Spatiotemporal Dynamics of CaMKI During Structural Plasticity of Single Dendritic Spines

by

Rohit Ramnath

Department of Neurobiology
Duke University

Date:_____

Approved:

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Ryohei Yasuda, Co-Supervisor

_____
Scott Soderling, Co-Supervisor

_____
James O. McNamara, Chair

_____
Fan Wang

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophyin the Department of
Neurobiology in the Graduate School
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ABSTRACT

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Abstract

Multifunctional calcium/calmodulin dependent protein kinases (CaMKs) are key regulators of spine structural plasticity and long-term potentiation (LTP) in neurons. CaMKs have promiscuous and overlapping substrate recognition motifs, and are distinguished in their regulatory role based on differences in the spatiotemporal dynamics of activity. While the function and activity of CaMKII in synaptic plasticity has been extensively studied, that of CaMKI, another major class of CaMK required for LTP, still remain elusive.

Here, we develop a Förster's Resonance Energy Transfer (FRET) based sensor to measure the spatiotemporal activity dynamics of CaMK1. We monitored CaMKI activity using 2-photon fluorescence lifetime imaging, while inducing LTP in single dendritic spines of rat (*Rattus Norvegicus*, strain Sprague Dawley*)* hippocampal CA1 pyramidal neurons using 2-photon glutamate uncaging. Using RNA-interference and pharmacological means, we also characterize the role of CaMKI during spine structural plasticity.

We found that CaMKI was rapidly and transiently activated with a rise time of ~0.3 s and decay time of ~1 s in response to each uncaging pulse. Activity of CaMKI spread out of the spine. Phosphorylation of CaMKI by CaMKK was required for this spreading and for the initial phase of structural LTP. Combined with previous data

showing that CaMKII is restricted to the stimulated spine and required for long-term maintenance of structural LTP, these results suggest that CaMK diversity allows the same incoming signal – calcium – to independently regulate distinct phases of LTP by activating different CaMKs with distinct spatiotemporal dynamics.

Dedication

This work is primarily dedicated to my family. To my mother, who always believed in me and pushed me to become a better version of myself constantly. To my grandparents, who have shown me nothing but love throughout my life. To Indu Chitti and Suresh Chitappa, Kumar Mama and Bhuvana Mami and to all the other family members. Every one of you has uniquely shaped my journey to and through graduate school, and I cannot thank you all enough for everything you have done for me. I am lucky to have people who have supported me from the very beginning, and continue to believe in me.

To my sister, Ramya. While we started off our relationship as siblings, you have grown to be a friend, confidante, housemate, peer and so much more. While you might have been envious of me through school, I think the tables have now turned and I am in awe of all the amazing things that you are doing and that you will do. To Sashvat, Sankarsh, Abhishek and Madhuri – We mostly spent our time being more destructive than constructive, and I continue to do that today.

To my father. You were taken from us too early, and I hope that I can live up to your ideals.

To all my friends. You all guided me through man facets of life and exposed me to many new ideas. To Greg- Yeah Buddy. To Ameya and Pranav- I did,

and I think I also said I did. There are so many others that I could name, and I would probably end up forgetting someone. I am honored and humbled to be part of all of your lives, and I hope our friendship continues to expand to new horizons and through new adventures.

To Catherine, my π. You were there with me through the brightest days and the blackest nights. Our journey navigating graduate school, adulthood and life has brought us closer together, and I look forward to the rest of my life with you.

To Rita – keep barking. To Tazzies – keep purring.

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Chapter 1. Introduction

1.1 A locus for learning and memory within single neurons -

A fundamental question in neurobiology is – "How are memories formed and maintained?" Humans can remember important events from a very young age through the rest of their lives, even though the situation may never present itself again. The long-lasting encoding of memory is a mystery that is still being resolved today. While cellular and circuit level processes contributing to learning and memory have been described, the mechanisms of memory encoding and retrieval remain unclear.

Ramon y Cajal, the father of modern neuroscience, proposed the hypothesis that learning must be accompanied by structural changes in the brain. Based on his neuroanatomical studies, he identified a neuronal compartment called dendritic spines as a locus of physical changes mirroring the acquisition of memory. It has since been shown that dendritic spines are postsynaptic structures whose structure and function can change in a long-lasting manner. It has also been shown that spine structural changes accompany behavioral changes in many model organisms under many learning paradigms. Investigation into the molecular mechanisms of structural changes to the spine have revealed a plethora of signaling cascades that regulate spine morphology, and play a role in learning and memory.

This dissertation seeks to characterize the activity and role of a particular protein, calcium/calmodulin dependent protein kinase 1 (CaMKI), in a cellular process that underlies some aspects of learning and memory – synaptic long term potentiation (LTP). Although this work does not propose a solution to the problem of memory formation, the conclusions of our study help elucidate the intracellular mechanisms that may underlie critical aspects of memory formation and maintenance.

1.1.1 Dendritic Spines are specialized structures for synaptic contact, and a substrate for memory encoding

Most neurons in the mammalian brain are polarized. Dendrites are major sources of synaptic input into the cell, whereas the axon (or axons) serve as the synaptic output. While axons can travel vast distances to find synaptic partners, dendrites tend to be shorter, and form tree-like arrays in order to collect and process synaptic input. In pyramidal neurons of the cortex and hippocampus, dendrites of excitatory neurons contain tiny protrusions called dendritic spines, which are the major sources of excitatory input onto the cells.

Dendritic spines contain the molecular machinery that is necessary to receive excitatory synaptic input from axons and depolarize the postsynaptic neuron. This machinery includes the neurotransmitter receptors for glutamate, such as AMPA and NMDA receptors, scaffolding proteins such as PSD-95, SAPAPs and Shanks, and signaling molecules including calcium/calmodulin dependent protein kinases (CaMKs),

Rho family GTPases, and others. Spine structure is maintained by a network of actin polymers which are anchored to microtubules in the parent dendrite at the base of the spine(Korobova and Svitkina, 2010). The actin network is dynamic, and as such spines can have a variety of shapes in the cell, such as filopodia-like thin spines or stubby spines or mushroom spines(Woolley et al., 1990). Spines are dynamic structures, and changes in spine shape and number underlie changes in the electrical properties of neurons. The plasticity of dendritic spines – changes in the shape, size and electrical properties of dendritic spines – is thought to underlie critical aspects of learning and memory.

Spine morphology changes have been associated with functional changes to neurons and neuroplasticity(Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Matsuzaki et al., 2004; Murakoshi et al., 2011; Oh et al., 2013). Changes to sensory stimulation have been used to understand plastic changes in neuronal responses(Grutzendler et al., 2002; Trachtenberg et al., 2002; Zuo et al., 2005; Alvarez and Sabatini, 2007; De Roo et al., 2008; Roberts et al., 2010). For instance, deprivation of input to one eye, or trimming whiskers in the mystacial pad alters cortical responses which result in remodeling of the representations of the visual or whisker fields, respectively(Majewska and Sur, 2003; Holtmaat et al., 2006). These changes are associated with an increase in spine turnover, as well as changes to spine morphology. Learning specific behavioral tasks also increases the number and morphology of

spines(Roberts et al., 2010). Thus, changes in spine volume are correlated with changes to neuronal activity, and changes to the behavior of animals.

A recent paper established a causal link between dendritic spine volume increase and learning(Hayashi-Takagi et al., 2015). The authors used a novel optogenetic probe using photoactivatable Rac1 (AS-paRac1) targeted to activated synapses on dendritic spines. The authors showed that training animals on a motor leaning task (rotarod training) recruits AS-paRac1 to activated spines. Following light treatment, the authors could artificially shrink these spines, and this shrinkage disrupted the memory trace of the learned task. This study suggested that increase in spine volume of activated synapses is a trace of memory, establishing dendritic spines as important structural memory stores.

The salience of spine shape in neuronal activity and behavior has prompted studies investigating molecular cascades regulating spine morphology and volume. Dendritic spines contain several signaling molecules that can alter actin dynamics and change the morphology of spines. Since many different mechanisms exist throughout the brain, in this dissertation, I will focus on long-lasting spine volume change in the hippocampus, a phenomenon called structural long term potentiation (sLTP). I will summarize research into hippocampal function in learning and memory, and describe calcium-responsive signaling molecules that play a role in spine sLTP. This will allow us to appreciate the role of CaMKI during spine sLTP.

1.2 The hippocampus and its role in learning and memory

The role of the hippocampus in learning and memory was first established by studies on a patient named Henry Molaison(Scoville, 1954; Dossani et al., 2015). A large region of Molaison's hippocampus and surrounding cortex was surgically removed as an epilepsy treatment. This surgery caused anterograde amnesia, an inability to form new memories. Molaison remembered events prior to the surgery, but could not form any new memories after it. Studies on Molaison and others demonstrated that the hippocampus is involved in memory formation, consolidation and retrieval.

The hippocampal circuit is a trisynaptic circuit (Figure 1), with 3 major regions within the hippocampus connected to each other through serial synaptic connections(Neves et al., 2008). Granule cells of the dentate gyrus, which receives incoming synaptic input from the entorhinal cortex, project to and synapse with cells in the CA3 region of the hippocampus through the mossy fiber pathway(Henze et al., 2000). The CA3 pyramidal cells, in turn, project their axons and synapse onto the CA1 pyramidal neurons through the Schaeffer collateral pathway. The CA1 neurons provide the hippocampal output through the subiculum back to the entorhinal cortex. The synapse between the CA3 and CA1 neurons of the hippocampus will be the primary focus of this study, since postsynaptic regions of the CA1 neurons undergo an

interesting plasticity process called long-term potentiation (LTP), which is thought to underlie memory formation.

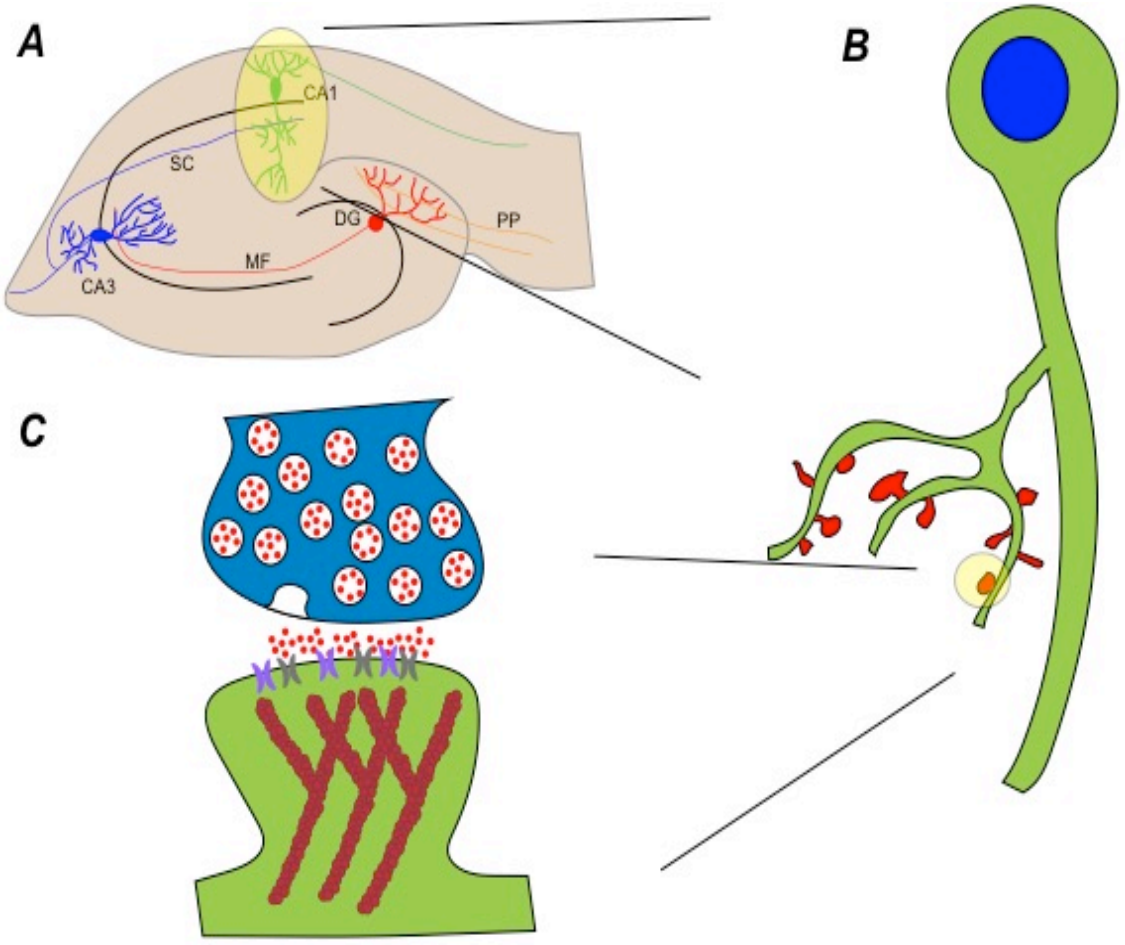


Figure 1: Schematic of the hippocampus and dendritic spines.

A. Schematic of the Hippocampus. The performant path axons (PP), neurons of the dentate gyrus (DG), mossy fiber axons (MF), CA3 pyramidal neurons (CA3), schaeffer collateral axons (SC) and CA1 pyramidal neurons (CA1) are shown. Note the trisynaptic circuit (DG-MF-CA3-SC-CA1).

B. Schematic of a CA1 pyramidal neuron. The large apical dendrite, and smaller secondary and tertiary dendrites are shown. Basolateral dendrites have been omitted. Dendritic spines are shown in red.

C. An excitatory synapse onto dendritic spines. Neurotransmitter vesicles on the presynapse (blue) release glutamate (red circle). The postsynaptic dendritic spine (green) composed primarily of actin (red) translate glutamate input into depolarization via AMPA and NMDA receptors (purple, gray).

1.2.1 Long-term Potentiation in the Hippocampus

Studies into mechanisms of hippocampal LTP were made possible by the development of an *ex vivo* slice model(Mathis et al., 2011). Hippocampal neurons are organized in a repeating array, such that the hippocampus can be removed from an animal and sectioned while preserving a majority of synaptic inputs. This allowed researchers to electrically stimulate hippocampal slices, while measuring neuronal electrical activity, to better understand the induction and expression of LTP.

One of the first studies to characterize LTP used a rabbit hippocampal model(Bliss and Lømo, 1973). Bliss and Lomo recorded extracellular postsynaptic currents from the hippocampus of anaesthetized rabbits. A recording electrode was placed on granule cells of the dentate gyrus, and a stimulating electrode on the incoming axonal input from the entorhinal cortex. Upon high frequency stimulation of the axons, they detected an increase in synaptic response from the dentate granule cells. Strikingly, even upon cessation of the high frequency stimulus, the increase in synaptic response of the granule cells was maintained. The increase lasted as long as the cells could be reliably recorded, and the phenomenon was thus named long-term potentiation (LTP).

Further studies on this phenomenon showed that the potentiation was input-specific(Nishiyama et al., 2000). If a potentiation stimulus was provided to one input,

and another input was left unperturbed, the potentiation was restricted to the stimulated synapse, suggesting that LTP must occur at the synapses, and not in the neuron as a whole. Later studies helped further elucidate the many molecular mechanisms utilized by a neuronal pair to induce long-term potentiation. LTP induction could have mechanisms that are either pre-synaptic or post-synaptic in origin, and could involve any of a plethora of molecular changes(Malenka and Bear, 2004).

The synapse we are interested in studying is the synapse between the CA3 and CA1 region of the hippocampus. The 2 regions are connected by the Schaeffer collateral pathway; high-frequency stimulation of the axons leads to a long-lasting change in the excitatory post synaptic potential (EPSP) recorded in the CA1 region. This change is termed electrical LTP (eLTP). In addition, one can also observe a change in the structure of dendritic spines on CA1 pyramidal neurons. The spines increase in volume upon undergoing LTP, and the increase in volume is maintained for a long period of time following induction of LTP. This latter change in termed structural LTP (sLTP) (Figure 2).

1.2.2 Dendritic spines as a locus of Long Term Potentiation in the Hippocampus

As described above, the discovery of hippocampal LTP provided a prominent cellular model of learning and memory, and research into the molecular mechanisms of LTP soon followed. There were many questions about LTP and its mechanisms – Were

structural and functional LTP linked? What are the major molecules responsible for LTP?

The induction and expression of LTP has been described at a wide variety of synapses throughout the brain. LTP has been thought to occur at possibly every excitatory synapse in the brain, and many of these mechanisms differ from each other(Morris et al., 1990; Doyère and Laroche, 1992; Bear and Malenka, 1994; Malenka and Bear, 2004). For the purposes of this dissertation, we will limit our study of LTP to that which occurs in the hippocampus (Schwartzkroin and Wester, 1975). Induction of LTP in the hippocampus can be accomplished by pairing a presynaptic stimulus and a post-synaptic depolarization in a near-simultaneous manner, and is called spike-timing dependent LTP(Markram et al., 1997; Bi and Poo, 1998). LTP can also be induced by High-frequency (~100Hz) stimulation of the Schaeffer collaterals at the CA3-CA1 synapse, Theta-burst stimulus or glutamate uncaging in single dendritic spines(Douglas and Goddard, 1975; Figurov et al., 1996; Matsuzaki et al., 2004). While there are differences in the mechanisms of LTP induction by these methods, certain properties of LTP induction and expression can be discerned by pharmacological inhibition of molecules involved in LTP.

Postsynaptic LTP in the hippocampus requires cellular signaling through N-Methyl-D-aspartate (NMDA)-type glutamate receptors(Coan et al., 1987; Collingridge and Bliss, 1987; Bashir et al., 1990). Addition of and NMDA-receptor inhibitor – 2-amino-

5-phosphonopentanoate (APV) – into the hippocampus prevents the induction of LTP(Collingridge et al., 1983). NMDA receptors are activated by binding of the neurotransmitter glutamate. However, at resting membrane potential, NMDA receptors are blocked by magnesium ions. This magnesium block can be relieved by depolarizing the neurons, leading to the description of NMDA receptors as "coincidence detectors" of both neurotransmitter release and postsynaptic depolarization(Bliss and Collingridge, 1993). Activation of NMDA receptors by coincident input of glutamate binding and removal of the magnesium block allows passage of depolarizing current into the cell. Additionally, NMDA receptors are permeable to calcium, and activation of NMDA receptors allows calcium entry into the cell(MacDermott et al., 1986; Jahr and Stevens, 1987).

Calcium signaling in neurons is crucial for induction of LTP(Sabatini et al., 2001). Using calcium imaging techniques, it was found that stimulation of dendritic spines increased the intracellular calcium. The increase in calcium concentration was dependent on NMDA-receptors(Koester and Sakmann, 1998; Yuste et al., 1999; Kovalchuk et al., 2000). Additionally, calcium chelators abolish LTP induction(Lynch et al., 1983). Since functional NMDA receptors are localized to dendritic spines(Kennedy, 1993, 2000), and calcium spread out of dendritic spines is limited(Holmes, 1990; Koch and Zador, 1993; Svoboda et al., 1996), it can be assumed that postsynaptic LTP induction mechanisms operate within or near the dendritic spines. While there are

signals that propagate along the dendrite or to the nucleus, the origin of the signal must be the spine(Zhai et al., 2013).

In addition to identifying molecular targets of LTP induction, it is also important to identify the locus and molecular mechanisms of LTP expression. LTP can be expressed postsynaptically, by increasing the number of receptors or increasing the conductance through the receptors. Alternatively, LTP can also be expressed presynaptically, by increasing the probability of neurotransmitter release from presynaptic terminals. In reality, a combination of these mechanisms might occur at different timescales in different synapses(Malenka and Nicoll, 1999). However, for the purposes of this dissertation, we will focus on changes occurring in the postsynaptic side following LTP induction.

One of the major changes occurring postsynaptically involves increasing the number of AMPA receptors at the synapse. Exocytosis of AMPA receptors occurs in dendritic spines following LTP induction mechanisms(Bredt and Nicoll, 2003). An increase in AMPA receptors at the surface of synapses allows increased ionic flow in response to glutamate release, leading to LTP. In addition to AMPA receptor exocytosis, there are also changes to the biophysical properties of AMPA receptors dependent on phosphorylation(Benke et al., 1998; Malinow and Malenka, 2002). LTP induction causes an increase in the single-channel conductance of AMPA receptors(Benke et al., 1998). These two mechanisms sum together to cause increased postsynaptic response to the

same amount of presynaptic input. These changes, along with others, to the electrical conductance of the postsynaptic neuron in response to presynaptic input are collectively called electrical LTP.

In addition to functional changes to the synapse, one can also observe changes to the structure of the postsynaptic dendritic spine (Figure 2). Dendritic spines are composed of an actin cytoskeleton, which is anchored to microtubules on the dendrites and interacts with synaptic scaffold proteins(Korobova and Svitkina, 2010). In response to LTP induction, there is an increase in actin polymerization, which causes the spines to undergo a process called structural LTP (sLTP)(Kim and Lisman, 1999; Krucker et al., 2000). sLTP is a process involving the increase in volume of the spine, and proceeds over 2 phases. The transient phase is characterized by a rapid and pronounced enlargement of the spine. This phase of structural LTP occurs within 1-5 minutes of LTP induction. The transient phase of sLTP then gives way to the sustained phase, characterized by a reduction in volume of the spine. During the sustained phase, the spine has a long-lasting but more modest increase in spine volume(Matsuzaki et al., 2004; Murakoshi and Yasuda,

2012).

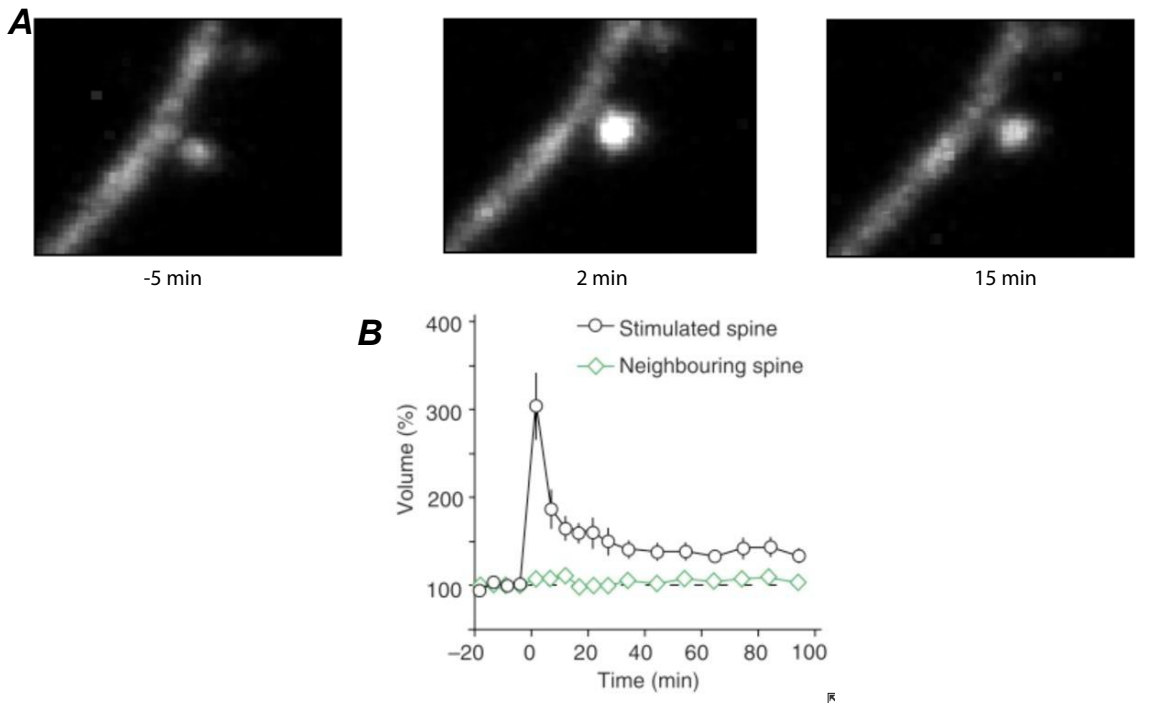


Figure 2: LTP of single dendritic spines

A. Structural LTP of a spine is depicted. Images show a dendrite of a CA1 pyramidal neuron undergoing LTP at a single dendritic spine. Time below the images is relative to LTP induction. Spine volume shows a transient pronounced increase following LTP induction (2min), and a long lasting modest increase in volume (15 min).

B. Quantification of structural plasticity of single dendritic spines. Note the spine-specificity of LTP (stimulated vs neighboring spine) and the transient and sustained phases. Image from Matsuzaki et al, 2004.

To better understand cellular signaling during LTP, we have limited ourselves to LTP induction and expression at single dendritic spines on the CA1 pyramidal neuron. We further simplified our LTP induction paradigm by using a chemically "caged" glutamate in order to bypass differences in function of the presynaptic CA3 region.

Within this reduced preparation, we can ask questions about the details of the molecular cascade beyond NMDA-receptor activation and calcium influx into the cell. The next section will focus on the signaling cascade downstream of calcium in the dendritic spines, namely calcium/calmodulin dependent kinases, or CaMKs.

1.3 Calcium/calmodulin dependent protein kinases – Structure-Function relationships and role in cellular signaling

Following influx into dendritic spines of hippocampal CA1 pyramidal neurons, calcium ions bind to and activate calmodulin (Figure 3). Binding of calmodulin to calcium exposes hydrophobic residues in the protein, that allow signaling by the calcium/calmodulin signaling complex (Ca/CaM). This allows the neuron to initiate biochemical changes within the cell in response to NMDA-receptor dependent calcium influx. The primary transducer of the Ca/CaM signals is a protein family of calcium/calmodulin dependent protein kinases (CaMKs). The focus of this dissertation is on CaMKI, and this section will discuss the activity of CaMKI in comparison to other members of the CaMK protein family.

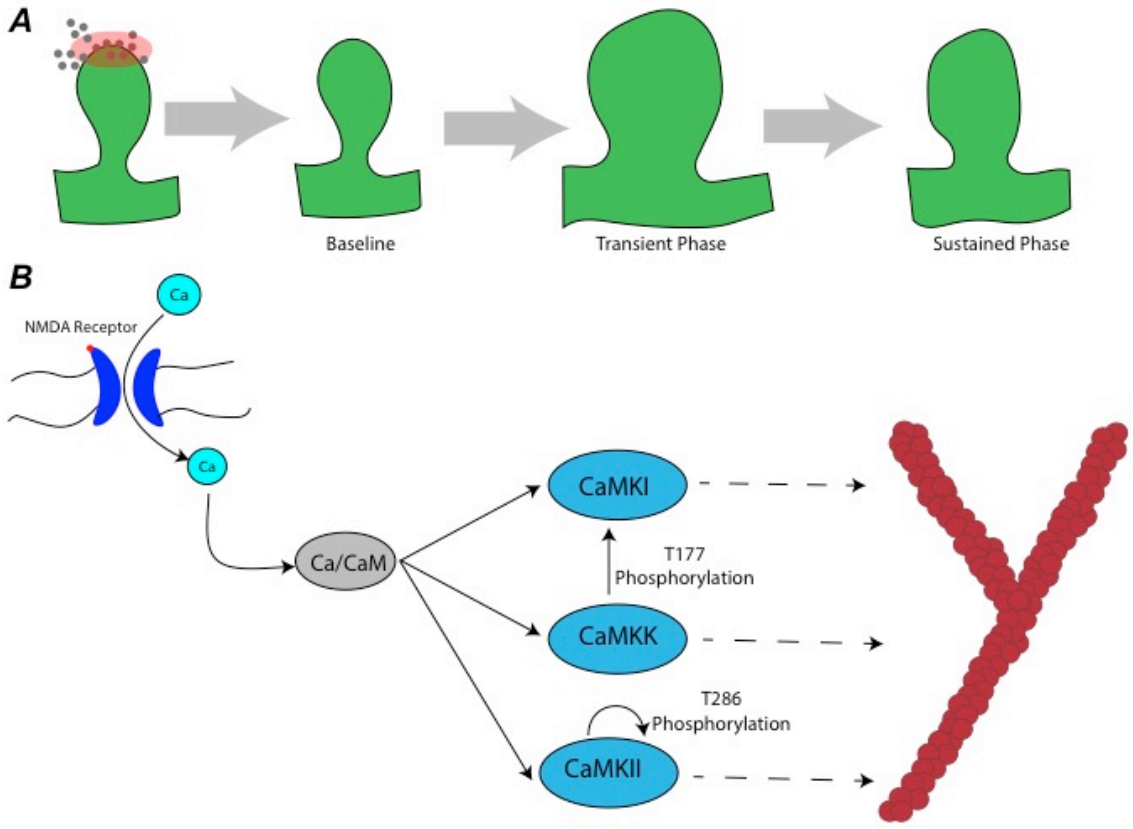


Figure 3: CaMK signaling during structural LTP

A. Schematic of a 2-photon glutamate uncaging experiment is shown. Activation of caged glutamate (gray circles) with 720nm light (red oval) stimulates induction of structural plasticity in a spine through the transient and sustained phases.

B. CaMK signaling cascade in spine structural LTP. Activation of NMDA receptors with glutamate (red circles), leads to influx of calcium into the cell. Upon entering the cell, calcium binds and activates calmodulin (gray oval) leading to the formation of the calcium calmodulin signaling complex. Ca/CaM can activate multiple CaMKs, including CaMKI, CaMKII and CaMKK (blue oval). Additionally, CaMKI is activated by phosphorylation from CaMKK, whereas CaMKII can autophosphorylate itself. Downstream effectors of CaMKs converge on the actin cytoskeleton to induce structural changes to the spine.

1.3.1 Calcium/calmodulin Dependent Protein Kinase 1 (CaMKI) - Structure/Function relationship and role in cellular signaling

CaMKI is a monomeric 42kD enzyme, and is present in a wide variety of cell types in the body(Nairn and Picciotto, 1994). There are 3 genes encoding the 4 different isoforms of CaMKI - α, β1, β2, and γ (β1 and β2 are differentially spliced version of the β gene)(Picciotto et al., 1993; Naito et al., 1997). In pyramidal neurons, CaMKI is expressed throughout the cytoplasm of the cell, but is notably absent from the nucleus(Picciotto et al., 1995; Ueda et al., 1999). In the cytoplasm, CaMKI is evenly distributed through the dendrites and spines, and does not show evidence of subcellular accumulation. Despite expression throughout the neuron, the activity of CaMKI may be confined to specific subcellular regions, since CaMKI activity is self-regulated by intramolecular interactions in the protein.

CaMKI has a bilobial catalytic domain, comprised of an upper ATP-binding lobe and a lower substrate binding lobe(Zha et al., 2012). Upon both lobes binding their respective partners, catalysis occurs by transferring a phosphate group from ATP to the substrate, thereby generating ADP and a phosphorylated substrate. Together, the lobes of CaMKI are referred to as the kinase domain, since this is the region of the protein primarily responsible for its kinase activity. However, the C-terminal region of the protein contains 2 other domains essential for self-regulation of CaMKI activity. The autoinhibitory domain (AID) of CaMKI contains 2 alpha helices that interact with both

the upper and lower lobes(Goldberg et al., 1996). Binding to the upper lobe distorts the ATP-binding pocket. In the lower lobe, the alpha helix mimics substrate binding, and thereby prevents interaction of CaMKI with downstream targets. These interactions render CaMKI catalytically inactive, since CaMKI can no longer effectively bind ATP or catalyze phosphorylation of substrates. This state of the protein is called a closed state, and is characterized by a close proximity of the C-terminus to the N-terminus of the protein.

CaMKI also contains a calmodulin binding domain (CBD) which is the site where calcium-bound calmodulin can interact with CaMKI. Binding of Ca/CaM to the CBD disrupts the interactions between the alpha helix and ATP-binding domain, thereby rendering the protein capable of binding ATP(Chin et al., 1997; Matsushita and Nairn, 1998). This conformational change also disrupts binding of the "pseudosubstrate" helix with the lower lobe of the kinase domain. Together, these changes cause CaMKI to become catalytically active, since it is now capable of binding both ATP and substrates. In this state, the alpha-helices of the C-terminus are no longer closely associated with the bilobial kinase domain, and the protein adopts an open conformation. Thus, a change in the functional state of CaMKI – from inactive to active – is accompanied by a change in the structure of CaMKI – from a closed state to an open one. This change in conformation can be measured using Förster's Resonance Energy Transfer (FRET) and will be used as the basis for generating a CaMKI activity sensor.

In addition to activation by calcium, CaMKI is also activated by calcium/calmodulin dependent protein kinase kinase (CaMKK). CaMKI contains a threonine residue at position 177(T177)(Haribabu et al., 1995), which is in a region called the "activation loop." Upon binding of Ca/CaM to CaMKI, the activation loop, which is normally buried in the protein, is exposed and can be bound by CaMKK. CaMKK binding to the activation loop phosphorylates T177. It is worth noting that T177 phosphorylation can only be accomplished upon calcium binding to both CaMKI and CaMKK(Hawley et al., 1995). Also, CaMKI remains dependent upon calcium for its activity even after phosphorylation. This is different from related kinases, such as CaMKII and CaMKIV(Watanabe et al., 1996; Giese et al., 1998), which acquire calcium-independent activity upon phosphorylation. While CaMKI remains dependent on Ca/CaM for activation, phosphorylation increases the affinity of CaMKI for downstream substrates *in vitro*. In addition, phosphorylation also increases the V_{max} of CaMKI 10-50 fold(Hook et al., 1999). Thus, phosphorylation of CaMKI by CaMKK primarily increases its calcium-dependent activity.

The studies described above were primarily done *in vitro* but what is the role of CaMKI in the neuron? What role does CaMKI play in LTP in the hippocampus? To answer this question, many groups have conducted studies manipulating or measuring CaMKI activity during specific cellular signaling cascades (Figure 4). Fortin et al, showed that long-term potentiation in the hippocampus can be modeled using a

primary neuronal culture system(Fortin et al., 2010). Isolated hippocampal pyramidal neurons were incubated on glass coverslips, and LTP was induced by removing magnesium in the bath and adding in the NMDA-receptor agonist glycine. Stimulation caused an increase in spine-head width and a decrease in spine length, indicative of structural LTP. Interestingly, application of STO-609 before glycine stimulation, or transfection of a dominant-negative CaMKI isoform abrogated the structural change, indicating that both CaMKK and CaMKI were involved in the LTP signaling cascade. The authors went on to show that the CaMKK/CaMKI signaling cascade was also required for AMPA receptor exocytosis.

The involvement of CaMKI in functional LTP was also demonstrated by Schmitt et al(Schmitt et al., 2005) (Figure 4). Theta-burst stimulation of acute hippocampal slices induced LTP and activated CaMKI. Application of STO-609 before LTP induction reduced that magnitude of LTP and the activation of CaMKI. The authors went on to show that CaMKK/CaMKI activity was required for Erk activation following LTP induction. These studies demonstrated the necessity of CaMKI for structural LTP and prompted the question – How does CaMKI affect structural LTP?

Since CaMKI is a kinase, identification of downstream substrates of CaMKI would provide a mechanistic understanding of the role of CaMKI in signaling during neuronal plasticity. *In vitro,* CaMKI can phosphorylate synapsin and the cystic fibrosis transmembrane regulator(Picciotto et al., 1992; Picciotto et al., 1993). However, CaMKI is

a multifunctional CaMK, with many substrates, and the complete list of substrates remains unknown. Insights into the targets of CaMKI have come from several studies measuring CaMKI activity during other cellular plasticity events. Saneyoshi et al, showed that CaMKI can phosphorylate βPIX *in vitro* at Serine516(Saneyoshi et al., 2008). They also showed that CaMKI phosphorylates βPIX in cultured hippocampal neurons upon depolarization. Phosphorylation of βPIX enhanced its interaction with Rac1, and enhanced its GEF activity towards Rac1. This was a key insight, since Rac1 is a known modulator of the cytoskeleton. Signaling through Rac1 can stimulate actin polymerization, which is necessary for LTP. The authors also showed that CaMKK and CaMKI contributed to activity-dependent spine formation of neurons. Inhibition of CaMKK using STO-609, and inhibition of CaMKI using a dominant-negative isoform decreased the density of spines in cultured neurons and hippocampal slices. This study was pivotal, because it showed that activity of CaMKI could affect actin polymerization through βPIX and Rac1, and that this activity is crucial for spine formation.

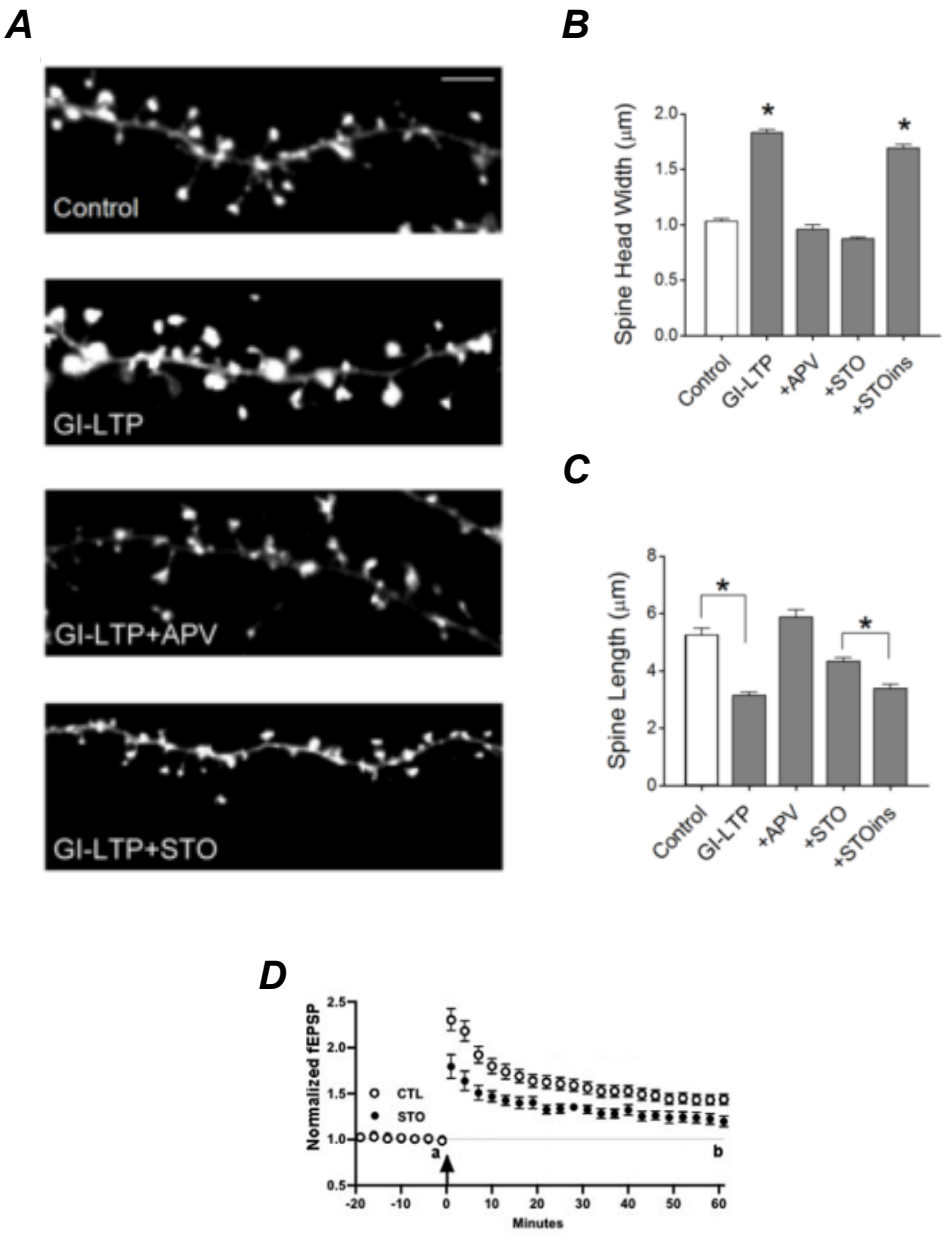


Figure 4: CaMKI and its Role in structural LTP

A. Dendrites and spines of cultured hippocampal neurons are shown. Induction of glycine-induced LTP (GI-LTP) results in an expansion of spine heads. Addition of APV (GI-LTP + APV) or STO-609 (GI-LTP + STO) abrogates the increase in spine head size.

B. Quantification of spine head width in images shown in A. No increase in spine head width is observed in the presence of APV or STO-609.

C. Quantification of spine length in images shown in A. No decrease in spine length is observed in the presence of APV or STO-609/ Images in A,B,C modified from Fortin et al, 2010.

D. Addition of STO-609 blocks the increase in EPSP slope observed upon LTP induction in acute hippocampal slices.

1.4 CaMKK CaMKII and CaMKIV – Structure and Function, Comparison to CaMKI and their role in LTP

A discussion of CaMKI and its role in cellular signaling and plasticity will be incomplete without comparing CaMKI regulation with that of other multifunctional CaMKs – namely CaMKII, CaMKK and CaMKIV (Figure 5). CaMKIII is not included in this discussion since it phosphorylates a single unique substrate and does not contain many canonical motifs of Ser/Thr kinases and is thus considered to be in a different protein kinase family. The other CaMKs have similar domain organization(Kobe et al., 1997), all containing a catalytic/kinase domain, an autoinhibitory domain and a calmodulin binding domain. However, there are important differences in subcellular targeting and protein regulation that serve to make each CaMK unique.

1.4.1 CaMKII

CaMKII is very similar to CaMKI in both domain organization and subcellular targeting. CaMKII is encoded by 4 genes (α, β, γ, and ∂)(Braun and Schulman, 1995).The

α and β isoforms are most prominent in the brain and comprise 2% of total protein in the hippocampus. Within the hippocampus, CaMKIIα accumulates in the spines of pyramidal neurons, due to associations with several ion channels, such as the NR2B subunit of NMDA receptors, and actin. CaMKII can phosphorylate the GluR1 subunit of AMPA receptors upon activation, leading to an increase in the channel's conductance. CaMKII can also phosphorylate GEFs of Rho family GTPases, leading to an increase in actin polymerization crucial for LTP induction.

The protein has an N-terminal catalytic domain, an autoinhibitory domain and a calmodulin binding domain. However, it also contains a C-terminal association domain, that allows CaMKII to form oligomeric dodecamers stacked as 2 hexamers(Kanaseki et al., 1991; Kolodziej et al., 2000). In the inactive state, the autoinhibitory domain of CaMKII acts as a "pseudosubstrate" binding to and inactivating the catalytic domain. Upon calcium/calmodulin binding to the CBD, the autoinhibitory domain is released, rendering the kinase active(Brocke et al., 1999). CaMKII is also regulated by phosphorylation, but unlike CaMKI, it is not trans-phosphorylated by CaMKK(Tokumitsu et al., 1995). CaMKII undergoes autophosphorylation on threonine 286(Braun and Schulman, 1995), whereby one monomer of the holoenzyme can phosphorylate an adjacent monomer(Hanson et al., 1994; Mukherji and Soderling, 1994). Phosphorylation of CaMKII causes 2 important changes to a monomer. Firstly, it reduces the dissociation rate for Ca/CaM by several orders of magnitude, and secondly,

the protein also acquires calcium-independent activity, retaining its function in the absence of calcium/calmodulin binding(Meyer et al., 1992).

In vitro the calcium-independent activity of CaMKII can allow the protein to produce a prolonged response to transient pulses of calcium(Lisman, 1994). It was thought that CaMKII activity would persist for hours or longer in response to a transient pulse of calcium, and that this property of CaMKII would allow encoding of an LTP-induction stimulus(De Koninck and Schulman, 1998; Lisman and Zhabotinsky, 2001), but work from our lab using a CaMKII sensor showed otherwise(Lee et al., 2009). We developed a FRET based sensor for CaMKIIα, and measured the spatial and temporal dynamics of CaMKII activation using 2-photon fluorescence lifetime imaging (2pFLIM). We induced LTP using 2-photon glutamate uncaging, so that the spatial and temporal spread of the LTP-induction signal were controlled. Using this technique, we showed that CaMKII activation lasts for 1-2 minutes after glutamate uncaging, but did not persist for hours as had previously been suggested. Decrease in activity of CaMKII after glutamate uncaging was primarily due to protein dephosphorylation, since phosphatase inhibitors allowed the protein to maintain its activity in neurons. In addition, the minutes-long temporal domain of activity required T286 phosphorylation, since mutating the residue to non-phosphorylatable alanine (T286A mutation) caused a large decrease in CaMKII activity. T286 phosphorylation, and the resultant calcium-

independent activity, allowed accumulation of CaMKII activity between iterative influxes of calcium into the cell, slowing the decay of CaMKII in the absence of calcium.

In conclusion, CaMKII shares many properties in coming with CaMKI. They have similar domain structures and are localized to the cytoplasm. However, CaMKII accumulates in spines due to its interaction with proteins in the post synaptic density, whereas CaMKI does not. Both proteins are regulated by calcium and phosphorylation, but unlike CaMKI, CaMKII is regulated by autophosphorylation which allows the protein to have calcium-independent activity.

1.4.2 CaMKIV

CaMKIV is a monomeric protein encoded by one gene, which has 2 splice variants. The α variant is expressed in mature neurons(Soderling, 1999; Means, 2000). Unlike CaMKI and CaMKK, CaMKIV has not been found in *Drosophila* or *C. elegans*. Unlike CaMKI, expression of CaMKIV is restricted to the nucleus(Jensen et al., 1991), where it regulates calcium-dependent gene transcription by phosphorylation of transcription factors such as CREB, SRF and MEF2(Enslen et al., 1994; Matthews et al., 1994; Blaeser et al., 2000).

CaMKIV has a domain structure similar to CaMKI, with an N-terminal catalytic domain, an auto-inhibitory domain and a calmodulin binding domain, however it has extended N and C termini. In addition to regulation by calcium, CaMKIV is also

regulated by CaMKK phosphorylation. Calcium binding to CaMKIV exposes an activation loop, and Threonine 196 (T196) in the activation loop can be phosphorylated by CaMKK. However, phosphorylation of T196 imbues CaMKIV with calcium-independent activity, and prevents subsequent binding and activation by Ca/CaM(Selbert et al., 1995). In addition, CaMKIV can also autophosphorylate itself at Serine 12 and Serine13, and autophosphorylation is required for full activity of CaMKIV(Okuno et al., 1995; Chatila et al., 1996). There is evidence to support an N-terminal autoinhibitory domain (S12 and S13) in addition to a C-terminal one (T196), and autophosphorylation of S12 and S13 disrupting the function of this second AID. It has been suggested that activation of CaMKIV by calcium can lead to a prolonged response, similar to CaMKII, but studies on the spatiotemporal dynamics of CaMKIV in neurons needs to be done to confirm this hypothesis.

1.4.3 CaMKK

CaMKK is encoded in mammalian neurons by 2 genes (α and β isoforms). Like CaMKI, CaMKK is monomeric, and the α isoform is also localized to the cytosol(Tokumitsu et al., 1995). The β isoform is also nuclear(Kitani et al., 1997; Anderson et al., 1998). CaMKK can phosphorylate and activate CaMKI and CaMKIV as described previously, but other targets for the protein, such as PKB, also exist(Yano et al., 1998). The activation of CaMKI and CaMKIV by CaMKK is only possible when

calcium is bound to both proteins. The advantage of the requirement of the same activator for adjacent members of a signaling cascade is not yet understood.

CaMKK has similar domain organization to CaMKI, with a N-terminal catalytic domain, a "pseudosubstrate" autoinhibitory domain and a calmodulin binding domain that disrupts the interaction between the AID and catalytic domain(Tokumitsu et al., 1997). CaMKK is different from other kinases in 2 major ways. First, CaMKKs do not have conserved acidic residues in their catalytic domains. Thus, it appears that CaMKK does not recognize basic residues in the primary sequence of its substrates, like other Ser/Thr Kinases. Secondly, CaMKK has an unusual Arginine-Proline rich insert (RP-insert). This RP-insert is critical for the activity of CaMKK towards CaMKI and CaMKIV. Deletion of this insert abolishes the ability of CaMKK to phosphorylate and activate CaMKI and CaMKIV(Tokumitsu et al., 1999).

CaMKK allows a practical advantage towards understanding the function of CaMKI, since a pharmacological inhibitor – STO-609 – has been developed that is specific for CaMKK(Tokumitsu et al., 2002; Tokumitsu et al., 2003). STO-609 is a cell-permeable inhibitor that binds to the ATP-binding pocket of CaMKK kinase domain, rendering the protein catalytically inactive(Kukimoto-Niino et al., 2011). In addition, STO-609 is specific to CaMKK, with little effect on the activities of related kinases such as CaMKI, CaMKII and PKA(Tokumitsu et al., 2002). This drug allows us to assay the effect of inhibition of CaMKK on single spine LTP, while minimally disturbing the

functions of other protein kinases with the dendritic

spine.

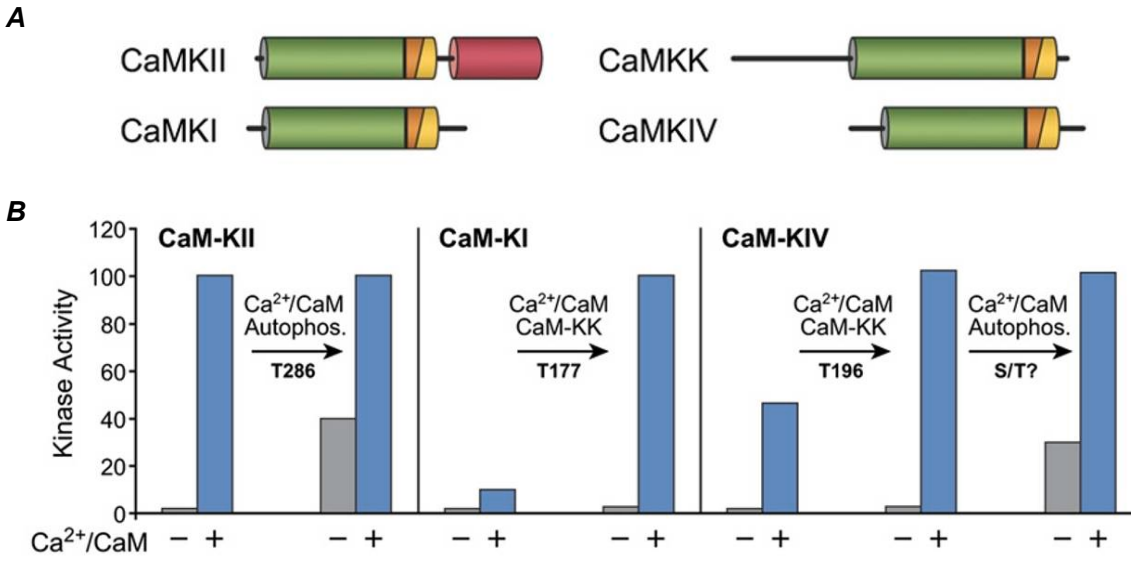


Figure 5: Structure Function Relationships and Effects of Phosphorylation of CaMKs

A. Structures of multifunctional CaMKs. All CAMKs have a kinase domain (green), an autoinhibitory domain (orange) and a calmodulin binding domain (yellow). In addition, CaMKII also has an association domain (red) allowing dodecamerization of the protein.

B. Phosphorylation of CaMKII (left) allows activity in the absence of calcium (gray bar). Phosphorylation of CaMKI (middle) increases its calcium-dependent activity (blue bar). Phosphorylation of CaMKIV (right) increases its calcium dependent activity (blue bar). CaMKIV can then autophosphorylate itself conferring calcium-independent activity (gray bar). Images taken from Wayman et al, 2008.

1.5 Aims of this dissertation:

The goal of this dissertation is to observe and explain the spatiotemporal dynamics of CaMKI during structural plasticity in single dendritic spines. I have presented here some background to help understand the goals of the study and impact of the results of this study. Dendritic spines are postsynaptic structures containing molecular machinery to receive excitatory neurotransmitter and depolarize the neuron. Spines are highly dynamic structures, and changes in the number and morphology of dendritic spines are correlated with changes in neuron function in response to various paradigms. Studying hippocampal structural LTP allows us to understand molecular cascades involved in regulating spine dynamics.

Postsynaptic LTP in the hippocampus depends on calcium signaling through NMDA receptors. Within the spine, calcium binds and activates calmodulin, and the calcium/calmodulin signaling complex can signal through calmodulin dependent kinases (CaMKs) in order to induce postsynaptic structural changes. CaMKs are multifunctional, and CaMKs can exert diverse signaling by responding to intracellular calcium with different spatiotemporal dynamics. Understanding the spatiotemporal dynamics of activity of CaMKI will distinguish its activity from that of other CaMKs in structural changes to the spine. In the next chapters, I will describe the study we

undertook to measure and spatiotemporal dynamics of CaMKI activity. The study aims to answer 3 major questions:

1. What are the spatial and temporal domains of CaMKI activity in response to stimulation of single dendritic spines?
2. What is the role of calcium and phosphorylation on the spatiotemporal dynamics?
3. What is the regulatory role of CaMKI on structural LTP in single dendritic spines?

Chapter 2. Materials and Methods

2.1 Introduction:

In this chapter, I describe the materials and methods used in this study to generate the experimental data in the following chapters.

2.2Reagents:

D-2 amino-5-phosphonovalerate (D-AP5), STO-609, Ionomycin and MNI-glutamate were purchased from Tocris.

2.3Constructs:

CaMKI sensor (dimVenus-CaMKIα-mEGFP) was generated by S.J. Lee. CaMKI T177A and CaMKI T177D were generated using site directed mutagenesis. siRNA against Rat CaMKIα was obtained from Dharmacon (smartpool M-098596-01-0005, D-001206-14-05)

2.4 Preparation:

HeLa cells were cultured in Dulbecco's modified Eagle's Medium, with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin in 5% CO_2. Transfection was performed at 50-90% cell confluency using Lipofectamine 2000 (Invitrogen) and 2 μg/ml total DNA/35mm dish. Cells were imaged under HEPES-buffered artificial cerebrospinal

fluid containing 130mM NaCl, 20mM HEPES, 2mN $NaHCO_3$, 25mM D-glucose, 2.5mM KCl, 1.25mM NaH_2PO_4.

Hippocampal slice cultures were prepared from postnatal day 6-9 rats of either sex as described previously (Lee et al., 2009), in accordance with animal care and use guidelines of the Duke University Medical Center. After 7-12 days in culture, cells were biolistically transfected with 1.6μm gold beads containing 30-50μg of CaMKI sensor or 30-50μg of CaMKI sensors with appropriate mutation, 25μg of paGFP-CaMKI(T177A) + 5μg mCherry, 25μg of paGFP-CaMKI(T177D) + 5μg mCherry, 30μg of GFP. Cells were incubated for 2-5 days to allow bright expression of the fluorescent constructs.

For siRNA experiments, bullets were prepared according to protocols described below: 160μl of 20μM smartpool siRNA along with 15μg mCherry and 5μg mEGFP (or appropriate rescue constructs) were precipitated onto 12.5mg of 1.6μm gold particles. After biolistic gene transfer, slices were incubated for 4-5 days before experiments to allow knockdown of endogenous protein and expression of fluorescence constructs.

All experiments were performed at room temperature, (~25°C) in artificial cerebrospinal fluid (ACSF) (127mM NaCl, 25mM $NaHCO_3$, 25mM D-glucose, 2.5mM KCl, 1.25mM NaH_2PO_4) containing 4 mM $CaCl_2$, 0 mM $MgCl_2$ and 1 μM TTX aerated with 95% O_2 and 5% CO_2. STO-609, AP-5 and vehicle were incubated for 30-60 minutes before glutamate uncaging. 2-5 spines from a single neuron underwent uncaging-induced LTP before addition of the drug. After application of the drug and the incubation time, a different secondary or tertiary branch of the same pyramidal neuron was chosen for

subsequent imaging experiments. After application of drug, up to 15 additional spines were imaged. All experiments measuring spine volume change following glutamate uncaging were performed in a pair-wise manner.

2.5 2-photon glutamate uncaging, 2-photon intensity imaging and 2-photon fluorescence lifetime imaging

We used a custom built 2-photon microscope with two Ti:Sapphire lasers (Spectra Physics MaiTai) (Figure 6). One laser was tuned to 920nm to excite mEGFP and mCherry. The other laser was tuned to 720nm and was used to uncage MNI-glutamate. Each lasers' intensity was controlled independently using electro-optical modulators (Pockels' cells, Conoptics). The beams were combined using a beam splitting cube, and passed through the same set of scan mirrors and objective (60x, 0.9NA, Olympus). mEGFP and mCherry signals were separated using a dichroic mirror (565nm) and band-pass filters (510/70, 620/60; Chroma), Fluorescence signals from cooled GaAsP sPMTs (H7422-40P, Hamamatsu) were acquired by ScanImage using a data acquisition board (PCI-6110, National Instruments). Images were taken using an intensity of 1.8-2.0mW. For 2-photon fluorescence lifetime imaging, signals from the PMT were passed through time-correlated single photon counting board (SPC-140; Becker and Hickl) controlled with custom software. For fast imaging experiments (Figures 2-4), images were acquired at ~8Hz (1 Frame/0.128 seconds). Each experiment contained 1024 frames, lasting ~2 minutes. 4s (32 frames) of baseline images were captured, followed by the onset of

uncaging. For slower imaging (Figures 5) images were acquired in a stack of 4 fluorescence intensity images each comprising 24 frames. Images were acquired every minute. For Figure 1, fluorescence lifetime images were acquired every 30 seconds. The stack contained 1-2 images and each image comprised 24 frames.

2- photon glutamate uncaging was performed in ACSF lacking Mg^{2+} and containing TTX and MNI-caged-L-glutamate. For uncaging the laser power was tuned to 4.5-6mW (720nm) with a pulse width of 6ms with 4mM-2mM caged glutamate in solution. During uncaging, the beam was parked at a spot ~0.5um from the spine head. 30 uncaging pulses were applied at ~0.5Hz.

2.6 Data Analysis:

All data analysis was performed in MATLAB (Mathworks). To measure fluorescence intensity or fluorescence lifetime, regions of interest (ROIs) were drawn around individual HeLa cells or the spine and adjacent dendritic segment. Another ROI was drawn in a region containing no fluorescence, to serve as a measurement of background intensity.

We measured the fluorescence lifetime from the mean photon arrival time as described previously. The fluorescence lifetime curve was fit with a double exponential function and this was used to calculate the fluorescence lifetime. For measurements of spine volume, we subtracted the fluorescence intensity of the spine from the

background. We then divided this result by the fluorescence intensity of the dendritic segment adjacent to the spine, to control for photobleaching or out-of focus movement.

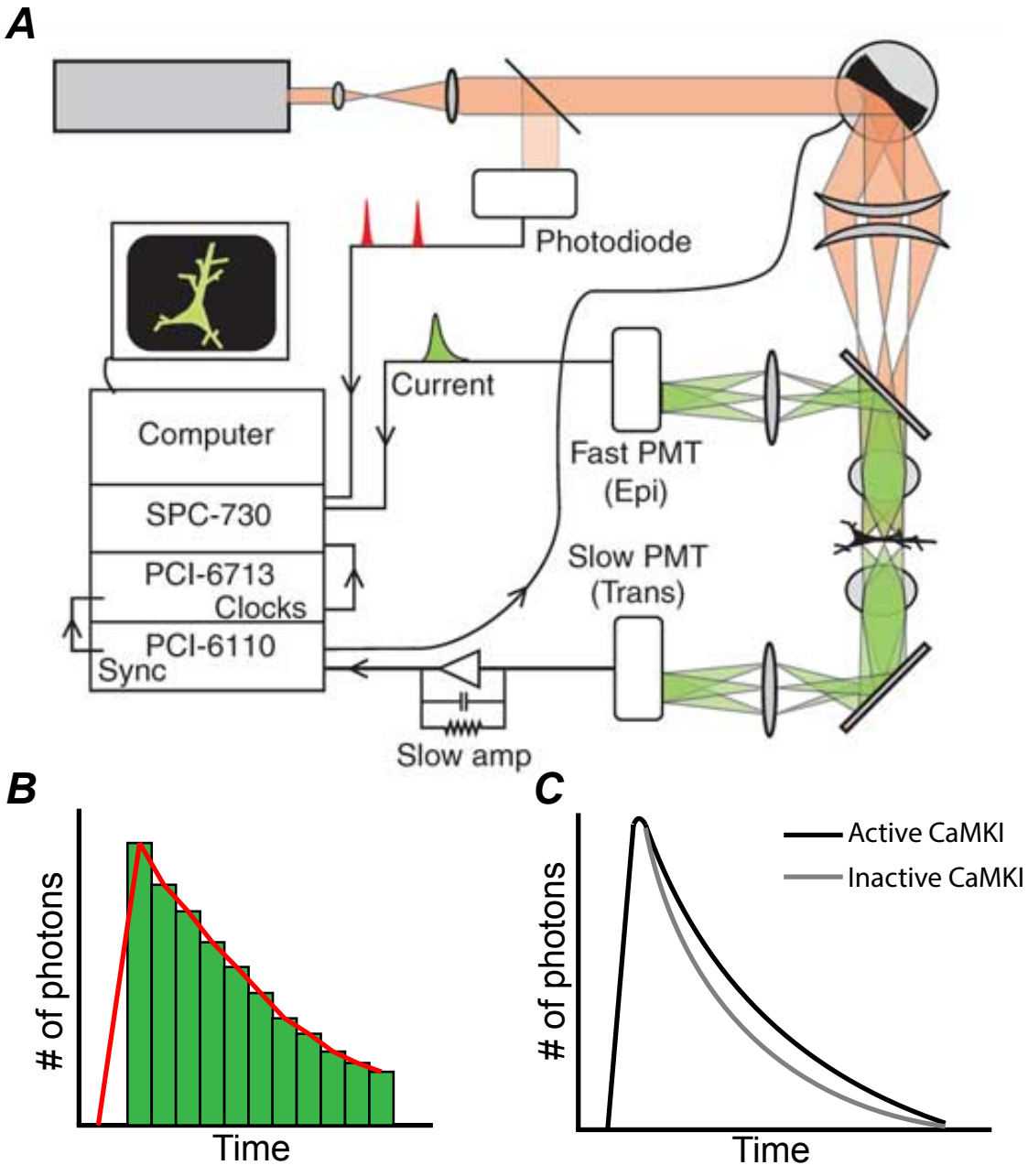


Figure 6: Two-photon Fluorescence Lifetime Imaging

A. Wiring of 2pFLIM setup. Laser pulses (red) are detected by a photodiode, and used to record the timing of incoming fluorescent photons (green). The timing of laser pulses, along with the position of scanning mirror allows us to generate the lifetime image.

B. Measurement of fluorescence lifetime. Incoming fluorescent photons are summed to generate a histogram (green bars). The histogram can be fit with a double exponential curve (red line) to measure fluorescence lifetime.

C. Activation of CaMKI reduces FRET efficiency between GFP and dimVenus and leads to an increase in fluorescence lifetime (black line) relative to inactive sensor (gray line).

Chapter 3. Measurement of the spatiotemporal Dynamics of CaMKI during glutamate uncaging evoked LTP

3.1Introduction

In the previous chapters, we established that dendritic spines are postsynaptic compartments that might serve as fundamental units of memory storage. Spines contain molecular machinery for receiving chemical information through neurotransmitters, and converting this information into postsynaptic depolarization. Additionally, spines contain a large array of molecular machinery that regulate the size of the spine and the expression of neurotransmitter receptors, thereby changing the response of the postsynaptic neuron to presynaptic input.

Dendritic spines on hippocampal pyramidal neurons undergo a phenomenon called structural LTP, whereby patterns of presynaptic input cause enlargement of the spine that is long-lasting(Matsuzaki et al., 2004). The increase in volume of the spine is correlated with a long-lasting functional change in synaptic transmission – larger spines cause a greater depolarization of the postsynaptic neuron in response to similar levels of neurotransmitters(Wayman et al., 2008). Signaling through the CaMK family of proteins is crucial for spine volume changes, but the diversity of this signaling cascade remains poorly characterized.

CaMKI and CaMKII are multifunctional, with broad substrate specificities(Soderling and Stull, 2001). Additionally, both CaMKs have been shown to regulate LTP. Inhibiting the function of CaMKI affects LTP, even though CaMKII is fully functional, suggesting that the mechanisms by which the two CaMKs regulate LTP must be unique(Schmitt et al., 2005; Fortin et al., 2010). Thus, there must be distinguishing features between CaMKI and CaMKII. Our hypothesis was that there must be a difference in the spatial and temporal dynamics of CaMKI compared to CaMKII. This would allow the protein to access similar substrates at different times or in different subcellular regions in order to have a distinctive effect on LTP. To test this hypothesis, we needed to compare the spatiotemporal dynamics of CaMKI to CaMKII.

The following chapter describes the results of our study describing the spatiotemporal dynamics of CaMKI. We chose to study CaMKI activity in a simplified system, so that we could look at a "fundamental unit" of CaMKI activity. In the native brain, neurons can receive a wide variety of synaptic inputs, and it would be difficult to interpret any changes in CaMKI activity that we observe. Thus, we chose to stimulate single dendritic spines in hippocampal slices in culture. The slices were incubated with TTX, that silenced action potentials from neurons. We provided synaptic stimulation using 2-photon uncaging of glutamate, that allowed us to precisely control the patterns of synaptic input to the spine. We used these techniques to precisely control input to CA1 pyramidal neurons, and used a sensor that we generated to measure CaMKI

activity. I will describe the procedure used to generate the sensor, followed by its characterization both in neurons and HeLa cells.

3.2 Results

3.2.1 Development of a sensor for CaMKI activity

The CaMKI sensor was designed based on insights into the structure-function relationship of the CaMKI protein. Inactive CaMKI has a 'closed' conformation (Figure 1A), whereby multiple residues in the autoinhibitory domain (AID) bind the kinase domain (KD) and inhibit its activity (Goldberg et al., 1996). Upon binding of calcium/calmodulin (Ca/CaM) to the calcium-binding domain (CBD), the interaction between the AID and the KD is disrupted, rendering the kinase active (Matsushita and Nairn, 1998) as well as adopting an "open conformation (Zha et al., 2012) (Figure 7).

We tagged CaMKI with monomeric enhanced GFP (meGFP) on the C-terminus and a non-radiative GFP variant (dimVenus) on the N-terminus. By measuring Förster Resonance Energy Transfer (FRET) efficiency between the 2-fluorophores using 2-photon fluorescence lifetime imaging (2pFLIM) (Yasuda, 2012), we can measure the activity of CaMKI by tracking its conformational change. In the "closed" conformation, the N-and C termini of the protein are in close proximity to each other. This positions GFP and dimVenus close to each other, leading to a high FRET efficiency between the pair (Figure 7). Upon an increase in activity of the protein, CaMKI adopts an "open"

conformation, which increases the distance between GFP and dimVenus. FRET efficiency is inversely proportional to distance, and so an increase in activity causes a decrease in FRET efficiency. We can measure the decrease in FRET efficiency as an increase in the fluorescence lifetime of the CaMKI sensor.

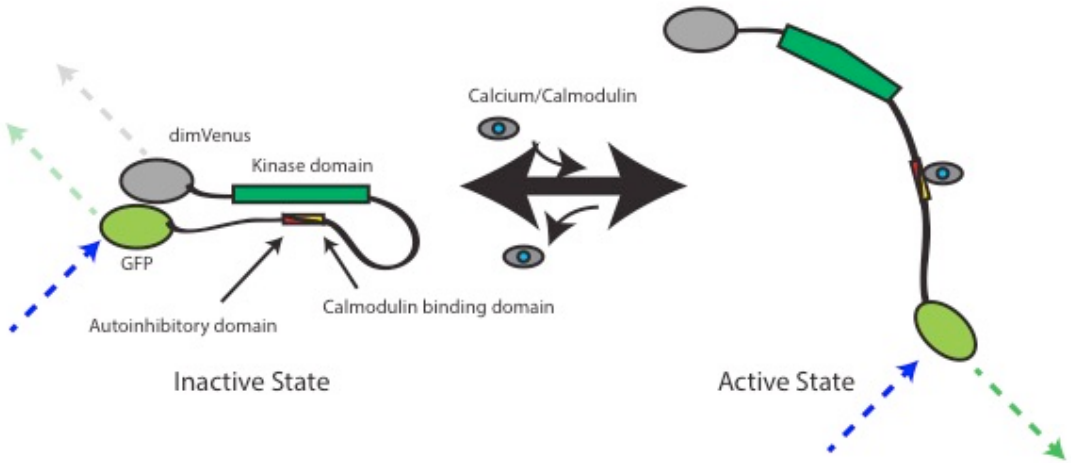


Figure 7: Schematic of the CaMKI Sensor

In the inactive state (left) The kinase domain is bound by the autoinhibitory domain, rendering the protein functionally inactive. The N-and-C termini are in close proximity, increasing FRET efficiency between GFP and dimVenus (dashed lines). The gray bar represents-FRET, but dimVenus is a low-radiative GFP variant and does not emit many photons. Binding of calcium/calmodulin causes a transition in the protein from the inactive to the active state (right). The autoinhibitory domain is no longer bound to the kinase domain, rendering the protein active. This decreases FRET efficiency between GFP and dimVenus (dashed lines)

3.2.2 Characterization of the CaMKI sensor in HeLa Cells

The CaMKI sensor was tested in HeLa cells in order to measure the response of the sensor to calcium influx. Addition of Ionomycin, a calcium ionophore, caused a significant increase in the fluorescence lifetime of the sensor (0.052 ± 0.003 ns) (Figure 8 A,B), indicating an increase in CaMKI activity. Increase in the activity of CaMKI in response to calcium has been previously observed (Haribabu et al., 1995). In agreement with this, addition of EGTA, a calcium chelator, completely abrogated the increase in activity of CaMKI upon addition ionomycin (Figure 8 A,B).

We also tested whether the activity of our CaMKI sensor was dependent on phosphorylation of CaMKI by CaMKK. Addition of STO-609, a specific blocker of CaMKK activity (Tokumitsu et al., 2002), (Tokumitsu et al., 2003), did not affect the initial increase in CaMKI activity upon ionomycin addition. However, the activity of CaMKI rapidly decayed back to baseline even in the presence of ionomycin (Figure 8 A,C). It is worth noting that STO-609 is a competitive antagonist of ATP-CaMKK binding (Tokumitsu et al., 2002), and the initial increase in CaMKI activity upon ionomycin addition might be a result of incomplete inhibition of CaMKK or phosphorylation-independent but calcium-dependent activity of CaMKI.

To further test the effect of phosphorylation on CaMKI activity, we generated two phospho mutant CaMKI sensors based on the known phosphorylation site of CaMKK on CaMKI – threonine 177. CaMKI T177A (phospho-dead CaMKI) was generated by replacing threonine 177 with uncharged alanine. CaMKI T177D (phospho-mimic CaMKI) was generated by substituting threonine 177 with negatively charged aspartate. Both the mutant forms of the sensor showed reduced magnitude of activation in response to ionomycin application (Figure 8 A,D). Additionally, the change in lifetime of the sensor decayed rapidly even in the presence of ionomycin and returned to baseline levels within 10 minutes (Figure 8 A,D). This decay is also observed in the presence of STO-609 (Figure 8 A,C). In previous studies, mutating threonine to either alanine or aspartate causes a decrease in the calcium-dependent specific activity of the protein *in vitro* (Haribabu et al., 1995).

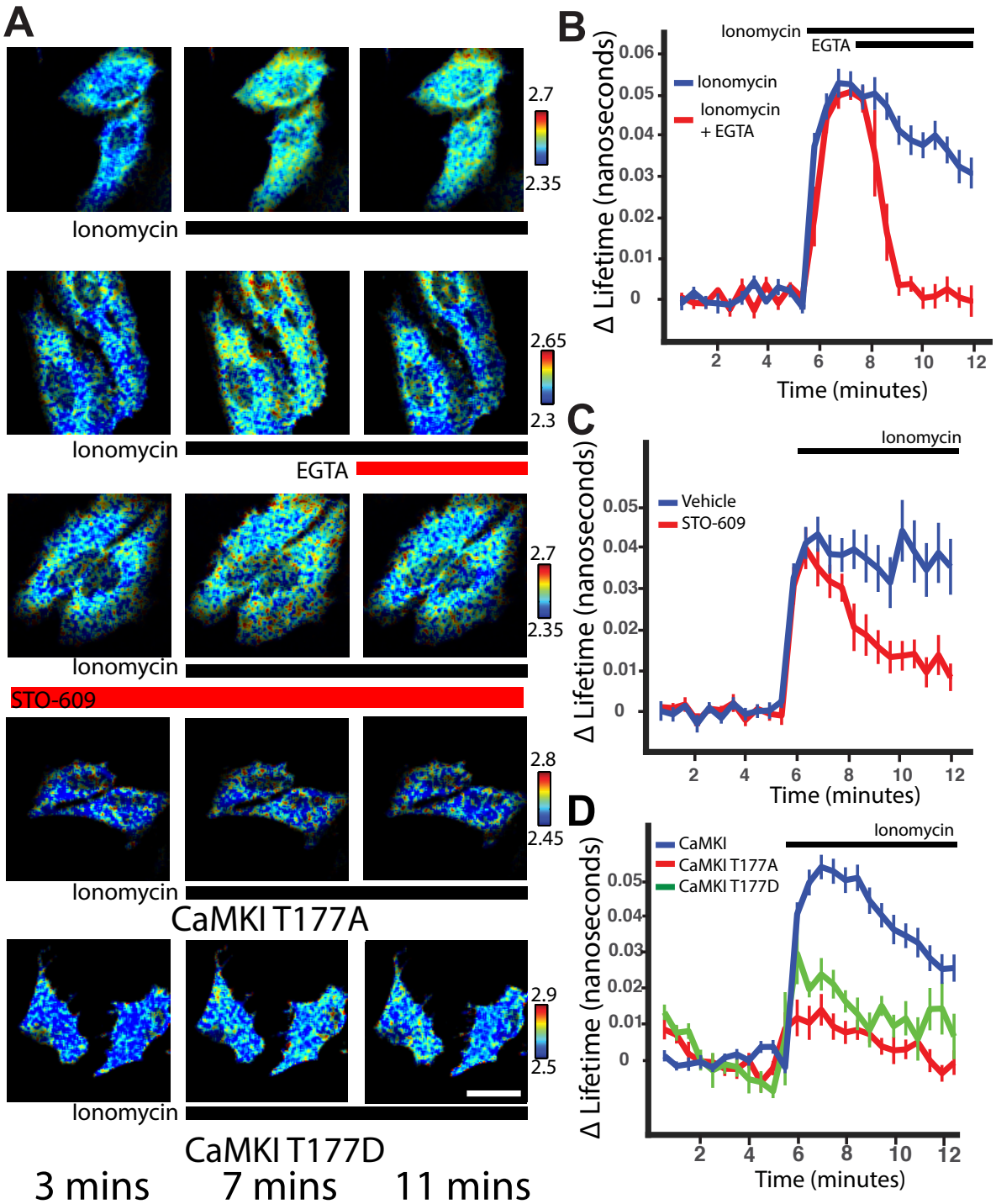


Figure 8: Characterization of CaMKI Sensor in HeLa Cells

A. HeLa cells were transfected with *CMV:GFP-CaMKI-dimVenus* using Lipofectamine and were imaged using 2-photon fluorescence lifetime imaging in HEPES-buffered

ACSF with 2mM calcium under various conditions. Fluorescence lifetime images are shown. The warmth of the color indicates the fluorescence lifetime, with warmer colors representing longer fluorescence lifetimes. The time and conditions of imaging are indicated under each figure.

B. Addition of the calcium ionophore ionomycin (blue) causes an increase in fluorescence lifetime of the CaMKI sensor. Addition of EGTA after ionomycin (red) completely abrogates the increase in fluorescence lifetime ($F_{1,3} = 6.301$, $p<0.005$)

C. Addition of STO-609(red) 30 minutes before ionomycin application does not affect CaMKI activation immediately following ionomycin application, but reduces the duration of CaMKI activation in the presence of ionomycin compared to vehicle treatment (blue). ($F_{1,4} = 5.539$, $p<0.005$)

D.Mutation of threonine 177 to alanine (T177A) (red) or aspartate(T177D) (green) causes a reduction in both the magnitude and duration of activation of the CaMKI sensor in the presence of ionomycin. ($F_{2,8} = 4.723$, $p<0.005$; Dunnett's Test $p<0.005$ for both CaMKI-T177A and CaMKI-T177D)

3.2.3 Imaging CaMKI activity in single dendritic neurons in response to LTP induction

To investigate the change in CaMKI activity associated with induction of structural LTP, we sparsely transfected the CaMKI sensor into CA1 pyramidal neurons of organotypic hippocampal slices using biolistics. (McAllister, 2000), (O'Brien and Lummis, 2013). CaMKIα is localized to the cytoplasm of neurons(Stedman et al., 2004), (Wayman et al., 2004). Consistent with this, fluorescence from the sensor was observed throughout the dendrites and spines, and no fluorescence was detected in the nucleus. To study the kinetics of CaMKI activity, we used 2-photon glutamate uncaging to

stimulate LTP at a single spine with very high spatial and temporal specificity

(Matsuzaki et al., 2004) while measuring the activity of CaMKI using 2pFLIM(Figure 9).

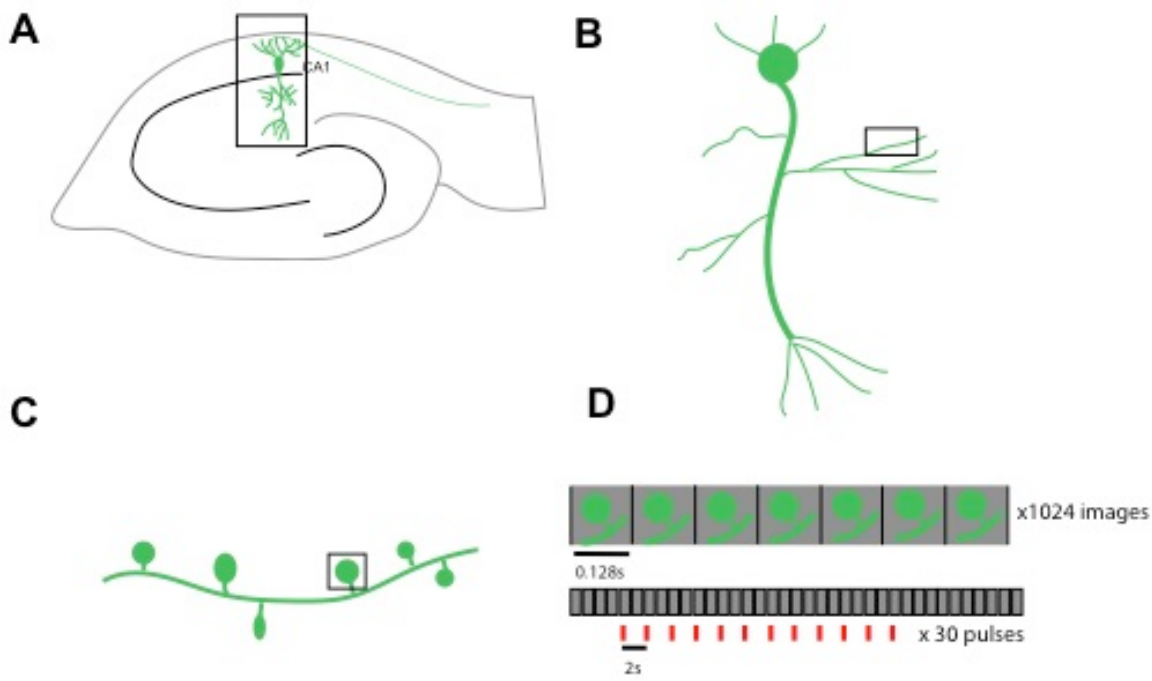


Figure 9: Experimental Schematic

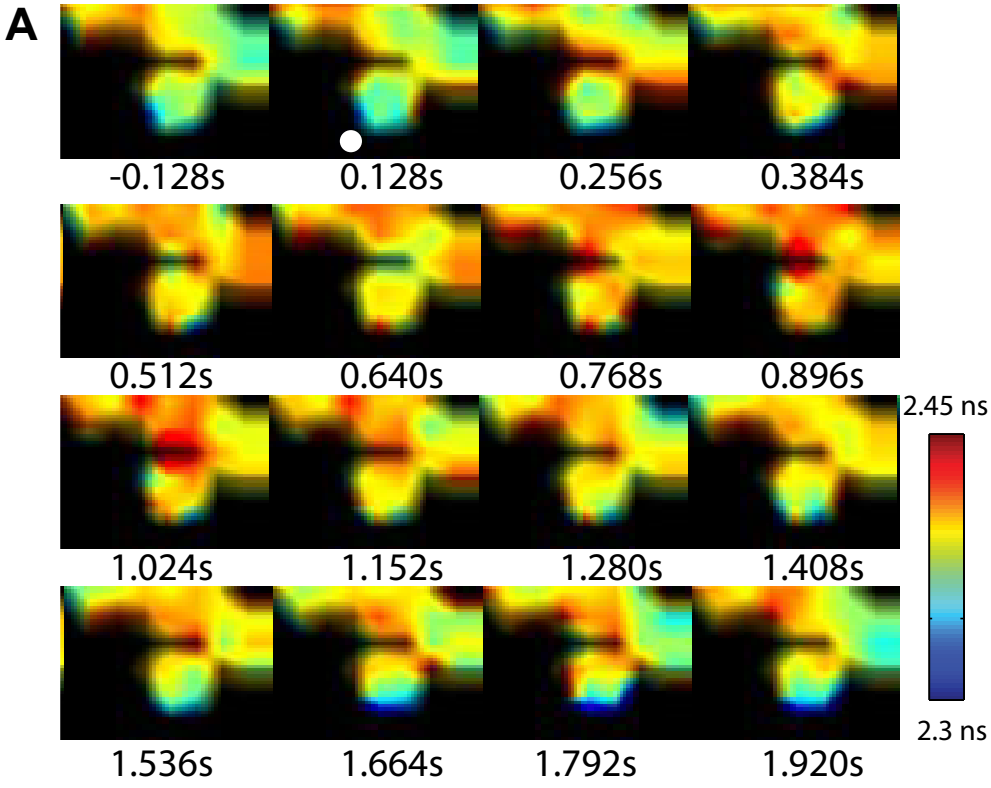
A. Biolistic transfection into cultured organotypic hippocampal slices sparsely labels neurons. One neuron is shown in the schematic, but a few might be labelled.

B. A transfected CA1 pyramidal neuron is chosen, and imaging experiments are conducted on dendritic spines of secondary and tertiary apical dendritic branches.

C. An apical dendritic branch containing dendritic spines are shown. Images are taken from one dendritic spine (black square) and adjacent dendritic segment.

D. Imaging is performed at ~8Hz (0.128 seconds per image). 1024 images are taken. Total imaging time is ~2 min per experiment. Uncaging is performed at ~0.5 Hz (2s between each pulse). 30 uncaging pulses are delivered. The uncaging pulses are synced to the imaging window, and imaging continues through uncaging.

Glutamate uncaging caused an increase in the fluorescence lifetime of the CaMKI sensor (average increase 0.018 ns ± 0.001ns). Uncaging was performed at 0.5Hz, and in response to each uncaging pulse, the lifetime of the sensor increased, plateaued and returned to baseline lifetime (Figure 10 A,B; Figure 11 A). Surprisingly, the increase in fluorescence lifetime of the sensor was not restricted to the spine but was also observed in the dendritic segment adjacent to the spine (Figure 10 A,C; Figure 11 B). The adjacent dendritic segment had very similar kinetics compared to the spine. In response to each pulse of glutamate, an increase, plateau and decrease to baseline was observed in the dendrite as well (Figure 11 C).


A
-0.128s
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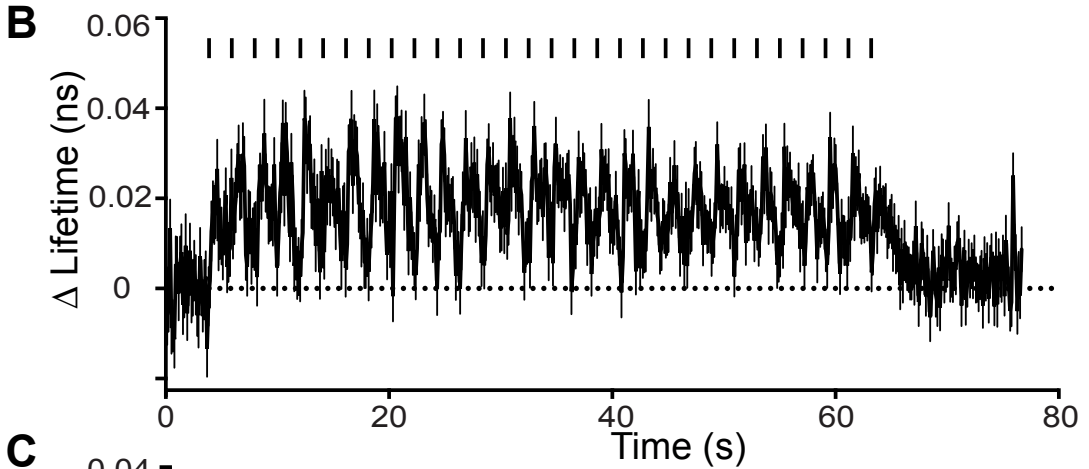
1.408s
1.536s
1.664s
1.792s
1.920s
2.45 ns
2.3 ns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Δ Lifetime (ns)
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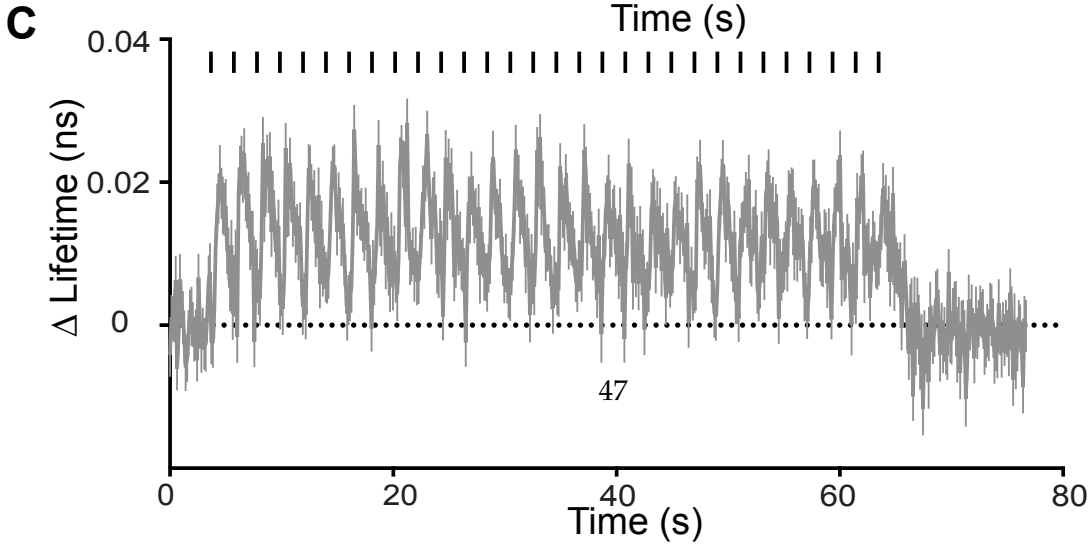
Time (s)


C
Δ Lifetime (ns)
0.04
0.02
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20
40
60
80
Time (s)

Figure 10: Spatiotemporal Dynamics of CaMKI Sensor in Single Dendritic Spines

A. CaMKI sensor was transfected into hippocampal CA1 pyramidal neurons using biolistics. Single dendritic spines on secondary and tertiary dendrites branching from the apical dendrite were imaged using 2-photon fluorescence lifetime imaging. Images were taken at 8Hz. LTP was induced using glutamate uncaging under nominally 0mM magnesium. The images shown are an average of the response of CaMKI to all 30 uncaging pulses. Each image is a smoothed average of 3 frames. The time after uncaging is indicated below each image. Scale bar, 1μm

B. CaMKI activity in the uncaged spine during LTP induction. The graph shows the change in fluorescence lifetime of CaMKI in response to glutamate uncaging in single dendritic spines. The time of uncaging pulses is indicated by the black bars above the graph. Fluorescence lifetime of CaMKI increase immediately following uncaging, and decreases to baseline before the onset of the next uncaging pulse. Following uncaging, no change in CaMKI lifetime is observed.

C. CaMKI activity in the adjacent dendritic segment during LTP induction. The graph shows the change in fluorescence lifetime of CaMKI in the imaged dendritic segment adjacent to the spine that undergoes glutamate-uncaging evoked LTP. Uncaging pulses are indicated in black bars above the graph. Fluorescence lifetime of the CaMKI sensor shows a similar time-course of activation and inactivation in the uncaged spine and adjacent dendritic segment.

To look for slowly developing trends in the activation of CaMKI, we overlaid the activation profile in response to each uncaging pulse. The activation and inactivation of CaMKI in the spine (Figure 11A) and adjacent dendritic segment (Figure 11B) was similar in response to each pulse and did not show any overall trends. We averaged the response of CaMKI to all uncaging pulses to generate the uncaging triggered average response of CaMKI (Figure 11C). In response to a pulse of glutamate, CaMKI activation was higher in the spine (0.018ns ± 0.001ns) than in the dendrite (0.014ns ± 0.0008ns). To

measure the rate of activation and inactivation of CaMKI, we fitted the uncaging triggered average with a double exponential curve (Figure 11D). CaMKI kinetics in the spine (time-constant of decay = 0.21s) and dendrite (time-constant of decay = 0.24s) were similar.

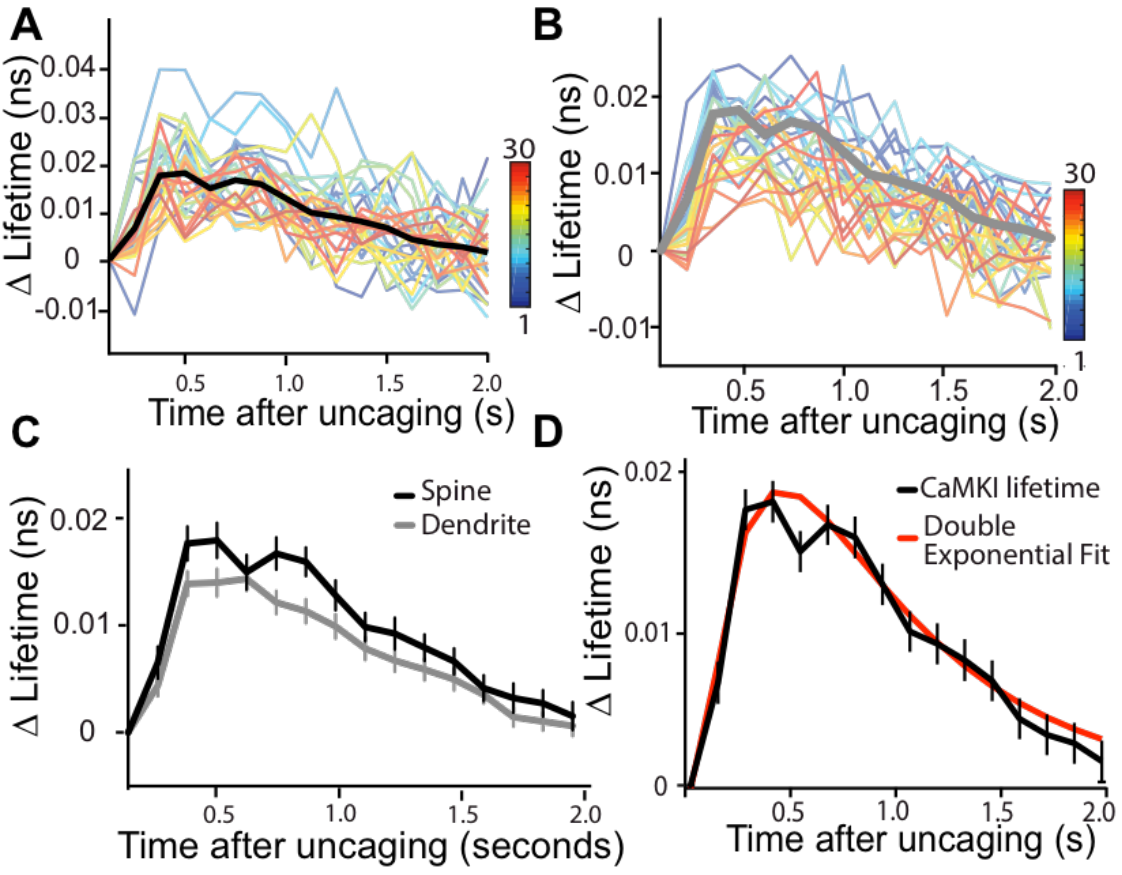

Figure 11: Uncaging Triggered Average of CaMKI Sensor

A. Uncaging-triggered average of CaMKI activity in the spine. The average change in CaMKI activation for each uncaging pulse is depicted by thin lines. The color of the line indicates the uncaging pulse number as shown by the color bar on the right. The thick line(black) indicates the uncaging-triggered average of CaMKI activation in the spine i.e. the average response of CaMKI to uncaging pulses in the spine.

B. Uncaging-triggered average of CaMKI activity in the dendrite. Thin lines depict average change in the fluorescence lifetime of CaMKI activity in the dendritic segment adjacent to the stimulated spine. The color of the line indicates uncaging pulse number as depicted by the color bar on the right. The thick line indicates the average change in fluorescence lifetime of CaMKI sensor in the dendrite in response to a glutamate uncaging pulse.

C. Uncaging triggered average of the fluorescence lifetime of the CaMKI sensor in spines (black) and adjacent dendritic segment (gray). Compared to the spine, the dendrite trends toward lower magnitude and duration of CaMKI activity.

D. The uncaging triggered average of CaMKI fluorescence lifetime in the spine (black) is shown, along with a double-exponential fit (red). The double exponential fit is used to calculate the time constant of activation and inactivation

3.3 Discussion:

In the previous section, I described a method for generating a sensor for CaMKI, and characterized its spatiotemporal dynamics in response to LTP-inducing stimuli in single dendritic spines. The sensor was generated by adding a dual-fluorophore tag to CaMKI. While GFP and its variants (dimVenus being one of them) are thought to be inert, it is still important to determine that the addition of the tags did not affect CaMKI function. To determine any effects the addition of fluorophores may have on CaMKI, we turned to HeLa cells, since the biochemistry of CaMKI, and the kinetics of CaMKI activity have been well characterized in those cells.

Most of the experiments on CaMKI kinetics were performed be investigating the phosphorylation of an artificial substrate (synapsin site I peptide and others) by CaMKI

in lysates from HeLa cells. This allows us to determine whether the fluorophores affected CaMKI activity by comparing the imaging results obtained with our sensor to biochemical results in HeLa cells. It has been shown that inhibition of CaMKK causes a reduction in the specific activity of CaMKI from7.89 μmol/min/mg to 0.32 μmol/min/mg(Haribabu et al., 1995). Our observations indicate that while the peak lifetime change of CaMKI was unaffected by STO-609, CaMKI activity was quickly reduced to baseline in the presence of STO-609 compared to vehicle treatment. These results are consistent with each other. Additionally, phosphorylation causes a decrease in the calcium-dependent increase in specific activity of CaMKI (7.89 μmol/min/mg-CaMKI, 0.12 μmol/min/mg-CaMKI T177A, 0.69 μmol/min/mg-CaMKI-T177D)(Haribabu et al., 1995), while we observed that both the level of CaMKI activation and its duration were affected by mutating threonine 177.

The spatiotemporal dynamics of CaMKI in neurons had not been previously described. We found the spatial activation profile of CaMKI is unique in comparison to CaMKII. We have previously measured CaMKII activity using a similar stimulating paradigm(Lee et al., 2009). In contrast to CaMKI, CaMKII is activated in a spine-specific manner in response to glutamate uncaging. CaMKII also has a slower time course of activation (~10 s) and inactivation (~2min) compared to CaMKI. This was an indication that CaMKI and CaMKII had distinct spatiotemporal dynamics. CaMKI activation was much more rapid that CaMKII, which allowed CaMKI-dependent phosphorylation to

proceed at a much more rapid rate than CaMKII. In addition, observation of CaMKI activity in the dendrite provided evidence for distinct spatial domains of CaMKI and CaMKII activity.

We next proceeded to characterize regulation of CaMKI signaling in neurons. CaMKI activity is governed by 2 major sources – the availability of calcium and phosphorylation of CaMKI by CaMKK. In the next chapter, I will discuss our findings related to the role of NMDA receptors, calcium and phosphorylation on CaMKI activity.

Chapter 4. Determination of the role of calcium and phosphorylation on CaMKI activity

4.1Introduction

In the previous chapter, we characterized the spatiotemporal dynamics of CaMKI activity in response to glutamate application in single dendritic spines. CaMKI was rapidly activated in dendritic spines, but the activity decayed to baseline within 2 seconds. CaMKI activity was also observed in the dendritic segment adjacent to the spine. Since CaMKI activity is governed by 2 major upstream factors – calcium influx and CaMKK phosphorylation, we proceeded to study the role of these factors in regulating the spatiotemporal dynamics of CaMKI activity.

In our preparation, glutamate uncaging was performed in a bath containing nominally 0mM magnesium. Since magnesium ions block NMDA receptor activity, removal of magnesium primes the NMDA receptors toward activation of glutamate(Ruppersberg et al., 1994). Thus, glutamate influx is sufficient for activation of NMDA receptors, and we would expect them to be highly active in our paradigm. We used a pharmacological manipulation to show that NMDA receptor activity is required for CaMKI activation in this paradigm.

Phosphorylation of CaMKI by CaMKK increases the affinity of CaMKI for Ca/CaM *in vitro*. However, it is unclear what role phosphorylation plays in the spatiotemporal dynamics of CaMKI in neurons. We have demonstrated in the previous

chapter that our sensor is rapidly activated in the dendrite and spines of neurons. Here, we test the role of phosphorylation on CaMKI activity using 2 different methods. We mutate the site of phosphorylation (threonine 177) to either either alanine (creating a phospho-dead mutant) or aspartate (creating a phospho-mimic mutant) within the context of the sensor. Additionally, we also apply the drug STO-609 in order to inhibit the upstream activating kinase, CaMKK. These two manipulations both affect a similar process through different methods, and thus provide orthogonal methods to assess the impact of CaMKI phosphorylation on it spatiotemporal activity.

4.2 Results

4.2.1 Calcium through NMDA receptors is required for CaMKI activation

To test the source of calcium necessary for CaMKI activation in the spine, we used a pharmacological approach. Blockade of NMDA receptors with (2R)-amino-5-phosphonovaleric acid (APV) caused an abrogation of CaMKI activation in the activated spine (Figure 12 A,C) and adjacent dendritic segment (Figure 12 B,D). No spine enlargement was detected while uncaging glutamate in the presence of APV. Thus, we concluded that calcium entry through NMDA receptors is necessary for activation of CaMKI in the spine and dendrite. Previous results (Fortin et al., 2010), (Schmitt et al., 2005) have also demonstrated the requirement of NMDA receptor activity in CaMKI activation.

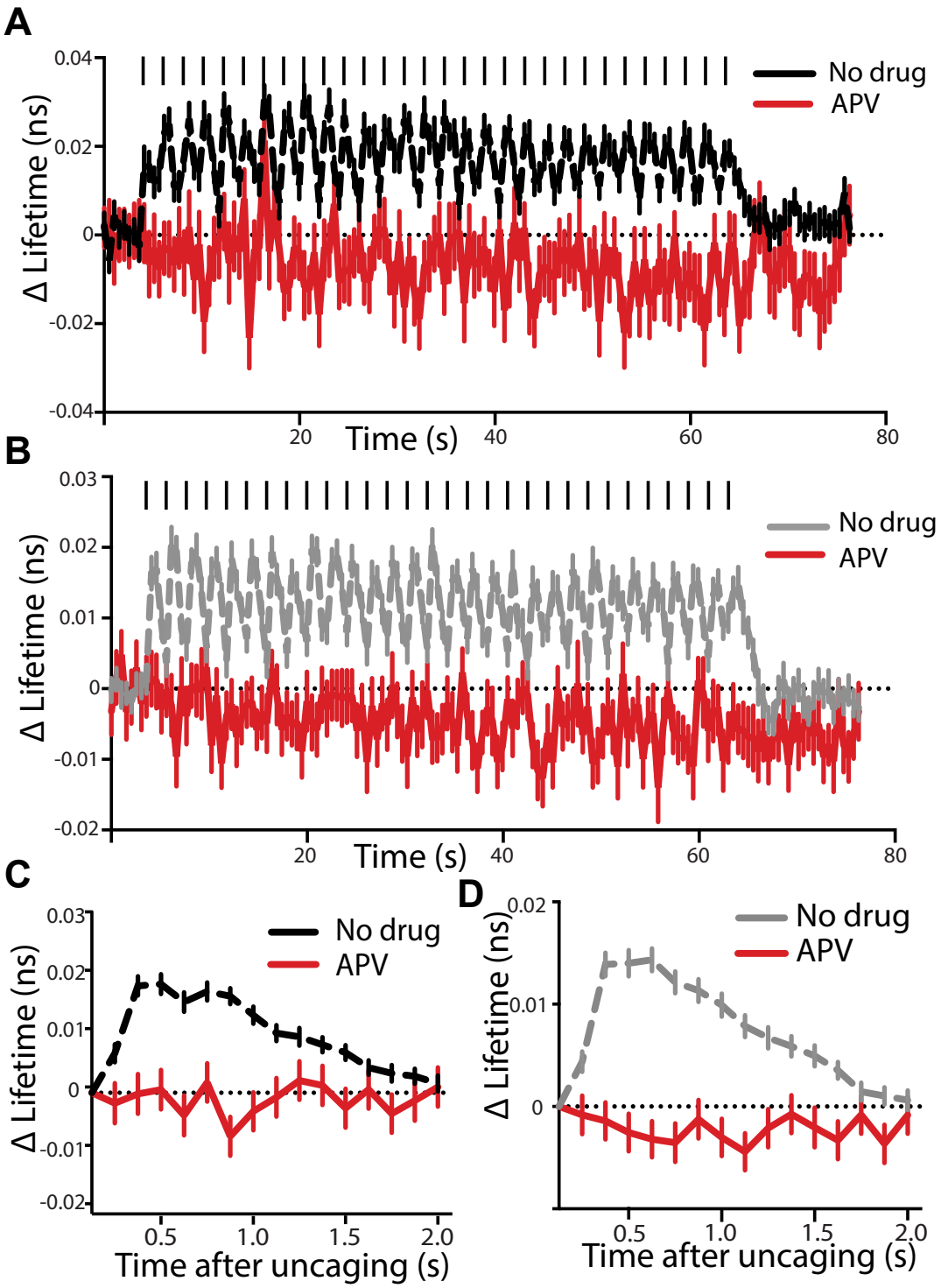

Figure 12: Activity of NMDA receptors is required for CaMKI activation

A. Application of APV 30 minutes before LTP induction causes a reduction of CaMKI activation in the stimulated spine in response to uncaging-evoked LTP. The graph indicates the change in fluorescence lifetime of the CaMKI sensor in the presence (red) or absence (dashed black line) of APV. There is a significant reduction in the fluorescence lifetime of the CaMKI sensor in response to glutamate uncaging in the uncaged spine in the presence of APV. ($F_{3,62}$ = 1.791, p<0.005; Dunnett'sTest, p<0.005)

B. Application of APV 30 minutes before LTP induction causes an abrogation of CaMKI activation in the dendrite in response to uncaging-evoked LTP. The graph indicates the change in fluorescence lifetime of the CaMKI sensor in the presence (red) or absence (dashed gray line) of APV. There is a significant reduction in the fluorescence lifetime of the CaMKI sensor in response to glutamate uncaging in the adjacent dendritic segment in the presence of APV. ($F_{3,61}$ = 2.185, p<0.005; Dunnett'sTest, p<0.005)

C. Uncaging-triggered average of CaMKI activity in the spine in the presence (red) or absence (dashed black line) of APV. APV causes an abrogation of CaMKI activity in the spine.($F_{4,12}$ = 20.906, p<0.005; Dunnett'sTest, p<0.005)

D. Uncaging-triggered average of CaMKI activity in the dendrite in the presence (red) or absence (dashed gray line) of APV. CaMKI activity in the dendrite is blocked in the presence of APV. ($F_{4,11}$ = 17.501, p<0.005; Dunnett'sTest, p<0.005)

We have shown previously that CaMKII can integrate the signal from iterative uncaging pulses(Lee et al., 2009). We were curious whether CaMKI had a similar potential. To test this hypothesis, we performed glutamate uncaging at 4Hz (interval between pulses = 250ms). This faster rate of uncaging causes CaMKI to reach a steady state of activation in both the spine and the dendrite (Figure 13) indicating that CaMKI does have the potential to integrate higher-frequency calcium influx signals. This suggests that CaMKI might detect higher frequency calcium signals in neurons.

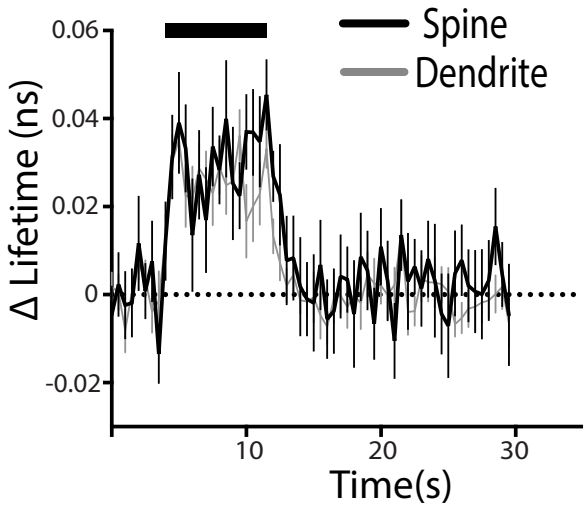

Figure 13: Calcium Integration of CaMKI Sensor

Calcium integration by CaMKI Sensor.Rapid uncaging of glutamate causes sustained activation of the CaMKI sensor. The graph shows the change in fluorescence lifetime of the CaMKI sensor in the spine (black) and adjacent dendritic segment (gray) in response to glutamate uncaging at 4Hz (black bar). CaMKI activity in both the spine and dendrite is sustained at this faster uncaging rate.

4.2.2 The Role of Phosphorylation on spatiotemporal dynamics of CaMKI

CaMKI is phosphorylated by CaMKK on Threonine 177 (Soderling and Stull, 2001). Studies have shown the requirement for both CaMKI activity and CaMKI phosphorylation by CaMKK in LTP induction ((Fortin et al., 2010), (Schmitt et al., 2005)). These studies have identified cellular targets that require activation of both CaMKK and CaMKI in an NMDA-receptor dependent manner. However, it remains unclear what the role of CaMKK phosphorylation plays in regulating CaMKI synaptic activity. We

addressed the question by measuring the spatiotemporal dynamics of CaMKI phospho-mutants (phospho-dead, CaMKI T177A; and phospho-mimic, CaMKI T177D) in response to glutamate uncaging.

4.2.3 Spatiotemporal dynamics of CaMKI phospho-mutants during uncaging-evoked LTP

Both phospho-mutants showed a similar subcellular localization compared to wild-type CaMKI upon biolistic transfection in neurons: the sensor was expressed in the cytoplasm and dendritic processes, and not in the nucleus. In response to glutamate uncaging, CaMKI T177A and CaMKI T177D showed similar activation profiles to CaMKI in the stimulated spine. Following an uncaging pulse, there was a rapid increase in CaMKI activity, regardless of phosphorylation, followed by a brief plateau and subsequent decay to baseline activity (Figure 14 A,B,C). The peak amplitude of CaMKI T177D was similar to that of WT, while that of CaMKI T177A trended toward smaller activation ($F_{3,62} = 1.791$, $p<0.005$; Dunnett'sTest, $p>0.1$). In addition, CaMKI T177A showed a dramatic reduction in activity in the adjacent dendritic segment in response to glutamate uncaging (Figure 14 A,D,E). CaMKI T177D responded in a manner similar to CaMKI in both the spine and the dendrite (Figure 14 A-E).

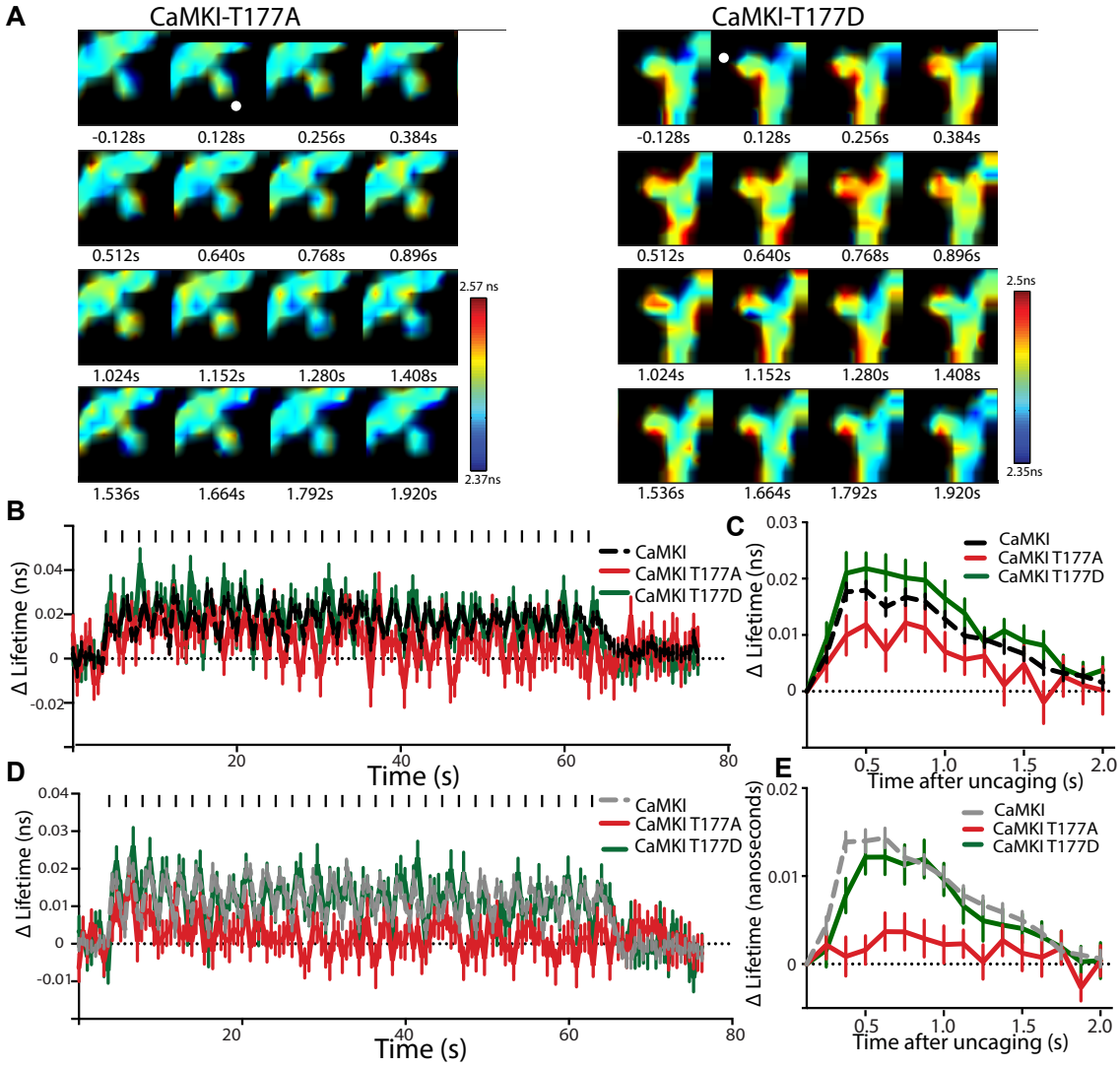

Figure 14: Phosphorylation of CaMKI is necessary for dendritic activity

A. Fluorescence lifetime images of CaMKI-T177A (left) and CaMKI-T177D (right) in response to glutamate uncaging. The images are of a single dendritic spine undergoing uncaging-induced LTP. The warmth of colors indicates the fluorescence lifetime as shown in the color bar. The images shown are an average of the response of CaMKI to all 30 uncaging pulses. Each image is a smoothed average of 3 frames. The time after uncaging is indicated below each image. CaMKI-T177A has reduced activity in the spine and does not show any change in activity in the adjacent dendrite. Scale bar, 1µm

B. Fluorescence lifetime of CaMKI (dashed black line), CaMKI-T177A (red) and CaMKI-T177D (green) in response to glutamate uncaging in a single dendritic spine. The time of uncaging is indicated by the black bars above the graphs. CaMKI-T177A trended towards reduced activity in response to glutamate uncaging in the spine whereas CaMKI-T177D trended towards greater activity, but the differences are not statistically significant ($F_{3,62} = 1.791$, $p<0.005$; Dunnett'sTest, $p>0.1$ for both CaMKI T177A and CaMKIT177D)

C. Uncaging-triggered average of CaMKI (dashed black line), CaMKI-T177A (red) and CaMKI-T177D (green) in dendritic spines. All three proteins have similar time-courses of activation and inactivation. CaMKI-T177A has reduced activity in response to glutamate uncaging in the spine. ($F_{4,12} = 20.906$, $p<0.005$; Dunnett'sTest, $p>0.1$ for both CaMKI T177A and CaMKIT177D)

D. Fluorescence lifetime of CaMKI (dashed black line), CaMKI-T177A (red) and CaMKI-T177D (green) in the adjacent dendritic segment of a single dendritic spine undergoing glutamate-uncaging induced LTP. CaMKI and CaMKI-T177D have similar time courses of activation and inactivation, whereas CaMKI-T177A shows a dramatic reduction of activity. ($F_{3,61} = 2.185$, $p<0.005$; Dunnett'sTest, $p<0.05$ for CaMKI T177A and $p>0.1$ for CaMKI T177D)

E. Uncaging-triggered average of CaMKI (dashed black line), CaMKI-T177A (red) and CaMKI-T177D (green) in the adjacent dendritic segment. CaMKI and CaMKI-T177A show activation and inactivation similar to the spine, but no activity is observed in CaMKI-T177A. ($F_{4,11} = 17.501$, $p<0.005$; Dunnett'sTest, $p<0.05$ for CaMKI T177A and $p>0.1$ for CaMKI T177D)

In order to further test whether CaMKK phosphorylation of CaMKI is required for its dendritic activity, we measured CaMKI activation 30-90 min after application of STO-609, a cell permeable CaMKK inhibitor (Tokumitsu et al., 2002). In response to glutamate uncaging in the presence of STO-609, the activity pattern was consistent with CaMKI T177A: the activity of CaMKI was smaller (but statistically not significant) than control in the spine (Figure 15A) but was abrogated in the dendrite (Figure 15B). Taken

together, the above results demonstrate that CaMKI phosphorylation by CaMKK is necessary for activation of CaMKI in the dendrite, and potentially for full activation in the spine, in response to glutamate uncaging induced LTP.

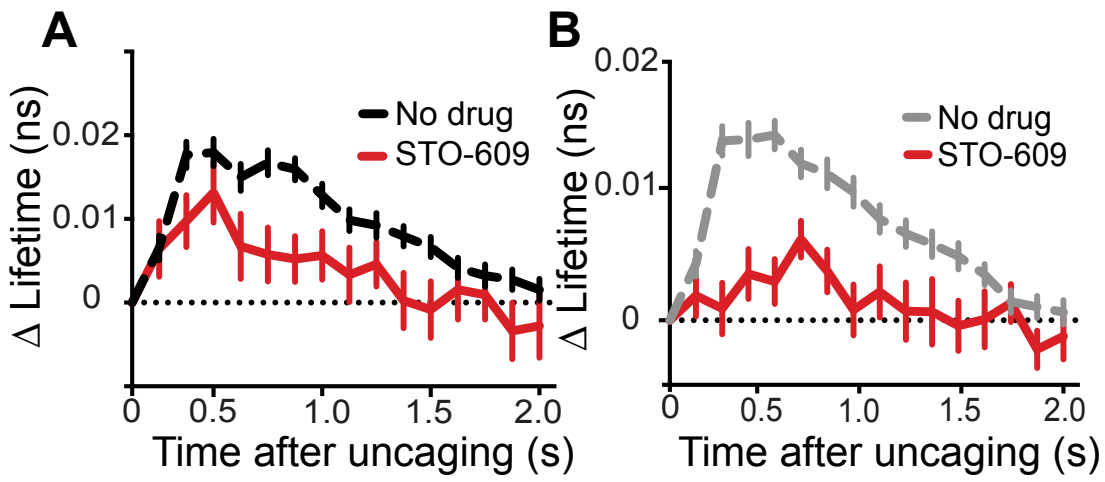

Figure 15: Effect of STO-609 on CaMKI Activity

A. Uncaging-triggered average of CaMKI activity in the presence (red) or absence (dashed black line) of STO-609 in single dendritic spines. STO-609 was applied 30 minutes prior to glutamate uncaging. CaMKI trended toward reduced activity in the presence of STO-609. ($F_{4,12}$ = 20.906, p<0.005; Dunnett'sTest, p>0.05)

B. Uncaging-triggered average of CaMKI activity in the presence (red) or absence (dashed gray line) of STO-609 in adjacent dendritic segment. STO-609 was applied 30 minutes prior to glutamate uncaging. Application of STO-609 dramatically reduced CaMKI activity in the dendrite. ($F_{4,11}$ = 17.501, p<0.005; Dunnett'sTest, p<0.05)

4.2.4 Diffusion of CaMKI phospho-mutants from the spine

Why is phosphorylation necessary for the activation of CaMKI in the dendrite? One possibility is that activity in the dendrite is due to diffusion of active CaMKI from the spine to the dendrite. Non-phosphorylated CaMKI might have a slower rate of diffusion, or might be trapped in the spine, leading to no detectable activity in the dendritic segment adjacent to the spine. To test this hypothesis, we tagged CaMKI T177A and CaMKI T177D with photoactivatable GFP (paGFP) (Patterson and Lippincott-Schwartz, 2002). We biolistically transfected hippocampal CA1 pyramidal neurons with either paGFP-CaMKI-T177A or paGFP-CaMKI-T177D along with mCherry to visualize the morphology of the neuron. paGFP fluorescence was activated using a 2-photon beam focused on spines of secondary and tertiary dendrites along the apical branch. pa-CamKI-T177A and pa-CamKI-T177D rapidly diffused out of the spine (Figure 16A) and the rates were not statistically different from each other under the basal condition (time-constant for paGFP-CamKI-T177A = 0.48s and time-constant for paGFP-CaMKI-T177D = 0.35s). In addition, in order to determine whether calcium influx affected the rate of diffusion of CaMKI in a phosphorylation-dependent manner, the experiment was repeated in the presence of caged glutamate (Figure 16B). Following LTP induction, the rate of diffusion of the CaMKI phospho-mutants was not statistically different from each other (time-constant for paGFP-CamKI-T177A = 0.33s and time-

constant for paGFP-CaMKI-T177D = 0.3s). Thus, it is unlikely that the loss of CaMKI T177A activity from the dendrite is a result of altered diffusion from the spine.

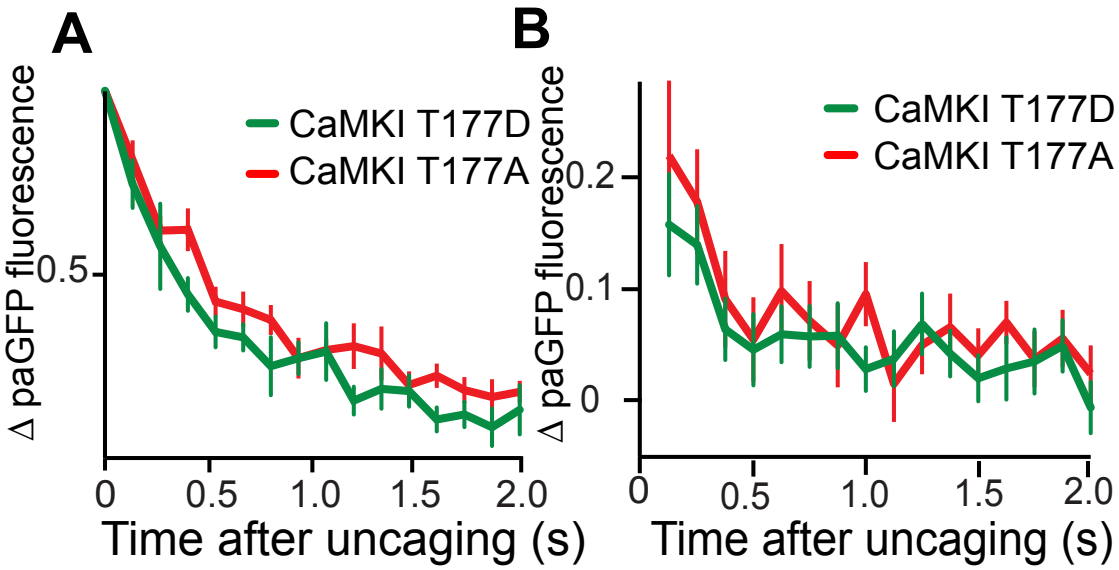

Figure 16: Phosphorylation does not Affect Diffusion of CaMKI

A. Decay of fluorescence of paGFP-CaMKI-T177A (red) and paGFP-CaMKI-T177D (green) in single dendritic spines in the absence of caged glutamate. Phosphorylation state of CaMKI does not alter diffusion of CaMKI out of the spine. ($F_{1,1}$ = 2.169, p>0.05)

B. Decay of fluorescence of paGFP-CaMKI-T177A (red) and paGFP-CaMKI-T177D (green) in single dendritic spines in the presence of caged glutamate. Calcium entry has does not have a phosphorylation-dependent effect on diffusion of CaMKI out of the spine. ($F_{1,8}$ = 0.312, p>0.05)

4.3 Discussion

In this chapter, we investigated the role of NMDA receptors and phosphorylation on the spatiotemporal dynamics of CaMKI. We demonstrated that blockade of NMDA receptor activity using APV abolished CaMKI activation in the spine

and dendrite. Further, we demonstrated that T177 phosphorylation of CaMKI by CaMKK was dispensable for CaMKI activation in the spine, but was required for CaMKI activity in the dendrite. Mutation of T177 to an uncharged alanine residue caused a dramatic reduction of CaMKI activity in the dendrite. Application of STO-609 had a similar effect on dendritic CaMKI activation, while activity of CaMKI in the stimulated spine remained at levels similar to control.

Blockade of CaMKI activity by APV served as a control to demonstrate that the observed CaMKI activity was a result of physiological activity of the dendrite, and not a stimulus artifact. This experiment demonstrated that changes to the fluorescence lifetime of the CaMKI sensor were not a result of optical changes to the slice or medium, but dependent on LTP-inducing calcium entry. This also tied in our work to research showing that NMDA-receptor dependent calcium is required for structural and functional LTP in the hippocampus. NMDA-receptor activity is also required for LTP in other regions of the brain, and NMDA receptor-dependent calcium might serve as a common mechanism for inducing CaMKI throughout the brain(Bear and Malenka, 1994; Malenka and Bear, 2004). Additionally, we also showed that CaMKI can integrate calcium signals at higher frequencies in order to remain at a sustained level of activation. This suggests that CaMKI might serve as a calcium sensor for high-frequency events in the neuron, whereas CaMKII activity would detect lower-frequency events.

While the importance of CaMKI phosphorylation by CaMKK had been previously established(Means, 2000), it was unclear what effect phosphorylation had on the spatiotemporal dynamics of CaMKI. Based on our results, phosphorylation is necessary for the dendritic activity of CaMKI, but is dispensable for CaMKI activity in the spine. This result was very surprising, since both CaMKK and CaMKI are present in the spines and dendrite, and are freely diffusible between the two subcellular compartments. Since phosphorylation does not affect the diffusibilty of CaMKI, we surmised that it must be affecting binding of Ca/CaM to CaMKI. Calcium diffusion into the dendrite is restricted due to the thin spine neck. Thus, glutamate uncaging-dependent calcium does not spread efficiently between the spine and dendrite. Our hypothesis is that CaMKK-dependent phosphorylation of CaMKI is required for dendritic activation because of the lower concentration of calcium in the dendritic environment.

CaMKI shows interesting differences in the kinetics of phosphorylation compared to CaMKII. CaMKII undergoes autophosphorylation on residue Threonine 286 which allows the kinase to maintain activity even in the absence of calcium/calmodulin (Lisman and Zhabotinsky, 2001). CaMKI phosphorylation occurs in trans by CaMKK (Lee and Edelman, 1994). Additionally, binding of calcium/calmodulin opens an “activation loop” exposing the threonine residue (T177) that is phosphorylated by CaMKK, and so phosphorylation does not confer calcium-independent activity. As a

result, CaMKI activation is transient, whereas CaMKII shows a comparatively sustained level of activity. CaMKII phosphorylation thus allows the enzyme to have a longer time-course of activation compared to CaMKI. Then why is it that CaMKII activation is spine-specific whereas CaMKI activity is also observed in the dendrite? CaMKII is a dodecameric protein and it takes more than 1 min for CaMKII to diffuse out of the spine (Lee et al., 2009). In contrast, CaMKI is a monomer (Soderling and Stull, 2001; Rosenberg et al., 2006) and diffuses out of the spine in ~0.3 s (Fig. 16 A,B). Differences in the spatial dynamics of CaMKI and CaMKII are accounted for by the differences in diffusion, which make up for the faster inactivation rate of CaMKI.

CaMKI activity in 2 different compartments of the cell (the spine and dendrite) are differentially regulated by phosphorylation. This finding is intriguing, since downstream targets of CaMKI might be differentially localized to the spine and dendrite. Substrates in the spine would depend on calcium and CaMKI but not CaMKK, whereas substrates in the dendrite would depend on activity of all 3 molecules. CaMKI targets include GEFs of Ras, Rac and Erk. We have shown previously that Ras activity spreads outward from the spine, and CaMKI activity and CaMKI phosphorylation might differentially regulate the dendritic activity of Ras.

Chapter 5. The role of CaMKI during structural plasticity

5.1 Introduction

CaMKI and CaMKK both have been previously shown to be involved in neuronal structural and functional LTP (Fortin et al., 2010), (Schmitt et al., 2005). However, the previous studies have either induced LTP using an electrical stimulus (Schmitt et al., 2005) or have used a chemical method of induction (Fortin et al., 2010). We have previously demonstrated that CaMKII is required for structural LTP using glutamate uncaging (Lee et al., 2009). To compare the role of the different CaMK signaling cascades in the same process, we performed experiments to determine the role of CaMKI activity, and CaMKI phosphorylation by CaMKK in the context of glutamate uncaging-induced structural LTP.

5.2 Results

5.2.1 The role of CaMKI activity and phosphorylation on structural plasticity

To determine whether CaMKI activity was necessary for LTP, we biolistically transfected neurons with siRNA against CaMKI or scrambled siRNA (negative control) and fluorescent markers to quantify the volume of the spine. Upon inducing LTP with caged glutamate, a marked reduction of volume was observed between 1-5 minutes after LTP induction in CaMKI siRNA transfected neurons when compared to controls (203 ± 18% versus 157 ± 13% relative to baseline volume). However, following this period, the

volume of the spines transfected with siRNA were indistinguishable from those transfected with control siRNA (140 ± 11% versus 118 ± 10% relative to baseline volume) (Figure 17A,B,C). Co-transfection of the siRNA-resistant CaMKI sensor along with the siRNA rescued the volume loss observed immediately following uncaging-induced LTP (Figure 17 A,B,C), demonstrating the siRNA effect was specific. Interestingly, co-transfection of siRNA-resistant CaMKI T177A sensor did not rescue the volume loss observed following uncaging induced LTP. Taken together, these data suggest that CaMKI is required for the immediate expansion of the spine following glutamate application, in the transient phase of structural LTP. Comparatively, we have shown previously (Lee et al., 2009), that CaMKII is also required for structural plasticity, but that it specifically regulates the volume in the later sustained phase of LTP.

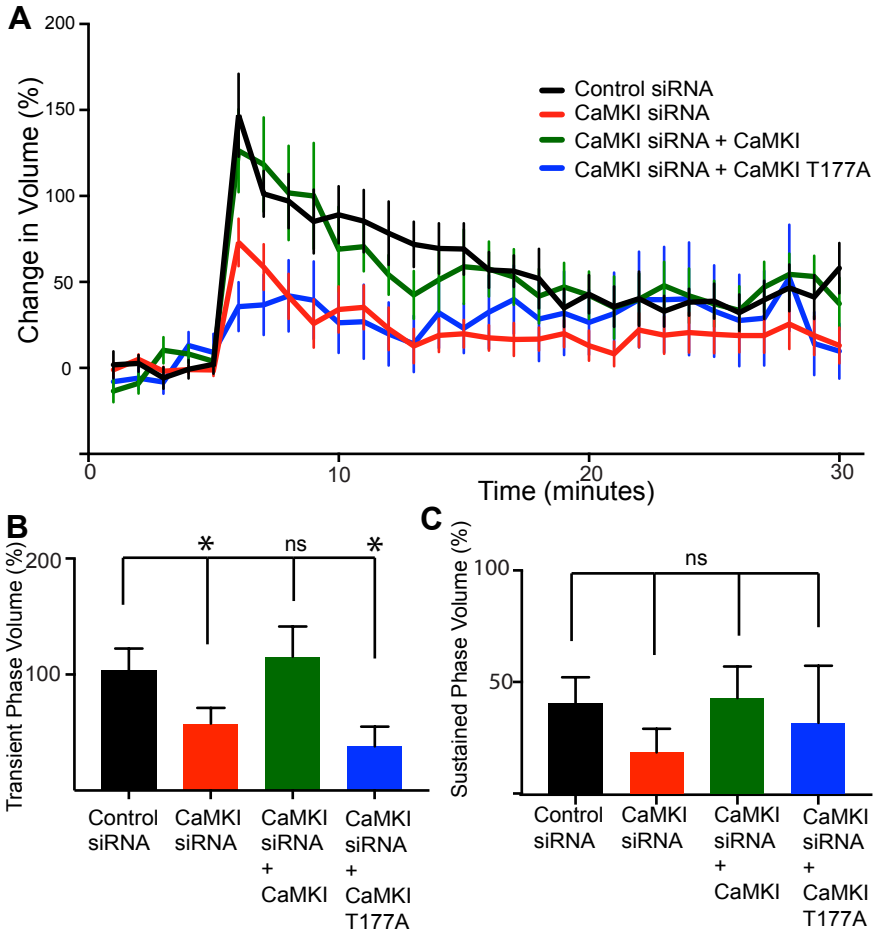

Figure 17: CaMKI is Required for Structural LTP

A. Time course of structural plasticity under different RNA interference conditions. CaMKI siRNA transfection (red) causes a reduction in rapid spine enlargement immediately following LTP compared to non-targeting siRNA transfection (black), but has no effect on the sustained volume increase following LTP. The reduction in rapid spine enlargement can be rescued by co-transfecting the CaMKI sensor (green) along with the siRNA construct. CaMKI-T177A (blue) does not rescue the reduction in rapid spine enlargement.

B. Bar graph representing spine volume immediately (1-5 minutes) following LTP induction (transient phase) ($F_{3,3} = 4.537$ $p<0.005$; Dunnett's test results are displayed on the graph *$p<0.05$, ns – not significant).

C. Bar graph representing spine volume >15 minutes following LTP induction (sustained phase) ($F_{3,3}$ = 0.808 p>0.05).

5.2.2 The role of CaMKK-dependent phosphorylation on structural LTP

To determine the role of CaMKK signaling more directly, we compared the change in volume of meGFP transfected neurons in the presence of STO-609. Application of STO-609 before glutamate uncaging-induced LTP also caused a marked reduction in volume immediately following glutamate application (Figure 18 A,B,C) with no effect on the volume in the later phase of LTP. This data is consistent with the previous siRNA and rescue results (Figure 18 A,B,C) and suggests that CaMKI activity, and CaMKI phosphorylation are involved in the regulation of the transient phase of LTP.

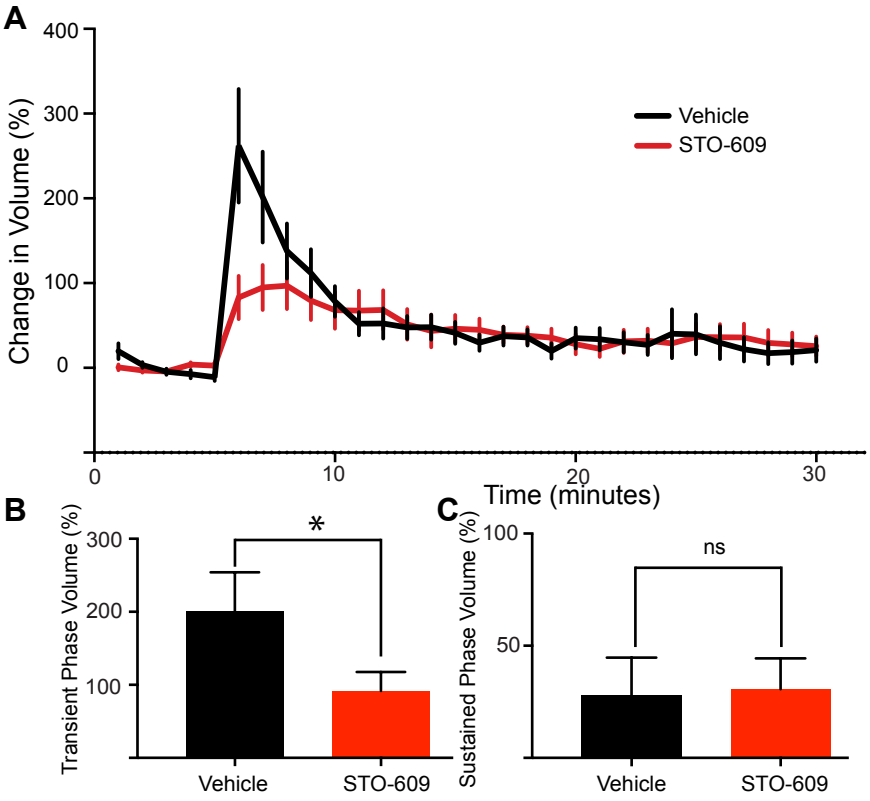

Figure 18: Effect of STO-609 on Structural LTP

A. Time course of volume change following glutamate uncaging-induced LTP. In the presence of STO-609 (red), there is a reduction in rapid spine enlargement following LTP induction compared to vehicle treatment (black).

B. Bar graph representing spine volume immediately (1-5 minutes) following LTP induction ($F_{1,1} = 4.267$, $p<0.05$)C. Bar graph representing spine volume >15 minutes following LTP induction. ($F_{1,1} = 4.267$, $p>0.05$)

5.3 Discussion:

In this chapter, we characterize the role of CaMKI in glutamate-uncaging dependent structural plasticity of single dendritic spines. While previous studies have established the role of CaMKI in chemical and electrical LTP, our studies show that CaMKI is required for immediate spine enlargement following LTP induction in single dendritic spines. This allows us to compare the role of CaMKI and CaMKII directly, since we performed similar experiments with CaMKII.

CaMKI and CaMKII regulate different phases of structural LTP. We have shown previously that inhibition of CaMKII abrogates the sustained volume change following LTP induction (Lee et al., 2009). However, it is important to note that inhibitors of calmodulin abrogate both the sustained volume change and also the large transient change following induction of LTP (Matsuzaki et al., 2004). Our data suggests that CaMKI is specifically required for the rapid increase in spine volume following LTP induction, but is dispensable for the sustained change in volume. Thus there appear to be 2 different signaling cascades regulating different temporal phases of LTP, with CaMKI and CaMKII serving as master regulators of these cascades.

CaMKI phosphorylation regulates the transient phase of structural LTP in addition to CaMKI activity. In the previous chapter, we provided evidence that CaMKI phosphorylation was required for dendritic activity of CaMKI, but was dispensable for CaMKI activity in the spine. These results together suggest that dendritic activity of CaMKI is required to regulate the transient phase of LTP, since CaMKI phosphorylation

does not severely affect CaMKI activity in the spine. It is unclear why dendritic signaling is required for the transient phase. One possibility is that downstream substrates of CaMKI required for actin polymerization might be localized to the dendrite. However, further experiments inhibiting CaMKI activity specifically in the dendrite need to be performed to confirm this finding. Additionally, LTP-inducing glutamate uncaging could be performed in neurons transfected with CaMKI siRNA and CaMKI-T177D. I expect that structural plasticity would have a large transient and normal sustained phase volumes, thereby confirming that phosphorylation of CaMKI, even at a constitutive level, would be sufficient for LTP.

Chapter 6. Discussion and Future Directions

In this study, we determined the spatiotemporal dynamics of CaMKI signaling in response to glutamate uncaging-evoked LTP in single dendritic spines. We developed a FRET-based sensor for CaMKI activity and used the sensor to track the activity of CaMKI in and near single dendritic spines. CaMKI activity was rapidly and transiently induced both in the spine and in the adjacent dendritic segment in response to glutamate application. Activation in both the spine and dendrite depended on calcium entry through NMDA receptors. Signaling in the dendrite also depended on phosphorylation of CaMKI by CaMKK. CaMKI activity and CaMKI phosphorylation were both required for the immediate spine volume increase during the transient phase of structural plasticity but were dispensable for sustained volume increase following LTP induction (Figure 19).

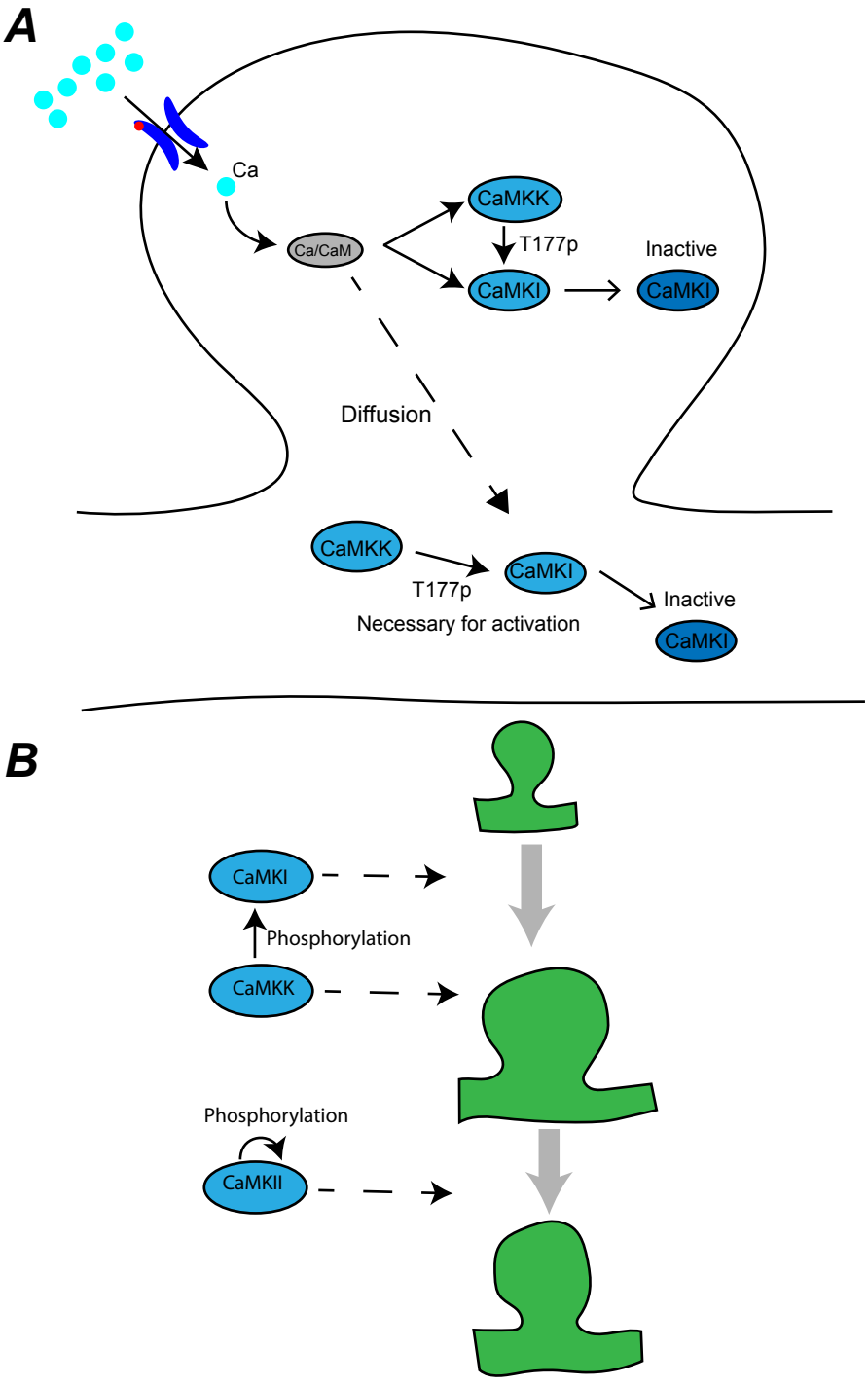

Figure 19: CaMKI Activity in Single Dendritic Spines

A. Calcium influx (blue circle) through NMDA receptors (dark blue) activates calmodulin in the dendritic spine. Ca/CaM rapidly activates CaMKI which is quickly inactivated. CaMKK phosphorylation occurs in the spine and dendrite, but is required for signaling only in the dendrite. Dendritic activation of CaMKI is driven by diffusion of Ca/CaM from the spine to the dendrite.

B. CaMKI activity and CaMKK phosphorylation of CaMKI are required for the transient phase of LTP, whereas CaMKII activity is required for the sustained phase of LTP.

6.1 Comparison of the activity of CaMKI and other CaMKs

We have previously characterized the activity of CaMKII using a similar paradigm and using a similar sensor(Lee et al., 2009). Comparison of the activity of CaMKI and CaMKII could provide insights into the roles of the various CaMKs in sLTP, plasticity, learning and memory. Both sensors are activated in dendritic spines of neurons in response to glutamate uncaging. CaMKII has a much longer time course of activation (~1min vs ~0.25s) and inactivation (~6 min vs~2s) compared to CaMKI. However, the dodecameric nature of CaMKII, along with its interactions with other PSD proteins reduces the rate of diffusion of CaMKII(Hudmon et al., 2005; Merrill et al., 2005), and so the activity of the protein is restricted to the spine. Thus, we can conclude that the 2 CaMKs have different spatial and temporal dynamics of activation.

Activation of CaMKII in the spine is severely reduced by mutation of the autophosphorylation site of CaMKII to non-phosphorylatable alanine. Autophosphorylation has been shown to confer calcium-independent activity of CaMKII *in vitro*, and allows CaMKII to integrate calcium signals from iterative NMDA receptor

stimulations during glutamate uncaging(Lisman and Zhabotinsky, 2001). Thus, phosphorylation of CaMKII extends the temporal duration of CaMKII activity. This is not the case for CaMKI, where phosphorylation serves to extend the spatial reach of the protein without altering the time-course of activation. This is likely because phosphorylation of CaMKI does not confer calcium-independent activity, but increase the affinity of CaMKI for Ca/CaM(Haribabu et al., 1995). This allows CaMKI to remain active in the dendrite, where the concentration of calcium is lower than that in the spine.

The slower time-course of CaMKII activation relative to CaMKI can also explain the differences between the 2 enzymes in regulation structural LTP. CaMKII is required for long-lasting spine changes, but not for the immediate increase in dendritic spine volume, following LTP induction(Lee et al., 2009). Since CaMKII has a slower time-course of activation, it is likely that downstream effectors of CaMKII are activated later following glutamate application, and therefore regulate later phases of LTP. CaMKI and CaMKII may have overlapping substrates. For instance, both proteins regulate activators of Rac1, and therefore converge on Rac1 signaling(Tolias et al., 2005; Saneyoshi et al., 2008). However, CaMKI would regulate Rac immediately following the uncaging events, whereas CaMKII-dependent Rac activation occurs with a slower time-course. The differences in the spatiotemporal dynamics of the two CaMKs could explain the differences in their role in regulating LTP.

The spatiotemporal dynamics of CaMKIV activation in response to single-spine LTP has not been studied. However, CaMKIV is restricted to the nucleus, and so does not play a role in single-spine LTP(Jensen et al., 1991). Major downstream targets of CaMKIV include transcription factors, and CaMKIV likely plays a role in protein-synthesis dependent late LTP(Enslen et al., 1994; Kang et al., 2001). CaMKK is also present in spines and dendrites, and has additional downstream targets independent of CaMKI(Yano et al., 1998). Since it is thought that CaMKK activity is not enhanced by phosphorylation or other post-translational modifications, it is likely that CaMKK activity is similar to CaMKI.

It is interesting to note that phosphorylation of CaMKI by CaMKK is required for both, dendritic activity of CaMKI and the transient phase of structural LTP. The experiments suggest an intriguing hypothesis whereby dendritic activation of CaMKI might be required for the transient phase of LTP. To test this hypothesis, future studies would have to be conducted to block dendritic activation of CaMKI and determine the effect of this blockade on structural LTP induction. For instance, a neuron could be transfected with a microtubule binding domain-tagged calcium buffer such as calcineurin. Transfection of this reagent would increase calcium buffering specifically in the dendrite while leaving the spine unaffected. The effects on CaMKI activity and structural plasticity in the presence of this reagent would provide insight into the role of dendritic CaMKI activity and its role in LTP induction.

6.2 Downstream Effectors of CaMKI

CaMKI regulates spine volume increase following induction of LTP. However, CaMKI is dispensable for long-lasting spine volume change, and regulates the immediate pronounced increase following LTP induction. Since spine volume changes depend on actin polymerization, it is likely that CaMKI regulates the activity of actin polymerization factors. Since CaMKI activity is observed in both the spine and adjacent dendritic segment, the downstream effectors of CaMKI activity are likely located near a spine.

The downstream effectors of CaMKI have been poorly characterized. Previous studies have shown that CaMKI can phosphorylate and activate βPix, a guanine nucleotide exchange factor (GEF) of Rac1 (Saneyoshi et al., 2008). CaMKI phosphorylates Serine 516 of βPix, which enhances its GEF activity. This enhanced GEF activity leads to increase in the activity of Rac1. In neurons, the authors showed that depolarization of neurons with KCl can activate this signaling cascade, and the enhanced Rac1 activity increases the number of dendritic spines. Thus, CaMKI has been shown to play a role in activity-dependent neuronal plasticity. Rac1 and CaMKI are both present in spines of neurons, and are likely activated by LTP-inducing calcium. Thus, βPix likely plays a role

in CaMKI-dependent Rac1 activation, with the latter leading to an enhancement of spine volume immediately following glutamate uncaging.

Studies have also demonstrated CaMKI phosphorylation of Ras-GRF1 during electrically induced LTP(Schmitt et al., 2004; Schmitt et al., 2005). In response to theta-bust stimulation, CaMKK and CaMKI are activated in an NMDA-receptor dependent manner. CaMKK-CaMKI dependent phosphorylation of Ras-GRF1 on Serine 916 activated the protein, which led to the activation of MEK and Erk. Blockade of CaMKK-CaMKI activation led to a protein-synthesis dependent reduction in eLTP expression. This study suggests that CaMKI activation of Ras regulates nuclear transcription and eLTP.

Further experiment on the activation of Ras by CaMKI can help elucidate the role of CaMKI in Ras signaling in neurons. We have previously generated and characterized a Ras sensor(Yasuda et al., 2006; Oliveira and Yasuda, 2013). Ras activation in response to LTP induction in single dendritic spines spreads from the spine along the dendrite(Harvey et al., 2008). Since CaMKI activity is also observed in the dendrite, an intriguing hypothesis that can be tested is that CaMKI phosphorylation of Ras increases the spreading of Ras and Ras effectors. Our lab has also generated a sensor to measure nuclear Erk signaling and shown that stimulation of a few spines can activate nuclear Erk(Zhai et al., 2013). These sensors, in combination with pharmacological or genetic

manipulations of CaMKK/CaMKI could be used to determine the effects of the CaMKK/CaMKI signaling cascade on the spatiotemporal dynamics of Ras-MEK-Erk signaling.

6.3 The role of CaMKK/CaMKI signaling cascade in neuronal function and behavior

What is the role of CaMKK/CaMKI activity on learning and memory? Previous analyses have shown that infusion of STO-609 to the perirhinal cortex inhibits object recognition memory in rats(Tinsley et al., 2012). In this study, rats were exposed to a constructed plastic toy. After 20 min or 24h, rats were exposed to the familiar plastic toy and a novel toy. Memory was assessed as a discrimination between the novel and familiar object. Infusion of STO-609 into the cortex lowered the discrimination between the 2 objects, suggesting that CaMKK activation was required for recognition memory. However, infusion of STO-609 was only effective if administered 15 min before or immediately after the "acquisition trial" with the familiar toy. The authors further demonstrated that CaMKI activation was lower during and ~40 min after the "acquisition time," but was not affected ~70 min after the "acquisition time." This suggests that CaMKK/CaMKI activity plays a role in long-term memory acquisition, but the activity of CaMKK and CaMKI are required only for a short period following the stimulus.

Genetic knockouts of both CaMKKα(Blaeser et al., 2006; Mizuno et al., 2006) and CaMKKβ(Peters et al., 2003) showed deficits in learning and memory. Interestingly, knockouts of CaMKKα showed deficits in contextual fear conditioning, but not in spatial memory. CaMKKβ knockouts showed deficits in spatial memory formation but not in contextual fear conditioning. Both sets of animals showed normal cued fear conditioning. Taken together, these studies suggest that CaMKK is involved in memory formation, but different isoforms might be involved in distinct types of memories. However, studies of the knockouts are further complicated since CaMKK isoforms could potentially compensate for the loss of function of either.

Although CaMKK has substrates other than CaMKI, these studies suggest that phospho-regulation of CaMKI could also be an essential component of learning and memory. Infusion of STO-609 inhibits phosphorylation of CaMKI. While not inhibiting CaMKI activity per se, phosphorylation of CaMKI is necessary for full activity of the protein, and may affect CaMKI activity in specific subcellular compartments. Further studies using genetic knockouts of CaMKI, or mutant CaMKI generated via CRISPR/Cas9 technology could be useful in determining the role of CaMKI at the level of neuronal circuits or behavior.

6.4 Future Directions

In this study, we have measured the spatiotemporal dynamics of CaMKI upon induction of LTP in single dendritic spines. CaMKI activity was not restricted to the stimulated spine, but spread to the adjacent dendritic segment. Calcium influx through NMDA receptors was required for activation of CaMKI. Phosphorylation of CaMKI by CaMKK was required for activation of CaMKI in the dendrite. CaMKI activity was required for immediate spine enlargement during the transient phase of LTP, but was dispensable for long-lasting structural changes to the spine during the sustained phase of LTP.

Structural plasticity of dendritic spines occurs on a variety of timescales, and spine formation, elimination and changes in morphology are correlated with changes in behavior of animals. CaMKI is involved in spine formation and elimination, and we have shown that it can also regulate the morphology of existing spines. Interestingly, CaMKI regulates the transient phase of LTP, but not the sustained phase. Sustained spine volume has been associated with memory formation, but the role of the transient phase remains unknown. To my knowledge, this is the first protein which exclusively regulates the transient phase of LTP. Further studies on the role of CaMKI in neuronal function, learning and memory can help elucidate the effects of the transient phase of LTP on learning and memory.

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Biography

Rohit Ramnath

Birthdate: July 17, 1987
Birthplace: Mumbai, India

Education

2005-2009
Carnegie Mellon University
Pittsburgh, Pennsylvania
BS in Biological Sciences with a focus in Neuroscience

2009-Present
Duke University
Durham, North Carolina
Ph.D. in Neurobiology

Publications

Hovis KR, Ramnath R, Dahlen JE, et al. Activity Regulates Functional Connectivity from the Vomeronasal Organ to the Accessory Olfactory Bulb. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2012;32(23):7907-7916. doi:10.1523/JNEUROSCI.2399-11.2012.

Posters and Presentations

2012-2015 Neurobiology Retreat, Wilmington NC

2015 Triangle SfN conference

2013,2015 Cell Biology Retreat, Beaufort, NC