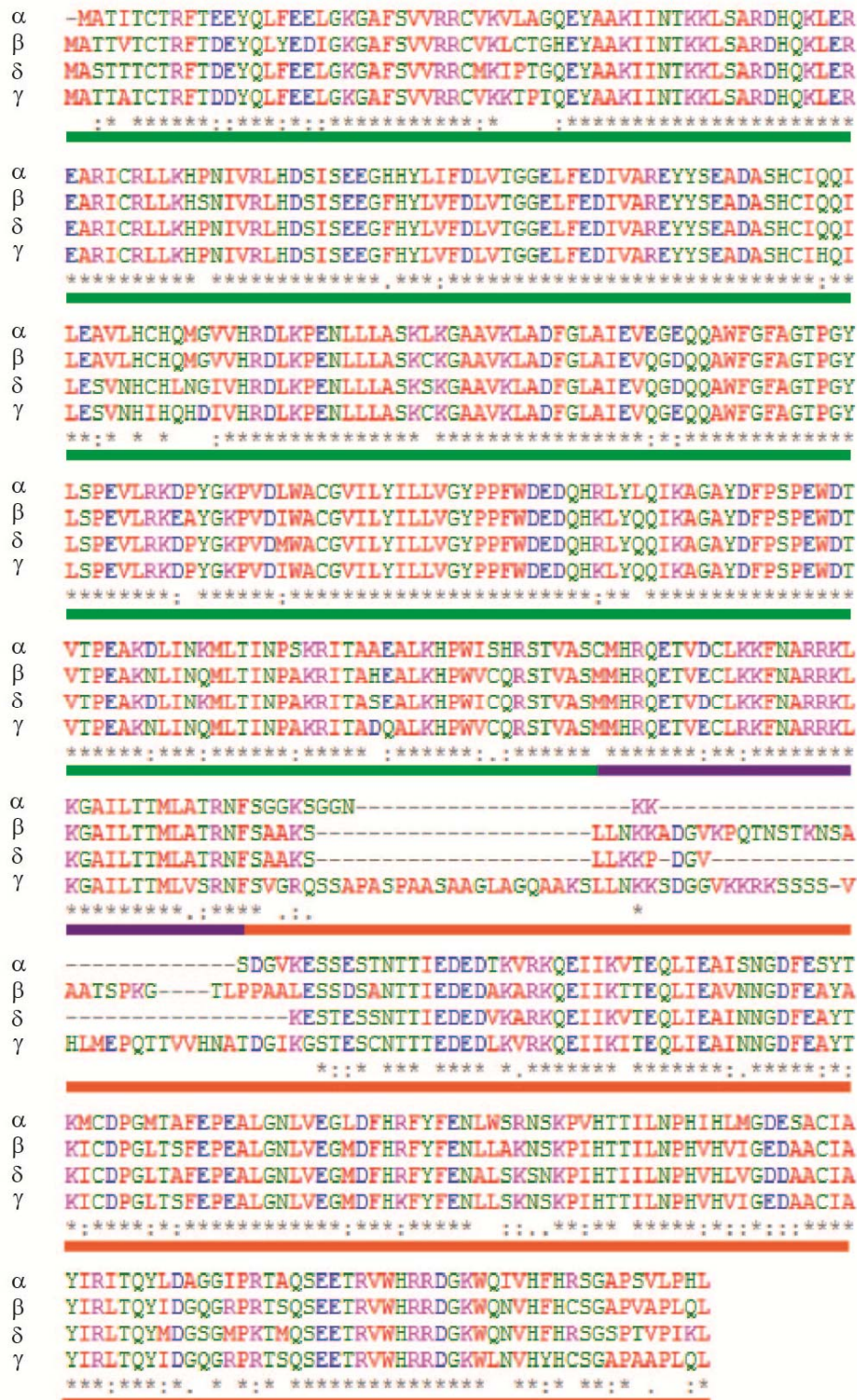
Figure 2: Sequence alignment of the four human isoforms of CaMKII.
 * indicates sequence identity while the : indicates conservation. The colored lines delineate the subunits of CaMKII: catalytic (green), autoregulatory (purple), and hub (orange).

is found in the lungs (Tobimatsu and Fujisawa, 1989, Karls et al., 1992). Within the nervous system, particularly within the brain, all four isoforms are expressed (Sakagami et al., 1992, Sakagami and Kondo, 1993, Bayer et al., 1999). The most abundant isoforms within neurons are αCaMKII and βCaMKII (Ouimet et al., 1984, Erondu and Kennedy, 1985, McGuinness et al., 1985, Burgin et al., 1990a). Meanwhile, δCaMKII and γCaMKII expression has also been observed, albeit at lower levels than αCaMKII and βCaMKII (Bayer et al., 1999). While αCaMKII and βCaMKII are the predominant isoforms in neurons, δCaMKII predominates within astrocytes (Takeuchi et al, 2000). Interestingly, because the CaMKII holoenzyme within the cell consists of twelve kinase subunits, co-assembly of various isoforms can occur (Vallano, 1989, Kolb et al., 1998, Brocke et al., 1999). Functional changes within the nervous system following CaMKII inhibitor application are often identified as αCaMKII-regulated changes because of the predominant expression of αCaMKII in excitatory neurons; however, it is important to recognize that these other isoforms may be contributing to observed effects.

Within the brain, there appears to be a general tissue-specific patterning of αCaMKII and βCaMKII expression. αCaMKII expression predominates in the cortex and βCaMKII expression predominates in the cerebellum (Erondu and Kennedy, 1985, McGuinness et al., 1985, Miller and Kennedy, 1985). Interestingly, there are specific cell types within each of these areas that do not conform to this pattern. Purkinje cells within the cerebellum largely expressed αCaMKII (Walaas et al., 1988). Moreover, within the dentate gyrus of the

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hippocampus, granule cells express βCaMKII (Churn et al., 1992b). Thus, while there does appear to be a pattern of expression within tissues, some cell types differ and express an isoform different than their counterparts within that tissue.

Despite different tissue and cellular expression patterns, not much is known about the influence this variance in isoforms has on substrate selection within cells. *In vitro* studies to date have not identified differences in substrate affinity depending on which isoform is present. We have observed that the phosphorylation motif on the NR2B subunit of the N-methyl-D-Aspartate receptor (NMDA-R), which was first identified as an αCaMKII substrate, is also a high-affinity δCaMKII substrate (personal communication with A Hudmon and D Johnson). However, differences in substrate targeting have been observed. βCaMKII has been shown to have an F-actin binding domain within the variable region that is not present in the other isoforms (Fink et al., 2003). Therefore, βCaMKII and not αCaMKII can bind this cytoskeletal protein. On the other hand, αCaMKII has been shown to bind substrates such as densin-180 which βCaMKII appears incapable of binding (Robison et al., 2005). This isoform-specific binding cannot be attributed to the variable domain as it appears that residues deep within the hub domain (residues 421-480, will be discussed later) confer αCaMKII binding to densin-180 (Robison et al., 2005). Another example of isoform-specific binding was observed with the ubiquitin proteasome system. Genetic knock-down of αCaMKII, and not βCaMKII, disrupted the recruitment of the ubiquitin proteasome system to post-synaptic densities in neurons (Bingol et al., 2010). Thus, there appear to be differences in the ability of isoforms to target and

regulate substrates *in situ*. While it is possible that structural differences between isoforms (specifically in the variable region) may contribute to binding substrate selection, the true mechanism underlying this difference is not known.

CAMKII STRUCTURE

CaMKII is a dodecameric protein composed of twelve 50 to 60 kDa kinase subunits (Kuret and Schulman, 1984, Chao et al., 2010). Thus, the size of the CaMKII holoenzyme within a cell is roughly 600 kDa (Bennett et al., 1983, Kuret and Schulman, 1984). Each subunit of the holoenzyme consists of an N-terminal catalytic domain, an autoregulatory domain, and a C-terminal hub domain (Figure 3). The catalytic domain contains the ATP binding pocket, catalytic cleft, and substrate targeting groove, making this domain the enzymatic mainstay of the CaMKII protein. The catalytic domain is tethered to the autoregulatory domain which is responsible for lying over the catalytic cleft thereby inhibiting kinase activity in the absence of Ca^{2+}/calmodulin (CaM) (Colbran et al., 1989) (Figure 3). CaM is a ubiquitously-expressed Ca^{2+}-binding protein that serves as an intermediate between cytosolic Ca^{2+} and downstream Ca^{2+}/CaM-effector proteins, such as CaMKII (Babu et al., 1985, Zhang et al., 1995, Tjandra et al., 1999). A CaM-binding motif is found on the C-terminal portion of the autoregulatory domain of CaMKII (Figure 3). When Ca^{2+}/CaM binds this motif, the helical autoregulatory domain is pulled away from the catalytic surface, allowing kinase activity (Rellos et al., 2010, Chao et al., 2011). Downstream of the CaM-binding motif, lay the hub domain of the kinase which is responsible for associating or interacting with other subunits to form the dodecameric
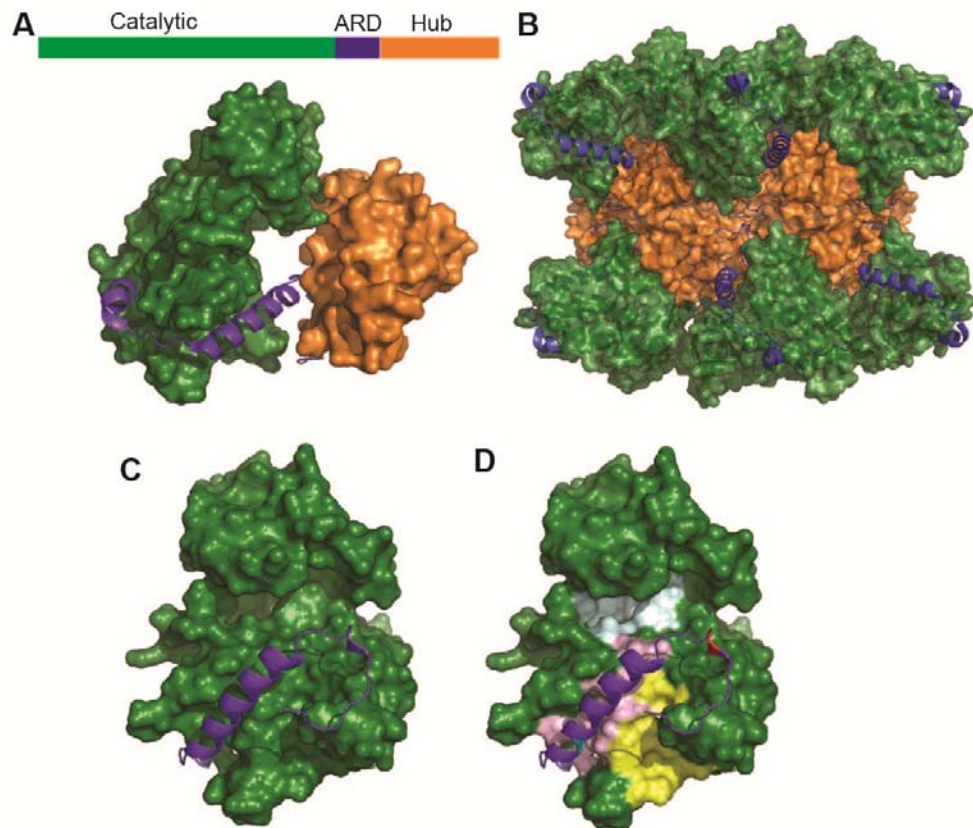
Figure 3: Structure of CaMKII. A, Linear schematic (top) and crystal structure (bottom) of one CaMKII subunit (derived from pdb 3SOA). The colors of the schematic correspond with the structures throughout the figure. **B,** The crystal structure of autoinhibited CaMKII holoenzyme (as solved by Chao et al., 2011, pdb 3SOA). **C,** Structure of the autoregulatory domain laying across the catalytic cleft. **D,** Catalytic surface of CaMKII with highlighted catalytic cleft (white), substrate binding groove (pink), inhibitory groove (yellow).

holoenzyme (Figure 3). Of note, the start of the hub domain is marked by the variable region which, as explained above, contains inserts found in different splice variants of CaMKII isoforms (Figures 2 and 3).

Much work has gone into understanding the unique architecture of CaMKII. Early studies using transmission electron microscopy indicated that CaMKII appeared to be a hexagonal ring with a hollow center (Woodgett et al., 1983). Subsequent cryoetching was in agreement with this, and further suggested that arm-like structures appeared to radiate out from the center ring (Kanaseki et al., 1991). Over ten years later, modern biophysical approaches, such as small-angle X-ray scattering and 3-D reconstruction of electron microscopy, suggested that the hub domains within the CaMKII holoenzyme are responsible for the formation of the ring-like structure from which the catalytic heads radiate outwards (Hoelz et al., 2003, Gaertner et al., 2004, Rosenberg et al., 2005, Rellos et al., 2010). This hypothesis was recently verified when the crystal structure for inactive human αCaMKII (with a β7-CaMKII variant linker) was solved (pdb# 3SOA)(Chao et al., 2011). The crystal structure confirmed that hub domains come together to form hexameric rings, one ring on top of another, with the connected catalytic subunits tightly packed towards the rings like 'petals' (Figure 3). This multivalent architecture allows intraholoenzyme autoregulation (will be discussed later). It is interesting to note that monomeric subunits of CaMKII are able to bind and phosphorylate substrates; thus, it is possible that this unique architecture of the dodecamer may also alter substrate selection or regulation within the cell.

ACTIVATION OF CAMKII BY CA^{2+}/CAM

As mentioned, the activation of CaMKII is dependent on the binding of Ca^{2+}-bound CaM to the autoregulatory domain of the kinase. A crystal structure of one active catalytic/autoregulatory domain has been solved (pdb #2WEL) and indicates that Ca^{2+}/CaM binding does lead to the exposure of the catalytic cleft by removal of the autoregulatory domain from the catalytic surface (Rellos et al., 2010). However, there is currently no crystal structure for an activated CaMKII holoenzyme. Thus, inferences on the how the entire holoenzyme structurally changes following activation remain largely speculative. Because the crystal structure of the inactive holoenzyme suggests that the CaM binding region of CaMKII appears to be buried within a tightly-packed interface between the catalytic and association domain, it is plausible that a dramatic structural rearrangement occurs in order to reach an active state (Hoffman et al., 2011).

Residues 293 and 310 within the autoregulatory domain are recognized as the CaM binding motif on CaMKII (Figure 3) (Colbran et al., 1988, Payne et al., 1988, Meador et al., 1993). A crystal structure of CaM bound to a peptide encompassing these residues indicates that the two lobes of CaM tightly wrap this binding domain (Meador et al., 1993). CaM binds in an antiparallel fashion with the C-terminal lobe of CaM binding on the N-terminal half of the binding domain, and the N-terminal lobe of CaM binding on the C-terminal portion of the binding domain (Evans and Shea, 2009). While both lobes ultimately bind, this binding occurs in a stepwise manner. The N-terminal lobe forms a contact with the autoregulatory domain and then if nucleotides are present, the C-terminal

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lobe will flex and form contacts as well (Jama et al., 2011). Recent biochemical studies have revealed that while both lobes may wrap the binding motif, the N-terminal lobe alone is able to bind and partially activate CaMKII (Shifman et al., 2006, Forest et al., 2008). While the C-lobe may make more contacts with the kinase autoregulatory domain, binding of the C-lobe is not necessary for kinase activation (Forest et al., 2008, Evans and Shea, 2009). However, the C-terminal lobe does increase the affinity of Ca^{2+}/CaM for the autoregulatory domain (Evans and Shea, 2009).

The binding kinetics of CaM to CaMKII exhibit positive cooperativity. Hill coefficients of 2 to 3 have been reported for CaM activation of CaMKII (Gaertner et al., 2004, Forest et al., 2008, Byrne et al., 2009, Chao et al., 2010, Chao et al., 2011). This indicates that the binding of Ca^{2+}/CaM to one subunit of the holoenzyme alters the activation state of nearby subunits that are not yet in contact with CaM. While the mechanism of this is not yet understood, it is thought that activation of one subunit induces conformational changes that can affect other components of the holoenzyme. Consistent with fact that the N-lobe is sufficient to activate the kinase, the N-lobe alone has also been shown to exhibit cooperative binding with a Hill coefficient of nearly 3 (Forest et al., 2008). The presence of nucleotides has been shown to decrease the cooperativity of CaM activation (Forest et al., 2008). This may be a consequence of the binding of the second lobe of CaM to CaMKII when nucleotides are present. It is possible that when the second lobe binds, there is another rearrangement within the corresponding catalytic subunit that decreases the ability of nearby subunits to

find CaM partners. Another possibility is that when nucleotides are present, a previously-identified autophosphorylation event (Thr286- will be discussed later) occurs that alters the holoenzyme conformation. Interestingly, introduction of extended linkers between the CaM binding region and the hub domain have been shown to significantly impact the cooperativity of Ca^{2+}/CaM activation (Chao et al., 2011). These data suggest that CaMKII relies on dynamic sampling of the environment in order to become activated and that the architecture of the holoenzyme plays a critical role in regulation of CaMKII activity.

Despite this cooperativity in CaM binding, CaMKII exhibits a weak affinity for CaM with the Kd of activation around 1 µM in the absence of ATP (Forest et al., 2008). However, the presence of ATP increases this affinity to around 20-100 nM (Gaertner et al., 2004, Forest et al., 2008). Ca^{2+}/CaM activation in the presence of saturating levels of ATP can lead to an autophosphorylation event (discussed below); this autophosphorylation further increases the affinity of CaMKII to CaM roughly 1000-fold, to 20 picomolar (Meyer et al., 1992). This means that CaMKII transitions from being one of the weaker CaM targets in cells to one of the best targets identified to date. Because of this, CaMKII is thought to 'trap' calmodulin (Meyer et al., 1992). The functional consequence of this CaM trapping is not fully understood; however, it is possible that CaMKII may serve as a CaM sink which, when activated, prevents the activation of other CaM signaling pathways. If this were true, then the trapping of CaM by CaMKII could impact a multitude of cellular processes.

As mentioned above, ATP can impact the binding affinity of Ca^{2+}/CaM to

CaMKII. Similarly, CaM binding can impact ATP binding affinity. For instance, the

Km of ATP for autophosphorylation is roughly 150 µM when Ca^{2+}/CaM is not

present (Colbran, 1993). When Ca^{2+}/CaM is present, the Km of ATP decreases

to 20 µM (Colbran, 1993). Thus, it appears as though the catalytic domain

structure impacts both CaM and ATP binding affinities. This ATP affinity

determined in the presence of activating Ca^{2+}/CaM is within a similar range for

other neuronal kinases in the presence of their activators, such as PKC (13 µM),

PKA (15 µM), and CaMKI (30 µM) (Cook et al., 1982, Nairn and Greengard,

1987, Spitaler et al., 2000). The ATP binding pocket, which is fully conserved

between the four CaMKII isoforms, is found on the face of the catalytic subunit,

near where the autoregulatory domain lays (Figure 3). Thus, it is possible that

there are interactions between the ATP binding pocket and the autoregulatory

domain that allow them to influence each other, as previously proposed (Smith et

al., 1992, Brickey et al., 1994, Praseeda et al., 2004, Pradeep et al., 2009).

CAMKII AUTOPHOSPHORYLATION

The unique architecture of the CaMKII holoenzyme affords opportunities

for intraholoenzyme modulation of one kinase subunit by another nearby subunit.

One example of this is the autophosphorylation of the autoregulatory domain.

This domain contains a classical CaMKII consensus sequence (R-X-X-S/T) with

the potential phosphorylation residue at Thr286. Indeed, CaMKII has been shown

to phosphorylate this residue following kinase activation (Lou and Schulman,

1989). Interestingly, this phosphorylation has been shown to be an

intraholoenzyme event in which one subunit within the holoenzyme phosphorylates a nearby subunit in the holoenzyme (Mukherji et al., 1994, Bradshaw et al., 2002). Phosphorylation of Thr286 leads to a unique enzymatic state in which the kinase is no longer dependent on Ca^{2+}/CaM for activity; a state known as autonomy, or Ca^{2+}/CaM-independent activity (Lai et al., 1986, Lou et al., 1986, Miller and Kennedy, 1986, Schworer et al., 1986, Schworer et al., 1988). It is important to point out that while autonomous activity is not dependent on Ca^{2+}/CaM, the autophosphorylation event that leads to autonomy is, as Thr286 is not accessible for phosphorylation in the absence of Ca^{2+}/CaM binding (Chao et al., 2011, Hoffman et al., 2011). Interestingly, even though CaMKII autophosphorylation at Thr286 renders the kinase autonomous of Ca^{2+}/CaM, this state is recognized as the high-affinity calmodulin binding state, ie CaM trapping state (Meyer et al., 1992).

Mutation of Thr286 has been shown to have dramatic effects on kinase function. Introducing the phosphomimetic Asp at Thr286 renders the kinase constitutively active (Fong et al., 1989, Waldmann et al., 1990). Mutation of Thr286 to non-phosphorylatable Ala prevents the induction of autonomous activity (Fong et al., 1989, Waxham et al., 1990). Furthermore, this mutation disrupts the ability of the kinase to trap CaM (Meyer et al., 1992). Mutant Thr286Ala also causes a loss of CaMKII substrate targeting in neurons (Shen et al., 2000). Importantly, while CaM trapping and substrate targeting are disrupted, mutant Thr286Ala appears to phosphorylate substrates normally, because Ca^{2+}/CaM can still activate the kinase (Fong et al., 1989). Thus, while

autophosphorylation may enhance CaMKII activity, it is not an absolute requirement for enzymatic phosphorylation of substrates.

Neuronal stimulation induces a rise in intracellular calcium which leads to increased CaMKII autophosphorylation (Fukunaga and Soderling, 1990, Fukunaga et al., 1992). The extent of autonomous activity can be used as a read-out for CaMKII autophosphorylation. For this, Ca^{2+}/CaM-independent activity can be compared to total CaMKII activity (in the presence of Ca^{2+}/CaM) *in vitro* (Saitoh and Schwartz, 1985). Using percent autonomy as a read-out of autophosphorylation, 10-20% of CaMKII is autophosphorylated under basal conditions, suggesting that some CaMKII activity is still present under resting conditions. Neuronal stimulation increases the extent of autonomy to nearly 50% of total CaMKII activity (Molloy and Kennedy, 1991, Ocorr and Schulman, 1991). Because Ca^{2+} levels within cells can rapidly change, it is thought that CaMKII autonomy serves as a molecular memory device for Ca^{2+} signals in cells even when the increased Ca^{2+} has subsided. This concept is central to my studies, as it is possible that CaMKII can continue to signal during neurodegenerative calcium signaling, further perpetuating the toxicity cascade.

Because phosphorylation of Thr286 renders the kinase autonomous of Ca^{2+}/CaM regulation, cells have devised multiple mechanisms for reversing this phosphorylation in order to properly reset CaMKII activity. First, CaMKII itself can reverse this phosphorylation. The presence of high levels of ADP *in vitro* can lead to dephosphorylation of Thr286, resulting in a subsequent loss of autonomous activity (Kim et al., 2001). Furthermore, several protein

phosphatases have been shown to dephosphorylate Thr286, including Ca^{2+}/CaM protein phosphatase, protein phosphatase 1, 2a, and 2c (Hashimoto et al., 1987, Fukunaga et al., 1993, Strack et al., 1997a). As expected, these phosphatases also reduce CaMKII autonomy (Hashimoto et al., 1987, Fukunaga et al., 1993). The presence of multiple avenues to dephosphorylate Thr286 suggesting that maintaining the proper level of CaMKII activity within the cell is critical for cellular physiology.

Thr286 is not the only residue shown to be autophosphorylated by CaMKII. Thr253, Ser279, Thr305, and neighboring Thr306 have all been shown to be phosphorylated by CaMKII as well (Hanson et al., 1989, Lou and Schulman, 1989, Patton et al., 1990, Hanson and Schulman, 1992a, Colbran, 1993). Recent studies suggest that while Thr253 phosphorylation does not have a direct effect on enzymatic activity, subcellular targeting of the enzyme is enhanced following this phosphorylation event (Migues et al., 2006). To date, the functional consequence of Ser279 phosphorylation is unknown, as it does not appear to effect enzymatic activity (Hanson et al., 1989). Unlike Ser279, phosphorylation of Thr305 and Thr306 plays a critical role in regulating kinase activity, as it prevents Ca^{2+}/CaM activation (Colbran and Soderling, 1990, Patton et al., 1990, Hanson and Schulman, 1992a, Colbran, 1993). These residues are particularly interesting as they lie within the CaM binding motif on the autoregulatory domain. Thus, when Ca^{2+}/CaM is bound, these residues are inaccessible. However, under a basal resting state, autophosphorylation of Thr305/Thr306 can occur which prevents Ca^{2+}/CaM activation (Colbran, 1993).

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Furthermore, Thr305/Thr306 autophosphorylation can reset CaMKII activity following CaM dissociation when intracellular Ca^{2+} resets following stimulation (Jama et al., 2009).

INACTIVATION OF CAMKII

A major portion of this dissertation is focused on understanding the physiological impact of CaMKII inactivation. Within cells, several mechanisms contribute to inactivation. First, as mentioned above, CaMKII is maintained in an inactive state in the absence of Ca^{2+}/CaM because basal phosphorylation of Thr305/Thr306 prevents Ca^{2+}/CaM binding. Therefore, following Ca^{2+}/CaM activation, kinase activity can be effectively reset by phosphorylation of Thr305/Thr306 following the dissociation of Ca^{2+}/CaM from the autoregulatory domain. Other forms of CaMKII inactivation have also been identified. Unlike activation of CaMKII under conditions of saturating Ca^{2+}/CaM and ATP, activation of the kinase under limiting ATP quickly induces inactivation (Lou et al., 1986, Colbran, 1993). The propensity to inactivate under limiting ATP is enhanced by elevated temperatures (Hudmon et al., 1996). It was later shown that saturating levels of ADP can prevent CaMKII inactivation, suggesting that the presence of nucleotides, not just ATP, following Ca^{2+}/CaM stimulation blocks inactivation. Interestingly, the presence of a CaMKII substrate that mimics the autoregulatory domain, termed Autocamtide (AC2), can prevent the induction of this inactivation (Ishida and Fujisawa, 1995). This suggests that the structure of the kinase becomes unstable when the catalytic domain is activated and autophosphorylation cannot occur which ultimately leads to CaMKII inactivation.

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Thus, conditions in which Ca^{2+}/CaM is present, nucleotides are limiting, and temperature is elevated are maximal for CaMKII inactivation (Lou and Schulman, 1989, Hudmon et al., 1996).

Inactivation of CaMKII is also instigated by the aggregation of kinase holoenzymes during periods of cellular distress. This process is an activity-dependent form of inactivation that is termed self-association as several CaMKII holoenzymes associate/aggregate together (Hudmon et al., 1996, Hudmon et al., 2005). The details entailing the intiation and influence of self-association will be discussed later. However, it is important to recognize that several of the conditions that prevent CaMKII inactivation (saturating ATP levels/ autophosphorylation of Thr286) are known to prevent self-association. Thus, it is possible that the inactivation that is observed under these conditions is due to self-association/aggregation of the enzyme.

REGULATORS OF CAMKII SIGNALING

Several post-translational modifications of CaMKII have been identified. As mentioned above, CaMKII autophosphorylates many residues within the catalytic subunit, most recognizably Thr286. Interestingly, other protein kinases, such as PKC, have been shown to phosphorylate CaMKII (Waxham and Aronowski, 1993). Scansite prediction software suggests that over 15 potential modulatory kinase phosphorylation/binding sites may exist in the CaMKII protein; however, most of these sites have not been validated experimentally. Several protein phosphatases play a key role in dephosphorylating these residues (Hashimoto et al., 1987, Fukunaga et al., 1993, Strack et al., 1997a). Recently,

several glycosylation sites on CaMKII have been identified (Trinidad et al., 2012). The effects of these glycosylation events are not known, as there appears to be no change in substrate targeting or enzymatic activity when the glycosylation groups are removed (Trinidad et al., 2012). It is interesting, however, that one of the residues observed to be glycosylated is Thr306, a residue which is critical for maintaining CaMKII in an inactive state in the absence of Ca^{2+}/CaM (Trinidad et al., 2012).

Oxidation has also been shown to modulate CaMKII activity. In lymphocytes and cardiomyocytes, oxidative stress is associated with increased autonomous CaMKII activity (Howe et al., 2004, Zhu et al., 2007, Erickson et al., 2008). Biochemical studies indicated that direct oxidation of Met281/Met282 results in CaMKII autonomy (Erickson et al., 2008). The initiation of this autonomy is still dependent on Ca^{2+}/CaM. However, unlike traditional autonomous activity, Thr286 phosphorylation is not required to maintain activity when these residues are oxidized, as best highlighted when the Thr286Ala mutant exhibited autonomous activity in the presence of oxidizing agents like H_2O_2 (Erickson et al., 2008). This was the first evidence of the generation of autonomous activity that bypassed Thr286 autophosphorylation, allowing the kinase to maintain activity under cellular distress; an effect which I hypothesize is detrimental to neuronal viability.

CAMKII INHIBITORS

Post-translational modifications are not the only avenues of regulating CaMKII activity *in situ*. Yeast two-hybrid screens for CaMKII-interacting proteins

identified a novel family of endogenous inhibitory proteins in the brain (Chang et al., 1998, 2001). These small 6.5kDa inhibitory proteins, termed CaMKIIN (two isoforms CaMKIINα and CaMKIINβ), are high-affinity CaMKII binding proteins. They are encoded by two separate genes on chromosome 1 and 3. CaMKIIN is capable of inhibiting both autonomous (Ca^{2+}/CaM independent) and total (Ca^{2+}/CaM dependent) activity in a dose-dependent manner, with an IC_{50} of 100-400 nM (Chang et al., 1998). The inhibitory domain of CaMKIIN was localized to 27 residues within the carboxy-terminal of the protein (Chang et al., 1998). Importantly, these 27 residues were identical between the two CaMKIIN isoforms. A peptide encompassing these residues was generated and termed CaMKIINtide (Chang et al., 1998). Similar to the parent protein, CaMKIINtide was a potent inhibitor of CaMKII activity (Chang et al., 1998). Subsequent biochemical studies have shortened this inhibitory peptide to 21 residues (termed CN21, (Vest et al., 2007)), and most recently 19 residues (termed CN19, (Coultrap and Bayer, 2011)), without significantly affecting the affinity of inhibition. Importantly, CaMKIIN and its peptide derivatives are highly-selective for CaMKII, with greater than 100-fold potency of CaMKII inhibition over CaMKIV, another CaM-dependent kinase family member, as well as PKC, PKA, and MAP-K1 (Vest et al., 2007).

While the regulation of CaMKIIN is not fully understood, there appears to be differential expression of the two CaMKIIN isoforms in differing brain regions. CaMKIINβ is highly expressed in the cerebellum, while CaMKIINα is highly-expressed in the cortex (Chang et al., 2001). Interestingly, the expression pattern

of these isoforms appears to mimic the expression pattern of αCaMKII and βCaMKII isoforms. Recent studies suggest that CaMKIIN expression is upregulated during the acquisition and consolidation of fear learning (Radwanska et al., 2010). Autonomous CaMKII activity has been shown to be critical for this form of learning and memory (Frankland et al., 2001, Frankland et al., 2004, Easton et al., 2011); thus, the authors propose that the upregulation of CaMKIIN, which can turn off this autonomous activity, is critical for the turnover of CaMKII activity for proper memory formation.

Other peptide and small molecule inhibitors of CaMKII have also been identified. Autocamtide-2 (AC2) is a high-affinity CaMKII substrate derived to mimic the autoregulatory domain surrounding Thr286. Interestingly, mutation of the phosphoacceptor site of AC2 to Ala, resulted in a high-affinity peptide inhibitor of CaMKII with an IC_{50} of around 50 nM (Ishida et al., 1995). This peptide, termed AIP (AC-2-related inhibitor peptide) is also able to inhibit both autonomous (Ca^{2+}/CaM independent) and total (Ca^{2+}/CaM dependent) CaMKII activity (Ishida et al., 1995). Because AIP is derived from a substrate peptide, it is a competitive inhibitor of substrate phosphorylation with a Ki of 320 nM (with respect to parent AC2) (Ishida et al., 1995). However, the autoregulatory domain from which AIP is derived is also known to inhibit other CaM-kinase family members, PKC and MLCK, at concentrations above 10 μM (Smith et al., 1990). KN-62 and KN-93 are allosteric small molecule CaMKII inhibitors (Tokumitsu et al., 1990, Sumi et al., 1991). Unlike their peptide inhibitor counterparts, these small molecule inhibitors prevent kinase activation and have no effect on

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autonomous activity (Tokumitsu et al., 1990, Sumi et al., 1991). The KN drugs are competitive inhibitors of CaM binding with a Ki of 900 nM for KN-62 and a Ki of 370 nM for KN-93 (Tokumitsu et al., 1990, Sumi et al., 1991). While these inhibitors are cell-permeable, there are a variety of off-target effects associated with the KN family, including inhibition of other CaM-kinase family members, voltage gated calcium channels, and purinergic receptors (Smith et al., 1990, Enslen et al., 1994, Ledoux et al., 1999, Gao et al., 2006). However, studies using KN-93 can be properly controlled for using the inactive control KN-92.

Because of off-target effects associated with the small molecule inhibitors, focus has shifted towards the use of the high-affinity, highly-selective peptide inhibitors such as CN21. While most peptide inhibitors are not inherently cell-permeable, penetrating motifs and the addition of protein modifications such as myristolation can allow for peptide uptake in cells (Nelson et al., 2007). Several recent studies have shown that the addition of positively-charged amino acids allow for CaMKII inhibitors such as CN21 and AIP to be taken up by neurons (Vest et al., 2007, Buard et al., 2010, Vest et al., 2010, Lisman et al., 2012). One sequence often used in these studies is a series of subsequent Lys and Arg residues found in the HIV-associated coat protein Tat. Inhibitors conjugated to the Tat motif exhibit rapid cellular uptake (Vives et al., 1997, Silhol et al., 2002, Brooks et al., 2005, Richard et al., 2005). Thus, highly-selective peptide inhibitors are easily manipulated to allow for cellular application, thereby affording an opportunity to examine the physiological effects of CaMKII signaling within the native cell background.

TARGETS OF CAMKII IN THE NERVOUS SYSTEM

  My studies are focused on understanding the physiological impact of aberrant fluctuations in CaMKII activity. The following section highlights CaMKII-regulated proteins that are critical for neuronal excitability and cell structure (see Figure 1). Understanding the impact that CaMKII regulation can have on the activity/stability of these proteins is essential to appreciate how dysregulation of CaMKII can affect cellular viability.

  Glutamate Receptors

  Within neurons, CaMKII is activated following increases in cytosolic calcium. Neurons express several voltage-gated and ligand-gated calcium channels that transduce calcium into the intracellular space. Receptors on intracellular stores of calcium can be activated to release calcium into the cytosol as well. While various sources of calcium may contribute to CaMKII activation, the most characterized pathway for activation is via the N-methyl-D-Aspartate receptor (NMDA-R). The NMDA-R is an ionotropic glutamate receptor widely-expressed in neurons. This receptor is tetrameric with two NR1 subunits and two NR2 subunits (NR2A and NR2B), all of which come together to create a pore-forming ion channel (Behe et al., 1995, Laube et al., 1998, Rosenmund et al., 1998). Following activation by glutamate and the co-factor glycine, the NMDA-R transduces extracellular Ca^{2+} (and some sodium) into the intracellular cytosol. Calcium influx through the NMDA-R has been shown to lead to Thr286 autophosphorylation, thereby inducing CaMKII autonomy (Shen and Meyer, 1999). Following this activation, CaMKII then translocates to a variety of cellular

compartments, targeting a variety of substrates. Interestingly, one of the substrates that CaMKII is shown to target after activation is the NMDA-R (Shen and Meyer, 1999, Shen et al., 2000, Strack et al., 2000, Bayer et al., 2001). Once the kinase has translocated to the NMDA-R, it is able to both bind and phosphorylate this receptor, which has been shown to enhance NMDA-R desensitization (Sessoms-Sikes et al., 2005). Thus, there is a reciprocal relationship in which NMDA-R leads to CaMKII activation which then causes CaMKII translocation back to the NMDA-R to enhance receptor desensitiziation.

The NR1, NR2A, and the NR2B subunits of the NMDA-R have all been shown to interact with CaMKII (Gardoni et al., 1999, Leonard et al., 1999, Bayer et al., 2001, Gardoni et al., 2001, Leonard et al., 2002). CaMKII phosphorylates both NR2A and NR2B (Gardoni et al., 1999). Furthermore, CaMKII has been shown to form a stable interaction with all three subunits (Gardoni et al., 1999, Leonard et al., 1999, Bayer et al., 2001, Leonard et al., 2002). Interestingly, the binding of CaMKII to the C-terminal portion of the NR2B subunit can affect the enzymatic function of CaMKII (Bayer et al., 2001). A peptide encompassing the binding region on NR2B has been shown to generate Ca^{2+}/CaM-independent CaMKII activity in the absence of Thr286 autophosphorylation (Bayer et al., 2001). When Ca^{2+}/CaM induces a conformational change in the autoregulatory domain, a substrate binding groove on the catalytic surface is exposed (Bayer et al., 2006). This groove is separate from the catalytic cleft and the associated substrate phosphorylation groove. If Ca^{2+}/CaM has removed the autoregulatory domain from the catalytic surface and the binding peptide from the NR2B subunit

is able to occupy the substrate binding groove, the catalytic surface is occluded and the autoregulatory domain is no longer able to re-bind (Bayer et al., 2006). The consequence of this is the generation of autonomous activity even in the absence of Thr286 phosphorylation. This is particularly interesting, as it suggests that CaMKII substrates can not only provide for feedback for their own regulation, they may also provide feed-forward modulation of proteins localized within the same subcellular compartment.

NMDA-Rs can be found within the post-synaptic densities (PSD) and extrasynaptic spaces in neurons. The post-synaptic density is a specialized area within dendrites that receives signals from pre-synaptic connections. The PSD is a tightly-packed subcellular compartment full of CaMKII and CaMKII substrates. Nearly 18% of the PSD is composed of CaMKII (Miller and Kennedy, 1985). Along with CaMKII, the PSD contains cytoskeletal proteins and an array of voltage-gated and ligand-gated channels/receptors. As mentioned, following Ca^{2+} influx via the NMDA-R, CaMKII translocates to NR2B. Because the PSD contains NMDA-Rs, CaMKII is recruited to the PSD following activation (Strack et al., 1997b). This recruitment is critical as a large number of CaMKII substrates are found within this small subcellular compartment. To date, over 30 CaMKII substrates have been identified in the PSD (Yoshimura et al., 2000, Yoshimura et al., 2002). Without proper translocation of the kinase to the PSD, the regulation of these substrates would be lost. In fact, *in situ* studies in HEK293 cells indicated that the presence of NR2B at the cell membrane induces translocation of CaMKII to the membrane which enhances the phosphorylation of other

membrane-bound proteins (Tsui et al., 2005). Thus, the dynamic interactions between CaMKII and the NMDA-R are essential for normal synaptic function.

<u>Ion Channels and Extracellular Receptors</u>

CaMKII has been shown to regulate an array of other voltage-gated and ligand-gated ion channels in neurons and astrocytes. Sodium channels, potassium channels, calcium channels, and purinergic receptors have all been shown to be regulated by CaMKII (Table 1). Connexins are also regulated by CaMKII. These hemichannels are particularly interesting as they serve as regulators of intracellular ion concentration between connected cells. For example, CaMKII has been shown to phosphorylate Connexin43 (found in myocytes and astrocytes) which subsequently results in decreased gap junction open probability (Dermietzel et al., 1989, Huang et al., 2011, Xu et al., 2012). Thus, CaMKII regulation of Connexin43 is critical for turning off the calcium flux from one cell to another. The loss of CaMKII activity would then result in dispersion of increased cytosolic calcium to nearby, unstimulated astrocytes.

Several metabotropic receptors, including glutamate and dopamine receptors, are also regulated by CaMKII. Table 1 summarizes several ionotropic and metabotropic receptors within neurons and astrocytes that CaMKII has been shown to regulate. CaMKII has also been shown to regulate several known channel/receptor modulators. This includes accessory subunits of these ion channels and G-coupled protein receptors as well as other signaling proteins that influence channel/receptor function, such as Stargazin, an accessory protein essential for AMPA-R insertion (Opazo et al., 2010). While Table 1 summarizes a

Ion Channel	Physiological Impact	Reference
AMPA-R	Increases current density, targets channel to synapse	Yakel et al., 1995, Tan et al., 1994
ASIC1a	Increases current density	Gao et al., 2005
BK	Increases open probability	Van Welie and du Lac, 2011
Cav1.2	Increases calcium-dependent facilitation	Hudmon et al., 2005
Cav2.1	Slows inactivation	Xiang et al., 2007
Cav3.2	Decreases threshold of activation	Wolfe et al., 2002
CLC-3	Activates channel	Cuddapah et al., 2010
GABA-A	Increases current density, targets channel to membrane	Houston and Smart, 2006, Houston et al., 2007
GABA-B1	Prevents internalization	Guetg et al., 2010
Kv1.4	Slows inactivation	Roeper et al., 1997
Kv4.2	Increases channel expression	Varga et al., 2004
Nav1.8	Increases current density	Hudmon et al., 2008
NMDA-R	Enhances receptor desensitization	Sessoms-Sikes et al., 2005
P2Y1	Internalizes receptor	Tulapurkar et al., 2006
TRPV1	Sensitizes receptor	Oh et al, 2004

Table 1: Neuronal ion channels and receptors regulated by CaMKII.

very small portion of the known CaMKII substrates, it is easy to appreciate that CaMKII signaling (or loss of it) can have dramatic effects on neuronal excitability and the ability of neurons to respond to external stimulation.

Cytoskeletal Proteins

CaMKII has also been shown to regulate a variety of cytoskeletal proteins in neurons and astrocytes. Cytoskeletal proteins are often dynamic in nature, assembling and disassembling in response to stimulation, allowing cell growth and motility. Furthermore, these proteins play a critical role in organelle trafficking throughout the cell. CaMKII phosphorylation can inhibit cytoskeleton dynamics. For example, CaMKII phosphorylation of α- and β-tubulin not only inhibits tubulin assembly, it also inhibits the interaction between tubulin and microtubule-associated protein II (MAP-2), a key structural component of neurons (Wandosell et al., 1986). Similarly, CaMKII phosphorylation of actin also inhibits actin polymerization (O'Leary et al., 2006). Furthermore, CaMKII phosphorylation of tau, a microtubule-associated protein in neurons, prevents the binding and stabilization of tau to microtubules (Singh et al., 1996, Sironi et al., 1998). This is particularly interesting as the destabilization of tau within neurons is associated with many neurodegenerative diseases such as Alzheimer's Disease and Parkinson's Disease (as reviewed by (Lee et al., 2001, Morris et al., 2011)).

CaMKII activity can also decrease cytoskeletal stability by leading to protein disassembly. For example, CaMKII phosphorylation of vimentin, a cytoskeletal protein in astrocytes, results in the disassembly of vimentin filaments *in vitro* and *in situ* (Inagaki et al., 1987, Oguri et al., 2006). Thus, in combination

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with influencing cell excitability, CaMKII phosphorylation of key cytoskeletal

proteins can greatly impact cell structure, and may ultimately affect processes

controlled by the cytoskeleton, such as neurotransmitter release and organelle

transport.

PHYSIOLOGICAL CAMKII SIGNALING

Synaptic Plasticity

CaMKII activity is critical for altering the ability of neurons to strengthen or

weaken synaptic connections in response to stimulation, a process termed

synaptic plasticity. Several studies have shown that glutamate stimulation-

induced CaMKII activity can lead to the enhancement of synaptic connections by

increasing excitatory AMPA-R signaling (reviewed by (Lisman et al., 2012)).

CaMKII targets both the AMPA-R and a regulatory protein of the AMPA-R to

directly and indirectly optimize AMPA-R signaling in the synapse. CaMKII

phosphorylation of Stargazin, a calcium channel accessory subunit shown to

interact with AMPA-R, results in mobilization of the AMPA-R (Tomita et al., 2005,

Opazo et al., 2010). Because Stargazin also interacts with PSD-95, a key post-

synaptic density cytoskeletal protein, the AMPA-R is recruited to the PSD (Bats

et al., 2007). The AMPA-R is an ionotropic glutamate receptor, thus subsequent

synaptic stimulation can activate both the NMDA-R and the newly-inserted

AMPA-R. CaMKII also phosphorylates AMPA-R (Tan et al., 1994, Yakel et al.,

1995). This phosphorylation event leads to increased channel conductance in

response to stimulation (Lledo et al., 1995, Barria et al., 1997a, Barria et al.,

1997b, Derkach et al., 1999). Thus, both the insertion of the AMPA-R at the

synapse and the increased ability of the channel to pass ions into the extrasynaptic neuron are induced by CaMKII activity. Recently, CaMKII has also been shown to downregulate the AMPA-R regulating protein AKAP79/150, which is known to decrease AMPA-R current (Nikandrova et al., 2010). By turning off the negative feedback, CaMKII activity is able to maintain AMPA-R availability in the PSD thereby increasing synaptic strength and initiating long-term potentiation.

The consequence of all of these modulatory events is the strengthening of the synaptic connection, a type of synaptic plasticity referred to as long-term potentiation (LTP). LTP is the cellular correlate for memory and learning and CaMKII activity plays a key role in this process (Malenka et al., 1989, Chen et al., 2001). Considering that αCaMKII is the predominant isoform of CaMKII within the hippocampus, it is not surprising that αCaMKII knock-out animals exhibit both a loss of long-term potentiation and severe deficits in memory and learning (Silva et al., 1992).

Not only does CaMKII signaling impact the initiation of LTP, it is also critical for its maintenance. Even after initiation, LTP is reversed by the addition of AIP and CN27/CN21 (Sanhueza et al., 2007, Sanhueza et al., 2011). It is proposed that CaMKII plays a structural role in the synapse. Because the maintenance phase of LTP is marked by changes in the structure of the synapse, it is possible that disrupting this role of CaMKII ultimately disrupts the maintenance of LTP. Recent studies have also identified a role for CaMKII in regulating protein turnover within the synapse, a process which is critical for

maintenance of LTP. Inhibitors of the ubiquitin proteasome system have been shown to reverse LTP (Dong et al., 2008). Following synaptic stimulation, the ubiquitin proteasome is recruited to the PSD to ensure proper turnover of proteins in this highly-active subcellular compartment. Interestingly, this translocation is dependent on CaMKII. CaMKII directly binds one of the proteasome subunits and as the kinase becomes activated and begins to translocate to NMDA-Rs in the PSD, it pulls the proteasome along with it (Bingol et al., 2010). Expression of a CaMKII mutant that does not translocate (Iso205Lys) results in a loss of proteasome recruitment to the PSD (Bingol et al., 2010). Thus, CaMKII may play a direct and indirect role in maintaining synaptic strength by contributing to the structure of the PSD as well as by recruiting proteins to the synapse that are essential for this maintenance phase.

Evolutionary Insight

Genetic manipulations of CaMKII within transgenic mice have alluded to a critical need for maintaining CaMKII homeostasis for normal physiology. βCaMKII knock-out mice exhibit severe motor deficits and disrupted synaptic plasticity within the cerebellum (van Woerden et al., 2009). As mentioned, αCaMKII knock-out animals display deficits in memory and learning. Furthermore, these mice are predisposed to neuronal damage following stroke (Silva et al., 1992). A gene dose-dependence of damage was observed when heterozygous and homozygous αCaMKII knock-out animals were subjected to middle cerebral artery occlusion. Additionally, αCaMKII knock-out mice exhibited increased hyperexcitability and were predisposed to epileptic seizures. Interestingly,

autoinhibitory-deficient transgenic mice (lacking Thr305/Thr306) also display enhanced susceptibility to seizure (Elgersma et al., 2002). Similarly, transgenic expression of constitutively-autonomous CaMKII (Thr286Asp) resulted in increased epilepsy, which was further associated with decreased life-span in males (Mayford et al., 1995). Thus, both a loss of CaMKII and an increase in CaMKII activity result in epilepsy and other pathophysiological outcomes, including deficits in memory and learning as well as decreased life-spans.

Many insights into the potential physiological role of CaMKII can also be ascertained when we examine the evolutionary homologs and orthologs of CaMKII. CaM-dependent kinases are found in nearly all cell types, from single-celled organisms to higher order multicellular systems. While nomenclature varies and there is a wide degree of sequence variability between different cell systems, CaMKII signaling proteins can be found from bacteria to plants to mammals. Several studies indicate that these CaMKII family members play critical roles in maintaining cellular function and viability. For example, overexpression of CMK2, the yeast (*Schizosaccharomyces pombe*) ortholog of CaMKII, locks the cell division cycle in the G2 (Gap 2) phase (Alemany et al., 2002). Ultimately, the consequence of this is the arrest of cell division (Alemany et al., 2002). Genetic knockdown of CMK2 sensitizes yeast to oxidative stress and the ensuing cell death that can occur from it (Sanchez-Piris et al., 2002). This phenomena is particularly interesting because under periods of distress, CMK2 becomes activated and phosphorylated at the onset of insult (Sanchez-Piris et al., 2002), analogous to the activation of CaMKII observed during

cerebral ischemia (will be discussed later). Moreover, the enhanced sensitization to further stress when CMK2 is inactive is analogous to the predisposition of neurons to ischemic death in the αCaMKII knock-out animals (Waxham et al., 1996).

Overexpression of CPK32, the thale cress (*Arabidopsis thaliana*) ortholog of CaMKII, is beneficial for plant survival following several insults. An example of this is the increased resistance to drought and decreased sensitivity to salt when CPK32 is overexpressed (Karve, 2009). Similar to CaMKII in neurons, cellular stress in these plants leads to activation and redistribution of CPK32 in the cell (Karve, 2009). Osmotic stress, wound formation, and biological stressors such as changes in climate all result in increased CPK32 activity (Karve, 2009). Based on the protective qualities associated with CPK32, it is inferred that CPK32 activation is cytoprotective during multiple insults. Interestingly, genetic knock-down of CPK32 results in an increased sensitivity of these plants to damage following insults (Karve, 2009). This is consistent with CMK2 knockdown in yeast and αCaMKII knock-out mice, which exhibit predisposition to death following excitatory insults (Waxham et al., 1996).

One of the most radical effects of increased CaMKII activity is observed within *Drosophila melanogaster*. Mutation of CaMKII to a constitutively active form leads to Drosophila death as a failure of action potential propogation in the insect neurons ultimately induces cardiac failure (Park et al., 2002). Loss-of-function CaMKII mutations in Drosophila also have profound effects on the insects. A loss of CaMKII activity decreases the ability to learn and detect

pheromone cues (Joiner MI and Griffith, 1997, Mehren and Griffith, 2004). There is also increased muscle excitability when CaMKII signaling is lost (Wang et al., 1994). On a cellular level, these changes in muscle excitability are due to increased motor neuron branching and increased excitatory synaptic release at neuromuscular junctions (Wang et al., 1994). This finding, in combination with predisposition to seizure observed in αCaMKII knock-out mice, suggests that a loss of CaMKII activity leads to hyperexcitability.

Neuronal function is also altered in *Caenorhabditis elegans* when CaMKII, or Unc-43, is aberrantly regulated. A gain-of-function mutation results in loss of neurotransmitter release, which consequently, impairs the motor system (Reiner et al., 1999, Robatzek and Thomas, 2000, Liu et al., 2007). As expected, *C elegans* exhibit a trend in general hyperexcitability when Unc-43 (CaMKII homolog) function is decreased (Reiner et al., 1999). Enteric muscle excitability is significantly increased with loss-of-function mutations, which results in increased defecation. There is also a significant increase in egg laying due to hyperexcitability. The most robust effect in *C elegans* is the increased sensitivity to seizure-like convulsions when Unc-43 is knocked down (Reiner et al., 1999, Williams et al., 2004). It has been reported that loss-of-function mutants spontaneously exhibit repeating muscle bursts akin to seizures. Furthermore, the threshold for seizure induction following stimulant treatment is drastically decreased in these mutant worms (Williams et al., 2004). This is similar to the increased propensity to generate epileptic seizures in the αCaMKII knock-out

mice and increased excitability in Drosophila (Wang et al., 1994, Butler et al., 1995).

Thus, evolutionary alterations in CaMKII are unfavorable for cellular viability. Increased CaMKII signaling in several of these examples is associated with cellular death and dramatic decreases in the excitability of neurons. A loss of CaMKII function appears to decrease the ability of organisms to respond to insults and in higher order systems, this loss of function leads to increased cellular excitability. From these evolutionary counterparts, we can begin to recognize that a proper balance of CaMKII signaling is required for maintaining cellular physiology.

Insight from other tissue systems

Several studies have examined the effects of aberrant CaMKII activity in other tissue systems. For example, increased CaMKII activity in cardiomyocytes is associated with increased cellular excitability and a predisposition to toxicity. The expression of δCaMKII, the predominant cardiac isoform, is significantly elevated during heart failure (Hoch et al., 1999, Kirchhefer et al., 1999, Zhang et al., 2003). Mechanistic studies have identified that when activated, CaMKII aberrantly regulates cardiac ion channels and initiates the transcription of several pro-hypertrophic and pro-inflammatory genes (as reviewed by (Singh and Anderson, 2011). Thus, inhibition of CaMKII during heart failure affords cardioprotection by decreasing these cardio-myopathies. Moreover, the δCaMKII knock-out mice are more resistant to heart failure (Backs et al., 2009). No changes in morphology or excitation were observed in these knock-outs,

indicating that a loss of CaMKII signaling in the heart does not have a detrimental effect on cardiac function.

Within the immune system, CaMKII signaling is required for cytokine production in T lymphocytes, activation of natural killer cells, and initiation of macrophage infiltration (Bui et al., 2000, Poggi et al., 2002a, Poggi et al., 2002b, Liu et al., 2008). Expression of constitutively-active CaMKII leads to increased cytokine production and release, while pharmacological and genetic inhibition of CaMKII is associated with a loss of cytokine signaling (Poggi et al., 2002b, Liu et al., 2008). Several of the cytokines that appear to be downstream of CaMKII signaling are implicated in autoimmune disorders and inflammation. Thus, studies suggest that alterations in CaMKII signaling may contribute to the pathophysiology of immune diseases. While changes in cellular viability of immune cells treated with constitutively-active CaMKII or CaMKII inhibitors has not been reported, it is evident that CaMKII signaling plays a key role in immune cell function.

Homeostatic regulation of CaMKII signaling is also critical within the reproductive system. CaMKII inhibition dramatically decreases sperm motility and negatively regulates the exocytotic mechanisms required for spermatozoa penetration of the ovum. (Schlingmann et al., 2007, Ackermann et al., 2009). Within females, a loss of CaMKII activity in Oocytes leads to apoptotic death. CaMKII phosphorylation of caspase-2 renders the pro-apoptotic caspase inactive to promote oocyte survival. Mutation of the phospho-acceptor site on caspase-2, Ser135, results in robust oocyte death within 8 hours (Nutt et al., 2005). Thus, a

loss of CaMKII activity in both male and female reproductive machinery can be detrimental to fertilization.

There have also been several studies connecting CaMKII signaling and cancer. In general, a loss of CaMKII signaling appears to be toxic for cancer cells. Pharmacological CaMKII inhibitors and genetic ablation of CaMKII have been shown to induce apoptosis in malignant glioma, melanoma, osteosarcoma, and prostate cancer cells (Xiao et al., 2005, Song et al., 2006, Rokhlin et al., 2007, Yuan et al., 2007). Furthermore, expression of the CaMKIIN inhibitory protein has been shown to arrest cell cycling and induce apoptosis in ovarian adenoblastomas (Ma et al., 2009). These findings are particularly interesting as fluctuations in CaMKIIN protein levels are associated with ovarian adenoblastomas (Ma et al., 2009). Robust levels of the CaMKIIN protein has been shown to be expressed in normal healthy ovarian tissue; however, adenocarcinomas are marked by a significant decrease in CaMKIIN protein (Ma et al., 2009). Thus, a reduction in the endogenous regulation of CaMKII may be a mechanism underlying how cancer cells evade death. These findings suggest that CaMKII activity is essential for the growth and survival of several variants of cancer.

Together, the literature overwhelmingly suggests that dysregulation of CaMKII is detrimental to cellular function and viability. Fluctuations in activity are correlated with altered excitability, enhanced sensitivity to insults, and increased toxicity. From this, we postulate that a proper balance of CaMKII activity is essential for maintaining neuronal viability.

PATHOLOGICAL CAMKII SIGNALING

Changes in CaMKII Signaling during Cerebral Ischemia

Ischemic events within the brain can be devastating to neurons. Neurons require oxygen and glucose in order to generate and maintain their excitable membrane. Therefore, a loss of blood flow associated with ischemia is detrimental to neurons as metabolically-dependent functions such as the regulation of ion pumps are no longer able to be maintained. Because ion pumps are critical for the maintenance of membrane potential, the loss of pump activity during ischemic insult has been shown to lead to an aberrant neuronal depolarization from which the neurons are not able to recover (as reviewed by (Lipton and Rosenberg, 1994, Aronowski et al., 2000, Mark et al., 2001)). It is well-understood that neuronal depolarization can lead to neurotransmitter release. Thus, ischemic insults and the resulting periods of decreased energy availability are also marked by an increase in neurotransmitter release. While several factors can contribute to neuronal death following stroke (such as reactive oxygen species generation, mitochondrial dysfunction, lipase activation, and macrophage infiltration), the dysregulation of neuronal excitability and aberrant neurotransmitter release are key mechanisms underlying ischemic neuronal death.

As mentioned, the terminal depolarization of ischemic neurons results in the release of neurotransmitter stores. The most abundant excitatory neurotransmitter in the cerebral cortex is the amino acid glutamate. Thus, extracellular concentrations of nearly 250 µM glutamate have been observed

within the ischemic tissue in a human stroke patient (Bullock et al., 1995). While glutamate is critical for excitatory synaptic signaling, the overabundance of extracellular glutamate in ischemia is detrimental to neuronal viability. In 1969, J.W. Olney first described the neurotoxic effects of increased glutamate in mice (Olney, 1969). Over the next 20 years, several groups identified that neuronal damage following ischemic stroke was correlated with excess glutamate signaling (reviewed by (Choi, 1988)). The hypothesis that excess glutamate played a causal role in neurotoxicity was supported by *in situ* analysis of neuronal viability in the hours following exogenous glutamate application. Neurons treated with high levels of glutamate exhibited the loss of neuritic processes, swollen somas, and increased membrane permeability (Olney et al., 1986, Choi et al., 1987, Finkbeiner and Stevens, 1988). The process of glutamate-induced neuronal death was termed 'excitotoxicity'; a term which highlights the connection between over-excitation and toxicity (Olney, 1969).

Further work has identified that excitotoxicity is largely induced by glutamate activation of the NMDA-R (Choi, 1987, Murphy et al., 1987). While neurons express several metabotropic and ionotropic glutamate receptors, antagonism of the ionotropic NMDA-R has been shown to prevent excitotoxic neuronal death *in situ* (Choi et al., 1988, Frandsen et al., 1989). Furthermore, NMDA-R antagonists have been shown to reduce neuronal damage in animal models of stroke (Gotti et al., 1988, Park et al., 1988a, b, Bullock et al., 1990, Uematsu et al., 1991). While these findings have been critical for understanding the mechanism underlying the initiation of excitotoxicity, the optimism for NMDA-

R antagonists in therapeutic treatment of ischemic stroke has been dampered by the inability of these inhibitors to be effective in clinical trials (as reviewed by (Ginsberg, 2008, 2009)). There are large disparities in the findings of these trials with some suggesting a neuroprotective effect, others suggesting no effect, and others suggesting that the negative side-effects of these drugs outweigh any potential benefits. Considering how efficacious these reagents were in animal models, the failure of these drugs to be neuroprotective in clinical trials is perplexing. As mentioned above, extracellular glutamate levels are significantly increased within minutes of insult (Benveniste et al., 1984, Globus et al., 1988, Butcher et al., 1990). While this extracellular glutamate has been shown to remain elevated for hours to days (Davalos et al., 1997), the therapeutic time window for NMDA-R antagonism appears to be much shorter. Because of this, recent studies have focused on examining the impact of signaling pathways downstream of the NMDA-R (Ginsberg, 2008).

NMDA-R activation results in Ca^{2+} influx into neurons. This Ca^{2+} influx has been shown to play a causal role in the neurotoxic effects of glutamate stimulation, as buffering of extracellular Ca^{2+} can prevent excitotoxicty *in vitro* (Choi, 1985). As mentioned earlier, intracellular Ca^{2+} levels impact numerous signaling pathways, including CaMKII which is activated following the onset of ischemic insult (Westgate et al., 1994). This excitotoxic-induced activation is short-lived as CaMKII enters an inactive state within minutes (Aronowski et al., 1992, Hanson et al., 1994, Aronowski and Grotta, 1996). This inactivation can last for hours to days, depending on the length of ischemic insult. Figure 4

summarizes the fluctuations in CaMKII activity in the time surrounding ischemic insult (based on previous literature (Aronowski et al., 1992, Hanson et al., 1994, Westgate et al., 1994)).

The Role of CaMKII Inactivation

These fluctuations in CaMKII activity during ischemic stroke are correlated with the extent of neuronal damage. In an animal model of stroke, CaMKII activity within the ischemic core was undetectable 24 hours following insult (Hanson et al., 1994). For reference, the ischemic core is the tissue that has directly lost blood supply. The area surrounding the ischemic core is known as the penumbra. This tissue retains partial blood supply; however, signals from the core render the penumbra sensitive to the insults that accompany stroke (glutamate and reactive oxygen species). Interestingly, CaMKII activity within the penumbral region is also decreased 24 hours following stroke (Hanson et al., 1994). There was a striking pattern between CaMKII activity levels and neuronal damage. While the core exhibited the largest extent of CaMKII inactivation, this loss of activity radiated from the core into the surrounding penumbra (Hanson et al., 1994). This pattern of radiation directly mimicked the pattern of cell death, with the largest extent in the core and radiation outward away from insult into the penumbra (Hanson et al., 1994). Importantly, proteolytic degradation of the kinase does not underlie this decreased activity (Aronowski et al., 1992, Westgate et al., 1994, Shackelford et al., 1995). Interestingly, neuroprotective strategies that block NMDA-R function have been shown to prevent the loss of
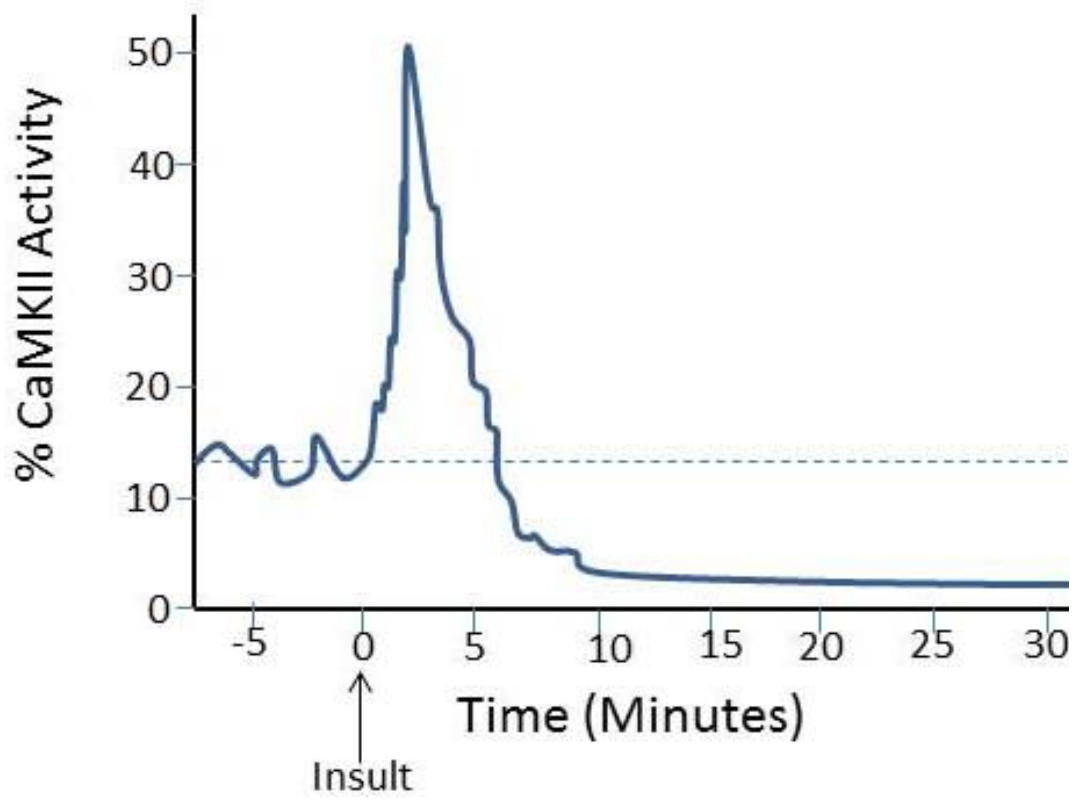
Figure 4: Schematic of CaMKII activity in the time surrounding excitotoxic insult.

CaMKII activity, further supporting the correlation between CaMKII inactivation

and the extent of neuronal death (Aronowski et al., 1993).

The concept that CaMKII inactivation is detrimental to neuronal survival

following stroke is also supported by studies in the αCaMKII knock-out mouse.

Interestingly, αCaMKII knock-out mice displayed greater infarct size than their

wild-type littermates (Waxham et al., 1996). Moreover, there was gene-dosage

sensitivity to stroke damage, with the greatest damage in homozygous knock-out

mice and moderate damage in heterozygous αCaMKII animals (Waxham et al.,

1996). Together, these data suggest that a loss of CaMKII activity could play a

detrimental role in the ability of neurons to survive an ischemic/excitotoxic insult.

The Role of Aberrant CaMKII Activation

While inactivation of CaMKII correlates with neuronal death, an increase in

CaMKII activity precedes this inactivation. Several studies have attempted to

better understand the influence of CaMKII activation during periods of cellular

distress. Application of small molecule inhibitor KN-93 prior to excitotoxic insult

has been shown to afford neuroprotection to cortical, hippocampal, and retinal

neurons (Hajimohammadreza et al., 1995, Takano et al., 2003, Vest et al., 2010).

Peptide inhibitors of CaMKII, such as tat-CN21 and tat-AIP, have also been

shown to be efficacious at reducing excitotoxicity (Laabich and Cooper, 2000,

Fan et al., 2006, Goebel, 2009, Vest et al., 2010). However, these studies have

been limited to inhibition of CaMKII prior to insult. Thus, our studies are focused

on understanding the impact of CaMKII signaling in the time surrounding insult,

by applying inhibitors at various time points before and after glutamate

stimulation. During the completion of our studies for Chapter 2, the Bayer lab indicated that injection of tat-CN21 an hour after the induction of middle cerebral artery occlusion in mice reduced infarct size 24 hours following insult (Vest et al., 2010). These findings indicated that targeting CaMKII during ischemic stroke may be therapeutically advantageous. As will be discussed in Chapter 2, we have expanded on their findings to better address enzymatic changes in CaMKII that occur when the inhibitors are applied in the time surrounding insult.

Self-Association

How can both aberrant activation and inactivation of CaMKII play a role in excitotoxic neuronal death? The answer may lie in the functional consequences that activation of CaMKII during periods of cellular distress has on CaMKII stability and activity. Nearly 20 years ago, differential centrifugation of whole brain lysates taken from animals subjected to cerebral ischemia indicated that CaMKII transitioned from soluble to sedimentable fractions following insult (Aronowski et al., 1992, Yamamoto et al., 1992, Kolb et al., 1995, Shackelford et al., 1995). This transition occurs rapidly, and directly correlates with a loss of CaMKII activity (Aronowski et al., 1992, Kolb et al., 1995, Shackelford et al., 1995). This translocation was consistent in hippocampal slices subject to ischemic insult *in vitro* (Kolb et al., 1995). Electron microscopy studies identified that ischemic-like insults induced the translocation of CaMKII to aggregate-like clusters in neurons (Dosemeci et al., 2000, Tao-Cheng et al., 2001, Tao-Cheng et al., 2002). These clusters were purified and mass spectrometry revealed that they were primarily composed of αCaMKII protein (Dosemeci et al., 2000).

Utilization of GFP-tagged αCaMKII allowed for real-time analysis of this CaMKII aggregation within neurons following excitotoxic glutamate stimulation (Hudmon et al., 2005). Interestingly, αCaMKII began to cluster within minutes of glutamate application, suggesting that excitotoxic insults rapidly cause CaMKII aggregation (Hudmon et al., 2005). To further understand this, purified αCaMKII was observed in ischemic-like conditions *in vitro* (limiting ATP, reduced pH; discussed below) (Hudmon et al., 1996, Hudmon et al., 2001, Hudmon et al., 2005). Light scattering analysis indicated that the shape and size of αCaMKII in solution was drastically different when the kinase was added to 'ischemic-like' solution compared to physiologically normal solution (pH 7.4 with saturating levels of ATP) (Hudmon et al., 2001). Transmission electron microscopy indicated that the size and shape difference between these two conditions was due to the formation of grape-like clusters of CaMKII holoenzymes (Hudmon et al., 2001). Because this process is an interholoenzyme association (*ie* multiple CaMKII holoenzymes cluster together) the process was termed 'self-association'.

Much work has gone into trying to understand the mechanism of CaMKII self-association. It is known that this process is activity-dependent in that it requires Ca^{2+}/CaM activation (Hudmon et al., 1996). However, Ca^{2+}/CaM binding is not sufficient to induce self-association, as under physiological conditions little CaMKII is self-associated. A role for ATP concentration has been established; self-association does not occur when free ATP levels are within 2-4 mM (Hudmon et al., 1996). However, when CaMKII is activated in limiting ATP, self-association is observed (Hudmon et al., 1996). Interestingly, the kinase is unable

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to self-associate when ATP is completely absent, suggesting a requirement for the presence of some nucleotides. Indeed, ADP and the ATP analog AMP-PNP are able to substitute for ATP and induce self-association when ATP is completely depleted (Vest et al., 2009). These data suggest that under periods of cellular distress that decrease ATP availability, CaMKII is sensitive to self-association. During ischemic stroke *in vivo*, ATP concentration has been shown to plummet within minutes of insult (Kobayashi et al., 1977, Onodera et al., 1986, Eleff et al., 1991). Along with this loss in ATP, an increase in AMP levels has been observed (Kobayashi et al., 1977, Onodera et al., 1986) as well as a transient increase in ADP levels (Kobayashi et al., 1977). *In situ* excitotoxic insults show a similar effect with significant decreases in intracellular ATP levels following insult (Mattson et al., 1993, Budd et al., 2000). Thus, ischemic events create an environment that fosters CaMKII self-association.

Another hallmark of ischemia that has been shown to regulate CaMKII self-association is a reduction in pH. Under physiological pH (7.3-7.4), αCaMKII does not self-associate, even when ATP levels are depleted (Hudmon et al., 1996, Hudmon et al., 2001); however, as the pH decreases towards pH 7.0 or pH 6.0, the sensitivity of the kinase to self-associate increases (Hudmon et al., 2001). Because intracellular pH within neurons is maintained by proper oxygen/glucose availability, ischemic insults result in rapid decreases in pH; *in vivo* stroke models have shown that pH can reach levels as low as pH 6.2 in minutes (Silver and Erecinska, 1992). Decreased ATP and decreased pH additively affect the sensitivity of αCaMKII self-association. As mentioned, high

levels of ATP (>1 mM) prevent self-association even when pH is significantly reduced (Hudmon et al., 1996). Furthermore, higher pH (pH 7.4) can prevent this aggregation from occurring even when ATP is depleted (Hudmon et al., 2001). While self-association is prevented at higher pH, the limiting levels of ATP still induce CaMKII inactivation (Hudmon et al., 1996, Hudmon et al., 2001). Together, a decrease in pH and a decrease in ATP lead to enhanced susceptibility to self-association.

Interestingly, autophosphorylation of Thr286 prevents self-association even under periods of decreased ATP and pH. While phosphorylation of this residue is most often recognized as being essential to rendering CaMKII autonomous, Thr286 phosphorylation also appears to provide stability to the kinase by preventing this aggregation (Hudmon et al., 2005). It is thought that this protection that autophosphorylation affords may explain the ability of high levels of ATP to prevent self-association; increased ATP availability will result in increased Thr286 phosphorylation. The concept that the autophosphorylation state of Thr286 determines the sensitivity to self-association is supported by *in situ* studies with Thr286 mutants. Overexpression of autophosphorylation deficient Thr286Ala resulted in significantly higher levels of self-association in neurons and HEK293 cells subjected to ischemic-like insults, compared to wild-type αCaMKII (Hudmon et al., 2005). The constitutively-autophosphorylated mutant Thr286Asp is more resistant to self-association than wild-type αCaMKII (Hudmon et al., 2005). Many theories exist as to how this residue may influence the stability/structure of the kinase; however, there is currently no crystal

structure available for autophosphorylated CaMKII. AC-2, the peptide mimetic of the autoregulatory domain, can prevent self-association (Hudmon et al., 2001). While the mechanism underlying self-association is not fully-understood, it has been hypothesized that the autoregulatory domain interacts with the surface of another holoenzyme causing the interholoenzyme aggregation (Hudmon et al., 2001).

Despite these speculations, the connection between self-association and CaMKII inactivation is apparent. Self-association results in the inactivation of CaMKII. It is important to point out that inactive kinase does not self-associate, rather self-association leads to inactivation. Phosphorylation of Thr305/Thr306 results in inactivation (see CaMKII Inactivation section). Phosphomimetics at these residues (Thr305Asp/Thr306Asp) have been shown to be resistant to self-association *in situ* (Hudmon et al., 2005). This is because CaMKII self-association is dependent on Ca^{2+}/CaM binding, which cannot occur when Thr305/Thr306 are phosphorylated. Thus, the loss of enzyme activity under ischemic-like conditions is likely due to inactivation subsequent to self-association. The impact of self-association/inactivation on neuronal viability is still poorly-understood.

RESEARCH GOALS

Ultimately, a better understanding of how CaMKII activity contributes to neuronal physiology is essential in order to fully-appreciate the role of CaMKII in normal and diseased states. Neurodegenerative diseases such as cerebral ischemia destabilize CaMKII activity while inducing cellular death. It is apparent

that CaMKII signaling is correlated with excitotoxic neuronal death; however, whether CaMKII plays a causal role, rather than a correlative one, is still unknown. The purpose of this dissertation is to address this knowledge gap and solidify the role of CaMKII signaling in regulating/maintaining neuronal viability.

I hypothesize that the large fluctuations in CaMKII activity- both the transient increase and prolonged inactivation observed following excitotoxic insult- play a causal role in the induction of neurotoxicity observed in the hours and days following insult. To address this, I will modulate CaMKII in the time surrounding excitotoxic insult to fully understand what impact overactivation of the kinase has on both neuronal viability and subsequent functional changes in CaMKII itself. Furthermore, we will also analyze the consequence of a rapid, yet prolonged decrease in neuronal CaMKII activity. Finally, we will examine the effect of CaMKII inhibition in astrocytes to identify how these cells can influence neuronal survival when CaMKII activity is decreased. These experiments will allow us to solidify a role for CaMKII activity as an important director of neuronal function and will highlight potential avenues for therapeutic targeting of CaMKII in neurodegenerative diseases.

PART I: EXCITOTOXIC NEUROPROTECTION AND VULNERABILITY WITH CAMKII INHIBITION

SUMMARY

Aberrant calcium signaling is a common feature of ischemia and multiple neurodegenerative diseases. While activation of Ca^{2+}/CaM-dependent protein kinase II (CaMKII) is a key event in calcium signaling, its role in excitotoxicity is controversial. Our findings demonstrate neuroprotection in neuronal cultures treated with the small molecule (KN-93) and peptide (tat-AIP and tat-CN21) inhibitors of CaMKII immediately prior to excitotoxic glutamate/glycine insult. Unlike KN-93 which blocks CaMKII activation, but not constitutively active forms of CaMKII, tat-CN21 and tat-AIP significantly reduced excitotoxicity in cultured neurons when applied post-insult. We observed that the neuroprotective effects of tat-CN21 are greatest when applied before the toxic glutamate challenge and diminish with time, with the neuroprotection associated with CaMKII inhibition diminishing back to control 3 hours post glutamate insult. Mechanistically, tat-CN21 inhibition of CaMKII resulted in an increase in CaMKII activity and the percentage of soluble αCaMKII observed in neuronal lysates 24 hours following glutamate stimulation. To address the impact of prolonged CaMKII inhibition prior to excitotoxic insult, neuronal cultures were treated with CaMKII inhibitors overnight and then subjected to a sub-maximal excitotoxic insult. In this model, CaMKII inhibition prior to insult exacerbated neuronal death, suggesting that a loss of CaMKII enhances neuronal vulnerability to glutamate. Although changes in αCaMKII or NR2B protein levels are not responsible for this enhanced

glutamate vulnerability, this process is blocked the protein translation inhibitor cycloheximide. Thus, the neuroprotection afforded by CaMKII inhibition can be seen as neuroprotective immediately surrounding the excitotoxic insult, whereas sustained CaMKII inhibition produced by excitotoxicity leads to neuronal death by enhancing neuronal vulnerability to glutamate.

INTRODUCTION

Finely-tuned transient increases in intracellular calcium are essential for neuronal development, communication and plasticity. However, dysregulated calcium signaling can produce neuronal death via necrotic and programmed cell death mechanisms (Portera-Cailliau et al., 1997, Dirnagl et al., 1999, Snider et al., 1999). Excitotoxicity is a hallmark of most neurodegenerative diseases; a process that leads to excessive accumulation of intracellular calcium via the over-activation of excitatory glutamate receptors. Because of its prominent role in neuronal calcium signaling, calcium/calmodulin-dependent protein kinase II (CaMKII) may contribute to excitotoxic neurodegeneration for the following reasons, 1) CaMKII is activated and autophosphorylated in stroke, brain trauma and epilepsy (Perlin et al., 1992, Churn et al., 1995, Morioka et al., 1995, Zalewska and Domanska-Janik, 1996), 2) the calmodulin inhibitor calmidazolium is a neuroprotective agent in ischemia (Pohorecki et al., 1990), and 3) ischemia induces CaMKII translocation (Aronowski et al., 1992, Morioka et al., 1995, Aronowski and Grotta, 1996, Hu et al., 1998, Dosemeci et al., 2001) and phosphorylation of key post-synaptic substrates (i.e. post-synaptic density) (Meng and Zhang, 2002, Takagi et al., 2003, Fu et al., 2004, Hao et al., 2005).

Previous studies using small molecule and peptide inhibitors of CaMKII such as KN-93 and autocamtide-2 inhibitory protein (AIP) have been shown to be neuroprotective when applied before an excitotoxic insult *in vitro* (Hajimohammadreza et al., 1995, Laabich and Cooper, 2000, Takano et al., 2003, Fan et al., 2006, Goebel, 2009, Vest et al., 2010). However, because CaMKII also regulates substrates involved in neuronal survival (e.g. L-type calcium channels, CREB and BCL-2 etc.), it is also possible that inhibiting CaMKII may exacerbate excitotoxic neuronal death (Dash et al., 1991, Bok et al., 2007, Wheeler et al., 2008). In support of this, an ischemic insult in αCaMKII knock-out mice leads to much greater neuronal death than in wild-type litter mates, suggesting that CaMKII activity is important for neuronal survival to excitotoxicity (Waxham et al., 1996).

To date, the discrepancies found between the *in vivo* knock-out model and *in situ* small molecule and peptide inhibitor experiments are not understood. While it possible that differences could be due to model systems (genetic knock-out versus pharmacological manipulation), the impact that both short-term and sustained CaMKII inhibition prior to excitotoxic insult have on neuronal survival has not been explored in one system concurrently. Furthermore, the small molecule and peptide inhibitors of CaMKII used in previous experiments have been shown to have a variety of off-target effectors. The family of KN-drugs has been shown to inhibit a variety of CaM-kinase family members as well as voltage-gated potassium and calcium channels (Enslen et al., 1994, Ledoux et al., 1999, Gao et al., 2006). AIP, which mimics the autoregulatory domain of

CaMKII, has also been shown to inhibit other CaM-kinase family members (Smith et al., 1990).

In order to determine whether CaMKII inhibition is neuroprotective, a battery of CaMKII inhibitors including those presented in earlier studies (KN-93 and AIP) as well as the highly-specific CaMKIINtide peptide inhibitor, CN21 (Vest et al., 2007), were applied either immediately before or after the onset of excitotoxic insult. KN-93, AIP, and CN21 all afforded neuroprotection when applied prior to the onset of an excitotoxic insult. Interestingly, only AIP and CN21, which inhibit the autonomous form of CaMKII (Ishida et al., 1995, Chang et al., 1998, Rose and Hargreaves, 2003), afforded neuroprotection when applied after insult. The translocation and loss of CaMKII activity observed 24 hours after glutamate excitotoxicity was prevented in a time-dependent manner by CaMKII inhibition. To examine the effect that prolonged loss of CaMKII activity has on neuronal sensitivity to excitotoxicity, neuronal cultures were treated with CaMKII inhibitors and were then subjected to a sub-maximal glutamate/glycine insult. Prolonged CaMKII inhibition (>8 hours) exacerbates neuronal death following an excitotoxic challenge; a process that requires protein synthesis. Taken together, these data indicate that acute inhibition of CaMKII is neuroprotective when applied immediately surrounding an excitotoxic insult, whereas prolonged inhibition enhances neuronal death to an excitotoxic challenge.

EXPERIMENTAL PROCEDURES

Materials. CN21 (KRPPKLGQIGRSKRVVIEDDR), CN21c (GQIGRSKVVIEDDRIDDVLK), CN21Ala (KAPAKAAWAAASKRVVIEDDR), AIP

(RKKLRRQEAFDAL) as well as tat (YGRKKRRQRR)-conjugated peptides and Fam-labeled peptides were synthesized and HPLC purified by Biopeptide Co, Inc. San Diego, CA, USA. KN-93 and KN-92 were purchased from Calbiochem, EMD Biosciences, La Jolla, CA. Culture-grade glutamate, glycine, and cycloheximide was purchased from Sigma, St. Louis, MO.

Embryonic cortical neuron culture. Cortical tissue from E18-E19 Sprague-Dawley rat pups was harvested according to approved IACUC guidelines as previously described (Hudmon et al., 2005) with the following modifications. Pelleted cortical cells were resuspended in neuronal growth media (Neurobasal media containing 2% NuSerum (BD Biosciences, San Jose, CA), 2%NS21 (Chen et al., 2008), and penicillin (10 units/mL), streptomycin (10 µg/mL), and L-glutamine (29.2 µg/mL) at a density of 2.5 million cells/mL and seeded on poly-D-lysine (50 µg/mL) coated 15mm coverslips (German glass Number 0) or 60mm dishes. Forty-eight hours after plating, cultures are treated with 5-fluor-2'-deoxyuridine(1.5 µg/mL) (Sigma) and Uridine (3.5 mg/mL) to kill mitotically active cells. Neurons were fed every 48 hours, with half of the conditioned media replaced with fresh media.

Immunocytochemistry of neuronal cultures. Neurons 7-8 days *in vitro* (DIV) were fixed in 4% paraformaldehyde (0.1 M phosphate buffer, pH 7.4) for 10 minutes and washed in phosphate buffered saline (PBS) three times. Cells were permeabilized in 0.5% Triton X-100 in PBS for 10 minutes at room temperature, washed in PBS three times, blocked for 1 hour in 2% BSA Fraction V, 20% normal goat serum, 0.1% Triton X-100 in PBS at room temperature, washed an additional three times in PBS. Cells were then incubated in primary antibodies,

monoclonal MAP-2 (1:1000 Sigma, St. Louis, MO), polyclonal GFAP (1:1000 Sigma), mouse monoclonal anti-αCaMKII antibody (CBα2; 1:5000), or polyclonal pan CaMKII (1:1000 Cell Signaling, Danvers, MA) for 2 hours at room temperature. After three washes, secondary antibodies (goat anti-mouse DyLight$_{800}$, 1:5000 or anti-rabbit Alexa$_{594}$, 1:5000 (Molecular Probes, Eugene, OR)) were incubated PBS for one hour at room temperature. Coverslips were mounted in Prolong Gold Antifade with DAPI mounting media (Molecular Probes) and neurons imaged using a Nikon Ti-E inverted microscope.

Excitotoxic stimulation. Neurons (7-8 or 14 DIV) were stimulated with varying amounts of glutamate/glycine as described in results. The standard excitotoxic condition used was 200 μM glutamate/20 μM glycine for 1 hour at 37°C. Half of the conditioned media is removed and fresh growth media containing 2x glutamate/glycine is added to each dish. Peptide inhibitors were diluted in fresh neuronal growth media from a 10 mM stock. When pretreatment with inhibitors is performed, media containing 2x peptide was added and allowed to incubate at 37°C for 20 minutes at concentrations described in results. Half of this media is removed and stimulation media containing 2x glutamate/glycine supplemented with 1x peptide is then added for stimulation. When inhibitors are added during the excitotoxic insult, half of the excitation media is removed and fresh neuronal growth media containing 1x glutamate/glycine and 2x peptide is added. For application of peptide following the excitotoxic insult, stimulation media was washed and replaced with fresh neuronal growth media. At the time of application, half of this media is removed and 2x peptide is added for 1 hours at

37°C. Following all treatments, stimulation media is washed with fresh neuronal growth media three times. Half of the media is removed and conditioned media is added back to the wells at the end of stimulation or drug treatment. Cultures are incubated at 37°C 24 hours following stimulation. Manipulation of the neuronal cultures (multiple washes consistent with experimental manipulations) produces roughly 10-20% cell death compared to the percentage of dying cells measured in the absence of manipulation.

Cell death assay. Twenty-four hours following glutamate/glycine stimulation, the coverslips were washed in PBS and stained using Live/Dead Cytotoxicity/Viability kit (Molecular Probes, Eugene, OR). Coverslips are incubated as directed by the manufacturer for 40 mins at room temperature, washed in PBS and immediately imaged on Nikon Ti-E inverted microscope (100x magnification). Each coverslip is imaged in three different fields using a Texas Red filter to detect cytotoxic cells and a FITC filter to detect viable cells. Cells were quantified using the automated counting software Nikon Elements 3.0. A threshold of cytotoxic nuclei was set to >20 μm^2, as determined with DAPI co-staining. A viability threshold was set at >50 μm^2, and anything smaller was disregarded as debris. Total cell number was determined by addition of cytotoxic and viable cells. Total cell number was not significantly different between experimental groups for each assay. Propidium iodide was also applied for a series of experiments to determine the rate of excitotoxic neuronal injury. Co-staining with DAPI allowed for the calculation of total cell number in these experiments. In some assays, cell death is normalized to excitotoxic stimulation control as indicated.

CaMKII activity assay. Recombinant αCaMKII was expressed in insect cells using baculovirus and purified as described previously (Bradshaw et al., 2002). CaMKII (25ngs) was incubated with 50 mM Hepes pH 7.4, 100 mM NaCl, 10 mM MgCl$_2$, 100 μM ATP, 2 mM CaCl$_2$, 5 μM CaM, 50 μM Syntide2 (PLARTLSVAGLPGKK), and [γP^{32}]-ATP (3 μCi per reaction) for 1 minute at 30°C. The solution was then transferred to P81 filter papers (Whatman, GE Healthcare, Piscataway, NJ), washed with 75 mM phosphoric acid 3 times for 5 minutes each, and counted in Beckman Beta Counter as described previously (Hudmon et al., 1996). The purified CaMKII routinely had a specific activity of 4-15 μmols/min/mg. For activity assays performed with neuronal lysates, cells were lysed in lysis buffer containing (50 mM Hepes pH 7.4, 4 mM EGTA, 10 mM EDTA, 15 mM Na$_4$P$_2$O$_7$-10H$_2$0, 100 mM β-glycerophosphate, 25 mM NaF, and protease inhibitor cocktail (#539137,Calbiochem)(Kolb et al., 1995)), sonicated, and incubated with 50 mM Hepes pH 7.4, 100 mM NaCl, 10 mM MgCl$_2$, 100 μM ATP, 2 mM CaCl$_2$, 5 μM CaM, 50 μM AC2 (KKALRRQETVDAL), and [γP^{32}]-ATP (3 μCi per reaction) for 3 minutes at 30°C. Preliminary experiments indicated the linear range of the reaction extended from 30 seconds to 4 minutes. Protein levels were assessed and activity was normalized to total protein using a DC protein assay kit (Biorad, Hercules, CA).

Peptide SPOTS arrays. Peptide arrays are constructed using the SPOTS-synthesis method (Frank and Overwin, 1996, Frank, 2002). Standard 9-fluorenylmethoxy carbonyl (Fmoc) chemistry is used to synthesize peptides on cellulose membranes prederivatized with a polyethylene glycerol spacer (Intavis

AG, Cologne, Germany). Fmoc protected and activated amino acids (Anaspec, Fremont, CA) are spotted in 20x30 arrays on 150 mm by 100 mm cellulose membranes using an Intavis robot (Intavis AG, Cologne, Germany). Peptides consist of CN21c (GQIGRSKVVIEDDRIDDVLK) and CN21Ala (KAPAKAAWAAASKRVVIEDDR) as well as a scanning mutagenesis (stepwise single amino acid replacement) of CN21 (KRPPKLGQIGRSKRVVIEDDR) with alanine. The ethanol dried membrane was stained in 0.1% bromophenol blue, washed, ethanol dried, and labeled for annotation. After imaging, the blot was deprotected in a solution of 49.9% trifluoroacetic acid, 49.9% dichloromethane, 0.03% triisopropylsilane, and 0.02% water for one hour at room temperature. Followed by 4 X 25 mL washes with dichloromethane, 4 X 25 mL washes with dimethylformamide, and 2 X 25 mL washes with ethanol. The peptide membrane is blocked at room temperature for 1 hour in binding buffer (20 mM Tris, pH 7.4, 200 mM NaCl, 1 mM EDTA, and 0.1% Tween-20) plus 5% BSA. Alexa$_{800}$-labeled CaMKII is activated in a pre-reaction mixture as described previously (Hudmon et al., 1996). Autophosphorylated CaMKII was diluted in binding buffer plus 1% BSA and added to the membrane for 5 minutes at room temperature. After 5 minutes, the blot was washed 3 times in binding buffer for 5 minutes each. CaMKII binding is visualized using a Licor imaging station. Intensity of binding is analyzed using Odyssey v1.2. CaMKII binding to blank areas (non-peptide containing spots) of the membrane were used to normalize for background and non-specific binding of CaMKII.

Measuring CaMKII translocation. Neurons were lysed in lysis Buffer (see above), sonicated, and protein concentration was quantified. Equal protein levels were centrifuged for 30 mins, 4 degrees, 15,000xg. The supernatant and pellet were resuspended in LDS/BME (final volume 250 μLs for all samples) and SDS-PAGE separation was performed. Western blotting was done using primary monoclonal αCaMKII (CBα2 1:1000) and βCaMKII (CBβ1 1:1000) antibodies followed by secondary DyLight$_{800}$-labeled antibody. Immunostaining was quantified using a Licor imaging station and Odyssey v1.2 was used for analysis.

Anisotropy. Fluorescent anisotropy was monitored in real-time using an AB-2 fluorimeter with a $\lambda_{excitation}$ of 500 nm, $\lambda_{emission}$ of 525 nm, and slit widths of 4 nm. An increase in anisotropic value indicates binding to the fluorescent-labeled NR2B peptide (Fam-MKAQKKNRNKLRRQHSYDTFDVDL). Baseline was attained for 30 seconds with anisotropy buffer (50 mM Tris pH 7.6 and 100 mM KCL), 100 nM Fam-NR2B, 10 mM $MgCl_2$, 1 mM ADP, 5 µM CaM, and 2 mM $CaCl_2$ (+/- inhibitor peptide), 1 µg purified αCaMKII was added, and 30 seconds later 5 mM EGTA was added to induce CaM-dissociation and reverse peptide binding.

Immunoblotting. Neurons were lysed in lysis buffer (see above), sonicated, and protein concentration was determined using a DC kit (Biorad). Protein lysate levels were normalized and suspended in LDS-PAGE buffer (Invitrogen). Monoclonal αCaMKII (1:1000), monoclonal NR2B (1:1000), and monoclonal GAPDH (1:1000) antibodies were used along with secondary goat anti-mouse DyLight$_{800}$-labeled and goat anti-rabbit Alexa$_{680}$-labeled (1:10,000) antibodies.

Detection and quantification were peformed using a Licor imaging station (Odyssey v1.2).

Fluorescent uptake assays. tat-CN21-Fam (KRPPKLGQIGRSKRVVIEDDR–Fam) and tat-CN21Ala-Fam (KAPAKAAWAAASKRVVIEDDR –Fam) peptides were diluted in fresh neuronal growth media and applied at a final concentration of 10 µM for varying lengths of time to 96-well plates containing neurons 7-8 DIV. After treatment, neurons were washed with PBS 3 times, all media was removed and WallacV3 microplate reader used to detect total fluorescence in each well. Fam-labeled peptides were only used to examine uptake.

Data analysis. One-way ANOVA with a subsequent Dunnett's Test was conducted to compare differences between the means of each group ($p < 0.05$, SEM) in all *in situ* cell death assays as well as many *in vitro* catalytic assays. Student t-test was also used when appropriate. Statistical analyses were performed using SigmaPlot 11 and statistical significance was accepted at $p < 0.05$.

RESULTS

Characterization of Neuronal Cultures and Their Death Following Glutamate-Glycine Application in Cortical Neurons

After 7-8 days *in vitro* (DIV), 97% of the cultured cortical cells are MAP2 positive. Less than 2% of the remaining cells stained with the astrocyte marker GFAP, indicating that these cultures are predominantly neurons (Figure 5A-5B). Over 90% of the cultured cells were also CaMKII positive, as detected by both a monoclonal αCaMKII and polyclonal pan-CaMKII antibody (Figure 5B). Neuronal

viability was assessed using a differential calcein-AM (FITC green) to label vital

cells and ethidium homodimer to label cytotoxic cells (Texas Red) (Figure 5C-D).

Dose-dependent applications of glutamate/glycine indicated that 200 μM

glutamate/20 μM glycine for one hour at 37°C is sufficient to induce maximal

cytotoxicity (Figure 5E). Time-dependent application of 200 μM glutamate/20 μM

glycine indicated that maximal death is achieved by one hour of stimulation

(Figure 6A). Under these conditions, neuronal death did not exceed 40%-50% of

total cell number. The inability to kill 100% of neurons in culture may be attributed

the composition of NMDA receptors and the subsequent sensitivity of the NMDA

receptors at this stage of neuronal development (Sinor et al., 2000). For

normalization, cell death is expressed as a percentage of the maximal death

observed 24 hrs following 200 μM glutamate/20 μM glycine for one hour, unless

noted otherwise. Pre-treatment with 20 μM MK-801, an NMDA-receptor

antagonist, completely prevents glutamate/glycine excitoxicity in this assay,

indicative of classical NMDA-dependent excitotoxicity (Figure 5E inset).

Morphologically, the neurons appear in brightfield as round and

swollen immediately following glutamate/glycine treatment and visible

degeneration of neuronal processes is observed within 4 hours (data not shown).

A time course of propidium iodide staining indicated that by 4 hours following

glutamate/glycine insult, 40-50% of cells have compromised membranes (Figure

6B). Similarly, 40-50% of cells are cytotoxic 24 hours following stimulation, as

detected by LIVE/DEAD staining. Consistent with necrotic death, cycloheximide
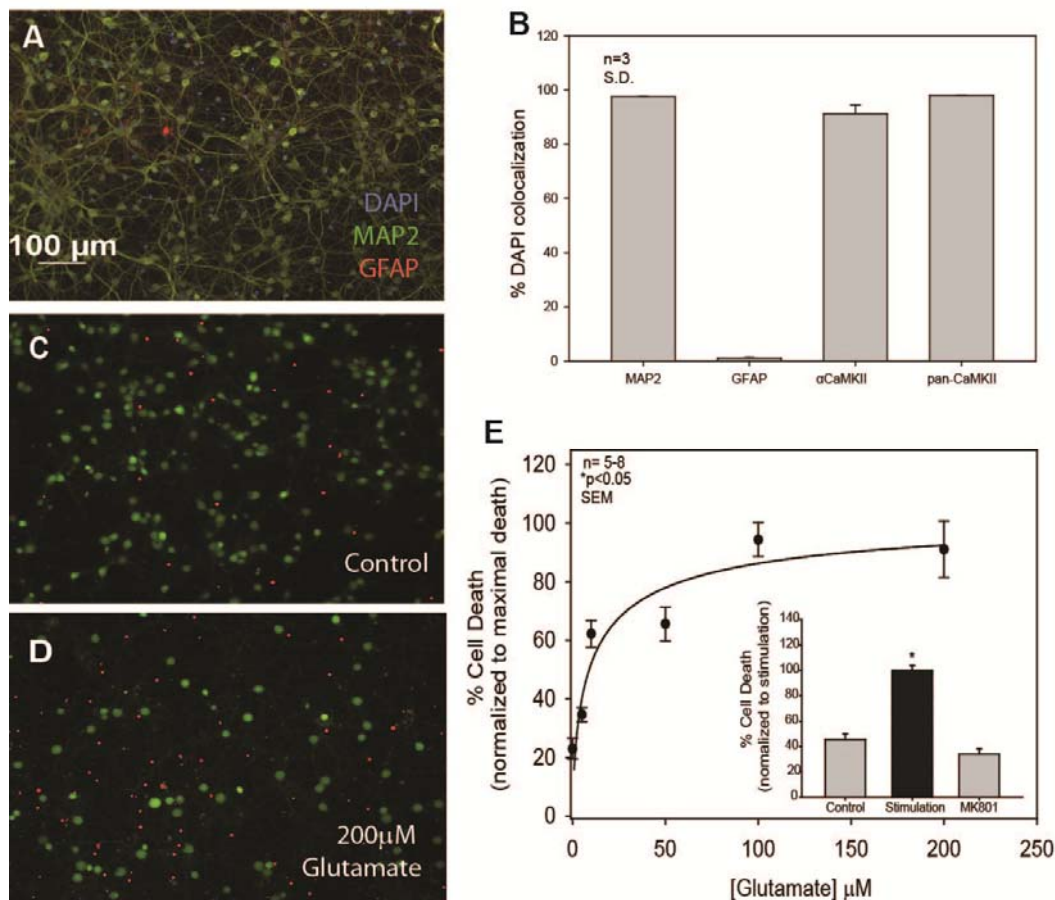
Figure 5: Glutamate-glycine induced excitotoxicity in cortical neuron cultures. A, Primary cortical neurons 7 DIV, stained with MAP-2 (green), GFAP (red), DAPI (blue). **B,** Average number of cells (±SD, n=3) stained for the neuronal marker (MAP-2), astrocyte marker (GFAP), αCaMKII, and pan-CaMKII at 7 DIV. **C-D**, Representative images of control cultures, **C**, and cultures treated with 200 µM glutamate/20 µM glycine for one hour, **D,** stained with LIVE/DEAD Viability/Cytotoxicity kit (Molecular probes) 24 hours following treatment. This differentially stains viable cells (green) and cytotoxic cells (red). **E,** Average cell death (normalized to maximal death, ±SEM, n=5-8) in control cultures and cultures treated with varying concentrations of glutamate. Inset, average cell death (±SEM, n=5-8) in control cultures, cultures stimulated with 200 µM glutamate/20 µM glycine, and cultures pretreated with 20 µM MK-801 followed by glutamate stimulation (*$p < 0.05$; One-way ANOVA, post-hoc Dunnett's test).
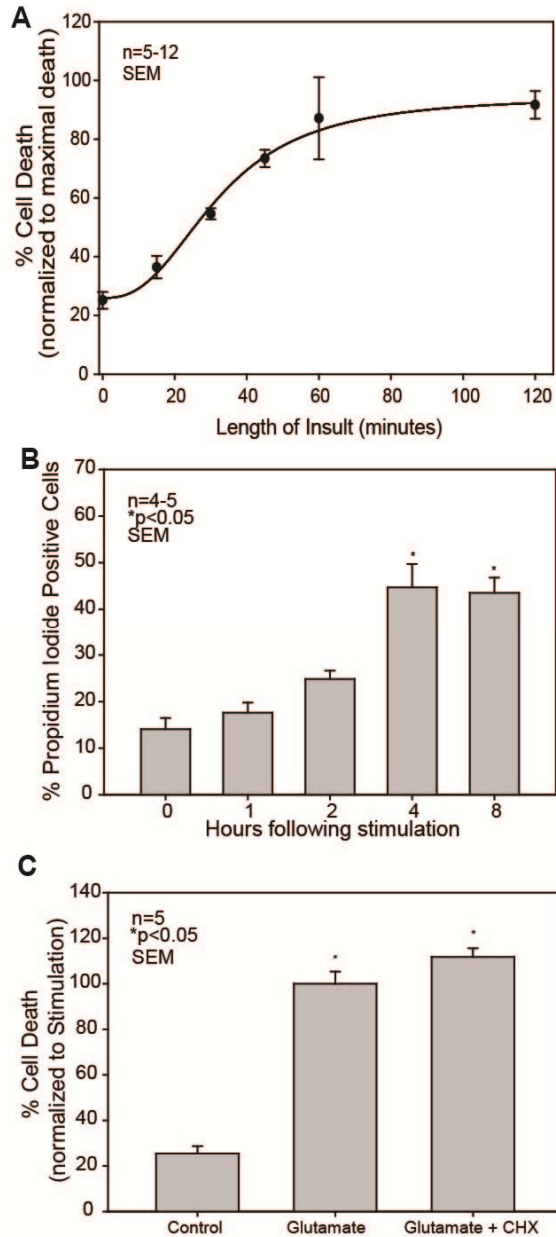
Figure 6: Characterization of glutamate/glycine induced excitotoxicity in 7-8 DIV cortical neurons. A, Average cell death (±SEM; n=5-12) with application of 200 µM glutamate/ 20 µM glycine for varying amounts of time as measured by Live/Dead Cytotoxicity/Viability kit. **B,** Average percentage of cells (±SEM; n=4-5) labeled with propidium iodide at varying times following 200 µM glutamate/20 µM glycine stimulation for 1 hour. The asterisk indicates a significant difference compared to control, no stimulation ($*p<0.05$; One-way ANOVA, post-hoc Dunnett's test). **C,** Average cell death (±SEM, n=5) following glutamate stimulation with and without co-treatment with 0.5 mg/mL cycloheximide as measured by Live/Dead Cytotoxicity/Viability kit ($*p<0.05$; One-way ANOVA, post-hoc Dunnett's test).

pretreatment did not prevent glutamate/glycine-triggered neuronal death (Figure

6C) (Deshpande et al., 1992, Lipton, 1999, Bredesen, 2007).

<u>Validation of CaMKII Inhibitors and Controls</u>

The role of CaMKII in neuronal excitotoxicity was characterized using

small molecule and peptide inhibitors. KN-93 is a membrane-permeable small

molecule that inhibits CaMKII activation with a K_i of 370 nM (Sumi et al., 1991). A

peptide derived from the autoregulatory domain of CaMKII, termed AIP, has also

been established as a highly-potent CaMKII inhibitor with an IC_{50} of 40 nM

(Ishida et al., 1995). However, like the KN-family of inhibitors, AIP has also been

shown to inhibit multiple members of the CaM-kinase family (Smith et al., 1990,

Enslen et al., 1994). We also examined the potential of peptides derived from the

endogenous CaMKII inhibitor, CaMKII-Ntide (Chang et al., 1998). The 21 amino

acid version of CaMKII-Ntide, termed CN21, has an IC_{50} of 100 nM (Vest et al.,

2007). A control for CN21, termed CN21c, had also been previously established

(Vest et al., 2007). CN21c is a 21 amino acid peptide that was produced by a C-

terminal shift of 5 residues from the CN21 peptide on the CaMKIIN protein. In

order to import these peptides into cells, we fused the cellular import sequence

from Tat (tat) to the N-terminus of the CN21 peptide, as described previously

(Vest et al., 2007), as well as the control CN21c peptide and the AIP peptide. We

first verified the effects of the unconjugated and tat-conjugated CN21 and control

CN21c in an *in vitro* CaMKII activity assay. Unconjugated tat alone did not have a

significant impact on CaMKII (Figure 7A). Consistent with previous literature

(Vest et al., 2007), CN21 effectively inhibited CaMKII phosphorylation of

Syntide2, a known substrate of CaMKII, in our *in vitro* catalytic assay (Figure 7A). Similarly, tat-CN21 also inhibited phosphorylation of Syntide2 (Figure 7A). CaMKII was not inhibited with unconjugated control CN21c, consistent the previous report (Vest et al., 2007). Surprisingly, unlike unconjugated CN21c, tat-CN21c significantly reduced CaMKII activity by 80% compared to control (Figure 7A). A full dose-response curve shows that while tat-CN21 exhibited a lower IC_{50} of CaMKII inhibition (0.0772 ± 0.012 µM), tat-CN21c also inhibited CaMKII activity at low doses, IC_{50} 2.5574 ± .138 µM (Figure 7B). Sequence analysis of CN21, CN21c and tat-CN21c indicated that the addition of the highly charged tat sequence restores 3 of the 5 residues removed in the development of CN21c (Figure 7C). In support of this observation, addition of the tat motif resulted in an increase in CaMKII binding to immobilized peptides of CN21c (Figure 8A). Thus, when CN21c is conjugated to tat, the highly-charged cell-penetrant motif partially restores inhibitory efficacy.

To develop a new control peptide for CN21, alanine (Ala) scanning mutagenesis was performed on the CN21 sequence to examine the impact that changes in amino acid size, charge and hydrophobicity has on CaMKII/CN21 interaction. Activated $Alexa_{800}$-labeled CaMKII was used to measure CaMKII binding to the immobilized peptide array (Figure 7D). The binding intensity of each peptide spot was compared to wild-type peptide; variance for the wild-type CN21 (n=3) is shown for comparison (black bar in Figure 7E). Analysis of the Alanine (Ala) scanning indicated seven residues in CN21 (Arg2, Pro4, Leu6, Gly7, Iso9, Gly10, Arg11) that reduced CaMKII binding by >40% (Figure 7E,
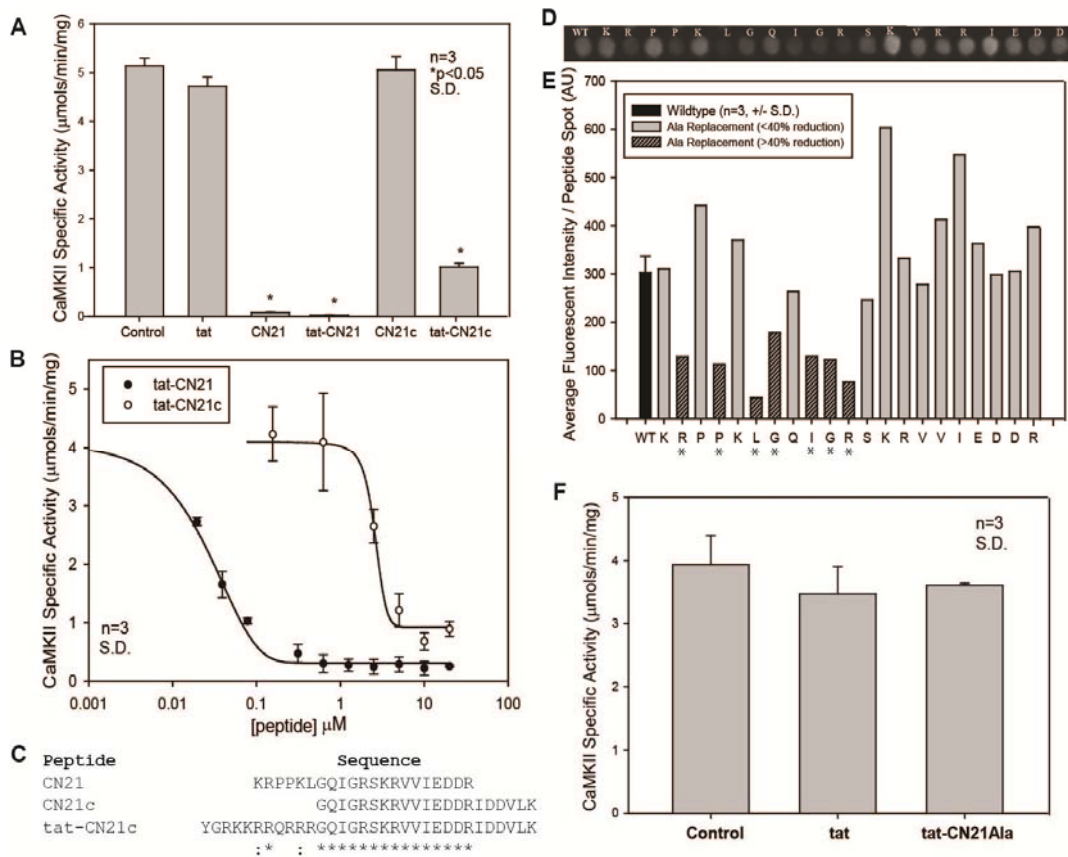
Figure 7: Effects of tat-conjugated inhibitory and control peptides on CaMKII substrate phosphorylation and identification of critical amino acids in CN21 required for CaMKII binding. A, Effects of 10 µM unconjugated and tat-conjugated inhibitory peptide CN21 as well as control CN21c on CaMKII phosphorylation of Syntide2 (±SD; n=3; *p<0.05; One-way ANOVA, post-hoc Dunnett's test). **B,** Dose-responses for tat-CN21 and tat-CN21c inhibition of CaMKII phosphorylation of Syntide2 (±SD; n=3). **C**, Clustal alignment of CN21, CN21c, and tat-CN21c. * indicates identical residues and **:** indicates conserved residues. **D,** Image of Alexa$_{800}$-labeled CaMKII bound to an immobilized peptide array of the CN21 peptide (wild-type) followed by Alanine (Ala) scanning. **E,** Quantification of Alexa$_{800}$-labeled CaMKII binding to CN21 and Ala mutants. The hatched bars represents residues with greater than 40 percent reduction of CaMKII binding when mutated, compared to wild-type (black bar). * indicates residues that were replaced to Ala in the CN21Ala control peptide. **F,** CaMKII phosphorylation of Syntide2 in the presence of control (DMSO), tat, or tat-CN21Ala (±SD; n=3; *p<0.05; One-way ANOVA, post-hoc Dunnett's test).
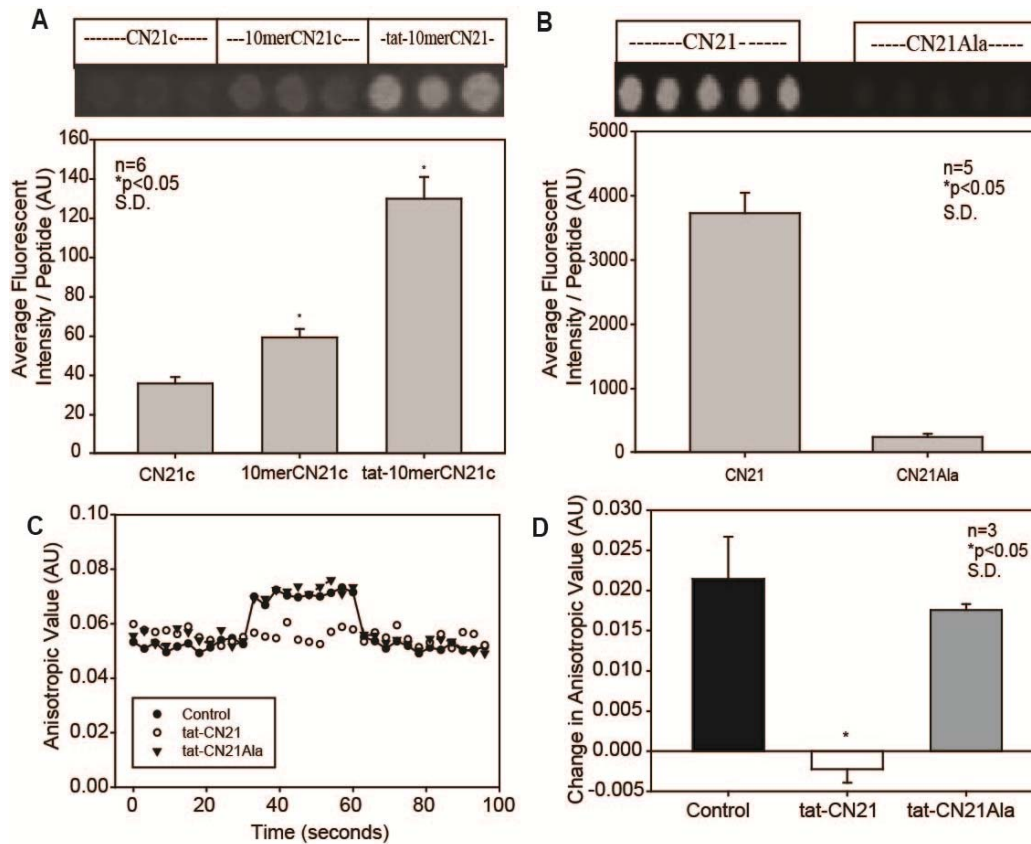
Figure 8: Characterization of tat-CN21, tat-CN21c and tat-CN21Ala binding to CaMKII. A, Image (top) of Alexa$_{800}$-labeled CaMKII bound to an immobilized peptide array containing CN21c, the first ten residues of CN21c (10merCN21c), and tat-10merCN21c, as indicated above. Bar graph indicates quantification of average fluorescent intensity (±SD; n=6). The asterisk indicates significant difference compared to CN21c (*p<0.05; One-way ANOVA, post-hoc Dunnett's test). **B,** Average fluorescent intensity (±SD; n=5) of Alexa$_{800}$-labeled CaMKII binding to immobilized CN21 or CN21Ala (*p<0.05; t-test). **C,** Fluorescent anisotropy of Fam-NR2Bs binding to CaMKII in real time in the presence and absence of 10 µM tat-CN21 and tat-CN21Ala. Baseline was determined (0-30 seconds) in the presence of Mg^{2+}/ATP and Ca^{2+}/CaM. 1 µg CaMKII was added (30-60 seconds) and the binding reaction was reversed with the addition of 5 mM EGTA (60-90 seconds). **D,** Average changes in anisotropic value (±SD; n=3) from Supplemental Figure 2C. The asterisk indicates significant difference compared to control (*p<0.05, One-way ANOVA, post-hoc Dunnett's test).

hatched boxes). Additional scanning with residues that would add variations in residue charge (Aspartate) and size/hydrophobicity (Phenylalanine) also indicated that mutation of these seven residues blocked CaMKII binding (data not shown). In development of the CN21c, Bayer and colleagues reported that multiple residues on the C-terminus of CN21 may also be important for binding to CaMKII (Vest et al., 2007). Our data indicate that the large pocket of charged amino acids in the C-terminus of CN21 (Glu-Asp-Asp-Arg) cannot be disrupted using single Ala replacements. A recent crystal structure of CN21 bound to CaMKII (PDB#3KL8) revealed that many of the residues identified by our biochemical/functional approach are in the appropriate orientation and position to interact with the catalytic surface of CaMKII (Chao et al., 2010). Accordingly, a new peptide containing Ala replacement of multiple key residues (Arg2Ala, Pro4Ala, Leu6Ala, Gly7Ala, Iso9Ala, Gly10Ala, Arg11Ala- marked with an asterisk in 7E) was synthesized and termed CN21Ala. CN21Ala reduced CaMKII binding to ~5% of wild-type CN21 (Figure 8B).

We then tested whether tat-CN21Ala inhibited CaMKII substrate phosphorylation in an *in vitro* kinase assay. Unlike, tat-CN21c, tat-CN21Ala did not significantly decrease CaMKII activity under similar conditions (Figure 7F). Similarly, tat-CN21, and not tat-CN21Ala, inhibited CaMKII binding to a peptide derived from the NMDA-receptor subunit, a known targeting substrate (Figure 8C-D). To verify that tat-CN21 and tat-CN21Ala were both taken up to a similar extent by neurons, we generated a C-terminal carboxyfluorescein-tagged (Fam) version of both peptides (termed tat-CN21-Fam and tat-CN21Ala-Fam) and

measured fluorescent uptake in a fluorescent microplate reader (Figure 8). Fluorescent labeling could be detected as early as 2 minutes and was maximal by 20 minutes in both tat-CN21-Fam and tat-CN21Ala-Fam groups (Figure 8), with no significant difference in peptide uptake detected.

Pretreatment with CaMKII Inhibitors Affords Neuroprotection from Excitotoxicity

We next examined if inhibition of CaMKII with tat-CN21 application prior to an excitotoxic insult is neuroprotective. Because uptake of tat-conjugated peptides was maximal by 20 minutes (Figure 9), neuronal cultures were pre-treated with varying doses of tat-CN21 for 20 minutes before application of glutamate/glycine (200 μM glutamate/20 μM glycine at 37°C for one hour). tat-CN21 afforded neuroprotection from the excitotoxic insult in a dose-dependent manner. The dose-response curve revealed that the optimal concentration of tat-CN21 for neuroprotection is approximately 2-10 μM (Figure 10A). Doses exceeding 10 μM were less efficacious, which may be attributed to inhibition of CaMKIV with high levels of tat-CN21 (Vest et al., 2007). We also examined cell death levels 48 hours following insult to see if cultures treated with tat-CN21 exhibited delayed degeneration compared to control cultures. Yet, even 48 hours later, neurotoxicity levels were no different than control (Figure 11A). Having determined that tat-CN21 is neuroprotective, we examined the impact of pretreatment with control peptides, tat-AIP, and the KN-drugs. Consistent with previous studies (Vaslin et al., 2009), 10 μM unconjugated tat provides slight, yet significant neuroprotection from excitotoxicity (Figure 10B). tat-CN21Ala (10 μM)
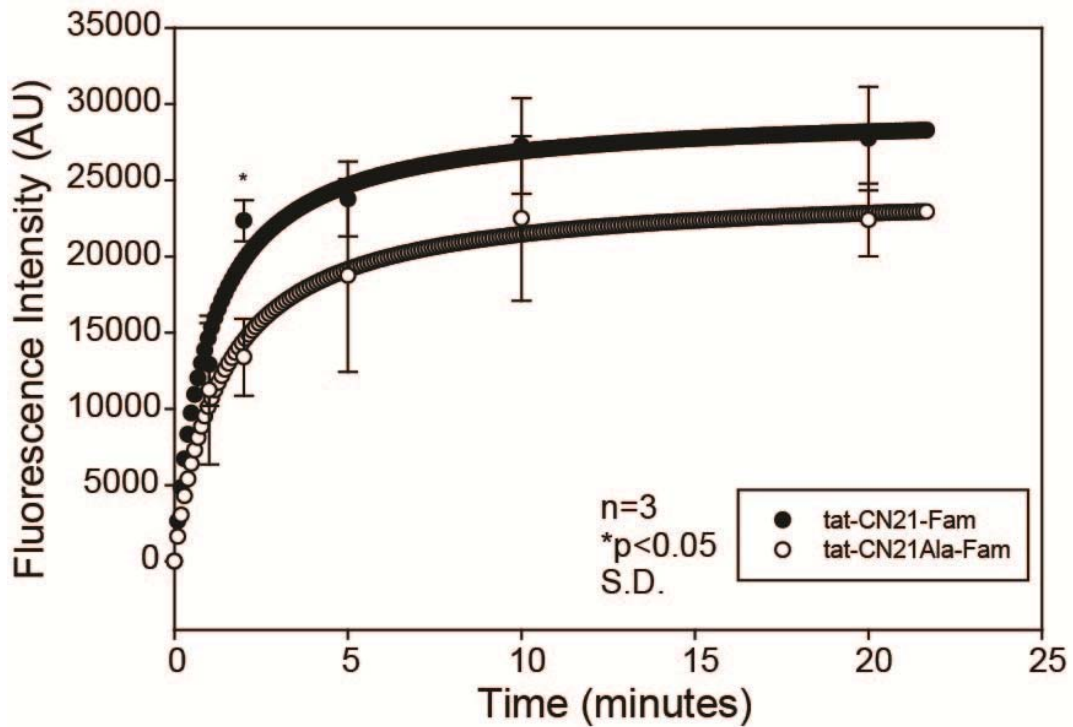
Figure 9: Characterization of tat-CN21-Fam and tat-CN21Ala-Fam uptake in neurons. Average fluorescent uptake (±SD; n=3) was measured using Wallac3V multiplate reader following incubations with 10 µM tat-CN21-Fam or tat-CN21Ala-Fam for varying times (0-25 minutes). The asterisk indicates significant difference compared to tat-CN21Ala-Fam (*p<0.05; t-test).
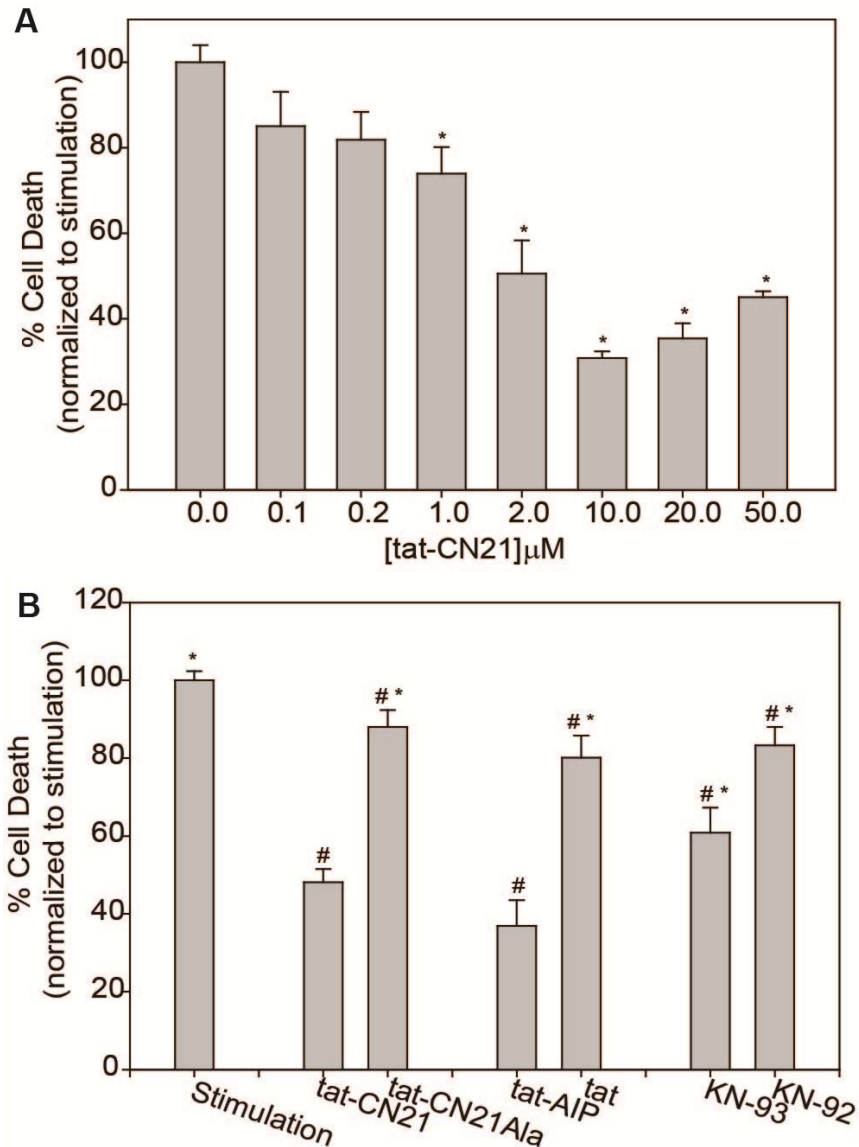
Figure 10: Neuroprotection from excitotoxicity using CaMKII inhibitors. A, Average cell death (±SEM; n=6-10) with application of varying concentrations of tat-CN21 prior to stimulation. Death is normalized to glutamate/glycine stimulation. The asterisk indicates significant difference compared to control, 0 μM (*p<0.05; One-way ANOVA, post-hoc Dunnett's test). **B,** Average cell death (±SEM; n=7-14) 24 hours following glutamate/glycine stimulation with and without pretreatment with various CaMKII inhibitors, as indicated. Death is normalized to glutamate/glycine stimulation. Experimental groups were treated with 10 μM tat-CN21, tat-CN21Ala, tat, and tat-AIP, as well as 1 μM KN-93 and KN-92. The pound symbol indicates significant difference compared to glutamate/glycine stimulation (#p<0.05, One-way ANOVA, post-hoc Dunnett's test). The asterisk indicates significant difference compared to non-stimulated control (*p<0.05; One-way ANOVA, post-hoc Dunnett's test).
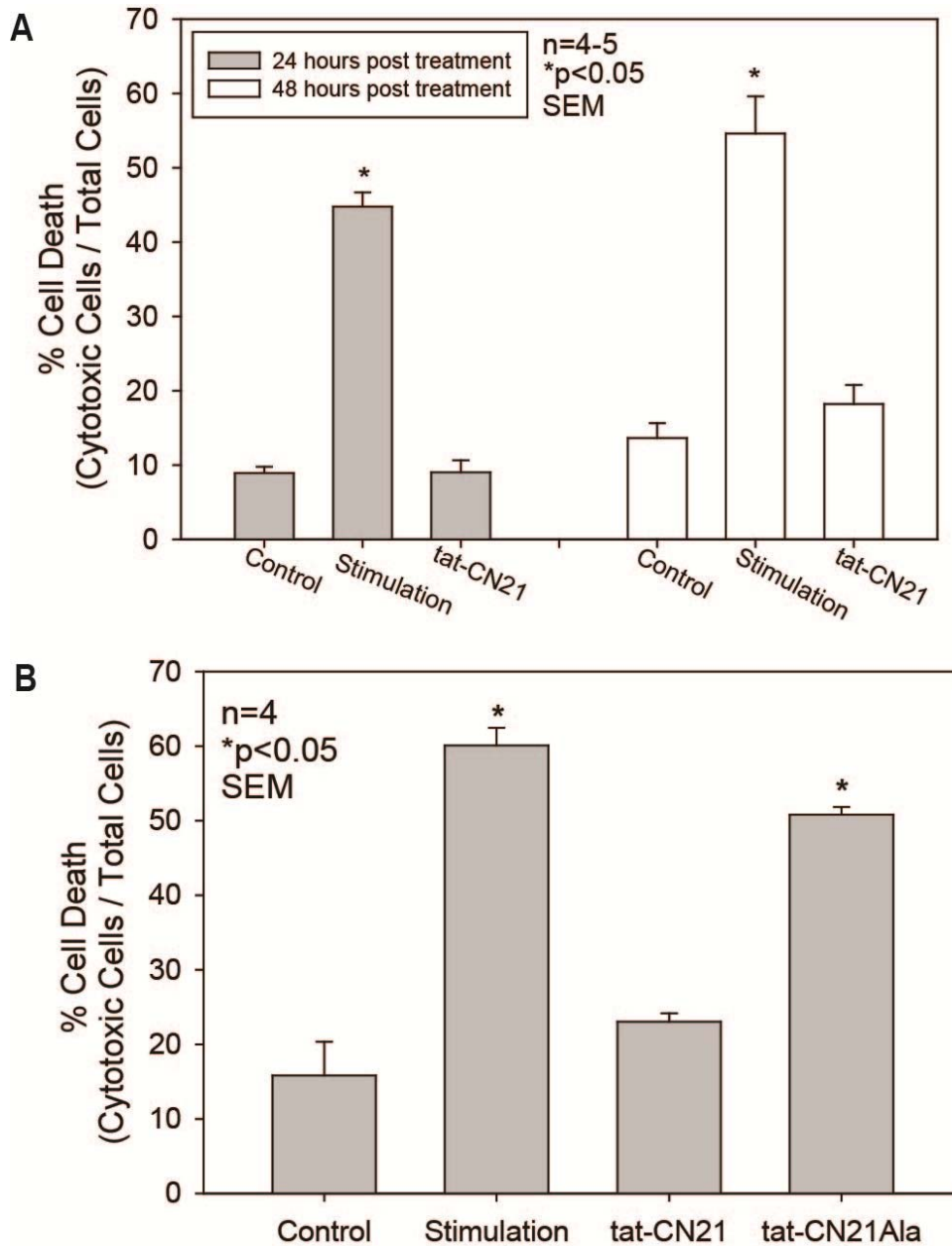
Figure 11: tat-CN21 neuroprotection at 14 DIV and 48 hours following stimulation. A, Average cell death (±SEM, n=4-5) in neuronal cultures 24 hours (gray bars) and 48 hours (white bars) following treatment as indicated. The asterisk indicates significant difference compared to the control of that time-point (*p<0.05, One-way ANOVA, post-hoc Dunnett's test). **B,** Average cell death (±SEM, n=4) in 14 DIV cortical cultures exposed to glutamate stimulation with/without 10 µM tat-CN21 or 10 µM control tat-CN21Ala. The asterisk indicates significant difference compared to non-stimulated control. (*p<0.05, One-way ANOVA, post-hoc Dunnett's test).

did not afford neuroprotection beyond that observed with unconjugated tat indicating that unlike tat-CN21, tat-CN21Ala is not neuroprotective (Figure 10B). The neuroprotective property of tat-CN21 pretreatment was also examined in older cultures (14 DIV). Despite an increase in excitotoxic vulnerability, neuronal cultures treated with tat-CN21, and not tat-CN21Ala, exhibited significantly reduced cell death, bringing cellular toxicity back to control levels (Figure 11B). Consistent with the *in vitro* kinase assay, tat-CN21c which maintained CaMKII inhibitory potency, also significantly reduced glutamate/glycine stimulated neuronal death to control levels (data not shown). Similar to tat-CN21, pretreatment with 10 μM tat-AIP completely abolished glutamate/glycine-induced excitotoxic neuronal death (Figure 10B). Pretreatment with 1 μM KN-93 significantly decreases neuronal death when applied 20 minutes prior to glutamate/glycine stimulation (Figure 10B). Unexpectedly, the inactive analog KN-92 also reduced glutamate/glycine toxicity in this assay (Figure 10B), which may be consistent with off-target effects previously ascribed (Enslen et al., 1994, Ledoux et al., 1999, Gao et al., 2006). Peptide inhibitors AIP and CN21 were also tethered to another cell-penetrating motif, antennepedia. However, these peptides proved to be toxic when applied for 80 minutes (time of pretreatment and stimulation) and were omitted from further use (data not shown).

CaMKII inhibitors Afford Neuroprotection when Applied Following an Excitotoxic Insult

CaMKII undergoes an autophosphorylation reaction that bestows a unique form of activity (i.e. autonomous) that no longer requires its activator,

calcium/CaM (Saitoh and Schwartz, 1985, Miller and Kennedy, 1986). Thus, sustained intracellular calcium potentiates CaMKII signaling to support both aberrant phosphorylation of CaMKII substrates as well as autonomous activity, which together may be detrimental to neuronal survival. Although CN21 is not a potent inhibitor of autophosphorylation (Vest et al., 2007), it does inhibit activity of both the calcium/CaM dependent and autonomous forms of CaMKII (Chang et al., 1998). In contrast, the KN-family of drugs (KN-62 and KN-93) only blocks CaMKII activation and therefore does not inhibit CaMKII autonomous activity (Sumi et al., 1991). Thus, we hypothesized that KN-93 would not be neuroprotective when applied after an excitotoxic insult, whereas CN21 would be neuroprotective after the insult. Application of tat-CN21 at different time points during or after the glutamate/glycine challenge (Figure 12A) revealed a significant decrease in cell death even when applied 2 hours following the excitotoxic insult (Figure 12B). However, the protection afforded by tat-CN21 under these conditions was lost when applied 3 hours after the onset of the excitotoxic insult. Although KN-93 pre-treatment is neuroprotective (Figure 10B), application of this drug one hour after the excitotoxic insult is not (Figure 12B) as cell death levels were equal to the glutamate/glycine challenge in the absence of any inhibitors. Like tat-CN21, tat-AIP, which also inhibits CaMKII in the presence or absence of calcium/calmodulin (Ishida et al., 1995, Rose and Hargreaves, 2003), was also neuroprotective when applied one hour after the excitotoxic insult (Figure 12B). The levels of tat-CN21 and tat-AIP neuroprotection seen at
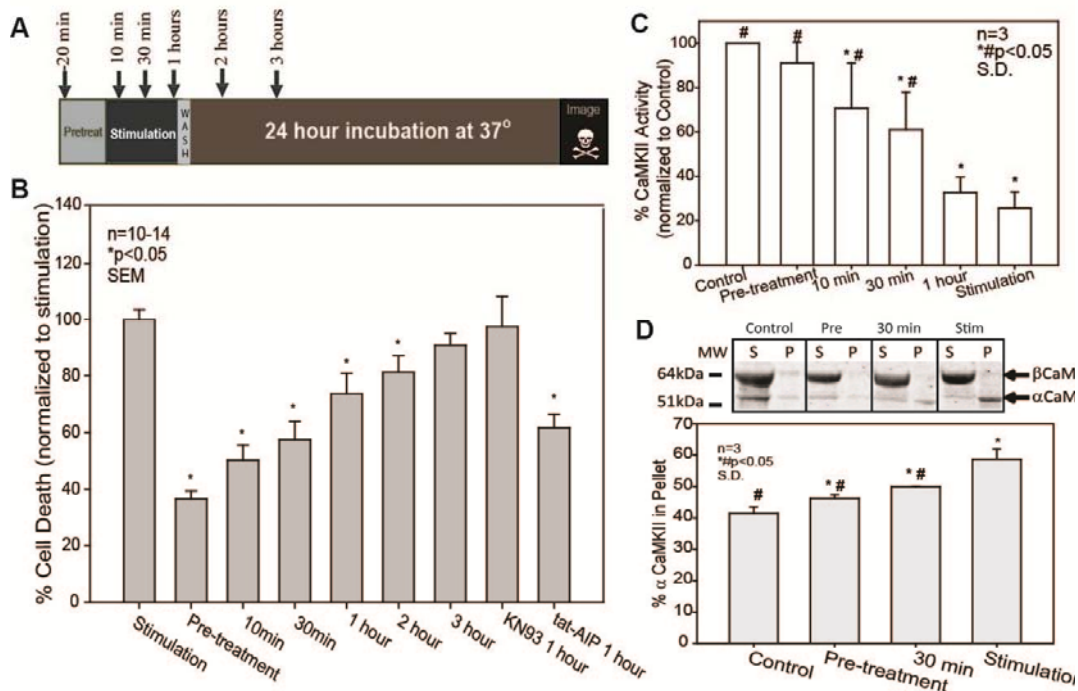
Figure 12: Time-dependent neuroprotection of tat-CN21 from excitotoxicity. **A,** Schematic timeline of inhibitor application and glutamate stimulation. **B,** Average cell death (±SEM; n=10-14) with varying time-points of 10 µM tat-CN21 application as well as 1 µM KN-93 and 10 µM tat-AIP at one hour following stimulation. All inhibitors were applied for one hour. The asterisk indicates significant difference compared to stimulation control (*p<0.05; One-way ANOVA, post-hoc Dunnett's test). **C,** Average CaMKII activity (±SD; n=3) in neuronal lysates 24 hours following glutamate stimulation and application of 10 µM tat-CN21 at various time-points after glutamate application. Activity was first normalized for equal protein amounts and then normalized to control, or non-treated cultures. The asterisk indicates significant difference compared to control, while the pound sign indicates significant difference compared to stimulation (*#p<0.05; One-way ANOVA, post-hoc Dunnett's test). **D,** Western blots (top) of αCaMKII and βCaMKII in the supernatant (S) and particulate (P) fractions 24 hours following stimulation and application of 10 µM tat-CN21 at various time points. Summary data (bottom) for particulate aMKII (±SD; n=3). The asterisk indicates a significant difference compared to control while the pound sign indicates a significant difference compared to stimulation (*#p<0.05, One-way ANOVA, post-hoc Dunnett's test).

one hour were not significantly different (t-test, *p>0.05). Thus, only inhibitors of activated CaMKII are neuroprotective following an excitotoxic glutamate insult.

Following cerebral ischemia, CaMKII has been shown to undergo periods of prolonged inactivation and the length of CaMKII inactivation has been correlated with extent neuronal death (Hanson et al., 1994). Therefore, we examined CaMKII activity within our neuronal cultures 24 hours after excitotoxic insult when tat-CN21 was applied at various points before and after the onset of stimulation. As expected, CaMKII activity was reduced 70% in stimulated cultures compared to control cultures (Figure 12C). However, CaMKII activity was significantly higher in cultures that tat-CN21 was applied to before and after the onset of insult. A time-dependent decrease in CaMKII activity correlates with the time-dependent increase in neuronal death (Figure 12B-C). Translocation of CaMKII from the supernatant to the particulate fraction is one key event that may result in prolonged inactivation of the kinase, a process that has been shown to occur under excitotoxic conditions (Aronowski et al., 1992, Kolb et al., 1995, Hudmon et al., 1996, Tao-Cheng et al., 2002) and may be due to self-association (Hudmon et al., 1996, Dosemeci et al., 2000, Hudmon et al., 2005). We hypothesized that tat-CN21 application was able to avert the induction of prolonged CaMKII inactivation, by preventing translocation of CaMKII. Compared to control conditions, glutamate stimulated cultures exhibited significantly more αCaMKII in the particulate fraction 24 hours following treatment (Figure 12D). At this stage of neuronal development, βCaMKII expression is predicted to be higher than αCaMKII (Burgin et al., 1990b, Fink et al., 2003); however, there was

no statistical change in levels of βCaMKII in the supernatant or particulate fractions (quantification not shown). Application of tat-CN21 prior to stimulation as well as up to 30 minutes after the onset of stimulation resulted in a significant decrease in the amount of αCaMKII found in the sedimentable, pelleted fraction (Figure 12D). Importantly, despite changes in activity and transitions to the particulate fraction, total αCaMKII levels were not changed in any treatment group (Figure 13). Thus, tat-CN21 decreased the levels of αCaMKII translocation and CaMKII inactivation. Together, this suggests that while inhibition of the transient increase in CaMKII activity may be neuroprotective, preventing a long-term loss of activity is also beneficial for neuronal viability.

CaMKII inhibition Predisposes Neurons to Excitotoxicity

While small molecule and peptide inhibitors have neuroprotective effects when applied immediately surrounding the excitotoxic insult, knock-out studies indicated that the loss of αCaMKII resulted in increased infarct size following arterial occlusion in knock-out animals compared to wild-type littermates (Waxham et al., 1996). Thus, a loss of CaMKII predisposed neurons to ischemic death. This, along with the correlation between the levels of activatable CaMKII and neuronal viability, led us examine what impact prolonged loss of CaMKII prior to an excitotoxic insult has on neuronal survival. Treatment with a sub-maximal excitotoxic insult (200 μM glutamate/20 μM glycine for 15 minutes) resulted in approximately 18% cell death after 48 hours (Figure 14B). Application of tat-CN21 for 24 hours, followed by wash and then further incubation for 24 hours resulted in approximately 18% cell death (Figure 14B). Combination of
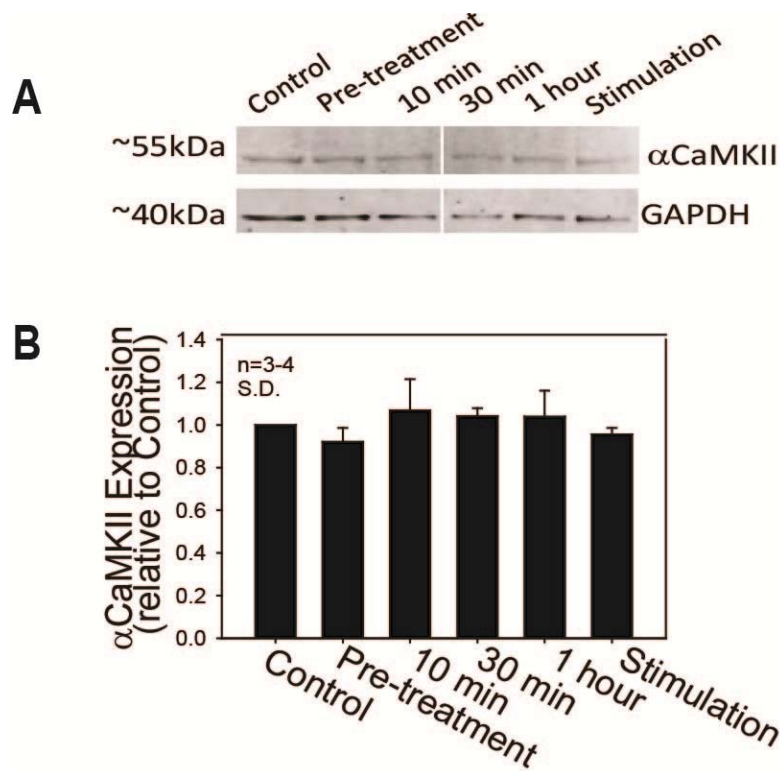
Figure 13: tat-CN21 application following onset of excitotoxic insult does not affect αCaMKII protein levels. A, Western blots of αCaMKII and loading control GAPDH in neuronal lysates 24 hours following stimulation with application of 10 µM tat-CN21 at various time-points surrounding glutamate/glycine stimulation. **B,** Summary data for normalized protein levels of αCaMKII quantified and plotted relative to GAPDH control (p>0.05, One-way ANOVA).

prolonged tat-CN21 application (24 hours) followed by sub-maximal glutamate/glycine stimulation synergistically increased neuronal toxicity to >50% which was significantly higher than glutamate/glycine alone or tat-CN21 treatment alone (Figure 14B). This exacerbation of neuronal death was not seen in cultures treated with control tat-CN21Ala for 24 hours prior to glutamate/glycine stimulation (Figure 14B). Interestingly, the application of sub-maximal glutamate followed by tat-CN21 overnight enhanced death compared to either treatment alone (Figure 14B), suggesting that CaMKII activity may be important for neuronal survival following excitotoxic insult.

The time-frame in which CaMKII inhibition prior to excitotoxic insult predisposes cells to excitotoxicity was examined by applying tat-CN21 for varying lengths of time prior to insult. Interestingly inhibition for 4 hours or less had no impact on neuronal sensitivity to glutamate (Figure 14C). However, tat-CN21 application for 8 or more hours resulted in a significant increase in neuronal death compared to glutamate/glycine stimulation alone (Figure 14C). Because this time frame would allow for potential changes in protein translation, we co-applied cycloheximide with tat-CN21. This co-treatment prior to stimulation significantly reduced neurotoxicity levels compared to 24 hour tat-CN21 alone (Figure 14D). This suggests that protein translation plays a key role in the switch to increased glutamate sensitivity. We examined the levels of CaMKII and the NR2B subunit of the NMDA receptor to determine whether these key players are upregulated following prolonged CaMKII inhibition, however there was no change
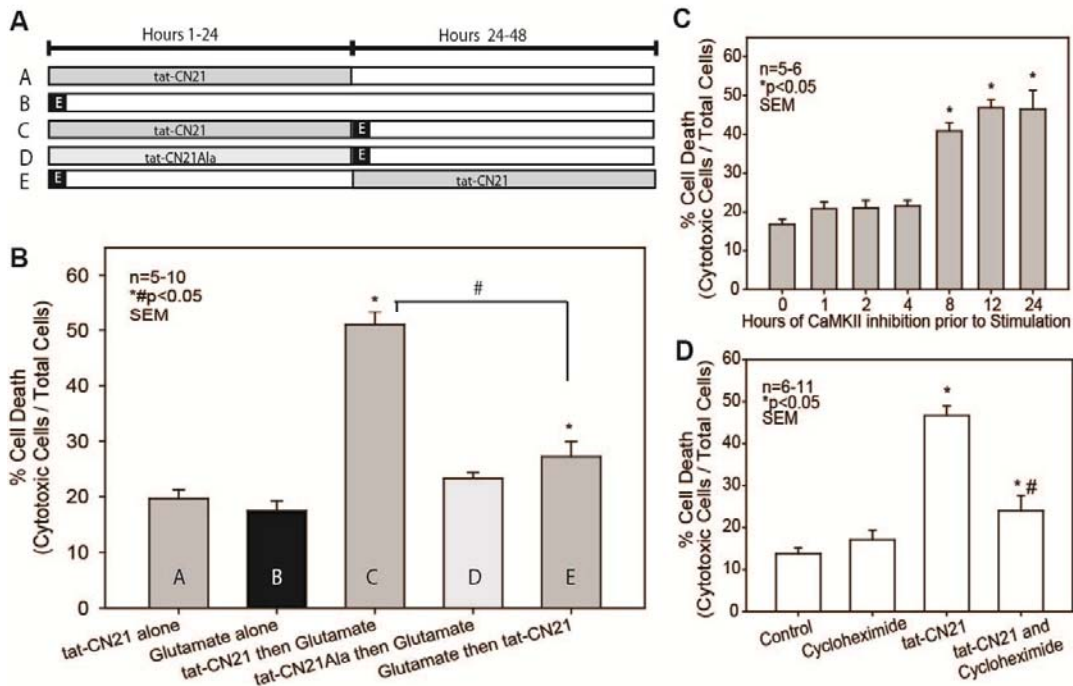
Figure 14: tat-CN21 enhances neuronal vulnerability to excitotoxicity.
A, Schematic indicating treatment protocols (**A-E**) used to characterize the effect of prolonged CaMKII inhibition prior to and before a sub-maximal glutamate stimulation (200 µM glutamate/glycine for 15 mins signified by black box ▣). **B,** Average cell death (±SEM; n=5-10) of neuronal cultures treated with tat-CN21 and tat-CN21Ala overnight before or after a sub-maximal glutamate/glycine insult, (**A-E**). The asterisk indicates significant difference compared to tat-CN21 treatment alone (*p<0.05, One-Way ANOVA, post-hoc Dunnett's test). The pound sign indicates significant difference between tat-CN21/glutamate vs glutamate/tat-CN21 (#p<0.05, t-test). **C,** Average cell death (±SEM, n=5-6) in cultures exposed to 10 µM tat-CN21 for varying amounts of time prior to a submaximal glutamate/glyine stimulation. Cell death is visualized 24 hours following stimulation. The asterisk indicates significant difference compared to control cultures (0 hours) which received submaximal stimulation alone with no inhibitor application. (*p<0.05, One-way ANOVA, post-hoc Dunnett's test). **D,** Average cell death (±SEM, n=5-6) in cultures exposed to 0.5mg/mL cycloheximide, 10 µM tat-CN21, or co-treatment with both 24 hours prior to submaximal stimulation. Cell death is visualized 24 hours following glutamate/glycine stimulation. The asterisk indicates significant difference compared to control while the pound sign indicates significant difference between co-treatment and tat-CN21 treatment alone. (*p<0.05, One-way ANOVA, post-hoc Dunnett's test; #p<0.05, t-test).

in the levels of either protein (Figure 15). The protein(s) responsible for this switch have yet to be determined.

DISCUSSION

Aberrant release of the excitatory neurotransmitter L-glutamate has long been hypothesized to contribute to neuronal death in stroke and neurodegenerative disorders *via* over-excitation and calcium accumulation (Olney, 1969, Olney and Sharpe, 1969, Olney, 1971, Choi, 1985). Based on the success of *in vitro* and animal models (Foster et al., 1987, Omae et al., 1996, Suzuki et al., 2003, Hoyte et al., 2004), many were disappointed to discover that drug therapies designed solely to block or reduce glutamate receptor activation did not prevent neurodegeneration in clinical trials (Albers et al., 1999, Morris et al., 1999, Davis et al., 2000, Hoyte et al., 2004). Thus, focus now lays on searching for new mechanisms to modulate calcium entry or the downstream targets activated by aberrant calcium signaling (Szydlowska and Tymianski, 2010). A key mediator of glutamate-induced calcium signaling is the multifunctional CaMKII; a Ser/Thr protein kinase implicated in synaptic plasticity (Hudmon and Schulman, 2002, Lisman et al., 2002). CaMKII is activated by excitotoxic calcium signaling (Westgate et al., 1994, Zalewska and Domanska-Janik, 1996) and has been implicated in regulating a number of different substrates linked to excitotoxic calcium signaling as well as neuronal survival. Earlier studies have shown that pre-incubation with the small molecule CaMKII inhibitors KN-62 or KN-93 produce neuroprotection when given before an
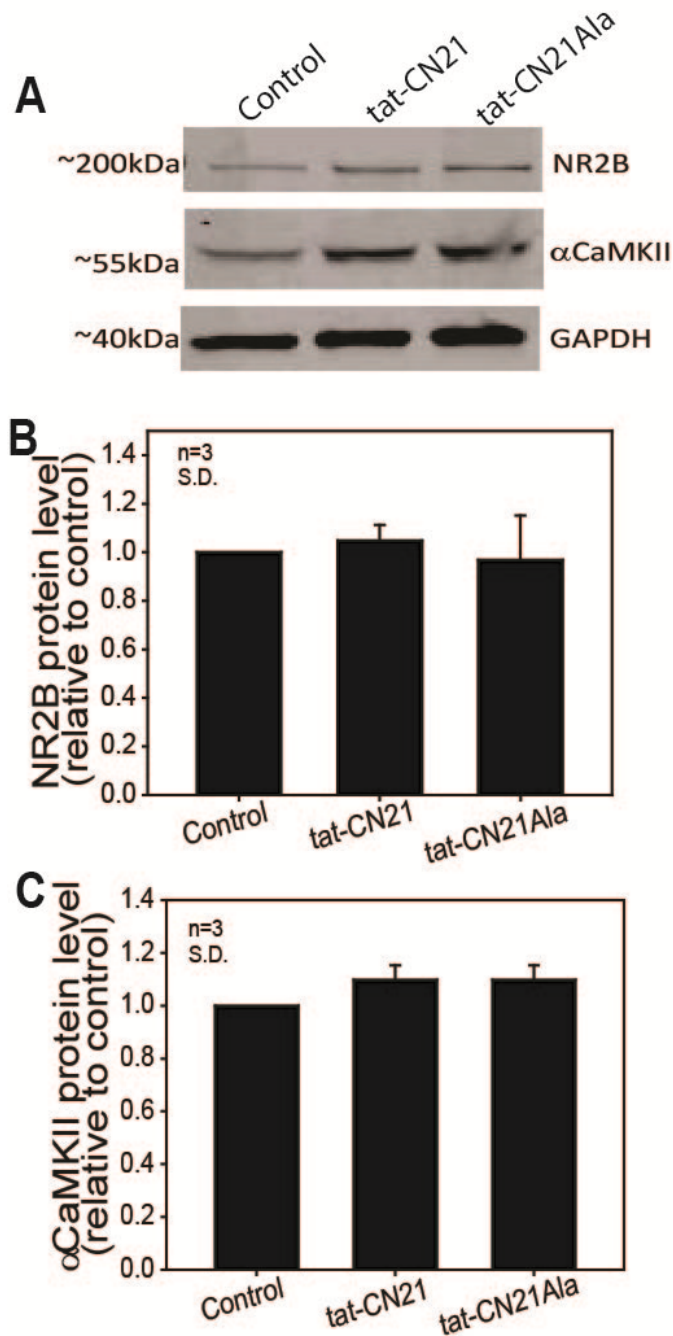
Figure 15: Prolonged tat-CN21 treatment does not affect αCaMKII or NR2B protein levels. A, Western blots of NR2B, αCaMKII and GAPDH in neuronal lysates 24 hours following application of 10 μM tat-CN21 or tat-CN21Ala. Summary data for normalized protein levels of NR2B, **B,** and αCaMKII, **C,** quantified and plotted relative to control (p>0.05).

excitotoxic challenge (Hajimohammadreza et al., 1995, Takano et al., 2003, Vest et al., 2010). We also observed neuroprotection with KN-93 pretreatment (Figure 10B), however, it was difficult to interpret that the neuroprotection was due to CaMKII inhibition because the inactive analog, KN-92, was also protective in our assays (Figure 10B). The myristoylated CaMKII peptide inhibitor AIP (Ishida et al., 1995) has also been shown to display neuroprotective activity *in vivo* and in cultured neurons exposed to toxic levels of NMDA (Laabich and Cooper, 2000, Fan et al., 2006, Goebel, 2009). We observed potent neuroprotection using the AIP peptide as well as a peptide derived from the endogenous CaMKII inhibitor, CN21, when tagged with the tat cellular import sequence to enhance membrane permeability (Figure 10B). Because recent data indicates that cellular import sequences can inhibit neurotoxicity to glutamate (Vaslin et al., 2009), we also validated the specificity of these peptide inhibitors to control peptides. We relied on tat alone to serve as a control for AIP yet optimized a control peptide for CN21 by identifying and mutating critical residues responsible for binding to CaMKII. Compared to the active peptides (tat-CN21 and tat-AIP), very little inhibition was observed for tat-CN21Ala or the tat peptide in either *in vitro* or cell based assays, suggesting that the neuroprotection observed with the active peptides is not due to the cellular import sequence nor to peptide sequences that do not interact with CaMKII. In total, our data favor the hypothesis that excitotoxic calcium signaling induces neurotoxic CaMKII activity. Interestingly, both tat-AIP and tat-CN21, which inhibit activated and/or autophosphorylated CaMKII (Ishida et al., 1995, Chang et al., 1998, Rose and Hargreaves, 2003), were also neuroprotective

when applied after the excitotoxic challenge (Figure 12B). These data are intriguing as KN-93, which can only inhibit CaMKII activation, was not neuroprotective when applied after an excitotoxic insult (Figure 12B); a result that suggests that only inhibitors of activated/autophosphorylated CaMKII are efficacious following the onset of excitotoxic calcium signaling.

During the preparation of this manuscript, it was reported that tat-CN21 was neuroprotective *in vitro* as well as in an animal model of stroke (Vest et al., 2010). However, in these studies, the neuroprotection afforded by tat-CN21 in cultured hippocampal neurons was not diminished even after 6 hours following the excitotoxic glutamate insult, the longest time-point reported in their study (Vest et al., 2010). We observed that the neuroprotective effects of tat-CN21 are greatest when applied before the toxic glutamate challenge and diminish with time (Figure 12B), with the neuroprotection associated with CaMKII inhibition diminishing back to control after 3 hours post glutamate insult. We found that this time window of neuroprotection correlates to changes in neuronal membrane integrity (see Figure 6B). Thus, our data favor a model whereby the pathological contributions of CaMKII to excitotoxic damage may be limited to a 2-3 hour period post excitotoxic injury. Potential reasons for different outcomes between our study and that of Vest *et al.*, include differences in the glutamate stimulation protocol (200 µM for 1 hour versus 400 µM for 5 mins) as well as the type of culture (7-8 DIV cortical neurons versus 7 DIV hippocampal neurons obtained from embryonic (highly pure) versus post-natal (mixed), respectively). However, it should be noted that the *in vivo* experiment performed by Bayer and colleagues

was, in fact, performed at one hour post-ischemia (Vest et al., 2010); a time point our study finds to be neuroprotective in cultured neurons.

The potential therapeutic utility of inhibiting CaMKII to enhance neuronal survival following an excitotoxic glutamate exposure is intriguing, as following activation in excitotoxic conditions (Westgate et al., 1994, Zalewska and Domanska-Janik, 1996), CaMKII undergoes functional changes that at face-value would appear to be designed to limit aberrant CaMKII activity. We observed a significant decrease in CaMKII activity 24 hours following an excitotoxic glutamate insult (Figure 12C). In addition, unlike βCaMKII, excitotoxic calcium signaling induces αCaMKII to translocate form the soluble to the particulate fraction (Figure 12D). Soluble to particulate accumulation of CaMKII may be due to activity-dependent translocation of CaMKII to specific subcellular compartments, like the post-synaptic density (Aronowski et al., 1992, Suzuki et al., 1994) and/or the formation of CaMKII aggregates (Dosemeci et al., 2000, Tao-Cheng et al., 2002, Hudmon et al., 2005). These functional changes in CaMKII activity and localization are not limited to our neuronal culture model, as both CaMKII inactivation and soluble to particulate translocations are observed *in vivo* and in slice models of ischemia (Aronowski et al., 1992, Hanson et al., 1994, Kolb et al., 1995, Aronowski and Grotta, 1996, Zalewska et al., 1996). Importantly, the glutamate-induced translocation and loss of CaMKII activity are reduced by tat-CN21 application in a time-dependent fashion (Figure 12C and 12D), suggesting that maintaining a soluble and activatable pool of CaMKII for 24 hours after an excitotoxic insult enhances neuronal survival (Hanson et al.,

1994). These data are consistent with a functional switch from CaMKII playing a neurotoxic role with the induction of the excitotoxic insult to a neuroprotective role 2-3 hours after the insult. One might envision that sustained loss of CaMKII activity induced by excitotoxic calcium signaling could be detrimental for neuronal survival, as a number of CaMKII substrates are pro-survival (e.g. CREB and L-type channels) (Dash et al., 1991, Wheeler et al., 2008). CaMKII regulation of substrates that limit neuronal excitability and glutamate signaling could function as a "brake" for subsequent excitotoxic calcium signaling. For example, CaMKII phosphorylation of NMDA-receptors leads to desensitization (Sessoms-Sikes et al., 2005) whereas CaMKII upregulation of GABA-receptors could be a form of feed-forward inhibition that could limit the activity of neuronal circuits (Houston and Smart, 2006). In these scenarios, sustained loss of CaMKII could be maladaptive for future recovery or susceptibility to excitotoxicity. In support of this model, we observed that inhibition of CaMKII for 8-24 hours prior to a sub-maximal glutamate/glycine challenge exacerbates neuronal death (Figure 14B-C). This enhanced neuronal vulnerability to an excitotoxic insult is also seen *in vivo*, as genetic knockdown of αCaMKII doubles the size of the infarct volume in knockout mice exposed to focal ischemia compared to wild-type littermates (Waxham et al., 1996). Changes in neither αCaMKII nor NR2B protein levels appear to be responsible for this enhanced glutamate vulnerability (Figure 15A-C), although this process does require protein synthesis (Figure 14D). Although the mechanism(s) of this shift from neuroprotection to enhanced neuronal vulnerability to glutamate following CaMKII inhibition warrants further

investigation, our data suggests that inhibition of CaMKII via tat-CN21/AIP prior to or immediately after an excitotoxic insult functions to limit aberrant CaMKII activity and reduce the long-term inactivation of CaMKII following aberrant calcium signaling (Figure 12C and 12D). Thus, the neuroprotection afforded by CaMKII inhibition can be seen as neuroprotective immediately surrounding the excitotoxic insult, whereas sustained CaMKII inhibition produced by excitotoxicity leads to neuronal death by enhancing neuronal vulnerability to glutamate.

In summary, our data supports the hypothesis that inhibition of CaMKII activity either at the onset or soon after an excitotoxic insult is neuroprotective. CaMKII activated by excitotoxic calcium signaling is neurotoxic and therefore, inhibition of this activity within a limited time-window around the excitotoxic insult enhances neuronal survival. However, these data also support a model whereby sustained loss of CaMKII activity predisposes neurons to glutamate excitotoxicity. The role of CaMKII in promoting neuronal survival and recovery from an excitotoxic challenge must be taken into consideration when pursuing CaMKII inhibition as a therapeutic intervention to ischemic stroke or other neurodegenerative diseases.

PART II: CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE II INHIBITION INDUCES NEUROTOXICITY VIA DYSREGULATION OF GLUTAMATE/CALCIUM SIGNALING AND HYPEREXCITABILITY

SUMMARY

Aberrant glutamate and calcium signaling are neurotoxic to specific neuronal populations. Calcium/calmodulin-dependent kinase II (CaMKII), a multifunctional serine/threonine protein kinase in neurons, is believed to regulate neurotransmission and synaptic plasticity in response to calcium signaling produced by neuronal activity. Importantly, several CaMKII substrates control neuronal structure, excitability, and plasticity. Here, we demonstrate that CaMKII inhibition for >4 hours using small molecule and peptide inhibitors induces apoptosis in cultured cortical neurons. The neuronal death produced by prolonged CaMKII inhibition is associated with an increase in TUNEL staining, caspase-3 cleavage, and is blocked with translation inhibitor cycloheximide. Thus, this neurotoxicity is consistent with apoptotic mechanisms, a conclusion that is further supported by dysregulated calcium signaling with CaMKII inhibition. CaMKII inhibitory peptides also enhance the number of action potentials generated by a ramp depolarization, suggesting increased neuronal excitability with a loss of CaMKII activity. Extracellular glutamate concentrations are augmented with prolonged inhibition of CaMKII. Enzymatic buffering of extracellular glutamate and antagonism of the NMDA subtype of glutamate receptors prevent the calcium dysregulation and neurotoxicity associated with prolonged CaMKII inhibition. However, in the absence of CaMKII inhibition,

elevated glutamate levels do not induce neurotoxicity, suggesting that a combination of CaMKII inhibition and elevated extracellular glutamate levels results in neuronal death. In sum, the loss of CaMKII observed with multiple pathological states in the central nervous system, including epilepsy, brain trauma, and ischemia, likely exacerbates programmed cell death by sensitizing vulnerable neuronal populations to excitotoxic glutamate signaling and inducing an excitotoxic insult itself.

INTRODUCTION

Precisely-regulated calcium signaling is essential for normal neuronal growth and survival. While slight fluctuations in intracellular calcium concentration are tolerated by neurons, and necessary for a variety of physiological processes, dysregulation of intracellular calcium leads to neuronal death. Intracellular calcium overload can lead to mitochondrial depolarization and the over-activation of the downstream signaling pathways regulated by calcium. One important signaling pathway that is activated by calcium signaling is calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII). Following activation with calcium-bound calmodulin, CaMKII targets to and phosphorylates a number of substrates in neurons, including voltage- and ligand-gated calcium channels, CREB, ERK, and voltage-gated sodium channels (as previously reviewed (Hudmon and Schulman, 2002, Colbran, 2004, Coultrap et al., 2011)).

Fluctuations in CaMKII activity have been associated with neuronal disease states that exhibit excitotoxic calcium dysregulation, such as stroke, epilepsy, and traumatic brain injury (Aronowski et al., 1992, Perlin et al., 1992,

Hanson et al., 1994, Westgate et al., 1994, Churn et al., 1995, Zalewska and Domanska-Janik, 1996, Schwarzbach et al., 2006, Folkerts et al., 2007). Immediately following the onset of excitotoxic stimulation, CaMKII is activated (Westgate et al., 1994, Zalewska and Domanska-Janik, 1996) and inhibition of CaMKII prior to excitotoxic insult prevents neuronal damage both *in vitro* and *in vivo* (Hajimohammadreza et al., 1995, Laabich and Cooper, 2000, Fan et al., 2006, Goebel, 2009, Vest et al., 2010, Ashpole and Hudmon, 2011). However, αCaMKII knock-out animals paradoxically exhibit a significant increase in neuronal damage following stroke compared to wild-type littermates (Waxham et al., 1996). Moreover, we recently showed that prolonged pharmacological inhibition of CaMKII actually exacerbated excitotoxicity following a submaximal glutamate challenge (Ashpole and Hudmon, 2011). Thus, while an acute loss of CaMKII may protect neurons from excitotoxic insult, a prolonged loss of CaMKII activity sensitizes neurons to glutamate toxicity; an observation we hypothesize contributes to programmed cell death in the penumbral region associated with ischemia and brain trauma. In support of this hypothesis, a loss of CaMKII activity has been shown to be spatially correlated with the extent of neuronal damage following focal ischemia (Hanson et al., 1994). The region immediately surrounding the infarct not only displays the greatest damage but also the greatest loss in CaMKII activity (Hanson et al., 1994). However, the ischemic environment is associated with complex biochemical changes that are associated with aberrant glutamate signaling, including enhanced ROS activity, acidosis, and a decrease in energy availability. Thus, we choose to investigate neuronal

survival, calcium signaling, and excitability following a loss of CaMKII activity

induced by a broad spectrum of CaMKII inhibitors in the absence of an

exogenous glutamate challenge. Our data supports a model whereby prolonged

inhibition of CaMKII produces apoptosis in cortical neurons by a feed-forward

process associated with neuronal hyperexcitabilty and dysregulated calcium and

glutamate signaling.

EXPERIMENTAL PROCEDURES

Materials. Unconjugated tat (YGRKKRRQRR), CN21

(KRPPKLGQIGRSKRVVIEDDR), CN21Ala (KAPAKAAWAAASKRVVIEDDR),

CN21C (GQIGRSKRVVIEDDRIDDVLK), tat-AIP (YGRKKRRQRR-

RKKLRRQEAFDAL), tat-CN21, tat-CN21Ala, as well as Fam-labeled versions of

these peptides were synthesized and HPLC purified by Biopeptide Co, Inc. San

Diego, CA, USA. Myristolated AIP (myr-AIP; 64929) was purchased from

Anaspec, Fremont, CA. KN-93 (422708) and KN-92 (422709) were purchased

from Calbiochem, EMD Biosciences, La Jolla, CA. STO-609 (1551) was

purchased from Tocris Bioscience, Ellisville, MO. MK-801 (M107), Nifedipine

(N7634), Nimodipine (N149), Tetrodotoxin (T8024), Omega-Conotoxin (C9915),

Ifenprodil (I2892), and Memantine (M9292) were purchased from Sigma Aldrich,

St. Louis, MO.

Neuronal cultures. Cortical neurons were harvested from E18-E19 Sprague-

Dawley rat pups according to approved IACUC guidelines as previously

described (Ashpole and Hudmon, 2011). Primary hippocampal neurons were

prepared from postnatal day 1 Sprague-Dawley rat pups as previously described

(Dubinsky, 1993, Brustovetsky et al., 2009). For most experiments, cortical

neurons were seeded at a density of 2.5 million cells/mL and seeded on poly-D-

lysine (50 µg/mL) coated 15 mm coverslips (German glass Number 0) or 60 mm

dishes. For experiments looking at neuronal viability at different stages of culture

development, neurons were seeded at 1.25 million cells/mL. Cultures were

treated with 5-fluor-2'-deoxyuridine (15 mg/mL) (Sigma, F0503) and Uridine (35

mg/mL) (Sigma, U3750) to kill mitotically active cells on day 2-4. Co-cultures of

neurons and astrocytes were not treated with these mitotic inhibitors.

Cell death assay. Following treatment, the coverslips were washed in PBS and

stained using Live/Dead Cytotoxicity/Viability kit (Molecular Probes, Eugene, OR)

as previously described (Ashpole and Hudmon, 2011). Each coverslip is imaged

in three different fields using a Texas Red filter to detect cytotoxic cells and a

FITC filter to detect viable cells on a Nikon Ti-E inverted microscope (100x

magnification). Cells were quantified using the automated counting software

Nikon Elements 3.0 as previously described (Ashpole and Hudmon, 2011). Total

cell number was determined by addition of cytotoxic and viable cells. Complete

media exchanges and washing conditions routinely induced cytotoxicity in about

5-10% of cultured neurons.

Immunocytochemistry of neuronal cultures. Neurons (8-9 DIV) treated with

CaMKII inhibitors were fixed in 4% paraformaldehyde (0.1 M phosphate buffer,

pH 7.4) for 10 minutes and washed in phosphate buffered saline (PBS) three

times. For labeling, cells were permeabilized in 0.5% Triton X-100 in PBS for 10

minutes at room temperature, washed in PBS three times, blocked for 1 hour in

2% BSA Fraction V, 20% normal goat serum, 0.1% Triton X-100 in PBS at room temperature, washed an additional three times in PBS. Cells were then incubated in primary polyclonal cleaved caspase 3 antibody (1:500, Cell Signaling (#9661), Beverly, MA) for 2 hours at room temperature. After three washes, secondary antibodies (anti-rabbit Alexa$_{594}$, 1:5000 (Molecular Probes, Eugene, OR)) were applied for one hour at room temperature. Coverslips were washed in PBS three times and were subsequently mounted in Prolong Gold Antifade with DAPI mounting media (Molecular Probes) and cells were imaged using a Zeiss Axio ObserverZ1 and processed with Axiovision 4.

CaMKII activity assay. Neuronal cultures were lysed in lysis buffer containing (50 mM HEPES pH 7.4, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7$-$10H_2O$, 100 mM β-glycerophosphate, 25 mM NaF, 1% Triton-X 100, and protease inhibitor cocktail (Calbiochem, 539137) as described previously (Kolb et al., 1995), sonicated, and incubated with 50 mM HEPES pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 100 μM ATP, 2 mM $CaCl_2$, 5 μM CaM, 50 μM AC2 (KKALRRQETVDAL), and [γP^{32}]-ATP (3μCi per reaction) for 3 minutes at 30°C. The linear range of the reaction extended from 30 seconds to 4 minutes. Protein levels were assessed and activity was normalized to total protein using DC protein assay kit (Biorad, Hercules, CA).

Calcium imaging. Hippocampal neurons 10-12 DIV were loaded with 2.6 μM Fura-2FF-AM (Molecular Probes, Invitrogen) and 1.7 μM Rhodamine 123 and subsequently imaged as previously described (Brustovetsky et al., 2009, Brustovetsky et al., 2011). During imaging, the neurons were maintained in a

bath solution containing 10 mM HEPES, pH 7.4, 139 mM NaCl, 3 mM KCl, 0.8 mM MgCl$_2$, 1.8 mM CaCl$_2$, 5 mM glucose and 65 mM sucrose. For Fluo-4 experiments, cortical neurons were incubated with 5 µM Fluo-4-AM (Molecular Probes, Invitrogen) diluted in rat physiological saline (138 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl$_2$, 1.06 mM MgCl$_2$, 12.4 mM HEPES pH 7.4, 5.6 mM glucose; final pH adjusted to 7.3) for 30 minutes at 37°C. Following incubation cells were washed with rat physiological saline 3 times for 5 minutes each. A Nikon Ti-E inverted microscope with a FITC filter was employed to monitor Fluo-4 levels once every thirty seconds. Baseline was monitored for 5 minutes. In all experiments with CaMKII inhibitor application, the inhibitor was added 10 minutes after start of imaging, some experiments called for the addition of other inhibitors as indicated. For these, the drugs were applied at the 5 minute mark in order to identify whether the drug itself had an impact on calcium levels prior to CaMKII inhibitor application at minute 10. Analysis was performed using Nikon Elements v3.0 in which Fluo-4 levels were measured in ~20 cells per field and 3 fields per coverslip. Neurons were identified at the start of the experiment with a 20 mM KCl depolarization with a subsequent wash. The fluorescent intensity of each cell was normalized to time 0, as the drug was applied.

Electrophysiology. Action potential studies were carried out using the whole cell patch-clamp technique under the current clamp mode. Whole cell voltages were recorded with HEKA software (HEKA electronic). The neuronal growth media on the cortical neurons (8-10 DIV) was gradually replaced by the extracellular recording solution before the patch clamp recording. The extracellular solution

consisted of rat physiological saline (as previously described). The intracellular

solution contains 140 mM potassium gluconate, 2 mM KCl, 3 mM $MgCl_2$, 10 mM

HEPES, 5 mM phosphocreatine, 2 mM K-ATP, 0.2 mM Na-GTP; final pH

adjusted to 7.4. Pipette resistance was 2-4 MΩ when filled with the internal

solution. Data were acquired and analyzed using the Pulsefit software following

previously established protocols (Wang et al., 2005, Zhang et al., 2008). Cortical

neurons were held at their resting potentials by injection of steady current

throughout the experiment. To determine the current threshold for action

potential initiation, neurons were injected with a series of depolarizing currents

with variable amplitudes for 200 ms. The sampling frequency was 10 kHz.

Following identification of the current threshold for action potential generation,

the neurons were injected with a 1 second depolarizing ramp current that

subsequently elicited one to two action potentials. Neurons that maintain initial

resting membrane potentials more negative than -50 mV after whole cell

configuration and had less than 3 action potentials at the starting point were

included in this study. We compared the change in the number of APs obtained

10 minutes after establishing the whole cell configuration to the number obtained

at the initial starting time point when the peptide had not diffused into the

intracellular environment. The current clamp studies were performed at room

temperature (~21°C).

Glutamate measurements. Glutamate concentrations in the neuronal media were

assessed using Amplex Red Glutamic Acid/Glutamate Oxidase Assay per

manufacturer protocol (Molecular Probes, Invitrogen). The glutamate detection

assay standard curve was linear from 180 nM to >6 µM glutamate. For high high-performance liquid chromatography quantification, glutamate was measured by HPLC separation and electrochemical detection of an OPA-mercaptoethanol derivative using modifications of the method of Donzanti and Yamamoto (Donzanti and Yamamoto, 1988). See Supplemental Figure 4 for more detail.

Data analysis. Electrophysiology data are presented as the mean ± S.E.M. Statistical significance between groups was determined using one-way ANOVA with a post-hoc Bonferroni's test. One-way ANOVA with a subsequent Dunnett's Test was conducted to compare differences between the means of each group in all *in situ* cell death assays, *in vitro* catalytic assays, and calcium imaging experiments. Student t-test was also used when appropriate. Statistical significance was accepted at $p < 0.05$. Analysis was performed using SigmaPlot 11 software.

RESULTS

Neurotoxicity with CaMKII Inhibition

To test the hypothesis that CaMKII inhibition induces neuronal death, we subjected cortical neurons (8 DIV) to acute (1 hour) and prolonged (24 hours) pharmacological inhibition of CaMKII using multiple inhibitors. Peptides derived from the endogenous inhibitor of CaMKII, termed CN21, and the autoinhibitory domain of CaMKII itself (AIP) were used because of their high specificity to inhibit CaMKII (Ishida et al., 1995, Chang et al., 1998, Vest et al., 2007). As previously described (Vest et al., 2007, Vest et al., 2010, Ashpole and Hudmon, 2011), conjugation of peptide inhibitors to the cell-penetrant motif, tat, affords the ability

to examine the effects of CaMKII inhibition with cell penetrating peptides in both

cultures and *in vivo*. In addition to the peptide inhibitors, we also utilized the

allosteric CaMKII inhibitor KN-93 (Sumi et al., 1991, Ishida et al., 1995, Vest et

al., 2007, Ashpole and Hudmon, 2011). We applied 10 µM tat-CN21, tat-AIP, and

1 µM KN-93 to cortical neuronal cultures for 1 or 24 hours and subsequently

examined viability/cytotoxicity levels 24 hours following the start of treatment

(Ashpole and Hudmon, 2011). To compensate for the decreased bioavailability

associated with tat-based peptides accumulating in non-cytoplasmic

compartments (Tunnemann et al., 2006), the concentrations of the peptide

inhibitors used in this study are roughly 100 fold above the *in vitro* IC_{50} for CN21

(77-100 nM) (Vest et al., 2007, Ashpole and Hudmon, 2011). To control for

potential off target effects, we applied KN-92, the inactive control for KN-93, or a

peptide control, tat-CN21-Ala (Ashpole and Hudmon, 2011). For each neuronal

death experiment, data was normalized by subtracting average neuronal death

observed in control cultures. The control cultures in each experimental group

consistently exhibited 5-10% toxicity. Cortical neuron viability was no different

than control cultures (DMSO treated) when CaMKII inhibitors were applied for

one hour (Figure 16A, gray bars). In contrast, all three CaMKII inhibitors (KN93,

tat-CN21, tat-AIP) significantly increased neuronal death 12-18% when applied

for 24 hours (Figure 16A, black bars). Compared to control cultures, lower

concentrations of tat-CN21 also induced neurotoxicity when applied overnight (1

µM tat-CN21 5.3% ± 2.4%; p<0.05). Importantly, cultures treated with the

inactivecontrols, tat-CN21Ala and KN-92, for 24 hours did not exhibit significant

changes in neuronal viability (Figure 16A), suggesting that the neurotoxic effects are specific to the application of active small molecule and peptide CaMKII inhibitors. Furthermore, the tat motif, itself, was not toxic when applied for 24 hours ($0.533 \pm 1.6\%$; $p > 0.5$). Utilization of a myristolated-AIP peptide inhibitor of CaMKII also induced neurotoxicity ($29.6 \pm 4.2\%$; $p < 0.05$). Thus, CaMKII inhibition using both small molecule and peptide inhibitors conjugated to tat and myristolated cell-penetrating motifs induce neuronal death when applied for 24 hours.

While a significant increase in neurotoxicity was observed within our neuronal cultures when CaMKII was inhibited at 8 DIV, we tested whether the age of the culture affected this neuronal death. While no differences were observed in neuronal viability between control groups at 8 DIV, 14 DIV and 21 DIV, 24 hour inhibition of CaMKII with tat-CN21 significantly enhanced neurotoxicity with respect to time in culture (Figure 16B). Compared to cultures 8 DIV, 14 DIV cultures exhibited nearly 25% toxicity while nearly 75% of cells were compromised when CaMKII was inhibited at 21 DIV. The cell death observed with CaMKII inhibition is not limited to highly enriched cortical cultures (>95% MAP-2 immunopositive (Ashpole and Hudmon, 2011), as a similar level of toxicity was observed in neurons cultured with glia (Figure 16C). In these co-cultures, a monolayer of GFAP positive astrocytes underlying MAP2 positive cortical neurons represent an astrocytes to neurons ratio of 3:2 (Figure 17). Thus, the toxicity produced by CaMKII inhibition is enhanced by culture age and
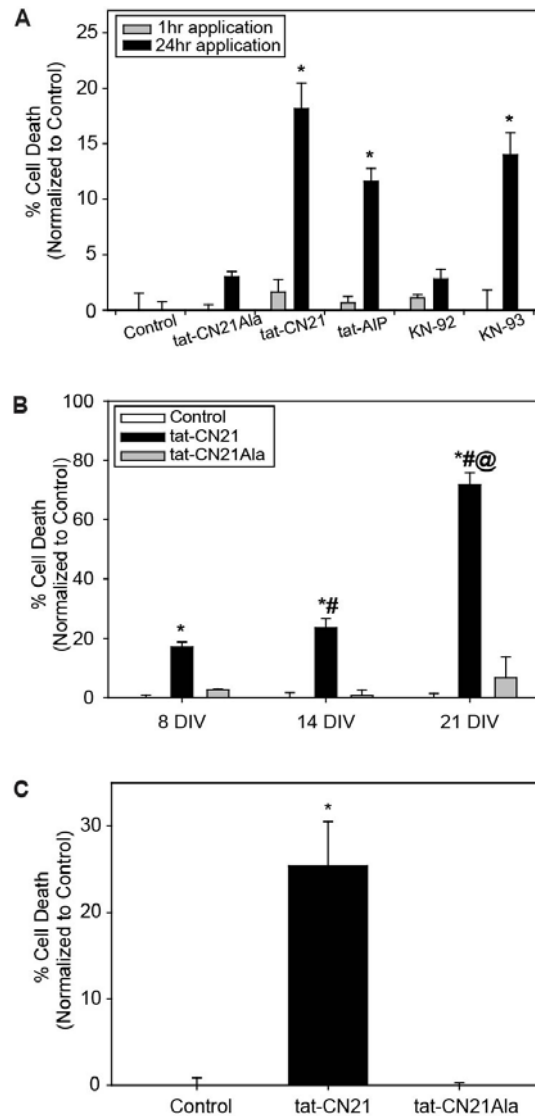
Figure 16: Neurotoxicity with CaMKII inhibition. A, Neuronal death (mean ± SEM, n=3-15) normalized to control when CaMKII inhibitors (10 µM peptide inhibitors and 1 µM small molecule inhibitors) were applied to neuronal cultures (8 DIV) for 1 hr (gray bars) or 24 hrs (black bars). *p<0.05 compared to control (One-Way ANOVA, post-hoc Dunnett's test). **B,** Neuronal death (mean ± SEM, n=4-17) normalized to control when 10 µM tat-CN21 or tat-CN21Ala was applied to neuronal cultures for 24 hours at 8, 14, and 21 DIV. *p<0.05 compared to control at that time point, #p<0.05 compared to 8 DIV tat-CN21 treatment (One-Way ANOVA, post-hoc Dunnett's test). @p<0.05 for 14 DIV tat-CN21 treatment vs 21 DIV tat-CN21 treatment (t-test). **C,** Neuronal death (mean ± SEM, n=4) normalized to control when 10 µM tat-CN21 or tat-CN21Ala was applied to co-cultures for 24 hours. *p<0.05 compared to control (One-Way ANOVA, post-hoc Dunnett's test).
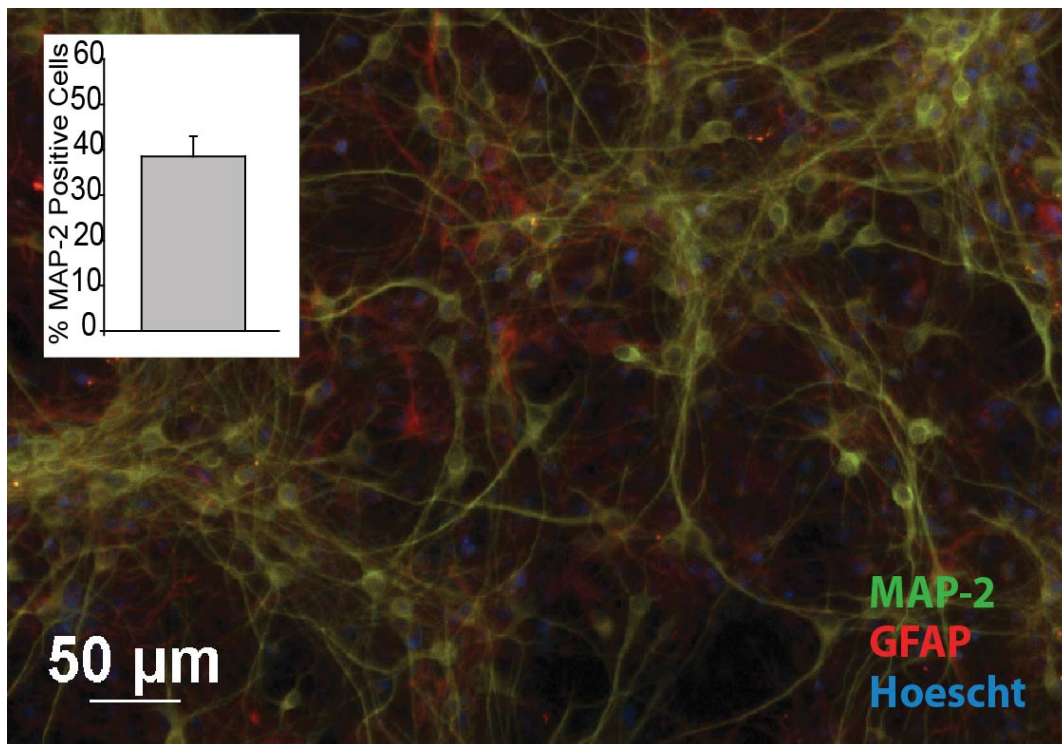
Figure 17: Characterization of co-cultures of neurons and astrocytes. Representative image of immunostained co-cultures with MAP2 positive neurons (green) and GFAP positive glia (red). The inset indicates average percentage of cells (± SEM; n=4) that are MAP2 positive.

observed in both highly pure cultures of cortical neurons as well as cortical neurons cultured with astrocytes.

In Figure 16A we report that CaMKII inhibition for acute (1 hour) versus prolonged (24 hours) differentially affects neuronal death. To determine the minimal time where CaMKII inhibition-induced cell death, we applied tat-CN21 for various times between 1 and 24 hours. In addition, we explored exposure times longer than 24 hours to determine if neurotoxicity was saturated. Once again, a 1 hour exposure of neurons to tat-CN21 failed to produce cell death (Figure 18A). CaMKII inhibition for 4 hours or less was also not toxic to neurons (Figure 18A). Progressively increasing neuronal death as a function of CaMKII inhibition time was observed up to 12 hours. However, longer incubation times (24 or 48 hrs) did not produce additional neurotoxicity (Figure 18A), suggesting that events occurring within the first 12 hours largely determine the neuronal death associated with CaMKII inhibition.

The extent of CaMKII inactivation produced by focal ischemia *in vivo* was previously shown to correlate with the extent of neuronal damage (Hanson et al., 1994). Because we observed a time-dependence in the neurotoxicity produced by CaMKII inhibition, we determined if neuronal death in cortical cultures is correlated with functional changes in CaMKII. We observed a time-dependent loss in the activatable pool of CaMKII that correlated with exposure time of the CaMKII inhibitor. Application of tat-CN21 for ≥4 hours resulted in a significant decrease in activatable CaMKII compared to vehicle-treated control neurons (Figure 18B). A maximal 50% loss of activity occurred when tat-CN21 was
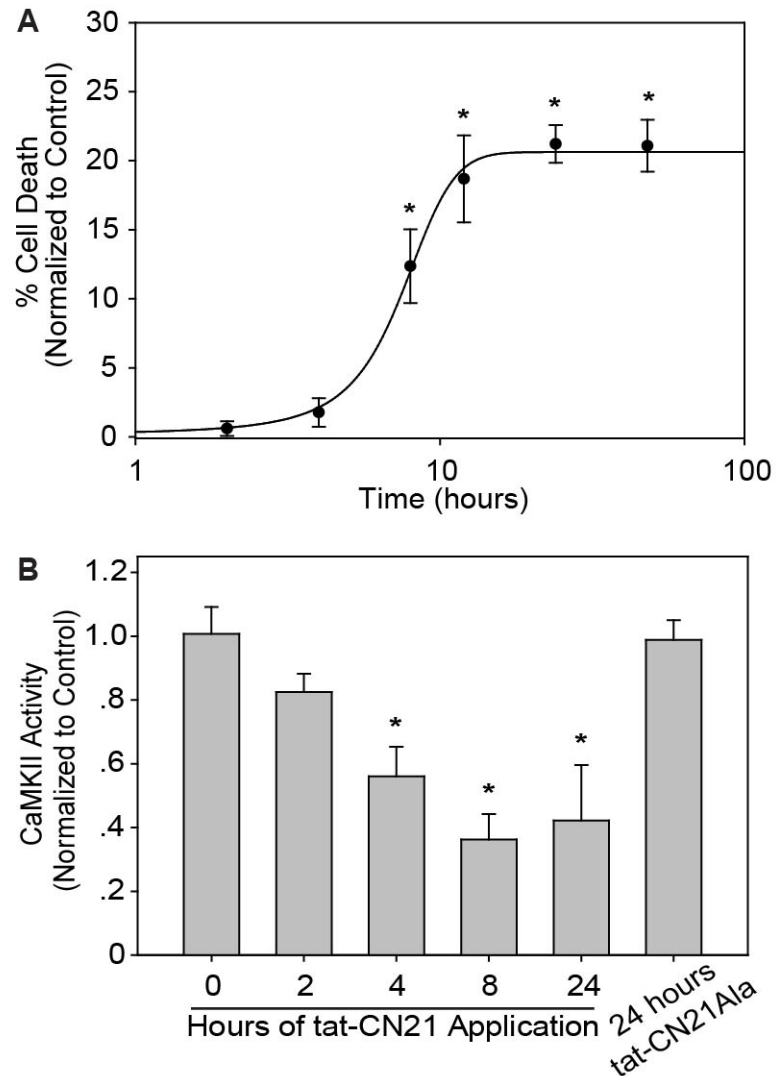
Figure 18: Time-dependence of neurotoxicity and CaMKII inactivation with CaMKII inhibition. A, Neuronal death (mean ± SEM, n=7-12) normalized to control when 10 µM tat-CN21 was applied to neuronal cultures for varying lengths of time. *$p<0.05$ compared to control (One-Way ANOVA, post-hoc Dunnett's test). **B,** Kinase activity (mean ± S.D., n=3-4) in neuronal lysates subjected to an *in vitro* CaMKII assay in the presence of calcium/calmodulin after 10 µM tat-CN21 (or tat-CN21Ala) was applied to cortical neurons for varying lengths of time. *$p<0.05$ compared to control (One-Way ANOVA, post-hoc Dunnett's test).

applied for 24 hours. Importantly, neurons treated with inactive tat-CN21Ala for 24 hours did not exhibit a decrease in activatable CaMKII (Figure 18B).

We previously showed that neuronal uptake of fluorescent carboxyfluorescein (Fam) labeled tat-CN21 and control tat-CN21Ala (Fam conjugated to the C-terminus) were similar in our cortical cultures (Ashpole and Hudmon, 2011). Using these Fam-labeled peptides, we measured peptide uptake and neuronal death in order to identify whether fluorescently labeled neurons are preferentially dying in our assay. Using fluorescent microscopy, we observed that ~25% of cells display substantial tat-CN21-Fam or tat-CN21Ala-Fam uptake (Figure 19). However, a large proportion of cells exhibit low, but still above background levels of fluorescent peptide, suggesting that CaMKII activity may be reduced in more than the 25% of cells (Figure 19). It is noteworthy that peptide uptake (~25%) correlates with neuronal death (~25%). The disconnect between maximal cell death (~25%) and the 50% decrease in CaMKII activity could be explained by the observation that not all cells exhibit the same levels of peptide uptake, suggesting that low levels of CaMKII inhibition may not reach a threshold that is required for neurotoxicity. While the mechanism behind the differential peptide uptake and loss of CaMKII activity is not known, there is a clear correlation with cytotoxicity and CaMKII inhibitor uptake as nearly 80% of cytotoxic cells exhibit tat-CN21-Fam co-localization (Figure 19).

CaMKII Inhibition Induces Apoptosis

The time-dependence of CaMKII inhibition (>4 hours) leading to toxicity is consistent with apoptosis. To test if neurons were undergoing apoptosis in
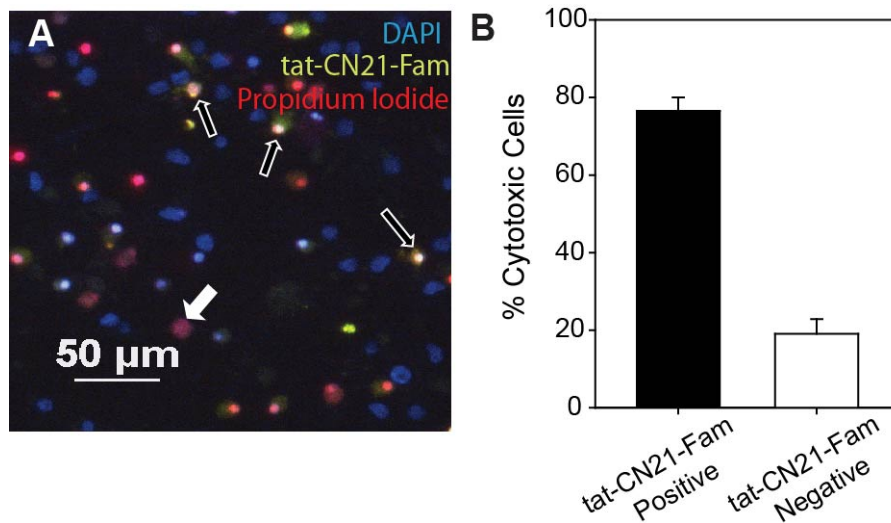
Figure 19: Neurotoxicity is predominantly found in neurons that have CaMKII inhibitor uptake. A, Representative image of neurons treated with tat-CN21-Fam (green) for 24 hours and stained with ethidium homodimer (red) and Hoescht staining (blue). **B,** Average number of cytotoxic cells (± SEM; n=8) per field found to co-localize or not co-localize with tat-CN21-Fam.

response to prolonged CaMKII inhibition, cultures were co-treated with tat-CN21 and the protein translation inhibitor cycloheximide. Co-application of 0.5 mg/mL cycloheximide blocked tat-CN21-induced neurotoxicity (Figure 20A). Similarly, co-treatment of tat-AIP and cycloheximide abolished the tat-AIP-induced toxicity (Figure 21). In further support of apoptosis, there was a significant increase in TUNEL staining of cultures treated with tat-CN21 for 24 hours compared to control and cultures co-treated with 0.5 mg/mL cycloheximide (Figure 20B). To confirm that the apoptosis occurred within cells that took up the CaMKII inhibitors, we next examined levels of activated caspase-3, a neuronal marker for apoptosis. Overnight application of tat-CN21-Fam was used in order to identify neuron uptake of the CaMKII inhibitor. Activated caspase-3 labeling was limited to cells that contained tat-CN21-Fam (Figure 20C). Not all neurons that were tat-CN21-Fam positive displayed caspase-3 activation. However, nearly all of the neurons that contained tat-CN21-Fam had pyknotic and fragmented nuclei, indicating that the neurons were compromised (Figure 20C). Thus, while some necrotic cell death may not be ruled out, CaMKII inhibition does lead to apoptosis.

<u>Calcium Dysregulation with CaMKII Inhibition</u>

One prominent mechanism underlying neurodegeneration is dysregulated calcium signaling (as reviewed by (Orrenius et al., 2003)). Cultured hippocampal neurons (DIV 14) were loaded with Fura-2FF-AM and intracellular calcium levels were measured using ratiometric fluorescent imaging. While tat-CN21Ala or tat did not alter intracellular calcium levels, tat-CN21 led to a gradual rise in
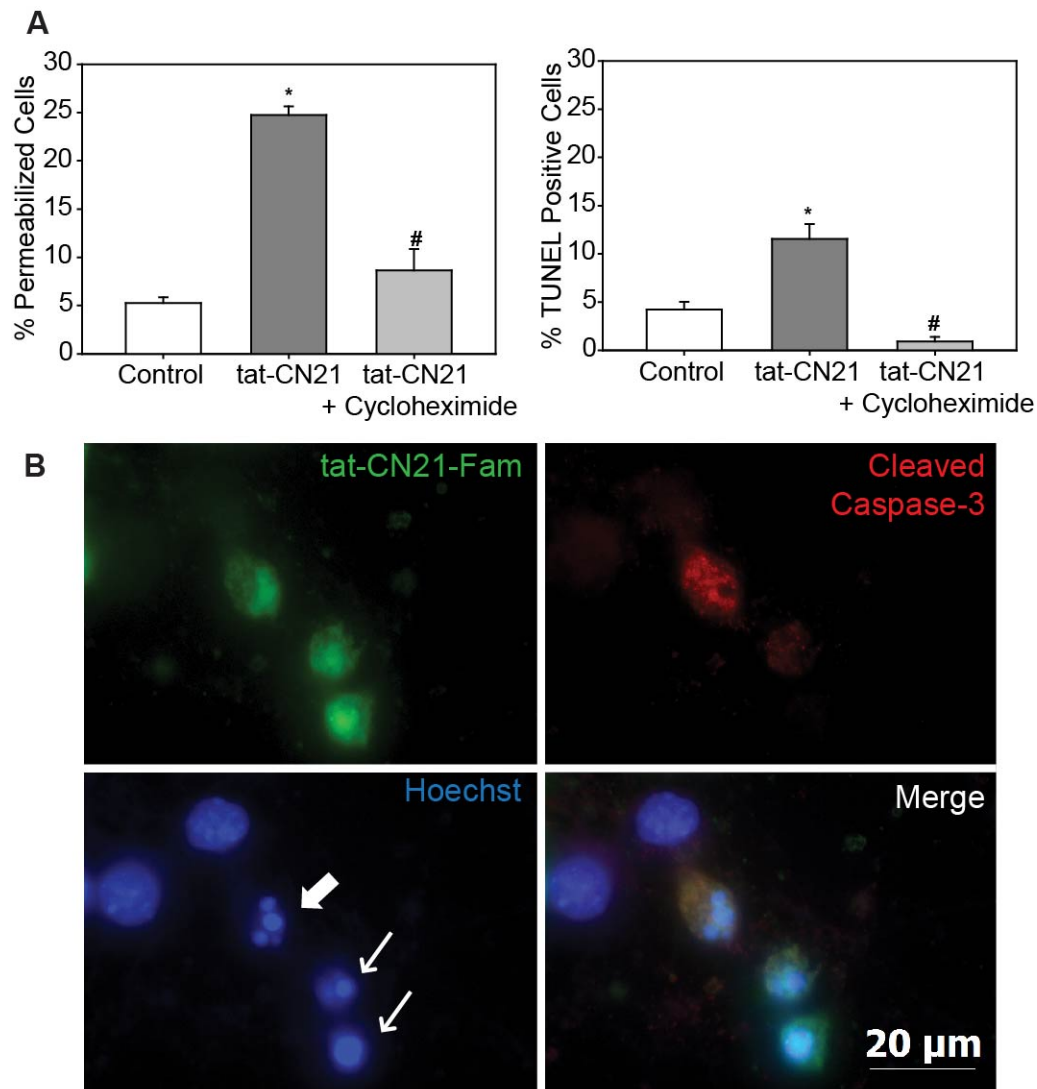
Figure 20: Neuronal apoptosis with CaMKII inhibition. Neuronal death (mean ± SEM, n=5-10) following treatment with tat-CN21 with or without 0.5 mg/mL cycloheximide as measured by **A,** ethidium homodimer membrane permeability dye, or **B,** TUNEL staining. *p<0.05 compared to control while #p<0.05 compared to tat-CN21 alone (One-Way ANOVA, post-hoc Dunnett's test). **C,** Representative image of a field of neurons treated with 10 µM tat-CN21-Fam for 24 hours (top left) immunostained for cleaved caspase-3 (top right), nuclear marker Hoechst (bottom left), and a merge of all three channels (bottom right). Arrows indicate fragmented or pyknotic nuclei.
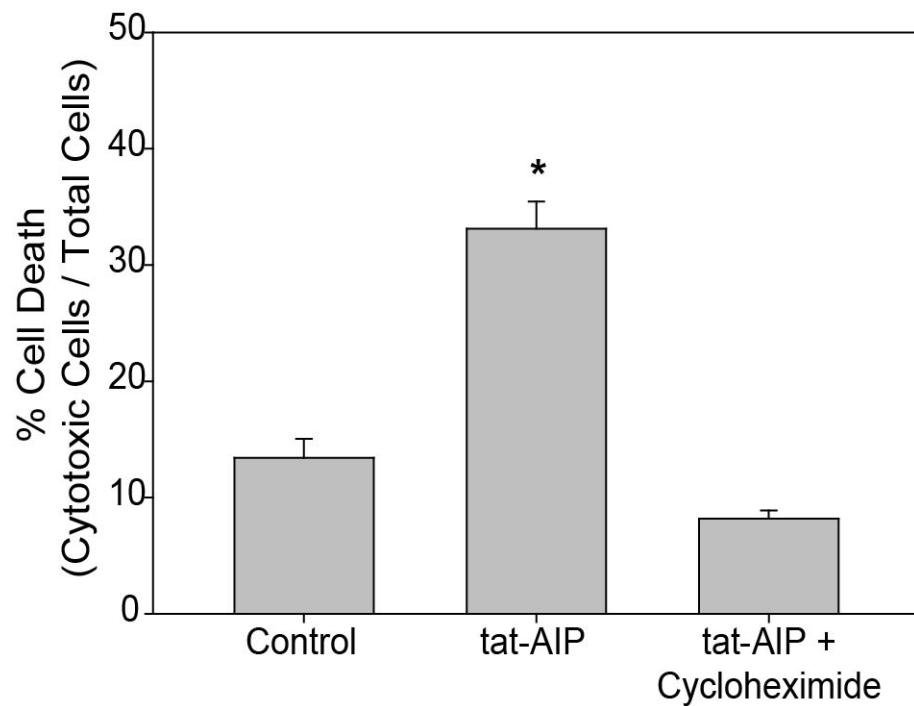
Figure 21: CaMKII inhibitor tat-AIP also induces apoptosis. Neuronal death (mean ± SEM; n=5-7) following 24 hour treatment with 10 µM tat-AIP in the absence or presence of 0.5 mg/mL cycloheximide. *p<0.05 compared to control (One-Way ANOVA, post-hoc Dunnett's test).

intracellular calcium (Figure 22B-D), suggesting that CaMKII inhibition leads to a slow, tonic increase in intracellular calcium. This calcium dysregulation is also observed in cortical neurons loaded with Fluo-4AM. Using this high affinity calcium indictor, we observed significantly elevated intracellular calcium levels occurring within 10 minutes exposure to tat-CN21 application (Figure 22E-F). Again, no changes in intracellular calcium were observed with tatCN21-Ala (Figure 22E). Similar to tat-CN21, 10 µM tat-AIP (data not shown) and 10 µM myristolated-AIP (Figure 22F) induced a significant increase in intracellular calcium concentration. In contrast, inhibition of the CaMKK pathway (CaMKI and CaMKIV (Tokumitsu et al., 2002, Schmitt et al., 2005)), using STO-609, does not induce calcium dysregulation (Figure 22F). Thus, acute CaMKII inhibition leads a slow increase in intracellular calcium levels.

Low extracellular calcium largely prevented the tat-CN21-induced calcium influx (Figure 22F), indicating that calcium is likely derived from extracellular sources. L-type voltage-gated calcium channels do not appear to contribute to this process because pretreatment with 10 µM nimodipine had no effect on tat-CN21 induced calcium dysregulation (Figure 22F). However, synaptic transmission may contribute to the increase in intracellular calcium, as 1 µM N-type calcium channel blocker omega-conotoxin abolished calcium dysregulation prior to tat-CN21 treatment. Because N-type calcium channels play a prominent role in synaptic activity, we tested if neuronal activity was essential for this process by inhibiting AMPA receptors. Indeed, inhibition of AMPA receptors,
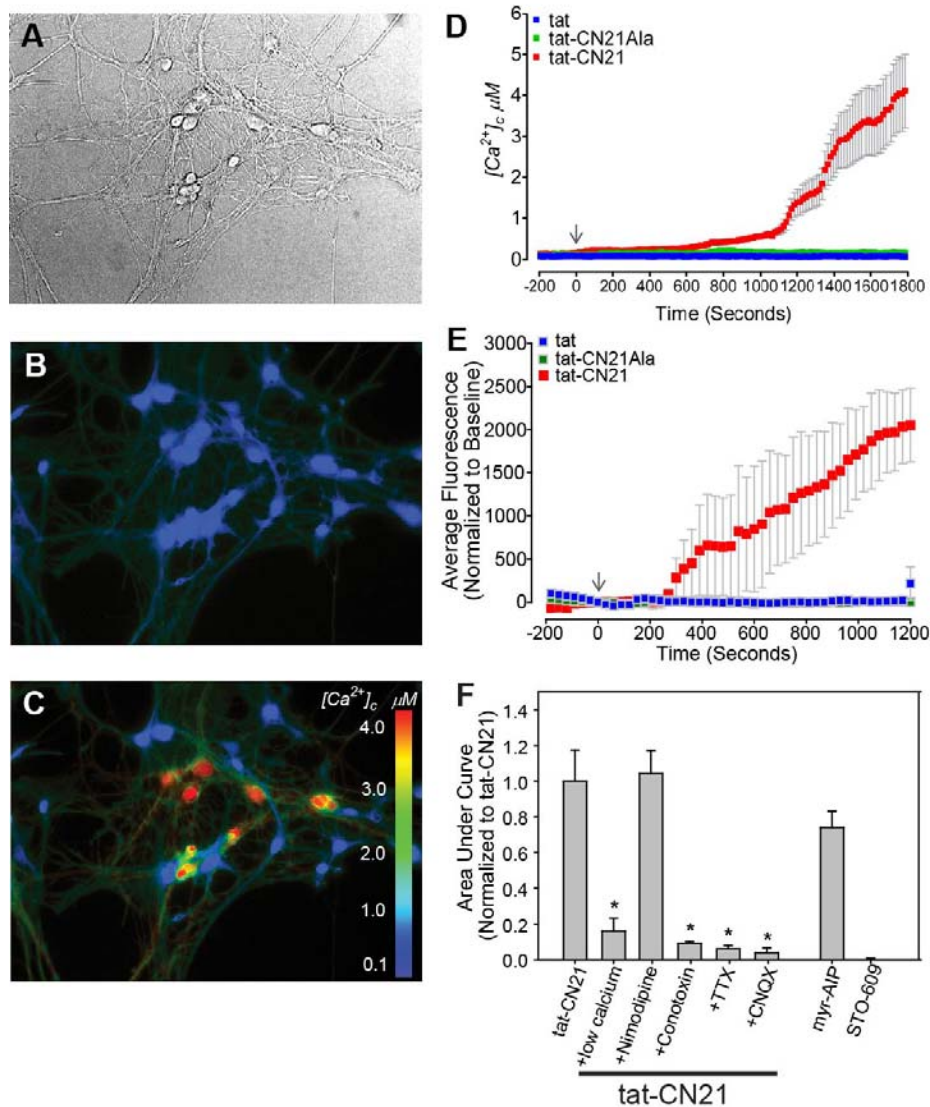
Figure 22: Calcium dysregulation with CaMKII inhibition in neurons.
A, Representative bright-field and, **B**-**C,** fluorescent images of Fura-2FF-loaded hippocampal neurons, **B,** before and, **C,** after treatment with 10 µM tat-CN21. **D**, Cytoplasmic calcium levels, $[Ca^{2+}]_C$, (mean ± SEM) before and after application of 10 µM tat-CN21, tat-CN21Ala, or tat. **E**, Neuronal intracellular calcium levels (mean ± SEM) before (-300 to 0 seconds) and after (0 to 1200 seconds) application of 10 µM tat-CN21, tat-CN21Ala, or tat, as measured by Fluo-4 (n=4). **F,** Average integral of fluorescent intensity from 0-1200 seconds in **E** (mean ± SEM, n=3-6) with application of CaMKII inhibitors with and without pharmacological blockers of neuronal excitability. The integral was normalized to the calcium influx observed with application of 10 µM tat-CN21. *$p<0.05$ compared to tat-CN21 alone (One-Way ANOVA, post-hoc Dunnett's test).

FURA Imaging Courtesy of the Brustovetsky Lab

using 10 µM CNQX, blocked calcium dysregulation following CaMKII inhibition. In further support for neuronal activity being essential for the tonic increase in intracelluar calcium observed with CaMKII inhibition, inhibition of voltage-gated sodium channels using TTX (1 µM) prior to tat-CN21 treatment completely abolished the calcium influx (Figure 22F). These data are consistent with neuronal activity being essential for the calcium influx associated with inhibition of CaMKII.

Enhanced Neuronal Excitability with CaMKII Inhibition

To address the potential for CaMKII inhibition to alter neuronal excitability, we employed whole cell current-clamp to measure action potential firing in response to a depolarizing voltage ramp. In these experiments we used lower peptide concentration (1 µM) because the patch pipette provides direct access to the cytosol. We used inhibitory peptides without the cell-penetrating tat motif to affect only the cell from which we were recording. Each cell served as its own control by determining the number of action potentials generated immediately after establishing whole-cell configuration versus 10 minutes later when the peptide inhibitors have had the opportunity to diffuse from the pipette to inhibit CaMKII. Cortical neurons exposed to 1 µM CN21 for 10 minutes exhibited a three-fold increase in the number of action potentials compared to neurons treated with 1 µM inactive peptide CN21Ala (Figure 23A-B). CN21C, another previously established control for CN21 (Vest et al., 2010, Ashpole and Hudmon, 2011), did not result in a significant increase in action potential number compared
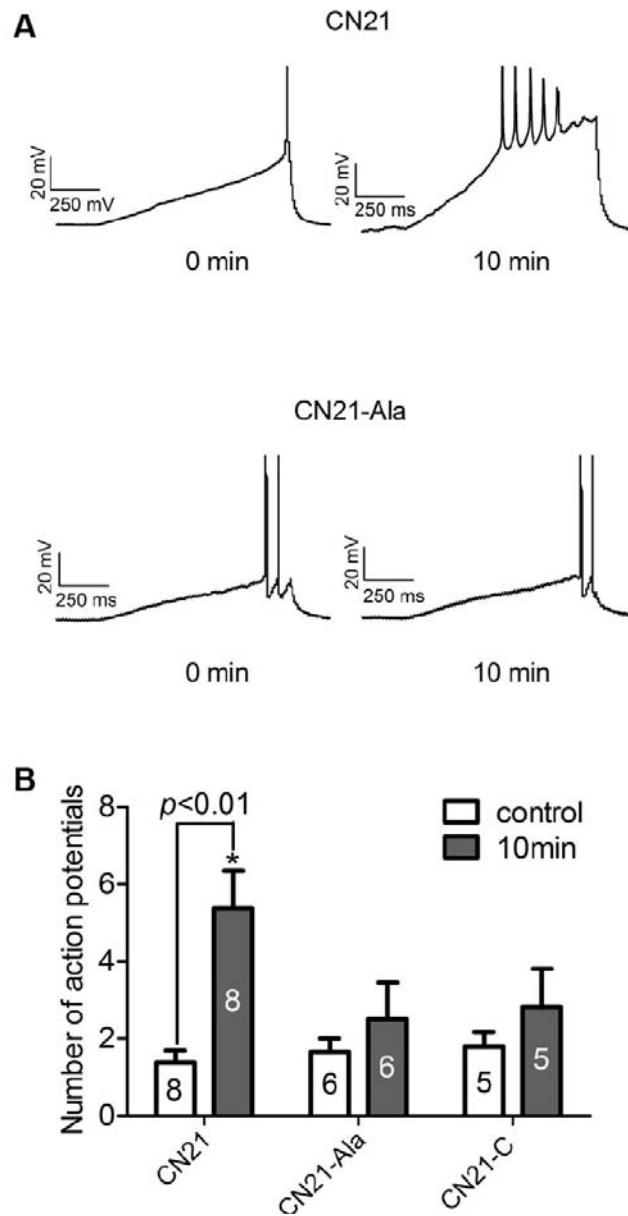
Figure 23: CaMKII inhibition augments neuronal excitability. A, Representative traces from cortical neurons at time 0 and 10 minutes following diffusion of either 1 µM CN21 or 1 µM CN21Ala. Neurons were held at their resting membrane potentials and injected with 1 second depolarizing current ramps to evoke action potentials. **B,** Number of action potentials (mean ± SD) evoked at time 0 or 10 minutes after whole cell configuration in the presence of CN21 or control CN21Ala or CN21C. *p<0.01 between the number of action potentials between time 0 and 10 minutes (One-Way ANOVA, post-hoc Bonferroni).

Courtesy of Weihua Song

to baseline. Overall, these data support the hypothesis that CaMKII inhibition enhances neuronal excitability.

<u>CaMKII Inhibition Predisposes Neurons to Excitotoxic Insults</u>

Our observed increase in neuronal excitability with a loss of CaMKII activity supports previous reports indicating that genetic αCaMKII knock-out animals are predisposed to epilepsy (Butler et al., 1995). We hypothesize that this increased excitability not only underlies the observed neurotoxicity, but also mechanistically underlies the decreased ability of the neurons to handle excitatory insults. As mentioned, αCaMKII knock-out animals also exhibit greater neuronal damage following middle cerebral artery occlusion than their wild-type littermates (Waxham et al., 1996). Similarly, overnight inhibition of CaMKII with tat-CN21 exacerbated cortical cell death following application of exogenous glutamate in an *in vitro* model of excitotoxicity (Ashpole and Hudmon, 2011). To further explore the role of CaMKII inhibition in sensitizing neurons to excitotoxic-related insults, we sought to identify whether prolonged CaMKII inhibition predisposed neurons specifically to NMDA-R activation and/or sensitized neurons to the deleterious effect of reactive oxygen species. Thus, 10 μM tat-CN21 was applied to cortical neurons for 24 hours. Following overnight inhibition of CaMKII, the neurons were subjected to submaximal levels of 100 μM NMDA/10 μM glycine or H_2O_2 for 5 minutes, washed, and 24 hours later cell viability assessed. Compared to cultures that were treated with NMDA for 5 minutes alone, cultures subjected to CaMKII inhibition prior to NMDA-R stimulation exhibited significantly increased neuronal death (15% vs 45%)

(Figure 24). Similarly, neurons treated with tat-CN21 also exhibited significantly higher levels of toxicity when treated with H_2O_2, compared to H_2O_2 alone (Figure 24). Neuronal sensitivity to Microcystin-LR, a cell-permeable protein phosphatase 1 and 2A inhibitor, was also assessed as CaMKII activity has been shown to be necessary for microcystin-induced apoptosis (35). Interestingly, prolonged CaMKII inhibition was not synergistic nor additive with microcystin toxicity (Figure 24), suggesting that CaMKII inhibition via tat-CN21 blocks neurotoxicity produced by microcystin treatment and that the neuronal death induced by CaMKII inhibition is not obstructed by protein phosphatase inhibitors. Together these data suggest that a prolonged loss of CaMKII sensitizes neurons to ROS and NMDA-R mediated excitotoxicity. Thus, CaMKII inhibition appears toxic to neurons both directly via inducing calcium dysregulation and hyperexcitabilty and indirectly through predisposing neurons to glutamate excitotoxicity.

Glutamate Dysregulation with CaMKII Inhibition

Because a loss of CaMKII predisposes neurons to glutamate excitotoxicity, we questioned whether CaMKII inhibition itself affected glutamate levels within our cultures. To address this question, cultures were treated with tat-CN21 for varying lengths of time and glutamate concentration in the media was assessed using a glutamate oxidase assay. There was a significant increase in glutamate concentration in the media as early as 20 minutes following tat-CN21 application (Figure 25A). Twenty-four hours following tat-CN21 application the concentration of glutamate in the bath solution was 4-5 µM while tat-CN21Ala failed to raise glutamate levels compared to control without treatment (0.5-1 µM).

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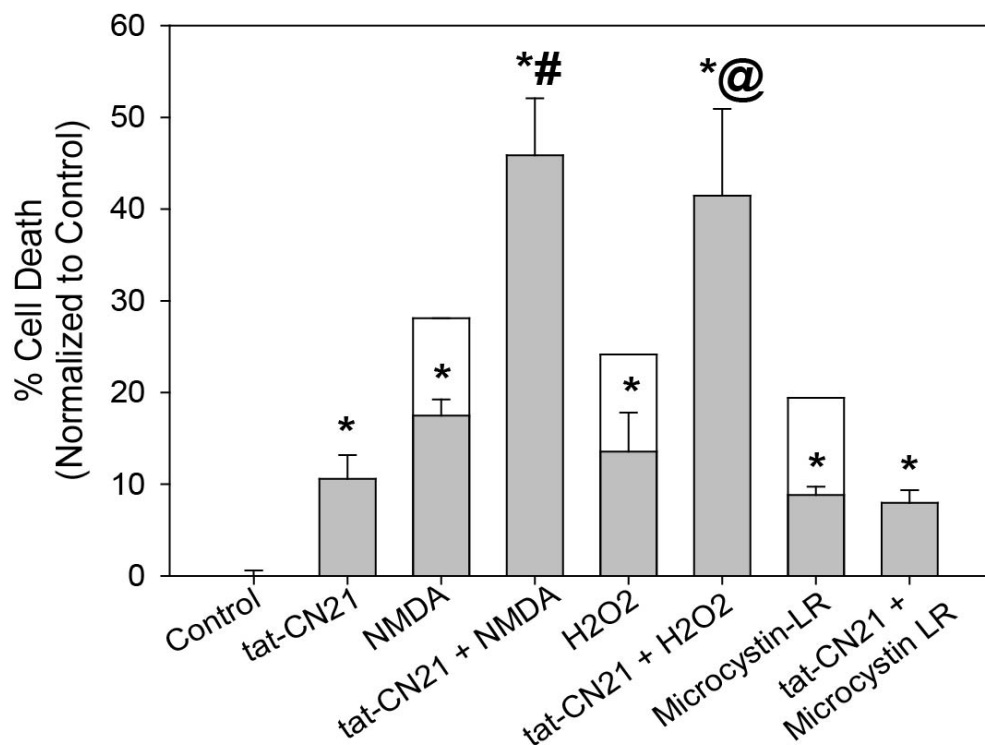
Figure 24: Prolonged CaMKII inhibition sensitizes neurons to excitotoxic-related insults. Neuronal death (mean ± SEM, n=3-7) following treatment with various combinations of tat-CN21, NMDA, H_2O_2, or microcystin-LR. All cell death measurements were made 48 hours from the start of treatments. Cultures were treated with NMDA, H2O2, or microcystin-LR independently or in combination with a 24 hour pre-treatment of 10 µM tat-CN21. The clear boxes highlight the potential levels of cytotoxicity if the average death induced by tat-CN21 treatment alone would be additive. *p<0.05 compared to control (One-Way ANOVA, post-hoc Dunnett's test). #p<0.05 compared to NMDA treatment alone (t-test). @p<0.05 compared to H_2O_2 alone (t-test).
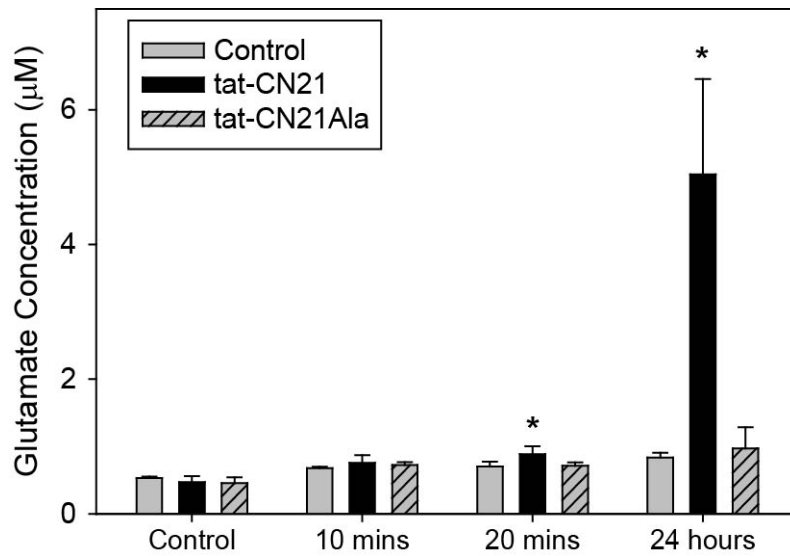
Figure 25: CaMKII inhibition results in increased glutamate in conditioned neuronal media. Glutamate concentration (mean ± SD, n=3-6) in neuronal media following incubation with 10 μM tat-CN21 or tat-CN21Ala for varying lengths of time, as measured by a glutamate oxidase assay. *p<0.05 compared to vehicle control (DMSO) (One-Way ANOVA, post-hoc Dunnett's test).

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Similarly, HPLC analysis of glutamate concentration in the media also indicated that 24 hour application of tat-CN21 resulted in more than a two-fold elevation in glutamate concentration compared to inactive tat-CN21Ala (Figure 26).

To determine whether this slight elevation in glutamate is important for calcium dysregulation and cell death, we sought to attenuate the glutamate rise enzymatically to dissociate CaMKII inhibition with the increased extracellular glutamate. Glutamate pyruvate transaminase (GPT), in the presence of pyruvate, catalyzes the conversion of glutamate to α-ketoglutarate and alanine (Beaton et al., 1957). Thus, we added 0.25 mg/mL GPT and 2 mM pyruvate in combination with the CaMKII inhibitory peptide tat-CN21 to the cortical neurons and measured extracellular glutamate levels using the oxidase assay. Twenty-four hour application of tat-CN21 resulted in a significant increase in glutamate in the media of the cultured cortical neurons (Figure 27A). Co-application of GPT and pyruvate with tat-CN21 brought glutamate levels back to control. When pyruvate was omitted from the treatment, a significant increase in glutamate concentration was seen with tat-CN21 (Figure 27A). Furthermore, when GPT was boiled prior to application, GPT and pyruvate failed to bring tat-CN21-induced glutamate release back to control levels (Figure 27A).

Having successfully buffered the prolonged rise in extracellular glutamate when CaMKII was inhibited, we measured acute changes in intracellular calcium concentrations with Fluo-4AM when GPT and pyruvate was present. As before, 10 µM tat-CN21 induced significant dysregulation of intracellular calcium concentrations within minutes of application (Figure 27B). Interestingly,

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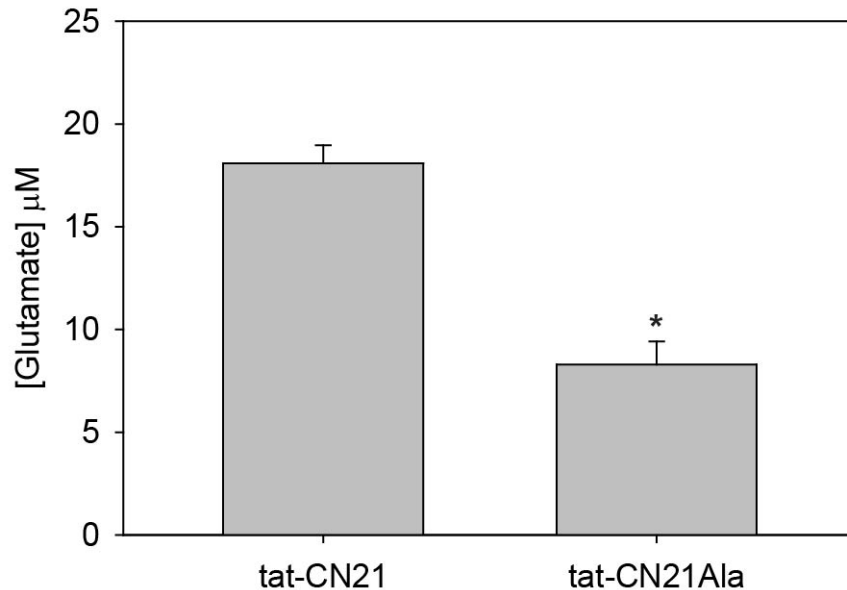
Figure 26: CaMKII inhibition results in increased glutamate in the media. Average concentration of glutamate in the media (± SEM, n=3) following treatment with CaMKII inhibitor tat-CN21 or control tat-CN21Ala, as measured by HPLC. *$p<0.05$ between the groups (t-test). For calculation of concentration, 5 µl of sample was transferred to assay tubes and placed in an autosampler tray at 8°C. Internal standard (20 µl homoserine) was added to each sample, then the sample was derivatized by the addition and mixing of 20 µl of the OPA/β-mercaptoethanol reagent. One minute and thirty seconds after the addition of the reagent 10 µl of the sample-reagent mixture was injected onto an HPLC column (HR-80; ESA, Chelmsford, MA). Separations were carried out isocratically with a mobile phase containing 0.1 M sodium phosphate dibasic (pH 6.75), and 25% methanol at a flow rate of 410 µl/minutes and a column temperature of 40°C. Electrochemical detection was performed by an ESA (Chelmsford, MA) Coulochem II detection system with a guard cell set at 700 mV, and dual electrodes set at 300 mV (E1), and 600 mV (E2) for oxidization of the glutamate derivative. Peak areas were calculated based on standard curves and adjusted for the value of the internal standard.

Courtesy of Dr. Eric Engleman
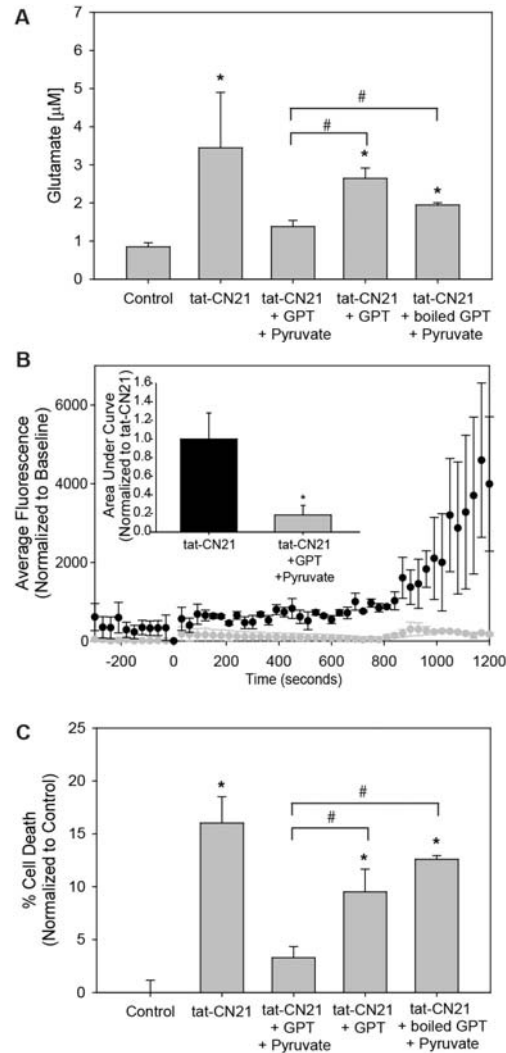
Figure 27: Enzymatic catalysis of glutamate prevents acute and prolonged effects of CaMKII inhibition. A, Glutamate concentration (mean ± S.D., n=4-8) in neuronal media following incubation with 10 µM tat-CN21 for 24 hours with and without co-application of glutamate pyruvate transaminase (GPT)/pyruvate, GPT alone, or boiled GPT/pyruvate. *p<0.05 compared to control while #p<0.05 compared to tat-CN21/GPT/pyruvate treatment (One-Way ANOVA, post-hoc Dunnett's test). **B,** Neuronal intracellular calcium levels (mean ± SEM, n=3) following application of tat-CN21 in the presence or absence of GPT/pyruvate. Bar graph inset indicates the average integral from 0-1200 seconds (mean ± SEM, n=3) for these treatment groups. **C,** Neuronal death (mean ± SEM, n=3-6) after 24 hour treatment with 10 µM tat-CN21 alone or co-application with GPT/pyruvate, GPT alone, or boiled GPT/pyruvate. *p<0.05 compared to control while #p<0.05 compared to tat-CN21/GPT/pyruvate treatment (One-Way ANOVA, post-hoc Dunnett's test).

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application of GPT and pyruvate prevented tat-CN21-induced calcium influx

(Figure 27B). Co-application of tat-CN21 with pyruvate alone did not alter calcium

influx with CaMKII inhibition (0.85 ± .24, normalized to tat-CN21).

Neuronal viability following CaMKII inhibition was also assessed with

extracellular glutamate buffering. Consistent with the glutamate experiments, the

tat-CN21-induced neurotoxicity was abolished by co-treatment with GPT and

pyruvate (Figure 27C). Cultures treated with GPT/tat-CN21 exhibited a statistical

increase in neuronal death, similar to cultures treated with tat-CN21 alone

(Figure 27C). Significant neurotoxicity was also observed when cultures were

treated with pyruvate and tat-CN21 (19.077 ±1.876%). Once more, both omission

of pyruvate and boiling of GPT prior to application resulted in a failure in

preventing tat-CN21-induced neurotoxicity (Figure 27C). Together, these data

indicate that enzymatically degrading the glutamate released after CaMKII

inhibition prevents calcium dysregulation and neuronal death.

We next examined if calcium entering through glutamate receptors,

specifically the NMDA receptors contributes to the observed calcium influx.

Application of 20 µM MK-801 reduced tat-CN21-induced calcium influx by ~80%

(Figure 28A). As mentioned earlier, the calcium influx induced by CaMKII

inhibition was also blocked by pharmacological antagonism of synaptic activity

(via blockade of VGSCs, AMPA-Rs, and VGCCs). To determine the contribution

of synaptic NMDA receptors in this calcium dysregulation, cortical neurons were

treated with bicuculline to induce synaptic activity by inhibiting GABAergic

signaling as described previously (Hardingham et al., 2002, Ivanov et al., 2006).
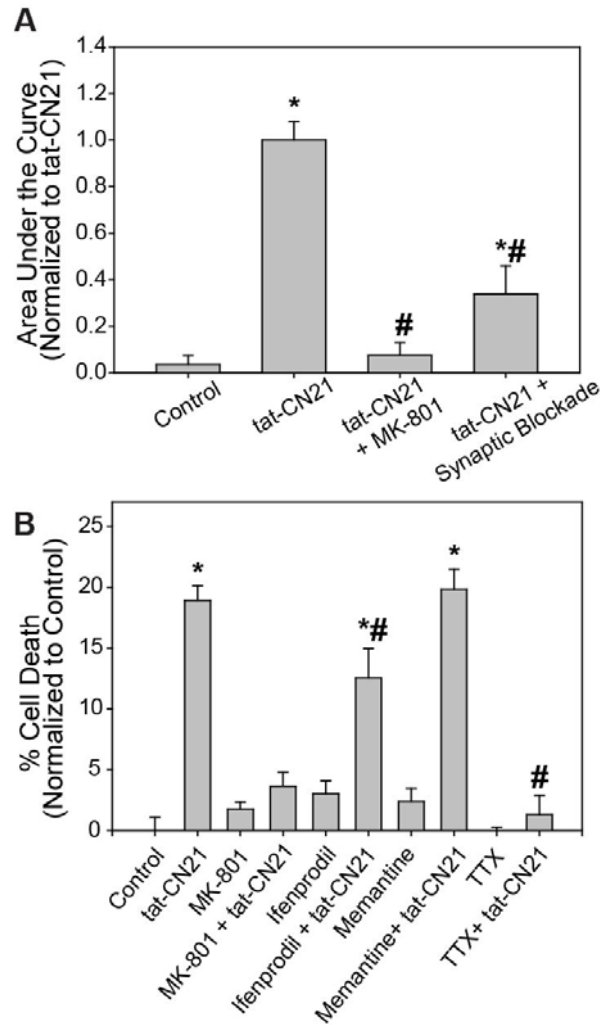
Figure 28: Pharmacological antagonism of the NMDA receptor prevents acute and prolonged effects of CaMKII inhibition. A, Average integral of fluorescent intensity from 0-1200 seconds (mean ± SEM, n=3-6) reflecting calcium influx in control neurons, or neurons subjected to treatment with tat-CN21 alone or in combination with 20 µM MK-801 or in combination with a prior synaptic NMDA-R blockade. To block synaptic NMDA-Rs before tat-CN21 treatment, 10 µM bicuculline was applied to allow synaptic activity, followed by the addition of 20 µM MK-801 to inhibit the synaptic NMDA-Rs opened as a result of this synaptic activity. *$p < 0.05$ compared to control while #$p < 0.05$ compared to tat-CN21 (One-Way ANOVA, post-hoc Dunnett's test). **B,** Neuronal death (mean ± SEM, n=4-24) after 24 hour treatment with 10 µM tat-CN21 alone or in the presence of 20 µM MK-801, 10 µM ifenprodil, 1 µM memantine, or 200 nM TTX. *$p < 0.05$ compared to control while #$p < 0.05$ compared to tat-CN21 (One-Way ANOVA, post-hoc Dunnett's test).

Rapid increases in cytoplasmic calcium were observed with bicuculline treatment. MK-801 was added immediately following exposure to bicuculline to block the open NMDA-Rs (Huettner and Bean, 1988) (Figure 29). The CaMKII inhibitor, tat-CN21, was then applied and subsequent changes in intracellular calcium concentration were monitored. Interestingly, this blockade of synaptic NMDA-Rs significantly blunted tat-CN21-induced calcium influx (Figure 28A). However, this treatment did not maintain levels of intracellular calcium to those observed in control, indicating that while much of the calcium influx observed with CaMKII inhibition was via the synaptic NMDA receptors, calcium entering through extrasynaptic NMDA-Rs may also contribute to this process.

Because antagonism of the NMDA receptor successfully blunted the acute increase in calcium influx, we hypothesized that MK-801 would also reduce the neurotoxicity induced by CaMKII inhibition. Compared to cultures treated with tat-CN21 alone, neurotoxicity was reduced nearly 80% when tat-CN21 was co-applied with 20 µM MK-801 (Figure 28B). We next attempted to block extrasynaptic NMDA-Rs to determine the influence of extrasynaptic versus synaptic NMDA-Rs to the neurotoxicity induced by CaMKII inhibition. Low levels of ifenprodil and memantine have been shown to be preferential antagonists to the NR2B-containing extrasynaptic NMDA-Rs (Thomas et al., 2006, Xia et al., 2010). There was still a significant increase in the observed neurotoxicity when tat-CN21 was co-applied with 10 µM ifenprodil or 1 µM memantine, with neither drug affecting viability alone (Figure 9B). However, ifenprodil does reduce the levels of toxicity below that observed with tat-CN21 alone (Figure 28B),
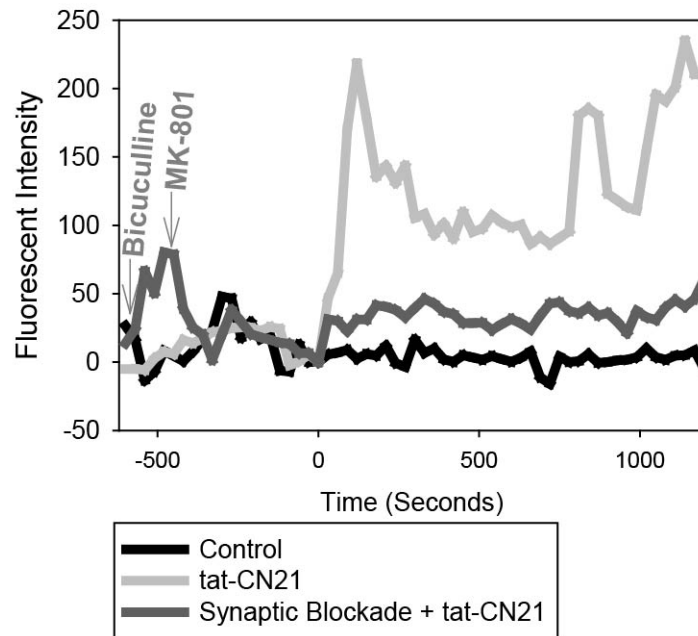
Figure 29: Calcium influx with CaMKII inhibition is blunted by synaptic blockade. Representative traces of intracellular calcium levels in a field of control cortical neurons or a field treated with tat-CN21 with/without prior synaptic blockade. To induce synaptic blockade (dark gray trace), 10 µM bicuculline was applied to the bath (see arrow). When intracellular calcium levels became elevated, 20 µM MK-801 was applied (see arrow). At -300 seconds, control and antagonist-treated cultures were washed with physiological saline before application of 10 µM tat-CN21 at time 0.

suggesting that extrasynaptic NMDA-Rs may play a partial role in the toxicity associated with the loss of CaMKII signaling. Increasing the dose of memantine to 10 µM, a level that inhibits not only extrasynaptic NMDA-Rs but also partially inhibits synaptic NMDA-Rs, does significantly reduce neuronal death (13.6 ± 4.6%). When cultures were treated with low levels of TTX (200 nM) to block action potential-induced synaptic activity, tat-CN21-induced toxicity was brought back to baseline (Figure 28B). Thus, while extrasynaptic NMDA-Rs cannot be ruled out, it is quite convincing that synaptic activity is necessary for both the calcium dysregulation and neurotoxicity associated with a loss of CaMKII signaling (Thomas et al., 2006, Xia et al., 2010). In total, these data suggest that CaMKII inhibition induces a slow-tonic excitotoxic event via calcium dysregulation, enhanced neuronal excitability, and augmented extracellular glutamate levels.

Finally, to determine if the accumulation of glutamate in the bath solution was solely responsible for this neurotoxicity, cortical neurons were treated for 24 hours with 10 µM tat-CN21. Then, the conditioned media was removed from these cultures and directly applied to naïve cortical neurons. Neurons treated with the conditioned media did not display a significant difference in viability compared to non-treated control cultures (Figure 30). We also did not observe significant neuronal death when 4 µM glutamate was applied to our cortical cultures for 24 hours (6.9 ± 3.1% vs control 5.8 ± 2.2%), suggesting that this glutamate concentration in the media is not sufficient to induce neuronal death in the absence of CaMKII inhibition. Thus, while the increased extracellular
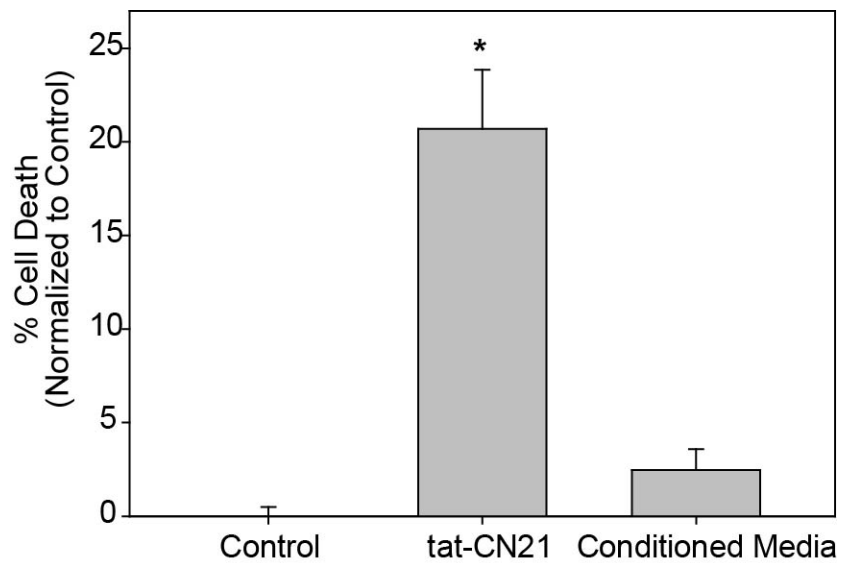
Figure 30: Conditioned media from neurons treated with CaMKII inhibitors does not induce neurotoxicity. Neuronal death (mean ± SEM, n=3-9) in neurons treated with 10 µM tat-CN21 for 24 hours or naïve neurons treated for 24 hours with media removed from tat-CN21-treated neurons. *p<0.05 compared to control (One-Way ANOVA, post-hoc Dunnett's test).

glutamate resulting from CaMKII inhibition is necessary for the observed

neurotoxicity, it is not sufficient to produce the toxicity itself. Together, these data

indicate that the combination of elevated glutamate with the loss of CaMKII

activity induces toxicity. Thus, the mechanism underlying the neurotoxicity with

CaMKII inhibition is the decreased ability of neurons to tolerate glutamate

stimulation, *including* the stimulation resulting from the low levels of glutamate

associated with the neuronal hyperactivity directly resulting from CaMKII

inhibition.

DISCUSSION

Prolonged CaMKII inhibition using both small molecule (KN-93) and

peptide (tat-AIP, myr-AIP, and tat-CN21) inhibitors is toxic to cultured neurons in

the presence and absence of astrocytes. Although an acute one hour exposure is

not toxic, all of these inhibitors produced neuronal death after 24 hours. We

elected to use pharmacological approaches over genetic knockdown of CaMKII

in order to better mimic the time-course of CaMKII inactivation associated with

ischemic brain trauma and other diseases associated with aberrant neuronal

activity. Pharmacological inhibitors afford the opportunity to determine acute

changes in neuronal activity and calcium homeostasis with CaMKII inhibition.

Plus, the CaMKII inhibitors used are not thought to display any isoform

specificity, avoiding potential complications associated with isoform

compensation associated with genetic knockdown. Multiple pharmacological

inhibitors (small molecule and peptide) employing different methodologies for cell

uptake (cell permeable small molecule versus tat and myristolated peptide import

strategies) were used to limit the possibility of off-target effects confounding our conclusions.

The concentration of tat-CN21 (10 µM) used throughout this study is similar previous studies using this CaMKII inhibitor to explore neurite extension (Vest et al., 2007) and neuroprotection to a glutamate-insult (Vest et al., 2010, Ashpole and Hudmon, 2011). Although these values are ~100 fold over the IC_{50} for CN21 *in vitro* (Vest et al., 2007, Ashpole and Hudmon, 2011), cell uptake and bioavailability can be limiting for intracellular peptide inhibitors (Tunnemann et al., 2006), making the peptide concentrations used in this and other studies reasonable pharmacological concentrations. Finally, previously established inactive controls for KN-93 (KN-92) (Sumi et al, 1991) and tat-CN21 (tat-CN21Ala) (Ashpole and Hudmon, 2011) did not induce neuronal death at the concentration of the active inhibitors that clearly produced toxicity, suggesting that the neuronal toxicity observed is due to CaMKII inactivation.

Prolonged CaMKII inhibition is consistent with features of both necrotic and apoptotic cell death. Apoptotic cell death is supported by the following observations: 1) application of the inhibitors required an incubation period of >8 hours to induce toxicity, 2) inhibitor application led to an increase in TUNEL staining, 3) colocalization between neurons that take up fluorescent tat-CN21 and cleaved caspase-3 was observed, and 4) cell death was prevented by the protein translation inhibitor, cycloheximide. The pro-apoptotic Bcl-2-associated death promoter (BAD) protein is inactivated by CaMKII phosphorylation. Thus, it is possible that the prolonged inhibition of CaMKII activity dysinhibits the BAD

cascade, leading to apoptosis (Bok et al., 2007). A population of neurons containing the CaMKII inhibitor did not exhibit caspase-3 staining, yet these neurons consistently exhibited morphological changes in the nucleus (condensation and fragmentation). It is unclear whether these neurons represent a continuum between necrosis and apoptosis, or whether this could be specifically related to apoptosis with secondary necrosis (Bonfoco et al., 1995).

CaMKII inhibition results in a slow sustained increase in intracellular calcium levels that is accompanied by elevated glutamate and enhanced neuronal excitability. Calcium dysregulation occurs within 10-20 minutes of exposure to tat-CN21; a time-course that correlates with maximal fluorescent uptake of tat-based peptides in our cortical cultures (Ashpole and Hudmon, 2011). Although L-type voltage-gated calcium channels do not appear to contribute to calcium dysregulation following CaMKII inhibition, ion channels regulating neuronal activity (voltage-gated sodium channels and N-type voltage-gated calcium channel) are critical for this process. Consistent with synaptic coupling required for calcium dysregulation, we observed that functional glutamate receptors (AMPA- and NMDA-subtypes) as well as elevated glutamate to be necessary for this process. While synaptic NMDA-Rs appeared to largely dictate calcium dysregulation and neuronal toxicity to CaMKII inhibition, we cannot rule out a contribution played by extrasynaptic NMDA-Rs in these processes. The involvement of both synaptic and extrasynaptic NMDA-Rs in calcium dysregulation and toxicity is reasonable considering that elevated levels of glutamate appear to accumulate in the media over time, with enhanced

extracellular glutamate levels observed as early as 20 minutes following exposure to CaMKII inhibitors. Thus, while increased synaptic transmission may be necessary, it is possible that once glutamate accumulates in the milieu, it can also activate receptors outside of the synaptic cleft. Interestingly, exposure to elevated glutamate using conditioned media or exogenous glutamate is not toxic in the absence of CaMKII inhibition, suggesting that glutamate by itself is not sufficient for neurotoxicity in the absence of CaMKII inhibition. However, the elevated extracellular glutamate in conjunction with CaMKII inhibition appears to be essential to both calcium dysregulation and neurotoxicity, as enzymatic buffering of extracellular glutamate or pharmacological inhibition of the AMPA or NMDA receptors prevents calcium dysregulation. The fact that media exchange after 4 hours prevents neuronal toxicity to CaMKII inhibition is consistent with these observations, further supporting an important functional and temporal association between CaMKII inhibition and neuronal activity in this form of neurotoxicity.

Although our data does not rule out the possibility that elevated extracellular glutamate is produced by cell lysis, the observation that an increase in the number of action potentials induced by a depolarizing current following CaMKII inhibition by localized delivery of the CN21 inhibitor to individual cortical neurons supports the hypothesis that CaMKII inhibition directly enhances neuronal excitability. This acute response in neuronal excitability to CaMKII inhibition is novel. However, genetic knock down of αCaMKII in mice (Butler et al., 1995) or neuronal cultures by siRNA (Carter et al., 2006) support these

findings. Thus, results from experiments examining the effect of CaMKII inhibition via pharmacological (this study) or genetic approaches (Butler et al., 1995, Carter et al., 2006) support the hypothesis that CaMKII is a major regulator of neuronal excitability.

Our experiments favor a model whereby the sustained inhibition of CaMKII activity instigated a vicious cycle of sustained increases in intracellular calcium due to sustained glutamate release. In essence, CaMKII inhibition initiates an excitotoxic cycle by increasing neuronal excitability which subsequently supports enhanced glutamate levels in the media; a feed forward cycle that can be broken by preventing neuronal activity, blocking calcium dysregulation or by removing extracellular glutamate. These data suggest that CaMKII may be viewed to function in neurons as a brake for glutamate-excitation and/or as a master regulator of neuronal excitability and calcium homeostasis. Finally, the observed results do not appear to be limited to highly purified cortical cultures, as co-cultures of cortical neurons with astrocytes also display neuronal death following CaMKII inhibition. We cannot rule out the potential of glial function or viability being altered following CaMKII inhibition in mixed cultures, as glial cells also express CaMKII (δCaMKII (Takeuchi et al., 2000)), thus on-going experiments will need to examine consequences of CaMKII inhibition in astrocyte function and survival.

How could CaMKII inhibition impact calcium-induced neuronal death during ischemia and other excitotoxic stimuli? CaMKII has been shown to inactivate in the core of an ischemic insult *in vivo* as well as in the surrounding

penumbral tissue (Hanson et al., 1994); a phenomena also observed following aberrant neuronal activity in epilepsy (Yamagata et al., 2006). The mechanism of CaMKII inactivation in these diseases is not well understood, but it is known that CaMKII proteolysis is preceeded by post-translational modifications (Churn et al., 1992a), including a soluble to particulate transition consistent with CaMKII aggregation following ischemia (Aronowski et al., 1992, Hanson et al., 1994, Tao-Cheng et al., 2002). Inactivation associated with aggregation is consistent with CaMKII self-association; a form of catalytic aggregation that requires calcium-CaM activation and is maximized under ischemic conditions (i.e. reduced energy) (Hudmon et al., 1996, Hudmon et al., 2005). In the current study, we have attempted to mimic one consequence of CaMKII self-association via pharmacological inhibition of enzymatic activity. Similar to *in vivo* studies characterizing functional changes in CaMKII associated with aberrant neuronal activity, we have observed a sustained loss of activatable CaMKII with long-term tat-CN21 exposure in cultured cortical neurons. This sustained inactivation and transition of αCaMKII from the soluble to particulate fractions has been previously shown to also accompany excitotoxic glutamate-glycine insults in neuronal cultures (Hudmon et al., 2005, Ashpole and Hudmon, 2011). An intriguing hypothesis is that the neuroprotection produced by acute exposure to CaMKII inhibitors may be limiting neurotoxicity to excitotoxic glutamate/glycine challenges by paradoxically preventing excitotoxic-induced CaMKII inactivation-aggregation.

Independent of this speculation, the present results indicate that sustained inactivation of CaMKII leads to neuronal cell death through engagement of apoptotic/necrotic pathways induced by calcium dysregulation and hyperexcitability, which contributes to the decreased capacity of neurons to cope with excitatory insults. These data may provide further mechanistic insight into the increased infarct size observed within αCaMKII knock-out animals (Waxham et al., 1996), and moreover, to the phenomenon of expanding neuronal damage in the ischemic penumbra. Peri-infarct depolarizations have been shown to underlie the progression of neuronal damage from the core throughout the penumbra (Mies et al., 1993, Ohta et al., 2001, Fujioka et al., 2004). Interestingly, these depolarizations have been shown to be calcium dependent and are significantly reduced by NMDA receptor antagonism (Ohta et al., 2001). These findings are consistent with the functional consequences of CaMKII inactivation highlighted in this study. Thus, our working hypothesis is that the extent of neuronal damage in the penumbral region is governed by the loss of CaMKII, which increases neuronal activity and heightens susceptibility to excitotoxic-related insults, such as glutamate and ROS activity.

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Part III: LOSS OF CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE II ACTIVITY IN CORTICAL ASTROCYTES INDUCES NEUROTOXIC ATP RELEASE

<u>SUMMARY</u>

The extent of calcium/calmodulin-dependent protein kinase II (CaMKII) inactivation in the brain following ischemia directly correlates with the extent of damage. We have previously shown that a loss of CaMKII in neurons is detrimental to neuronal viability. In the current study, we detail functional changes in cortical astrocytes with CaMKII inhibition along with examining how these changes impact neurons. CaMKII inhibition in cultured astrocytes using either a small molecule (KN-93) or a peptide inhibitor (tat-CN21) is associated with calcium oscillations and dysregulated intracellular calcium levels. Surprisingly, this calcium influx could be blocked by the N-type calcium channel antagonist, omega-conotoxin. While the function of N-type calcium channels within astrocytes is controversial, these voltage-gated calcium channels have been linked to calcium-dependent vesicular gliotransmitter release. When extracellular glutamate and ATP levels were measured following CaMKII inhibition, we observed glutamate levels were not altered, whereas ATP levels in the extracellular environment increased with CaMKII inhibition. Extracellular ATP accumulation associated with CaMKII inhibition contributes both to calcium dysregulation within astrocytes and cortical neuron toxicity. Thus, CaMKII inhibition within astrocytes dysregulates calcium signaling supporting ATP

release – a process that fuels calcium oscillations and dysregulation in astrocytes along with toxic gliotransmitter activity in neurons.

INTRODUCTION

Astrocytes play a critical role in regulating neuronal function and plasticity by providing structural support, releasing neurotrophic factors, tuning synaptic transmission, and buffering neurotoxic ions/neurotransmitters produced by normal/aberrant neuronal activity (Banker, 1980, Rosenberg and Aizenman, 1989, Pfrieger and Barres, 1997, Barres, 2008, Ransom and Ransom, 2012). The excitatory neurotransmitter glutamate is essential for normal synaptic activity amongst a majority of neuronal synapses; however, the aberrant neuronal activity and damage that accompanies ischemia and traumatic brain injury lead to toxic levels of extracellular glutamate – a process classically defined as excitotoxicity. Importantly, astrocytes have been shown to decrease the sensitivity of neurons to glutamate excitotoxicity 100-fold in culture systems, presumably by their ability to take-up and buffer extracellular glutamate (Rosenberg and Aizenman, 1989). We have previously shown that inhibition of calcium/calmodulin-dependent protein kinase II (CaMKII) within cultured cortical neurons leads to the induction of excitotoxic glutamate release. Inclusion of astrocytes in these neuronal cultures did not alleviate neuronal toxicity, suggesting that astrocyte-neuronal communication is compromised when CaMKII is inhibited.

A significant decrease in CaMKII is seen within the core and penumbral tissue regions following a stroke (Hanson et al., 1994). The extent in the loss of

CaMKII signaling directly correlates with the extent of tissue damage, supporting the hypothesis that CaMKII inhibition may be detrimental to neuronal survival (Hanson et al., 1994). This model is further supported by data showing that pharmacological and genetic CaMKII inhibition compromises neuronal survival to excitotoxic insults (Waxham et al., 1996, Ashpole and Hudmon, 2011, Ashpole et al., 2012). However, what role or contribution that CaMKII inhibition plays in astrocytes and other support cells to glial-neuronal function and communication is unknown.

Although CaMKII is best known for its function in neurons, CaMKII is expressed throughout several cell types in the brain, including glial and endothelial cells (Fukunaga et al., 1988, Takeuchi et al., 2000). For example, while αCaMKII is the predominant isoform in neurons (Ouimet et al., 1984), the δCaMKII variant appears to be the predominant isoform within astrocytes (Takeuchi et al., 2000). Within astrocytes, CaMKII signaling regulates cytoskeletal networks (Yano et al., 1994, Ogawara et al., 1995), gene expression (Yano et al., 1996), and apoptosis (Kubes et al., 1998, Song et al., 2006). Thus, it is conceivable that the loss of CaMKII activity within ischemic tissue represents CaMKII inactivation in neurons as wells as astrocytes and other supporting cells. In the current study, we used acute and chronic application of CaMKII inhibitors to investigate calcium signaling and gliotransmitter release in cultured cortical astrocytes. These data support a feed-forward model by which CaMKII inhibition in astrocytes compromises astrocyte calcium homeostasis and ultimately neuronal survival via ATP release. Thus, we propose a model whereby a loss

CaMKII activity in astrocytes further compromises neuronal survival to aberrant glutamate signaling through extracellular accumulation of ATP; a process that could greatly impact the spread and severity of the neuronal death in the penumbral and border zones of an ischemic insult.

EXPERIMENTAL PROCEDURES

Materials. Peptide inhibitors including tat-CN21 (YGRKKRRQRR-KRPPKLGQIGRSKRVVIEDDR) and tat-CN21Ala (YGRKKRRQRR-KAPAKAAQAAASKRVVIEDDR) as well as Fam-labeled versions of these peptides were synthesized by Biopeptide Co. Inc, San Diego, CA. KN-93 (422708) and KN-92 (422709) were purchased from Calbiochem. Myristolated AIP (64929) was purchased from Anaspec, Fremont, CA. MRS 2179 (0900), A 740003 (3701), and ARL 67156 (1283) were purchased from Tocris. MK-801 (M107), CNQX (C239), nifedipine (N7634), omega-conotoxin (C9915), and suramin (S2671) were purchased from Sigma.

Neuron and astrocyte cultures. Mixed co-cultures of neurons (both cortical and hippocampal) and astrocytes were derived from E18 to E19 Sprague-Dawley rat pups according to approved IACUC guidelines as described previously (Ashpole et al., 2012). Pure astrocytes were derived from postnatal day 1-3 Sprague-Dawley rat pups following methods established by McCarthy and de Vellis (McCarthy and de Vellis, 1980). Following dissociation, digestion, and titruation, cortical cells were resuspended in growth media (DMEM containing 2% NuSerum, penicillin (10 units/mL), streptomycin (10 µg/mL), and L-glutamine (29.2 µg/mL)) at a density of 2.5 million cells/mL and seeded on poly-d-lysine

(50 μg/mL) coated 10 cm dishes. Cells were feed every 3-4 days, with half of the conditioned media being replaced with fresh growth media. When the cultures became confluent (7-8 DIV), the plates were shaken to remove oligodendrocytes. Following a wash, the astrocytes were then split (using trypsin), and seeded on 12 mm or 15 mm coverslips coated in poly-d-lysine. The cultures were then grown in neuronal growth media (Neurobasal containing 2% NuSerum, 2% NS21, penicillin (10 units/mL), streptomycin (10 μg/mL), and L-glutamine (29.2 μg/mL) until treatment (following an additional 3-4 DIV). Pure cortical neurons were grown on 12 mm or 15 mm poly-d-lysine coverslips as previously described (Ashpole et al., 2012) for 8-10 DIV until treatment.

Calcium imaging. Co-cultures of neurons and astrocytes (10-12 DIV) as well as cultures of pure astrocytes were loaded with Fluo-4AM or 2.6 μm Fura-2FF-AM and subsequently imaged as described previously (Ashpole et al., 2012). The use of Fluo-4AM or Fura-2FF-AM is highlighted in the Results. During imaging, the cultures were incubated in rat physiological saline (138 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.06 mM $MgCl_2$, 12.4 mM HEPES, pH 7.4, 5.6 mM glucose; final pH adjusted to 7.3) as described (Ashpole et al., 2012). A Nikon Ti-E inverted fluorescent microscope was utilized to monitor fluorescent intensity once every 5-10 seconds. Baseline was monitored for 2-5 minutes. To identify neurons in the co-culture experiments, a 20mM KCl depolarization at the start of the imaging was employed. Neurons were identified as cells that immediately responded to KCl with a robust increase in calcium. In all experiments with CaMKII inhibitor application, the inhibitor was added 5 minutes after start of

imaging. For experiments requiring various receptor/channel antagonists, the drugs were applied at the 2 minute mark to identify whether the drug itself had an impact on calcium levels prior to CaMKII inhibitor application. Analysis was performed using Nikon Elements v3.0 in which fluorescent intensity measured in at least 10 cells per field. The fluorescent intensity of each cell was normalized to time 0 (or the 5 minute mark), as the CaMKII inhibitor was applied.

Glutamate uptake. Glial cultures (3-4 DIV following split unto coverslips) were treated with CaMKII inhibitors in combination with 1.5 µCi/mL H^3-glutamate and 0.5 µM unlabeled glutamate for various lengths of time, as indicated. Cultures were then washed in cold PBS and lysed in 20 mM Tris, pH 7.4, 200 mM NaCl, 0.1 mM EDTA, and 2X protease inhibitor mixture (Calbiochem, 539137) as described previously (Ashpole and Hudmon, 2011, Ashpole et al., 2012). The lysate was then diluted in Ready Safe liquid scintillation cocktail (Beckman Coulter), vortexed, and H^3 was measured using a liquid scintillation β-counter.

Immunocytochemistry of astrocyte cultures. Glial cultures (3-4 DIV following split unto coverslips) were immunostained as previously described (Ashpole and Hudmon, 2011, Ashpole et al., 2012). Following fixation, permeabilization, and blocking, the cultures were incubated in polyclonal anti-GFAP, monoclonal anti-OX-42, polyclonal anti-pan-CaMKII, or monoclonal anti-vimentin overnight at 4 degrees. After three washes, secondary antibodies (anti-rabbit Alexa$_{680}$ or anti-mouse Alexa$_{594}$, 1:5000 (Molecular Probes)) were applied for 1 h at room temperature. Coverslips were washed in PBS three times and were subsequently mounted in Prolong Gold Antifade with DAPI mounting media (Molecular

Probes), and cells were imaged using a Zeiss Axio ObserverZ1 and processed with Axiovision 4.

CaMKII activity assay. Astrocyte cultures were lysed in 20mM Tris, pH 7.4, 200 mM NaCl, 0.1 mM EDTA, and 2X protease inhibitor mixture (Calbiochem, 539137) as described previously (Ashpole and Hudmon, 2011, Ashpole et al., 2012), sonicated, and incubated with 0.1% Triton-X-100 for 5 minutes. To measure total CaMKII activity, a portion of the lysate was then incubated with 50 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}P]ATP (3 µCi per reaction) for 3 minutes at 30°C. To measure autonomous CaMKII activity, a portion of the lysate was incubated in 50 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 100 µM ATP, 5 mM EGTA, 50 µm AC-2, and [γ-^{32}P]ATP (3 µCi per reaction) for 3 minutes at 30°C. The linear range of the phosphorylation reactions extended from 1 to 10 minutes. Protein levels were assessed using the DC protein assay kit (Bio-RAD) and activity was normalized to total protein.

Inhibitor uptake analysis. Fluorescently conjugated peptides (tat-CN21-Fam and tat-CN21Ala-Fam) were diluted in fresh neuronal growth media and applied to astrocyte cultures at a final concentration of 10 µM for varying lengths of time (0-2 min). After treatment, coverslips were washed 3 times in PBS before blotting and were then mounted in Prolong Gold Antifade with DAPI mounting media. Coverslips were then imaged in three different fields with a Zeiss Axio Observer Z1 and processed with Axiovision 4. The total cell number (DAPI staining) and the total number of cells containing the fluorescent peptides (FITC detection)

were then quantified. No fluorescence was detected in cultures that were not treated with the fluorescent peptides.

ATP measurements. ATP concentrations in the media were assessed using the Enlighten ATP Assay System per manufacturer's protocol (Promega). The luciferase was detected and quantified using a Victor V3 plate reader. The ATP detection assay standard curve was linear from 0.001 nM to 0.1 μM.

Cell death measurements. Neuronal coverslips were stained using Live/Dead Cytotoxicity/Viability kit (Molecular Probes) as previously described (Ashpole and Hudmon, 2011, Ashpole et al., 2012). The cells were imaged using a Zeiss Axio Observer Z1 and processed with Axiovision 4 (x100 magnification). Each coverslip was imaged in three different fields. The images were exported and automated cell counting software (Nikon Elements v3.0) was used to quantify cytotoxic cells (Texas red filter), viable cells (FITC filter), or total cell number (DAPI filter).

Data analysis. Statistical analysis was performed using SigmaPlot v11 software. One-way ANOVA with a subsequent Dunnett's test was used to compare differences between the means of each group in the *in situ* calcium imaging experiments, *in vitro* catalytic assays, and *in situ* cell death assays. When appropriate, a Student's *t* test was also performed.

RESULTS

Calcium Dysregulation with CaMKII inhibition in Neuronal/Glial Cultures

The degree of CaMKII inactivation within the brain following stroke directly correlates with the extent of neuronal damage (Hanson et al., 1994). We have

previously shown that inhibition of CaMKII in cultured cortical neurons induced an excitotoxic neuronal death cascade (Ashpole et al., 2012). This neuronal death was also observed in co-cultures of neurons and astrocytes, suggesting that perhaps the neuroprotective function of astrocytes was compromised when CaMKII was inhibited (Ashpole et al., 2012). Because aberrant neuronal calcium influx contributed to the neurotoxicity induced by CaMKII inhibition, we first examined whether similar patterns of calcium dysregulation were observed in the co-cultures of neurons and astrocytes following application of CaMKII inhibitors as previously observed in our pure neuronal cultures. For this, we optically monitored intracellular calcium levels using Fluo-4AM in our co-cultures of neurons and astrocytes. Previously, we characterized the cellular content of these co-cultures and identified that roughly 40% of cells in these cultures were MAP-2 positive neurons and nearly 60% were GFAP-positive astrocytes (Ashpole et al., 2012). To inhibit CaMKII, we used the specific CaMKII inhibitor, tat-CN21 (Vest et al., 2007, Ashpole and Hudmon, 2011, Ashpole et al., 2012). CN21 is a peptide inhibitor derived from the endogenous CaMKII inhibitory protein in the brain originally termed CAMKII-N (Chang et al., 1998). Because the peptide inhibitor is not membrane permeable, it was conjugated to the cell-penetrant motif, tat, for intracellular delivery of the peptide inhibitor as described previously (Vest et al., 2010, Ashpole and Hudmon, 2011, Ashpole et al., 2012). As shown previously in highly enriched neuronal cultures (Ashpole et al., 2012), we observed that application of tat-CN21 peptide resulted in increased intracellular calcium levels (Figure 1A); however, the kinetics of this increase in

intracellular calcium appeared markedly different than the slow-onset previously observed in our enriched cortical cultures. The increase in intracellular calcium levels seen with the active CaMKII inhibitor tat-CN21 were not seen using our control peptide, tat-CN21Ala (Ashpole and Hudmon, 2011). To tease apart the contribution of changes in Fluo-4AM fluorescence within the mixed culture system, a KCl depolarization stimulation was used to identify the fluorescent calcium signatures of neurons. Because nearly all of the cells that were not MAP-2 positive in these co-cultures were GFAP-positive (Ashpole et al., 2012), the cells that did not immediately respond to the KCl were considered astrocytes. Using this method to deconvolute the calcium signatures of neurons versus glial cells, we found that as we described previously (Ashpole et al., 2012), neurons undergo a characteristic delayed calcium dysregulation (Figure 31B). Astrocytes appear quite different in both the time of onset and oscillatory nature of the kinetics for changes in intracellular calcium levels (Figure 31B-C). In addition, unlike the dysregulated calcium levels seen in neurons, the dysregulated intracellular calcium levels did not appear to keep increasing over time in the glial cells (Figure 31C). These findings in mixed cortical cultures were consistent when co-cultures of hippocampal neurons and astrocytes were loaded with Fura-2FF as the calcium indicator and subjected to CaMKII inhibitor application (Figure 32). These data demonstrate that the calcium dysregulation seen in cultured cortical neurons and hippocampal neurons is accompanied by aberrant
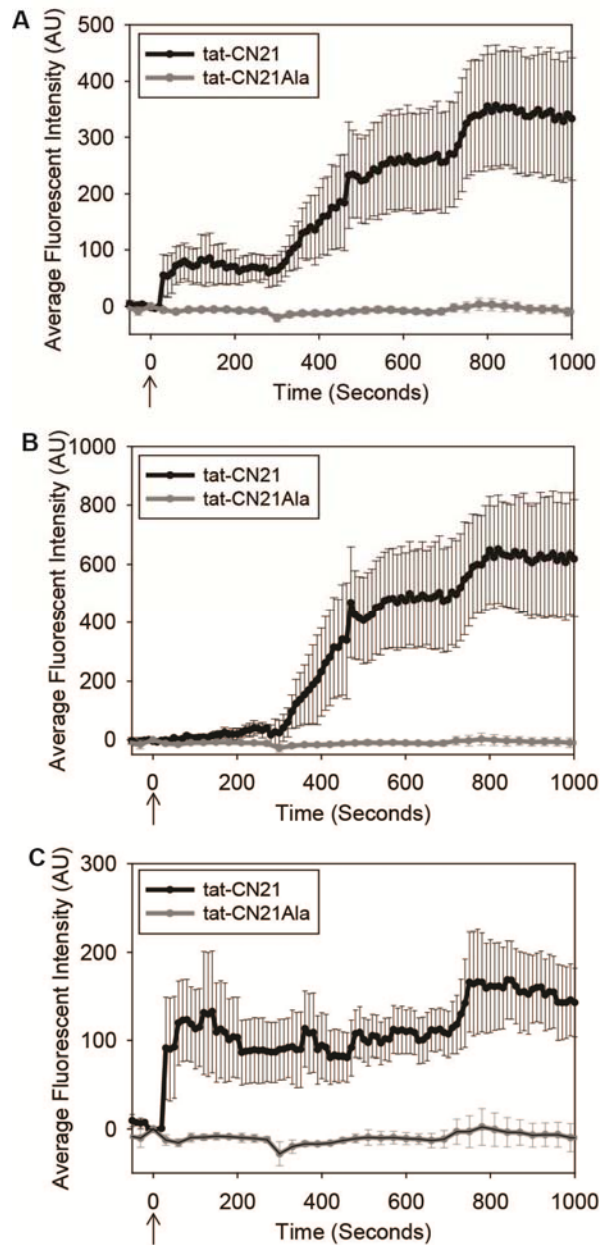
Figure 31: Calcium dysregulation in neuronal/astrocyte cultures with CaMKII inhibition. A, Average trace (+/- SEM, n=3) of calcium response in mixed cultures of cortical neurons and astrocytes treated with 10 µM tat-CN21 or tat-CN21Ala, as measured by Fluo-4AM. **B,** Average trace (+/- SEM, n=3) of calcium response with tat-CN21 or tat-CN21Ala application in cells within the field of interest that responded to a depolarizing 20 mM KCl pulse at time -300 seconds (considered neurons). **C,** Average trace (+/- SEM, n=3) of calcium response with tat-CN21 or tat-CN21Ala application in cells that did not respond to the KCl pulse at time -300 seconds(considered astrocytes).
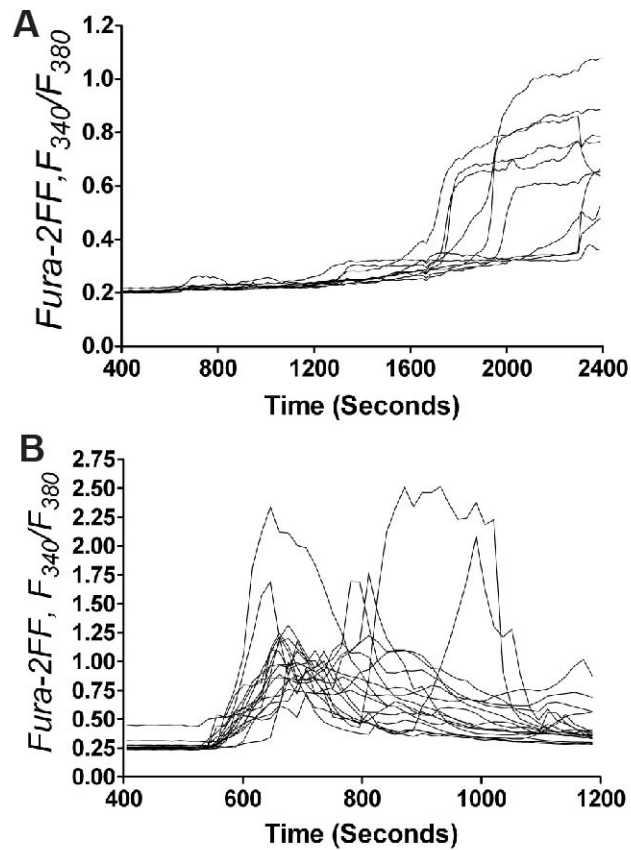
Figure 32: CaMKII inhibition dysregulates calcium in neurons and astrocytes. A, Representative traces of Fura-2FF calcium response in hippocampal neurons (identified by KCl pulse at time 0) following 10 µM tat-CN21 application. **B,** Representative traces of Fura-2FF calcium response in astrocytes (which did not respond to KCl at time 0) following tat-CN21 application.

Courtesy of the Brustovetsky Lab

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calcium signaling of different kinetics (faster onset, oscillations, and decay) than the surrounding astrocytes.

CaMKII Inhibition in Astrocytes

In order to better understand the impact of CaMKII inhibition specifically within the one population of neuronal support cells, cortical astrocytes devoid of neurons were cultured. Although we employed a well-characterized methodology for astrocyte cultures (McCarthy and de Vellis, 1980)(*see Methods*), we used immunohistochemistry to further characterize the cellular content of specific cellular markers within these cultures. Fluorescent immunostaining shows that 93.9 +/- 10.8 % (n = 6) of these cells were GFAP positive and 78.9 +/- 21.2% (n = 6) were vimentin positive (Figure 33A-C), while only 2.3 +/- 3.9% (n = 6) were OX42 positive, suggesting these cultures were predominantly reactive astrocytes with little microglial contamination. We do not see any MAP-2 immunostaining in these enriched astrocyte cultures. In addition, 93.0 +/- 9.8% of our astrocytes in culture exhibited CaMKII staining using a pan-CaMKII primary antibody (Figure 33B-C).

In addition, because one study has previously published that a significant fraction of CaMKII existed in the activated (i.e. autophosphorylated) state in cultured astrocytes (Song et al., 2006), we also measured the Ca^{2+}/CaM-dependent and Ca^{2+}/CaM-independent CaMKII activity, using the highly specific CaMKII peptide, AC-2, in our well-characterized *in vitro* kinase assays (Hudmon et al., 1996, Ashpole et al., 2012). The CaMKII activity measured in the presence of Ca^{2+}/CaM is defined as the total pool of CaMKII activity, whereas, the CaMKII
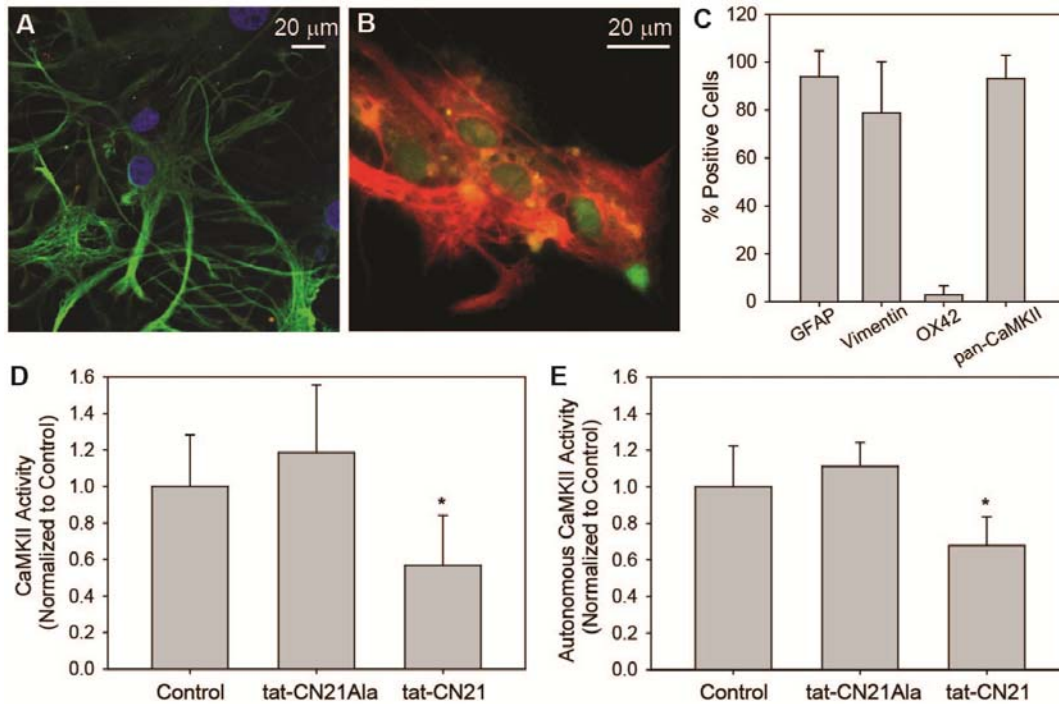
Figure 33: CaMKII expression and activity in cultured cortical astrocytes. A, Representative image of a field of astrocytes immunostained with GFAP (green), OX42 (red), and Hoecsht (blue). **B,** Representative image of field of astrocytes immunostained with vimentin (red) and pan-CaMKII (green). **C,** Average number of cells (n=3, +/- SEM) positively stained with GFAP, vimentin, OX42, and CaMKII. **D,** Average Ca^{2+}/CaM-stimulated (total) CaMKII activity within astrocyte lysates treated with 10 µM tat-CN21 or tat-CN21Ala. Inhibitors were added to the cultures 10 minutes before lysis and activity was measured *in vitro* via P-32 incorporation unto AC-2, a known CaMKII substrate. The asterisk indicates significant difference compared to control (*$p<0.05$, One-Way ANOVA, post-hoc Dunnett's test). **E,** Average CaM-independent (autonomous) CaMKII activity within astrocyte lysates treated with 10 µM tat-CN21 or tat-CN21Ala as described in **D**. The asterisk indicates significant difference compared to control (*$p<0.05$, One-Way ANOVA, post-hoc Dunnett's test).

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activity measured in the absence of Ca^{2+}/CaM is defined as the pool of autonomous, or Thr286 autophosphorylated pool of CaMKII (Lai et al., 1986, Lou et al., 1986, Schworer et al., 1988). The fraction of CaMKII autophosphorylated at Thr286 is believed to represent the pool of CaMKII activated *in situ*. We observed that under basal conditions, 14.0 +/- 2.6% (n = 4) of the total CaMKII activity within our cultured astrocytes was autonomous. Although these results differ from a previous study showing that CaMKII is largely autophosphorylated in cultured astrocytes (Song et al., 2006), it is important to note that the percentage of this autophosphorylated activity can be influenced by the "state" of the astrocytes as well during the tissue processing required to make the measurement. Thus, we do not see fully autophosphorylated CaMKII within our highly enriched astrocyte cultures; however, as shown in neurons and other cell types, our data favor the hypothesis that astrocyte activation and calcium signaling can further enhance the extent of activated-autophosphorylated CaMKII.

Next, we examined the effect of the high-affinity CaMKII inhibitor, tat-CN21 (Vest et al., 2007, Ashpole and Hudmon, 2011), on CaMKII activity in the astrocyte cultures. To address this, we measured Ca^{2+}/CaM-stimulated and autonomous CaMKII activity in the astrocytes after 10 minute exposure to the active and control tat-CN21 inhibitor. As expected, tat-CN21 significantly reduced autonomous CaMKII activity. We observed a 40.8 +/- 19.9% and 38.7 +/- 13.5% decrease in autonomous CaMKII activity in tat-CN21 cultures compared to cultures treated with DMSO or control tat-CN21Ala (Figure 33D-E). In an effort to

correlate the reduction in CaMKII activity with the uptake of the CaMKII inhibitors, we applied the carboxyfluorescein-tagged tat-CN21 to astrocytes and measured cellular uptake using fluorescent microscopy. Interestingly, tat-CN21-Fam uptake was maximal within minutes (Figure 34), with 46.3 +/- 7.4% of astrocytes exhibiting robust inhibitor uptake (tat-CN21Ala taken up in 44.8 +/- 3.1% of cells). Remarkably, fluorescent peptide uptake is optically seen by 30 seconds (Figure 34). These data show that both the uptake of the control CaMKII inhibitor (tat-CN21Ala-Fam) and the active CaMKII inhibitor (tat-CN21-Fam) is seen in rapidly (within seconds) and within 50% of the cells. Together, these data indicate that there is a correlation between the percentage of astrocytes that take up the peptide inhibitor (45%) and the extent of CaMKII inhibition (40%). The uptake of the peptide inhibitors in cultured astrocytes is faster than we previously observed in cortical neurons (Ashpole and Hudmon, 2011), suggesting that the rate of calcium dysregulation in both cell types is correlated to the rate of peptide inhibitor uptake.

Decreased Glutamate Uptake in Astrocytes

Our previous studies indicated that inhibition of glutamate signaling prevented neurotoxicity associated with CaMKII inhibition. Because the inclusion of astrocytes in the neuronal cultures did not prevent this neurotoxicity or the calcium dysregulation associated with CaMKII inhibition, we hypothesized that CaMKII inhibitors were negatively impacting glutamate uptake in astrocytes. To address this, we applied H^3-glutamate to astrocytes and measured its uptake in the presence and absence of the CaMKII inhibitor tat-CN21. As expected, 10 µM
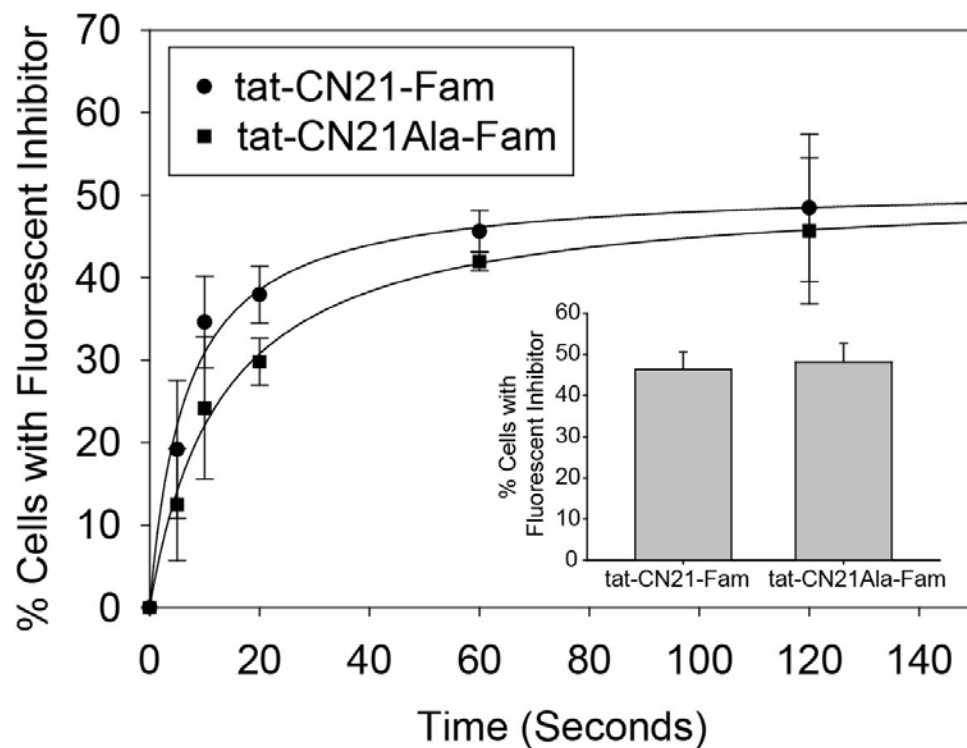
Figure 34: CaMKII inhibitors are rapidly taken up by astrocytes.
Fluorescently-conjugated tat-CN21 and tat-CN21Ala (10 μM) were applied to astrocytes for varying lengths of time. The average number of cells that exhibited uptake was examined using fluorescent microscopy. Total cell number was determined by Hoecsht staining. **Inset**, Average number of cells with fluorescently conjugated tat-CN21 and tat-CN21Ala 20 minutes after application, identified as described above.

tat-CN21 significantly reduced the level of H^3-glutamate uptake in astrocytes compared to control cultures, and cultures treated with inactive tat-CN21Ala (Figure 35). Glutamate uptake was reduced when CaMKII inhibitors were applied for 4 hours (73.3 +/- 0.96 %), 8 hours (75.9 +/- 7.4%), or 24 hours (78.8 +/- 1.75%), suggesting that a loss of CaMKII activity in astrocytes negatively affects the ability of astrocytes to buffer extracellular glutamate.

Calcium Dysregulation in Astrocytes

Having identified that CaMKII inhibition effects astrocyte homeostasis in our enriched astrocyte cultures, we next aimed to identify mechanisms that contribute to the calcium dysregulation in these cultures when CaMKII is inhibited. As in the mixed cultures, application of tat-CN21 rapidly induced oscillatory increases in the intracellular calcium levels within astrocytes. Calcium transients in a single imaged astrocyte is shown in Figure 36A, whereas time traces for multiple astrocytes are shown in Figure 36B. In contrast to the mixed culture glia, the intracellular calcium dysregulation seen in pure astrocyte cultures peaks (~30 seconds following application) and is slowly reduced over a period of several minutes (Figure 36C). As before, only the active CaMKII inhibitor tat-CN21 and not tat-CN21Ala the inactive control peptide dysregulated intracellular calcium levels in astrocytes (Figure 36C-D). Not all cells exhibited this calcium dysregulation with tat-CN21 application, which is likely attributed to the limited uptake of the inhibitors within the cultures (Figure 34). Although we were initially surprised at how quickly we observed calcium dysregulation in astrocytes compared to what was observed previously in neurons, the rapid calcium
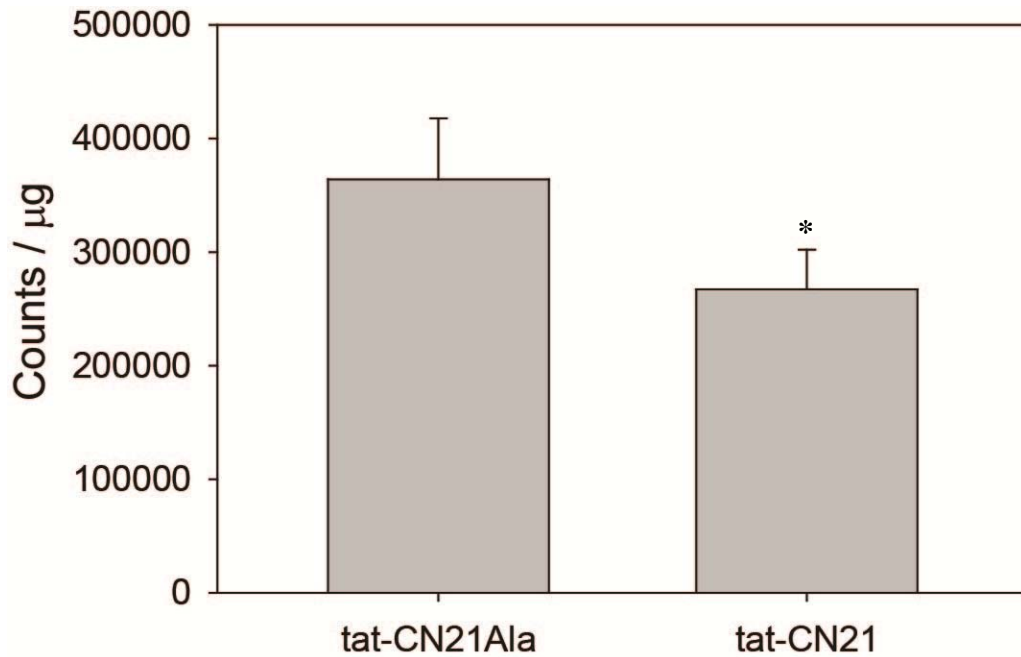
Figure 35: CaMKII inhibition decreases glutamate uptake in Astrocytes. Astrocytes were treated with 10 µM tat-CN21 or tat-CN21Ala for 20 minutes following which 1.5 µCi/mL [H^3]-glutamate/ 0.5 µM unlabeled-glutamate were applied for 2 hours. Following incubation, liquid scintillation counting of cellular lysates indicated levels of H3-glutamate in the cells. Counts were normalized for protein concentration. * indicates significant difference compared to tat-CN21Ala (*p<0.05, t-test).
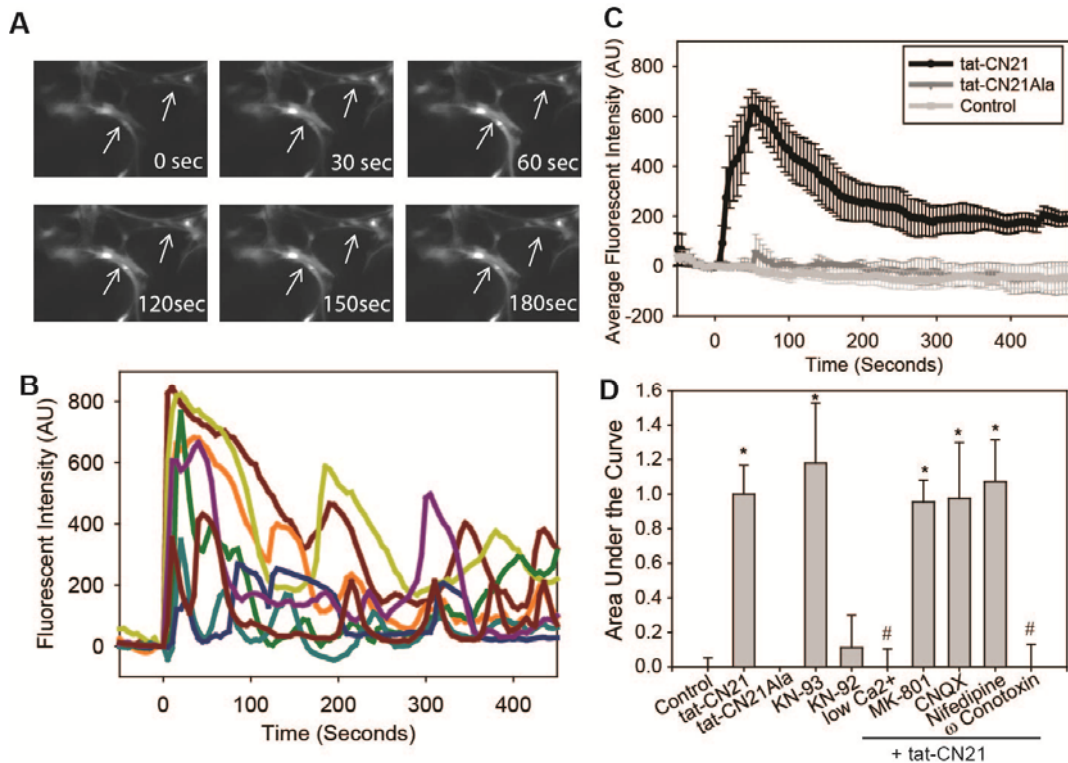
Figure 36: Calcium dysregulation in astrocytes with CaMKII inhibition.
A, Representative image of an astrocyte loaded with Fluo-4AM responding to 10 µM tat-CN21 application. **B,** Representative traces of a field of astrocytes in response to tat-CN21 application at time 0. **C**, Average trace (+/-SEM, n=3) of calcium response in astrocytes treated with DMSO control, 10 µM tat-CN21, or tat-CN21Ala. **D,** Average area under the curve from time 0-500 seconds (+/- SEM, n=3-5) following treatment with tat-CN21 and/or co-treatment with various other pharmacological inhibitors, as indicated. Pharmacological inhibitors were added at -120 sec; none of the inhibitors altered baseline. Asterisk indicates significant difference compared to control while the pound sign indicates a significant difference compared to tat-CN21 (*#$p < 0.05$, One-way ANOVA, post-hoc Dunnett's test).

response induced by CaMKII inhibition is consistent with the rate of peptide inhibitor uptake we observed using fluorescently labeled peptides (Figure 34). The small membrane permeable inhibitor of CaMKII, KN-93, also produced a rapid increase in intracellular calcium concentrations while KN-92 (inactive control) had no effect (Figure 36D). Thus, both peptide and small molecule inhibitors of CaMKII rapidly dysregulate astrocyte calcium homeostasis. Because the calcium dysregulation induced by CaMKII inhibition is rapid and was blocked by low extracellular calcium (Figure 36D), we probed the role of multiple CaMKII substrates within the plasma membrane that are known modulators of calcium signaling.

Although the functional role of many of the voltage- and ligand-gated channels in the astrocyte plasma membrane is not fully appreciated, astrocytes express several of these proteins, including L-type ($Ca_V1.2$) and N-type ($Ca_V2.2$) calcium channels and the NMDA receptor (Latour et al., 2003, D'Ascenzo et al., 2004, Zhou et al., 2010). Thus, pharmacological antagonists of these receptors were applied to the astrocytes two minutes before tat-CN21 was administered. Pretreatment with MK-801, the NMDA-receptor blocker, had no effect on the calcium influx induced by tat-CN21 (Figure 36D). Furthermore, CNQX, the AMPA-receptor blocker, and Nifedipine, the L-type calcium channel antagonist, did not reduce the calcium influx associated with the CaMKII inhibitors (Figure 36D). The N-type calcium channel antagonist, omega-conotoxin, completely prevented the calcium influx associated with tat-CN21 application (Figure 36D). While the contribution of N-type calcium channels in astrocyte signaling is not

fully understood, it has been shown that gliotransmitter release (ATP and glutamate) from astrocytes is dependent on calcium/SNARE-dependent exocytosis (Araque et al., 2000, Parpura and Zorec, 2010, Yaguchi and Nishizaki, 2010, Liu et al., 2011, Yasuda et al., 2011). The potential for the gliotransmitter ATP being aberrantly released by CaMKII inhibition is particularly intriguing, as this glia-neurotransmitter has been shown to regulate calcium signaling in neurons and calcium oscillations in astrocytes (McCarthy and Salm, 1991, Salter and Hicks, 1994, Centemeri et al., 1997, Guthrie et al., 1999).

<u>CaMKII Inhibition Induces ATP Release</u>

To test the hypothesis that perhaps aberrant gliotransmitter release is produced during CaMKII inhibition, we first determined whether CaMKII inhibition led to the accumulation of extracellular glutamate in the astrocyte media after 24 hours. Previously we had used both a glutamate oxidase assay and HPLC chromatography to show that glutamate levels are elevated in the neuronal media as soon as 20 minutes following CaMKII inhibition in neurons (Ashpole et al., 2012). However, in astrocytes, neither the peptide inhibitor tat-CN21 nor the small molecule inhibitor KN-93 were observed to alter the levels of extracellular glutamate compared to control astrocytes (Figure 37A). Next we measured changes in ATP accumulation in the media using a luciferase assay to measure ATP levels in the media. ATP has been shown previously to be one of the most abundant gliotransmitters within cortical astrocytes (Guthrie et al., 1999, Cotrina et al., 2000, Coco et al., 2003) where its release has been shown to modulate a number of different purinergic receptors in neurons and astrocytes (Inoue et al.,

2007, Koles et al., 2011). Unlike extracellular glutamate, we observed that both

tat-CN21 and KN-93 significantly increased extracellular ATP concentration

compared to their matched control inhibitors (i.e. tat-CN21Ala and KN-92) (Figure

37B). Furthermore, a myristolated version of the autoinhibitory protein of CaMKII,

termed myr-AIP, also significantly increased extracellular ATP with a 1.88 +/-

0.50 fold increase over control, suggesting that both small molecule and peptide

inhibitors of CaMKII with various cell-penetrant motifs are able to induce ATP

accumulation in the extracellular media.

Because N-type calcium channel inhibition blocked intracellular calcium

dysregulation, we pretreated astrocytes with omega-conotoxin and again

measured extracellular ATP content following CaMKII inhibition. Application of

omega-conotoxin indeed prevented the accumulation of extracellular ATP when

CaMKII was inhibited (Figure 37C). This data suggests that CaMKII inhibition

leads to calcium influx through the N-type voltage-gated calcium channel and

ATP exocytosis. Because purinergic signaling within astrocytes has been shown

to induce further release of ATP, we measured extracellular ATP levels when tat-

CN21 was applied to astrocytes in combination with various purinergic receptor

antagonists. As expected, suramin, the non-selective purinergic receptor blocker,

decreased extracellular ATP levels compared to cultures treated with tat-CN21

alone (Figure 37C). However, the small increase in ATP levels were significantly

different than control astrocytes (Figure 37C), suggesting that the initial phase of

CaMKII-induced ATP release produced downstream of N-type calcium channel-

activity is initially independent of puringeric receptors. While astrocytes express
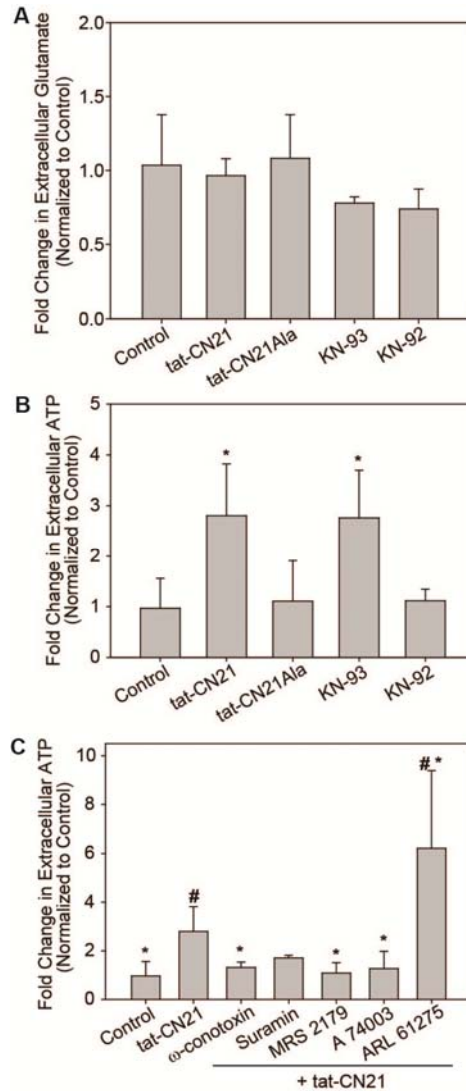
Figure 37: Aberrant gliotransmitter release with CaMKII inhibition. A, Average change in extracellular glutamate concentration (n=6, St Dev) in astrocyte cultures following 24 hour application of various CaMKII inhibitors and controls ($p > 0.05$, One-Way ANOVA). **B,** Average change in extracellular ATP concentration (n=6, St Dev) in astrocyte cultures following 24 hour application of various CaMKII inhibitors and control. The asterisk indicates significant difference compared to control (*$p < 0.05$, One-way ANOVA, post-hoc Dunnett's test). **C,** Average change in extracellular ATP concentration when tat-CN21 was applied alone or in combination with various pharmacological modulators of purinergic signaling and the N-type calcium channel blocker, omega-conotoxin. The asterisk indicates significant difference compared to tat-CN21 while the pound sign indicates significant difference compared to control (*#$p < 0.05$, One-way ANOVA, post-hoc Dunnett's test).

several purinergic receptors, CaMKII signaling has been previously linked to P2Y1 and P2X7 receptors (Leon et al., 2006). Thus, we next examined whether pharmacological antagonists of these subtypes of purinergic receptors reduced the aberrant increase extracellular ATP concentration induced by CaMKII inhibition. Interestingly, co-application of tat-CN21 with either MRS 2179, the P2Y1 antagonist, or A 74003, the P2X7 antagonist, led to a significant reduction in extracellular ATP concentrations compared to tat-CN21 alone (Figure 37C). Importantly, none of the purinergic receptor antagonists had an effect on basal ATP concentration (i.e. without tat-CN21) (Figure 38). Although we observed a statistical difference in the levels extracellular ATP after 24 hrs following CaMKII inhibition, we went ahead and determined the contribution of ectoATPases on the level of ATP measured in the astrocyte media following CaMKII inhibition. We observed a robust increase in level of extracellular ATP under these conditions (Figure 37C), suggesting that CaMKII inhibition within astrocytes negatively impacts astrocyte homeostasis by leading to calcium dysregulation and release of the gliotransmitter ATP.

To further connect the immediate dysregulation of intracellular calcium homeostasis observed with CaMKII inhibition (Figure 36) to the long-term accumulation of extracellular ATP (Figure 37), intracellular calcium levels were monitored when purinergic receptors were antagonized prior to tat-CN21 application. Interestingly, suramin, MRS 2179, and A 74003 all significantly reduced the total calcium influx induced by CaMKII inhibition (Figure 39). The inability of these inhibitors to reduce calcium influx back to baseline is not
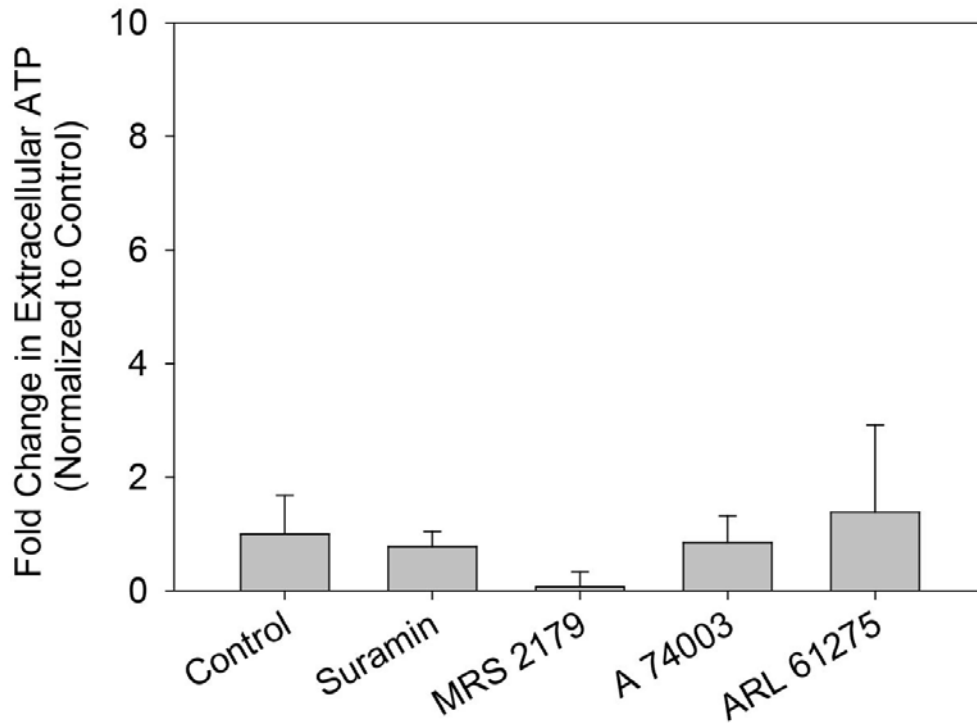
Figure 38: Pharmacological purinergic signaling modulators had no effect on basal extracellular ATP. Average fold change in extracellular ATP levels (n=3-4, St Dev) when astrocytes were treated with various pharmacological modulators of purinergic signaling, compared to control (p>0.05, One-Way ANOVA).

surprising, as the N-type calcium channels appear to be responsible for the initial

phase of calcium dysregulation and release of ATP. Interestingly, when multiple

calcium wave forms for the individual astrocytes are plotted rather than simply

integrating the are under the curve as shown in Figure 5A, it become quite

apparent that the oscillations of calcium observed with tat-CN21 (Figure 39A-B)

are absent in the presence of purinergic antagonists (Figure 39B). Together,

these data support the model that CaMKII inhibition in astrocytes leads to

aberrant activation of N-type calcium channels which in turn leads to ATP release

and a feed-forward loop of subsequent calcium dysregulation supported by ATP

release.

<u>CaMKII Inhibition in Astrocytes is Detrimental for Neuronal Viability</u>

Astrocytes play a critical role in maintaining neuronal viability, thus

alterations in astrocyte homeostasis could have dire consequences on neuronal

survival. Thus, we examined neuronal viability when neurons were treated with

astrocyte-conditioned media following astrocyte exposure to CaMKII inhibitors.

Compared to control media, conditioned media from astrocytes treated with tat-

CN21 significantly increased levels of neurotoxicity (Figure 40). Importantly,

conditioned media from astrocytes treated with the inactive control, tat-CN21Ala,

did not induce neuronal death (Figure 40). As described earlier, astrocytes

treated with tat-CN21 in combination with P2Y1 or P2X7 antagonists (MRS 2179

or A 74003, respectively) reduced extracellular ATP concentrations compared to

tat-CN21 alone. Similarly, these combinatorial treatments reduced neuronal
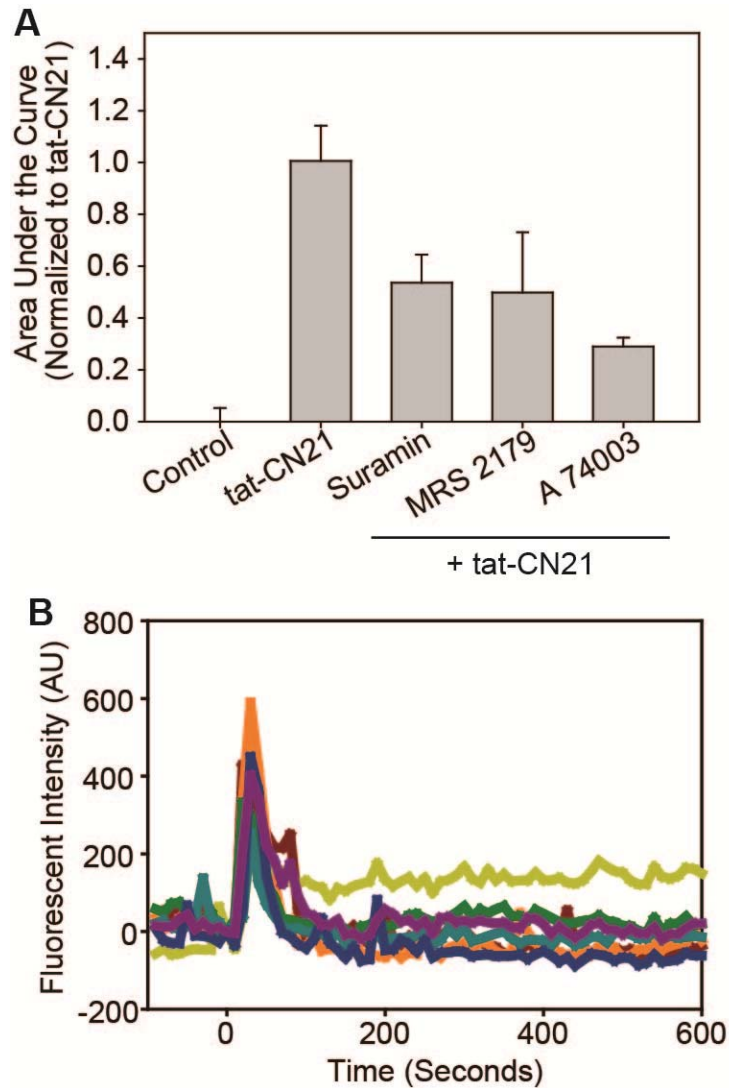
Figure 39: ATP signaling contributes to calcium dysregulation with CaMKII inhibition. A, Average area under the curve (+/- SEM, n=3-5) for calcium influx following treatment with tat-CN21 and/or co-treatment with various other pharmacological inhibitors, as indicated. Pharmacological inhibitors were added at -120 sec; none of the inhibitors altered baseline. Asterisk indicates significant difference compared to control while the pound sign indicates a significant difference compared to tat-CN21 (One-way ANOVA, post-hoc Dunnett's test, *#$p<0.05$). **B,** Representative traces of astrocytic calcium response following tat-CN21 application at time 0 with suramin pre-treatment at time -120 seconds.
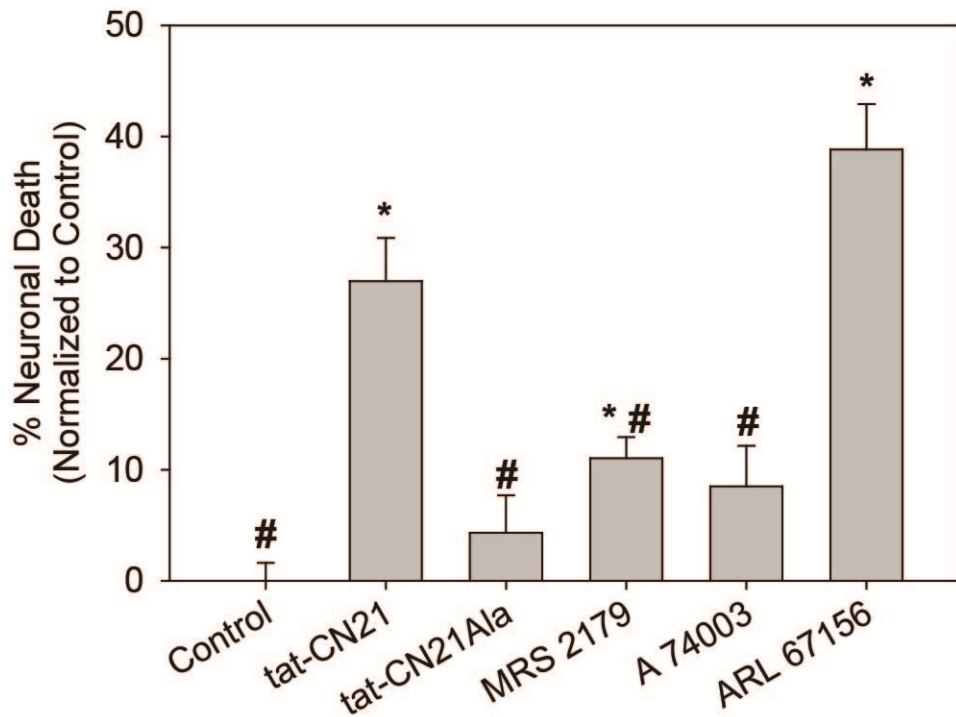
Figure 40: Neuronal death with conditioned media from astrocytes treated with CaMKII inhibitors. Average neuronal death (n=5-8, ± SEM) in neurons treated (24 hr) with conditioned media from astrocytes subjected to 10 µM tat-CN21Ala or tat-CN21 alone, or in combination with MRS 2179, A 74003, or ARL 67156 for 24 hr. The asterisk indicates significant difference compared to control while the pound sign indicates significant difference compared to tat-CN21 alone (*#$p<0.05$, One-Way ANOVA, post-hoc Dunnett's test).

death (Figure 40). In Figure 5B, we show that ectoATPase treatment significantly increased extracellular ATP concentrations compared to tat-CN21 alone. In parallel, we see that this media with the highest ATP concentration results in the highest level of neuronal death (Figure 40). Thus, there is a direct correlation between the level of extracellular ATP released by astrocytes following CaMKII inhibition and the extent of neuronal death. Together, these data indicate that CaMKII inhibition in astrocytes dysregulates calcium homeostasis and leads to ATP release, which not only exacerbates the calcium dysregulation in astrocytes, also ultimately induces neuronal death.

DISCUSSION

Following an ischemic stroke, astrocytes are activated and accumulate within the ischemic core and penumbral regions (Petito et al., 1998, Kajihara et al., 2001, Schmidt-Kastner et al., 2005, Zamanian et al., 2012). This infiltration and activation has been shown to have both beneficial and harmful effects. Neuroprotection can be seen when astrocytes are properly functioning and are able to take up glutamate, buffer K+, and scavenge reactive oxygen species. However, astrocytes within this region can also contribute to neuronal death when these neuroprotective effects are reversed and glutamate, reactive oxygen species, and ATP are released from the astrocytes (Takahashi et al., 1997, Parpura et al., 2004, Zhang et al., 2007). Thus, the dysregulation of astrocyte homeostasis within the core and penumbral regions contributes to the subsequent neuronal death. Interestingly, the extent of damage observed within these regions is also correlated with a decrease in CaMKII activity. We have

previously shown that CaMKII inhibition within neurons is detrimental to neuronal viability by inducing an excitotoxic-like cascade. We now show that inhibition of CaMKII in the supporting astrocytes can also negatively affect neuronal viability. CaMKII inhibitors induced an aberrant calcium influx in astrocytes immediately after entering the cells. While the initial calcium response appears to be the largest, the intracellular calcium concentration continues to remain elevated over time and oscillations of calcium are prevalent throughout the culture. Calcium oscillations and wave propagation from one astrocyte to a nearby astrocyte are dependent on purinergic receptor activation (Cotrina et al., 2000, Suadicani et al., 2006). Consistent with this, we saw that blockade of purinergic receptors resulted in a decrease in the calcium influx and ablation of the calcium oscillations induced by CaMKII inhibition. Furthermore, an increase in extracellular ATP concentration was observed. Importantly, there was a direct correlation between the aberrant calcium influx and ATP accumulation; antagonists that reduced calcium influx, also reduced extracellular ATP concentrations. These findings are in line with the well-understood cyclic relationship between intracellular calcium and extracellular ATP within astrocytes. Increases in cytotosolic calcium underlie vesicular ATP release (Coco et al., 2003, Pryazhnikov and Khiroug, 2008), which then activates purinergic receptors to induce further influx of calcium (McCarthy and Salm, 1991, Salter and Hicks, 1994, Centemeri et al., 1997, Guthrie et al., 1999) thereby inducing a cascade of calcium influx and ATP release.

Increased purinergic signaling has long-been implicated in neurodegeneration (reviewed by (Franke and Illes, 2006)). Upregulation of

purinergic receptors and increased extracellular ATP have been associated with ischemia (Phillis et al., 1994, Volonte et al., 2003, Franke et al., 2004), traumatic brain and spinal cord injury (Ryu et al., 2002, Wang et al., 2004), epilepsy, Parkinson's disease, and Alzheimer's disease (reviewed by (Franke and Illes, 2006)). Antagonism of purinergic receptors has been shown to decrease infarct size following stroke (Lammer et al., 2006, Kuboyama et al., 2011), and improve functional recovery after stroke and spinal cord injury (Wang et al., 2004, Kuboyama et al., 2011, Lammer et al., 2011). Although a decrease in overall ATP availability is seen within the ischemic core and penumbral regions, there is a significant increase in extracellular ATP concentration, suggesting that either ATP release is increased or ATP breakdown mechanisms are impaired (Melani et al., 2005). Our data suggest that purinergic signaling initiated by CaMKII inhibition negatively impacts both astrocytes and neurons. Within astrocytes, it sustains the calcium dysregulation initiated by N-type calcium channels. The increased ATP in the astrocyte-conditioned media then instigates neurotoxicity when applied to cultured cortical neurons. While neurons and astrocytes express a variety of purinergic receptors, we chose to focus on two receptors, P2Y1 and P2X7; both of which are known to play a role in maintaining astrocyte function and regulating neuronal viability. P2X7 receptor activation has been shown to produce gliotransmitter release (Sperlagh et al., 2002, Duan et al., 2003, Suadicani et al., 2006) and is often associated with cellular death signaling in both neurons and astrocytes (Schulze-Lohoff et al., 1998, Ferrari et al., 1999). P2Y1 signaling has also been implicated in gliotransmitter release (Jourdain et

al., 2007), apoptosis (Sellers et al., 2001, Mamedova et al., 2006), and calcium dysregulation (Gallagher and Salter, 2003). Thus, it was not surprising when the highly-selective antagonists of both P2Y1 and P2X7 were effective at reducing the dysregulation observed in both astrocyte and neuronal cultures. However, we initially did not expect both MRS 2179 (P2Y1 antagonist) and A 74003 (P2X7 antagonist) to be equally effective at preventing ATP accumulation within the astrocyte media. This may be explained by the fact that both receptor subtypes influence intracellular calcium concentrations and ATP release (Hamilton et al., 2008) and perhaps blunting one of the effectors is sufficient to block the regenerative cascade of calcium influx and ATP release.

Purinergic receptor signaling has been shown to activate a variety of downstream signaling pathways, including the CaMKII pathway. Both P2Y1 and P2X7 receptor activation has been shown to lead to the phosphorylation and activation of CaMKII (Leon et al., 2006). Not only is CaMKII activated by the calcium influx following P2 activation, CaMKII also regulates purinergic receptor signaling. For example, P2Y1 receptor internalization secondary to receptor activation is dependent on CaMKII activity (Tulapurkar et al., 2006). It is interesting to note that several CaMKII substrates (P2Y1, GluN2B, AMPA-R) are also known to play a role in regulating CaMKII activity. It is possible that the cellular response to a loss of CaMKII activity is to attempt to re-instate the loss of this calcium-sensor by enhancing further calcium flux. Our data is consistent with this theory. We previously showed that CaMKII inhibition induces glutamate release from neurons which subsequently increases intracellular calcium. Here,

we show that CaMKII inhibition induces ATP release from astrocytes which also subsequently increases intracellular calcium. While excessive levels of extracellular glutamate have been shown to lead to CaMKII inactivation, it is not known whether excessive extracellular ATP induces CaMKII inactivation as well.

However, several studies suggest that excessive calcium influx under circumstances of decreased intracellular ATP availability lead to the inactivation and/or aggregation of CaMKII. Thus, it is possible that CaMKII becomes inactivated when P2 signaling is excessively increased. If this were the case, our data suggests that CaMKII inactivation would continue the vicious cycle of extracellular ATP accumulation and aberrant calcium influx. Regardless of this speculation, our data supports our previous model in which a loss of CaMKII activity is detrimental to neuronal survival. From this study we can add to our model that CaMKII inhibition in astrocytes is also neurotoxic as inhibition leads to calcium influx and ATP release from astrocytes which inevitably induces neurotoxicity. These data suggest that the loss of CaMKII activity within the core and penumbral regions of an ischemic stroke is detrimental for neuronal survival by altering neuronal-neuronal and glial-neuronal communications. Therefore, avenues of restoring CaMKII activity within these ischemic tissues may be imperative for affording neuroprotection and stopping the expansion of cellular death away from the core following the insult.

*This manuscript is under review *

Ashpole NM, Martin MP, Brustovetsky T, Brustovetsky N, Hudmon A

DISCUSSION

GENERAL CONCLUSIONS

Over thirty years of work has gone into understanding the role of CaMKII signaling within the brain. Since it was first identified, CaMKII has been shown to regulate neurotransmitter release, membrane excitability, cellular metabolism, and a variety of other cellular processes (as reviewed by (Colbran, 1992, Hudmon and Schulman, 2002)). The most recognized role of CaMKII signaling is the one it serves as a master regulator of LTP. Following activation, CaMKII transduces the incoming calcium signals into long-lasting effects with neurons, thereby enhancing synaptic plasticity (see Figure 41). My findings expand the role of CaMKII signaling in neurons, identifying that in addition to enhancing synaptic plasticity, CaMKII serves as a regulator of neuronal viability. Activation of CaMKII during periods of cellular distress leads to prolonged inactivation (≥8 hours) of the kinase and ultimately induces neuronal death (Figure 41).

Fluctuations in CaMKII activity have long-been associated with excitotoxic insults such as ischemic stroke (Aronowski et al., 1992, Westgate et al., 1994, Zalewska and Domanska-Janik, 1996, Zalewska et al., 1996). CaMKII is known to be activated following the onset of ischemia (Westgate et al., 1994, Zalewska and Domanska-Janik, 1996, Zalewska et al., 1996). This activation is followed by a rapid inactivation (Aronowski et al., 1992, Zalewska et al., 1996). The extent of CaMKII inactivation in the hours following insult is temporally correlated with the extent of neuronal death (Hanson et al., 1994). Despite these correlations,
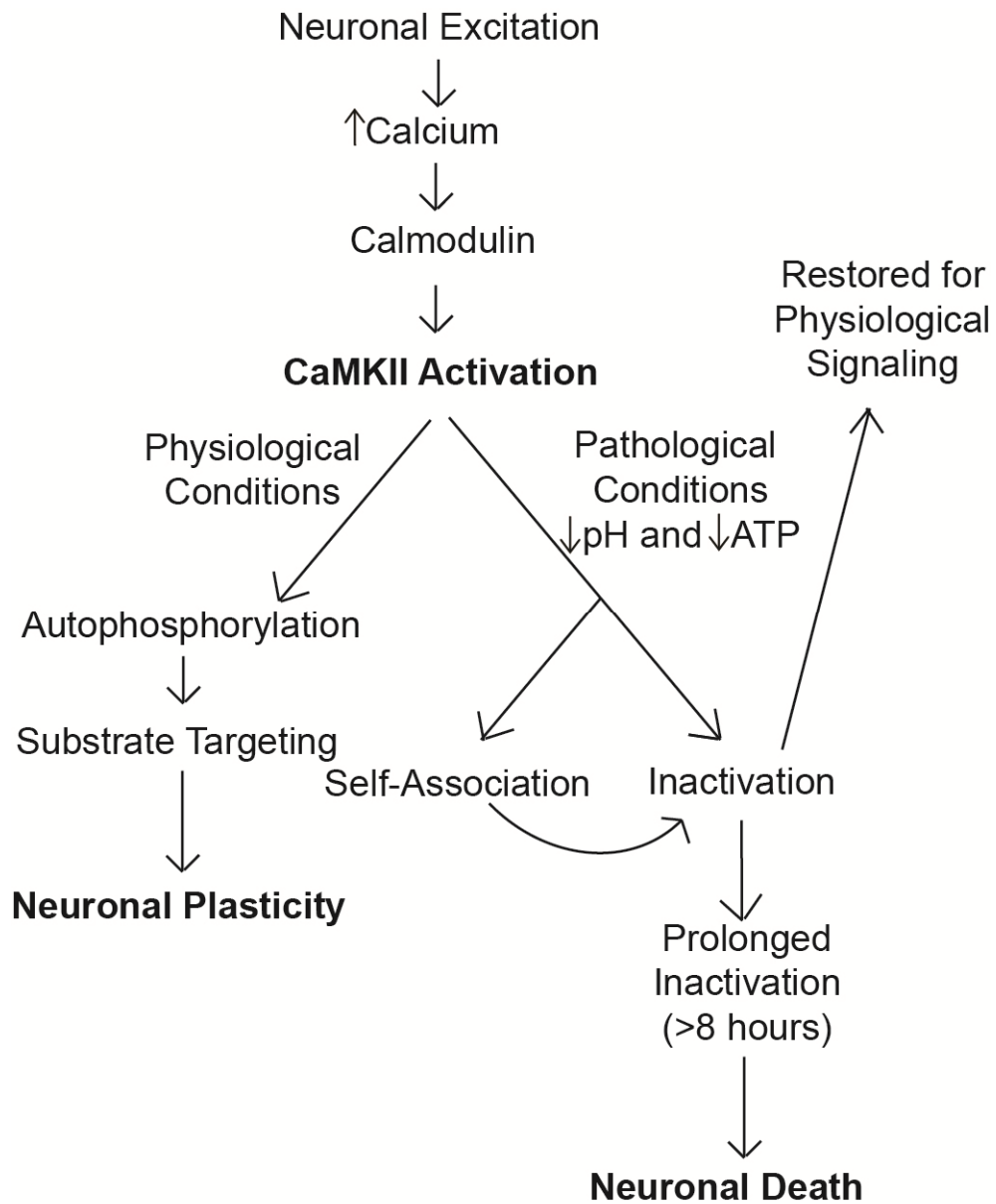
Figure 41: Working model of CaMKII signaling following activation under physiological and pathophysiological conditions. Under physiological conditions, CaMKII activation leads to substrate targeting and synaptic plasticity. Excitotoxic insults can lead to decreased ATP availability and decreased pH, which support the prolonged inactivation and self-association of CaMKII and ultimately result in neuronal death.

chemical and genomic inhibition studies had yielded conflicting results as to whether or not it is toxic or beneficial to inhibit CaMKII during excitotoxic calcium signaling. Small molecule and peptide inhibitor studies suggest CaMKII activity is detrimental to neuronal survival, as inhibition of the kinase during insult is neuroprotective (Hajimohammadreza et al., 1995, Hou et al., 2009, Vest et al., 2010). Yet, αCaMKII knock-out animals exhibit increased infarct size following stroke, suggesting the loss of αCaMKII activity predisposes neurons in the ischemic area to death (Waxham et al., 1996). While the *in vitro* studies suggested increased CaMKII activity was harmful and the *in vivo* studies suggested decreased CaMKII activity was harmful, one theme was consistent: aberrant fluctuations in CaMKII are tied to neuronal death. Thus, we decided to examine both the aberrant activation and inactivation of CaMKII in one simplified model system, allowing us to identify the contributions of CaMKII activity in neuronal death associated with excitotoxic calcium signaling.

The data presented here unites the concepts that both aberrant activation and inactivation of CaMKII contribute to neuronal death. Small molecule and highly-specific cell-permeable peptide inhibitors of CaMKII were neuroprotective when applied prior to excitotoxic insult. This is consistent with a recent report indicating that the peptide inhibitor tat-CN21 is neuroprotective within an excitotoxic insult *in situ* and an *in vivo* animal model of ischemic stroke (Vest et al., 2010). Because aberrant CaMKII activation during times of cellular distress is known to lead to aggregation and prolonged inactivation of the kinase, we questioned whether these secondary effects were altered by the application of

the CaMKII inhibitors during excitotoxic insult. Interestingly, we observed that pharmacological inhibition of CaMKII during excitotoxic insult prevented both the aggregation and prolonged inactivation of the kinase. These findings led to the hypothesis that the activation of CaMKII during excitotoxic conditions is detrimental to neuronal viability because it leads to a prolonged loss of CaMKII activity, which ultimately underlies neuronal death. While we can not separate that aberrant CaMKII activation causes neuronal death because it leads to inactivation, we present strong evidence that the long-term inhibition which is known to occur following activation under these conditions predisposes neurons to excitotoxicity. Moreover, CaMKII inhibition in the absence of excitotoxic insult induced cortical neuron apoptosis by dysregulating intracellular calcium homeostasis and increasing excitatory glutamate signaling. This led to the accumulation of extracellular glutamate which perpetuated a slow-induced excitotoxic cascade within the cortical neurons. Blockade of the NMDA-receptors and enzymatic degradation of the extracellular glutamate signal afforded neuroprotection from CaMKII inhibition-induced toxicity. Surprisingly, co-cultures of neurons with glutamate-buffering astrocytes still exhibited toxicity with CaMKII inhibition. This was a result of CaMKII inhibitors reducing the ability of astrocytes to buffer extracellular glutamate. Furthermore, CaMKII inhibition dysregulated calcium homeostasis in astrocytes and led to increased ATP release. This increased ATP was neurotoxic when applied to naïve cortical neurons, suggesting that, similar to our observations in neurons, a loss of CaMKII activity

in astrocytes leads to calcium dysregulation with subsequent neurotoxic transmitter release.

Together, these findings suggest that activation of CaMKII during periods of cellular distress is toxic as it leads to aggregation and prolonged inactivation of the kinase. Without CaMKII activity, neurons and astrocytes release stores of transmitters and perpetuate the neurotoxic signals. Thus, the role of CaMKII signaling in neurons extends beyond a modulator of synaptic plasticity; CaMKII serves as a critical regulator of neuronal viability by functioning as a feedback mechanism that maintains glutamate/calcium homeostasis.

BENEFITS AND LIMITATIONS OF THE MODEL SYSTEM

Several neurodegenerative diseases are marked by excitotoxicity, including ischemic stroke. A majority of ischemic strokes occur within the middle cerebral artery (del Zoppo et al., 1992). This artery is responsible for providing blood supply to the surface of the cerebral cortex and the basal ganglia. Ischemic stroke patients often manifest a loss in language expression and comprehension because the middle cerebral artery is essential for blood supply to the Broca's and Wernicke's language areas of the frontal and temporal lobes. Furthermore, the blood supply of the motor cortex and sensory cortex are also supplied by the middle cerebral artery, which underlies the deficits in movement and sensation in stroke patients. Thus, several of the phenotypical characteristics of ischemic stroke are produced by the occlusion of blood supply from the middle cerebral artery to the cerebral cortex. Because of this, cortical cultures are an optimal

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avenue for uncovering mechanisms underlying the excitotoxicity that ensues following stroke.

The development of techniques to derive neurons from embryonic mouse and rat cortex has afforded the ability of using cortical cultures to model excitotoxicity for over 20 years (Dichter, 1978, Choi et al., 1987). While *in vivo* models mimic the pathological disease state, they inherently contain more variables than a reduced culture system. Our culture model allowed us to effectively answer our questions regarding the physiological role of CaMKII signaling in neurons and astrocytes without complications from exogenous variables such as immune cell activation and infiltration. While we opted to use cortical cultures because the cortex is most-often the site of ischemic stroke damage, many studies also use hippocampal cultures to model excitotoxic-related insults. Hippocampal neurons, particularly within the CA1 region, are extremely vulnerable to excitotoxicity (Benveniste et al., 1984, Kirino and Sano, 1984, Mattson and Kater, 1989). However, the hippocampus is also marked by neuronal populations within dentate gyrus that are resistant to this toxicity (Kirino and Sano, 1984, Mattson and Kater, 1989). Therefore, hippocampal cultures contain a mixture of excitotoxic-sensitive and excitotoxic-resistant neurons. Several of our key findings were replicated in hippocampal cultures. For example, tat-CN21 was recently reported to display neuroprotection in hippocampal neurons subjected to excitotoxic insult in a dose- and time-dependent manner, similar to my findings (Vest et al, 2010). Moreover, in collaboration with our lab, the Brustovetsky lab identified that neurons and

astrocytes derived from the hippocampus also displayed robust calcium dysregulation when CaMKII was inhibited. Thus, both model systems indicate that aberrant activation and inactivation of CaMKII can be detrimental to neuronal physiology.

Proper neuron-astrocyte communication allows for regulation of neuronal excitability, synaptic plasticity, and viability. Because excitotoxic insults are marked by both neuron and astrocyte dysfunction (and subsequent changes in excitability and viability), it was critical to examine the effects of CaMKII independently in cultures of neurons or astrocytes, and together within co-cultures of both cell types. Our *in situ* model system also afforded the ability to manipulate the levels of astrocytes within the neuronal cultures so we could address the effects of CaMKII inhibition both in the absence and presence of astrocytes. Moreover, this system allowed us to perform live measurements in order to determine the immediate cellular response to CaMKII activation and inactivation in neurons and astrocytes. Thus, there were several advantages to using acutely-dissociated cortical cultures.

Excessive glutamate stimulation in dissociated neurons is known to induce significant levels of neuronal death by activating the NMDA-R and causing calcium overload (Choi, 1985, 1987, Choi et al., 1987). Our findings were consistent with previous reports indicating that the glutamate concentration which induced neurotoxicity is within the range of glutamate observed within ischemic brain tissue (Benveniste et al., 1984, Kanthan et al., 1995). The rapid neuronal death observed following our stimulation protocol is consistent with the induction

of necrosis often observed following excitotoxic glutamate stimulation (Figure 42). Lower levels of glutamate are known to induce a slow-induced excitotoxic apoptosis when applied to neurons for prolonged length of time (Cheung et al., 1998). The data presented here suggests that CaMKII inhibition in naïve cortical neurons resulted in the accumulation of 2-4 µM extracellular glutamate, which induced apoptotic cell death (Figure 42). These levels are in line with previous concentrations identified to induce apoptosis. Thus, our experimental models and acquired data that identify the concentration of glutamate required to induce necrosis or apoptosis within neurons are consistent with previous literature.

Both genetic and pharmacological approaches can be used to determine the physiological role of CaMKII signaling in neurons. As stated previously, αCaMKII knock-out mice exhibit a predisposition to stroke damage. Furthermore, these animals are more prone to epilepsy. Studies using siRNA against αCaMKII indicate that knock-down increases basal calcium concentration and reduces the ability of hippocampal neurons to restore intracellular calcium concentration in response to stimulation (Carter et al., 2006). Thus, these genetic studies have yielded similar to results to ours (increased excitability, dysregulated calcium homeostasis, predisposition to toxicity); however, genetic studies have largely been focused on αCaMKII. While αCaMKII is the predominant isoform within the mature cortex (Ouimet et al., 1984, Erondu and Kennedy, 1985), other isoforms of CaMKII are expressed in the brain. There are robust levels of βCaMKII expression throughout development; the mature brain still cortex still maintains
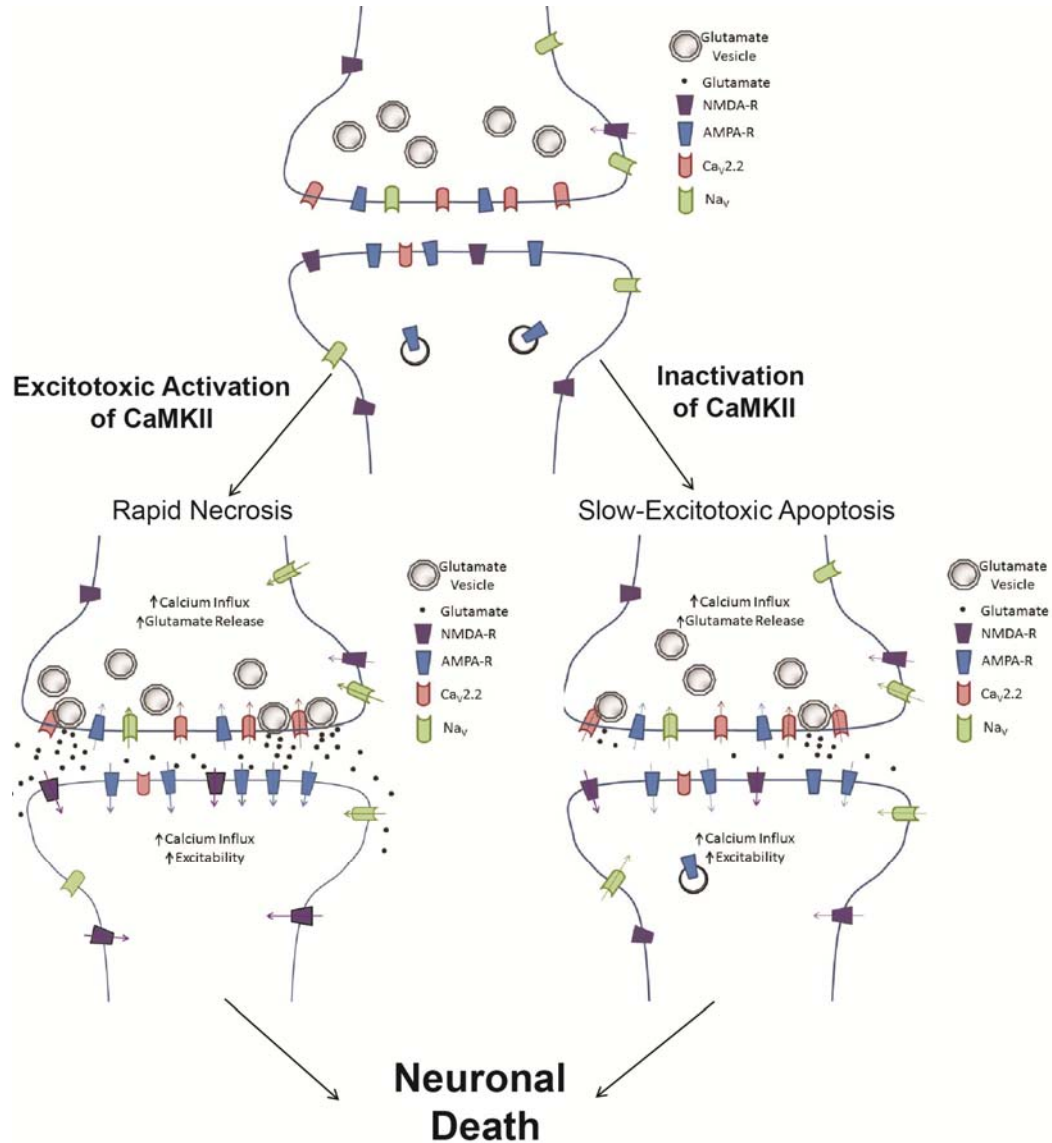
Figure 42: Model of dysregulated CaMKII signaling following excitotoxic activation or inactivation in neurons. Under resting conditions (top), intracellular calcium is maintained at a low level and vesicles of glutamate are ready for release. Excitotoxic stimulation (left), which activates CaMKII, induces rapid necrosis by increasing sodium and calcium influx and subsequently resulting in hyperexcitability and the release of stores of glutamate. On the post-synaptic side, CaMKII activation can lead to increased AMPA-R insertion and the perpetuation of increased neuronal excitability and increased calcium influx. Prolonged inactivation of CaMKII (right) induces a slow-excitotoxic cascade by leading to increased Cav2.2 calcium influx and increased glutamate release. This can subsequently increase the excitability of post-synaptic neurons and further dysregulate intracellular calcium. The effects of both activation and inactivation lead to neuronal death either via necrotic or apoptotic mechanisms.

low levels of βCaMKII expression as well. Furthermore, δCaMKII and γCaMKII are ubiquitously expressed, thus low levels of these two isoforms are also found throughout the brain, with δCaMKII predominating in astrocytes (Ouimet et al., 1984, Erondu and Kennedy, 1985, McGuinness et al., 1985, Burgin et al., 1990a). Thus, genetic knock-down studies are hampered by the presence of multiple CaMKII isoforms in cells. Further studies using genetic knock-down techniques may provide insight into the contribution of each isoform during excitotoxic calcium signaling.

We opted to use pharmacological CaMKII inhibitors to examine the role of CaMKII activity in aberrant calcium signaling. This was advantageous for multiple reasons. First, these pharmacological inhibitors presumably target all CaMKII isoforms. Furthermore, pharmacological inhibitors have a distinct advantage for potential therapeutic use. While we went on to show multiple disadvantages to inhibition of CaMKII, we initially observed that inhibiting CaMKII up to two hours after the onset of excitotoxic insult was neuroprotective, potentially within a therapeutic time window for ischemic stroke patients. The pharmacological inhibitors also allowed us to inhibit CaMKII activity at several different time points before and after the onset of excitotoxic insult. This allowed for a better understanding of the contribution of CaMKII activity in the hours following insult. Furthermore, application of pharmacological inhibitors allowed us to quickly reduce CaMKII activity within cells and monitor rapid changes in cellular physiology as CaMKII became inhibited. This was particularly beneficial for modeling the loss of CaMKII activity observed during excitotoxic insults without

having the confounding increased activity that is observed at the onset of insult. Thus, pharmacological inhibitors allowed us to tease apart the contributions of the aberrant activation and inactivation of CaMKII in a controlled manner. Together, I believe our model system was optimal for uncovering the influence that changes in CaMKII activity have on neuronal viability.

SUBSTRATES DYSREGULATED DURING ABBERANT CAMKII SIGNALING

CaMKII has been shown to regulate a number of proteins associated with synaptic function and neuronal excitability. Our data suggests that aberrant CaMKII activity dysregulates critical ion channels, leading to dysregulated calcium and glutamate homeostasis as well as disrupted neuronal excitability. CaMKII activity contributes to excitotoxic signaling following neuronal glutamate stimulation. While we did not fully-delineate which downstream substrates were responsible for this, increased AMPA-R signaling likely contributes, as illustrated in Figure 42. Pharmacological antagonists of the AMPA-R have been shown to be neuroprotective in animal models of stroke (Sheardown Nielsen Hansen Science 1990, Graham Chen Simon 1996). As described earlier, CaMKII phosphorylation of AMPA-R and its accessory protein Stargazin result in increased AMPA-R trafficking and increased channel conductance. Thus, it is possible the initial activation of CaMKII during excitotoxic Ca^{2+}-signaling leads to increased AMPA-R signaling, which further contributes to over-excitation, calcium dysregulation, and neuronal death (Figure 42).

Conversely, inactivation of CaMKII also dysregulates ion channel signaling. Pharmacological inhibition of CaMKII led to increased calcium influx. While

several voltage-gated calcium channels have been linked to CaMKII signaling (See Table 1), only pharmacological antagonism of the N-type calcium channel ($Ca_V2.2$) prevented the aberrant increase in calcium associated with CaMKII inhibition. This is particularly interesting, as CaMKII regulation of $Ca_V2.2$ has not yet been reported. $Ca_V2.2$ plays a critical role in neurotransmitter release following neuronal depolarization; thus, it was not surprising when the calcium influx associated with CaMKII inhibition correlated with increased neuronal excitability and increased neurotransmitter release. While we do not know which substrate(s) was essential for the initiation of the neurotoxic cascade, our data suggests that increased $Ca_V2.2$ activity led to increased calcium accumulation and increased release of excitotoxic glutamate. This glutamate subsequently activated NMDA-Rs and AMPA-Rs to further perpetuate the neurotoxic signal (Figure 42). Together, these data indicate that CaMKII serves as a brake for substrates regulating neuronal activity, therefore CaMKII inactivation results in aberrant excitability.

It is interesting that $Ca_V2.2$ appears to be dysregulated when CaMKII is inhibited within astrocytes as well. Our working model suggests that similar to neurons, this increase in calcium influx through $Ca_V2.2$ is also accompanied by increased transmitter release (Figure 43). However, unlike in neurons, the neurotransmitter that is released is ATP, not glutamate. While our data indicates that $Ca_V2.2$ activity is increased when CaMKII is inactivated, we cannot rule out that aberrant regulation of other synaptic release machinery, such as members of the SNARE complex, play a role in the increased transmitter release with CaMKII
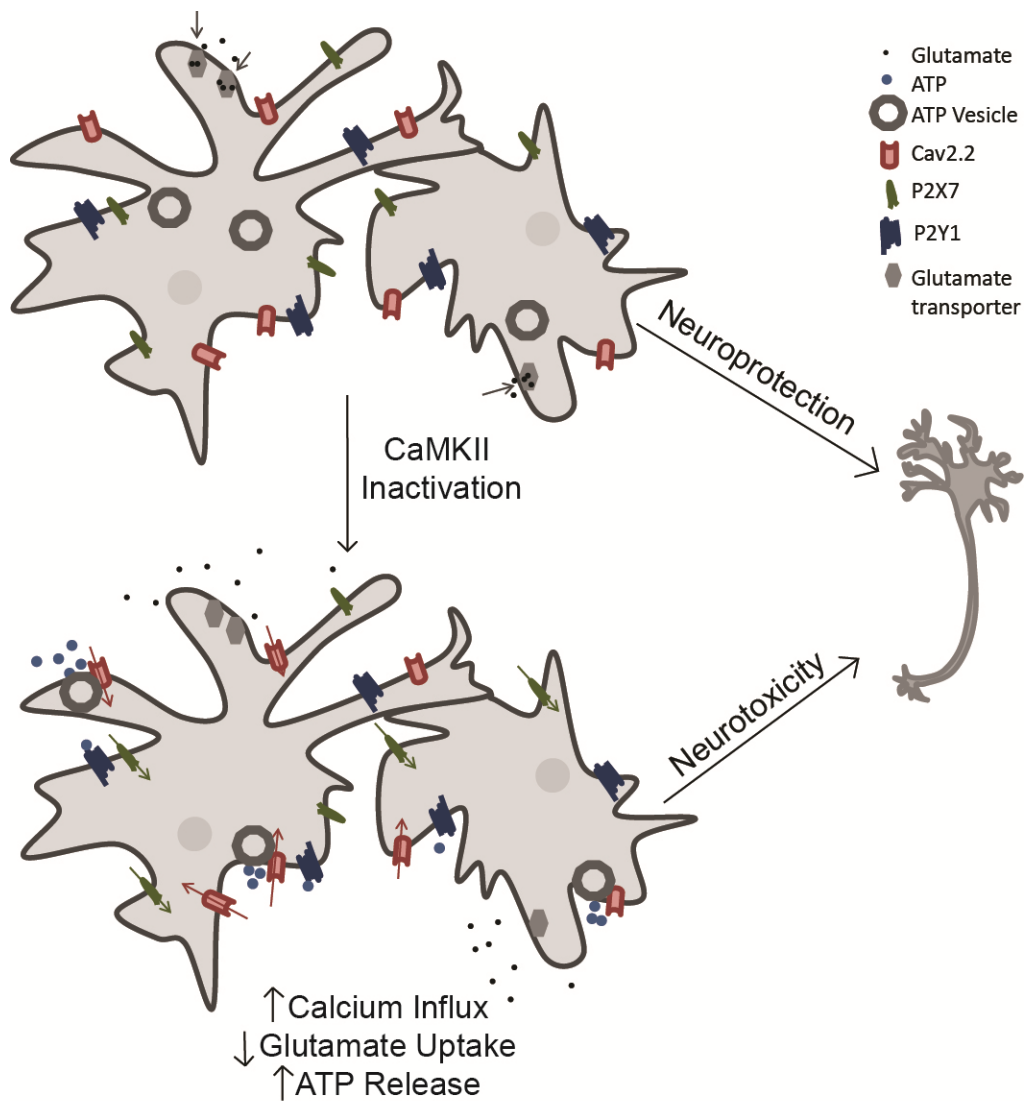
Figure 43: Model of CaMKII inactivation in astrocytes and subsequent effects on neuronal viability. Under resting conditions (top), intracellular calcium is maintained at a low level, extracellular glutamate is readily taken up, and vesicles of ATP are ready for release. Prolonged inactivation of CaMKII (bottom) reduces glutamate uptake, increases Cav2.2 calcium influx, and increases inter-astrocyte calcium flux. ATP release is also increased with CaMKII inactivation, which leads to increased purinergic receptor signaling that further dysregulates calcium homeostasis. While under physiological conditions astrocytes buffer neurotoxic signals and release neurotrophic factors, the decreased glutamate uptake and increased ATP release associated with CaMKII inhibition can lead to neurotoxicity.

inhibition. Indeed, CaMKII has been shown to regulate syntaxin and synaptobrevin, two critical proteins within the SNARE complex (Greengard et al., 1987, Nielander et al., 1995, Verona et al., 2000, Ohyama et al., 2002). Thus, it is possible that transmitter release is increased via multiple avenues, including dysregulated transmitter machinery and increased calcium channel activity.

Glutamate uptake mechanisms in astrocytes are also negatively regulated when CaMKII is inhibited. Further studies may identify which component of the glutamate uptake system is directly affected by CaMKII inhibition as, to date, glutamate transporters have not been identified as CaMKII substrates. The increased ATP release is associated with increased purinergic signaling within the astrocytes. Previously, P2X7 and P2Y1 have been linked to CaMKII signaling. Our data suggests that inhibition of these purinergic signaling pathways (P2X7 and P2Y1) reduces calcium dysregulation following ATP stimulation. Together, all of these examples highlight that multiple ion channels/receptors and other cellular proteins may be dysregulated by aberrant CaMKII activity (Figures 42-43), which subsequently negatively effect neuronal viability.

A NEED FOR UNDERSTANDING MECHANISMS OF SELF-ASSOCIATION

As previously discussed, it is well-documented that CaMKII undergoes prolonged inactivation after the onset of ischemia (Aronowski et al., 1992, Hanson et al., 1994, Zalewska et al., 1996). Several mechanisms may underlie this inactivation. While it was first hypothesized that proteolysis may account for the loss of enzymatic activity, only prolonged ischemia in one animal model

(gerbil) exhibited a significant reduction in CaMKII protein levels (Yamamoto et al., 1992) whereas other rodent models of stroke (rats) do not see this reduction in protein levels (Aronowski et al., 1992, Hanson et al., 1994). Similarly, I did not see a reduction in overall CaMKII protein 24 hours following excitotoxic insult. However; I did observe a significant reduction in CaMKII activity, suggesting that CaMKII was enzymatically inactivating without being proteolyzed.

The activation of CaMKII under conditions of limiting ATP has also been shown to lead to inactivation of the kinase (Hudmon et al., 1996). Saturating levels of ADP and the non-hydroloyzable ATP analog AMP-PNP are able to prevent this inactivation, suggesting that occupation of the nucleotide binding pocket affords stability of activated enzyme (Hudmon et al., 1996, Vest et al., 2009). While the mechanisms underlying this inactivation are not fully-understood, it is interesting that autophosphorylation of Thr253 coincides with activation of CaMKII in limiting ATP. It is possible that a structural rearrangement occurs during inactivation that allows Thr253 to be available for phosphorylation. Importantly, mutagenesis of Thr253 to non-phosphorylatable Ala does not prevent CaMKII inactivation, indicating that this phosphorylation event is simply correlated with inactivation in low ATP but is not necessary to induce inactivation. Recently, an antibody against phospho-Thr253 was used to characterize CaMKII inactivation in an animal model of stroke (Skelding et al., 2012). Because the phosphorylation of Thr253 appears to be a hallmark of inactivation, this phospho-antibody opens the possibility of characterizing CaMKII inactivation states in other neurodegenerative diseases.

The early studies that showed CaMKII isolated from animal models of stroke exhibited a loss of enzymatic activity also indicated that CaMKII translocated from the supernatant fractions to the pellet following differential centrifugation (Aronowski et al., 1992, Kolb et al., 1995). In fact, this phenomenon also led to a gross over-estimation of the levels of CaMKII within the post-synaptic density (which is isolated using differential centrifugation) (Suzuki et al., 1994). Immunohistochemical analysis demonstrated the formation of CaMKII clusters within individual neurons following ischemic-like stimulation (Tao-Cheng et al., 2002, Tao-Cheng et al., 2007). These changes in subcellular localization correlated with an aggregation of CaMKII holoenzymes (Hudmon et al., 2001). Biochemical studies illustrated an activity-dependent aggregation, termed self-association, when purified αCaMKII was exposed to an ischemic-like environment – reduced pH, reduced ATP availability, and increased calcium (Hudmon et al., 1996, Hudmon et al., 2001). My studies suggest that self-association-induced inactivation is detrimental to neuronal survival, as inhibitors that prevent self-association are neuroprotective in excitotoxic insults and a prolonged loss of activity (as is seen with self-association) leads to neurotoxicity. We directly show that application of CaMKII inhibitors during excitotoxic glutamate insult prevent neurotoxicity, αCaMKII self-association, and inactivation.

While αCaMKII is sensitive to self-association under ischemic conditions, βCaMKII exhibits resistance (Hudmon et al., 2001). It is interesting that αCaMKII expression is limited in tissues that are less sensitive to excitotoxicity. As mentioned before, robust αCaMKII expression predominates in the cortex and

βCaMKII expression predominates in the cerebellum (Erondu and Kennedy, 1985, McGuinness et al., 1985, Miller and Kennedy, 1985). Cerebellar neurons (βCaMKII) are more resistant to glutamate toxicity in culture than cortical neurons (αCaMKII). Moreover, the CA1 region of the hippocampus is predominantly marked by αCaMKII expression and is highly-vulnerable to excitotoxicity. However, the granule cells of the dentate gyrus in the hippocampus which is resistant to excitotoxicity express βCaMKII (Churn et al., 1992b). Thus, cells that are sensitive to excitotoxicity predominantly express αCaMKII; the isoform that is predisposed to inactivation via self-association.

This concept that the expression of αCaMKII underlies neuronal sensitivity to excitotoxic calcium signaling is further supported when looking at the developmental regulation of CaMKII expression. As neurons mature, they become increasingly more vulnerable to excitotoxic damage. Within the nervous system, βCaMKII is also largely expressed during development. This is true even in tissue types such as the cortex and hippocampus that are largely recognized as areas dominated by αCaMKII expression. In early post natal days, αCaMKII expression begins to increase (Scholz et al., 1988, Burgin et al., 1990a). By the time the tissue is fully matured, αCaMKII will out-number βCaMKII 3 to 1 in the hippocampus while during embryonic development, βCaMKII out-numbers αCaMKII (Miller and Kennedy, 1985, Burgin et al., 1990a). Several other factors may influence the increased sensitivity of neurons to excitotoxicity throughout development, such as NMDA receptor expression patterns; however, it is

possible that prevalence of a self-association sensitive isoform of CaMKII plays a critical role in this vulnerability.

The mechanism underlying the ability of αCaMKII to self-associate while βCaMKII cannot, has not been identified, although we hypothesize that the molecular variations between these two isoforms play a key role in this sensitivity. The variable domain is typically asserted as the primary difference among the CaMKII isoforms (Hudmon and Schulman, 2002); however, we have recently identified many residues in both the autoregulatory and catalytic domains that vary between αCaMKII and βCaMKII, which may produce this isoform-specific sensitivity to aggregation. Recent crystallographic data indicates that several of these residues lie in regions that may be important for anchoring the regulatory domain to the catalytic domain during activation (pdb #2WEL and #2VN9). In the future, it would be interesting to mutate these residues between the catalytic surface and autoregulatory domain in αCaMKII to the residues seen in βCaMKII in hopes to disrupt αCaMKII self-association in an animal model. Preliminary data indicates that mutation of Ser272/His273 in αCaMKII to βCaMKII residues (Cys/Gln) prevents self-association *in vitro* (Ashpole and Hudmon, unpublished). Further *in situ* analysis is required to identify whether these residues are the mechanisms that underlie isoform-dependent sensitivity to self-association. Once the molecular mechanism has been deciphered, these effects of self-association on neuronal viability can be assessed. For this, endogenous CaMKII in neurons could be replaced with mutants that are more resistant or more vulnerable to self-association and viability could be measured

in the absence and presence of excitotoxic insults. A mechanistic understanding of the role that CaMKII self-association plays in neuronal viability will illuminate long-standing questions associated with the changes in CaMKII localization and activity during ischemia. Importantly, these studies may identify a mechanism to therapeutically target CaMKII in the time following ischemia/excitotoxic insult.

My data support the idea that therapeutic intervention should aim at restoring the availability of CaMKII after insult rather than inhibiting it. While pharmacological inhibitors afforded neuroprotection when applied in the time immediately surrounding insult, I believe this was because they prevented prolonged inactivation, which induces toxicity. However, it is also conceivable that these inhibitors prevented the phosphorylation of key proteins that destabilize neuronal excitability and survival during excitotoxic calcium signaling. The therapeutic time window for CaMKII inhibitors may be relatively brief, as CaMKII is already inactivating and aggregating in the time following insult. To date, no inhibitor has been shown to be able to restore activity or reverse aggregation. As suggested above, deciphering the mechanism behind isoform-dependent self-association may provide key information as to how to therapeutic target this process following an ischemic event. Furthermore, because protein aggregation has become a hallmark of many disease states, we believe self-association may provide additional insights into how protein aggregation influences neuronal survival and function following cellular stress.

IMPLICATIONS TOWARDS OTHER NEURODEGENERATIVE DISEASES

Excitotoxicity that accompanies several neurodegenerative diseases is marked by dysregulated calcium signaling, limited ATP availability, decreased pH, and the generation of reactive oxygen species. While roles for ATP and pH in CaMKII self-association and inactivation have been established, the role oxidative stress plays is not understood. As mentioned before, oxidation of residues within the autoregulatory domain (Met281/Met282) has been shown to increase CaMKII activity in other cell systems (Erickson et al., 2008). Interestingly, oxidative stress has been associated with aggregation and inactivation within synaptosomes (Shetty et al., 2008). Based on these findings, we hypothesize that increased reactive oxygen species may aberrantly increase CaMKII activity during periods of cellular stress such as excitotoxic insult, thereby enhancing the sensitivity of αCaMKII to self-associate. Consistent with this, we have preliminary data correlating increased reactive oxygen species generation in neurons following excitotoxic stimulation and the presence of self-associated αCaMKII (Ashpole and Hudmon, unpublished). Moreover, *in vitro* analysis indicates that H_2O_2 treatment induces αCaMKII aggregation (Ashpole and Hudmon, unpublished). Thus, oxidative stress during excitotoxic insult could contribute to self-association and inactivation of CaMKII.

Based on the findings presented here, it is likely that CaMKII is aberrantly-regulated within several neurodegenerative diseases, as they are associated with increased intracellular calcium, decreased energy availability, and reduced intracellular pH. Aberrant increases in CaMKII activity have been identified

following traumatic brain injury (Atkins et al., 2006, Folkerts et al., 2007).

Inhibition of CaMKII signaling with small molecule KN-62 reduced the extent of

neuronal damage following traumatic brain injury (Zhang et al., 2012).

Interestingly, isoforms of CaMKII has been shown to be differentially regulated

following traumatic brain injury. αCaMKII levels are significantly lower in animal

models of traumatic brain injury while δCaMKII levels are significantly increased

in the days following injury (Schwarzbach et al., 2006, Zhang et al., 2012).

Together, these findings indicate that CaMKII expression and activity are altered

during traumatic brain injury and may contribute to the damage observed

following insult.

Alzheimer's disease is marked by the formation of cellular plaques and

tangles, and is associated with a subsequent excitotoxic neurodegeneration.

Interestingly, the Alzheimer's associated protein β-amyloid has been shown to

decrease Thr286 autophosphorylation when applied to neurons *in situ* (Zhao et

al., 2004). Furthermore, application of the β-amyloid peptide 1-42 resulted in a

redistribution of CaMKII away from the dendritic process to the soma (Reese et

al., 2011). Similarly, the distribution of CaMKII is altered in human patients with

mild cognitive impairment and late stage Alzheimer's disease (Reese et al.,

2011). The hallmark senile plaques observed in Alzheimer's disease have been

shown to co-localize with αCaMKII (Xiao et al., 1996). While a direct link between

CaMKII self-association and β-Amyloid plaques has not yet been made, it is

interesting that Alzheimer's disease is marked by a decrease in phospho-Thr286,

as this decrease would render αCaMKII susceptible to self-association (a process I propose contributes to neuronal death).

A commonality between disease states with aberrant CaMKII activity is their association with glutamate-induced neurodegeneration. Ischemia, traumatic brain injury, and Alzheimer's disease are all marked by increased glutamate release and neuronal excitation. Several other disease states, such as Huntington's disease, amyotrophic lateral sclerosis, and Parkinson's disease, are all associated glutamate toxicity as well. While CaMKII signaling has not yet been implicated in these diseases, it is possible that because aberrant glutamate signaling dramatically impacts CaMKII function, CaMKII dysregulation occurs with these diseases as well. Further characterization of CaMKII in these disease states is necessary. As mentioned above, the phospho-Thr253 antibody may be beneficial as an indicator of CaMKII inactivation in these disease states.

Several HIV-related proteins have also been shown to lead to increased neuronal activity, increased glutamate release, and dysregulated intracellular calcium. As a consequence, neuronal dysfunction is prevalent within patients infected with HIV. One HIV protein that has been implicated in these devastating changes is the HIV-coat protein, Tat. Immunological studies have identified that many CaMKII signaling pathways are disrupted by HIV-Tat (Poggi et al., 2002a, Poggi et al., 2002b). Recent unpublished findings from our lab indicate that HIV-Tat interacts with CaMKII in neurons and is an effective CaMKII inhibitor (Ashpole and Hudmon, unpublished). The portion of Tat that is responsible for CaMKII inhibition (a region upstream of the cell-penetrant motif used throughout

my studies) has also been shown to dysregulate calcium homeostasis and induce neurotoxicity (Ashpole and Hudmon, unpublished). Thus, it is possible that some of the deficits associated with HIV-Tat or other HIV-associated proteins are due to the disruption of CaMKII signaling. It will be interesting to see if disease models of HIV exhibit reduced CaMKII activity. Together, these studies would solidify a role for CaMKII inactivation as a global initiator of neuronal death.

CONCLUSIONS

The findings of this dissertation deepen the understanding of the physiological and pathophysiological role of CaMKII signaling in neurons. We have strong evidence that aberrant fluctuations in CaMKII activity play a causal role in neuronal death. The increases in activity during periods of neuronal stress are detrimental as they lead to a prolonged inactivation of the kinase,which increases neuronal excitabililty, dysregulates calcium homeostasis, induces toxicity, and predisposes neurons to other cellular stressors. These findings are consistent with previous studies in other cell systems (bacteria, plants, and invertebrates) indicating that the dysregulation of CaMKII leads to toxicity and predisposes cells to subsequent toxic insults. Thus, my findings support a role for CaMKII within neurons as a master regulator of viability, and universal role for CaMKII as a governor of cellular responsiveness to insults.

REFERENCES

Ackermann F, Zitranski N, Borth H, Buech T, Gudermann T, Boekhoff I (2009) CaMKIIalpha interacts with multi-PDZ domain protein MUPP1 in spermatozoa and prevents spontaneous acrosomal exocytosis. J Cell Sci 122:4547-4557.

Albers GW, Clark WM, Atkinson RP, Madden K, Data JL, Whitehouse MJ (1999) Dose escalation study of the NMDA glycine-site antagonist licostinel in acute ischemic stroke. Stroke 30:508-513.

Alemany V, Sanchez-Piris M, Bachs O, Aligue R (2002) Cmk2, a novel serine/threonine kinase in fission yeast. FEBS Lett 524:79-86.

Araque A, Li N, Doyle RT, Haydon PG (2000) SNARE protein-dependent glutamate release from astrocytes. J Neurosci 20:666-673.

Aronowski J, Grotta JC (1996) Ca2+/calmodulin-dependent protein kinase II in postsynaptic densities after reversible cerebral ischemia in rats. Brain Res 709:103-110.

Aronowski J, Grotta JC, Waxham MN (1992) Ischemia-induced translocation of Ca2+/calmodulin-dependent protein kinase II: potential role in neuronal damage. J Neurochem 58:1743-1753.

Aronowski J, Strong R, Kang HS, Grotta JC (2000) Selective up-regulation of I kappaB-alpha in ischemic penumbra following focal cerebral ischemia. Neuroreport 11:1529-1533.

Aronowski J, Waxham MN, Grotta JC (1993) Neuronal protection and preservation of calcium/calmodulin-dependent protein kinase II and protein kinase C activity by dextrorphan treatment in global ischemia. J Cereb Blood Flow Metab 13:550-557.

Ashpole NM, Hudmon A (2011) Excitotoxic neuroprotection and vulnerability with CaMKII inhibition. Mol Cell Neurosci 46:720-730.

Ashpole NM, Song W, Brustovetsky T, Engleman EA, Brustovetsky N, Cummins TR, Hudmon A (2012) Calcium/calmodulin-dependent protein kinase II (CaMKII) inhibition induces neurotoxicity via dysregulation of glutamate/calcium signaling and hyperexcitability. J Biol Chem 287:8495-8506.

Atkins CM, Chen S, Alonso OF, Dietrich WD, Hu BR (2006) Activation of calcium/calmodulin-dependent protein kinases after traumatic brain injury. J Cereb Blood Flow Metab 26:1507-1518.

Babu YS, Sack JS, Greenhough TJ, Bugg CE, Means AR, Cook WJ (1985) Three-dimensional structure of calmodulin. Nature 315:37-40.

Backs J, Backs T, Neef S, Kreusser MM, Lehmann LH, Patrick DM, Grueter CE, Qi X, Richardson JA, Hill JA, Katus HA, Bassel-Duby R, Maier LS, Olson EN (2009) The delta isoform of CaM kinase II is required for pathological cardiac hypertrophy and remodeling after pressure overload. Proc Natl Acad Sci U S A 106:2342-2347.

Banker GA (1980) Trophic interactions between astroglial cells and hippocampal neurons in culture. Science 209:809-810.

Barres BA (2008) The mystery and magic of glia: a perspective on their roles in health and disease. Neuron 60:430-440.

Barria A, Derkach V, Soderling T (1997a) Identification of the Ca2+/calmodulin-dependent protein kinase II regulatory phosphorylation site in the alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate-type glutamate receptor. J Biol Chem 272:32727-32730.

Barria A, Muller D, Derkach V, Griffith LC, Soderling TR (1997b) Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. Science 276:2042-2045.

Bats C, Groc L, Choquet D (2007) The interaction between Stargazin and PSD-95 regulates AMPA receptor surface trafficking. Neuron 53:719-734.

Bayer KU, De Koninck P, Leonard AS, Hell JW, Schulman H (2001) Interaction with the NMDA receptor locks CaMKII in an active conformation. Nature 411:801-805.

Bayer KU, LeBel E, McDonald GL, O'Leary H, Schulman H, De Koninck P (2006) Transition from reversible to persistent binding of CaMKII to postsynaptic sites and NR2B. J Neurosci 26:1164-1174.

Bayer KU, Lohler J, Schulman H, Harbers K (1999) Developmental expression of the CaM kinase II isoforms: ubiquitous gamma- and delta-CaM kinase II are the early isoforms and most abundant in the developing nervous system. Brain Res Mol Brain Res 70:147-154.

Beaton GH, Curry DM, Veen MJ (1957) Alanine-glutamic transaminase activity and protein metabolism. Arch Biochem Biophys 70:288-290.

Behe P, Stern P, Wyllie DJ, Nassar M, Schoepfer R, Colquhoun D (1995) Determination of NMDA NR1 subunit copy number in recombinant NMDA receptors. Proc Biol Sci 262:205-213.

Bennett MK, Erondu NE, Kennedy MB (1983) Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. J Biol Chem 258:12735-12744.

Benveniste H, Drejer J, Schousboe A, Diemer NH (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. J Neurochem 43:1369-1374.

Bingol B, Wang CF, Arnott D, Cheng D, Peng J, Sheng M (2010) Autophosphorylated CaMKIIalpha acts as a scaffold to recruit proteasomes to dendritic spines. Cell 140:567-578.

Bok J, Wang Q, Huang J, Green SH (2007) CaMKII and CaMKIV mediate distinct prosurvival signaling pathways in response to depolarization in neurons. Mol Cell Neurosci 36:13-26.

Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci U S A 92:7162-7166.

Bradshaw JM, Hudmon A, Schulman H (2002) Chemical quenched flow kinetic studies indicate an intraholoenzyme autophosphorylation mechanism for Ca2+/calmodulin-dependent protein kinase II. J Biol Chem 277:20991-20998.

Bredesen DE (2007) Key note lecture: toward a mechanistic taxonomy for cell death programs. Stroke 38:652-660.

Brickey DA, Bann JG, Fong YL, Perrino L, Brennan RG, Soderling TR (1994) Mutational analysis of the autoinhibitory domain of calmodulin kinase II. J Biol Chem 269:29047-29054.

Brocke L, Chiang LW, Wagner PD, Schulman H (1999) Functional implications of the subunit composition of neuronal CaM kinase II. J Biol Chem 274:22713-22722.

Brooks H, Lebleu B, Vives E (2005) Tat peptide-mediated cellular delivery: back to basics. Adv Drug Deliv Rev 57:559-577.

Brustovetsky T, Brittain MK, Sheets PL, Cummins TR, Pinelis V, Brustovetsky N (2011) KB-R7943, an inhibitor of the reverse Na+ /Ca2+ exchanger, blocks N-methyl-D-aspartate receptor and inhibits mitochondrial complex I. Br J Pharmacol 162:255-270.

Brustovetsky T, Li V, Brustovetsky N (2009) Stimulation of glutamate receptors in cultured hippocampal neurons causes Ca2+-dependent mitochondrial contraction. Cell Calcium 46:18-29.

Buard I, Coultrap SJ, Freund RK, Lee YS, Dell'Acqua ML, Silva AJ, Bayer KU (2010) CaMKII "autonomy" is required for initiating but not for maintaining neuronal long-term information storage. J Neurosci 30:8214-8220.

Budd SL, Tenneti L, Lishnak T, Lipton SA (2000) Mitochondrial and extramitochondrial apoptotic signaling pathways in cerebrocortical neurons. Proc Natl Acad Sci U S A 97:6161-6166.

Bui JD, Calbo S, Hayden-Martinez K, Kane LP, Gardner P, Hedrick SM (2000) A role for CaMKII in T cell memory. Cell 100:457-467.

Bullock R, Graham DI, Chen MH, Lowe D, McCulloch J (1990) Focal cerebral ischemia in the cat: pretreatment with a competitive NMDA receptor antagonist, D-CPP-ene. J Cereb Blood Flow Metab 10:668-674.

Bullock R, Zauner A, Woodward J, Young HF (1995) Massive persistent release of excitatory amino acids following human occlusive stroke. Stroke 26:2187-2189.

Burgin KE, Waxham MN, Rickling S, Westgate SA, Mobley WC, Kelly PT (1990a) In situ hybridization histochemistry of Ca2+/calmodulin-dependent protein kinase in developing rat brain. J Neurosci 10:1788-1798.

Burgin KE, Waxham MN, Rickling S, Westgate SA, Mobley WC, Kelly PT (1990b) *In situ* hybridization histochemistry of Ca^{2+}/calmodulin-dependent protein kinase in developing rat brain. J Neurosci 10:1788-1798.

Butcher SP, Bullock R, Graham DI, McCulloch J (1990) Correlation between amino acid release and neuropathologic outcome in rat brain following middle cerebral artery occlusion. Stroke 21:1727-1733.

Butler LS, Silva AJ, Abeliovich A, Watanabe Y, Tonegawa S, McNamara JO (1995) Limbic epilepsy in transgenic mice carrying a Ca2+/calmodulin-dependent kinase II alpha-subunit mutation. Proc Natl Acad Sci U S A 92:6852-6855.

Byrne MJ, Putkey JA, Waxham MN, Kubota Y (2009) Dissecting cooperative calmodulin binding to CaM kinase II: a detailed stochastic model. J Comput Neurosci 27:621-638.

Carter DS, Haider SN, Blair RE, Deshpande LS, Sombati S, DeLorenzo RJ (2006) Altered calcium/calmodulin kinase II activity changes calcium homeostasis that underlies epileptiform activity in hippocampal neurons in culture. J Pharmacol Exp Ther 319:1021-1031.

Centemeri C, Bolego C, Abbracchio MP, Cattabeni F, Puglisi L, Burnstock G, Nicosia S (1997) Characterization of the Ca2+ responses evoked by ATP and other nucleotides in mammalian brain astrocytes. Br J Pharmacol 121:1700-1706.

Chang BH, Mukherji S, Soderling TR (1998) Characterization of a calmodulin kinase II inhibitor protein in brain. Proc Natl Acad Sci U S A 95:10890-10895.

Chang BH, Mukherji S, Soderling TR (2001) Calcium/calmodulin-dependent protein kinase II inhibitor protein: localization of isoforms in rat brain. Neuroscience 102:767-777.

Chao LH, Pellicena P, Deindl S, Barclay LA, Schulman H, Kuriyan J (2010) Intersubunit capture of regulatory segments is a component of cooperative CaMKII activation. Nat Struct Mol Biol 17:264-272.

Chao LH, Stratton MM, Lee IH, Rosenberg OS, Levitz J, Mandell DJ, Kortemme T, Groves JT, Schulman H, Kuriyan J (2011) A mechanism for tunable autoinhibition in the structure of a human Ca2+/calmodulin- dependent kinase II holoenzyme. Cell 146:732-745.

Chen HX, Otmakhov N, Strack S, Colbran RJ, Lisman JE (2001) Is persistent activity of calcium/calmodulin-dependent kinase required for the maintenance of LTP? J Neurophysiol 85:1368-1376.

Chen Y, Stevens B, Chang J, Milbrandt J, Barres BA, Hell JW (2008) NS21: re-defined and modified supplement B27 for neuronal cultures. J Neurosci Methods 171:239-247.

Cheung NS, Pascoe CJ, Giardina SF, John CA, Beart PM (1998) Micromolar L-glutamate induces extensive apoptosis in an apoptotic-necrotic continuum of insult-dependent, excitotoxic injury in cultured cortical neurones. Neuropharmacology 37:1419-1429.

Choi DW (1985) Glutamate neurotoxicity in cortical cell culture is calcium dependent. Neurosci Lett 58:293-297.

Choi DW (1987) Ionic dependence of glutamate neurotoxicity. J Neurosci 7:369-379.

Choi DW (1988) Glutamate neurotoxicity and diseases of the nervous system. Neuron 1:623-634.

Choi DW, Koh JY, Peters S (1988) Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. J Neurosci 8:185-196.

Choi DW, Maulucci-Gedde M, Kriegstein AR (1987) Glutamate neurotoxicity in cortical cell culture. J Neurosci 7:357-368.

Churn SB, Limbrick D, Sombati S, DeLorenzo RJ (1995) Excitotoxic activation of the NMDA receptor results in inhibition of calcium/calmodulin kinase II activity in cultured hippocampal neurons. J Neurosci 15:3200-3214.

Churn SB, Taft WC, Billingsley MS, Sankaran B, DeLorenzo RJ (1992a) Global forebrain ischemia induces a posttranslational modification of multifunctional calcium- and calmodulin-dependent kinase II. J Neurochem 59:1221-1232.

Churn SB, Yaghmai A, Povlishock J, Rafiq A, DeLorenzo RJ (1992b) Global forebrain ischemia results in decreased immunoreactivity of calcium/calmodulin-dependent protein kinase II. J Cereb Blood Flow Metab 12:784-793.

Coco S, Calegari F, Pravettoni E, Pozzi D, Taverna E, Rosa P, Matteoli M, Verderio C (2003) Storage and release of ATP from astrocytes in culture. J Biol Chem 278:1354-1362.

Colbran RJ (1992) Regulation and role of brain calcium/calmodulin-dependent protein kinase II. Neurochem Int 21:469-497.

Colbran RJ (1993) Inactivation of Ca2+/calmodulin-dependent protein kinase II by basal autophosphorylation. J Biol Chem 268:7163-7170.

Colbran RJ (2004) Targeting of calcium/calmodulin-dependent protein kinase II. Biochem J 378:1-16.

Colbran RJ, Fong YL, Schworer CM, Soderling TR (1988) Regulatory interactions of the calmodulin-binding, inhibitory, and autophosphorylation domains of Ca2+/calmodulin-dependent protein kinase II. J Biol Chem 263:18145-18151.

Colbran RJ, Smith MK, Schworer CM, Fong YL, Soderling TR (1989) Regulatory domain of calcium/calmodulin-dependent protein kinase II. Mechanism of inhibition and regulation by phosphorylation. J Biol Chem 264:4800-4804.

Colbran RJ, Soderling TR (1990) Calcium/calmodulin-independent autophosphorylation sites of calcium/calmodulin-dependent protein kinase II. Studies on the effect of phosphorylation of threonine 305/306 and serine 314 on calmodulin binding using synthetic peptides. J Biol Chem 265:11213-11219.

Cook PF, Neville ME, Jr., Vrana KE, Hartl FT, Roskoski R, Jr. (1982) Adenosine cyclic 3',5'-monophosphate dependent protein kinase: kinetic mechanism for the bovine skeletal muscle catalytic subunit. Biochemistry 21:5794-5799.

Cotrina ML, Lin JH, Lopez-Garcia JC, Naus CC, Nedergaard M (2000) ATP-mediated glia signaling. J Neurosci 20:2835-2844.

Coultrap SJ, Bayer KU (2011) Improving a natural CaMKII inhibitor by random and rational design. PLoS One 6:e25245.

Coultrap SJ, Vest RS, Ashpole NM, Hudmon A, Bayer KU (2011) CaMKII in cerebral ischemia. Acta Pharmacol Sin 32:861-872.

D'Ascenzo M, Vairano M, Andreassi C, Navarra P, Azzena GB, Grassi C (2004) Electrophysiological and molecular evidence of L-(Cav1), N- (Cav2.2), and R- (Cav2.3) type Ca2+ channels in rat cortical astrocytes. Glia 45:354-363.

Dash PK, Karl KA, Colicos MA, Prywes R, Kandel ER (1991) cAMP response element-binding protein is activated by Ca2+/calmodulin- as well as cAMP-dependent protein kinase. Proc Natl Acad Sci U S A 88:5061-5065.

Davalos A, Castillo J, Serena J, Noya M (1997) Duration of glutamate release after acute ischemic stroke. Stroke 28:708-710.

Davis SM, Lees KR, Albers GW, Diener HC, Markabi S, Karlsson G, Norris J (2000) Selfotel in acute ischemic stroke : possible neurotoxic effects of an NMDA antagonist. Stroke 31:347-354.

del Zoppo GJ, Poeck K, Pessin MS, Wolpert SM, Furlan AJ, Ferbert A, Alberts MJ, Zivin JA, Wechsler L, Busse O, et al. (1992) Recombinant tissue plasminogen activator in acute thrombotic and embolic stroke. Ann Neurol 32:78-86.

Derkach V, Barria A, Soderling TR (1999) Ca2+/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. Proc Natl Acad Sci U S A 96:3269-3274.

Dermietzel R, Traub O, Hwang TK, Beyer E, Bennett MV, Spray DC, Willecke K (1989) Differential expression of three gap junction proteins in developing and mature brain tissues. Proc Natl Acad Sci U S A 86:10148-10152.

Deshpande J, Bergstedt K, Linden T, Kalimo H, Wieloch T (1992) Ultrastructural changes in the hippocampal CA1 region following transient cerebral ischemia: evidence against programmed cell death. Exp Brain Res 88:91-105.

Dichter MA (1978) Rat cortical neurons in cell culture: culture methods, cell morphology, electrophysiology, and synapse formation. Brain Res 149:279-293.

Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 22:391-397.

Dong C, Upadhya SC, Ding L, Smith TK, Hegde AN (2008) Proteasome inhibition enhances the induction and impairs the maintenance of late-phase long-term potentiation. Learn Mem 15:335-347.

Donzanti BA, Yamamoto BK (1988) An improved and rapid HPLC-EC method for the isocratic separation of amino acid neurotransmitters from brain tissue and microdialysis perfusates. Life Sci 43:913-922.

Dosemeci A, Reese TS, Petersen J, Tao-Cheng JH (2000) A novel particulate form of Ca(2+)/calmodulin-dependent [correction of Ca(2+)/CaMKII-dependent] protein kinase II in neurons. J Neurosci 20:3076-3084.

Dosemeci A, Tao-Cheng JH, Vinade L, Winters CA, Pozzo-Miller L, Reese TS (2001) Glutamate-induced transient modification of the postsynaptic density. Proc Natl Acad Sci U S A 98:10428-10432.

Duan S, Anderson CM, Keung EC, Chen Y, Swanson RA (2003) P2X7 receptor-mediated release of excitatory amino acids from astrocytes. J Neurosci 23:1320-1328.

Dubinsky JM (1993) Intracellular calcium levels during the period of delayed excitotoxicity. J Neurosci 13:623-631.

Easton AC, Lucchesi W, Schumann G, Giese KP, Muller CP, Fernandes C (2011) alphaCaMKII autophosphorylation controls exploratory activity to threatening novel stimuli. Neuropharmacology 61:1424-1431.

Eleff SM, Maruki Y, Monsein LH, Traystman RJ, Bryan RN, Koehler RC (1991) Sodium, ATP, and intracellular pH transients during reversible complete ischemia of dog cerebrum. Stroke 22:233-241.

Elgersma Y, Fedorov NB, Ikonen S, Choi ES, Elgersma M, Carvalho OM, Giese KP, Silva AJ (2002) Inhibitory autophosphorylation of CaMKII controls PSD association, plasticity, and learning. Neuron 36:493-505.

Enslen H, Sun P, Brickey D, Soderling SH, Klamo E, Soderling TR (1994) Characterization of Ca2+/calmodulin-dependent protein kinase IV. Role in transcriptional regulation. J Biol Chem 269:15520-15527.

Epigenomics (2012) National Center for Biotechnology Information. U.S. National Library of Medicine.

Erickson JR, Joiner ML, Guan X, Kutschke W, Yang J, Oddis CV, Bartlett RK, Lowe JS, O'Donnell SE, Aykin-Burns N, Zimmerman MC, Zimmerman K, Ham AJ, Weiss RM, Spitz DR, Shea MA, Colbran RJ, Mohler PJ, Anderson ME (2008) A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. Cell 133:462-474.

Erondu NE, Kennedy MB (1985) Regional distribution of type II Ca2+/calmodulin-dependent protein kinase in rat brain. J Neurosci 5:3270-3277.

Evans TI, Shea MA (2009) Energetics of calmodulin domain interactions with the calmodulin binding domain of CaMKII. Proteins 76:47-61.

Fan W, Agarwal N, Cooper NG (2006) The role of CaMKII in BDNF-mediated neuroprotection of retinal ganglion cells (RGC-5). Brain Res 1067:48-57.

Ferrari D, Los M, Bauer MK, Vandenabeele P, Wesselborg S, Schulze-Osthoff K (1999) P2Z purinoreceptor ligation induces activation of caspases with distinct roles in apoptotic and necrotic alterations of cell death. FEBS Lett 447:71-75.

Fink CC, Bayer KU, Myers JW, Ferrell JE, Jr., Schulman H, Meyer T (2003) Selective regulation of neurite extension and synapse formation by the beta but not the alpha isoform of CaMKII. Neuron 39:283-297.

Finkbeiner S, Stevens CF (1988) Applications of quantitative measurements for assessing glutamate neurotoxicity. Proc Natl Acad Sci U S A 85:4071-4074.

Folkerts MM, Parks EA, Dedman JR, Kaetzel MA, Lyeth BG, Berman RF (2007) Phosphorylation of calcium calmodulin-dependent protein kinase II following lateral fluid percussion brain injury in rats. J Neurotrauma 24:638-650.

Fong YL, Taylor WL, Means AR, Soderling TR (1989) Studies of the regulatory mechanism of Ca2+/calmodulin-dependent protein kinase II. Mutation of threonine 286 to alanine and aspartate. J Biol Chem 264:16759-16763.

Forest A, Swulius MT, Tse JK, Bradshaw JM, Gaertner T, Waxham MN (2008) Role of the N- and C-lobes of calmodulin in the activation of Ca(2+)/calmodulin-dependent protein kinase II. Biochemistry 47:10587-10599.

Foster AC, Gill R, Kemp JA, Woodruff GN (1987) Systemic administration of MK-801 prevents N-methyl-D-aspartate-induced neuronal degeneration in rat brain. Neurosci Lett 76:307-311.

Frandsen A, Drejer J, Schousboe A (1989) Direct evidence that excitotoxicity in cultured neurons is mediated via N-methyl-D-aspartate (NMDA) as well as non-NMDA receptors. J Neurochem 53:297-299.

Frank R (2002) The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports--principles and applications. J Immunol Methods 267:13-26.

Frank R, Overwin H (1996) SPOT synthesis. Epitope analysis with arrays of synthetic peptides prepared on cellulose membranes. Methods Mol Biol 66:149-169.

Franke H, Gunther A, Grosche J, Schmidt R, Rossner S, Reinhardt R, Faber-Zuschratter H, Schneider D, Illes P (2004) P2X7 receptor expression after ischemia in the cerebral cortex of rats. J Neuropathol Exp Neurol 63:686-699.

Franke H, Illes P (2006) Involvement of P2 receptors in the growth and survival of neurons in the CNS. Pharmacol Ther 109:297-324.

Frankland PW, Bontempi B, Talton LE, Kaczmarek L, Silva AJ (2004) The involvement of the anterior cingulate cortex in remote contextual fear memory. Science 304:881-883.

Frankland PW, O'Brien C, Ohno M, Kirkwood A, Silva AJ (2001) Alpha-CaMKII-dependent plasticity in the cortex is required for permanent memory. Nature 411:309-313.

Fu XZ, Zhang QG, Meng FJ, Zhang GY (2004) NMDA receptor-mediated immediate Ser831 phosphorylation of GluR1 through CaMKIIalpha in rat hippocampus during early global ischemia. Neurosci Res 48:85-91.

Fujioka H, Kaneko H, Suzuki SS, Mabuchi K (2004) Hyperexcitability-associated rapid plasticity after a focal cerebral ischemia. Stroke 35:e346-348.

Fukunaga K, Goto S, Miyamoto E (1988) Immunohistochemical localization of Ca2+/calmodulin-dependent protein kinase II in rat brain and various tissues. J Neurochem 51:1070-1078.

Fukunaga K, Kobayashi T, Tamura S, Miyamoto E (1993) Dephosphorylation of autophosphorylated Ca2+/calmodulin-dependent protein kinase II by protein phosphatase 2C. J Biol Chem 268:133-137.

Fukunaga K, Soderling TR (1990) Activation of Ca(2+)/calmodulin-dependent protein kinase II in cerebellar granule cells by N-methyl-d-aspartate receptor activation. Mol Cell Neurosci 1:133-138.

Fukunaga K, Soderling TR, Miyamoto E (1992) Activation of Ca2+/calmodulin-dependent protein kinase II and protein kinase C by glutamate in cultured rat hippocampal neurons. J Biol Chem 267:22527-22533.

Gaertner TR, Kolodziej SJ, Wang D, Kobayashi R, Koomen JM, Stoops JK, Waxham MN (2004) Comparative analyses of the three-dimensional structures and enzymatic properties of alpha, beta, gamma and delta isoforms of Ca2+-calmodulin-dependent protein kinase II. J Biol Chem 279:12484-12494.

Gallagher CJ, Salter MW (2003) Differential properties of astrocyte calcium waves mediated by P2Y1 and P2Y2 receptors. J Neurosci 23:6728-6739.

Gao L, Blair LA, Marshall J (2006) CaMKII-independent effects of KN93 and its inactive analog KN92: reversible inhibition of L-type calcium channels. Biochem Biophys Res Commun 345:1606-1610.

Gardoni F, Bellone C, Cattabeni F, Di Luca M (2001) Protein kinase C activation modulates alpha-calmodulin kinase II binding to NR2A subunit of N-methyl-D-aspartate receptor complex. J Biol Chem 276:7609-7613.

Gardoni F, Schrama LH, van Dalen JJ, Gispen WH, Cattabeni F, Di Luca M (1999) AlphaCaMKII binding to the C-terminal tail of NMDA receptor subunit NR2A and its modulation by autophosphorylation. FEBS Lett 456:394-398.

Ginsberg MD (2008) Neuroprotection for ischemic stroke: past, present and future. Neuropharmacology 55:363-389.

Ginsberg MD (2009) Current status of neuroprotection for cerebral ischemia: synoptic overview. Stroke 40:S111-114.

Globus MY, Busto R, Dietrich WD, Martinez E, Valdes I, Ginsberg MD (1988) Intra-ischemic extracellular release of dopamine and glutamate is associated with striatal vulnerability to ischemia. Neurosci Lett 91:36-40.

Goebel DJ (2009) Selective blockade of CaMKII-alpha inhibits NMDA-induced caspase-3-dependent cell death but does not arrest PARP-1 activation or loss of plasma membrane selectivity in rat retinal neurons. Brain Res 1256:190-204.

Gotti B, Duverger D, Bertin J, Carter C, Dupont R, Frost J, Gaudilliere B, MacKenzie ET, Rousseau J, Scatton B, et al. (1988) Ifenprodil and SL 82.0715 as cerebral anti-ischemic agents. I. Evidence for efficacy in models of focal cerebral ischemia. J Pharmacol Exp Ther 247:1211-1221.

Greengard P, Browning MD, McGuinness TL, Llinas R (1987) Synapsin I, a phosphoprotein associated with synaptic vesicles: possible role in regulation of neurotransmitter release. Adv Exp Med Biol 221:135-153.

Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB (1999) ATP released from astrocytes mediates glial calcium waves. J Neurosci 19:520-528.

Hajimohammadreza I, Probert AW, Coughenour LL, Borosky SA, Marcoux FW, Boxer PA, Wang KK (1995) A specific inhibitor of calcium/calmodulin-dependent protein kinase-II provides neuroprotection against NMDA- and hypoxia/hypoglycemia-induced cell death. J Neurosci 15:4093-4101.

Hamilton N, Vayro S, Kirchhoff F, Verkhratsky A, Robbins J, Gorecki DC, Butt AM (2008) Mechanisms of ATP- and glutamate-mediated calcium signaling in white matter astrocytes. Glia 56:734-749.

Hanson PI, Kapiloff MS, Lou LL, Rosenfeld MG, Schulman H (1989) Expression of a multifunctional Ca2+/calmodulin-dependent protein kinase and mutational analysis of its autoregulation. Neuron 3:59-70.

Hanson PI, Schulman H (1992a) Inhibitory autophosphorylation of multifunctional Ca2+/calmodulin-dependent protein kinase analyzed by site-directed mutagenesis. J Biol Chem 267:17216-17224.

Hanson PI, Schulman H (1992b) Neuronal Ca2+/calmodulin-dependent protein kinases. Annu Rev Biochem 61:559-601.

Hanson SK, Grotta JC, Waxham MN, Aronowski J, Ostrow P (1994) Calcium/calmodulin-dependent protein kinase II activity in focal ischemia with reperfusion in rats. Stroke 25:466-473.

Hao ZB, Pei DS, Guan QH, Zhang GY (2005) Calcium/calmodulin-dependent protein kinase II (CaMKII), through NMDA receptors and L-Voltage-gated channels, modulates the serine phosphorylation of GluR6 during cerebral ischemia and early reperfusion period in rat hippocampus. Brain Res Mol Brain Res 140:55-62.

Hardingham GE, Fukunaga Y, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5:405-414.

Hashimoto Y, Schworer CM, Colbran RJ, Soderling TR (1987) Autophosphorylation of Ca2+/calmodulin-dependent protein kinase II. Effects on total and Ca2+-independent activities and kinetic parameters. J Biol Chem 262:8051-8055.

Hoch B, Meyer R, Hetzer R, Krause EG, Karczewski P (1999) Identification and expression of delta-isoforms of the multifunctional Ca2+/calmodulin-dependent protein kinase in failing and nonfailing human myocardium. Circ Res 84:713-721.

Hoelz A, Nairn AC, Kuriyan J (2003) Crystal structure of a tetradecameric assembly of the association domain of Ca2+/calmodulin-dependent kinase II. Mol Cell 11:1241-1251.

Hoffman L, Stein RA, Colbran RJ, McHaourab HS (2011) Conformational changes underlying calcium/calmodulin-dependent protein kinase II activation. EMBO J 30:1251-1262.

Hou ST, Jiang SX, Aylsworth A, Ferguson G, Slinn J, Hu H, Leung T, Kappler J, Kaibuchi K (2009) CaMKII phosphorylates collapsin response mediator protein 2 and modulates axonal damage during glutamate excitotoxicity. J Neurochem 111:870-881.

Houston CM, Smart TG (2006) CaMK-II modulation of GABA(A) receptors expressed in HEK293, NG108-15 and rat cerebellar granule neurons. Eur J Neurosci 24:2504-2514.

Howe CJ, Lahair MM, McCubrey JA, Franklin RA (2004) Redox regulation of the calcium/calmodulin-dependent protein kinases. J Biol Chem 279:44573-44581.

Hoyte L, Barber PA, Buchan AM, Hill MD (2004) The rise and fall of NMDA antagonists for ischemic stroke. Curr Mol Med 4:131-136.

Hu BR, Park M, Martone ME, Fischer WH, Ellisman MH, Zivin JA (1998) Assembly of proteins to postsynaptic densities after transient cerebral ischemia. J Neurosci 18:625-633.

Huang RY, Laing JG, Kanter EM, Berthoud VM, Bao M, Rohrs HW, Townsend RR, Yamada KA (2011) Identification of CaMKII phosphorylation sites in Connexin43 by high-resolution mass spectrometry. J Proteome Res 10:1098-1109.

Hudmon A, Aronowski J, Kolb SJ, Waxham MN (1996) Inactivation and self-association of Ca2+/calmodulin-dependent protein kinase II during autophosphorylation. J Biol Chem 271:8800-8808.

Hudmon A, Kim SA, Kolb SJ, Stoops JK, Waxham MN (2001) Light scattering and transmission electron microscopy studies reveal a mechanism for calcium/calmodulin-dependent protein kinase II self-association. J Neurochem 76:1364-1375.

Hudmon A, Lebel E, Roy H, Sik A, Schulman H, Waxham MN, De Koninck P (2005) A mechanism for Ca2+/calmodulin-dependent protein kinase II clustering at synaptic and nonsynaptic sites based on self-association. J Neurosci 25:6971-6983.

Hudmon A, Schulman H (2002) Neuronal CA2+/calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function. Annu Rev Biochem 71:473-510.

Huettner JE, Bean BP (1988) Block of N-methyl-D-aspartate-activated current by the anticonvulsant MK-801: selective binding to open channels. Proc Natl Acad Sci U S A 85:1307-1311.

Inagaki M, Nishi Y, Nishizawa K, Matsuyama M, Sato C (1987) Site-specific phosphorylation induces disassembly of vimentin filaments in vitro. Nature 328:649-652.

Inoue K, Koizumi S, Tsuda M (2007) The role of nucleotides in the neuron--glia communication responsible for the brain functions. J Neurochem 102:1447-1458.

Ishida A, Fujisawa H (1995) Stabilization of calmodulin-dependent protein kinase II through the autoinhibitory domain. J Biol Chem 270:2163-2170.

Ishida A, Kameshita I, Okuno S, Kitani T, Fujisawa H (1995) A novel highly specific and potent inhibitor of calmodulin-dependent protein kinase II. Biochem Biophys Res Commun 212:806-812.

Ivanov A, Pellegrino C, Rama S, Dumalska I, Salyha Y, Ben-Ari Y, Medina I (2006) Opposing role of synaptic and extrasynaptic NMDA receptors in regulation of the extracellular signal-regulated kinases (ERK) activity in cultured rat hippocampal neurons. J Physiol 572:789-798.

Jama AM, Fenton J, Robertson SD, Torok K (2009) Time-dependent autoinactivation of phospho-Thr286-alphaCa2+/calmodulin-dependent protein kinase II. J Biol Chem 284:28146-28155.

Jama AM, Gabriel J, Al-Nagar AJ, Martin S, Baig SZ, Soleymani H, Chowdhury Z, Beesley P, Torok K (2011) Lobe-specific functions of Ca2+.calmodulin in alphaCa2+.calmodulin-dependent protein kinase II activation. J Biol Chem 286:12308-12316.

Joiner Ml A, Griffith LC (1997) CaM kinase II and visual input modulate memory formation in the neuronal circuit controlling courtship conditioning. J Neurosci 17:9384-9391.

Jourdain P, Bergersen LH, Bhaukaurally K, Bezzi P, Santello M, Domercq M, Matute C, Tonello F, Gundersen V, Volterra A (2007) Glutamate exocytosis from astrocytes controls synaptic strength. Nat Neurosci 10:331-339.

Kajihara H, Tsutsumi E, Kinoshita A, Nakano J, Takagi K, Takeo S (2001) Activated astrocytes with glycogen accumulation in ischemic penumbra during the early stage of brain infarction: immunohistochemical and electron microscopic studies. Brain Res 909:92-101.

Kanaseki T, Ikeuchi Y, Sugiura H, Yamauchi T (1991) Structural features of Ca2+/calmodulin-dependent protein kinase II revealed by electron microscopy. J Cell Biol 115:1049-1060.

Kanthan R, Shuaib A, Griebel R, Miyashita H (1995) Intracerebral human microdialysis. In vivo study of an acute focal ischemic model of the human brain. Stroke 26:870-873.

Karls U, Muller U, Gilbert DJ, Copeland NG, Jenkins NA, Harbers K (1992) Structure, expression, and chromosome location of the gene for the beta subunit of brain-specific Ca2+/calmodulin-dependent protein kinase II identified by transgene integration in an embryonic lethal mouse mutant. Mol Cell Biol 12:3644-3652.

Karve R (2009) FUNCTIONAL CHARACTERIZATION OF CALCIUM DEPENDENT PROTEIN KINASE 32 FROM ARABIDOPSIS. In: Genetics, vol. Doctor of Philosophy, p 165 Clemson: Clemson Universeity.

Kim SA, Hudmon A, Volmer A, Waxham MN (2001) CaM-kinase II dephosphorylates Thr(286) by a reversal of the autophosphorylation reaction. Biochem Biophys Res Commun 282:773-780.

Kirchhefer U, Schmitz W, Scholz H, Neumann J (1999) Activity of cAMP-dependent protein kinase and Ca2+/calmodulin-dependent protein kinase in failing and nonfailing human hearts. Cardiovasc Res 42:254-261.

Kirino T, Sano K (1984) Selective vulnerability in the gerbil hippocampus following transient ischemia. Acta Neuropathol 62:201-208.

Kobayashi M, Lust WD, Passonneau JV (1977) Concentrations of energy metabolites and cyclic nucleotides during and after bilateral ischemia in the gerbil cerebral cortex. J Neurochem 29:53-59.

Kolb SJ, Hudmon A, Ginsberg TR, Waxham MN (1998) Identification of domains essential for the assembly of calcium/calmodulin-dependent protein kinase II holoenzymes. J Biol Chem 273:31555-31564.

Kolb SJ, Hudmon A, Waxham MN (1995) Ca2+/calmodulin kinase II translocates in a hippocampal slice model of ischemia. J Neurochem 64:2147-2156.

Koles L, Leichsenring A, Rubini P, Illes P (2011) P2 receptor signaling in neurons and glial cells of the central nervous system. Adv Pharmacol 61:441-493.

Kubes M, Cordier J, Glowinski J, Girault JA, Chneiweiss H (1998) Endothelin induces a calcium-dependent phosphorylation of PEA-15 in intact astrocytes: identification of Ser104 and Ser116 phosphorylated, respectively, by protein kinase C and calcium/calmodulin kinase II in vitro. J Neurochem 71:1307-1314.

Kuboyama K, Harada H, Tozaki-Saitoh H, Tsuda M, Ushijima K, Inoue K (2011) Astrocytic P2Y(1) receptor is involved in the regulation of cytokine/chemokine transcription and cerebral damage in a rat model of cerebral ischemia. J Cereb Blood Flow Metab 31:1930-1941.

Kuret J, Schulman H (1984) Purification and characterization of a Ca2+/calmodulin-dependent protein kinase from rat brain. Biochemistry 23:5495-5504.

Laabich A, Cooper NG (2000) Neuroprotective effect of AIP on N-methyl-D-aspartate-induced cell death in retinal neurons. Brain Res Mol Brain Res 85:32-40.

Lai Y, Nairn AC, Greengard P (1986) Autophosphorylation reversibly regulates the Ca2+/calmodulin-dependence of Ca2+/calmodulin-dependent protein kinase II. Proc Natl Acad Sci U S A 83:4253-4257.

Lammer A, Gunther A, Beck A, Krugel U, Kittner H, Schneider D, Illes P, Franke H (2006) Neuroprotective effects of the P2 receptor antagonist PPADS on focal cerebral ischaemia-induced injury in rats. Eur J Neurosci 23:2824-2828.

Lammer AB, Beck A, Grummich B, Forschler A, Krugel T, Kahn T, Schneider D, Illes P, Franke H, Krugel U (2011) The P2 receptor antagonist PPADS supports recovery from experimental stroke in vivo. PLoS One 6:e19983.

Latour I, Hamid J, Beedle AM, Zamponi GW, Macvicar BA (2003) Expression of voltage-gated Ca2+ channel subtypes in cultured astrocytes. Glia 41:347-353.

Laube B, Kuhse J, Betz H (1998) Evidence for a tetrameric structure of recombinant NMDA receptors. J Neurosci 18:2954-2961.

Ledoux J, Chartier D, Leblanc N (1999) Inhibitors of calmodulin-dependent protein kinase are nonspecific blockers of voltage-dependent K+ channels in vascular myocytes. J Pharmacol Exp Ther 290:1165-1174.

Lee VM, Goedert M, Trojanowski JQ (2001) Neurodegenerative tauopathies. Annu Rev Neurosci 24:1121-1159.

Leon D, Hervas C, Miras-Portugal MT (2006) P2Y1 and P2X7 receptors induce calcium/calmodulin-dependent protein kinase II phosphorylation in cerebellar granule neurons. Eur J Neurosci 23:2999-3013.

Leonard AS, Bayer KU, Merrill MA, Lim IA, Shea MA, Schulman H, Hell JW (2002) Regulation of calcium/calmodulin-dependent protein kinase II docking to N-methyl-D-aspartate receptors by calcium/calmodulin and alpha-actinin. J Biol Chem 277:48441-48448.

Leonard AS, Lim IA, Hemsworth DE, Horne MC, Hell JW (1999) Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-D-aspartate receptor. Proc Natl Acad Sci U S A 96:3239-3244.

Li X, Nghiem P, Schulman H, Francke U (1994) Localization of the CAMKG gene encoding gamma isoforms of multifunctional calcium/calmodulin-dependent protein kinase (CaM kinase) to human chromosome 10 band q22 and mouse chromosome 14. Cytogenet Cell Genet 66:113-116.

Lipton P (1999) Ischemic cell death in brain neurons. Physiol Rev 79:1431-1568.

Lipton SA, Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 330:613-622.

Lisman J, Schulman H, Cline H (2002) The molecular basis of CaMKII function in synaptic and behavioural memory. Nat Rev Neurosci 3:175-190.

Lisman J, Yasuda R, Raghavachari S (2012) Mechanisms of CaMKII action in long-term potentiation. Nat Rev Neurosci 13:169-182.

Liu Q, Chen B, Ge Q, Wang ZW (2007) Presynaptic Ca2+/calmodulin-dependent protein kinase II modulates neurotransmitter release by activating BK channels at Caenorhabditis elegans neuromuscular junction. J Neurosci 27:10404-10413.

Liu T, Sun L, Xiong Y, Shang S, Guo N, Teng S, Wang Y, Liu B, Wang C, Wang L, Zheng L, Zhang CX, Han W, Zhou Z (2011) Calcium triggers exocytosis from two types of organelles in a single astrocyte. J Neurosci 31:10593-10601.

Liu X, Yao M, Li N, Wang C, Zheng Y, Cao X (2008) CaMKII promotes TLR-triggered proinflammatory cytokine and type I interferon production by directly binding and activating TAK1 and IRF3 in macrophages. Blood 112:4961-4970.

Lledo PM, Hjelmstad GO, Mukherji S, Soderling TR, Malenka RC, Nicoll RA (1995) Calcium/calmodulin-dependent kinase II and long-term potentiation enhance synaptic transmission by the same mechanism. Proc Natl Acad Sci U S A 92:11175-11179.

Lou LL, Lloyd SJ, Schulman H (1986) Activation of the multifunctional Ca2+/calmodulin-dependent protein kinase by autophosphorylation: ATP modulates production of an autonomous enzyme. Proc Natl Acad Sci U S A 83:9497-9501.

Lou LL, Schulman H (1989) Distinct autophosphorylation sites sequentially produce autonomy and inhibition of the multifunctional Ca2+/calmodulin-dependent protein kinase. J Neurosci 9:2020-2032.

Ma S, Yang Y, Wang C, Hui N, Gu L, Zhong H, Cai Z, Wang Q, Zhang Q, Li N, Cao X (2009) Endogenous human CaMKII inhibitory protein suppresses tumor growth by inducing cell cycle arrest and apoptosis through down-regulation of the phosphatidylinositide 3-kinase/Akt/HDM2 pathway. J Biol Chem 284:24773-24782.

Malenka RC, Kauer JA, Perkel DJ, Mauk MD, Kelly PT, Nicoll RA, Waxham MN (1989) An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. Nature 340:554-557.

Mamedova LK, Gao ZG, Jacobson KA (2006) Regulation of death and survival in astrocytes by ADP activating P2Y1 and P2Y12 receptors. Biochem Pharmacol 72:1031-1041.

Mark LP, Prost RW, Ulmer JL, Smith MM, Daniels DL, Strottmann JM, Brown WD, Hacein-Bey L (2001) Pictorial review of glutamate excitotoxicity: fundamental concepts for neuroimaging. AJNR Am J Neuroradiol 22:1813-1824.

Mattson MP, Kater SB (1989) Development and selective neurodegeneration in cell cultures from different hippocampal regions. Brain Res 490:110-125.

Mattson MP, Zhang Y, Bose S (1993) Growth factors prevent mitochondrial dysfunction, loss of calcium homeostasis, and cell injury, but not ATP depletion in hippocampal neurons deprived of glucose. Exp Neurol 121:1-13.

Mayford M, Wang J, Kandel ER, O'Dell TJ (1995) CaMKII regulates the frequency-response function of hippocampal synapses for the production of both LTD and LTP. Cell 81:891-904.

McCarthy KD, de Vellis J (1980) Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J Cell Biol 85:890-902.

McCarthy KD, Salm AK (1991) Pharmacologically-distinct subsets of astroglia can be identified by their calcium response to neuroligands. Neuroscience 41:325-333.

McGuinness TL, Lai Y, Greengard P (1985) Ca2+/calmodulin-dependent protein kinase II. Isozymic forms from rat forebrain and cerebellum. J Biol Chem 260:1696-1704.

Meador WE, Means AR, Quiocho FA (1993) Modulation of calmodulin plasticity in molecular recognition on the basis of x-ray structures. Science 262:1718-1721.

Mehren JE, Griffith LC (2004) Calcium-independent calcium/calmodulin-dependent protein kinase II in the adult Drosophila CNS enhances the training of pheromonal cues. J Neurosci 24:10584-10593.

Melani A, Turchi D, Vannucchi MG, Cipriani S, Gianfriddo M, Pedata F (2005) ATP extracellular concentrations are increased in the rat striatum during in vivo ischemia. Neurochem Int 47:442-448.

Meng F, Zhang G (2002) Autophosphorylated calcium/calmodulin-dependent protein kinase II alpha induced by cerebral ischemia immediately targets and phosphorylates N-methyl-D-aspartate receptor subunit 2B (NR2B) in hippocampus of rats. Neurosci Lett 333:59-63.

Meyer T, Hanson PI, Stryer L, Schulman H (1992) Calmodulin trapping by calcium-calmodulin-dependent protein kinase. Science 256:1199-1202.

Mies G, Iijima T, Hossmann KA (1993) Correlation between peri-infarct DC shifts and ischaemic neuronal damage in rat. Neuroreport 4:709-711.

Migues PV, Lehmann IT, Fluechter L, Cammarota M, Gurd JW, Sim AT, Dickson PW, Rostas JA (2006) Phosphorylation of CaMKII at Thr253 occurs in vivo and enhances binding to isolated postsynaptic densities. J Neurochem 98:289-299.

Miller SG, Kennedy MB (1985) Distinct forebrain and cerebellar isozymes of type II Ca2+/calmodulin-dependent protein kinase associate differently with the postsynaptic density fraction. J Biol Chem 260:9039-9046.

Miller SG, Kennedy MB (1986) Regulation of brain type II Ca2+/calmodulin-dependent protein kinase by autophosphorylation: a Ca2+-triggered molecular switch. Cell 44:861-870.

Molloy SS, Kennedy MB (1991) Autophosphorylation of type II Ca2+/calmodulin-dependent protein kinase in cultures of postnatal rat hippocampal slices. Proc Natl Acad Sci U S A 88:4756-4760.

Morioka M, Fukunaga K, Nagahiro S, Kurino M, Ushio Y, Miyamoto E (1995) Glutamate-induced loss of Ca2+/calmodulin-dependent protein kinase II activity in cultured rat hippocampal neurons. J Neurochem 64:2132-2139.

Morris GF, Bullock R, Marshall SB, Marmarou A, Maas A, Marshall LF (1999) Failure of the competitive N-methyl-D-aspartate antagonist Selfotel (CGS 19755) in the treatment of severe head injury: results of two phase III clinical trials. The Selfotel Investigators. J Neurosurg 91:737-743.

Morris M, Maeda S, Vossel K, Mucke L (2011) The many faces of tau. Neuron 70:410-426.

Mukherji S, Brickey DA, Soderling TR (1994) Mutational analysis of secondary structure in the autoinhibitory and autophosphorylation domains of calmodulin kinase II. J Biol Chem 269:20733-20738.

Murphy SN, Thayer SA, Miller RJ (1987) The effects of excitatory amino acids on intracellular calcium in single mouse striatal neurons in vitro. J Neurosci 7:4145-4158.

Nairn AC, Greengard P (1987) Purification and characterization of Ca2+/calmodulin-dependent protein kinase I from bovine brain. J Biol Chem 262:7273-7281.

Nelson AR, Borland L, Allbritton NL, Sims CE (2007) Myristoyl-based transport of peptides into living cells. Biochemistry 46:14771-14781.

Nielander HB, Onofri F, Valtorta F, Schiavo G, Montecucco C, Greengard P, Benfenati F (1995) Phosphorylation of VAMP/synaptobrevin in synaptic vesicles by endogenous protein kinases. J Neurochem 65:1712-1720.

Nikandrova YA, Jiao Y, Baucum AJ, Tavalin SJ, Colbran RJ (2010) Ca2+/calmodulin-dependent protein kinase II binds to and phosphorylates a specific SAP97 splice variant to disrupt association with AKAP79/150 and modulate alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptor (AMPAR) activity. J Biol Chem 285:923-934.

Nutt LK, Margolis SS, Jensen M, Herman CE, Dunphy WG, Rathmell JC, Kornbluth S (2005) Metabolic regulation of oocyte cell death through the CaMKII-mediated phosphorylation of caspase-2. Cell 123:89-103.

O'Leary H, Lasda E, Bayer KU (2006) CaMKIIbeta association with the actin cytoskeleton is regulated by alternative splicing. Mol Biol Cell 17:4656-4665.

Ocorr KA, Schulman H (1991) Activation of multifunctional Ca2+/calmodulin-dependent kinase in intact hippocampal slices. Neuron 6:907-914.

Ogawara M, Inagaki N, Tsujimura K, Takai Y, Sekimata M, Ha MH, Imajoh-Ohmi S, Hirai S, Ohno S, Sugiura H, et al. (1995) Differential targeting of protein kinase C and CaM kinase II signalings to vimentin. J Cell Biol 131:1055-1066.

Oguri T, Inoko A, Shima H, Izawa I, Arimura N, Yamaguchi T, Inagaki N, Kaibuchi K, Kikuchi K, Inagaki M (2006) Vimentin-Ser82 as a memory phosphorylation site in astrocytes. Genes Cells 11:531-540.

Ohta K, Graf R, Rosner G, Heiss WD (2001) Calcium ion transients in peri-infarct depolarizations may deteriorate ion homeostasis and expand infarction in focal cerebral ischemia in cats. Stroke 32:535-543.

Ohyama A, Hosaka K, Komiya Y, Akagawa K, Yamauchi E, Taniguchi H, Sasagawa N, Kumakura K, Mochida S, Yamauchi T, Igarashi M (2002) Regulation of exocytosis through Ca2+/ATP-dependent binding of autophosphorylated Ca2+/calmodulin-activated protein kinase II to syntaxin 1A. J Neurosci 22:3342-3351.

Olney JW (1969) Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. Science 164:719-721.

Olney JW (1971) Glutamate-induced neuronal necrosis in the infant mouse hypothalamus. An electron microscopic study. J Neuropathol Exp Neurol 30:75-90.

Olney JW, Price MT, Samson L, Labruyere J (1986) The role of specific ions in glutamate neurotoxicity. Neurosci Lett 65:65-71.

Olney JW, Sharpe LG (1969) Brain lesions in an infant rhesus monkey treated with monsodium glutamate. Science 166:386-388.

Omae T, Hasegawa Y, Ogata J, Tamaki K, Minematsu K, Yamaguchi T (1996) The competitive NMDA antagonist, CGS-19755, improves postischemic hypoperfusion in selectively vulnerable regions in gerbils. J Neurol Sci 138:105-113.

Onodera H, Iijima K, Kogure K (1986) Mononucleotide metabolism in the rat brain after transient ischemia. J Neurochem 46:1704-1710.

Opazo P, Labrecque S, Tigaret CM, Frouin A, Wiseman PW, De Koninck P, Choquet D (2010) CaMKII triggers the diffusional trapping of surface AMPARs through phosphorylation of stargazin. Neuron 67:239-252.

Orrenius S, Zhivotovsky B, Nicotera P (2003) Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol 4:552-565.

Ouimet CC, McGuinness TL, Greengard P (1984) Immunocytochemical localization of calcium/calmodulin-dependent protein kinase II in rat brain. Proc Natl Acad Sci U S A 81:5604-5608.

Park CK, Nehls DG, Graham DI, Teasdale GM, McCulloch J (1988a) Focal cerebral ischaemia in the cat: treatment with the glutamate antagonist MK-801 after induction of ischaemia. J Cereb Blood Flow Metab 8:757-762.

Park CK, Nehls DG, Graham DI, Teasdale GM, McCulloch J (1988b) The glutamate antagonist MK-801 reduces focal ischemic brain damage in the rat. Ann Neurol 24:543-551.

Park D, Coleman MJ, Hodge JJ, Budnik V, Griffith LC (2002) Regulation of neuronal excitability in Drosophila by constitutively active CaMKII. J Neurobiol 52:24-42.

Parpura V, Scemes E, Spray DC (2004) Mechanisms of glutamate release from astrocytes: gap junction "hemichannels", purinergic receptors and exocytotic release. Neurochem Int 45:259-264.

Parpura V, Zorec R (2010) Gliotransmission: Exocytotic release from astrocytes. Brain Res Rev 63:83-92.

Patton BL, Miller SG, Kennedy MB (1990) Activation of type II calcium/calmodulin-dependent protein kinase by Ca2+/calmodulin is inhibited by autophosphorylation of threonine within the calmodulin-binding domain. J Biol Chem 265:11204-11212.

Payne ME, Fong YL, Ono T, Colbran RJ, Kemp BE, Soderling TR, Means AR (1988) Calcium/calmodulin-dependent protein kinase II. Characterization of distinct calmodulin binding and inhibitory domains. J Biol Chem 263:7190-7195.

Perlin JB, Churn SB, Lothman EW, DeLorenzo RJ (1992) Loss of type II calcium/calmodulin-dependent kinase activity correlates with stages of development of electrographic seizures in status epilepticus in rat. Epilepsy Res 11:111-118.

Petito CK, Olarte JP, Roberts B, Nowak TS, Jr., Pulsinelli WA (1998) Selective glial vulnerability following transient global ischemia in rat brain. J Neuropathol Exp Neurol 57:231-238.

Pfrieger FW, Barres BA (1997) Synaptic efficacy enhanced by glial cells in vitro. Science 277:1684-1687.

Phillis JW, Smith-Barbour M, O'Regan MH, Perkins LM (1994) Amino acid and purine release in rat brain following temporary middle cerebral artery occlusion. Neurochem Res 19:1125-1130.

Poggi A, Carosio R, Rubartelli A, Zocchi MR (2002a) Beta(3)-mediated engulfment of apoptotic tumor cells by dendritic cells is dependent on CAMKII: inhibition by HIV-1 Tat. J Leukoc Biol 71:531-537.

Poggi A, Carosio R, Spaggiari GM, Fortis C, Tambussi G, Dell'Antonio G, Dal Cin E, Rubartelli A, Zocchi MR (2002b) NK cell activation by dendritic cells is dependent on LFA-1-mediated induction of calcium-calmodulin kinase II: inhibition by HIV-1 Tat C-terminal domain. J Immunol 168:95-101.

Pohorecki R, Becker GL, Reilly PJ, Landers DF (1990) Ischemic brain injury in vitro: protective effects of NMDA receptor antagonists and calmidazolium. Brain Res 528:133-137.

Portera-Cailliau C, Price DL, Martin LJ (1997) Excitotoxic neuronal death in the immature brain is an apoptosis-necrosis morphological continuum. J Comp Neurol 378:70-87.

Pradeep KK, Cheriyan J, Suma Priya SD, Rajeevkumar R, Mayadevi M, Praseeda M, Omkumar RV (2009) Regulation of Ca2+/calmodulin-dependent protein kinase II catalysis by N-methyl-D-aspartate receptor subunit 2B. Biochem J 419:123-132, 124 p following 132.

Praseeda M, Pradeep KK, Krupa A, Krishna SS, Leena S, Kumar RR, Cheriyan J, Mayadevi M, Srinivasan N, Omkumar RV (2004) Influence of a mutation in the ATP-binding region of Ca2+/calmodulin-dependent protein kinase II on its interaction with peptide substrates. Biochem J 378:391-397.

Pryazhnikov E, Khiroug L (2008) Sub-micromolar increase in [Ca(2+)](i) triggers delayed exocytosis of ATP in cultured astrocytes. Glia 56:38-49.

Radwanska K, Tudor-Jones AA, Mizuno K, Pereira GS, Lucchesi W, Alfano I, Lach A, Kaczmarek L, Knapp S, Giese KP (2010) Differential regulation of CaMKII inhibitor beta protein expression after exposure to a novel context and during contextual fear memory formation. Genes Brain Behav 9:648-657.

Ransom BR, Ransom CB (2012) Astrocytes: multitalented stars of the central nervous system. Methods Mol Biol 814:3-7.

Reese LC, Laezza F, Woltjer R, Taglialatela G (2011) Dysregulated phosphorylation of Ca(2+) /calmodulin-dependent protein kinase II-alpha in the hippocampus of subjects with mild cognitive impairment and Alzheimer's disease. J Neurochem 119:791-804.

Reiner DJ, Newton EM, Tian H, Thomas JH (1999) Diverse behavioural defects caused by mutations in Caenorhabditis elegans unc-43 CaM kinase II. Nature 402:199-203.

Rellos P, Pike AC, Niesen FH, Salah E, Lee WH, von Delft F, Knapp S (2010) Structure of the CaMKIIdelta/calmodulin complex reveals the molecular mechanism of CaMKII kinase activation. PLoS Biol 8:e1000426.

Richard JP, Melikov K, Brooks H, Prevot P, Lebleu B, Chernomordik LV (2005) Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors. J Biol Chem 280:15300-15306.

Robatzek M, Thomas JH (2000) Calcium/calmodulin-dependent protein kinase II regulates Caenorhabditis elegans locomotion in concert with a G(o)/G(q) signaling network. Genetics 156:1069-1082.

Robison AJ, Bass MA, Jiao Y, MacMillan LB, Carmody LC, Bartlett RK, Colbran RJ (2005) Multivalent interactions of calcium/calmodulin-dependent protein kinase II with the postsynaptic density proteins NR2B, densin-180, and alpha-actinin-2. J Biol Chem 280:35329-35336.

Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Soliman EZ, Sorlie PD, Sotoodehnia N, Turan TN, Virani SS, Wong ND, Woo D, Turner MB (2012) Heart disease and stroke statistics--2012 update: a report from the American Heart Association. Circulation 125:e2-e220.

Rokhlin OW, Taghiyev AF, Bayer KU, Bumcrot D, Koteliansk VE, Glover RA, Cohen MB (2007) Calcium/calmodulin-dependent kinase II plays an important role in prostate cancer cell survival. Cancer Biol Ther 6:732-742.

Rose AJ, Hargreaves M (2003) Exercise increases Ca2+-calmodulin-dependent protein kinase II activity in human skeletal muscle. J Physiol 553:303-309.

Rosenberg OS, Deindl S, Sung RJ, Nairn AC, Kuriyan J (2005) Structure of the autoinhibited kinase domain of CaMKII and SAXS analysis of the holoenzyme. Cell 123:849-860.

Rosenberg PA, Aizenman E (1989) Hundred-fold increase in neuronal vulnerability to glutamate toxicity in astrocyte-poor cultures of rat cerebral cortex. Neurosci Lett 103:162-168.

Rosenmund C, Stern-Bach Y, Stevens CF (1998) The tetrameric structure of a glutamate receptor channel. Science 280:1596-1599.

Ryu JK, Kim J, Choi SH, Oh YJ, Lee YB, Kim SU, Jin BK (2002) ATP-induced in vivo neurotoxicity in the rat striatum via P2 receptors. Neuroreport 13:1611-1615.

Saitoh T, Schwartz JH (1985) Phosphorylation-dependent subcellular translocation of a Ca2+/calmodulin-dependent protein kinase produces an autonomous enzyme in Aplysia neurons. J Cell Biol 100:835-842.

Sakagami H, Kondo H (1993) Differential expression of mRNAs encoding gamma and delta subunits of Ca2+/calmodulin-dependent protein kinase type II (CaM kinase II) in the mature and postnatally developing rat brain. Brain Res Mol Brain Res 20:51-63.

Sakagami H, Watanabe M, Kondo H (1992) Gene expression of Ca2+/calmodulin-dependent protein kinase of the cerebellar granule cell type or type IV in the mature and developing rat brain. Brain Res Mol Brain Res 16:20-28.

Salter MW, Hicks JL (1994) ATP-evoked increases in intracellular calcium in neurons and glia from the dorsal spinal cord. J Neurosci 14:1563-1575.

Sanchez-Piris M, Posas F, Alemany V, Winge I, Hidalgo E, Bachs O, Aligue R (2002) The serine/threonine kinase Cmk2 is required for oxidative stress response in fission yeast. J Biol Chem 277:17722-17727.

Sanhueza M, Fernandez-Villalobos G, Stein IS, Kasumova G, Zhang P, Bayer KU, Otmakhov N, Hell JW, Lisman J (2011) Role of the CaMKII/NMDA receptor complex in the maintenance of synaptic strength. J Neurosci 31:9170-9178.

Sanhueza M, McIntyre CC, Lisman JE (2007) Reversal of synaptic memory by Ca2+/calmodulin-dependent protein kinase II inhibitor. J Neurosci 27:5190-5199.

Schlingmann K, Michaut MA, McElwee JL, Wolff CA, Travis AJ, Turner RM (2007) Calmodulin and CaMKII in the sperm principal piece: evidence for a motility-related calcium/calmodulin pathway. J Androl 28:706-716.

Schmidt-Kastner R, Aguirre-Chen C, Saul I, Yick L, Hamasaki D, Busto R, Ginsberg MD (2005) Astrocytes react to oligemia in the forebrain induced by chronic bilateral common carotid artery occlusion in rats. Brain Res 1052:28-39.

Schmitt JM, Guire ES, Saneyoshi T, Soderling TR (2005) Calmodulin-dependent kinase kinase/calmodulin kinase I activity gates extracellular-regulated kinase-dependent long-term potentiation. J Neurosci 25:1281-1290.

Scholz WK, Baitinger C, Schulman H, Kelly PT (1988) Developmental changes in Ca2+/calmodulin-dependent protein kinase II in cultures of hippocampal pyramidal neurons and astrocytes. J Neurosci 8:1039-1051.

Schulze-Lohoff E, Hugo C, Rost S, Arnold S, Gruber A, Brune B, Sterzel RB (1998) Extracellular ATP causes apoptosis and necrosis of cultured mesangial cells via P2Z/P2X7 receptors. Am J Physiol 275:F962-971.

Schwarzbach E, Bonislawski DP, Xiong G, Cohen AS (2006) Mechanisms underlying the inability to induce area CA1 LTP in the mouse after traumatic brain injury. Hippocampus 16:541-550.

Schworer CM, Colbran RJ, Keefer JR, Soderling TR (1988) Ca2+/calmodulin-dependent protein kinase II. Identification of a regulatory autophosphorylation site adjacent to the inhibitory and calmodulin-binding domains. J Biol Chem 263:13486-13489.

Schworer CM, Colbran RJ, Soderling TR (1986) Reversible generation of a Ca2+-independent form of Ca2+(calmodulin)-dependent protein kinase II by an autophosphorylation mechanism. J Biol Chem 261:8581-8584.

Sellers LA, Simon J, Lundahl TS, Cousens DJ, Humphrey PP, Barnard EA (2001) Adenosine nucleotides acting at the human P2Y1 receptor stimulate mitogen-activated protein kinases and induce apoptosis. J Biol Chem 276:16379-16390.

Sessoms-Sikes S, Honse Y, Lovinger DM, Colbran RJ (2005) CaMKIIalpha enhances the desensitization of NR2B-containing NMDA receptors by an autophosphorylation-dependent mechanism. Mol Cell Neurosci 29:139-147.

Shackelford DA, Yeh RY, Hsu M, Buzsaki G, Zivin JA (1995) Effect of cerebral ischemia on calcium/calmodulin-dependent protein kinase II activity and phosphorylation. J Cereb Blood Flow Metab 15:450-461.

Shen K, Meyer T (1999) Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. Science 284:162-166.

Shen K, Teruel MN, Connor JH, Shenolikar S, Meyer T (2000) Molecular memory by reversible translocation of calcium/calmodulin-dependent protein kinase II. Nat Neurosci 3:881-886.

Shetty PK, Huang FL, Huang KP (2008) Ischemia-elicited oxidative modulation of Ca2+/calmodulin-dependent protein kinase II. J Biol Chem 283:5389-5401.

Shifman JM, Choi MH, Mihalas S, Mayo SL, Kennedy MB (2006) Ca2+/calmodulin-dependent protein kinase II (CaMKII) is activated by calmodulin with two bound calciums. Proc Natl Acad Sci U S A 103:13968-13973.

Silhol M, Tyagi M, Giacca M, Lebleu B, Vives E (2002) Different mechanisms for cellular internalization of the HIV-1 Tat-derived cell penetrating peptide and recombinant proteins fused to Tat. Eur J Biochem 269:494-501.

Silva AJ, Paylor R, Wehner JM, Tonegawa S (1992) Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science 257:206-211.

Silver IA, Erecinska M (1992) Ion homeostasis in rat brain in vivo: intra- and extracellular [Ca2+] and [H+] in the hippocampus during recovery from short-term, transient ischemia. J Cereb Blood Flow Metab 12:759-772.

Singh MV, Anderson ME (2011) Is CaMKII a link between inflammation and hypertrophy in heart? J Mol Med (Berl) 89:537-543.

Singh TJ, Wang JZ, Novak M, Kontzekova E, Grundke-Iqbal I, Iqbal K (1996) Calcium/calmodulin-dependent protein kinase II phosphorylates tau at Ser-262 but only partially inhibits its binding to microtubules. FEBS Lett 387:145-148.

Sinor JD, Du S, Venneti S, Blitzblau RC, Leszkiewicz DN, Rosenberg PA, Aizenman E (2000) NMDA and glutamate evoke excitotoxicity at distinct cellular locations in rat cortical neurons in vitro. J Neurosci 20:8831-8837.

Sironi JJ, Yen SH, Gondal JA, Wu Q, Grundke-Iqbal I, Iqbal K (1998) Ser-262 in human recombinant tau protein is a markedly more favorable site for phosphorylation by CaMKII than PKA or PhK. FEBS Lett 436:471-475.

Skelding KA, Spratt NJ, Fluechter L, Dickson PW, Rostas JA (2012) alphaCaMKII is differentially regulated in brain regions that exhibit differing sensitivities to ischemia and excitotoxicity. J Cereb Blood Flow Metab.

Smith MK, Colbran RJ, Brickey DA, Soderling TR (1992) Functional determinants in the autoinhibitory domain of calcium/calmodulin-dependent protein kinase II. Role of His282 and multiple basic residues. J Biol Chem 267:1761-1768.

Smith MK, Colbran RJ, Soderling TR (1990) Specificities of autoinhibitory domain peptides for four protein kinases. Implications for intact cell studies of protein kinase function. J Biol Chem 265:1837-1840.

Snider BJ, Gottron FJ, Choi DW (1999) Apoptosis and necrosis in cerebrovascular disease. Ann N Y Acad Sci 893:243-253.

Song JH, Bellail A, Tse MC, Yong VW, Hao C (2006) Human astrocytes are resistant to Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. J Neurosci 26:3299-3308.

Sperlagh B, Kofalvi A, Deuchars J, Atkinson L, Milligan CJ, Buckley NJ, Vizi ES (2002) Involvement of P2X7 receptors in the regulation of neurotransmitter release in the rat hippocampus. J Neurochem 81:1196-1211.

Spitaler M, Villunger A, Grunicke H, Uberall F (2000) Unique structural and functional properties of the ATP-binding domain of atypical protein kinase C-iota. J Biol Chem 275:33289-33296.

Strack S, Barban MA, Wadzinski BE, Colbran RJ (1997a) Differential inactivation of postsynaptic density-associated and soluble Ca2+/calmodulin-dependent protein kinase II by protein phosphatases 1 and 2A. J Neurochem 68:2119-2128.

Strack S, Choi S, Lovinger DM, Colbran RJ (1997b) Translocation of autophosphorylated calcium/calmodulin-dependent protein kinase II to the postsynaptic density. J Biol Chem 272:13467-13470.

Strack S, McNeill RB, Colbran RJ (2000) Mechanism and regulation of calcium/calmodulin-dependent protein kinase II targeting to the NR2B subunit of the N-methyl-D-aspartate receptor. J Biol Chem 275:23798-23806.

Suadicani SO, Brosnan CF, Scemes E (2006) P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca2+ signaling. J Neurosci 26:1378-1385.

Sumi M, Kiuchi K, Ishikawa T, Ishii A, Hagiwara M, Nagatsu T, Hidaka H (1991) The newly synthesized selective Ca2+/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. Biochem Biophys Res Commun 181:968-975.

Suzuki T, Okumura-Noji K, Tanaka R, Tada T (1994) Rapid translocation of cytosolic Ca2+/calmodulin-dependent protein kinase II into postsynaptic density after decapitation. J Neurochem 63:1529-1537.

Suzuki Y, Takagi Y, Nakamura R, Hashimoto K, Umemura K (2003) Ability of NMDA and non-NMDA receptor antagonists to inhibit cerebral ischemic damage in aged rats. Brain Res 964:116-120.

Szydlowska K, Tymianski M (2010) Calcium, ischemia and excitotoxicity. Cell Calcium 47:122-129.

Takagi Y, Takagi N, Besshoh S, Miyake-Takagi K, Takeo S (2003) Transient global ischemia enhances phosphorylation of the GluR1 subunit of the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor in the hippocampal CA1 region in rats. Neurosci Lett 341:33-36.

Takahashi S, Shibata M, Fukuuchi Y (1997) Effects of increased extracellular potassium on influx of sodium ions in cultured rat astroglia and neurons. Brain Res Dev Brain Res 104:111-117.

Takano H, Fukushi H, Morishima Y, Shirasaki Y (2003) Calmodulin and calmodulin-dependent kinase II mediate neuronal cell death induced by depolarization. Brain Res 962:41-47.

Takeuchi Y, Yamamoto H, Fukunaga K, Miyakawa T, Miyamoto E (2000) Identification of the isoforms of Ca(2+)/Calmodulin-dependent protein kinase II in rat astrocytes and their subcellular localization. J Neurochem 74:2557-2567.

Tan SE, Wenthold RJ, Soderling TR (1994) Phosphorylation of AMPA-type glutamate receptors by calcium/calmodulin-dependent protein kinase II and protein kinase C in cultured hippocampal neurons. J Neurosci 14:1123-1129.

Tao-Cheng JH, Gallant PE, Brightman MW, Dosemeci A, Reese TS (2007) Structural changes at synapses after delayed perfusion fixation in different regions of the mouse brain. J Comp Neurol 501:731-740.

Tao-Cheng JH, Vinade L, Pozzo-Miller LD, Reese TS, Dosemeci A (2002) Calcium/calmodulin-dependent protein kinase II clusters in adult rat hippocampal slices. Neuroscience 115:435-440.

Tao-Cheng JH, Vinade L, Smith C, Winters CA, Ward R, Brightman MW, Reese TS, Dosemeci A (2001) Sustained elevation of calcium induces Ca(2+)/calmodulin-dependent protein kinase II clusters in hippocampal neurons. Neuroscience 106:69-78.

Thomas CG, Miller AJ, Westbrook GL (2006) Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. J Neurophysiol 95:1727-1734.

Tjandra N, Bax A, Crivici A, Ikura M (eds.) (1999) Calmodulin Structure and Target Interaction. Oxford: Oxford University Press.

Tobimatsu T, Fujisawa H (1989) Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. J Biol Chem 264:17907-17912.

Tokumitsu H, Chijiwa T, Hagiwara M, Mizutani A, Terasawa M, Hidaka H (1990) KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazi ne, a specific inhibitor of Ca2+/calmodulin-dependent protein kinase II. J Biol Chem 265:4315-4320.

Tokumitsu H, Inuzuka H, Ishikawa Y, Ikeda M, Saji I, Kobayashi R (2002) STO-609, a specific inhibitor of the Ca(2+)/calmodulin-dependent protein kinase kinase. J Biol Chem 277:15813-15818.

Tomita S, Adesnik H, Sekiguchi M, Zhang W, Wada K, Howe JR, Nicoll RA, Bredt DS (2005) Stargazin modulates AMPA receptor gating and trafficking by distinct domains. Nature 435:1052-1058.

Trinidad JC, Barkan DT, Gulledge BF, Thalhammer A, Sali A, Schoepfer R, Burlingame AL (2012) Global Identification and Characterization of Both O-GlcNAcylation and Phosphorylation at the Murine Synapse. Mol Cell Proteomics 11:215-229.

Tsui J, Inagaki M, Schulman H (2005) Calcium/calmodulin-dependent protein kinase II (CaMKII) localization acts in concert with substrate targeting to create spatial restriction for phosphorylation. J Biol Chem 280:9210-9216.

Tulapurkar ME, Zundorf G, Reiser G (2006) Internalization and desensitization of a green fluorescent protein-tagged P2Y nucleotide receptor are differently controlled by inhibition of calmodulin-dependent protein kinase II. J Neurochem 96:624-634.

Tunnemann G, Martin RM, Haupt S, Patsch C, Edenhofer F, Cardoso MC (2006) Cargo-dependent mode of uptake and bioavailability of TAT-containing proteins and peptides in living cells. FASEB J 20:1775-1784.

Uematsu D, Araki N, Greenberg JH, Sladky J, Reivich M (1991) Combined therapy with MK-801 and nimodipine for protection of ischemic brain damage. Neurology 41:88-94.

Vallano ML (1989) Separation of isozymic forms of type II calcium/calmodulin-dependent protein kinase using cation-exchange chromatography. J Neurosci Methods 30:1-9.

van Woerden GM, Hoebeek FE, Gao Z, Nagaraja RY, Hoogenraad CC, Kushner SA, Hansel C, De Zeeuw CI, Elgersma Y (2009) betaCaMKII controls the direction of plasticity at parallel fiber-Purkinje cell synapses. Nat Neurosci 12:823-825.

Vaslin A, Rummel C, Clarke PG (2009) Unconjugated TAT carrier peptide protects against excitotoxicity. Neurotox Res 15:123-126.

Verona M, Zanotti S, Schafer T, Racagni G, Popoli M (2000) Changes of synaptotagmin interaction with t-SNARE proteins in vitro after calcium/calmodulin-dependent phosphorylation. J Neurochem 74:209-221.

Vest RS, Davies KD, O'Leary H, Port JD, Bayer KU (2007) Dual mechanism of a natural CaMKII inhibitor. Mol Biol Cell 18:5024-5033.

Vest RS, O'Leary H, Bayer KU (2009) Differential regulation by ATP versus ADP further links CaMKII aggregation to ischemic conditions. FEBS Lett 583:3577-3581.

Vest RS, O'Leary H, Coultrap SJ, Kindy MS, Bayer KU (2010) Effective post-insult neuroprotection by a novel Ca(2+)/ calmodulin-dependent protein kinase II (CaMKII) inhibitor. J Biol Chem 285:20675-20682.

Vives E, Brodin P, Lebleu B (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem 272:16010-16017.

Volonte C, Amadio S, Cavaliere F, D'Ambrosi N, Vacca F, Bernardi G (2003) Extracellular ATP and neurodegeneration. Curr Drug Targets CNS Neurol Disord 2:403-412.

Walaas SI, Lai Y, Gorelick FS, DeCamilli P, Moretti M, Greengard P (1988) Cell-specific localization of the alpha-subunit of calcium/calmodulin-dependent protein kinase II in Purkinje cells in rodent cerebellum. Brain Res 464:233-242.

Waldmann R, Hanson PI, Schulman H (1990) Multifunctional Ca2+/calmodulin-dependent protein kinase made Ca2+ independent for functional studies. Biochemistry 29:1679-1684.

Wandosell F, Serrano L, Hernandez MA, Avila J (1986) Phosphorylation of tubulin by a calmodulin-dependent protein kinase. J Biol Chem 261:10332-10339.

Wang J, Renger JJ, Griffith LC, Greenspan RJ, Wu CF (1994) Concomitant alterations of physiological and developmental plasticity in Drosophila CaM kinase II-inhibited synapses. Neuron 13:1373-1384.

Wang X, Arcuino G, Takano T, Lin J, Peng WG, Wan P, Li P, Xu Q, Liu QS, Goldman SA, Nedergaard M (2004) P2X7 receptor inhibition improves recovery after spinal cord injury. Nat Med 10:821-827.

Wang Y, Nicol GD, Clapp DW, Hingtgen CM (2005) Sensory neurons from Nf1 haploinsufficient mice exhibit increased excitability. J Neurophysiol 94:3670-3676.

Waxham MN, Aronowski J (1993) Ca2+/calmodulin-dependent protein kinase II is phosphorylated by protein kinase C in vitro. Biochemistry 32:2923-2930.

Waxham MN, Aronowski J, Westgate SA, Kelly PT (1990) Mutagenesis of Thr-286 in monomeric Ca2+/calmodulin-dependent protein kinase II eliminates Ca2+/calmodulin-independent activity. Proc Natl Acad Sci U S A 87:1273-1277.

Waxham MN, Grotta JC, Silva AJ, Strong R, Aronowski J (1996) Ischemia-induced neuronal damage: a role for calcium/calmodulin-dependent protein kinase II. J Cereb Blood Flow Metab 16:1-6.

Westgate SA, Brown J, Aronowski J, Waxham MN (1994) Activity of Ca2+/calmodulin-dependent protein kinase II following ischemia: a comparison between CA1 and dentate gyrus in a hippocampal slice model. J Neurochem 63:2217-2224.

Wheeler DG, Barrett CF, Groth RD, Safa P, Tsien RW (2008) CaMKII locally encodes L-type channel activity to signal to nuclear CREB in excitation-transcription coupling. J Cell Biol 183:849-863.

Williams SN, Locke CJ, Braden AL, Caldwell KA, Caldwell GA (2004) Epileptic-like convulsions associated with LIS-1 in the cytoskeletal control of neurotransmitter signaling in Caenorhabditis elegans. Hum Mol Genet 13:2043-2059.

Woodgett JR, Davison MT, Cohen P (1983) The calmodulin-dependent glycogen synthase kinase from rabbit skeletal muscle. Purification, subunit structure and substrate specificity. Eur J Biochem 136:481-487.

Xia P, Chen HS, Zhang D, Lipton SA (2010) Memantine preferentially blocks extrasynaptic over synaptic NMDA receptor currents in hippocampal autapses. J Neurosci 30:11246-11250.

Xiao C, Yang BF, Song JH, Schulman H, Li L, Hao C (2005) Inhibition of CaMKII-mediated c-FLIP expression sensitizes malignant melanoma cells to TRAIL-induced apoptosis. Exp Cell Res 304:244-255.

Xiao J, Perry G, Troncoso J, Monteiro MJ (1996) alpha-calcium-calmodulin-dependent kinase II is associated with paired helical filaments of Alzheimer's disease. J Neuropathol Exp Neurol 55:954-963.

Xu Q, Kopp RF, Chen Y, Yang JJ, Roe MW, Veenstra RD (2012) Gating of connexin 43 gap junctions by a cytoplasmic loop calmodulin binding domain. Am J Physiol Cell Physiol 302:C1548-1556.

Yaguchi T, Nishizaki T (2010) Extracellular high K+ stimulates vesicular glutamate release from astrocytes by activating voltage-dependent calcium channels. J Cell Physiol 225:512-518.

Yakel JL, Vissavajjhala P, Derkach VA, Brickey DA, Soderling TR (1995) Identification of a Ca2+/calmodulin-dependent protein kinase II regulatory phosphorylation site in non-N-methyl-D-aspartate glutamate receptors. Proc Natl Acad Sci U S A 92:1376-1380.

Yamagata Y, Imoto K, Obata K (2006) A mechanism for the inactivation of Ca2+/calmodulin-dependent protein kinase II during prolonged seizure activity and its consequence after the recovery from seizure activity in rats in vivo. Neuroscience 140:981-992.

Yamamoto H, Fukunaga K, Lee K, Soderling TR (1992) Ischemia-induced loss of brain calcium/calmodulin-dependent protein kinase II. J Neurochem 58:1110-1117.

Yano S, Fukunaga K, Takiguchi M, Ushio Y, Mori M, Miyamoto E (1996) Regulation of CCAAT/enhancer-binding protein family members by stimulation of glutamate receptors in cultured rat cortical astrocytes. J Biol Chem 271:23520-23527.

Yano S, Fukunaga K, Ushio Y, Miyamoto E (1994) Activation of Ca2+/calmodulin-dependent protein kinase II and phosphorylation of intermediate filament proteins by stimulation of glutamate receptors in cultured rat cortical astrocytes. J Biol Chem 269:5428-5439.

Yasuda K, Itakura M, Aoyagi K, Sugaya T, Nagata E, Ihara H, Takahashi M (2011) PKC-dependent inhibition of CA2+-dependent exocytosis from astrocytes. Glia 59:143-151.

Yoshimura Y, Aoi C, Yamauchi T (2000) Investigation of protein substrates of Ca(2+)/calmodulin-dependent protein kinase II translocated to the postsynaptic density. Brain Res Mol Brain Res 81:118-128.

Yoshimura Y, Shinkawa T, Taoka M, Kobayashi K, Isobe T, Yamauchi T (2002) Identification of protein substrates of Ca(2+)/calmodulin-dependent protein kinase II in the postsynaptic density by protein sequencing and mass spectrometry. Biochem Biophys Res Commun 290:948-954.

Yuan K, Chung LW, Siegal GP, Zayzafoon M (2007) alpha-CaMKII controls the growth of human osteosarcoma by regulating cell cycle progression. Lab Invest 87:938-950.

Zalewska T, Domanska-Janik K (1996) Brain ischaemia transiently activates Ca2+/calmodulin-independent protein kinase II. Neuroreport 7:637-641.

Zalewska T, Zablocka B, Domanska-Janik K (1996) Changes of Ca2+/calmodulin-dependent protein kinase-II after transient ischemia in gerbil hippocampus. Acta Neurobiol Exp (Wars) 56:41-48.

Zamanian JL, Xu L, Foo LC, Nouri N, Zhou L, Giffard RG, Barres BA (2012) Genomic analysis of reactive astrogliosis. J Neurosci 32:6391-6410.

Zhang M, Shan H, Gu Z, Wang D, Wang T, Wang Z, Tao L (2012) Increased expression of calcium/calmodulin-dependent protein kinase type II subunit delta after rat traumatic brain injury. J Mol Neurosci 46:631-643.

Zhang M, Tanaka T, Ikura M (1995) Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. Nat Struct Biol 2:758-767.

Zhang T, Maier LS, Dalton ND, Miyamoto S, Ross J, Jr., Bers DM, Brown JH (2003) The deltaC isoform of CaMKII is activated in cardiac hypertrophy and induces dilated cardiomyopathy and heart failure. Circ Res 92:912-919.

Zhang YH, Chi XX, Nicol GD (2008) Brain-derived neurotrophic factor enhances the excitability of rat sensory neurons through activation of the p75 neurotrophin receptor and the sphingomyelin pathway. J Physiol 586:3113-3127.

Zhang Z, Chen G, Zhou W, Song A, Xu T, Luo Q, Wang W, Gu XS, Duan S (2007) Regulated ATP release from astrocytes through lysosome exocytosis. Nat Cell Biol 9:945-953.

Zhao D, Watson JB, Xie CW (2004) Amyloid beta prevents activation of calcium/calmodulin-dependent protein kinase II and AMPA receptor phosphorylation during hippocampal long-term potentiation. J Neurophysiol 92:2853-2858.

Zhou Y, Li HL, Zhao R, Yang LT, Dong Y, Yue X, Ma YY, Wang Z, Chen J, Cui CL, Yu AC (2010) Astrocytes express N-methyl-D-aspartate receptor subunits in development, ischemia and post-ischemia. Neurochem Res 35:2124-2134.

Zhu W, Woo AY, Yang D, Cheng H, Crow MT, Xiao RP (2007) Activation of CaMKIIdeltaC is a common intermediate of diverse death stimuli-induced heart muscle cell apoptosis. J Biol Chem 282:10833-10839.

CURRICULUM VITAE

Nicole M. Ashpole

Education

Ph.D.   Indiana University
              Stark Neuroscience Research Institute
              Indianapolis, IN
              Advisor: Andy Hudmon, Ph.D.

B.A.   Concordia College
              Bachelor of Biological Sciences
              Moorhead, MN

Honors and Awards

2012	Larry Kays Fellowship
2011	Educational Enhancement Grant
2010	Educational Enhancement Grant
2008-2009	Stark Scholar Fellowship
2008	Educational Enhancement Grant
2003-2007	Excellence Scholarship
2005	CC Women's League
2004	Friends of ND Scholarship
2003	Don Walter Memorial Scholarship

Manuscripts (*co-first author)

2012 Song W, Xiao Y, Chen H, **Ashpole NM**, Piekarz AD, Ma P, Hudmon A, Cummins TR, and Shou W. *The Human Nav1.5 F1486 Deletion Associated with Long QT Syndrome Leads to Impaired Sodium Channel Inactivation and Reduced Lidocaine Sensitivity.* [accepted 7-23-2012, J Physiology]

2012 **Ashpole NM***, Herren A*, Ginsburg K, Brogan J, Johnson D, Cummins T, Bers D, and Hudmon A. *Mechanisms underlying Ca2+/calmodulin-dependent protein kinase II (CaMKII) Regulation of Cardiac Sodium Channel Nav1.5 Gating.* Journal of Biological Chemistry 287(24):19856-69.

2012 **Ashpole NM**, Song W, Brustovetsky T, Engelman E, Brustovetsky N, Cummins T, Hudmon A. *Calcium/calmodulin-dependent protein kinase II (CaMKII) inhibition induces neurotoxicity via dysregulation of glutamate/calcium signaling and hyperexcitability.* Journal of Biological Chemistry, 287(11):8495-506.

2011 Brittain J**,** Chen L, Broustovetsky T, Gao X, **Ashpole NM**, Wilson S, Hudmon A, White F, Brustovetsky N, Chen J, Khanna R. *Neuroprotection against traumatic brain injury by a peptide derived from the collapsin response mediator protein 2 (CRMP-2).* Journal of Biological Chemistry. Oct 28;286(43):37778-92.

2011 Coultrap S, Vest R, **Ashpole NM**, Hudmon A, and Bayer KU. *CaMKII and Cerebral Ischemia.* Acta Pharmacologica Sinica, 32 (7):861-72.

2011 Brittain J, Duarte D, Wilson S, Zhu W, Ballard C, Johnson P, Liu N, Xiong W, Ripsch M, Wang Y, Fehrenbacher J, Fitz S, Khanna M, Park C, Schmutzler B, Cheon B, Due M, Brustovetsky T, **Ashpole NM**, Hudmon A, Meroueh S, Hingtgen C, Brustovetsky N, Ji R, Hurley J, Jin X, Shekhar A, Xu XM, Oxford G, Vasko M, White F, Khanna R. *Suppression of inflammatory and neuropathic pain by uncoupling CRMP-2 from the presynaptic Ca2+ channel complex.* Nature Medicine, 17(7):822-9.

2011 Li L, Khanna M, Jo I, Wang F, **Ashpole NM**, Hudmon A, and Meroueh S. *Target-Specific Support Vector Machine Scoring in Structure-Based Virtual Screening: Computational Validation, in Vitro Testing in Kinases, and Effects on Lung Cancer Cell Proliferation.* Journal of Chemical Information and Modeling, 51(4):755-9.

2011 **Ashpole NM** and Hudmon A. *Excitotoxic Neuroprotection and Vulnerability with CaMKII Inhibition.* Molecular and Cellular Neuroscience, 46(4):720-30.

Manuscripts in preparation or under review

Ashpole NM, Martin MT, Brustovetsky T, Brustovetsky N, and Hudmon A. *CaMKII Inhibition in Astrocytes Induces Neurotoxic Gliotransmitter Release.* [under review]

Ashpole NM, Martin TL, Martin MT, Johnson DE, Hudmon A. *HIV-Tat inhibits Calcium/Calmodulin-Dependent Protein Kinase II.* [in preparation, to be submitted 11/31/2012]

Posters

2012 **Ashpole NM**, Song W, Brustovetsky T, Brustovetsky N, and Hudmon A. *CaMKII Regulation of Calcium Homeostasis and Neuronal Activity.* Biophysical Society National Conference, San Diego, CA, February 28th.

2011 Brittain JM, Chen L, Brustovetsky T, Gao X, Wilson SM, **Ashpole NM**, Molosh AI, You H, Hudmon A, Brustovetsky N, Chen J, Khanna R. *Neuroprotection against Traumatic Brain Injury by a Peptide Derived from the Collapsin Response Mediator Protein 2 (CRMP2).* Society for Neuroscience Meeting, Washington D.C, Nov 14th.

2011 Zhu W, **Ashpole NM**, Johnson D, Oxford G, Hudmon A. *Modulation of desensitization of TRPV1 by direct p38 phosphorylation is mainly through T731.* Society for Neuroscience Meeting, Washington, DC, November 14th.

2011 **Ashpole NM**, Johnson D, and Hudmon A. *The Role of Calcium/Calmodulin-dependent Protein Kinase II Signaling in Neuronal Excitotoxicity.* {National Graduate Student Honors Research Conference, Bethesda, MD, October 18th} also presented at {Indiana University School of Medicine Biochemistry Research Day, Indianapolis, March 30th, **2012**-Awarded Honorable Mention} and at {Indiana Spinal Cord and Brain Injury Research Day **2012**}.

2011 Brittain JM, Duarte DB, Wilson SM, Ballard C, Johnson PL, Liu N, Xiong W, Ripsch MS, Wang Y, Fehrenbacher JC, Fitz JD, Khanna M, Park CK, **Ashpole NM**, Hudmon A, Meroueh SO, Ji RR, Hurley JH, Jin X, Shekhar A, Xu XM, Oxford GS, Vasko MR, White FA, Khanna R. *Suppression of inflammatory and neuropathic pain by uncoupling CRMP-2 from the presynaptic Ca^{2+} channel complex.* Midwest Pain Interest Group, Chicago, Il, July 22nd.

2011 **Ashpole NM**, Brustovetsky T, Brustovetsky N, Hudmon A. *Neuroprotection and Neurotoxicity with CaMKII Inhibition* {Kentucky Spinal Cord and Head Injury Research Trust Symposium, Louisville, May 19th} also presented at {Indiana Clinical and Translational Institute Annual Meeting, Indianapolis, April 25th}

2011 **Ashpole NM**, Johnson D, and Hudmon A. *Molecular Mechanism of Alpha-CaMKII Self-Association.* Biophysical Society National Conference, Baltimore, March 6th.

2011 Herren A, **Ashpole NM**, Ginsburg K, Brogan J, Cummins T, Bers D, and Hudmon A. *CaMKII Regulates Cardiac Sodium Channel Nav1.5 by Phosphorylation in the Loop between Domain I and II.* Biophysicial Society National Conference, Baltimore, March 8th.

2010 Zhu W, **Ashpole NM**, Oxford G, Hudmon A. *Phosphorylation by activated p38 MAPK reduces functional desensitization of TRPV1.* Society for Neuroscience Annual Meeting, San Diego, CA, November.

2010 **Ashpole NM** and Hudmon A. *The Role of Calcium Signaling and CaMKII in Excitotoxicity.* IUPUI Research Day, Indianapolis, April 9, 2010 and Gill Symposium, Bloomington, September 8th.

2008 **Ashpole NM**, Brogan J, Sheets P, Cummins T, Hudmon A. *CaMKII regulation of Cardiac Voltage-Gated Sodium Channel.* Biophysical Society Discussion Session, Asilomar, CA, October 30– November 2nd.

2007 **Ashpole NM**, Duppler L, Johnson I. *Fluctuations of Blood Glucose and Lactate Levels in Spermophilus Tridecemlineatus.* Concordia College Biology Honors Session, Moorhead, MN, April.

Presentations

2011 *The Role of Calcium/Calmodulin-Dependent Protein Kinase II Signaling in Excitotoxicity.* Thomas Reese Lab, National Institutes of Health, Bethesda, MD, October 18th.

2011 *Neurotoxicity with CaMKII Inhibition.* Spinal Cord and Brain Injury Research Forum, Indiana University School of Medicine, Indianapolis, IN, September 17th.

2010 *CaMKII and Excitotoxicity: Potential Therapeutic Target?* Spinal Cord and Brain Injury Research Forum, Indiana University School of Medicine, Indianapolis, IN, May 12th.

2007 *Preparing for Hibernation, Changes in the Blood Metabolite Levels of Spermophilus Tridecemlineatus.* Concordia College, Moorhead, MN, April.

Current Memberships in Scholarly Societies
2008- 2012   Biophysical Society
2008- 2012   Medical Neuroscience Graduate Student Group
2010- 2011   American Heart Association
2008- 2010   Society for Neuroscience