ROLE OF DENSIN AND ALPHA-ACTININ IN REGULATING Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE II

By

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DEDICATION

To my husband, Dhananjay Sakrikar for all the love, support and guidance through the years

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ABBREVIATIONS

AID…… Autoinhibitory Domain

AKAP……..……A-Kinase anchoring Protein

AMPAR……α-Amino-3-hydroxy-5-Methylisoxazolepropionate Receptors

CaM……..Calmodulin

CaMKII……..Calcium/Calmodulin-dependent Protein Kinase II

CAMKAP……..CaMKII-associated protein

CTD…….. C-Terminal Domain

LTD……..Long-term Depression

LTP……..….Long-term Potentiation

NMDAR……..…N-methyl-D-aspartate Receptors

PIP2…….. Phosphatidyl Inositol-4,5 bisphosphate

PKA……Protein Kinase A

PP……..Protein Phosphatase

PSD……..Postsynaptic Density

CHAPTER I

INTRODUCTION

As adults we can remember incidents from our childhood. The ability to acquire and retain information, defined as learning and memory, respectively, is a prerequisite for the full and comprehensive life of any individual in our society. The great mystery of the human brain lies in its ability to integrate multiple sensory inputs, motor outputs, and cognitive processes into conglomerations that can be consolidated and stored for decades in an apparently infinite number of memories. Understanding the molecular mechanisms underlying normal learning and memory processes are fundamental to neuroscience research. Although the mechanisms are far from solved, the past 50-60 years have seen significant progress in the formation of models that, at least in part, may explain the complicated process of memory formation and storage. The importance of hippocampus for normal memory function became evident in 1957, when a patient, known as H.M., had undergone bilateral medial temporal lobe resection (Scoville and Milner, 1957). This procedure performed to relieve the patient from medically intractable seizures, resulted in amnesia; although it did reduce the seizure frequency (Corkin, 2002).

The hippocampus is an integral part of the limbic system, a group of brain regions that contribute to a variety of behaviors, especially emotional responses, olfaction, and memory formation. Hippocampal function depends on

communication between neurons through specialized structures called synapses. These are the regions at which neurotransmitters released from one neuron activate signaling on another neuron. The excitatory glutamatergic synapse is the site of changes in the strength of neuronal connectivity that are believed to underlie learning and memory. An experiment in 1970 showed that when these excitatory synapses were stimulated repetitively, it lead to enhancement of synaptic transmission for hours, even days, after the initial stimulus (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1970). This persistent increase in synaptic strength is referred to as Long-Term Potentiation (LTP). A cognate reduction in synaptic strength resulting from low frequency stimulation is known as Long-Term Depression (LTD). These activity-dependent regulations in the strength of synapses are known as synaptic plasticity, a cellular correlate of learning and memory.

Long–term Potentiation:

Synaptic plasticity is studied in many brain regions, but much of our understanding derives from examination of LTP in synapses formed between the Schaffer collateral and commissural axons and the apical dendrites of CA1 pyramidal cells (Malenka and Nicoll, 1999) (Fig.1A). These are excitatory glutamatergic synapses, and often are associated with postsynaptic morphological specializations called dendritic spines, ~1 μm^3 protrusions onto which the axons synapse.

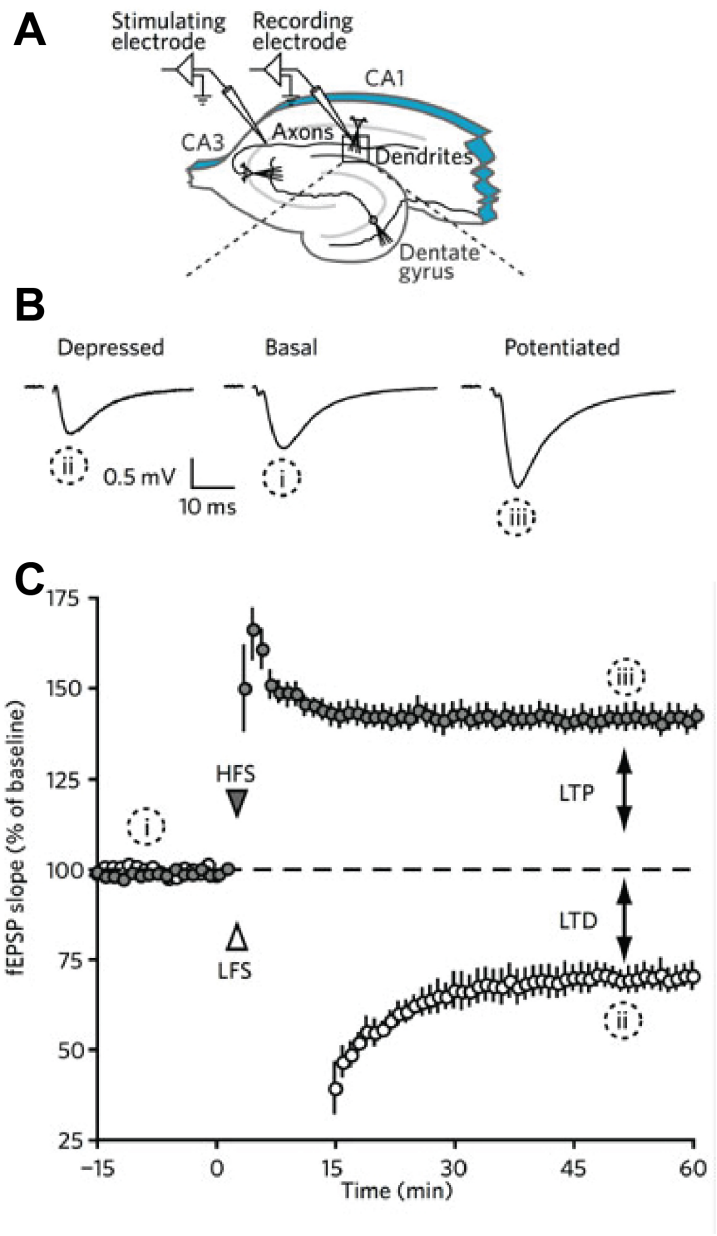

Figure 1.1 Long-term potentiation and long-term depression in hippocampus
A. Schematic of cellular architecture of a hippocampus showing the dentate gyrus, the CA1 and CA3 axons. A stimulating electrode is placed in the region of axons and is used to activate synapses. A recording electrode is placed in a field of activated synapses to measure the change in voltage or current. **B.** Examples of field excitatory postsynaptic potentials (fEPSPs), measured from synapses under (i) basal, (ii) depressed and (iii) potentiated conditions. **C.** Representation of typical LTP (closed circles) and LTD (open circles) experiments induced following brief periods of high frequency or low frequency stimulation (HFS and LFS, respectively). The amplitude or slope of the fEPSP (circles, see B) is measured once every minute. *Adapted from* (Fleming and England, 2010)

LTP is observed by electrically stimulating axons and recording postsynaptic responses in the cell body layer of CA1 region (Fig. 1B and C). LTP can be induced using several stimulation paradigms including high frequency like tetanus train of 100Hz, and "theta burst" stimulation (4 stimuli at 100 Hz repeated several times at 200 ms time intervals), which is reminiscent of normal physiological hippocampal firing patterns (Albensi et al., 2007) . Although LTP can be triggered rapidly (within seconds), it can lasts for hours *in vitro* to days *in vivo*. Also in the CA1, LTP is normally input-specific; LTP at one synapse does not increase the strength of neighboring synapses. This suggests that mechanisms normally supporting LTP are compartmentalized (Malenka and Nicoll, 1999). LTP is considered to contribute to memory formation by inducing long-lasting, sometimes permanent changes to the neural circuitry. Hence neuroscience research has further explored the molecular mechanism underlying this phenomenon.

Molecular Mechanisms of LTP*:*

There has been a hot debate on whether LTP occurs via pre- or postsynaptic mechanisms. Most researchers now believe that CA1 LTP occurs through a postsynaptic mechanism (Nicoll, 2003). Triggering LTP requires synaptic activation of receptors, which lie in the postsynaptic density (PSD). The PSD is an electron-dense thickening underneath the postsynaptic membrane at the excitatory synapses containing receptors, structural proteins linked to the actin cytoskeleton and signaling elements such as kinases and phosphatases (Kennedy, 1997). Upon depolarization of the presynaptic neuron, vesicles

release the stored neurotransmitter, glutamate, into the synapse, which traverses through the synaptic cleft to activate the glutamate receptors localized at the PSD (Fig 1.2). Amongst the large number of PSD proteins, the most critical signaling elements are glutamate-gated ion channels (α-amino-3-hydroxy-5-methylisoxazolepropionate receptors (AMPARs) and N-methyl-D-aspartate receptors (NMDARs)), various scaffolding and structural molecules (PSD-95, SAP97, AKAP79/150, spinophilin/neurabin, densin-180, and α-actinin), and regulatory molecules like kinases (PKA, CaMKII, PKC) and phosphatases (PP1 and PP2B). The shape, size and composition of PSDs may correlate with the efficiency of synaptic transmission. Through protein/protein interactions and cross talk, this conglomeration of 400-1000 proteins are able to spatially and

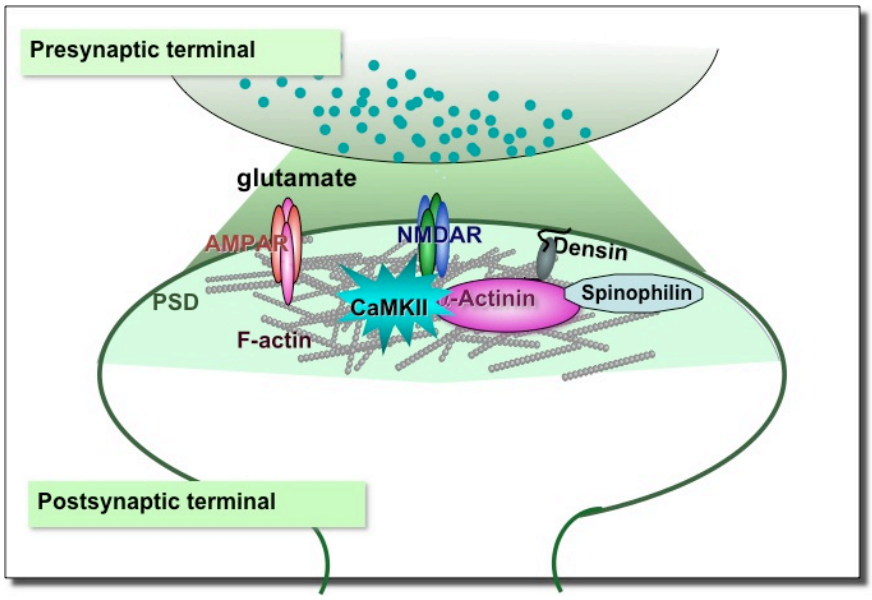

Figure 1.2 Structure of the excitatory synapse and the post-synaptic terminal

Schematic of a glutamatergic synapse with the presynaptic terminal containing vesicles filled with neurotransmitter, glutamate released in the synaptic cleft and the major signaling proteins at the postsynaptic density (PSD).

temporally organize signaling complexes at the PSD for efficient and specific dissemination of presynaptic messages (Sheng and Hoogenraad, 2007). It is hypothesized that altered expression, localization, or activation of PSD proteins that affect these protein/protein interactions can lead to learning and memory dysfunction.

Glutamate receptors:

Glutamate (Glu) is the major excitatory neurotransmitter in the hippocampus as well as the entire central nervous system (CNS). Glutamate released by presynaptic axon terminals activates a number of receptors on the postsynaptic membrane, including metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (iGluRs). mGluRs are G-protein coupled receptors (GPCRs), which are able to alter intracellular signaling cascades upon Glu binding. iGluRs function as ion channels, opening in response to glutamate activation (Danbolt, 2001). For the purposes of this dissertation I will focus on the ionotropic family of glutamate receptors.

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR):

AMPARs have a fundamental role in synaptic plasticity, learning and memory. These are the primary receptors responsible for fast excitatory synaptic transmission in the brain. Functional AMPARs exists as a tetramer composed of a combination of subunits that are encoded by four separate genes, namely, GluA1-GluA4 (old nomenclature was GluR1-GluR4). In the mature hippocampus GluA1, GluA2, and GluA3 are the major subunits expressed with GluA4 primarily expressed in the immature brain (Shepherd and Huganir, 2007). These subunits

combine to primarily form heteromers of either GluA1/GluA2 homodimers or GluA2/GluA3 homodimers (Wenthold et al., 1996). Structurally each subunit has a large extracellular N-terminus, three transmembrane domains, one pore forming intramembrane region, and a cytoplasmic C-tail (Gouaux, 2004). These subunits are very similar in the extracellular and transmembrane domains but differ in the intracellular C-tail. GluA1, GluA4 along with an alternatively spliced form of GluA2 (GluA2L) have long C-tails. The GluA2, GluA3 and alternatively spliced form of GluA4 (GluA4S) have shorter cytoplasmic tails (Shepherd and Huganir, 2007). Post-translational modification and protein/protein interactions in the C-terminal domain can direct trafficking and subcellular localization of the receptors, as well as affect function of the receptors (Song and Huganir, 2002).

Insertion or removal of AMPARs from the postsynaptic membrane dictates the strength of the synapse (Bredt and Nicoll, 2003; Greer et al., 2010; Malinow and Malenka, 2002; Nicoll, 2003). It is known that GluA2/3 AMPARs recycle into and out of the postsynaptic membrane constitutively, but GluA1/2 AMPARs are driven into the membrane by synaptic activity (Shi et al., 2001). Additionally, phosphorylation of the C-terminus by various kinases also plays an important role in AMPAR function, trafficking, and localization at the synapse. Specifically, GluA1 phosphorylation at Ser845 enhances synaptic insertion, open probability of the channel, and Ser831 phosphorylation increases channel conductance (Banke et al., 2000; Barria et al., 1997a; Barria et al., 1997b). Dephosphorylation at Ser845, along with phosphorylation at Ser880 can remove AMPARs from the synapse (Chung et al., 2000; Lee et al., 2000). An additional phosphorylation at

Ser818 can modulate plasticity dependent synaptic insertion of AMPAR (Boehm et al., 2006). Recently a new site, Ser567 in the intracellular loop of GluA1, phosphorylated by CaMKII was shown to regulate trafficking of AMPAR to the synapse (Lu et al., 2010). Changes in phosphorylation of the receptor affect AMPAR binding to PDZ domain proteins and are thought to lead to altered receptor localization (Chung et al., 2000). When activated by Glu binding, AMPARs are able to rapidly conduct Na^+ and K^+ ions, leading to a depolarization of the postsynaptic neuron. This depolarization is prerequisite for opening of another glutamate receptor, N-methyl-D-aspartate receptor (NMDAR), which influxes Ca^{2+} required for LTP.

N-methyl-D-aspartate receptor (NMDAR):

NMDARs are voltage-gated and ligand-gated cation channels that play important roles in synaptic plasticity and learning and memory (Bliss and Collingridge, 1993; Morris et al., 1986). Additionally these receptors are crucial in brain development and synaptogenesis (Cull-Candy et al., 2001; Stephenson et al., 2008).

The functional NMDAR has a tetrameric structure assembled from two GluN1 (formally known as NR1) and GluN2 (NR2) subunits. The GluN1 subunit is a product of one gene, and is highly expressed in most neurons of the brain. Alternative splicing of exons 5, 21 and 22 in the pre-mRNA gives rise to 8 splice variants (Durand et al., 1993; Zukin and Bennett, 1995). There are four GluN2 subunits (A-D), products of separate genes with varying expression across development and different brain regions. Another gene product GluN3 exists

mainly in immature brain, in contrast to GluN1 and GluN2 (A and B) subunits, which predominate in adult hippocampus (Cavara and Hollmann, 2008; Cull-Candy et al., 2001; Hollmann et al., 1989). Each subunit of the NMDA receptor contains an extracellular agonist-binding N-terminal domain, three transmembrane domains with an intracellular reentrant loop between transmembrane domains 1 and 2, forming the channel pore, and an intracellular C-terminal domain important for posttranslational modification, trafficking, and protein/protein interactions (Fig 1.3). Functional NMDARs are formed by the association of two obligatory GluN1 subunits, which contain an extracellular

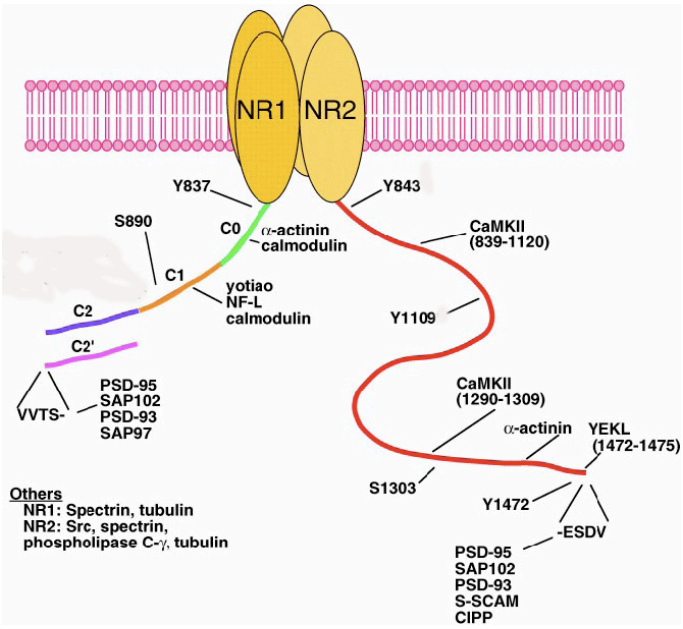

Figure 1.3 Proteins interacting with the NR1 (GluN1) and NR2B (GluN2B) subunits of the NMDAR

The splice variant GluN1-1 (residues 834–938) is illustrated along with an alternatively spliced cassette, C2'. Various phosphorylation sites on the C-tails of both the subunits by different kinases are depicted. Also highlighted are the numerous proteins that bind to the C-tail of both the subunits. The phosphorylation and protein-protein interactions regulate the function, synaptic delivery, endocytosis, and assembly of the NMDA receptors. *Adapted from* (Wenthold et al., 2003)

binding pocket for the co-agonist, glycine and two additional GluN2 subunits, which contain the extracellular glutamate binding domain (Anson et al., 1998; Anson et al., 2000; Banke and Traynelis, 2003; Forrest et al., 1994; Ivanovic et al., 1998; Laube et al., 1998; Monyer et al., 1992).

The PSD localization of NMDAR is dictated through protein–protein interactions. NMDAR subunits associate with a variety of scaffolding proteins, kinases, phosphatases, cytoskeletal and cell-adhesion molecules (Husi et al., 2000). The GluN2A and GluN2B interact with PSD-95 and other members of the membrane associated guanylate kinase (MAGUK) family of scaffolding proteins that are enriched in the PSD. GluN1 subunits along with the MAGUK family of proteins also bind α-actinin, an actin-binding protein enriched in dendritic spines, which can tether NMDAR to the actin cytsokeleton (Dunah et al., 2000; Wyszynski et al., 1998; Wyszynski et al., 1997). Stable localization of NMDA receptors at postsynaptic and extrasynaptic membranes is vital for receptor regulation and neuronal function (Barria and Malinow, 2002; Kohr, 2006; Mohrmann et al., 2002).

NMDAR acts as coincidence detector. In the inactive state, the pore of the channel is blocked by a Mg^{2+} ion, thus depolarization of the postsynaptic membrane is required for removal of the Mg^{2+} block. Coincident binding of Glu to the GluN2 subunits (released upon activation of presynaptic cell), as well as the co-agonist glycine to the GluN1 subunits, is also needed to activate the receptor (Kohr, 2006). Simultaneous binding of four agonist molecules (two glutamate and two glycine) to all four subunits promotes a conformational change of the

receptor allowing ion influx (Dingledine et al., 1999). The NMDAR is permeable to Ca^{2+}, Na^{+} and K^{+}. Ca^{2+} influx via the NMDAR is vital for modulating synaptic plasticity. Compared to other glutamate receptors, NMDAR has relatively slow gating properties, with slow opening and deactivation kinetics. The continued presence of agonist evokes NMDAR desensitization that additionally can be regulated by extracellular glycine, intracellular Ca^{2+} and intracellular proteins like CaMKII, calmodulin, and α-actinin (Krupp et al., 1999; Sessoms-Sikes et al., 2005; Zhang et al., 1998). The time course of receptor deactivation is much longer than the half-life of glutamate in the synaptic cleft, allowing prolongation of synaptic currents after neurotransmitter is cleared from the synapse. The specific parameters of NMDAR function in neurons depend on differential subunit expression and alternative splicing (Cull-Candy et al., 2001; Dingledine et al., 1999).

Calcium Signaling:

General Overview:

Eukaryotic cells adapt to the changing environments through a coordinated myriad of molecular activities. This requires intracellular communication possible through complex signaling pathways. Many of these pathways rely upon diffusible signals called second messengers to communicate across the cell. Of the many second messengers, the ubiquitous cation, Ca^{2+} essentially supports many biological processes involved in the adaptation of cells to ever changing environments. It is important in the basic operation of neurons, vesicle fusion,

neurotransmitter synthesis, gene transcription, cell cycle progression, apoptosis, and muscle physiology.

The basal intracellular concentration of Ca^{2+} in most mammalian cells is in the range of 10-50 nM, and stimulated concentrations can range from 500-1000 nM or more in subcellular compartments. The levels of intracellular Ca^{2+} can be controlled by a balance between "ON" and "OFF" processes (Berridge et al., 2000). The "ON" reactions increase the cytosolic Ca^{2+} and the "OFF" reactions remove Ca^{2+} from the cytosol. Cells have evolved tools like channels on the plasma membrane that regulate the "ON" process by influxing Ca^{2+} from the extracellular space. There are also channels that release Ca^{2+} from the intracellular stores like the endoplasmic or sarcoplasmic reticulum (Berridge et al., 2003). Cells have similarly employed various mechanisms to regulate the "OFF" process. These include Ca^{2+} -ATPases, Na/Ca^{2+} exchangers, and fast-acting Ca^{2+} buffering proteins such as calbindin, calretinin, and parvalbumin. Abnormality in these well-orchestrated changes in Ca^{2+} can lead to pathophysiology such as excitotoxicity, ischemic damage, heart failure, hypertension, diabetes, and Alzheimer's disease (Berridge et al., 2003; Berridge et al., 2000). Although, most of the tissues in the body exploit Ca^{2+}-signaling, this dissertation will emphasize Ca^{2+} signaling in neurons.

Calcium Signaling in neurons:

Changes in the intracellular concentration of Ca^{2+} in neurons are involved in neurite growth, development, and remodeling, regulation of neuronal excitability, increases and decreases in the strength of synaptic connections that underlie

learning and memory, and the activation of survival and programmed cell death pathways (Berridge, 1998; Berridge et al., 2000; Ghosh and Greenberg, 1995).

Ca^{2+} channels at the membranes of neurons can determine not only their spontaneous activity but also their responsiveness to stimuli. In addition, Ca^{2+} is a trigger for neurotransmitter release that is essential for transmission of impulses at chemical synapses. The efficacy of synaptic transmission can be altered, in a Ca^{2+}-dependent manner, by certain intensities and patterns of stimulation. Enhancements and reductions of synaptic transmission efficacy are the hallmarks of LTP and LTD respectively, the major forms of synaptic plasticity (see Fig 1.1C). The effects of Ca^{2+} on neurotransmission can be mediated by changes in a plethora of processes, including the synthesis and release of neurotransmitters, the abundance or modification of synaptic proteins, and changes in neuronal cytoskeleton. Consolidation of learning and memory is also thought to involve activation of gene expression by Ca^{2+}.

Calmodulin:

Although Ca^{2+} can directly interact with and modulate the function of many proteins, most of its intracellular effects are mediated by the ubiquitous Ca^{2+} sensor, calmodulin (CaM). CaM belongs to the EF-hand family of Ca^{2+}-binding proteins. It is a 17kDa protein and is highly conserved throughout all species. Structurally, CaM is made up of two globular Ca^{2+}-binding domains (lobes) connected by a flexible linker (Meador et al., 1992, 1993). Each of the lobes can bind up to two Ca^{2+} ions (Chin and Means, 2000). Thus, depending on the intracellular Ca^{2+} concentration, CaM may bind one, two, three, or four Ca^{2+} ions

simultaneously. The C-lobe has higher affinity for Ca^{2+} with faster dissociation kinetics than the N-lobe (Xia and Storm, 2005). Binding of Ca^{2+} to each lobe also exhibits positive cooperativity, in that; Ca^{2+} binding to one site increases the affinity of other site for Ca^{2+}. These properties place CaM as a unique decoder of Ca^{2+} signals to initiate the appropriate intracellular responses.

Although CaM can interact with some proteins in the absence of Ca^{2+}(apo-CaM), binding of Ca^{2+} causes a structural change that exposes hydrophobic surfaces necessary for interaction with most CaM targets (Hoeflich and Ikura,

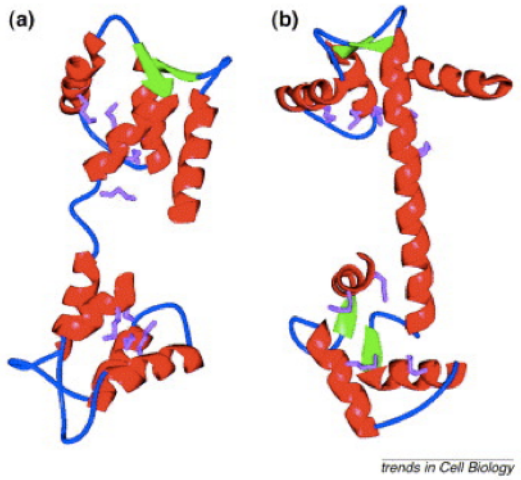

Figure 1.4 Structure of apo and Ca^{2+}-bound calmodulin (CaM)

The main chain structure of apo **(a)** and $Ca^{2+}{}_4$-bound CaM **(b)** show the Ca^{2+}-regulated conformational change in a CaM molecule. Highlighted in purple are the methionine residues that denote the hydrophobic pocket in each lobe of CaM. Ca^{2+} binding in each lobe results in large changes in helices of both the domains exposing the otherwise buried hydrophobic residues to interact with the target protein. *Adapted from* (Chin and Means, 2000)

2002) (Fig 1.4). In most cases, CaM binds to an α-helix within the target protein, essentially wrapping around the helix, such that the exposed hydrophobic regions of each lobe make direct contact with opposite sides of the α-helix in the

target proteins (Crivici and Ikura, 1995).

Binding to the target proteins also changes the affinity of CaM for Ca^{2+} and sensitizes the CaM-effector complex to the changes in Ca^{2+}-concentration (Chin and Means, 2000). Ca^{2+}/CaM complex can thus modulate activity and function of various classes of proteins and enzymes such as adenylate cyclases, phosphodiesterases, protein kinases, the protein phosphatase calcineurin, ion channels, and ryanodine receptors.

CaM Kinases:

Protein kinases are a prominent class of ~500 proteins that alter cellular function by phosphorylation of other proteins at Ser, Thr, or Tyr residues, a process that is specific, rapid, and reversible. Several of these kinases utilize CaM as a Ca^{2+} sensor. The CaM-dependent protein kinases (CaMKs) are well-recognized effectors of CaM. The CaMKs are serine/threonine (Ser/Thr) kinases, denoting their specificities for phosphorylation of Ser or Thr residues in substrate proteins. CaMKs are critical for proper cellular function as they influence a variety of processes such as gene transcription, metabolism, motor function, cell survival/death, cytoskeletal reorganization, and learning and memory (Soderling and Stull, 2001; Swulius and Waxham, 2008). Although the CaMKs vary in expression level, subcellular localization, and substrate specificity, they have similar domain organizations (Fig 1.5). They contain an autoinhibitory domain (AID) that interferes with the catalysis by blocking the catalytic site. Ca^{2+}/CaM binding to a domain (CaM-binding domain, CBD) that overlaps with the AID relieves this inhibition, thus activating the kinase. The kinases are further

regulated by phosphorylation - either autophosphorylation or phosphorylation by other kinase (Soderling and Stull, 2001).

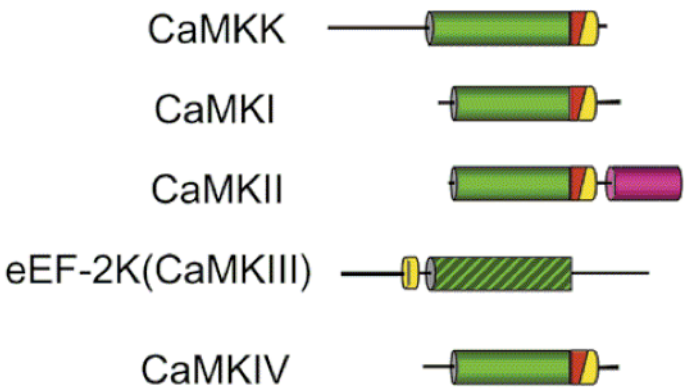

Figure 1.5 Schematic of CaMK domain organization

The domains shown are: catalytic domain, dark green; autoinhibitory domain (AID), red; Ca^{2+}/CaM-binding domains (CBD), yellow; association domain of CaM-KII, magenta. The catalytic core of eEF-2K, formerly known as CaMKIII, bears no homology to other CaMKs and hence is represented with black hatching. The different isoforms for some CaMKs are not shown. *Modified from* (Soderling and Stull, 2001)

CaMKK:

CaMK kinase (CaMKK), an upstream member of the CaMK cascade, and originally characterized as an "activating factor" from brain extracts can dramatically increase the activity of CaMKI and CaMKIV (Soderling, 1999). In the cell CaMKK resides in both the cytosol and nucleus where it is poised to respond to changing Ca^{2+} concentrations. Two isoforms, α and β, have been cloned, and have similar domain organizations as other CaMKs (Tokumitsu et al., 1995). Monomeric in nature, CaMKK has a catalytic domain followed by the AID and CBD (Tokumitsu et al., 1997). Although CaMKKs contain the AID and CBD, the catalytic domain has two unique properties: it lacks the acidic residues present in

other CaMKs to recognize basic residues in the substrate and they have an unusual Arg-Pro (RP) rich insert (Tokumitsu et al., 1999). Thus, instead of recognizing the primary sequence consisting of basic residues for phosphorylation, CaMKK utilizes the RP-insert for substrate recognition. Besides the presence of RP-insert, Ca^{2+}/CaM binding to both the CaMKK and its substrate (CaMKI or IV) is required for phosphorylation to occur. CaMKK can be regulated by protein kinase A (PKA), which inhibits CaMKK by phosphorylating Ser458 in the CBD of CaMKK (Wayman et al., 1997).

Transgenic and knockout animal studies have implicated CaMKK in learning and memory. For example, deletion of CaMKKβ affects hippocampus-dependent long-term memory (Peters et al., 2003), whereas CaMKKα-deficient mice displayed deficits in fear conditioning (Blaeser et al., 2006).

CaMKI:

CaMKI is a ubiquitous and monomeric cytoplasmic enzyme that is enriched in brain, liver and intestine. The 42 kDa protein is expressed from three genes encoding α, β, and γ isoforms with alternative splicing to form β1 and β2 (Soderling and Stull, 2001). The best-characterized substrates of CaMKI are synapsin 1 and the cAMP response element binding protein (CREB) (Nairn and Greengard, 1987; Sheng et al., 1991). Through synapsin 1 phosphorylation, CaMKI regulates vesicle mobility in the presynaptic terminal and thus neurotransmitter release. CREB phosphorylation by CaMKI facilitates binding of transcription factor to cAMP response element (CRE) and regulates CREB-dependent gene transcription. CaMKI was the first member of the CaMK family to

have a structure solved by X-ray diffraction (Goldberg et al., 1996). Like other protein kinases it has a bilobal catalytic domain containing the ATP- and substrate- binding site. In an inhibited state the AID contacts the catalytic domain and distorts the ATP-binding site (Goldberg et al., 1996). Interaction of Ca^{2+}/CaM with the CBD induces conformational changes that allows for ATP binding and kinase activation. CaMKK-dependent phosphorylation of Thr177 in the activation loop of CaMKI further increases its activity 10-20 fold (Haribabu et al., 1995). However, for this Ca^{2+}/CaM must be bound to both the CaMKI and CaMKK, perhaps permitting synergism in kinase activation. CaMKI is implicated both in dendritic and axonal growth during neuronal development, as CaMKI knockout animals show defects in axonal elongation (Ageta-Ishihara et al., 2009; Neal et al., 2010; Takemoto-Kimura et al., 2007).

CaMKIV:

The α splice variant of CaMKIV is expressed largely in neuronal tissues, testis, and T cells, whereas the β splice variant is expressed in the cerebellum during development (Soderling and Stull, 2001). Like CaMKI and CaMKK, CaMKIV is monomeric with similar domain organization. It is also activated by the multistep process of Ca^{2+}/CaM binding and CaMKK-dependent phosphorylation of Thr196 in the activation loop. However, unlike CaMKI, once phosphorylated by CaMKK, CaMKIV can undergo autophosphorylation in the Ser/Thr rich N-terminus (Okuno et al., 1995). This event not only maximally activates CaMKIV, but also generates Ca^{2+}/CaM-independent activity that allows the kinase to retain functionality beyond the Ca^{2+} transient. Additionally CaMKIV can autophosphorylate at

Ser332 in the CBD, which prevents subsequent Ca^{2+}/CaM binding until this site is dephosphorylated by a phosphatase (Watanabe et al., 1996). CaMKIV is found both in cytosol and nucleus where it is poised to regulate Ca^{2+}-dependent gene transcription via phosphorylation of various transcription factors (e.g. CREB, Serum Response Factor, and histone deacetylase 4). In addition to regulating gene transcription through CREB, CaMKIV also phosphorylates CREB-binding protein (CBP), which is a co-activator of CREB (Enslen et al., 1994; Impey et al., 2002). Protein phosphatase, PP2A, forms a signaling complex with CaMKIV, negatively regulating CaMKIV by dephosphorylation. This complex is a self-regulating prototype for kinase-phosphatase cross-talk in cell signaling (Anderson et al., 2004). In cytosol CaMKIV phosphorylates oncoprotein18 preventing its association with tubulin, thus modulating microtubule assembly (Marklund et al., 1994).

CaMKIV knockout mice show impairments in CREB activation, hippocampal LTP induction, and cerebellar LTD (Ho et al., 2000; Ribar et al., 2000). Overexpression of a dominant-negative form of CaMKIV in the mouse forebrain impaired LTP due to attenuation in CREB phosphorylation (Kang et al., 2001). In contrast, transgenic mice overexpressing CaMKIV in the forebrain showed enhancements in LTP and long-term memory (Steenland et al., 2010; Wu et al., 2008).

CaMKII:

CaMKII is the most intensely investigated member of the CaMK family and is also the focus of this dissertation. Although not a component of the CaMK

cascade, CaMKII plays an equally integral role in translating intracellular Ca^{2+} signals to a variety of downstream targets. It was first found in 1980 by gel filtration of CaM-dependent protein kinases of rat brain (Yamauchi and Fujisawa, 1980). It is implicated in regulation of diverse physiological processes such as neurotransmitter synthesis and release, cytoskeletal organization, intracellular Ca^{2+} homeostasis, gene transcription, synaptic plasticity, exocytosis, and metabolism (Colbran et al., 1989). Four separate genes encode the α, β, γ and δ isoforms of CaMKII, varying in molecular weight from 50-60KDa (Colbran and Soderling, 1990). These isoforms are also subjected to alternative splicing resulting in more than 20 variants of the kinase, showing distinct cellular and tissue distribution. The α and β isoforms are brain specific and comprise approximately 1% of total brain protein and up to 2% of total protein in hippocampus (Cheng et al., 2006; Kennedy et al., 1983). The γ and δ isoforms are present throughout the body at much lower concentrations (Tobimatsu and Fujisawa, 1989). The overall subunit domain organization is similar to other CaMKs, however, the striking difference between CaMKII and other CaMKs is the extended C-terminus that is involved in CaMKII subunit association to form a holoenzyme consisting of twelve subunits (Fig. 1.6 A). This dodecameric structure of CaMKII is compact and arranged as two stacked hexameric rings as determined by low resolution transmission electron microscopy and recently an atomic resolution structure (Chao et al., 2011; Kanaseki et al., 1991; Kolodziej et al., 2000) (Figs. 1.6C and1.8). The C-terminal association domains of each subunit combine to form the central core of each ring with the N-terminal catalytic

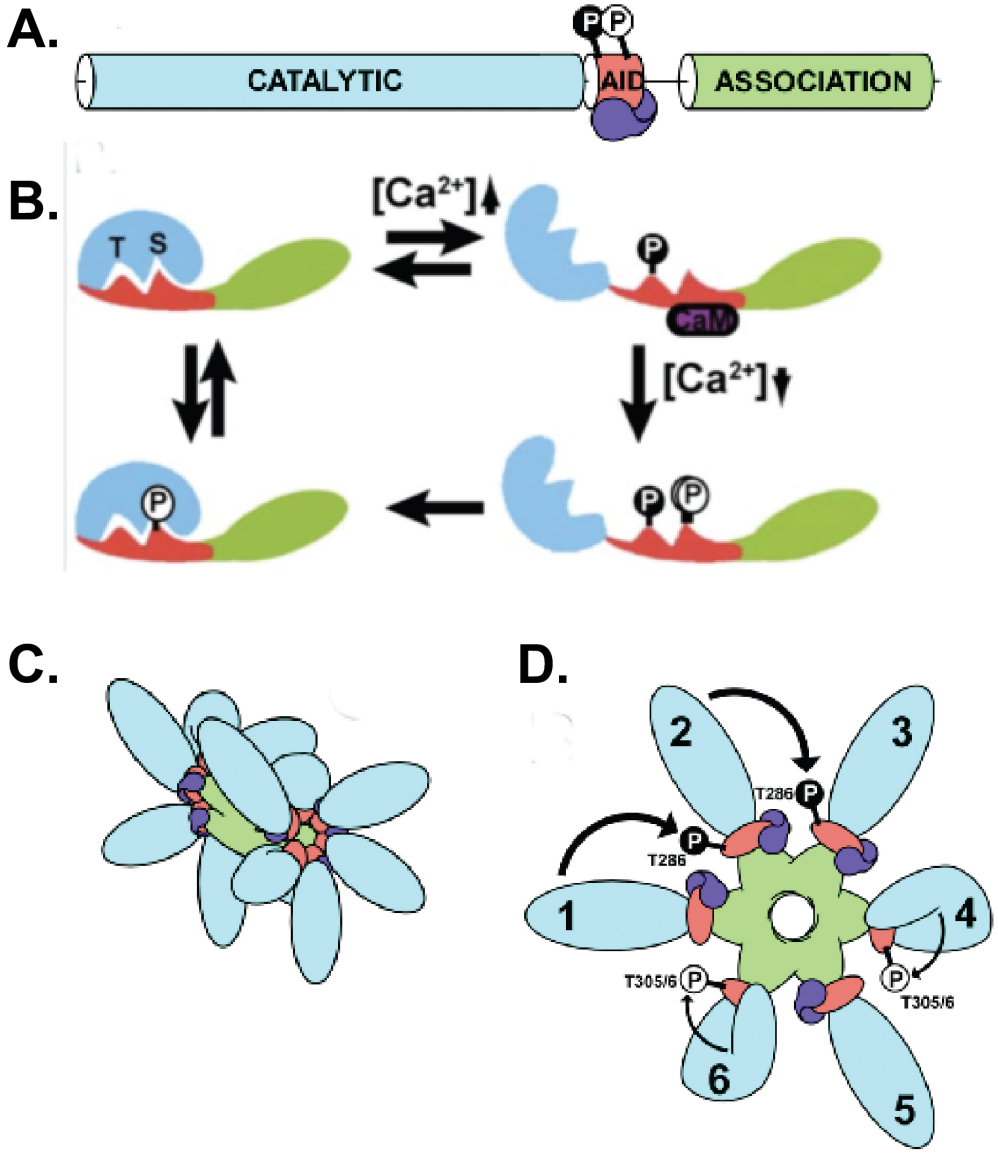

Figure 1.7 Atomic resolution structures of CaMKII catalytic domain in different states

A. Structure of the kinase and the regulatory domain of CaMKIIδ in autoinhibited form (PDB: 2VN9) (Rellos et al., 2010). Catalytic domain shown in blue reveals classic bilobal kinase architecture with the regulatory domain (red) making contacts with the catalytic domain thereby keeping the kinase autoinhibited. The CaM-binding domain is highlighted in purple. Shown are the three autophosphorylation sites, Thr286 (black), Thr305 and Thr306 (orange). **B.** Crystal structure of CaMKII bound to Ca^{2+}/CaM (PDB: 2WEL) (Rellos et al., 2010). Binding of Ca^{2+}/CaM (yellow, Ca^{2+} ions in green) induces helical conformation of the regulatory domain thereby disrupting contacts between the catalytic and the regulatory domain. The structure is cleaved at the end of the catalytic domain and is separated by unresolved data. **C.** Structure of CaMKII as an enzyme-substrate complex (PDB: 3KK8) (Chao et al., 2010a) depicting trans-autophosphorylation at Thr286 (black). The regulatory segment of the substrate "kinase" (light blue) is presented in the catalytic cleft of the neighboring "enzyme" kinase (dark blue) for phosphorylation.

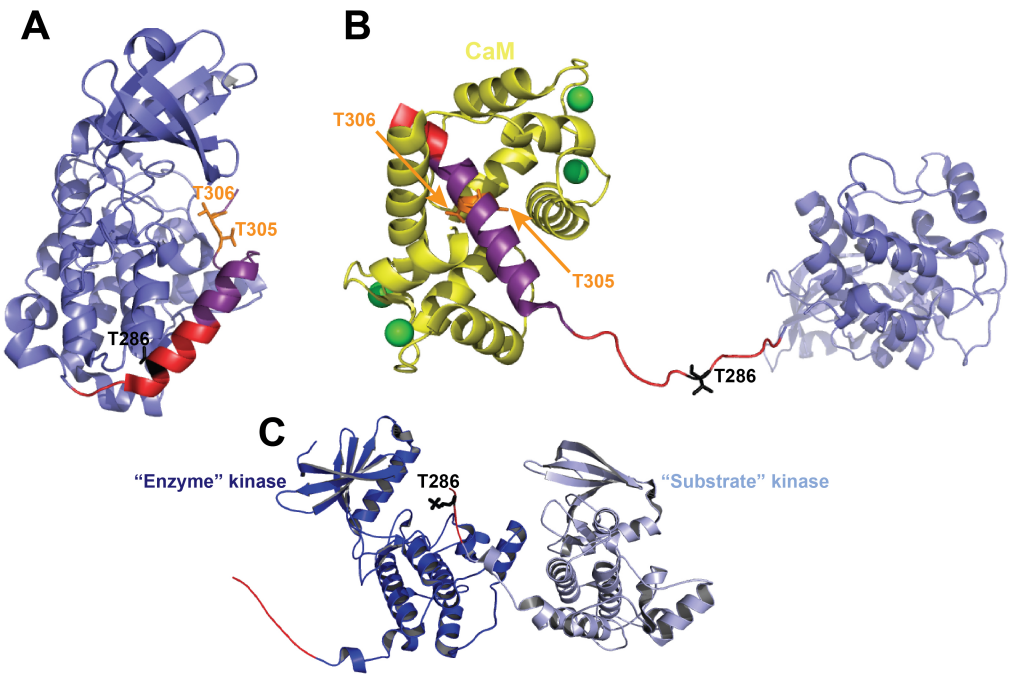

Figure 1.6 CaMKII domain architecture and regulation

A. Domain organization of CaMKII isoforms showing the N-terminal catalytic domain (blue), autoinhibitory domain (AID, red), and a C-terminal association domain (green). The regulatory domain contains a CaM-binding motif (purple) as well as Ca^{2+}/CaM-dependent (Thr286, black circle) and Ca^{2+}-independent (Thr305/6, white circle) autophosphorylation sites. **B.** Mechanism of CaMKII regulation by Ca^{2+}/CaM binding and autophosphorylation (see main text for details). T and S denote the T-site (region in the catalytic domain where Thr286 docks) and S-site (classical substrate binding site) **C.** Physiologically CaMKII exist as a stacked pair of the hexameric rings. **D.** A single hexameric ring showing the intersubunit (Thr286) and intrasubunit (Thr305/6) autophosphorylation mechanism of CaMKII. *Adapted and modified from* (Colbran, 2004)

domains extending radially outwards (Figs. 1. 6D, 1.8 A and B). Inside the cell, subunits of CaMKII can mix to form heteromeric CaMKII holoenzymes composed of varying ratios of different isoforms (Bennett et al., 1983; Miller and Kennedy, 1985), adding to the complexity of structural configurations and diversity of its biological functions.

Like other CaMKs, CaMKII initially exists in a quiescent state as the AID

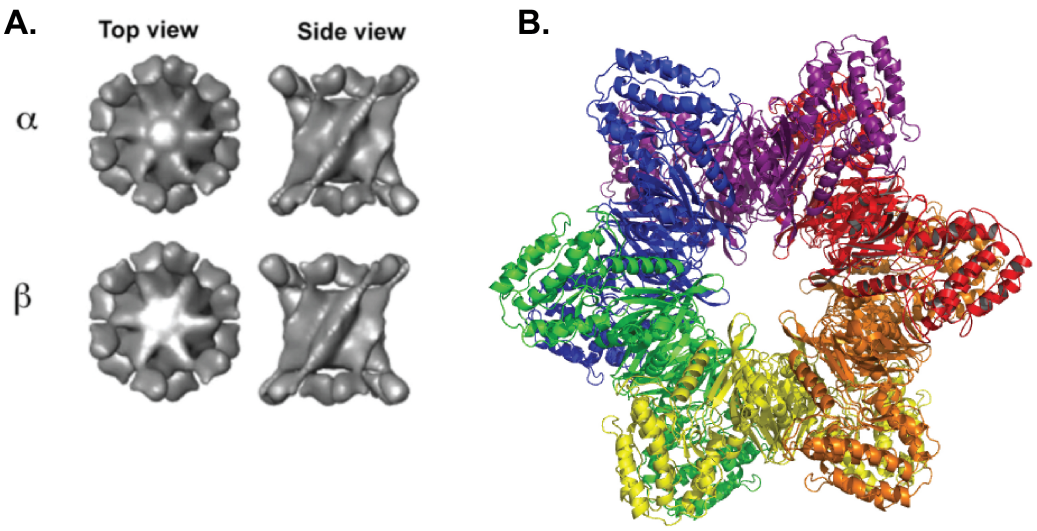

Figure 1.8 CaMKII holoenzyme structures

A. Top and side views of the 3D-reconstruction of cryo EM images of CaMKIIα and β showing the dodecameric structures (Gaertner et al., 2004). **B.** Crystal structure of the CaMKII holoenzyme (PDB: 3SOA) (Chao et al., 2011), showing resemblance to the 3D-reconstruction in A.

blocks the catalytic activity. Structures of the isolated kinase domain of various CaMKII isoforms (Rellos et al., 2010) show how the regulatory segment (red in Fig 1.7A) makes extensive contacts with the kinase domain thereby controlling kinase activity (Fig. 1.7A). Ca^{2+}/CaM binding to a region overlapping with the AID (purple in Fig. 1.7A) relieves the block exposing the catalytic and the nucleotide-binding site (Figs. 1.6B and 1.7B). Interestingly, the sensitivity of CaMKII to Ca^{2+}/CaM activation is dictated by the subunit composition (Brocke et al., 1999).

The unique holoenzyme structure of CaMKII endows it with a regulatory mechanism for sensing and transducing the intracellular Ca^{2+} signals. Ca^{2+}/CaM can independently activate each subunit in a holoenzyme. When adjacent subunits bind Ca^{2+}/CaM, they can transautophosphorylate at Thr286 (Bradshaw

et al., 2002; Chao et al., 2010a; Hanson et al., 1994) as shown in Figs. 1.6D and 1.7C. This modification of CaMKII has important regulatory consequences: 1) it increases the affinity of CaMKII for Ca^{2+}/CaM by more than 1000-fold, known as CaM-trapping (Meyer et al., 1992), and 2) it generates the autonomous state of CaMKII, meaning CaMKII retains partial Ca^{2+}-independent activity after dissociation of Ca^{2+}/CaM (Ohsako et al., 1991). Because of this unique property of CaMKII to remain activated beyond the time frame of a Ca^{2+} transient that initially switched on the molecule, CaMKII is referred to as “memory molecule” (Griffith, 2004; Lisman, 1985; Mullasseril et al., 2007). The prolonged response to transient elevation of intracellular Ca^{2+} through constitutive activity of autophosphorylated CaMKII is critical for certain physiological functions of CaMKII (Giese et al., 1998; Gustin et al., 2011). This is pertinent in brain regions where frequency of synaptic stimulation can alter the efficiency of synaptic transmission at glutamatergic synapses.

The self-regulated mechanism of CaMKII also includes autophosphorylation at residues Thr305 and/or Thr306, in the CBD (Hanson and Schulman, 1992). Slow autophosphorylation at Thr306 can occur in the basal state blocking Ca^{2+}/CaM binding and hence rendering the kinase insensitive to Ca^{2+} transients (basal autophosphorylation) (Colbran, 1993). Alternatively, removal of Ca^{2+}/CaM from Thr286 autophosphorylated CaMKII (autonomously active CaMKII) promotes rapid autophosphorylation at either Thr305 or Thr306 (sequential autophosphorylation), favoring kinase inhibition upon dephosphorylation of Thr286 (Hashimoto et al., 1987). Autophosphorylation at

Thr305/6 regulates synaptic plasticity by modulating CaMKII localization, and/or activity in neurons (Elgersma et al., 2002; Pi et al., 2010a; Pi et al., 2010b).

Methionine residues in the catalytic/regulatory domain of CaMKII can also be oxidized and this imparts Ca^{2+}-independent activity to the kinase, leading to apoptosis in cardiomyocytes (Erickson et al., 2008). Not much is known about CaMKII oxidation in neurons, although exposure of the neuronal isoform CaMKIIα to the oxidizing reagent, hydrogen peroxide, also imparts Ca^{2+}-independent activity suggesting that CaMKIIα can be oxidized (Nidhi Jalan-Sakrikar and Roger J. Colbran, unpublished observations).

In vivo roles of CaMKII:

There are at least 40-50 known substrates of CaMKII *in vitro*, and many of these may be involved in physiological functions. In the presynaptic terminals of a neuron, CaMKII phosphorylates synapsin1, thereby decreasing its affinity for synaptic vesicles and facilitating exocytosis (Colbran et al., 1989). In dendrites, MAP-2 (microtubule-associated protein 2) is phosphorylated by CaMKII, which inhibits MAP-2 stimulated microtubule assembly (Colbran and Soderling, 1990). CaMKII has also been implicated as a physiological catalyst for phosphorylation and activation of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis. In the heart CaMKII functions to regulate Ca^{2+} dynamics by modulating Ca^{2+} influx through L-type Ca^{2+} channels (Ca^{2+}-dependent facilitation), phosphorylating phospholamban to regulate Ca^{2+} uptake by Ca^{2+}-ATPase, phosphorylating ryanodine receptors to regulate SR Ca^{2+} release (Grueter et al., 2007). CaMKII inhibition has protective effects against cardiac hypertrophy in

response to adrenergic stimulation and myocardial infarction (Zhang et al., 2005).

Postsynaptic CaMKII enhances excitatory synaptic transmission through phosphorylation of Ser831 in the GluA1 subunit of the AMPAR (see AMPAR above). Phosphorylation by CaMKII increases single channel conductance of AMPAR. CaMKII also increases the number of AMPARs by facilitating the incorporation of new receptor subunits into the postsynaptic membrane (Benke et al., 1998; Derkach et al., 1999; Hayashi et al., 2000). Mice lacking GluA1 or harboring mutations in CaMKII (see below) show deficits in LTP, supporting prominent roles for CaMKII and AMPAR in LTP at CA1 synapses of hippocampus (Giese et al., 1998; Zamanillo et al., 1999). Several other PSD proteins are good substrates of CaMKII. CaMKII can phosphorylate the GluN2B subunit of NMDAR at Ser1303 (Omkumar et al., 1996), which enhances NMDAR desensitization ((Sessoms-Sikes et al., 2005), Suzanne Sessoms-Sikes and Roger Colbran, unpublished observations). Other known substrates of CaMKII involved in dendritic spine regulation include SynGAP, nNOS, SAP-97, kalirin-7, β2a subunit of the Ca^{2+} channels (Abiria and Colbran, 2010; Chen et al., 1998; Nikandrova et al., 2010; Rameau et al., 2004; Xie et al., 2008).

CaMKIIα null mice exhibited reduced hippocampal LTP and hippocampus-dependent learning (Silva et al., 1992a; Silva et al., 1992b). In mice, the knock-in mutation of Thr286 to Ala to prevent autophosphorylation resulted in a significant reduction of NMDAR-dependent hippocampal LTP and spatial learning (Giese et al., 1998). A similar effect was observed in mice harboring a knock-in mutation mimicking autophosphorylation of the inhibitory Thr305 site (T305D). In contrast,

a double knock-in mutation of Thr305/306 to prevent inhibitory autophosphorylation (TT305/306VA) resulted in enhanced hippocampal-dependent learning and a decreased threshold for LTP induction (Elgersma et al., 2002). Since mutations at autophosphorylation sites affect Ca^{2+}-independent activity, binding to CaM, and other proteins, (see below) as well as kinase localization, it remains unclear which aspects of kinase function play critical roles in learning. Recently, a new autophosphorylation site in CaMKII has been discovered, Thr253. Phosphorylation at this site does not alter CaMKII activity, unlike Thr286 and Thr305/6, but appears to alter CaMKII targeting to the PSD and interactions with binding partners (Migues et al., 2006; Skelding et al., 2010). Although Thr253 phosphorylation occurs *in vivo*, the importance of this phosphorylation is yet to be demonstrated.

Imbalances in CaMKII autophosphorylation have been linked to altered synaptic plasticity and neuronal dysfunction in disease models. For example, increased phosphorylation at both Thr286 and Thr305/306, and defects in hippocampal LTP and learning were observed in a mouse model of Angelman's mental retardation syndrome (AS) (Weeber et al., 2003). AS is a severe neuro-genetic disorder caused by deletion or inactivation of UBE3A gene encoding E6-AP ubiquitin ligase (Matsuura et al., 1997; Sutcliffe et al., 1997). A functional link between E6-AP and CaMKII remains unclear. However, the reduction in CaMKII activity and localization at the PSD are believed to contribute to molecular mechanisms underlying this disorder, given that the mutation of inhibitory phosphorylation sites (TT305/306VA) rescued the neurological deficits in AS

mice (van Woerden et al., 2007). The rescue of AS phenotypes by enhancing CaMKII actions at the synapse may result from CaMKII-dependent enhancement of AMPAR function at the synapses (reviewed in (Kessels and Malinow, 2009)). Alternatively, the recent findings by (Bingol et al., 2010) suggest that another effect of increasing PSD association of CaMKII may be to increase the degradation of synaptic proteins like Arc by targeting the ubiquitin-proteasome system to the synapse. Arc is synaptic protein, which promotes endocytosis of AMPAR (Greer et al., 2010). Ube3A regulates synaptic levels of Arc; in the Ube3A knockout mice there is accumulation of Arc, resulting in reduced synaptic AMPAR levels, accompanied by defects in synaptic transmission (Greer et al., 2010). Since the TT305/6DD mutation of CaMKIIα prevents activity-dependent translocation of CaMKIIα itself (Shen and Meyer, 1999) and also of proteasomes (Bingol et al., 2010), one might hypothesize that the mutation of the Thr305/6 sites to Val/Ala to prevent autophosphorylation can increase synaptic targeting of proteasomes and thereby increase the degradation of “negative regulators” of synaptic plasticity (like Arc). Thus, CaMKIIα may rescue synaptic transmission in AS mice by synergistically increasing the surface expression and function of AMPARs, and by promoting degradation of Arc to limit AMPAR internalization.

In a rat model of Parkinson’s disease, where dopamine is depleted in the nigrostriatal neurons, Thr286 phosphorylation of CaMKII is increased without any change in protein levels (Brown et al., 2005; Picconi et al., 2004). Moreover, in the transgenic mouse model of Alzheimer disease, CaMKII subcellular localization is changed resulting in reduced synaptic pools of CaMKII, which

further causes loss in synaptic AMPARs (Gu et al., 2009). Studies have also suggested the autonomous activity of CaMKII as a drug target for neuroprotection post cerebral ischemia *in* a mouse model of stroke or glutamate excitotoxicity in neuronal cultures (Ashpole and Hudmon, 2011; Vest et al., 2010).

Taken together, these findings emphasize an essential role for CaMKII in the physiology and pathology of the brain. As indicated above, each of the observed phenotypes was due to changes in Ca^{2+}-dependent or -independent activity and/or subcellular localization of CaMKII. Hence, the specific subcellular localization of CaMKII must be coordinated with the Ca^{2+} signals in order to elicit the appropriate response. Synaptic activation stimulates differential magnitudes and dynamics of Ca^{2+} changes in dendritic shafts and spines (Sabatini et al., 2002; Yuste et al., 2000). A great deal of evidence exists to support the idea that proper targeting of CaMKII plays an essential role in regulating its functions.

CaMKII Targeting:

Signal transduction occurs in dimensions of time and space. The intracellular response to the extracellular signals involves many broad substrate-specific kinases and protein phosphatases. Hence it is important to spatially organize these signaling molecules to evoke functional specificity in response to the extracellular cues. Many enzymes are targeted to the specific location through protein-protein interactions. Proteins that function to localize the enzymes are called anchoring proteins. These proteins can convey signal specificity by binding both the enzyme and its substrates, hence localizing them in each other's vicinity. Targeting proteins can also localize the enzymes close to the source of the

activating second messenger signal (e.g. Ca^{2+} channels). One of the most well studied classes of anchoring proteins is the A Kinase Anchoring Protein (AKAP) family, that serves to anchor protein kinase A (PKA) and often other proteins to diverse subcellular localization (Pawson and Scott, 2010; Sim and Scott, 1999).

Multifunctional CaM kinases need to coordinate the responses to Ca^{2+} stimuli by phosphorylation of various substrates in diverse cellular localizations. Targeting of CaMKII can have diverse effects on signaling: 1) by localizing the kinase to various sites of Ca^{2+} signals for proper response, 2) by localizing the kinase to specific substrates, thus effectively directing kinase activity, and 3) by localizing the kinase close to or away from phosphatases, thus modulating CaMKII autophosphorylation and associated kinase activity (Colbran, 2004).

The identity of CaMKII isoform and splice variants plays a role in modulating subcellular localization. Splice variants of α and δ CaMKII (αB and δB) contain a nuclear localization signal (NLS) and proper nuclear targeting of δB-CaMKII is essential for Ca^{2+}-dependent gene expression in cardiac myocytes (Ramirez et al., 1997). Nuclear and non-nuclear isoforms (like CaMKIIβ) can co-assemble when co-expressed, and recruit the normally non-nuclear isoform to the nucleus. The nuclear CaMKIIα isoform (αB) has restricted regional distribution in the brain (Brocke et al., 1995). Nuclear targeting of this splice variant is regulated by phosphorylation of a Ser that is adjacent to the NLS. Phosphorylation by CaMKI or CaMKIV, or introduction of a negatively charged residue at this position blocked nuclear targeting of αB (Heist et al., 1998). αKAP, another splice variant of CaMKIIα serves as an anchoring protein because the

catalytic domain is replaced by a hydrophobic anchoring sequence. With the full association domain it can still stably integrate into CaMKII holoenzymes, possibly with subunits containing normal kinase domain. It is predominantly expressed in skeletal muscle and can target CaMKII heteromers to the membrane of the SR (Bayer et al., 1998; Bayer et al., 1996).

Some CaMKIIβ isoforms have an F-actin binding domain, which specifically allows for direct association with the actin cytoskeleton. This interaction occurs in the basal state and is antagonized by Ca^{2+}/CaM binding to CaMKIIβ (Shen et al., 1998). Targeting domains of γ and δ isoforms also differentially influence CaMKII co-localization with filamentous actin (Caran et al., 2001).

In addition to isoform dictated targeting, CaMKII exhibits a dynamic translocation dependent on activation of NMDARs (Shen and Meyer, 1999). Early studies showed enrichment of CaMKII in PSD fractions, as much as 20-50% of the total protein in isolated PSDs (Kelly et al., 1984; Kennedy et al., 1983). Such high concentration of a protein for a purely enzymatic function was not reasonable, suggesting that CaMKII may play a structural role at the PSD. Immuno-electron microscopy studies indicated that the amount of CaMKII in PSDs is variable, and can be influenced by post-mortem accumulation. Thus, CaMKII is likely to be only 2-10% of the total protein in PSDs under physiological conditions (Fukunaga et al., 1988; Ouimet et al., 1984; Strack et al., 1997; Suzuki et al., 1994).

Although isolated PSDs contain substantial amounts of CaMKII, they

retain the capacity to bind additional CaMKII in a saturable manner dependent on Thr286 autophosphorylation. Furthermore, pharmacological agents that lead to an enhancement in CaMKII activity/autophosphorylation in hippocampal slices resulted in up to a 2.2-fold increase in the PSD-associated CaMKIIα and CaMKII β (Merrill et al., 2005; Strack et al., 1997; Yamauchi and Yoshimura, 1998). Neuronal depolarization or glutamate application induces a ~2-fold thickening of the PSDs and a 5-fold enhancement in CaMKII immunogold labeling, both of which were reversible upon glutamate removal (Dosemeci et al., 2001). Some studies also suggest that accumulation of CaMKII at the PSDs is partially due to formation of CaMKII "clusters" (Dosemeci et al., 2000; Tao-Cheng et al., 2002; Tao-Cheng et al., 2001), and may be a physiological correlate of CaMKII holoenzyme self- association seen *in vitro* (Hudmon et al., 1996; Hudmon et al., 2001). Elegant studies using GFP-tagged CaMKII in cultured hippocampal neurons revealed a more detailed temporal picture of CaMKII translocation and association with the PSD (Bayer et al., 2006; Shen and Meyer, 1999). GFP-CaMKII showed cytosolic distribution under basal conditions, but becomes punctate and co-localizes with the PSD within 20s of glutamate application. These changes in CaMKII properties were dependent on activation of NMDA receptors and CaM binding. To simulate more physiological synaptic release, localized glutamate puffs or high frequency stimulus to induce LTP were applied; these alterations also induced GFP-CaMKII translocation (Shen et al., 2000). Agonist removal caused dissociation of GFP-CaMKIIα over a time course of few minutes; dissociation was accelerated by mutation of Thr286 to Ala whereas it

was prolonged by mutation of Thr286 to Asp or by inhibition of protein phosphatase (Shen and Meyer, 1999; Shen et al., 2000). Not only does Thr286 regulate PSD association of CaMKII, but autophosphorylation at Thr305/6 also regulated the interaction of CaMKII with PSD. Double Ala mutations at Thr305 and Thr306 resulted in slower dissociation of GFP-CaMKIIα from the PSD(Shen et al., 2000), suggesting that phosphorylation at these sites destabilizes CaMKII from the PSD. Consistent with these observations, transgenic knock-in mice harboring Val/Ala mutation at Thr305/6 have higher levels of CaMKII in the PSD, compared to mice harboring Asp mutation at Thr305 that shows decreased association with the PSD (Elgersma et al., 2002). Also as mentioned above, a mouse model of Angelman Syndrome has low levels of CaMKII at the PSD associated with increased levels of Thr305/6 autophosphorylation (Weeber et al., 2003).

Hence a major role of Thr305/6 autophosphorylation is to promote CaMKII dissociation from the PSD. However, the molecular mechanisms underlying this effect are unclear. Ca^{2+}/CaM binding is essential for CaMKII interactions with some PSD proteins (see below) and Thr305/6 phosphorylation blocks CaM binding, suggesting dissociation of Ca^{2+}/CaM is necessary for CaMKII dissociation from the PSD. Alternatively, Thr305/6 phosphorylation itself may antagonize CaMKII interactions with the PSD or PSD proteins. Data presented in this dissertation presents biochemical evidence for a role for α-actinin in regulating CaMKII targeting and may inform future *in vivo* studies to dissect the molecular basis for prolonged association of CaMKII with the PSD.

CaMKAPs:

Subcellular targeting of protein kinases is mediated by protein-protein interactions (see above), with PKA targeting by AKAP being the most well studied example. Based on a similar analogy, our lab has focused much of its work in identifying CaMKAPs (**CaMK**II **A**ssociated **P**roteins) that may play a role in CaMKII signaling. With the promiscuous role for CaMKII in cell signaling, it is no doubt that there will be increasing numbers of interacting proteins discovered in the future (see table 1.1).

Table 1.1 CaMKII-associated proteins

Proteins italicized are discussed in detail. GluN1, 2A, 2B: NMDAR subunits; VGCC: voltage-gated Ca^{2+}-channels

Binding Protein	Domain in CaMKII	References
GluN1	Catalytic	(Leonard et al., 1999; Merrill et al., 2007)
GluN2A	Catalytic	(Gardoni et al., 1999)
GluN2B	Catalytic	(Bayer et al., 2001; Strack and Colbran, 1998; Strack et al., 2000a)
Densin-180	Association and Catalytic	(Jiao Jalan-Sakrikar et al., 2011; Strack et al., 2000b)
α-Actinin	Regulatory	(Dhavan et al., 2002; Robison et al., 2005b; Walikonis et al., 2001)
F-actin	Variable region	(O'Leary et al., 2006)
CaMKIIN	Catalytic	(Chang et al., 1998; Vest et al., 2007)
SAP-97	Catalytic	(Nikandrova et al., 2010)
β1b and β2a subunit of VGCC	Catalytic	(Grueter et al., 2008)
Cdk5 activators: p35 and p39	Catalytic/regulatory	(Dhavan et al., 2002)
Kalirin-7	?	(Xie et al., 2007)
Ubiquitin Proteasome System (UPS)	?	(Bingol et al., 2010)

NMDA receptor subunits:

The Ca^{2+}-permeable cation channel is highly enriched in the hippocampal PSD and is necessary for CA1 LTP (see above). The cytosolic C-terminal domains of NMDAR subunits interact with diverse proteins providing a structural framework for the PSD (Fig 1.3). CaMKII in hippocampus is mainly regulated by Ca^{2+}-flow through the NMDAR (Thalhammer et al., 2006); thus, CaMKII association with NMDAR is an attractive notion.

Initial screening to identify CaMKAPs using overlay assays revealed a strong signal for PSD-enriched protein of 180-190kda (McNeill and Colbran, 1995). This molecular mass was similar to that of GluN2A and GluN2B subunits of the NMDAR. Further studies showed high affinity interaction between the GluN2B subunit and Thr286 autophosphorylated CaMKII (Strack and Colbran, 1998). Since then other labs identified apparently weaker interactions of CaMKII with GluN2A and GluN1 subunits (Gardoni et al., 1998; Leonard et al., 2002; Leonard et al., 1999).

There are two CaMKII binding sites on GluN2B: the initially identified sites comprising of residues 1290-1309 and a membrane proximal region of residues 839-1120 (Bayer et al., 2001; Leonard et al., 1999; Strack et al., 2000a). Binding to both sites require Thr286 autophosphorylation of CaMKII (Bayer et al., 2001; Strack and Colbran, 1998), however, some labs reported initial binding can be stimulated by Ca^{2+}/CaM which is further potentiated by Thr286 autophosphorylation (Bayer et al., 2001; Leonard et al., 1999). This discrepancy could be due to the differences in the binding assays used. Experiments from our

lab by a former graduate student, AJ Robison, tried to resolve the apparent conflict. He showed that in the presence of nucleotides, Ca^{2+}/CaM could stimulate GluN2B binding to CaMKII without Thr286 autophosphorylation (Robison et al., 2005a). Bayer and colleagues used cell lysates expressing GluN2B for their binding assays that may carry nucleotides in them (Bayer et al., 2001). Recent work from Bayer and colleagues did show that indeed nucleotides enhanced CaMKII binding to GluN2B in presence of Ca^{2+}/CaM (O'Leary et al., 2011). Binding to GluN2B 1290-1309 occurs with an apparent affinity of 140 nM CaMKII subunit, i.e. ~12 nM holoenzyme (Strack and Colbran, 1998). This high affinity and stoichiometric binding required Thr286 autophosphorylation of CaMKII, whilst Thr305/6 autophosphorylation has a modest effect to reduce binding (Leonard et al., 2002). Not much is known about the role and regulation of CaMKII binding to the membrane-proximal region of GluN2B (residues 839-1120).

The actual affinity of CaMKII for GluN2A is unknown; but a comparison with GluN2B showed ~10-fold weaker binding (Strack and Colbran, 1998). Residues 1349-1464 at the extreme C-terminus of GluN2A are involved with residues 1412-1419 being critical for the interaction (Gardoni et al., 1999). This binding is potentiated by Thr286 autophosphorylation of CaMKII, but not by Ca^{2+}/CaM. PKC phosphorylation at Ser1416 in GluN2A antagonizes CaMKII binding, and the interaction is competitive with PSD-95 (Gardoni et al., 2001a; Gardoni et al., 2001b).

CaMKII binding to GluN1 subunit also requires Thr286

autophosphorylation (Leonard et al., 2002). Binding occurs in the C0 region of GluN1, which precedes a number of alternatively spliced cassettes, but is itself present in all GluN1 splice variants. The affinity and the stoichiometry of the interaction are not yet evaluated. Residues 845-861 are sufficient for interaction, and overlap with the Ca^{2+}/CaM and α-actinin-binding domain in the C0 region. Apo CaM and α-actinin can simultaneously bind to the C0 region, however, Ca^{2+} influx through NMDAR displaces α-actinin through Ca^{2+}-bound CaM. This displacement of α-actinin by Ca^{2+}/CaM promotes CaMKII binding to the C0 region of GluN1, allowing simultaneous interaction of CaMKII and CaM with GluN1 (Merrill et al., 2007). Thus Ca^{2+} - influx through NMDARs plays an intricate role in governing complex interactions of CaMKII with NMDAR subunits.

GluN2B is also a high affinity substrate for CaMKII with Ser1303 being the phosphorylation site (Omkumar et al., 1996). Phosphorylation at this site inhibits CaMKII-GluN2B complex and promotes dissociation of the preformed complex (Raveendran et al., 2009; Strack et al., 2000a). The functional significance of Ser1303 phosphorylation in GluN2B by CaMKII is not understood yet, however, unpublished data from our lab suggest it is required for CaMKII-dependent NMDAR desensitization (Sessoms-Sikes et al., 2005).

The high affinity CaMKII binding domain in GluN2B (residues 1290-1309) bears striking similarity with the autoinhibitory domain of CaMKII. Thus the peptide inhibitors modeled after the autoinhibitory domain can compete with GluN2B for binding to CaMKII (Strack et al., 2000a). Furthermore, syntide-2, a classical substrate for CaMKII failed to compete with GluN2B 1290-1309 for

interaction with CaMKII. These data suggested that GluN2B bound CaMKII within the T-site of the catalytic domain (T-site is the region in the catalytic domain of CaMKII where the AID containing Thr286 docks, Fig. 1.6). Incubation of CaMKII with Ca^{2+}/CaM and a peptide based on the 1290-1309 region of GluN2B can "trap" CaMKII in a Ca^{2+}-independent active conformation without Thr286 autophosphorylation (Bayer et al., 2001). This effect of GluN2B on CaMKII was proposed to occur due to binding of GluN2B at the T-site in presence of Ca^{2+}/CaM, and preventing the reinstatement of the AID to the T-site after removal of Ca^{2+}/CaM. As GluN2B peptide lacks the inhibitory pseudosubstrate sequence present in the AID, it allows the exposure of S-site for substrate binding and catalysis (Bayer et al., 2001). On the other hand, studies from our lab shows that GluN2B can inhibit Ca^{2+}/CaM-dependent and autonomous activity of CaMKII. This inhibition is competitive with respect to autocamtide-2 (AC-2), a T-site substrate, non-competitive with syntide-2, the classical S-site substrate and uncompetitive with ATP (Robison et al., 2005a). Thus, interaction with GluN2B modulates the kinetic parameters for ATP binding to CaMKII and catalysis (Cheriyan et al., 2011; Pradeep et al., 2009).

CaMKII associates with NMDARs in cells (Bayer et al., 2001; Bayer et al., 2006; Leonard et al., 1999; Strack and Colbran, 1998). CaMKII targeting to the NMDAR is dependent on receptor activation, Ca^{2+} influx and is stabilized by Thr286 autophosphorylation (Bayer et al., 2001; Bayer et al., 2006; Leonard et al., 2002; Strack and Colbran, 1998). Conditions that promote Thr286 autophosphorylation increase the co-immunoprecipitation of CaMKII with GluN2B,

including high frequency tetanic stimulation and ischemia (Gardoni et al., 2001b; Leonard et al., 1999; Meng et al., 2003; Meng and Zhang, 2002). In HEK293 cells, GluN2B, but not GluN2A subunit supports the redistribution of CaMKII upon NMDA stimulation (Strack and Colbran, 1998), suggesting GluN2B plays a dominant role in CaMKII association with NMDA receptors. Interference with CaMKII-GluN2B interaction by overexpression of the GluN2B C-terminal domain or mutated GluN2B strongly reduces CaMKII targeting and LTP induction (Barria and Malinow, 2005; Zhou et al., 2007). Recent studies indicate that this complex also plays a role in LTP maintenance (Sanhueza et al., 2011).

Taken together, these data make the GluN2B subunit of NMDAR an excellent candidate responsible for stimulus-induced CaMKII translocation to the PSD. However, interaction with GluN2B alone cannot account for all the CaMKII at the PSD. Recent proteomics studies have shown that there are ~ 20-fold more CaMKII holoenzymes in the PSD than NMDAR subunits on a molar basis (Cheng et al., 2006). Another recent study suggest that only 2% of CaMKII may be bound to the NMDARs in the PSD and of that only 10% may be held active by GluN2B subunit (Feng et al., 2011). Also autophosphorylation at Thr305/6 promotes CaMKII dissociation from the PSD; yet appear to have modest effects on direct interactions with GluN2B (Leonard et al., 2002; Robison et al., 2005a). Hence, we postulate that CaMKII targeting to the PSD is a reflection of GluN2B interactions along with additional interactions with other PSD proteins. The work presented in this dissertation explores the role of other CaMKAPs like densin and α-actinin in regulating CaMKII activity and/or localization in the cells.

Densin-180:

Densin-180 (densin) was originally identified as a PSD-associated protein with leucine-rich repeats (LRR) at the N-terminus and a PDZ domain at the C-terminus (Fig. 1.9). It was suggested to be an O-sialoglycoprotein with a single

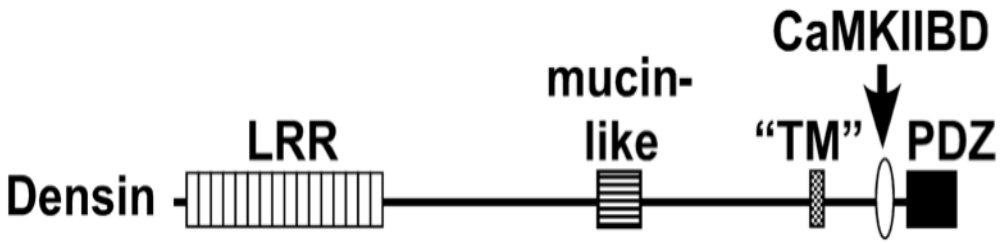

Figure 1.9 Domain structure of Densin

Domain organization of densin; LRR: leucine-rich repeat, "TM": originally proposed putative transmembrane domain, CaMKIIBD: originally identified CaMKII binding domain. PDZ domain is involved in interaction with α-actinin.

putative transmembrane domain (Apperson et al., 1996). Based on this topology the N-terminal domain was proposed to be extracellular and the C-terminal domain to be cytoplasmic. More recent studies indicate that densin is an intracellular protein that associates with plasma membrane, at least in part via the LRR domain (Izawa et al., 2002; Jiao et al., 2008; Thalhammer et al., 2009). Densin was identified as a CaMKAP by two laboratories (Strack et al., 2000b; Walikonis et al., 2001). Of four developmentally regulated C-terminal domain splice variants of densin, A-D, three variants (A, B, and D) interacted with CaMKII (Strack et al., 2000b). Moreover, the C-terminal CaMKII binding domain in densin was sufficient to confer membrane localization of expressed CaMKII in HEK293 cells. Densin can also be alternatively spliced in the N-terminal region. These

variants are expressed differentially during rat brain development; exhibit distinct subcellular localization and effects on cell morphology (Jiao et al., 2008). CaMKIIα and densin co-immunoprecipitate from brain extracts and also co-localize in cultured neurons, suggesting physiological association of these proteins (Jiao, Jalan-Sakrikar et al., 2011; Robison et al., 2005b; Walikonis et al., 2001).

The C-terminal CaMKII binding domain has no apparent sequence similarity with CaMKII binding domains in NMDAR subunits. Hence, GluN2B cannot compete with densin for binding to CaMKII ((Strack et al., 2000b) however see Chapter III). Moreover, binding of this C-terminal domain of densin to CaMKII is independent of CaMKII activation (Strack et al., 2000b; Walikonis et al., 2001), unlike GluN2B, which requires active CaMKII (Robison et al., 2005a), but see chapter III and (Jiao Jalan-Sakrikar et al., 2011). Interestingly initial studies identified densin as a CaMKIIα specific CaMKAP, binding to the C-terminal association domain (Walikonis et al., 2001). Results presented in this dissertation (Chapter III) identify a new CaMKII binding domain in densin which interacts with both the α and β isoforms of CaMKII.

Several other postsynaptic proteins can interact with the PDZ domain of densin (δ-catenin, MAGUIN-1, L-type Ca^{2+} channels) (Izawa et al., 2002; Jenkins et al., 2010; Ohtakara et al., 2002). Most significantly to this dissertation, the C-terminus of α-actinin (also a CaMKAP, see below) binds to the PDZ domain in densin (Robison et al., 2005b; Walikonis et al., 2001). These various interactions with several proteins makes densin an archetypical scaffolding protein to

assemble multi-protein complexes, thereby increasing the specificity and efficiency of postsynaptic signaling. A homozygous knockout mouse of densin displays phenotypes of schizophrenia and autism spectrum disorders (Carlisle et al., 2011). These mice also show reduced total levels of α-actinin, and metabotropic glutamate receptors. Furthermore, deletion of densin results in impairment of synaptic plasticity and spine morphology.

CaMKII Inhibitor protein (CaMKIIN):

Yeast-2- hybrid screen of rat brain cDNA using the catalytic domain of CaMKII as bait protein, identified two isoforms of CaMKIIN (α and β)(Chang et al., 1998, 2001). This naturally occurring 79 amino acid protein binds and potently inhibits CaMKII activity (IC_{50} of 50-100 nM). The inhibitory action of CaMKIIN is specific for CaMKII, with little or no effect on other tested kinases such as CaMKI, CaMKIV, PKA and PKC (Chang et al., 1998). The subcellular distribution of CaMKIIN isoforms correlates with CaMKII immunoreactivity (Chang et al., 2001). In cultured neurons, CaMKIIN colocalizes with CaMKII in cell bodies and dendrites, but not in dendritic spines (Chang et al., 2001). Thus, CaMKIIN may serve to negatively regulate CaMKII signaling in dendrites and soma, restricting its activity to the spines and PSD.

The inhibitory determinants for CaMKIIN were localized in a 27 amino acid domain near the C-terminal region (termed CN27 or CaMKIINtide) (Chang et al., 1998). Further truncations in the C-terminal region of this domain revealed a 21 amino acid peptide that contained the minimal inhibitory region, residues 43-63 (termed CN21) (Vest et al., 2007). CN21 can inhibit the Ca^{2+}-stimulated and

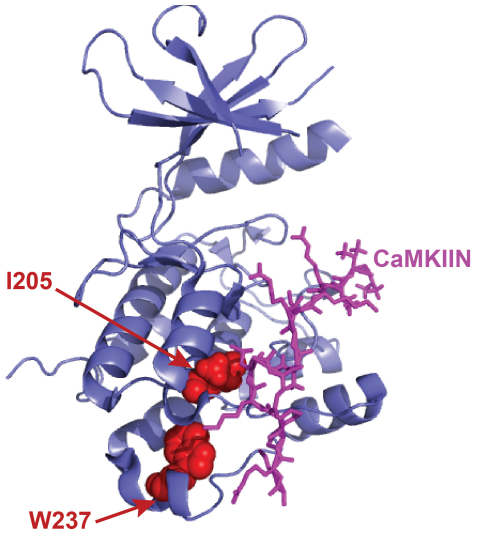

Figure 1.10 Structure of CaMKII-CaMKIIN complex
CaMKIIN (magenta) bound to the catalytic domain of CaMKII (PDB: 3KL8). Highlighted in red are the T-site residues shown to be involved in interaction with CaMKIIN peptide, CN21.

autonomous activity of CaMKII and also autophosphorylation at Thr305 (Vest et al., 2007). Mutagenesis studies showed that CaMKIIN binds to the CaMKII catalytic domain at the T-site (Vest et al., 2007), which was later shown in a crystal structure of the CaMKII-CaMKIIN complex, PDB: 3KL8 (Chao et al., 2010a) (Fig 1.10). Consistent with the T-site binding, CN21 was able to compete with GluN2B (T-site binding protein) for binding to CaMKII (Vest et al., 2007). Recently Bayer and colleagues further reduced the CN21 peptide to the CN19 peptide, which still retains the full inhibitory potency and specificity for CaMKII (Coultrap and Bayer, 2011).

The CN 19 peptide was recently used in hippocampal slice preparations to show that CaMKII-NMDAR complex is important for maintenance of synaptic strength (Sanhueza et al., 2011). Physiologically, CaMKIIN expression is

regulated in response to learning (Lepicard et al., 2006; Radwanska et al., 2010). Furthermore, CN peptides are neuroprotective when applied after glutamate insults in culture, or a stroke model in mouse (Ashpole and Hudmon, 2011; Vest et al., 2010).

F-actin:

Actin is a highly dynamic and prominent cytoskeletal protein that serves as a framework for mechanical stability of dendritic spine structure (Cingolani and Goda, 2008; Dillon and Goda, 2005). It also serves as a scaffold for recruiting several postsynaptic proteins. Actin exists in a dynamic equilibrium between two forms, the monomeric globular form (G-actin) and the filamentous form (F-actin), which is modulated during LTP induction (Okamoto et al., 2009). CaMKII isolated from rat brains can bind filamentous actin (F-actin) *in vitro* and Ca^{2+}/CaM antagonizes this binding (Ohta et al., 1986). The ability of CaMKIIβ, but not CaMKIIα to bind and bundle F-actin *in vitro* appears to be dependent on a specific splice insert (O'Leary et al., 2006; Okamoto et al., 2007; Sanabria et al., 2009). Under basal conditions, overexpressed GFP-CaMKIIβ associates with actin cytoskeleton in various cell types, whereas GFP-CaMKIIα shows diffused localization in the cytoplasm (Shen and Meyer, 1999). In cultured hippocampal neurons, transfection of CaMKIIβ with CaMKIIα resulted in F-actin colocalization of CaMKIIα, suggesting CaMKIIβ can act as a targeting module for CaMKIIα (Shen et al., 1998). Overexpression of CaMKIIβ, but not CaMKIIα, increases the neurite extensions and formation of new synapses in dissociated neuronal cultures (Fink et al., 2003). RNAi-mediated knockdown of CaMKIIβ leads to a

reduction in dendritic spine head volume, whereas overexpression of CaMKIIβ reduced the actin turnover rate in the spine head (Okamoto et al., 2007). Moreover, CaMKIIβ binding stabilized F-actin rich structures in cortical neurons, whereas disruption of binding reduced the F-actin filaments, again reinforcing the role of CaMKIIβ in maintaining polymerized actin (Lin and Redmond, 2008). Collectively, this suggests that CaMKII serves as an unusual signaling molecule with a pivotal role in structural changes during synaptic plasticity.

Besides direct interaction of CaMKIIβ with F-actin, multiple CaMKII isoforms can bind to the actin-binding protein, α-actinin (Robison et al., 2005b; Walikonis et al., 2001) (see below). The role of α-actinin in regulating CaMKII localization and function is a major focus of this dissertation.

α-Actinin:

A member of the spectrin super family consisting of spectrin and dystrophin, α-actinin is a cytoskeletal actin bundling protein. It is localized in multiple subcellular regions including cell-cell and cell-matrix contact sites, cellular protrusions, lamellipodia, and stress fiber dense regions (Otey and Carpen, 2004). The subcellular distribution of α-actinin suggests that it has important roles linking the cytoskeleton to various transmembrane proteins at different junctional structures.

Structurally, α-actinin has an N-terminal actin-binding domain followed by four spectrin repeats, and two putative C-terminal EF-hands. It is a rod-shaped antiparallel dimer; dimerization being mediated by the spectrin repeats. The four

isoforms (α-actinin-1 through -4) have been broadly divided into skeletal muscle and non-muscle isoforms (Blanchard et al., 1989). The main functional difference between the isoforms is that the non-muscle isoforms (1 and 4) are Ca^{2+}-sensitive, whereas the muscle isoforms (2 and 3) are Ca^{2+}-insensitive (Burridge and Feramisco, 1981). Although α-actinin is generally homodimeric, heterodimerization of α-actinin-2 and -3 has been observed *in vitro* and *in vivo* (Chan et al., 1998). The actin-binding domain, as the name suggests, plays an important role in bundling F-actin and shaping the actin cytoskeleton. The rod-domain comprised of spectrin repeats serves as a hub for protein-protein interactions leading to assembly of complex, multiprotein structures involved in either cytoskeletal regulation or signal transduction (Djinovic-Carugo et al., 2002).

α-Actinin-1 and -4 are commonly found at focal adhesion sites and stress fibers (Otey and Carpen, 2004). The spectrin repeat of α-actinin mediates the interaction with transmembrane adhesion molecules such as β integrin and intracellular adhesion molecule-1 (ICAM-1). This interaction serves many purposes: it provides structural stability for the adhesion site thereby maintaining cell shape, works as a scaffold to integrate signaling molecules at the adhesion sites, and also aids in clustering of adhesion molecules thereby enhancing adhesion (Otey and Carpen, 2004). In highly motile cells α-actinin-4 is highly localized in dorsal ruffles, whereas, α-actinin-1 is evenly distributed along F-actin stress fibers (Sjoblom et al., 2008). α-Actinin-4 deficient mice display abnormal podocyte morphology in the kidney, which results in severe glomerular disease (Kos et al., 2003). Point mutations in α-actinin-4 gene have been identified in

multiple human patients suffering from a familial kidney disease, focal and segmental glomeruloscelrosis (Kaplan et al., 2000). α-Actinin-4 also binds endothelial nitric oxide synthase (eNOS) competitively with Ca^{2+}/CaM to inhibit eNOS activity, thus regulating vascular function (Hiroi et al., 2008). In macrophages α-actinin-4 has been shown to contribute to mechanics of both phagocytosis and macropinocytosis (Araki et al., 2000; Washington and Knecht, 2008).

On the other hand, α-actinin-2 and -3 are the major isoforms in the cardiac and skeletal muscles (Blanchard et al., 1989). They form an important component of the contractile machinery at the Z-lines in striated muscles and dense bodies in smooth muscles. Several proteins of the contractile machinery directly bind α-actinin-2 such as the protein kinase titin, nebulin, actin-capping protein CapZ, Z-disk associated proteins (ZASP) (Sjoblom et al., 2008). This suggests that α-actinin plays a critical role in striated muscle sarcomere and thin filament organization as well as interaction between the sarcomeric cytoskeleton and the muscle membrane. Hence, it is not surprising, that α-actinin-2 has been implicated in myopathies such as hypertrophic and dilated cardiomyopathy (Chiu et al., 2010; Mohapatra et al., 2003). In contrast, the absence of α-actinin-3 protein due to homozygosity of a premature stop codon in 20% of the general population has no associated clinical disease phenotype (North et al., 1999). However, a genetic variant of α-actinin-3 (ACTN3-R577X) influences muscle metabolism in mice and athletic performance in humans (MacArthur and North, 2007; MacArthur et al., 2007).

α-Actinin also interacts with many other transmembrane proteins such as the adenosine A2 receptor, vinculin, and multiple potassium channels. Binding of α-actinin to ion channels like the $K_v1.5$, $Na_v1.5$, Ca^{2+}-activated K^+ channels (SK2 channel), or acid-sensing ion channels modulates the surface expression and/or function of these channels (Lu et al., 2009; Lu et al., 2007; Maruoka et al., 2000; Sadeghi et al., 2002; Schnizler et al., 2009). Interaction of α-actinin with metabotropic glutamate receptor 5 (mGluR5) was identified by yeast-two-hybrid and confirmed in heterologus cells and also in rat striatum (Cabello et al., 2007). Moreover, α-actinin increased the cell surface expression of mGluR5 receptors, an effect that was dependent on α-actinin binding to F-actin. In densin knockout mice, levels of mGluR5 were reduced in the PSD (Carlisle et al., 2011). The authors of this study suggest that since densin has not been shown to directly bind mGuR5, it could be attributed to the reduced levels of α-actinin in these mice. Functionally, α-actinin also modulated mGluR5-mediated activation of mitogen-activated protein kinase pathway (Cabello et al., 2007). α-Actinin-2 associates with regulator of G protein signaling 9-2 (RGS9-2) in striatum. This interaction can selectively modulate α-actinin-2-dependent regulation of NMDAR in HEK293 cells (Bouhamdan et al., 2006)

More relevant to this dissertation, α-actinin-2 is enriched at the PSD and localizes to dendritic spines (Wyszynski et al., 1998; Wyszynski et al., 1997). Overexpression of α-actinin-2 increases the length and density of dendritic protrusions in hippocampal neurons (Hoe et al., 2009; Nakagawa et al., 2004). The spectrin repeats of α-actinin interact with the NMDAR GluN1 and GluN2B

subunits. The interaction with GluN1 is competitive with Ca^{2+}/CaM (Wyszynski et al., 1997) and plays an important role in regulating calcium-dependent inhibition (CDI) of NMDAR (Krupp et al., 1999; Rycroft and Gibb, 2004; Zhang et al., 1998). Ca^{2+}/CaM-dependent displacement of α-actinin from the GluN1, also allows for CaMKII binding to the GluN1 C0 region (Merrill et al., 2007). The C-terminal domain of α-actinin also interacts with a PDZ domain in densin forming a ternary complex with densin and CaMKIIα (Robison et al., 2005b; Walikonis et al., 2001).

The F-actin bundling capability of α-actinin is reduced upon phosphorylation by focal-adhesion kinase (FAK) on Tyr12 (Izaguirre et al., 2001) or by Ca^{2+} binding to the EF-hands of α-actinin-1 and -4 (Burridge and Feramisco, 1981; Witke et al., 1993). The N-terminal F-actin binding domain of α-actinin also has lipid-binding sites (Fukami et al., 1996). In the naive state, the α-actinin dimer is proposed to be in closed conformation due to intersubunit interaction between the neck region connecting the ABD and spectrin repeats of one subunit with the C-terminal domain of another subunit (Young and Gautel, 2000). Binding of anionic lipids such as PIP2 or phosphatidylserine in the ABD causes a conformational change and relieves this interaction to induce an open conformation of the dimeric protein, that can now interact with other proteins via the free C-terminal domain (CTD) (Fig 1.11). Such a mechanism has been shown to regulate titin binding to α-actinin-1 and -2 (Young and Gautel, 2000). It has also been shown that phospholipids stimulate the binding of full-length α-actinin to another serine-threonine kinase, PKN (Mukai et al., 1997). PIP2 binding to α-actinin has also been shown to regulate NMDAR activity by keeping

the channel in the open position, an effect requiring α-actinin's simultaneous interaction with GluN2B and PIP2 (Michailidis et al., 2007).

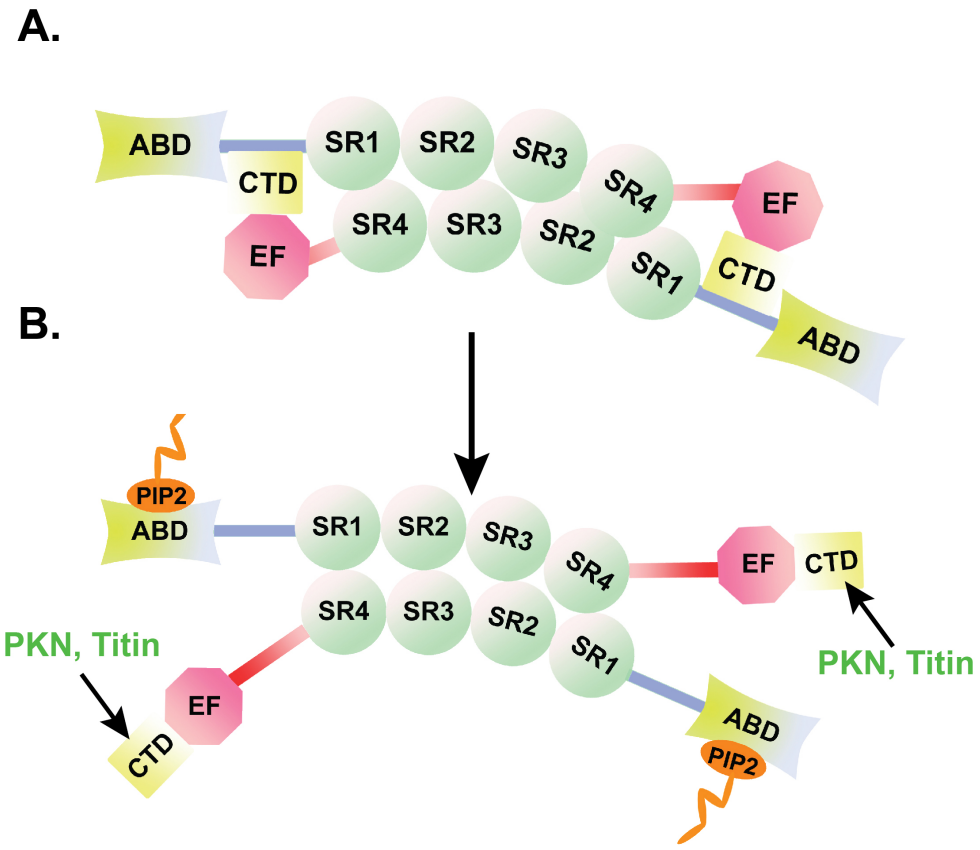

Figure 1.11 α-Actinin domain organization and regulation

α-Actinin exists as an anti-parallel dimer. Each molecule within a dimer consists of an N-terminal actin-binding domain (ABD), followed by a neck region (blue) which connects ABD to the rod-domain comprised of four spectrin repeats (SR1-SR4), EF hands and a C-terminal domain (CTD). **A.** In the closed conformation, CTD binds the neck region. **B.** PIP2 binding to the ABD relieves this interaction, freeing the CTD domain to bind other proteins like PKN and titin. *Modified from* (Young and Gautel, 2000).

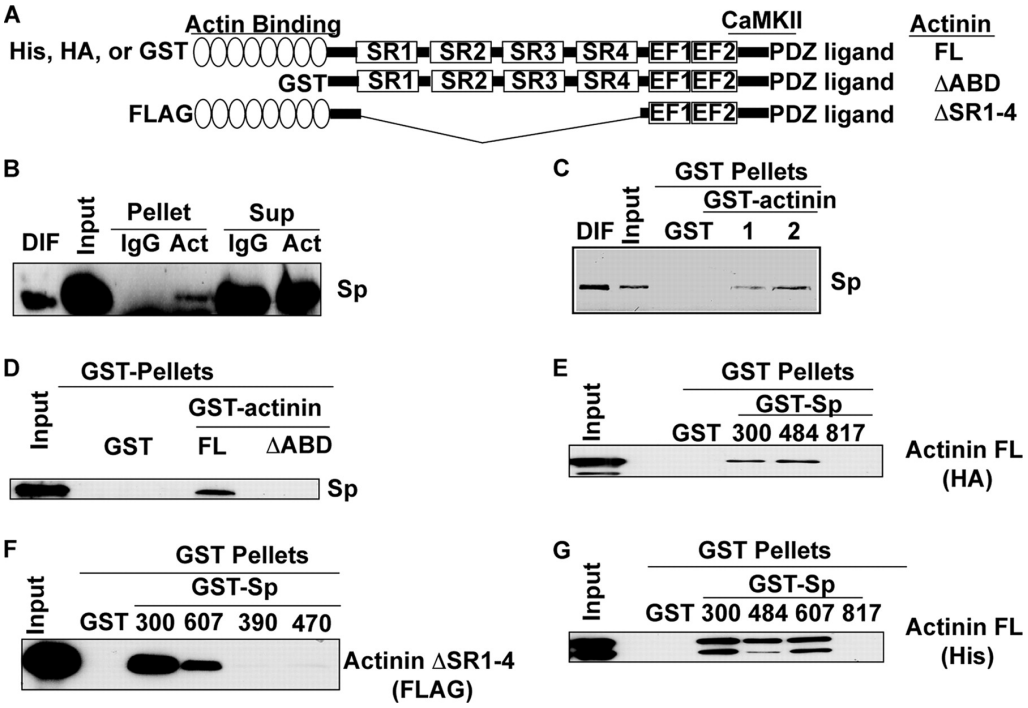

Fig 1.12 Characterization of α-actinin and spinophilin interaction

A. Domain structure of α-actinin and constructs used in the experiments. **B.** Spinophilin specifically co-immunoprecipitates with α-actinin (Act) but not with control (IgG) antibody from mouse brain. **C.** Full-length (FL) GST-α-actinin-1 and -2 associate with spinophilin from mouse brain extracts. **D.** Myc-spinophilin expressed in HEK293 cells can bind FL but not ΔABD GST-α-actinin. **E.** GST spinophilin 1-300(300) and 151-484(484), but not 446-817(817) interact with FL HA-α-actinin expressed in HEK293 cells. **F.** GST spinophilin 300 and 151-607(607) but not 286-390(390) and 427-470(470) bind ΔSR1-4 FLAG α-actinin expressed in HEK293 cells. **G.** GST-spinophilin 300, 484, and 607 but not 817 directly interact with purified FL His-α-actinin. *Adapted from* (Baucum et al., 2010)

In collaboration with a postdoctoral fellow in our lab, Dr. AJ Baucum, I found that α-actinin also directly interacts with spinophilin. Spinophilin is an F-actin and protein phosphatase 1 (PP1) binding protein. Spinophilin is highly expressed in brain and is localized to the PSD where it can target PP1 to regulate synaptic plasticity (Feng et al., 2000). Using a proteomics approach, Dr. Baucum identified several new spinophilin-associated proteins including α-actinin and densin (Baucum et al., 2010). I contributed to this study by showing that

there is a direct interaction between the N-terminal ABD of α-actinin and residues 151-300 of spinophilin (Fig 1.12).

α-Actinin also interacts with another CaMKAP, Cdk5, via its activators, p35 and p39 (Dhavan et al., 2002). Furthermore, α-actinin co-localizes with Cdk5 and CaMKII in hippocampal neurons, suggesting α-actinin could potentially regulate synaptic localization of both kinases.

Pharmacological disruption of the actin cytoskeleton results in a complete loss of both CaMKIIα and α-actinin-2 from the PSD but not of NMDAR (Allison et al., 2000; Allison et al., 1998). This suggests that localization of CaMKIIα to PSDs may in part depend on the F-actin cytoskeleton via α-actinin. Indeed several labs have independently identified α-actinin-1, -2 and -4 as CaMKII interacting proteins (Dhavan et al., 2002; Robison et al., 2005b; Walikonis et al., 2001). It was further shown that the catalytic/regulatory domain of CaMKII interacts with the C-terminal domain (CTD) of α-actinin-2 (residues 819-894) and that this interaction is competitive with Ca^{2+}/CaM and blocked by Thr305/6 autophosphorylation *in vitro* (Robison et al., 2005a; Robison et al., 2005b). Thus, the CTD of α-actinin-2 inhibits Ca^{2+}/CaM-dependent activation of CaMKII. These initial *in vitro* characterizations of CaMKII-α-actinin interaction used the isolated CTD of α-actinin. However, not much is known about the direct effect of full-length α-actinin on CaMKII regulation especially in intact cells. Data presented in this dissertation unravels an exciting new role for α-actinin in regulating CaMKII activity.

Hypothesis and Specific Aims:

General Hypothesis:

CaMKAPs dynamically regulate CaMKII activity and localization.

Roles for CaMKAPs such as GluN2B and CaMKIIN in regulating CaMKII actions *in vivo* have started to emerge. These studies were possible with groundwork laid by *in vitro* studies characterizing the interactions of the proteins with CaMKII. However, the role of other known CaMKAPs in modulating CaMKII functions are yet unknown. This dissertation takes a step toward characterizing the functional roles of two CaMKAPs.

Aim 1: To understand the impact of a newly identified CaMKII-binding domain in densin, densin-IN, on CaMKII activity.

Aim 2: To investigate the role of α-actinin in modulating CaMKII activity, localization, and interactions with NMDA receptors.

CHAPTER II

MATERIALS AND METHODS

Bacterial and mammalian expression constructs for α-actinin, densin, CaMKII, AMPAR and NMDAR subunits:

The α-actinin-1 cDNA (human) was made by Dr. Carol Otey (Edlund et al., 2001) and obtained from Dr. Donna Webb at Vanderbilt University. The α-actinin-2 cDNA (human) was a generous gift from Dr. Alan Beggs (Harvard). α-Actinin-2 EF (547-894) and CTD (819-894) were described before (Robison et al., 2005b). Other desired fragments of α-actinin-1 or -2 (Table 2.1) were amplified by PCR using EcoRI and XhoI, or SmaI restriction enzyme sites respectively. PCR products were ligated into the pGEX-2T/4T-1 (Amersham Pharmacia Biotech) or pET-28b(+) (Novagen) vectors and transformed into BL21-DE3 Gold *E. coli* bacteria. After induction of protein expression, glutathione-S-transferase (GST)- or His6-fusion proteins were purified using Glutathione-Agarose (Sigma) or His-Select Nickel Affinity Gel (Qiagen), according to the manufacturer's protocol.

GST-D-CTA (densin) contains residues 1247-1542(**Δ**1292-1337) as described (Robison et al., 2005b; Strack et al., 2000b). Other GST-densin constructs are described in (Jiao Jalan-Sakrikar et al., 2011). All purified proteins were quantified using Bradford (BioRad) or bicinchonic acid (BCA, Pierce) assays.

Vectors for expressing full-length α-actinin with a N-terminal HA-tag and untagged CaMKIIα in mammalian cells were described (Baucum et al., 2010; Jiao et al., 2008). Constructs to express FLAG-tagged α-actinin were made similar to the HA-tagged constructs. The GluA1 construct was provided by Dr. T. Soderling (Oregon Health Sciences University). GluN1, GluN2A and GluN2B expression constructs were described (Sessoms-Sikes et al., 2005). Site-directed mutagenesis was confirmed by sequencing. All constructs were purified using Maxi-Prep kits (Invitrogen, Carlsbad, CA).

Name of the construct	Residues in α-actinin-1	Residues in α-actinin-2
FL	1-892	1-894
Δ ABD	248-892	255-894
SR1	274-892	281-894
EF	540-892	547-894
CTD	816-892	819-894

Table 2.1: α-Actnin-1 and -2 fragments used in the thesis.

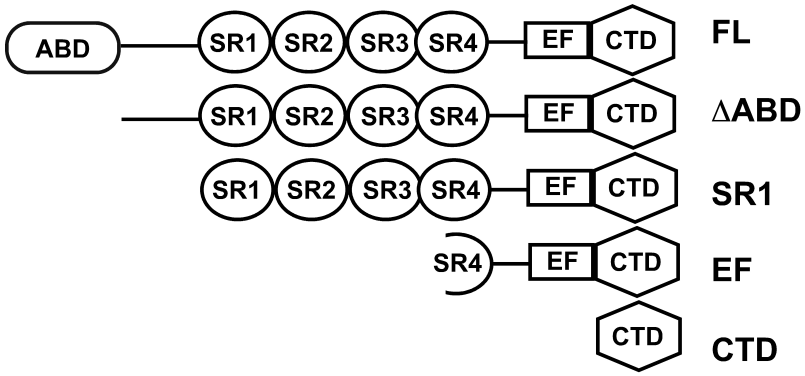

Figure 2.1 α-Actinin domain organization and truncations
Full-length (FL) α-actinin contains an N-terminal ABD, a neck region connecting ABD to the four spectrin repeats (SR), followed by a EF hand domain and the C-terminal domain (CTD). N-terminal deletion constructs were made as follows: ΔABD: Construct lacking ABD, SR1: construct lacking ABD and neck region, EF: lacking ABD, neck region and SR1, SR2 and half of SR3, CTD: domain after the EF hands. See table 2.1 for amino acid numbers in α-actinin-1 and -2.

CaMKII isoforms:

Murine CaMKIIα (WT, T305A, and T306A), Xenopus CaMKIIβ, porcine CaMKIIγB and rat CaMKIIδ2 were purified from baculovirus-infected Sf9 cells as described (Brickey et al., 1990; Colbran, 1993; McNeill and Colbran, 1995; Robison et al., 2005b).

CaMKII Autophosphorylation:

CaMKII isoforms were autophosphorylated essentially as described (McNeill and Colbran, 1995). Briefly, selective Thr286/7 autophosphorylation was achieved by incubating CaMKII (5 μM subunit) on ice for 90 seconds with 50 mM HEPES pH 7.5, 10 mM magnesium acetate, 1.5 mM $CaCl_2$, 10 mM CaM, 40 μM ATP, and the reaction was stopped with ethylenediamine-tetraacetic acid (EDTA: 12.5 mM, final). For sequential autophosphorylation of Thr286, Thr305/6 and other sites, Ca^{2+}/CaM-dependent autophosphorylation (as above, except using 500 μm ATP) was terminated using ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, 4 mM final) and the Ca^{2+}-independent reaction was continued for 2 minutes at 30^{o}C. For basal autophosphorylation at Thr305/6 and other sites, CaMKII (5 μM subunit) was incubated for 60 min at 30°C with 50 mM HEPES pH 7.5, 10 mM magnesium acetate, 1 mM EGTA, and 500 μM ATP, and the reaction was stopped with EDTA (12.5 mM, final).

GST Cosedimentation Assays:

Purified GST-fusion proteins or GST alone (≈250 nM full-length protein) were incubated with CaMKII (WT or mutant) (≈250 nM in the indicated autophosphorylation state) and glutathione beads (Sigma; 40 μl of 1:1 slurry) in

pulldown (PD) buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.5% Triton X-100 (v/v)) to a final volume of 500 ml for 2 hours at 4°C. Beads were sedimented by centrifugation and washed in PD buffer 4-5 times, with the beads transferred to a new microfuge tube during the second wash. After the last wash, proteins were eluted with SDS-PAGE sample buffer, resolved by SDS-PAGE and electrotransferred to nitrocellulose membrane. Proteins on the membrane were visualized by staining with Ponceau S and immunoblotted for indicated proteins. Amounts of CaMKIIα sedimented on the beads were normalized to the recovered GST-fusion protein, and background binding to GST alone was subtracted.

Synthetic peptides

Peptides were custom synthesized by Macromolecular Resources (University of Colorado, Fort Collins, CO) or by Global Peptide Services (Fort Collins, CO). GluN2B peptide (AQKKNRNKLRRQHSYDTFVD) is an analog of residues 1290-1309 of GluN2B. N2Btide is same as GluN2B peptide with the Ser103 replaced with Ala. Syntide-2: PLARTLSVAGLPGKK. Autocamtide-2: KKALRRQETVDAL. N-tide (KRPPKLGQIGRSKRVVIEDDRIDDVLK) is a peptide analog of residues 43-69 of the CaMKIINα inhibitor protein.

Kinase Assays:

Activation assay: Purified CaMKIIα (10 nM) was incubated with indicated concentrations of CaM or His_6-A2-CTD in presence of 50 mM HEPES pH 7.5, 1 mg/ml bovine serum albumin, 10 mM magnesium acetate, 0.5 mM $CaCl_2$ or 1mM EGTA, 1 mM DTT and a model peptide substrate (AC-2, syntide-2 or NR2B; 100

μM each). Reactions were started by adding [γ-^{32}P]ATP (0.4 mM; 500-1000 cpm/pmole) and incubated at 30°C for 10 min. Aliquots were spotted on phosphocellulose paper (Whatman P81), washed with water and analyzed by liquid scintillation counting.

Inhibition Assay: GST-D-IN, GST-D IN(L815E), N-tide, or a GST control were diluted into ice-cold 50 mM HEPES pH 7.5, 1 mg/ml bovine serum albumin, 10 mM magnesium acetate, 0.5 mM CaCl2, 1 μM CaM, 1 mM DTT and 0.4 mM [γ-^{32}P]ATP (≈500 cpm/pmole) containing the indicated substrate: syntide-2 (20 μM), GSTGluA1(2 μM) or GST-N2Bs (2 μM). Reactions were initiated by addition of CaMKII (0.75 nM for syntide-2) or autophosphorylated CaMKII (10 nM for GST protein phosphorylation) and incubated at 30°C for 10 min. Aliquots were spotted on phosphocellulose paper (Whatman P81), washed with water, rinsed with ethanol and dried for scintillation counting in non-aqueous scintillant. Remaining GST-GluA1 and GST-N2Bs reactions were quenched with SDS, resolved by SDS-PAGE and stained with Coomassie blue prior to autoradiography.

Cell culture and transfection

HEK293 cells maintained at 37°C in 5% CO_2 in modified Eagle medium (MEM) containing 10% fetal bovine serum (Gibco, Carlsbad, CA), penicillin-streptomycin (Sigma, St. Louis, MO) and 2 mM glutamate (Sigma) were transfected using Fugene (Roche, Indianapolis, IN) or PolyJet (SignaGen Laboratories, Gaithersburg, MD) (3 μl/μg DNA). Cells expressing NMDAR subunits were cultured with 1 mM (2*R*)-amino-5-phosphonovaleric acid; (2*R*)-amino-5-

phosphonopentanoate (AP-5) (Ascent Scientific) or 500 μM AP-5 plus 5 mM $MgCl_2$.

HEK293 cell lysis, immunoprecipitation and pulldown:

For immunoprecipitation, HEK293 cells (24-48 hrs after transfection) were rinsed with cold PBS twice and lysed on ice with 1.6 ml per 10 cm plate of Buffer A (2 mM Tris-HCl pH 7.5, 1% (v/v) Triton X-100; 0.1 mM PMSF, 1 mM benzamidine, 5 mg/l leupeptin, 20 mg/l soybean trypsin inhibitor). After sonication, lysates were incubated at 4°C for 1 hr and then centrifuged for 10 min at 10,000 X g. NaCl (150 mM final concentration) was added to the supernatants and equal aliquots were incubated overnight at 4°C with the indicated antibody. After addition of GammaBind Plus-Sepharose (Amersham Biosciences, Piscataway, NJ) (40 μl, 1:1 slurry) and continued incubation for 2 h at 4°C, samples were centrifuged and washed ≥4 times with 1 ml of Buffer B (50 mM Tris-HCl, 150 mM NaCl, 1% (v/v) Triton X-100). The Sepharose was transferred to new microcentrifuge tubes during the second wash. Immune complexes were solubilized in SDS-PAGE sample buffer for immunoblot analysis.

HEK293 cells expressing CaMKII (WT or mutants) alone (48 hrs after transfection) were processed for GST co-sedimentation assays essentially as for immunoprecipitations. The supernatants containing 150 mM NaCl were incubated for 2 hr at 4°C with 20 μg of GST proteins and glutathione-agarose (40 μl of 1:1 slurry) or 20 μg of calmodulin-agarose beads (Sigma). Agarose was collected and washed at least four times with 1 ml of Buffer B, transferring to a new tube at the 2^{nd} wash. Complexes were resolved by SDS-PAGE and

transferred to nitrocellulose membranes. CaMKII co-sedimented on the beads was detected by Ponceau-S staining or by immunoblotting.

Preparation of mouse brain extracts for immunoprecipitation

T305D, TT305/6VA, and corresponding WT forebrains were gift from Dr. Ype Elgersma (Erasmus University Rotterdam, The Netherlands). Indicated brain regions (forebrain, hippocampus, or striatum) from adult (3-4 months old) WT, T305D, TT305/6VA, or T286A mice were homogenized in low-ionic strength buffer or used for subcellular fractionation as described below and in ((Gustin et al., 2010). For experiments utilizing a low-ionic condition, brains were homogenized in Buffer C (2 mM Tris-HCl pH 7.5, 0.5% (v/v) Triton X-100, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 1 mM benzamidine, 10 µg/ml leupeptin, 10 µM pepstatin, and 1 µM microcystin-LR) in a Teflon-glass Wheaton tissue grinder with motorized plunger and incubated at 4ºC for 30-60 min. Total protein concentrations were adjusted to 2.4 mg/ml and samples were centrifuged at 9,000 X g for 10 min at 4ºC. Supernatants were mixed with goat anti-CaMKIIα (2 µg) or rabbit anti-α-actinin (8 µg) antibody, or an IgG_1 control, and incubated at 4°C overnight. After addition of GammaBind Plus-Sepharose (Amersham Biosciences) (40 µl of 1:1 slurry), incubations were continued for 2 h at 4°C. Beads were collected by microcentrifugation and washed ≥3 times with 1 ml of Buffer C containing 50 mM Tris-HCl pH 7.5 and 150 mM NaCl, transferring samples to a new tube during the first wash. Immune complexes were solubilized in SDS-PAGE sample buffer for immunoblotting.

Subcellular fractionation:

Forebrain, hippocampus, or striatum tissue was homogenized in homogenization buffer (150 mM KCl, 50 mM Tris-HCl pH 7.5, 1 mM DTT, 0.2 mM PMSF, 1 mM benzamidine, 1 μM pepstatin, 10 μg/ml leupeptin, and 1 μM microcystin-LR) using Wheaton Teflon tissue grinder at 4°C. The homogenate was passed through 22-gauge syringe twice to break any clumps. Total homogenate was then rocked for 30 minutes at 4°C and spun down at 10,000 x g for 10 minutes yielding an S1 fraction (soluble cytosolic protein pool) and a P1 pellet (insoluble fraction). P1 was resuspended in homogenization buffer containing 1% (v/v) Triton X-100 using a pipette and rocked for 30 minutes at 4°C. The homogenate was then centrifuged at 10,000 x g for 10 minutes at 4°C yielding an S2 fraction (membrane-associated protein pool) and a P2 pellet (Triton insoluble fraction). The P2 was sonicated at 4°C in homogenization buffer containing 1% (v/v) Triton X-100 and 1% (W/V) sodium deoxycholate and rocked for 30 minutes at 4°C. This is the S3/P3 fraction. Immunoprecipitation from S2 and S3/P3 fractions with anti-goat CaMKIIα, IgG, and rabbit anti-α-actinin was performed as described above.

Immunoblotting

SDS-polyacrylamide gels were transferred to nylon-backed nitrocellulose membranes in 10 mM N-Cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer. After blocking in TTBS (50 mM Tris-HCl pH 7.5, 0.1% (v/v) Tween-20, 150 mM NaCl) containing 5% Carnation nonfat milk, membranes were incubated for either 2 hr at room temperature or overnight at 4°C with primary antibodies diluted in

TTBS with 5% milk. Membranes were washed 5 times in TTBS and incubated for 1 hr at room temperature with secondary antibodies conjugated to either alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA), horseradish peroxidase (Promega or Santa Cruz Biotechnology), or infrared dyes (LiCor Biosciences, Lincoln, NE) in TTBS with 5% milk. After extensive washing, blots were developed with NBT/BCIP, by enhanced chemiluminescence (Perkin-Elmer, Waltham, MA), or using an Odyssey system (LiCor Biosciences) respectively.

Antibodies

Primary antibodies used for immunoprecipitation and immunoblotting:

CaMKII:

Goat anti-CaMKIIα (McNeill and Colbran, 1995), mouse anti-CaMKIIα (Affinity BioReagents, Golden, CO), mouse anti-CaMKIIβ (Invitrogen), rabbit anti-phospho-Thr286-CaMKII (Santacruz Biotechnology), and rabbit anti-phospho-Thr305/306-CaMKII (BIOMOL or Millipore).

α-Actinin:

Rabbit anti α-actinin H300 (Santacruz Biotechnology), mouse EA53 (Santacruz Biotechnology or Abcam), mouse BM75.2 (Sigma or Abcam), mouse A1-0.T.02 (Abcam), mouse AT6.172 (Abcam), mouse 7A4 (Abcam). The specificity and selectivity of these antibodies is characterized below.

NMDAR:

Mouse anti-GluN1 (BD Pharmingen), mouse anti-GluN2B (Transduction Laboratories), rabbit anti-phospho-Ser1303 GluN2B (Upstate), and rabbit anti-GluN2A (Millipore).

AMPAR:

Rabbit anti-GluA1 (Abcam), mouse GluA1 (Santacruz Biotechnology) and rabbit anti-phospho-Ser831-GluR1 (PhosphoSolutions).

Densin:

Rabbit R-300 was from Santa Cruz Biotechnology, Ab450 and Ab650 were prepared as described previously (Jiao et al., 2008)

Others:

Mouse anti-HA (Vanderbilt monoclonal antibody core), goat anti-HA (Bethyl Labs).

Structural Alignment

The structure of CaM bound to CaMKII regulatory domain (PDB:1CM1) was aligned with that of α-actinin-2 bound to the titin z-repeat (1H8B) using the align command of PyMOL (DeLano Scientific LLC).

Immunofluorescence co-localization

HEK293 cells expressing TT305/6AA-CaMKIIα with or without WT or Y861R HA-α-actinin-2 were fixed 24 hrs after transfection, labeled with mouse anti-CaMKIIα (1:500) and goat anti-HA (1:500) antibody. The corresponding secondary antibodies were used at 1:1000 dilution. For F-actin staining, phalloidin conjugated to Alexa fluor 647 at 1:500 dilution was used. The cells were imaged for intensity correlation (ICQ) analysis as described (Baucum et al., 2010).

Quantification and Statistics

CaMKII associated with GST proteins was quantified using ImageJ from digital scans of Ponceau-S-stained membranes, immunoblotted membranes, X-ray films, or Odyssey system. Binding of purified CaMKII was normalized to recovered GST-fusion protein, and background binding to GST alone was subtracted. Data were analyzed by one-way or two-way ANOVA with post-hoc Tukey or Dunnett's tests. CaMKII activation curves were fitted to one-site binding curve using GraphPad Prism. CaMKII inhibition curves (plotted as a % of the "no inhibitor" control) were fitted to sigmoidal dose-response models using GraphPad Prism, constraining top and bottom values to 100 and >0, respectively.

Characterization of α-actinin antibodies:

There are 4 isoforms of α-actinin (see Introduction), of which, α-actinin-1 and -2 are shown to be present in brain. α-Actinin-3 is not detected in the brain using immunocytochemistry or immunoblotting (Wyszynski et al., 1998). It is not clear if α-actinin-4 is present in the brain. To better understand the role of each isoform in regulating CaMKII, we wanted to test isoform specificity of available antibodies. Most of the prior studies have used α-actinin antibodies for immunofluorescence studies, while few studies have utilized these antibodies for immunoprecipitation or immunoblotting. Hence I wanted to test the isoform specificity of various commercial antibodies in immunoblotting and immunoprecipitation. I used a panel of purified GST-tagged α-actinin-1 and -2 proteins along with brain extracts and cell lysates to determine the isoform specificity (Fig. 2.2). Moreover, these tools allowed for identification of the region on α-actinin that is recognized by the

different antibodies. The rabbit α-actinin H-300 antibody recognizes both the

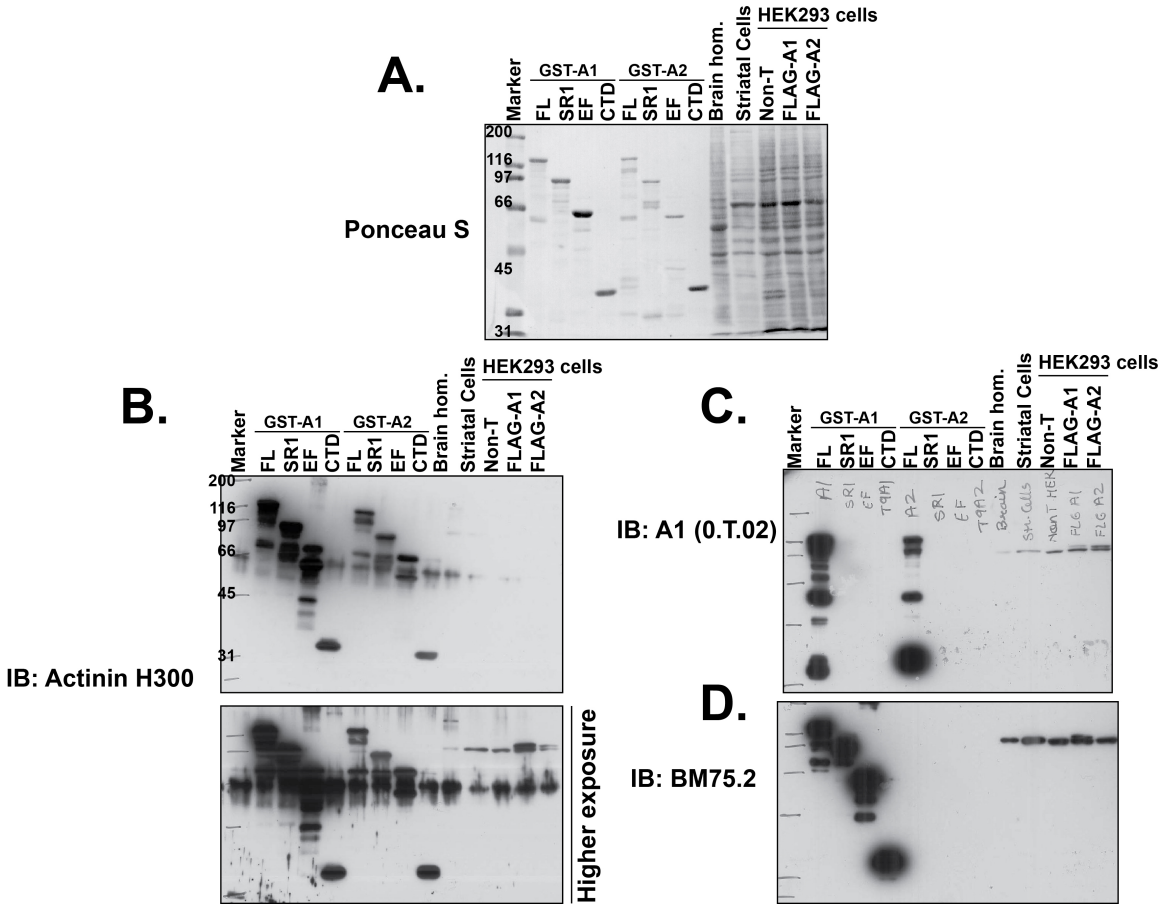

Figure 2.2 Characterization of α-Actinin antibodies

A. Purified fragments of GST α-Actinin-1 (A1) and α-actinin-2 (A2), along with brain homogenate (hom.), cell extracts from striatal cells, non-transfected (Non-T) HEK293 cells, FLAG-A1 or FLAG-A2 transfected cells were resolved by SDS-PAGE and stained for total protein (Ponceau S) and then analyzed by immunoblotting with different α-actinin antibodies **B.** rabbit α-actinin-H300 (1:1000), **C.** mouse A1-0.T.02 (1:2000), **D.** mouse BM 75.2 (1:1000). Findings are summarized in table 2.2.

isoforms (1 and 2), and also all the truncation mutants of α-actinin, confirming the epitope for this antibody is in the extreme C-terminus of the protein (Fig. 2.2B). This antibody also recognized a band at ~100Kd from brain extracts, striatal cells and non-transfected HEK293 cells. Two bands corresponding to the endogenous

α-actinin and transfected FLAG-tagged α-actinin-1 and -2 can be seen in the transfected cell lysates lanes. On the other hand, although the mouse α-actinin antibody A1-0.T.02 recognized both the isoforms α-actinin-1 and -2, it could only bind to the full-length protein, suggesting the epitope is in the N-terminal region of the protein (Fig. 2.2C). While both the H300 and A1-0.T.02 antibodies recognized α-actinin-1 and -2, the mouse BM75.2 antibody recognized only α-actinin-1. Moreover, the epitope appears to be localized to the C-terminus as all the truncation mutants of α-actinin-1 were detected by this antibody. There is endogenous α-actinin in HEK293 cells as detected by various antibodies in non-transfected cell lysates (Fig. 2.2). Thus, with the H300 and A1-0.T.02 antibody, two bands corresponding to the endogenous and transfected α-actinin were observed (Fig. 2.2B and C). The specificity of BM75.2 antibody to α-actinin-1 is also evident from two bands detected in FLAG-A1 transfected cell lysate versus a single band detected in FLAG-A2 transected (Fig. 2.2D). Thus, HEK293 cells endogenously express α-actinin-1, or other isoform but not α-actinin-2.

Next, I transfected HEK293 cells with HA-α-actinin-1 (HA-A1) or HA-α-actinin-2 (HA-A2) alone or in combination with untagged α-actinin-2 (A2) and GFP-tagged α-actinin-1 (GFP-A1) respectively (Fig. 2.3). The cell lysates were immunoprecipitated with control (IgG) or HA antibody. Protein stain of the immune complex shows approximately equal HA protein precipitated across different conditions. Analysis of the blots indicated that α-actinin-1 and -2 can heterodimerize in the cells (co-precipitation of GFP-A1 with HA-A2). Furthermore, the specificity of the BM75.2 antibody observed with purified proteins, was also

evident with transfected proteins as there was a stronger band when HA-A1 was immunoprecipitated compared to immunoprecipitated HA-A2. The mouse EA53 antibody also showed specificity for α-actinin-2 over α-actinin-1 (band detected for HA-A2 immunoprecipitate versus no band for HA-A1). However, the mouse AT6.172 as well as the H300 antibody recognized both isoforms (Fig. 2.3).

Further I wanted to test the ability of different antibodies to immunoprecipitate endogenous and overexpressed α-actinin from HEK293 cells. Hence non-transfected, HA-A1, and HA-A2 expressing cell lysates were immunoprecipitated with control (IgG) or mouse AT6.172, BM75.2, EA-53, and HA antibodies (Fig. 2.4). Upon protein staining and immunoblotting with the α-actinin H300 antibody (recognizes both isoforms and endogenous actinin), it was apparent that only the EA53 antibody could immunoprecipitate α-actinin, and specifically HA-A2 (Fig. 2.4). The amount of α-actinin immunoprecipitated with this antibody was comparable to that with the HA antibody.

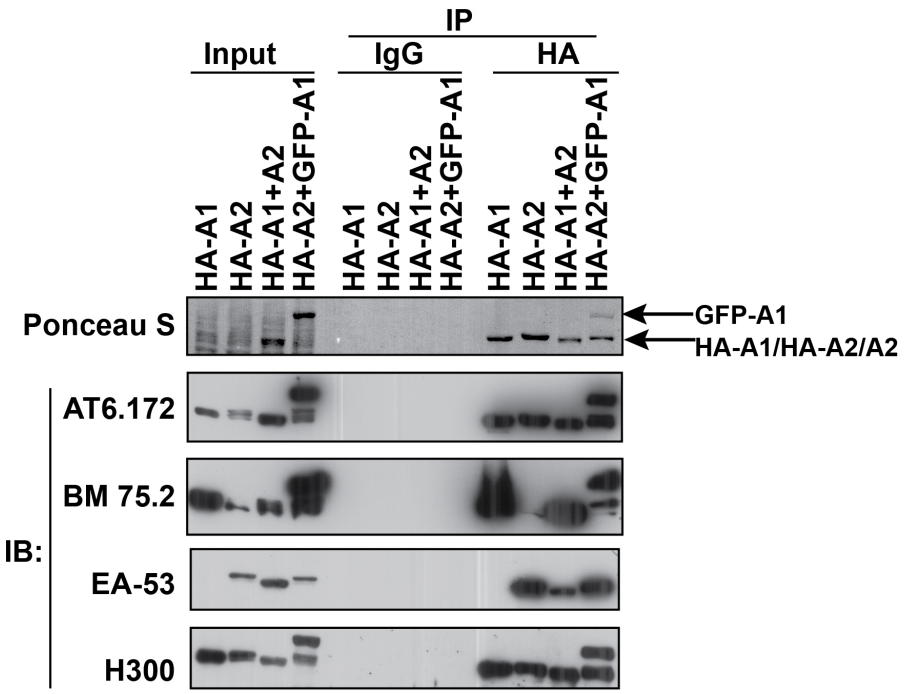

Figure 2.3 Heterodimerization of α-actinin-1 and -2 in HEK293 cells

HEK293 cell lysates (Input) expressing HA-α-actinin-1 (HA-A1) or HA-α-actinin-2 (HA-A2) alone or with untagged α-actinin-2 (A2) and GFP-α-actinin-1 (GFP-A1) respectively were immunoprecipitated with control (IgG) or HA antibody. Immune complexes isolated on Protein G beads were stained for total protein (Ponceau S) or immunoblotted with various α-actinin antibodies (AT6.172, BM75.2, EA-53, and H300).

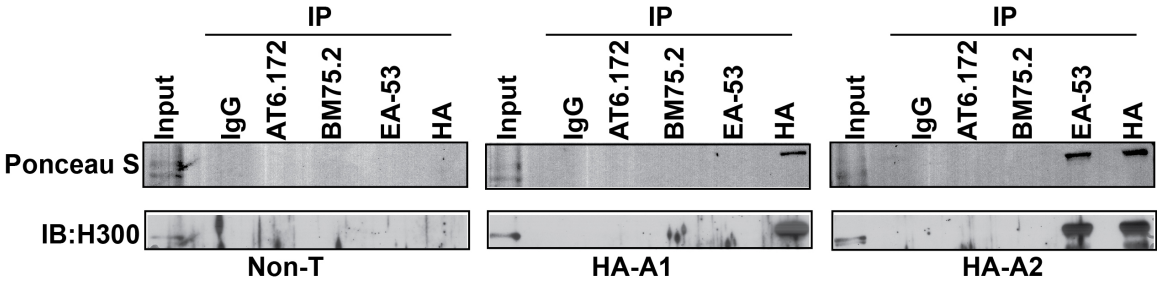

Figure 2.4 Immunoprecipitation of α-actinin with different antibodies

Lysates (Input) of HEK293 cells either non-transfected (Non-T) or transfected with HA-actinin-1 (HA-A1), or HA-actinin-2 (HA-A2) were immunoprecipitated with control (IgG), various α-actinin antibodies (AT6.172, BM75.2, EA-53), or HA antibody. Immune complexes isolated on protein G beads were stained for total protein (ponceau S) or immunoblotted (IB) with rabbit α-actinin H300 antibody.

In summary, the α-actinin antibodies characterized here could detect endogenous α-actinin in different cell lysates. Moreover, some antibodies are isoform specific. Also these experiments suggest that the endogenous α-actinin in HEK293 cells is α-actinin-1 or -4, as both are non-muscle isoform) (Figs. 2.2, 2.3 and 2.4). I did not have α-actinin-4 specific antibody to test this, however, there is one available that can be used. I also show that α-actinin-1 and -2 can heterodimerize in cells (Fig. 2.3) as was previously reported for α-actinin-2 and -3 (Chan et al., 1998). These results are summarized in Table 2.2.

	Immunoblot		**Immunoprecipitation**	
Primary Antibody	**α-Actinin-1**	**α-Actinin-2**	**α-Actinin-1**	**α-Actinin-2**
Rabbit H-300 (Santacruz)	✔	✔	✔	✔
Mouse A1 (0.T.02) (Abcam)	✔	✔	?	?
Mouse BM75.2 (Abcam)	✔		✖	✖
Mouse EA53 (Santacruz or Abcam)		✔	✖	✔
Mouse AT6.172 (Abcam)	✔	✔	✖	✖
Mouse 7A4 (Abcam)	?	?	?	?

Table 2.2 Summary of α-actinin antibodies characterization

CHAPTER III

CHARACTERIZATION OF A CENTRAL CaMKIIα/β-BINDING DOMAIN IN DENSIN THAT SELECTIVELY MODULATES GLUTAMATE RECEPTOR SUBUNIT PHOSPHORYLATION

This project was done in collaboration with a postdoctoral fellow in our lab, Dr. Yuxia Jiao. Here I will include mostly the data collected by me, with some of Yuxia's data. The work is published in (Jiao Jalan-Sakrikar et al., 2011)

Introduction:

Activation of N-methyl D-Aspartate receptors (NMDARs) and/or voltage-gated Ca^{2+}-channels induces postsynaptic Ca^{2+} signals that vary in frequency, duration and amplitude. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) decodes these signals to elicit multiple cellular responses, especially during learning and memory (see Chapter I). Precise targeting of CaMKII to specific subcellular compartments containing upstream activators, other modulators and/or specific substrates is thought to modulate the specificity and efficiency of CaMKII actions (Colbran, 2004; Schulman, 2004). Neuronal postsynaptic densities (PSDs) contain several CaMKII-associated proteins (CaMKAPs), such as NMDAR GluN2B subunits, α-actinin and densin, that presumably collaborate to dynamically control CaMKII targeting to, and perhaps within, the PSD during synaptic activation. Whereas CaMKII binding to GluN2B is required for some forms of LTP (Barria and Malinow, 2005; Zhou et al., 2007), specific roles of other CaMKAPs are less well understood.

Densin was identified as a PSD-enriched protein that can bind CaMKII (Apperson et al., 1996; Strack et al., 2000b; Walikonis et al., 2001) (Apperson et al., 1996; Strack et al., 2000b; Walikonis et al., 2001). Like other LAP protein family members, densin contains **L**eucine-rich repeats (LRRs) at the N-terminus **a**nd a C-terminal **P**DZ domain (Apperson et al., 1996). The LRR domain was originally proposed to be extracellular, but more recent studies indicate that densin is entirely intracellular and associates with plasma membranes, at least in part via the LRR domain (Izawa et al., 2002; Jiao et al., 2008; Thalhammer et al., 2009). Several postsynaptic proteins can interact with the densin PDZ domain (e.g., α-actinin, L-type calcium channel (LTCC) $Ca_V1.3$ α_1 subunit) (Jenkins et al., 2010; Robison et al., 2005b; Walikonis et al., 2001) or other C-terminal domains (e.g., CaMKIIα, SHANK, δ-catenin) (Izawa et al., 2002; Jiao et al., 2008; Quitsch et al., 2005; Strack et al., 2000b). These data suggest that densin is an archetypical scaffolding protein that assembles multi-protein complexes to enhance postsynaptic signaling specificity and efficiency at the plasma membrane. Consistent with this model, densin is required for CaMKIIα to elicit a novel form of $Ca_V1.3$ LTCC facilitation (Jenkins et al., 2010).

CaMKII genes (α, β, γ, δ) are differentially expressed in most if not all mammalian tissues and share 80-90% amino acid sequence identity in their catalytic and regulatory domains (Tobimatsu and Fujisawa, 1989). Consequently, their intrinsic catalytic and regulatory properties are very similar, with the most notable difference being ≈10-fold variations in the affinity for Ca^{2+}/calmodulin (Brocke et al., 1999). However, they are more divergent in C-terminal association

domains that are responsible for assembly of dodecameric CaMKII holoenzymes. CaMKII isoforms also may be differentially targeted in cells, for example to the F-actin cytoskeleton or to the nucleus (Brocke et al., 1995; Shen et al., 1998; Srinivasan et al., 1994). Initial studies found that the C-terminal association domain of CaMKIIα, but not CaMKIIβ, interacts with a CaMKII binding site immediately preceding the C-terminal PDZ domain of densin (Strack et al., 2000b; Walikonis et al., 2001), suggesting that densin is a CaMKIIα-specific CaMKAP. Indeed, this C-terminal CaMKII-binding site is sufficient to associate with and target CaMKIIα in intact cells (Jiao et al., 2008; Strack et al., 2000b). However, densin variants are expressed in embryos and in the early postnatal period, prior to expression of CaMKIIα (Jiao et al., 2008). Moreover, densin-dependent facilitation of $Ca_V1.3$ LTCCs by CaMKIIα is ablated by deletion of a large intracellular domain in densin (Δ483-1377), even though this protein retains the LRR, C-terminal CaMKIIα-binding, and PDZ domains. Surprisingly, this internal deletion also reduced the association of densin with CaMKIIα (Jenkins et al., 2010).

Here we show that densin associates with both CaMKIIα and CaMKIIβ in the brain and in transfected HEK293 cells. These interactions involve a second central/internal CaMKII binding site, the densin-IN domain, which displays 50% sequence similarity to the CaMKII inhibitor protein, CaMKIIN. The densin-IN domain interacts with catalytic domains of either isoform, but the densin C-terminal domain interacts efficiently only with CaMKIIα. Further the densin-IN domain differentially modulates CaMKII phosphorylation of AMPAR GluA1

subunits and NMDAR GluN2B subunits, suggesting a unique mechanism for fine-tuning CaMKII activity towards different substrates.

Results:

Densin associates with either CaMKIIα or CaMKIIβ

Initial *in vitro* studies found that densin is a CaMKIIα-specific interaction partner, and also that it binds in the association domain of CaMKIIα (Jiao et al., 2008; Strack et al., 2000b; Walikonis et al., 2001). However, analysis of CaMKII immune complexes isolated from CaMKIIα-knockout mouse brain with isoform specific antibodies show similar amounts of densin co-precipitating with CaMKIIβ (Fig. 3.1), suggesting that densin can associate with other CaMKII isoforms *in vivo*. Upon further investigation we found that a peri-natal densin-FLC variant

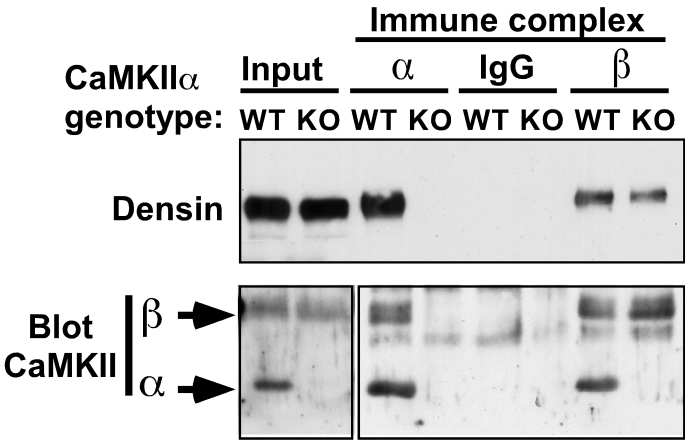

Figure 3.1 Densin associates with CaMKIIβ in mouse forebrain
Forebrain extracts from WT or CaMKIIα knockout (KO) mice were immunoprecipitated with antibodies to CaMKIIα, CaMKIIβ or control (IgG). Immune complexes were then blotted for both isoforms of CaMKII and co-precipitated densin.

lacking the C-terminal CaMKII binding domain also bound CaMKIIα in HEK293 cells (Fig. 2A and B in (Jiao Jalan-Sakrikar et al., 2011). Furthermore, CaMKIIβ and δ also co-immunoprecipitated with densin-FLA or –FLC when co-expressed in HEK293 cells, again implying that multiple CaMKII isoforms can associate with full-length densin independent of the known C-terminal domain CaMKII-binding domain (Jiao Jalan-Sakrikar et al., 2011), suggesting that densin contains an additional CaMKII-binding domain.

Characterization of a second CaMKII-binding domain in densin

Since there is no detectable interaction of CaMKIIα with the N-terminal LRR domain of densin (densin-T482 splice variant) (Jiao et al., 2008), CaMKII may interact somewhere between the LRR and C-terminal domains, in addition to the originally identified C-terminal CaMKII-binding domain. Consistent with this hypothesis, deletion of a large central domain (residues 483-1377) in a naturally occurring densin splice variant substantially reduced the binding of CaMKIIα, even though this variant retains the C-terminal CaMKIIα binding domain (Jenkins et al., 2010). Therefore, we expressed a family of GST fusion proteins spanning the large central domain of densin in bacteria (Fig. 3.2). Using this panel of GST-fusion proteins spanning the central domain of densin, we identified residues 793-824 as a novel **IN**ternal CaMKII-binding domain that was termed the densin-IN domain (refer Figs. 3C and D in (Jiao Jalan-Sakrikar et al., 2011)). Further we found that CaMKII activation by Ca^{2+}/CaM, or Thr286/7 (CaMKIIα/β) autophosphorylation in CaMKII is required for interaction with the densin-IN domain, unlike the C-terminal domain, which can associate with CaMKII

independent of the kinase activation (Fig. 4 (Jiao Jalan-Sakrikar et al., 2011)). The requirement of CaMKII activation for binding to the densin-IN domain is similar to that for GluN2B binding to CaMKII (Robison et al., 2005a). Parallel analysis with GST-GluN2B revealed similarities with subtle differences between GluN2B and densin-IN domain for binding to CaMKII.

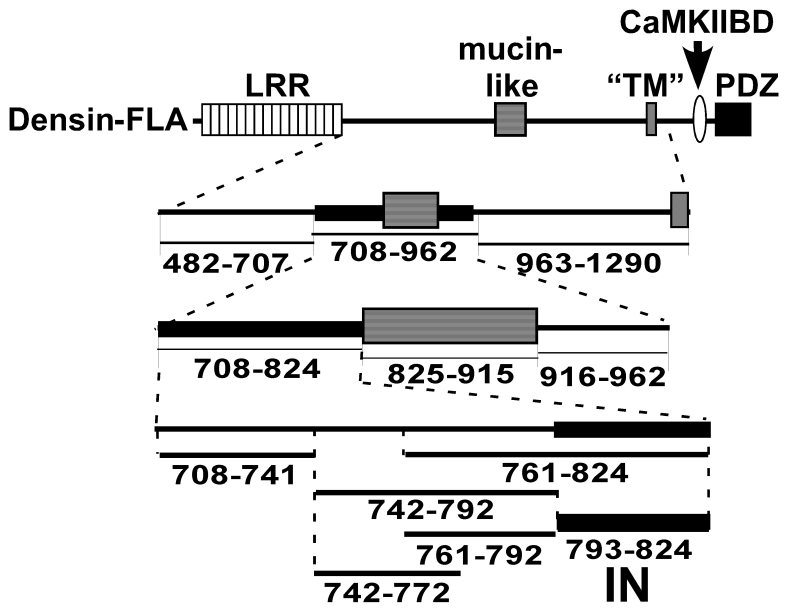

Figure 3.2 Novel CaMKII-binding domain in densin
Summary of truncation mutants created to identify the densin-IN domain. Thicker lines denote the major CaMKII-binding fragments.

In order to further characterize the role of the catalytic domain of CaMKII in binding to the densin-IN domain, several synthetic peptides known to interact with the CaMKII catalytic domain were tested as potential competitors. Syntide-2, a model substrate peptide based on glycogen synthase, had no effect. In contrast, binding was modestly, but significantly, reduced by ≈25% in the presence of autocamtide-2 (AC-2), a substrate peptide based on the Thr286 autophosphorylation site in CaMKIIα, or N2B-tide, a homologous peptide based

on the Ser1303 phosphorylation site in GluN2B (Fig. 5B in (Jiao Jalan-Sakrikar et al., 2011)). Moreover, N-tide, an inhibitory peptide based on the naturally occurring CaMKIIN inhibitor protein (Chang et al., 1998), almost completely blocked binding to GST-D-IN. These data suggest that the densin-IN domain interacts with a region of the CaMKIIα catalytic domain that overlaps the CaMKIIN-binding site, perhaps also in proximity to binding sites for AC-2 and GluN2B.

A region of the CaMKIIα catalytic domain, termed the T-site (see chapter I) appears to be critical for binding to CaMKIIN, GluN2B and the CaMKII autoinhibitory domain (Bayer et al., 2001; Bayer et al., 2006; Vest et al., 2007; Yang and Schulman, 1999). The role of T-site in mediating the interactions with densin-IN domain was investigated using T-site mutants (I206K and D239R in CaMKIIβ or I205K in CaMKIIα). Loss of binding to the Ile to Lys mutant suggested that both densin-IN and GluN2B interact at the T-site of CaMKIIα or CaMKIIβ. However, the Asp to Arg mutant indicated that interactions of densin-IN and GluN2B with the catalytic domain of CaMKII are somewhat distinct (Fig. 5C in (Jiao Jalan-Sakrikar et al., 2011)), supporting the interpretation of studies comparing the regulation of CaMKII binding and peptide competition studies (see above).

The densin-IN domain is homologous to CaMKIIN

The amino acid sequence of the C-terminal region of the densin-IN domain is similar to that of central/C-terminal regions of N-tide, with 11 identical or similar

residues (Fig. 3.3A). Possible roles of selected conserved charged or hydrophobic residues in densin were probed by site-directed mutagenesis.

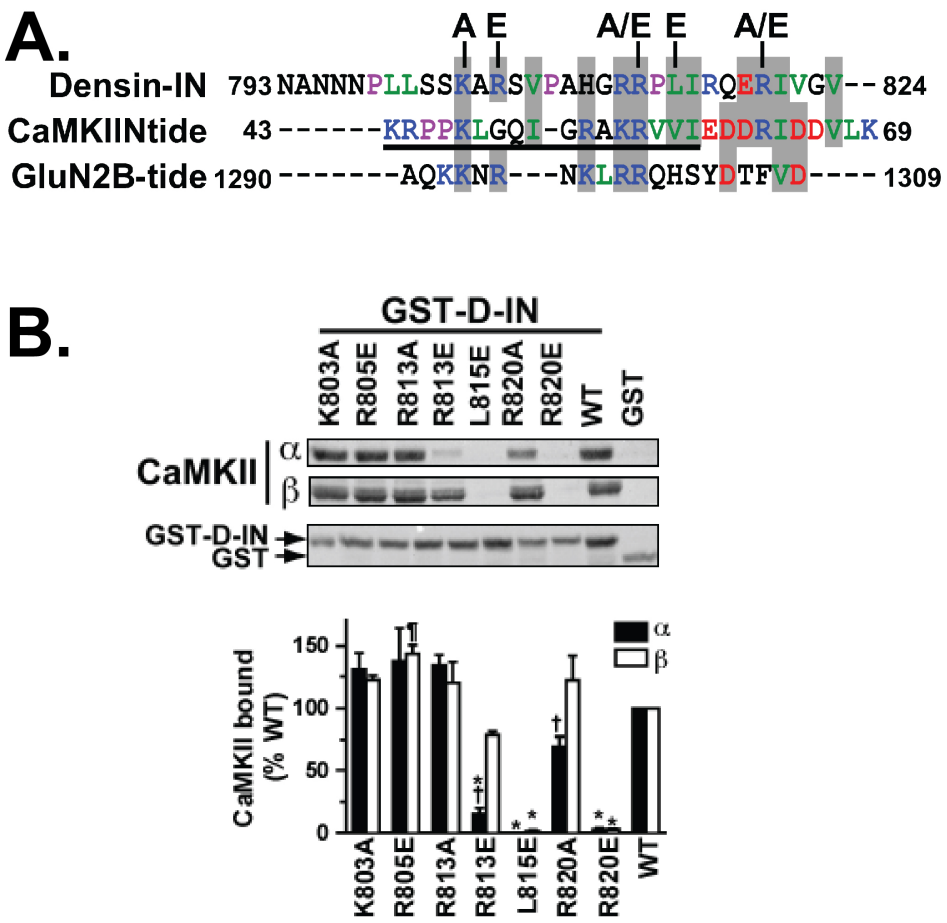

Figure 3.3 Densin-IN domain is homologous to CaMKIIN
A. Aligned amino acid sequences of the densin-IN domain, the inhibitory domain peptide of CaMKIINα and a CaMKII-binding domain peptide from GluN2B, with identical or similar residues in gray boxes. Residues are color coded for basic (blue), acidic (red), or hydrophobic (green) side chains. Pro residues are in purple. The underlined region of CaMKIIN-tide was resolved in an X-ray crystal structure of the CaMKII-CaMKIIN-tide complex (PDB:3KL8)(Chao et al., 2010a). Densin-IN domain mutants characterized in B are indicated above. **B.** Thr286/7-autophosphorylated CaMKIIα or β were incubated with GST-D-IN (WT or mutants) or GST. Complexes isolated on glutathione-agarose beads were analyzed by protein staining. CaMKII bound is expressed as a percentage of binding to WT (mean ± sem, n=3). †, $p<0.05$ compared to binding of CaMKIIβ (2-way ANOVA with Bonferoni post-test).*, p,<0.001 compared to WT (1-way ANOVA with Dunnett's multiple comparison test). ¶, $p<0.05$ compared to WT (1-way ANOVA with Dunnett's multiple comparison test).

Replacement of Leu815 or Arg820 with an acidic Glu in GST-D-IN reduced the binding of autophosphorylated CaMKIIα or CaMKIIβ by >95% (Fig. 3.3B). However, binding was modestly enhanced ≈40% by an R805E mutation. Mutations that only removed basic charge (K803A, R813A, or R820A) had no statistically significant effect on interactions with either isoform in this set of three experiments. However, there was a significant difference in the relative effects of R820A and R813E mutations on binding of CaMKII isoforms ($p<0.05$). Binding of CaMKIIα to the R813E mutant was significantly reduced by ≈85%, but the modest ≈20% reduction in binding of CaMKIIβ to the R813E mutant was not statistically significant (Fig. 3.3B). R820A mutation had no significant effect on binding of CaMKIIβ (Fig. 3.3B), but combining data from Fig. 6B with data from additional experiments comparing effects of all mutations on CaMKIIα binding alone, showed that R820A mutation significantly, if modestly, reduced binding of CaMKIIα to 74±4% of the WT ($N=8$; $p<0.05$. Dunnett's). Taken together, these data suggest an important role for hydrophobic interactions between the CaMKII catalytic domain and residues surrounding Leu815 in the densin-IN domain that are conserved in CaMKIIN. Furthermore we showed that the Leu815 residue is critical for interaction with CaMKIIα or β in the context of full-length densin in HEK293 cells (Figs. 6C and D in (Jiao Jalan-Sakrikar et al., 2011)).

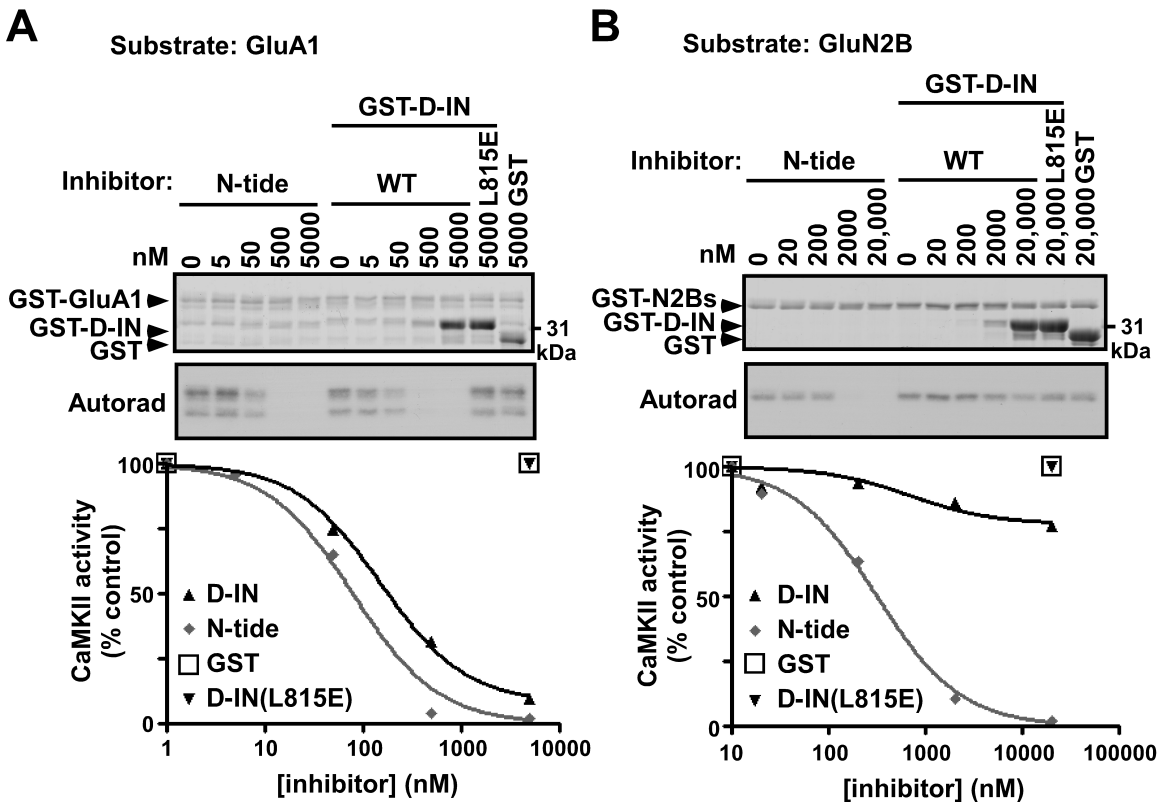

Figure 3.4 Densin-IN potently inhibits CaMKII phosphorylation of GluA1 but not GluN2B

A. GST-GluA1 was incubated with Thr286-autophosphorylated CaMKIIα, [γ^{32}P]ATP and the indicated concentrations of GST-D-IN (WT or L815E), N-tide, or GST. *Top:* Reaction aliquots were analyzed by SDS-PAGE followed by coomassie blue staining and autoradiography. *Bottom:* Quantitative analysis using scintillation counting: the mean of duplicates is plotted. Data are representative of four independent experiments. **B.** Similar to A, except with GST-GluN2B as the substrate.

The densin-IN domain is a substrate-dependent CaMKII inhibitor

Since CaMKIIN inhibits CaMKII (Chang et al., 1998), we hypothesized that densin would also inhibit CaMKII. Indeed, GST-D-IN potently and completely inhibited the phosphorylation of syntide-2, a model peptide substrate, by purified CaMKIIα or CaMKIIβ with comparable potencies (EC_{50}'s 49±12 nM, n=2 or 56±13 nM, n=3, respectively) similar to those of N-tide (a peptide analog of CaMKIIN) in parallel studies (EC_{50}'s 87±10 nM, n=2 or 67±24 nM n=3,

respectively). We then tested the effect of densin-IN on CaMKIIα phosphorylation of physiologically relevant synaptic substrates. Phosphorylation of GST-GluA1, containing residues 816-889 of GluA1 including the Ser831 phosphorylation site (Barria et al., 1997a), was potently and >90% inhibited by both GST-D-IN and N-tide (Fig. 3.4A) (EC_{50}'s 100±23 and 72±22 nM, respectively; n=4 similar experiments). Moreover, inhibition by GST-D-IN was completely prevented by the L815E mutation, which disrupts CaMKII binding. On the other hand, phosphorylation of GST-N2Bs, containing residues 1260-1309 of GluN2B including the Ser1303 phosphorylation site (Omkumar et al., 1996; Strack et al., 2000a), was only modestly inhibited by even micromolar concentrations of GST-D-IN, but was completely inhibited by N-tide (Fig. 3.4B), albeit with somewhat reduced potency (EC_{50} 380±42 nM, n=3). Technical limitations restricted use of higher concentrations of densin-IN. The extrapolated CaMKII activity at saturating concentrations of GST-D-IN was 79±5% (n=3) with an estimated half-maximal effective concentration of 571±268 nM, not significantly different from the potency of N-tide toward GluN2B phosphorylation. (The low efficacy of inhibition presumably accounts for the increased variability in the estimated half-maximal effective concentration.) Thus, the efficacy of densin-IN, but not CaMKIIN, as a CaMKII inhibitor appears to be highly substrate-dependent.

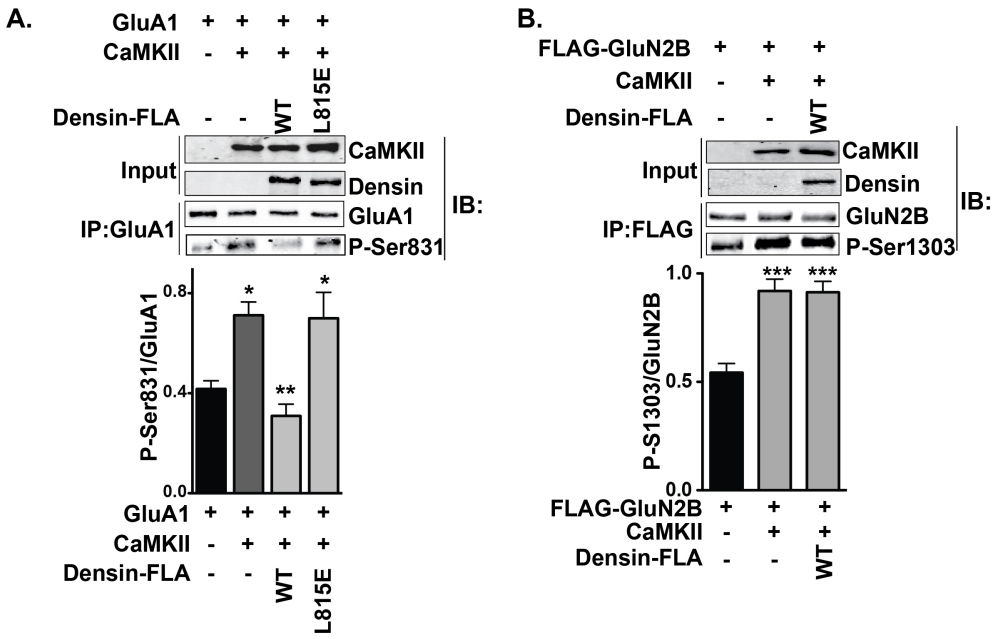

Figure 3.5 Densin inhibits phosphorylation of GluA1, but not GluN2B, in intact cells

Triton-soluble fractions (Inputs) were prepared from HEK293 cells expressing GluA1 or FLAG-GluN2B with or without CaMKIIα and/or densin as indicated and then immunoprecipitated. **A.** GluA1 immune complexes or inputs were immunoblotted for total GluA1, phospho-Ser831 GluA1 (P-Ser831), CaMKII, or densin as indicated. The graph summarizes analyses of Ser831 phosphorylation normalized to GluA1 from 5 similar experiments. *, $p<0.05$ compared to GluA1 alone. **, $p<0.01$ compared to GluA1+CaMKII. ##, $p<0.01$ compared to GluA1+CaMKII+L815E-densin. **B.** FLAG-GluN2B complexes or inputs were immunoblotted for total GluN2B, phospho Ser1303 GluN2B (P-S1303), CaMKII, or densin, as indicated. The graph summarizes analyses of Ser1303 phosphorylation normalized to total GluN2B from 4 similar experiments. ***, $p<0.0001$ compared to GluN2B alone.

Selective inhibition of glutamate receptor phosphorylation by densin in intact cells

In order to begin to explore the effects of the major adult splice variant of densin on CaMKII activity in cells, we compared phosphorylation of full-length GluA1 and GluN2B by CaMKII in transfected HEK293 cells in the absence and presence of densin-FLA.

Co-expression of CaMKIIα significantly enhanced the phosphorylation of GluA1 at Ser831. Densin-FLA significantly reduced the effect of over-expressed CaMKIIα on Ser831 phosphorylation, but L815E mutation in the densin-IN domain essentially abrogated this effect (Fig. 3.5A). In contrast, densin-FLA had no effect on CaMKII-stimulated phosphorylation of Ser1303 in GluN2B (Fig. 3.5B). Moreover, densin-FLA had no effect on the level of Thr286 autophosphorylation of CaMKIIα, or on the amount of CaMKIIα that co-

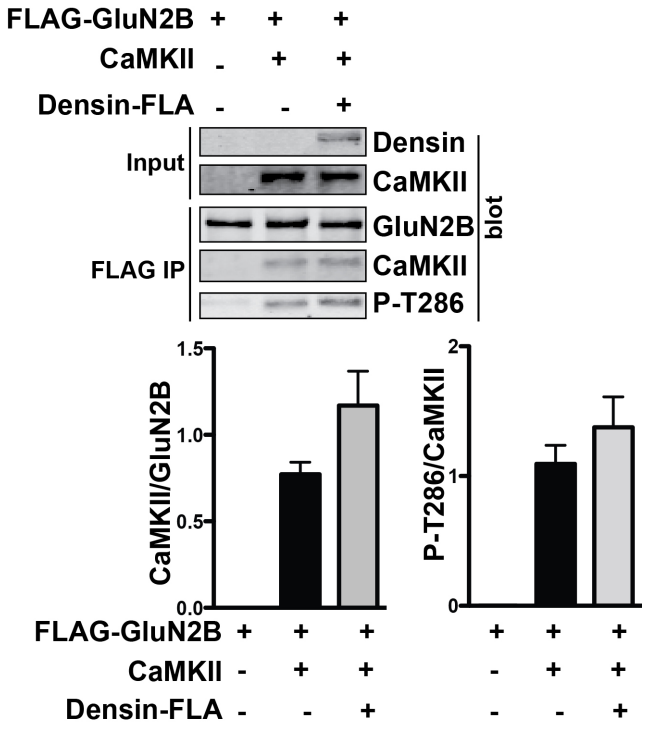

Figure 3.6 CaMKII association with GluN2B in presence of densin-FLA
HEK293 cell lysates expressing FLAG-GluN2B alone or with CaMKII in presence or absence of densin-FLA were immunoprecipitated (IP) with FLAG antibody. Input and protein complexes isolated on Protein G beads were analyzed by immunoblotting for indicated proteins (Top). *Below:* Graphs showing the amount of CaMKII co-immunoprecipitated with FLAG-GluN2B (left) and levels of CaMKII phospho-Thr286 (right) in presence or absence of densin-FLA, n=5.

precipitated with the FLAG-GluN2B (Fig. 3.6). These data show that densin-FLA can selectively inhibit CaMKII activity toward some, but not all, substrates in intact cells.

Discussion:

Precise physiological control of cell signaling depends on scaffolding and/or anchoring proteins (Pawson and Scott, 2010). For example, conserved amphipathic α-helical domains in a large family of A-kinase anchoring proteins (AKAPs) competitively interact with type II regulatory subunits to target PKA subpopulations to complexes that also contain diverse upstream regulators and downstream targets (Wong and Scott, 2004). Similarly, PP1 targeting subunits containing "RVXF" sequence motifs compete for a conserved hydrophobic groove on the surface of PP1 catalytic subunits to target phosphatase activity (Bollen et al., 2010). However, CaMKAPs are mechanistically more diverse. NMDAR GluN2B subunits and voltage-gated calcium channel β_{2a}/β_{1b} subunits contain CaMKII-binding domains that share sequence similarity with the autoinhibitory/regulatory domain of CaMKII. These domains compete for binding to the CaMKII catalytic domain, and each contains a key phosphorylation site that weakens the interaction (GluN2B: Ser1303. β_{2a}/β_{1b}: Thr498. CaMKIIα/β: Thr286/287) (Bayer et al., 2001; Grueter et al., 2006; Grueter et al., 2008; Strack et al., 2000a). SAP97 also binds to the CaMKII catalytic domain (Nikandrova et al., 2010), even though there is no obvious sequence similarity with GluN2B, β_{2a}/β_{1b} or CaMKIIα/β. In contrast, α-actinin appears to bind the calmodulin-binding domain of CaMKII (Robison et al., 2005b). Moreover, a C-terminal

domain in densin was shown to interact with the association domain of CaMKIIα, but not CaMKIIβ (Strack et al., 2000b; Walikonis et al., 2001), indicating that densin is a CaMKIIα-selective CaMKAP. These diverse interaction mechanisms allow dodecameric CaMKII holoenzymes to form a scaffold for assembly of multi-protein complexes (Robison et al., 2005b). Indeed, CaMKII can have synaptic roles that require scaffolding functions that are independent of ongoing kinase activity (but may be modulated by autophosphorylation) (Bingol et al., 2010; Hojjati et al., 2007; Pi et al., 2010). The present studies provide new insights into the complexity and function of protein-protein interaction mechanisms employed by CaMKII.

Our data show that rather than being CaMKIIα-specific; densin can target multiple CaMKII isoforms *in vivo* (Fig. 3.1). CaMKIIα is first expressed at about postnatal day 5 in rodents, and in adulthood is highly expressed in many, but not all, neurons in the forebrain, as well as in cerebellar Purkinje neurons. In contrast, CaMKIIβ splice variants are broadly expressed in many neuronal subtypes throughout development. The ratio of CaMKII isoform expression is thought to dictate the subunit composition of CaMKII holoenzymes. Long-term changes in synaptic activity antagonistically regulate CaMKIIα and CaMKIIβ expression levels (Thiagarajan et al., 2002). Indeed, local CaMKIIα protein synthesis in neuronal dendrites is controlled by synaptic activity and is necessary for normal synaptic plasticity, learning and memory (Aakalu et al., 2001; Ouyang et al., 1999). Thus, neurons likely contain distinct mixtures of CaMKII holoenzymes (CaMKIIβ-specific, CaMKIIα-specific or with varying isoform ratios), depending

on developmental stage and specific cell type. For example, α:β isoform ratios in CaMKII holoenzymes purified from adult forebrain and cerebellum are ≈4:1 and ≈1:4, respectively (McGuinness et al., 1985; Miller and Kennedy, 1985). Thus, investigations of physiological roles for densin in modulating CaMKII signaling need to be expanded to include neurons and other cell types that do not express CaMKIIα.

In addition to showing that the major adult densin-FLA variant can associate with CaMKIIβ, deletion of the known C-terminal CaMKII-binding domain (in the naturally-occurring densin-FLC variant) has only a modest effect on the binding of either CaMKII isoform. These observations were explained by identification of a second **in**ternal CaMKII-binding domain in densin (residues 793-824) (Fig. 3.2), which appears to interact with the catalytic domain of both CaMKII isoforms, but only following kinase activation. Although CaMKIIα holoenzymes can also interact with the densin-CTA domain (at least partially independent of activation), mutagenesis studies suggest that the densin-IN domain plays a dominant role in binding to both CaMKIIα and CaMKIIβ in heterologous cells (Jiao Jalan-Sakrikar et al., 2011). Numerous densin mRNA splice variants are differentially expressed across brain development (Jiao et al., 2008; Strack et al., 2000b). Several splice variants expressed during embryonic or early postnatal development lack the densin-IN and/or -CTA domains, presumably indicating that densin can have diverse roles, in some cases independent of CaMKII. For example, although densin-FLC may be expressed only in the peri-natal period (Strack et al., 2000b), prior to CaMKIIα expression,

the IN domain may allow densin-FLC to modulate CaMKIIβ. In addition, we previously showed that a natural densin splice variant lacking a large central region containing the densin-IN domain is unable to support CaMKIIα-dependent facilitation of $Ca_V1.3$ LTCCs (Jenkins et al., 2010). A deeper understanding of the expression of densin splice variants in different cell types during development is required in order to fully appreciate the implications of these findings for CaMKII isoform regulation.

Activation of CaMKII isoforms is required for interactions with both the densin-IN domain and with GluN2B, in contrast to CaMKIIα binding to the densin-CTA domain. Subtle differences in requirements for CaMKIIα/β binding to densin-IN and GluN2B (Fig 4 in (Jiao Jalan-Sakrikar et al., 2011) may relate to the fact that CaMKIIβ has an ≈10-fold higher affinity for Ca^{2+}/calmodulin binding than does CaMKIIα (Brocke et al., 1999). These differences may be relevant to understanding the role of densin in targeting the two CaMKII isoforms in intact cells. In addition, they suggest subtle differences in the mechanisms by which GluN2B and densin-IN bind to CaMKII isoforms (see below).

The densin-IN domain has significant amino acid sequence similarity with the core inhibitory domain of CaMKIIN, a naturally occurring CaMKII inhibitor protein (Chang et al., 1998). N-tide, a peptide analog containing residues 43-69 of CaMKIIN (Chang et al., 1998) (termed CN27 by Vest et al (Vest et al., 2007)), competes with densin-IN for binding CaMKII *in vitro*. In a crystal structure of CaMKII bound to an N-tide variant (Chao et al., 2010b), amino acid side chains in the N-terminal and central regions of N-tide (underlined in Fig. 3.3A:

corresponding to the CN17a peptide described in (Vest et al., 2007)) interact with a series of binding pockets on the catalytic domain that can also be partially occupied by the regulatory domain in autoinhibited CaMKII (Rellos et al., 2010; Rosenberg et al., 2005), and that have been collectively termed the T-site (Vest et al., 2007). Consistent with this structure, potent inhibition by N-tide is highly dependent on N-terminal Lys^{43}-Arg-Pro residues (Vest et al., 2007): however, these residues are not conserved in densin-IN (Leu^{799}-Leu-Ser). Interestingly, N-tide residues beyond amino acid 59 are not resolved in the crystal structure, suggesting that residues 60-63 are not tightly associated with the catalytic domain, even though functional studies show that residues 59-63 (IEDDR) are important for potent inhibition (Vest et al., 2007). Although central and C-terminal regions of N-tide are most similar to the densin-IN domain (residues 803-824), the densin-IN domain contains two Pro residues (808 and 814) that are not present in N-tide (Fig. 3.3A), presumably constraining the conformations that can be adopted when interacting with CaMKII. In addition, N-tide contains 5 acidic amino acids (red in Fig. 3.3A), with only one conserved in the densin-IN domain. We identified two basic residues (Arg813, Arg820; blue in Fig. 3.3A) and an intervening hydrophobic residue (Leu815; green in Fig. 3.3A) in the densin-IN domain that are important for binding (Fig. 3.3B) and inhibition (Fig. 3.4) of CaMKII. These residues are conserved as Arg56, Val58 and Arg63 in N-tide, and truncation mutagenesis studies suggested these residues are important for potent inhibition by N-tide (Vest et al., 2007). Removal of these basic charges from densin-IN (R813A or R820A mutation) had only a modest effect, but

insertion of acidic residues (L815E or R820E mutation) almost completely abrogated binding of CaMKIIα and CaMKIIβ to GST-D-IN. Surprisingly, the R813E and R820A mutations selectively reduced binding of CaMKIIα but not CaMKIIβ, for reasons that are not clear (Fig. 3.3B). In addition, we found that the L815E mutation severely disrupted the association of CaMKIIα or CaMKIIβ with full-length densin in HEK293 cells. Taken together, these data suggest that binding of CaMKII isoforms to the densin-IN domain is primarily driven by hydrophobic interactions, in part involving Leu815.

Although CaMKIIN competes with GluN2B or densin-IN for binding to the CaMKII catalytic domain (Bayer et al., 2006; Jiao Jalan-Sakrikar et al., 2011; Strack et al., 2000a; Vest et al., 2007), a GluN2B peptide is a poor competitor for densin-IN binding to CaMKII (Fig 5B in (Jiao Jalan-Sakrikar et al., 2011). Moreover our data indicated that while interactions of the densin-IN domain and N-tide with CaMKII are broadly similar, there are clear differences. Additional mutagenesis and structural studies will be required to fully understand these differences.

CaMKIIN completely and potently inhibits CaMKII activity toward all substrates tested (Fig. 3.4) (Chang et al., 1998; Vest et al., 2007). The densin-IN domain also potently and essentially completely inhibited CaMKII phosphorylation of either syntide-2, a model peptide substrate, or of the GluA1 AMPA receptor subunit at Ser831, with potencies similar to those of N-tide (EC_{50}'s 50-100 nM) (Fig. 3.4A). However, densin-IN was an ineffective inhibitor of CaMKII activity toward Ser1303 in GluN2B (Fig. 3.4B), correlating with the weak

competition by N2B-tide for CaMKII-binding to densin-IN. Studies in HEK293 cells confirmed that full-length densin-FLA effectively inhibits phosphorylation of GluA1, but not GluN2B, by CaMKII, and that the inhibition of GluA1 phosphorylation required a functional densin-IN domain (Fig. 3.5). Thus, while CaMKIIN competes with GluN2B for stable interaction with the CaMKII catalytic domain and blocks GluN2B phosphorylation, it appears that the densin-IN domain does not prevent efficient GluN2B phosphorylation or CaMKII binding to GluN2B.

In summary, the present findings substantially expand our understanding of densin. Rather than functioning as a CaMKIIα-selective targeting protein, our data show that densin can target multiple CaMKII isoforms. Binding of densin appears to have a novel modulatory role; to interfere with phosphorylation of GluA1-AMPARs, and favor phosphorylation of GluN2B-NMDARs. Thus, densin may direct CaMKII actions toward discrete subsets of potential downstream targets in dendritic spines in response to synaptic activity, potentially modulating key mechanisms underlying synaptic plasticity. The roles of densin in neurons clearly warrant further investigation. However, it is worth noting that the design and interpretation of such studies needs to consider the diversity of CaMKAPs likely to be present at individual synapses and their overlapping interaction mechanisms, as well as the likely presence of multiple densin splice variants in some systems.

CHAPTER IV

SUBSTRATE-SELECTIVE AND CALCIUM-INDEPENDENT ACTIVATION OF CAMKII BY α-ACTININ

Introduction:

Transient changes in intracellular Ca^{2+} concentrations can be decoded by the ubiquitous Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII) to elicit diverse physiological responses. Binding of Ca^{2+}/CaM activates CaMKII and Thr286 autophosphorylation, creating an autonomously active form of CaMKII that is a molecular memory of transient Ca^{2+} signals. Dissociation of Ca^{2+}/CaM allows for rapid autophosphorylation at Thr305 or Thr306 (Thr305/6), blocking re-association of Ca^{2+}/CaM such that CaMKII is desensitized to subsequent Ca^{2+} signals (reviewed in (Colbran, 2004; Hudmon and Schulman, 2002; Soderling and Stull, 2001; Swulius and Waxham, 2008), also see Introduction of the thesis). These autophosphorylation reactions are key for CaMKII-dependent modulation of synaptic glutamate receptors and other neuronal functions related to long-term synaptic plasticity, learning and memory (Elgersma et al., 2002; Giese et al., 1998; Gustin et al., 2011; Pi et al., 2010a; Pi et al., 2010b)

As for many signaling proteins, precise targeting of CaMKII is crucial for its actions (Bayer and Schulman, 2001; Griffith et al., 2003). Neuronal Ca^{2+} signals can be tightly compartmentalized in dendritic spines (Yuste et al., 2000), F-actin rich synaptic structures that contain postsynaptic densities (PSDs) enriched in

glutamate receptors and other signaling proteins, including CaMKIIα. Newly synthesized CaMKIIα appears to be initially cytosolic, but Ca^{2+}/CaM binding initiates translocation to dendritic spines; a recent study suggests that only a small fraction of CaMKII in spines is tightly associated with PSDs (Feng et al., 2011). Retention of CaMKII in the spines is modulated by autophosphorylation; Thr286 autophosphorylation stabilizes spine localization, whereas Thr305/6 autophosphorylation promotes CaMKII dissociation from spines (Shen and Meyer, 1999). N-methyl-D-Aspartate (NMDA)-type glutamate receptor (NMDAR) GluN2B subunits are the most widely recognized binding partner for CaMKII; this interaction modulates CaMKII activity and is important for synaptic plasticity (Barria and Malinow, 2005; Bayer et al., 2001; Robison et al., 2005a; Sanhueza et al., 2011; Zhou et al., 2007). GluN2B selectively binds activated CaMKII, perhaps contributing to CaMKII translocation and retention in spines. However, PSDs contain ~20-fold more CaMKII holoenzymes than NMDAR subunits (Cheng et al., 2006), suggesting that direct interactions with NMDARs account for only a fraction of spine-localized CaMKII holoenzymes (Feng et al., 2011). Thus, the overall dynamics of CaMKII targeting presumably reflect integration of NMDAR interactions with CaMKII binding to several other proteins (Colbran, 2004). Indeed, CaMKII can simultaneously interact with α-actinin, GluN2B, and densin (Robison et al., 2005b). Importantly, pharmacological disruption of F-actin interferes with synaptic clustering of CaMKIIα and actin-binding proteins such as α-actinin without changes in NMDAR localization (Allison et al., 2000; Allison et al., 1998). Synaptic CaMKII targeting therefore depends on an intact F-actin

cytoskeleton.

The four isoforms of α-actinin (1-4) link various signaling molecules to the cytoskeleton by virtue of conserved modular protein domains: an N-terminal F-actin-binding domain, four spectrin-like repeat domains (responsible for antiparallel dimerization), an EF hand domain (that binds divalent cations only in α-actinin-1 and α-actinin-4), and a carboxy-terminal domain (CTD) (Blanchard et al., 1989; Sjoblom et al., 2008). For example, α-actinin links NMDAR GluN1 and GluN2B subunits to the actin cytoskeleton and modulates NMDAR activity by diverse mechanisms (Krupp et al., 1999; Michailidis et al., 2007; Wyszynski et al., 1998; Wyszynski et al., 1997; Zhang et al., 1998). CaMKII also associates with multiple α-actinin isoforms in brain (Dhavan et al., 2002; Robison et al., 2005b; Walikonis et al., 2001). Our previous studies defined the CTD of α-actinin-2 (A2-CTD; residues 819-894) as the minimal CaMKII binding domain (Robison et al., 2005b). *In vitro*, A2-CTD interaction with CaMKII is Ca^{2+}-independent, competitive with Ca^{2+}/CaM, and disrupted by Thr305/6 autophosphorylation (Robison et al., 2005a). However, the mechanism and functional consequences of CaMKII interaction with full-length α-actinin are poorly understood. Here, we show that α-actinin-2 mimics CaM in binding to the CaMKII regulatory domain, and targets CaMKII to the actin cytoskeleton. *In vitro* and intact cell studies show that α-actinin-2 is a Ca^{2+}-independent, substrate-selective CaMKII activator, which also enhances CaMKII interaction with GluN2B, but not GluN2A-containing NMDARs.

Results:

Substrate-selective activation of CaMKII by α-actinin

The CaMKII-binding A2-CTD (residues 819-894) is highly conserved in all α-actinin isoforms and is similar to the C-terminal domain (lobe) of CaM (residues 73-149) (Fig. 4.1A). Since the C-terminal lobe of CaM is sufficient to partially activate CaMKII in the presence of Ca^{2+} (Shifman et al., 2006), we tested the effects of a His_6-tagged A2-CTD on CaMKII activity. We detected a concentration-dependent activation of purified CaMKIIα using a model peptide substrate, autocamtide-2 (AC-2). This activation was Ca^{2+}-independent (-Ca^{2+}: V_{max} 1.5±0.2 μmol/min/mg, K_a 8.6±1.6 μM; +Ca^{2+}: V_{max} 1.3±0.2 μmol/min/mg, K_a 6.7±1.7 μM), whereas CaM is a Ca^{2+}-dependent activator (+Ca^{2+}: V_{max} 6.1±0.1 μmol/min/mg, K_a 97±17 nM) (Fig. 4.1B). Moreover, Ca^{2+}/CaM stimulated the phosphorylation of all substrates tested, whereas CaMKII activation by A2-CTD was detected with AC-2 and GluN2B-1290-1309, but not with syntide-2 (Fig. 4.1C). Thus, the A2-CTD is a Ca^{2+}-independent and substrate-selective activator of CaMKII.

Identification of CaMKII-binding determinants in α-actinin-2

The different affinities and maximal activation by CaM and A2-CTD probably reflect distinct interactions with the α-helical CaMKII regulatory domain (residues 293-310). The N-and C-terminal lobes of full length CaM interact with both sides of this helix (Meador et al., 1993) (Fig. 4.2A). Interestingly, the A2-CTD interacts with a single face of a regulatory domain helix in the giant muscle protein kinase,

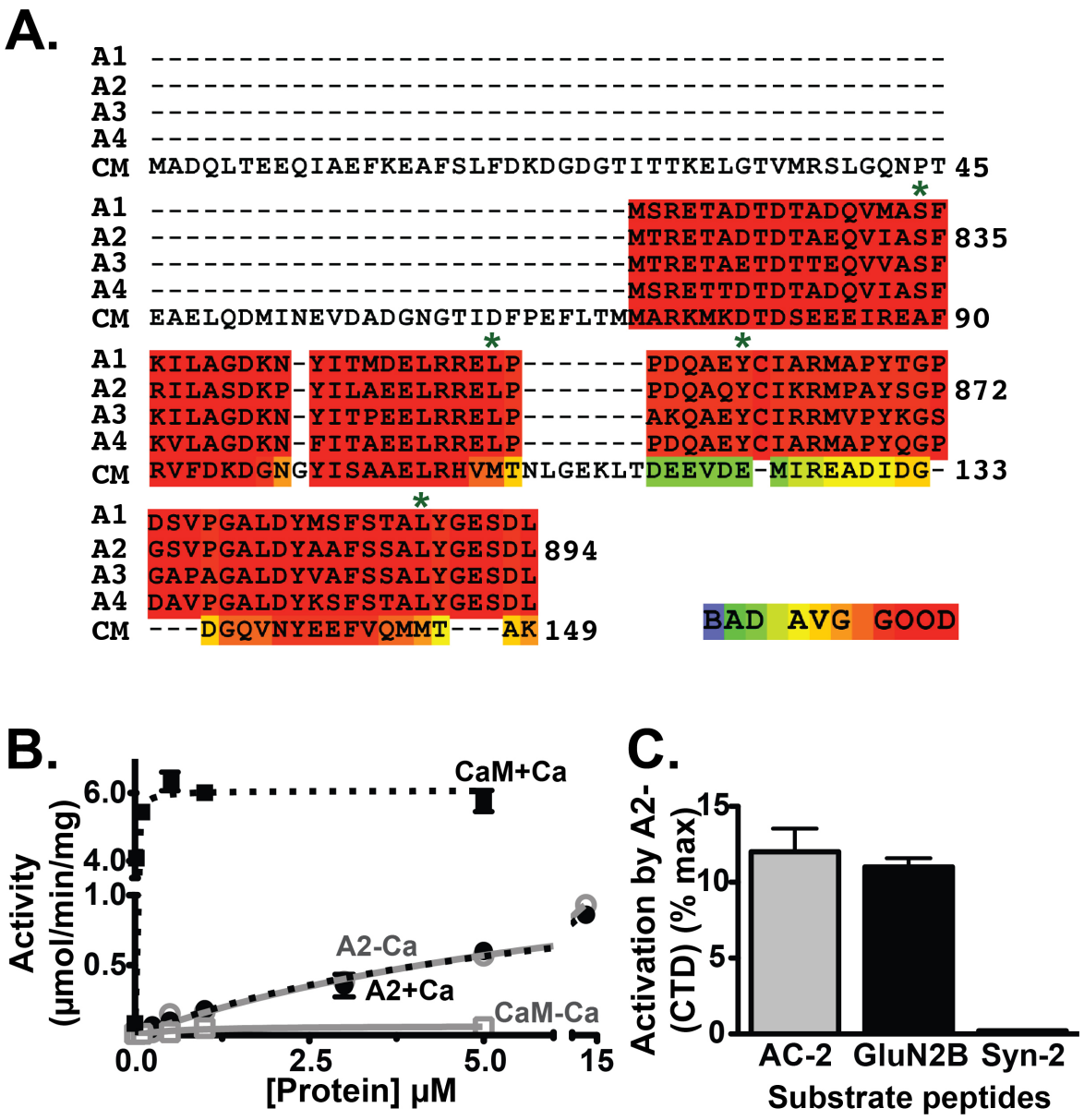

Figure 4.1 Ca^{2+}-independent activation of CaMKII by α-actinin

A. Alignment of C-terminal domain (CTD) residues 819-894 from human α-actinin-2 (A2) with corresponding domains in α-actinin-1, 3 and 4 (A1, A3, A4) and full-length human CaM (CM) using Expresso 3D-Coffee (Armougom et al., 2006), with relative alignment strength indicated on a color coded scale (lower right). Asterisks above the sequences indicate residues targeted for mutagenesis in α-actinin-2. **B.** Concentration-dependent effects of CaM (squares) and His_6-A2-CTD (circles) on CaMKII phosphorylation of autocamtide-2 in the presence of $CaCl_2$ (+Ca; black/dashed lines) or EGTA (-Ca; gray/solid) (mean±sem, n=3), thanks to Dr. Ryan K. Bartlett for this data. **C.** Ca^{2+}-independent activation of CaMKII by His_6-A2-CTD (14 µM) is detected with autocamtide-2 (AC2) and GluN2B(1290-1309), but not syntide-2. Data plotted as a percentage of maximum activation by Ca^{2+}/CaM (mean± S.E., n=3).

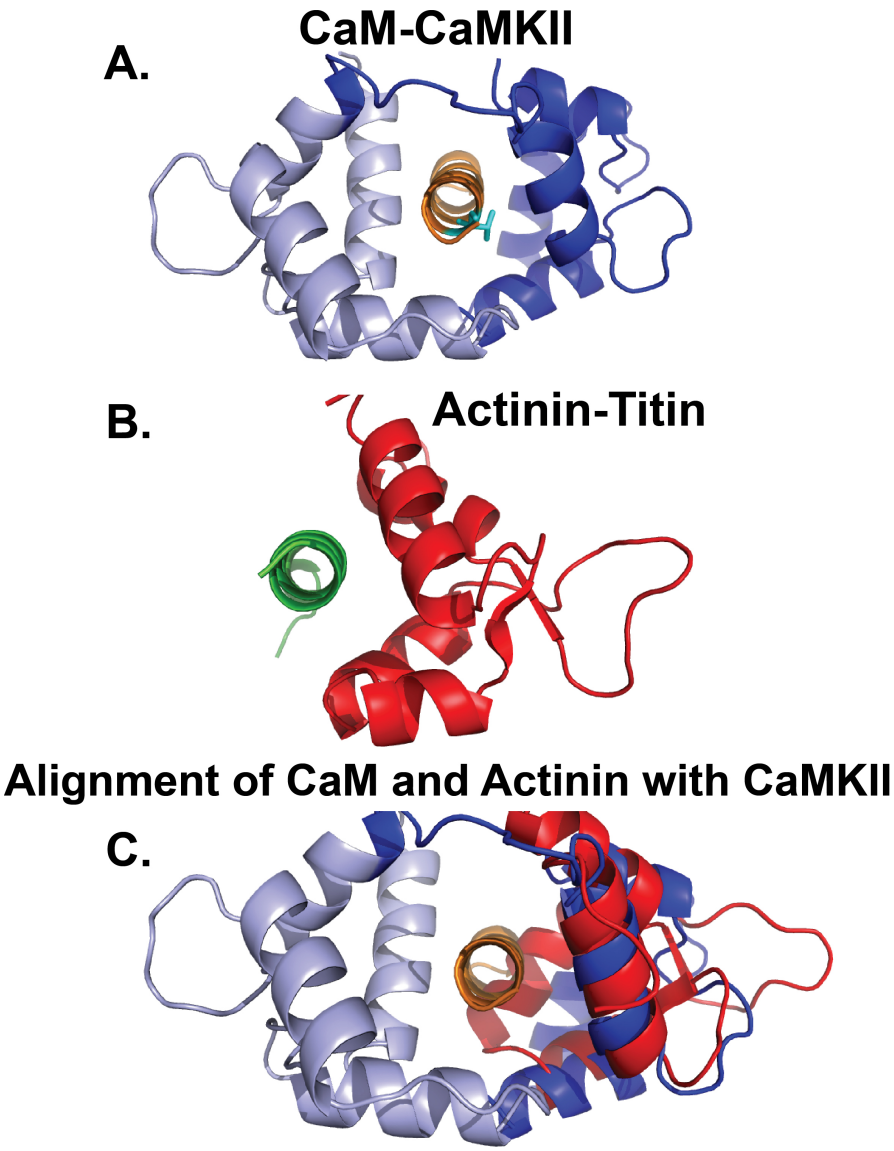

Figure 4.2 Structural similarity between CaM and α-actinin

A. Structure of Ca^{2+}/CaM bound to the CaMKII regulatory domain peptide (290-314) (PDB:1CM1). The N- and C-terminal domains (lobes) of CaM (residues 1-72 and 73-149) are shown in light and dark blue, respectively. The CaMKII peptide is in orange, with Thr306 in cyan. **B.** Structure of the α-actinin-2 CTD (residues 818-894) (red) bound to a z-repeat regulatory domain peptide from titin kinase (green) (PDB:1H8B). **C.** Alignment showing structural similarity between the α-actinin CTD (red) and the C-terminal lobe of Ca^{2+}/CaM in the CaM-CaMKII structure (dark blue), made using PyMol.

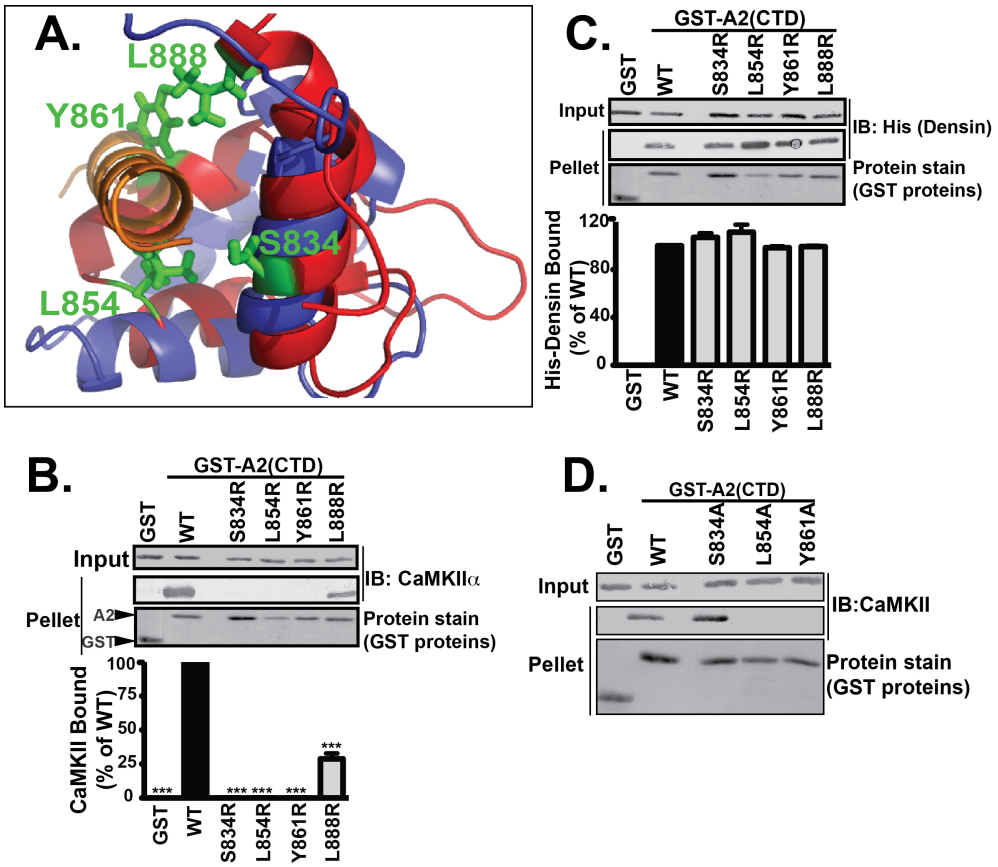

Figure 4.3 α-Actinin mimics CaM in binding to the CaMKII regulatory domain
A. Structural alignment of the A2-CTD with the C-terminal lobe of CaM bound to CaMKII (from Fig. 4.2C). Effects of mutating residues in α-actinin-2 highlighted in green to arginine **(B)** or Ala **(D)** on CaMKIIα binding to GST-A2-CTD were determined by glutathione-agarose cosedimentation assays. **B.** Introduction of charge in the predicted hydrophobic binding pocket disrupted CaMKIIα binding, as seen by protein staining for GST proteins and immunoblotting (IB) for CaMKII. Binding is expressed as a percentage of binding to WT (mean±S.E., n=4. ***, p<0.0001 vs. WT; 1-way ANOVA, Bonferroni's post-test). **C.** Mutations of Ser834, Leu854, Tyr861 or Leu888 to Arg in the CTD have no effect on interactions with densin, even though they disrupt CaMKII binding. **D)** Mutations of CTD residues Leu854 or Tyr861, but not Ser834, to Ala also interfere with CaMKII binding.

titin (Atkinson et al., 2001) (Fig. 4.2B). Given the amino acid sequence similarity between the A2-CTD and the C-lobe of CaM (Fig.4.1A), we aligned these two structures (Fig. 4.2C). The alignment (RMS 2.97 Å) predicts a hydrophobic interface between one face of the CaMKII regulatory domain helix and a concave binding "pocket" in A2-CTD formed by Ser834, Leu854, Tyr861, Leu888 and

other residues (Fig. 4.3A). In order to test this alignment as a model for CaMKII-binding to the A2-CTD, we introduced charge in the predicted hydrophobic binding pocket. S834R, L854R, and Y861R mutations completely abrogated CaMKII binding to GST-A2-CTD, and the L888R mutation reduced binding by ≈70% (Fig. 4.3B). However, none of these mutations affected binding of GST-A2-CTD to the densin PDZ domain in parallel experiments (Fig. 4.3C). Additional mutations reduced hydrophobicity without introducing charge; L854A and Y861A mutations completely abrogated CaMKII binding (like L854R and Y861R mutations), but S834A mutation had a minimal effect (Fig. 4.3D). In combination, these findings support our model for CaMKII interaction with the hydrophobic binding pocket in the CTD of α-actinin-2.

Determinants in CaMKII for binding to α-actinin

Ca^{2+}-independent autophosphorylation blocks CaMKII binding to both GST-A2-CTD and Ca^{2+}/CaM, and this loss of binding can be prevented by mutation of both Thr305 and Thr306 in CaMKIIα to Ala (Colbran, 1993; Robison et al., 2005b). Our alignment model indicates that the Thr306 side chain is buried in the A2-CTD interaction site, whereas the Thr305 side chain is solvent exposed (Fig. 4.4A), suggesting that phosphorylation at Thr306, but not Thr305, should disrupt binding. Autophosphorylation of wild type (WT) CaMKIIα can occur at both Thr305 and Thr306, whereas T305A- and T306A-CaMKIIα mutants can be autophosphorylated at only one residue (Thr306 and Thr305, respectively) (Colbran, 1993). Ca^{2+}-independent autophosphorylation reduced the binding of

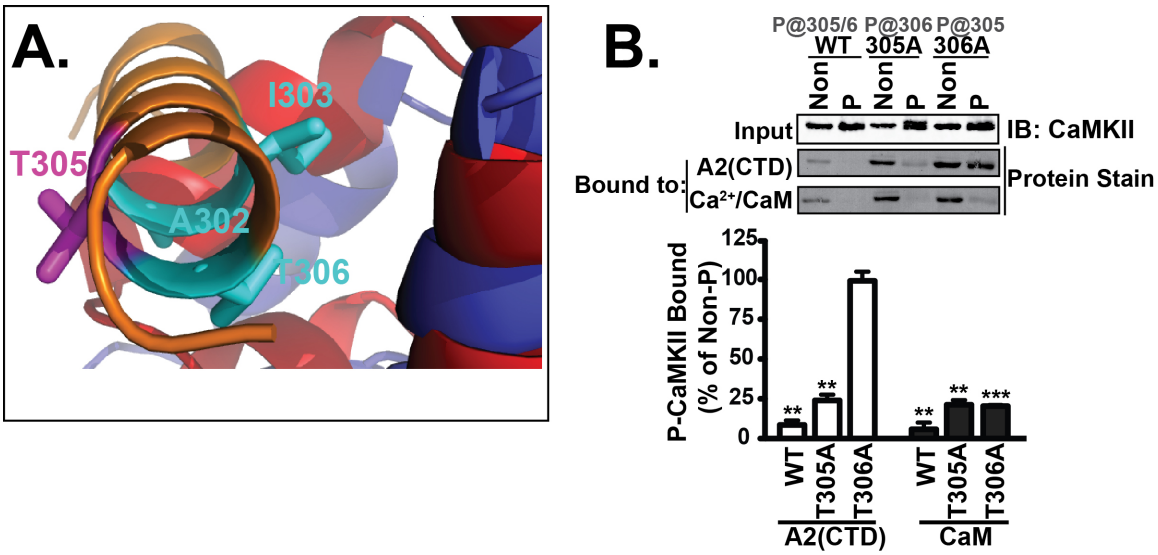

Figure 4.4 Thr305 and 306 autophosphorylation differentially affect α-actinin and CaM binding to CaMKII

A. Close-up of CaMKII regulatory domain helix showing residues in CaMKII targeted for mutagenesis (cyan or magenta) (from Fig. 4.2C). **B)** Effects of sequential autophosphorylation of WT, T305A- or T306A-CaMKIIα (P) on binding to Ca^{2+}/CaM or GST-A2-CTD. Levels of CaMKII in inputs were compared by immunoblot (top). Isolated complexes were analyzed by protein staining. Binding of autophosphorylated CaMKIIs was expressed as a percentage of binding of corresponding non-phosphorylated control CaMKII (mean±S.E., n=3. Two-way ANOVA, $p<0.0001$. **$p<0.001$, ***$p<0.0001$ vs. 100% by post-hoc 1-column t-test).

both purified WT and T305A-CaMKIIα to GST-A2-CTD by ≥80%, but had no effect on the binding of purified T306A-CaMKIIα to GST-A2-CTD (Fig. 4.4B). In contrast, Ca^{2+}-independent autophosphorylation reduced binding of WT, T305A- and T306A-CaMKIIα to Ca^{2+}/CaM-agarose by ≥80% (Fig. 4.4B), showing that the remaining native Thr residue in both mutated kinases was effectively autophosphorylated. Thus, CaMKIIα binding to α-actinin is sensitive to autophosphorylation at Thr306, but not at Thr305, consistent with our model for α-actinin binding to one face of the CaMKII regulatory domain helix.

In order to identify additional interaction determinants, we tested the binding of GST-A2-CTD to additional CaMKIIα mutants that had been expressed

in HEK293 cells. Initial studies found that WT CaMKIIα is basally phosphorylated at Thr305/6 in HEK293 cells (Fig. 4.5A) and that this basal autophosphorylation suppressed the interaction with both A2-CTD and Ca^{2+}/CaM (Fig. 4.5B). Moreover, we found that the level of Thr305/6 phosphorylation was altered by some mutations within the CaMKII regulatory domain. Thus, to avoid possible indirect effects on CaMKII binding to A2-CTD, we analyzed the effects of additional regulatory domain mutations on a background of a T305A/T306A double mutant kinase (TT305/6AA-CaMKIIα). A302R and I303R mutations had similar effects to reduce or block the binding of TT305/6AA-CaMKIIα to either A2-CTD or Ca^{2+}/CaM (Fig. 4.5C), consistent with the hydrophobic interactions predicted by our alignment model. In contrast, Thr310 in CaMKIIα contacts the N-lobe but not C-lobe of CaM (Meador et al., 1993), and a T310D mutation disrupted binding to Ca^{2+}/CaM, with no apparent effect on binding to A2-CTD (Fig. 4.5D). In combination, these findings show that α-actinin interacts with a subset of residues in the CaMKIIα regulatory domain used for interaction with Ca^{2+}/CaM.

CaMKII association with the CTD in α-actinin-2 dimers

Our *in vitro* studies with the A2-CTD constructs suggest that α-actinin has the potential to profoundly affect CaMKII activity and/or localization in cells. However,

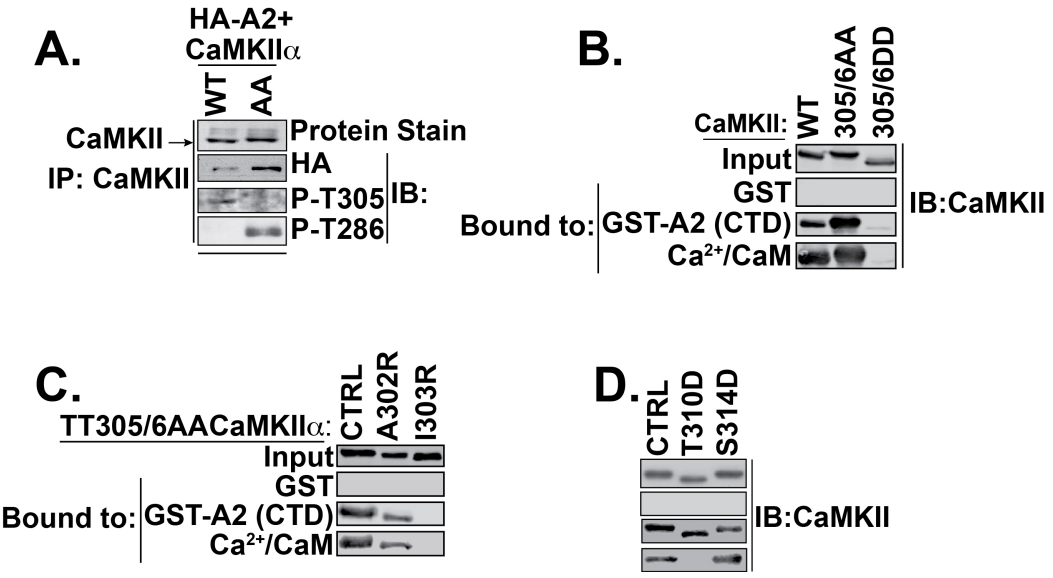

Figure 4.5 Binding of α-actinin or Ca^{2+}/CaM to CaMKII is modulated by phosphorylation or mutation within the CaMKII regulatory domain.

A. Basal autophosphorylation of WT CaMKII at Thr305/6 in HEK293 cells reduces binding to α-actinin-2 *in situ*. CaMKII immune complexes from extracts of HEK293 cells expressing WT or TT305/6AA-CaMKIIα with HA-actinin-2 were analyzed by protein staining to detect total CaMKII, and by immunoblotting for HA, phospho-Thr305 (P-T305) and phosphor-Thr286 (P-T286). **B-D.** Effects of mutations in the regulatory domain on CaMKII binding to GST-A2-CTD *in vitro*. Lysates of HEK293 cells expressing CaMKIIα (WT or with indicated mutations) were incubated with GST or GST-A2-CTD and glutathione-agarose, or with Ca^{2+}/CaM-agarose. Complexes were isolated and analyzed by immunoblotting. **(B)** Specific CaMKII binding to both GST-A2-CTD and Ca^{2+}/CaM is enhanced by TT305/6AA mutation and decreased by TT305/6DD mutation. **(C)** Interactions with GST-A2-CTD and Ca^{2+}/CaM are similarly affected by A302R and I303R mutations on a TT305/6AA background. **(D)** S314D mutation on a TT305/6AA background has little effect on interactions with GST-A2-CTD or Ca^{2+}/CaM, but T310D mutation disrupts binding to Ca^{2+}/CaM, with little effect on binding to GST-A2-CTD.

CaMKII interactions with full-length, dimeric α-actinins have not been directly characterized. Therefore, we co-expressed full-length HA-tagged α-actinin-2 (HA-actinin-2) with CaMKIIα in HEK293 cells. HA-actinin-2 was readily detected in CaMKII immune complexes isolated from lysates of cells expressing WT CaMKIIα, and mutation of Thr305 and Thr306 to Ala substantially enhanced the interaction (Fig. 4.5A). Subsequent studies used TT305/6AA-CaMKIIα to avoid potential confounding effects of variations in basal phosphorylation at Thr305/6

(see above). Importantly, L854R or Y861R mutations in the CTD that prevent CaMKII binding *in vitro* (Fig. 4.3B) substantially reduced the amount of HA-actinin-2 that co-immunoprecipitated with TT305/6AA-CaMKIIα (Fig. 4.6A). Thus, the CTD of full-length α-actinin-2 appears to be critical for the association of CaMKII in intact cells.

The cellular localization of WT/mutated HA-actinin-2 and TT305/6AA-CaMKIIα were also compared using immunofluorescence microscopy. In the absence of HA-actinin-2, TT305/6AA-CaMKIIα filled the cell cytoplasm relatively uniformly (Fig. 4.6B). Full length HA-actinin-2 was targeted to the sub-membrane cortical cytoskeleton (Fig. 4.6B), as shown previously (Khoory et al., 1993; Washington and Knecht, 2008). In the presence of co-expressed WT HA-actinin-2, the distribution of TT305/6AA-CaMKIIα became non-uniform. Moreover, there was a partial, but significant, co-localization of WT HA-actinin-2 with TT305/6AA-CaMKIIα, as revealed by yellow coloration in an overlay of images displayed in the red and green channels, respectively, and also by calculation of the intensity correlation quotient (ICQ) for the two channels (Fig. 4.6D). Importantly, this colocalization was disrupted by Y861R mutation of HA-actinin-2. Additional studies using phalloidin to label the F-actin cytoskeleton revealed little co-localization of TT305/6AA-CaMKIIα. This suggested that endogenous α-actinin-1 or -4 in HEK293 cells is not effective in targeting CaMKII to the F-actin.

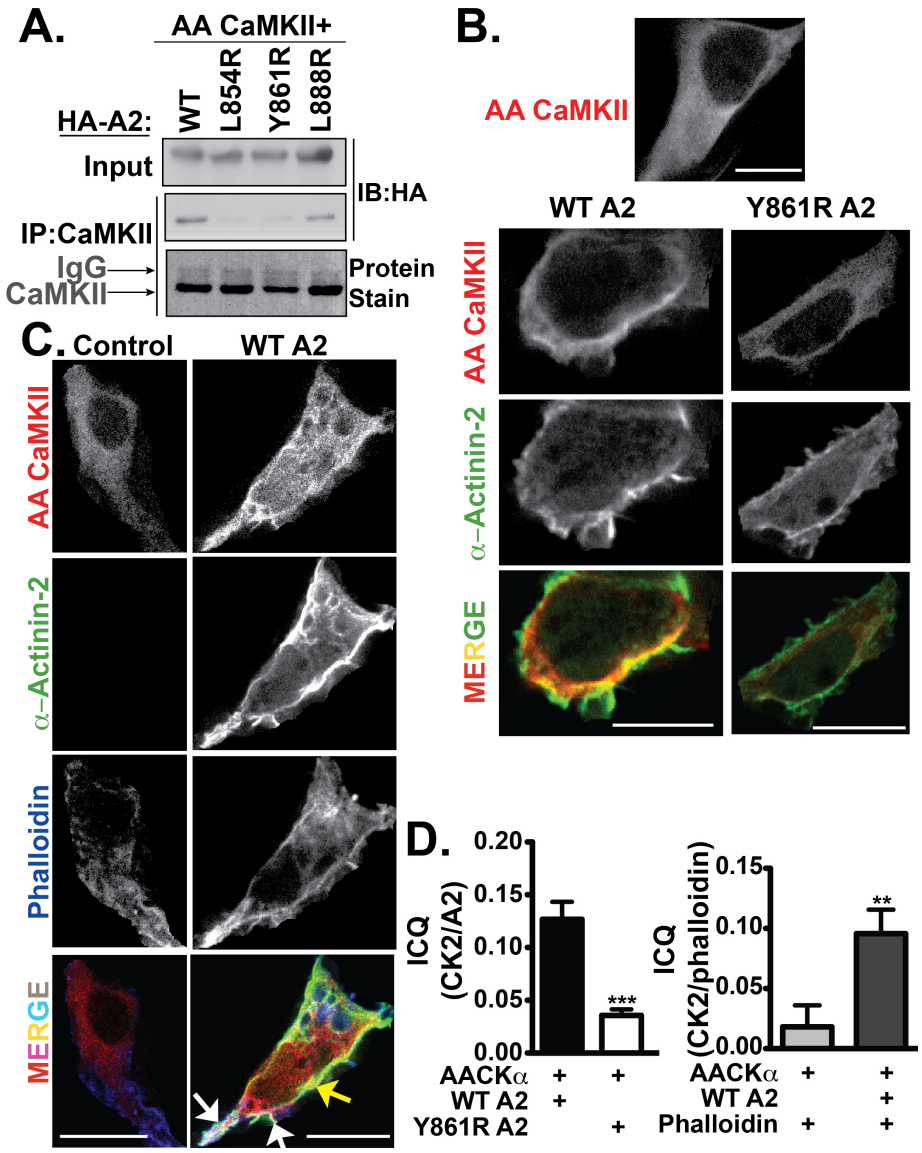

Figure 4.6 CaMKIIα is targeted to F-actin by the α-actinin-2 CTD in cells.
A) CaMKII immune complexes isolated from extracts of HEK293 cells co-expressing TT305/6AA-CaMKIIα with full-length HA-actinin-2 (HA-A2; WT or mutated) were analyzed by protein stain for CaMKII and immunoblotting for HA. **B)** Immunofluorescence microscopy of HEK293 cells expressing TT305/6AA-CaMKIIα (AA-CaMKII, red) alone (top) or with HA-actinin-2 (WT or Y861R; green). **C)** Localization of AA-CaMKII and F-actin (phalloidin staining; blue) in the absence and presence of WT HA-actinin-2 (green). Arrows indicate colocalization of HA-A2 and AA-CaMKII alone (yellow) or HA-A2, AA-CaMKII and phalloidin (white). **D)** Intensity correlation quotient (ICQ) analysis of colocalization between AA-CaMKII and WT or Y861R HA-actinin-2 (left) or AA-CaMKII and phalloidin in the presence and absence of WT HA-actinin-2 (right) (n=8 cells; ***p<0.0001 vs. WT by t-test, **p<0.005 vs. control by t-test, n=6 cells).

However, co-expression of HA-actinin-2 significantly enhanced the targeting of TT305/6AA-CaMKIIα to F-actin-enriched regions, primarily on the periphery of these cells (Fig. 4.6C), as revealed by a significant increase in ICQ between fluorescent signals for CaMKII and phalloidin (Fig. 4.6D). Taken together, these data show that CaMKIIα is targeted to the F-actin cytoskeleton by binding to the CTD of full-length α-actinin-2 dimers in intact cells.

α-Actinin modulation of CaMKII interactions with NMDARs in cells

Previous studies showed that CaMKII associates with multiple α-actinin isoforms in brain (Dhavan et al., 2002; Robison et al., 2005b; Walikonis et al., 2001). We expanded these analyses to demonstrate that these complexes also contain NMDAR GluN1 and GluN2B subunits (Fig. 4.7A). In order to determine the role of α-actinin-2 in formation of this complex, we co-expressed GluN1, GluN2B and CaMKIIα phosphorylation site mutants in HEK293 cells, with or without HA-actinin-2. We again used TT305/6AA-CaMKIIα to avoid potential confounding effects of varying levels of Thr305/6 phosphorylation (see above). Both NMDAR subunits associate with TT305/6AA-CaMKIIα in the absence of co-expressed HA-actinin-2 (Fig. 4.7B). This association was partially due to basal Thr286 phosphorylation because additional mutation of Thr286 to Ala (T286A/TT305/6AA-CaMKIIα) resulted in a significant ≈4-fold reduction in levels of co-precipitated NMDAR subunits (Fig. 4.7B). Co-expression of HA-actinin-2 significantly enhanced the associations of GluN1 and GluN2B with TT305/6AA-CaMKIIα by ≈1.8-fold, and with T286A/TT305/6AA-CaMKIIα by ≈4-fold, relative

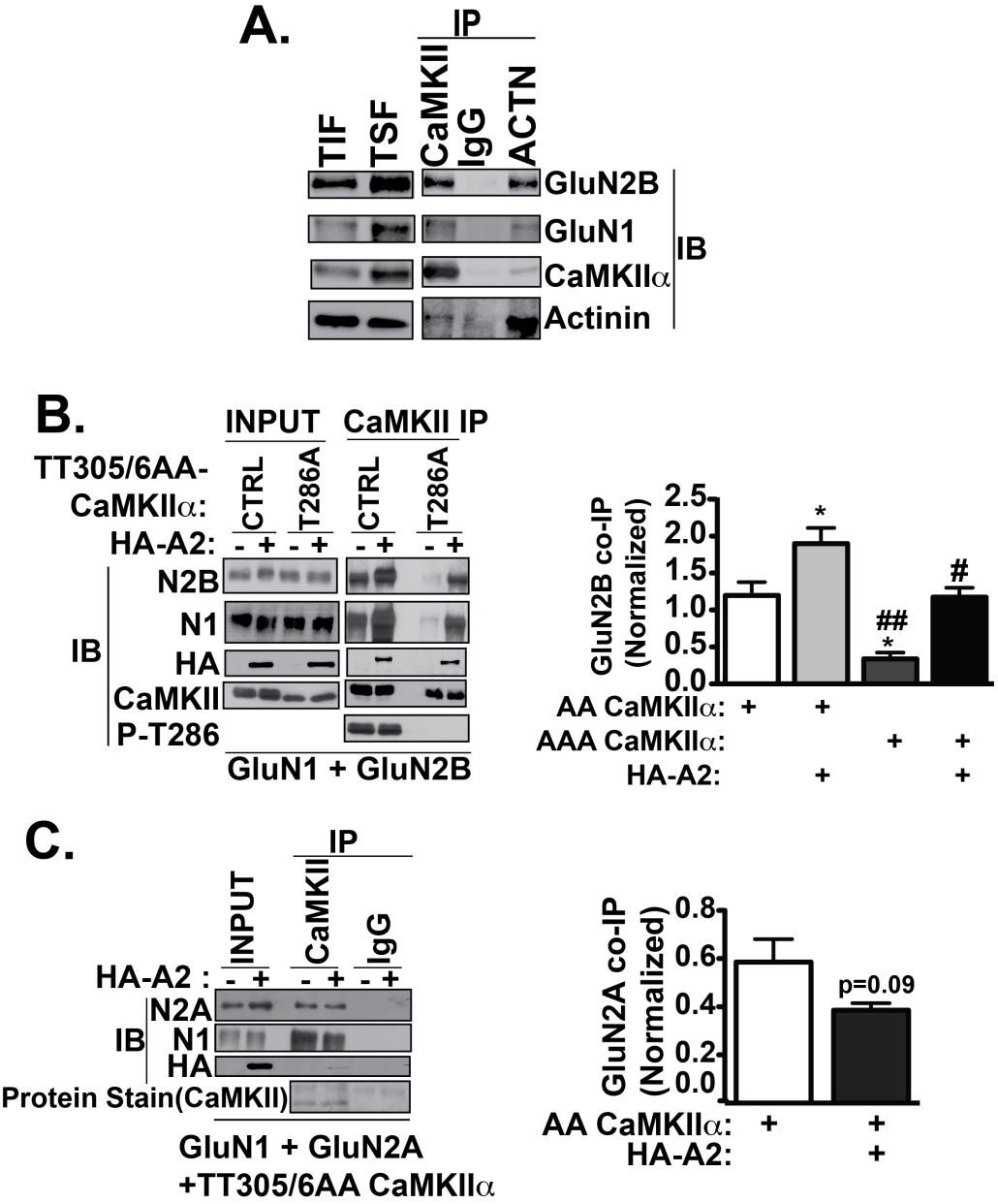

Figure 4.7 α-Actinin-2 enhances CaMKII association with GluN2B-NMDARs
A. A mouse forebrain Triton-soluble fraction (TSF) was immunoprecipitated (IP) using CaMKII, α-actinin, or control IgG. The Triton-insoluble fraction (TIF), TSF and immune complexes were immunoblotted for GluN2B, GluN1, α-actinin and CaMKII as indicated. **B.** CaMKII was immunoprecipitated from extracts of HEK293 cells (input) expressing GluN1, GluN2B and CaMKIIα (TT305/6AA or T286A/TT305/6AA; AA-CaMKIIα or AAA-CaMKIIα, respectively) with or without HA-α-actinin-2. Input and CaMKII immune complexes were immunoblotted for GluN1, GluN2B, HA, CaMKII and phospho-Thr286 (P-T286). Levels of co-immunoprecipitated GluN2B were normalized to input and plotted as mean±S.E. (n=5). *, $p<0.05$ vs. GluN1+GluN2B+AACK. ##, $p<0.01$ vs. GluN1+GluN2B+AACK+HA-A2. #, $p<0.05$ vs. GluN1+GluN2B+AAACK. **C.** Similar to B, except GluN2B is replaced with GluN2A.

to corresponding levels in the absence of HA-actinin-2 (Fig. 4.7B). Indeed, co-expression of HA-actinin-2 largely rescued the deficit in association of GluN2B-NMDARs that was induced by T286A mutation. In contrast, there was a trend (p=0.09) for HA-actinin-2 to decrease the association of GluN2A-NMDARs with TT305/6AA-CaMKIIα (Fig. 4.7C). Collectively, these data show that α-actinin-2 specifically enhances CaMKIIα targeting to GluN2B-NMDARs.

α-Actinin-2 differentially modulates CaMKII phosphorylation of glutamate receptors in cells

The CTD of α-actinin-2 activates T-site substrate phosphorylation in the presence or absence of Ca^{2+} (Figs. 4.1B/C), but inhibits Ca^{2+}-dependent phosphorylation of S-site substrates such as syntide-2 due to competition with Ca^{2+}/CaM (Robison et al., 2005a). These *in vitro* data lead us to hypothesize that α-actinin modulates the innate substrate-selectivity of CaMKII in cells. Therefore, we compared the effects of expressing HA-actinin-2 on CaMKII phosphorylation of two physiologically important substrates; Ser831 in AMPA-type glutamate receptor GluA1 subunits and Ser1303 in NMDAR GluN2B subunits (Barria et al., 1997a; O'Leary et al., 2011; Strack et al., 2000a). Phosphorylation of Ser831 in GluA1 in HEK293 cells was enhanced ≈3-fold by co-expression of TT305/6AA-CaMKIIα, but additional expression of HA-actinin-2 blocked this increase (Fig. 4.8A). In contrast, phosphorylation of Ser1303 in GluN2B by TT305/6AA-CaMKII was significantly enhanced by co-expression of HA-actinin-2 (Fig. 4.8B). Thus, α-actinin-2 modifies the innate substrate selectivity of CaMKII in intact cells,

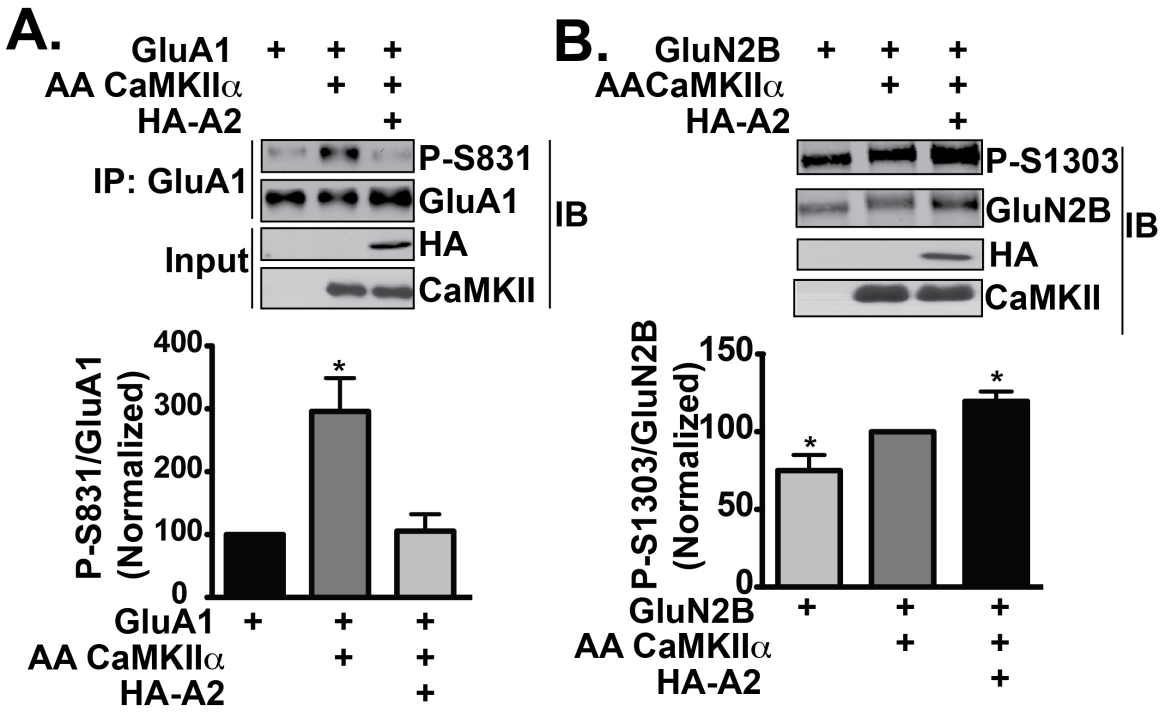

Figure 4.8 α-Actinin modulates phosphorylation of GluA1 and GluN2B by CaMKII.

A) GluA1 was immunoprecipitated from lysates of HEK293 cells expressing GluA1 with or without TT305/6AA-CaMKIIα and HA-actinin-2 (WT or SR1) and immunoblotted for total GluA1 and phospho-Ser831 (P-S831). **B)** Lysates of HEK293 cells expressing GluN2B with or without TT305/6AA-CaMKIIα and HA-actinin-2 were directly immunoblotted for total GluN2B, phospho-Ser1303 (P-S1303), CaMKII and HA as indicated. Graphs plot phospho-protein levels as a ratio to corresponding total protein after normalization to total GluA1 alone (A) or total GluN2B+AACK (B) (mean±S.E., n=4. *, $p<0.01$ by 1-way ANOVA with Bonferoni post-test).

favoring phosphorylation of T-site substrates such as GluN2B over S-site substrates, such as GluA1.

DISCUSSION:

Subcellular targeting and activity of CaMKII are dynamically regulated by synaptic activity and by autophosphorylation at multiple sites. CaMKII

interactions with several synaptic proteins are regulated by CaMKII activation and autophosphorylation, but their specific contributions to overall targeting and modulation of kinase activity are poorly understood (see Chapter I). The present findings show that α-actinin can function as a novel modulator of CaMKII targeting and kinase activity by mimicking CaM.

α-Actinin as a surrogate for CaM

CaMKII is conventionally activated by Ca^{2+}-dependent interaction of the two lobes of CaM with both sides of the α-helical regulatory domain (Meador et al., 1993). However, the two lobes of CaM can independently activate CaMKII, albeit with weaker affinity and to reduced levels (Evans and Shea, 2009; Forest et al., 2008; Jama et al., 2011; Shifman et al., 2006). The current studies were prompted by primary sequence and secondary/tertiary structural similarity between the C-terminal domain of α-actinin-2 (A2-CTD) bound to a z-repeat peptide from titin and Ca^{2+}/CaM bound to a CaMKII regulatory domain peptide (Figs. 4.1A, 4.2A, and B). Our data show that Ca^{2+}-independent interaction of A2-CTD with one side of the regulatory domain helix can activate CaMKII (Fig. 4.1B). The weaker apparent affinity and partial activation by A2-CTD compared to full length Ca^{2+}/CaM (K_a 6.7 vs. 0.1 μM; V_{max} 1.3 vs. 6.1 μmol/min/mg) presumably results from occupation of a reduced surface area on the regulatory domain by the A2-CTD and from only partial displacement of the regulatory domain from the catalytic site. Such a partial conformational effect may also account for the fact that activation by A2-CTD is selective for substrates with primary sequences resembling that surrounding the Thr286 autophosphorylation site, such as

GluN2B Ser1303 and the AC-2 model peptide (Fig. 4.1C). Besides binding in the catalytic site to be phosphorylated, such substrates also mimic the regulatory domain in binding to the modulatory T-site on the catalytic domain (Bayer et al., 2006). Peptides that bind to the T-site competitively displace the regulatory domain from the catalytic domain, thereby helping to "open" the catalytic site for substrate binding (Hoffman et al., 2011). Moreover, occupation of the T-site by GluN2B has also been shown to enhance the affinity for ATP binding to the catalytic site (Pradeep et al., 2009). Thus, we posit that binding of α-actinin allows for peptide binding at the T-site, which further displaces the regulatory domain to enhance both substrate access to the catalytic site and ATP-binding. The regulatory domain is not displaced to the same extent as Ca^{2+}/CaM, accounting for the reduced V_{max}, and seems insufficient to allow Thr286 to be autophosphorylated by a neighboring subunit in the CaMKII holoenzyme (data not shown). However, our data cannot exclude the possibility that the low binding affinity limits simultaneous binding of α-actinin to adjacent CaMKII subunits to stimulate Thr286 autophosphorylation.

Role of Thr305/Thr306 autophosphorylation

Most prior studies have treated Thr305 and Thr306 as functionally redundant because autophosphorylation at either Thr305 or Thr306 can block Ca^{2+}/CaM binding (Colbran, 1993). However, both Thr305 and Thr306 are strictly conserved among all CaMKII isoforms from *Hydra* to *H. sapiens* (Fig. 4.9). The evolutionary pressure to retain both sites may suggest that each residue has a

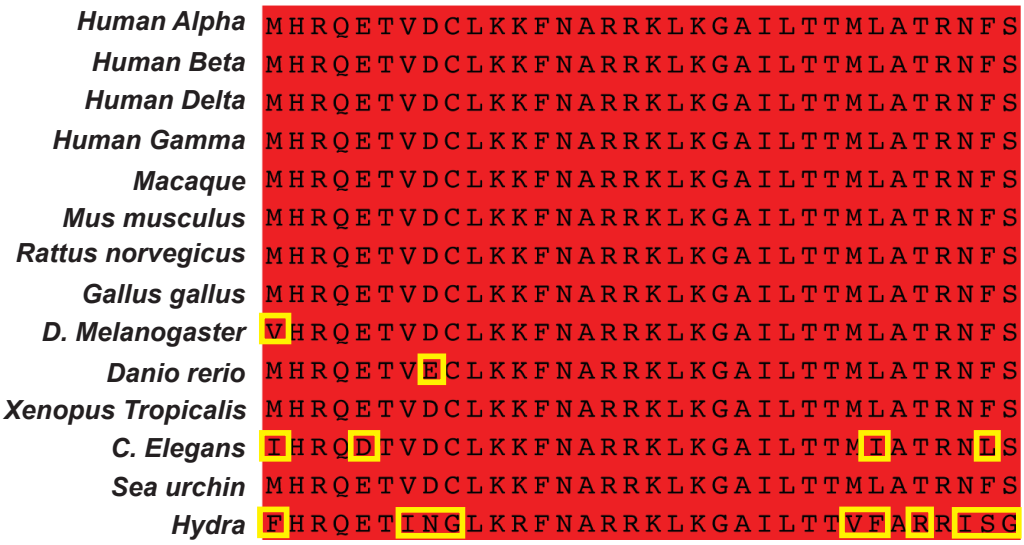

Figure 4.9 Thr305 and Thr306 in CaMKII are absolutely conserved through evolution

Primary sequence alignment of regulatory domains from human α, β, γ, and δ CaMKII isoforms and from CaMKII in various species. Yellow boxes highlight non-conserved residues. Human CaMKIIα :Q9UQM7, Human CaMKIIβ: Q13554, human CaMKIIδ: Q13557, Human CaMKIIγ: Q13555, macaque CaMKII: F7H4G9, mouse CaMKII: P11798, rat CaMKII: P11275, chicken CaMKII: Q9YHB8, drosophila CaMKII: Q00168, zebrafish CaMKII: Q32PV2, xenopus CaMKII: A4IIE5, C-elegans CaMKII: O62305-4, sea-urchin CaMKII: Q6UVK0, hydra CaMKII: 100200906

unique functional roles or that dual phosphorylation of both sites is functionally important. The current data favor the first possibility; Thr306 autophosphorylation prevents binding to both Ca^{2+}/CaM and α-actinin, whereas Thr305 phosphorylation prevents binding to Ca^{2+}/CaM, but not to α-actinin (Fig. 4.4B). Unfortunately, the specific role of each site in controlling interactions with α-actinin is not recapitulated by T305D and T306D mutations, which individually disrupt CaMKII binding to Ca^{2+}/CaM and A2-CTD (see chapter VI). Consequently disruption of CaMKII targeting to dendritic spines by replacement of Thr305 with an Asp may be due to loss of interaction with α-actinin, especially because Thr305/6 phosphorylation appears to have relatively modest effects on direct

interactions with GluN2B or other binding partners (Leonard et al., 2002; Robison et al., 2005b). Comparisons of functional effects of expressing mutated kinases with a single Thr mutated to an acidic residue provide limited insights into the specific role of α-actinin. However, mutation of Thr305 and Thr306 to Ala (or Val) to remove both autophosphorylation sites stabilizes synaptic targeting and appears to dictate the role of CaMKII in regulating synaptic strength and plasticity (Elgersma et al., 2002; Pi et al., 2010a; Pi et al., 2010b; Shen and Meyer, 1999), but it is unclear whether these changes involve altered interactions with α-actinin. Our data suggest that Thr306 phosphorylation makes a specific contribution to modulating CaMKII that is mediated by disruption of α-actinin interactions and that is distinct from the effects of phosphorylation at Thr305. Thus, it will be important to assess the specific phosphorylation at both Thr305 and Thr306 and the interactions with α-actinin in order to understand CaMKII regulation in cells.

CaMKII targeting to actin cytoskeleton and NMDA-type glutamate receptors:

Recent studies highlighted important roles for direct interactions of certain CaMKIIβ splice variants with F-actin (Fink et al., 2003; Lin and Redmond, 2008; Okamoto et al., 2007; Shen et al., 1998). However, CaMKIIα is the major isoform present in many adult forebrain neurons (Kennedy et al., 1983) and CaMKIIα homomers are not directly targeted to F-actin (Fink et al., 2003). Nevertheless, postsynaptic CaMKIIα localization is dependent on an intact F-actin cytoskeleton (Allison et al., 2000). This is consistent with the present observation that the CTD of α-actinin-2 can target CaMKIIα to F-actin (Fig. 4.6) and the fact that CaMKIIα

associates with multiple α-actinin isoforms in brain (Dhavan et al., 2002; Robison et al., 2005b; Walikonis et al., 2001) (Fig. 4.7A). Collectively, these data indicate that α-actinin plays an important role in targeting homomeric CaMKIIα holoenzymes that is independent of direct CaMKIIα interactions with F-actin.

Activated CaMKII translocates to dendritic spines and binds NMDAR GluN2B subunits (Bayer et al., 2001; Bayer et al., 2006; Strack and Colbran, 1998). CaMKII interactions with GluN2B are critical for synaptic plasticity, learning and memory (Barria and Malinow, 2005; Sanhueza et al., 2011; Zhou et al., 2007). NMDAR GluN1 and GluN2B subunits, but not GluN2A, also interact with α-actinin (Wyszynski et al., 1997), tethering NMDARs to the actin cytoskeleton to modulate channel activity (Michailidis et al., 2007; Zhang et al., 1998). Pharmacological destabilization of F-actin disrupts the colocalization of both α-actinin and CaMKII with synaptic NMDARs (Allison et al., 2000; Allison et al., 1998). Our data show that α-actinin selectively stabilizes CaMKII targeting to GluN2B-NMDARs (Fig. 4.7B), apparently due to the combined effects of activating CaMKII to interact directly with GluN2B and of bridging indirect CaMKII interactions with GluN2B and/or GluN1. The trend for α-actinin to reduce CaMKII targeting to GluN2A-NMDARs may be explained by the lack of α-actinin interaction with GluN2A, the low affinity of direct CaMKII-GluN2A interactions, and/or competition between CaMKII and α-actinin for binding GluN1 (Merrill et al., 2007; Strack and Colbran, 1998; Wyszynski et al., 1997). A key role for α-actinin in CaMKII targeting to GluN2B-NMDARs also may explain previous observations that Thr305/6 autophosphorylation, or mutation to acidic residues, destabilizes

synaptic targeting (Elgersma et al., 2002; Shen and Meyer, 1999), even though direct CaMKII-NMDAR subunit interactions are relatively insensitive to Thr305/6 autophosphorylation (Leonard et al., 2002; Robison et al., 2005b).

Differential modulation of glutamate receptor phosphorylation

In addition to targeting CaMKII, the CTD of α-actinin has complex effects on CaMKII activity, not only *in vitro* (Fig. 4.1) but also toward physiologically relevant CaMKII substrates in intact cells (Fig. 4.8). Phosphorylation of Ser831 in GluA1 subunits increases the conductance of synaptic AMPARs during LTP (Barria et al., 1997b; Kristensen et al., 2011), and we found that α-actinin suppressed CaMKII phosphorylation of Ser831 (Fig. 4.8A). GluA1 is considered to be a "pure" S-site substrate and α-actinin failed to directly activate S-site substrate phosphorylation (Fig. 4.1C). Therefore, we posit that α-actinin suppresses GluA1 phosphorylation by competing for Ca^{2+}/CaM binding to the regulatory domain, in agreement with prior findings (Robison et al., 2005a). In contrast, CaMKII is a negative-feedback modulator of Ca^{2+} influx via GluN2B-NMDARs (Sessoms-Sikes et al., 2005) and α-actinin enhanced CaMKII phosphorylation of Ser1303 in GluN2B NMDAR subunits (Fig. 4.8B). Ser1303 in GluN2B is a typical T-site substrate, and, as discussed above, interactions of GluN2B at the T-site may help α-actinin activate Ser1303 phosphorylation. This cooperation may relate to a previous report that GluN2B can "trap" CaMKII in an autonomously active conformation in the absence of Thr286 autophosphorylation (Bayer et al., 2001). Interestingly, α-actinin also interacts with the PDZ domain of densin, forming a ternary complex with CaMKII (Robison et al., 2005b; Walikonis et al., 2001), and

densin contains a novel CaMKII inhibitory domain that effectively blocks phosphorylation of GluA1 but not GluN2B (Jiao Jalan-Sakrikar et al., 2011). Thus, densin and α-actinin may collaborate to target neuronal CaMKII, favoring phosphorylation of the associated GluN2B-NMDARs at the expense of GluA1-AMPARs. In this way, α-actinin may act as a PSD "phosphostat", maintaining basal phosphorylation of a subset of CaMKII targets to control synaptic activity. It will be important to investigate the impact of α-actinin isoforms on the phosphorylation of CaMKII substrates at synapses and in other subcellular compartments.

Chapter V

CAMKII INTERACTIONS WITH α-ACTININ DIMERS

Introduction:

α-Actinin is a major F-actin crosslinking protein in both muscle and non-muscle cells, providing great mechanical stability. The two major forms of α-actinin: muscle (α-actinin-2 and -3) and non-muscle (α-actinin-1 and -4) differ in their ability to bind Ca^{2+} in the EF-hands: non-muscle but not muscle α-actinins can bind Ca^{2+} (Blanchard et al., 1989). The F-actin bundling capability of α-actinin-1 and -4 is reduced upon Ca^{2+}-binding (Burridge and Feramisco, 1981; Witke et al., 1993). The interactions of actin with other proteins like vinculin and gelsolin can also be regulated by anionic phospholipids like phosphatidyl inositol 4,5 bisphosphate (PIP2) (Fukami et al., 1994; Hartwig and Yin, 1988). Similarly, PIP2 and other anionic lipids regulate α-actinin interactions with actin (Fraley et al., 2005; Fukami et al., 1992).

α-Actinin has recently emerged as a major platform mediating interactions between cytoskeletal and regulatory proteins (Sjoblom et al., 2008). Although the majority of the interactions are at the spectrin repeats, the C-terminal domain (CTD) also interacts with diverse proteins, such as the Z-disk associated PDZ-domain protein ZASP, the LIM-protein hCLIM1, or the z-repeat of titin kinase (Otey and Carpen, 2004; Sjoblom et al., 2008). Hence, the mechanisms by which the interactions with CTD are regulated are of general importance for

understanding the diverse ligand interactions of α-actinin in the actin cytoskeleton. Studies with PKN and titin show that PIP2 can modulate their binding to the CTD of α-actinin (Mukai et al., 1997; Young and Gautel, 2000). In the naive state, the α-actinin dimer is proposed to be in closed conformation due to intrasubunit interaction between the neck region connecting the ABD and spectrin repeats of one subunit with the C-terminal domain of another subunit (Young and Gautel, 2000). Hence protein binding to the CTD is prevented by this "pseudoligand" interaction between the subunits of an α-actinin dimer. Binding of anionic lipids such as PIP2 or phosphatidylserine in the ABD causes conformational change and relieves this interaction to induce an open conformation of the dimeric protein in which the free C-terminal domain can now interact with other proteins.

The CTD of α-actinin interacts with CaMKII, thereby, regulating its localization and activity towards glutamate receptors (chapter IV). Most of the current understanding of the CaMKII-α-actinin interaction is obtained with the C-terminal fragment (residues 819-894 in α-actinin-2). However, it is important to understand the role of interactions with the intact protein. Initial studies failed to detect binding of purified CaMKII to purified full-length α-actinin *in vitro*. However, CaMKII co-precipitates with full-length α-actinin following co-expression in HEK293 cells (Chapter IV). These observations could be explained by intrinsic problem of the *in vitro* binding assay used, or it may also reflect a genuine property of the full-length dimeric molecule. It could also mean that cellular factors like Ca^{2+} or membrane lipids play a role in modulating this interaction.

In this chapter I attempt to gain insight into the mechanisms regulating CaMKII-α-actinin interaction, and show that intramolecular interactions within an α-actinin dimer can indeed regulate CaMKII binding to the CTD of α-actinin.

Results:

Modulation of CaMKII binding to the CTD in an α-actinin dimer

In the GST-cosedimentation assay, purified full-length GST-tagged α-actinin-1 or -2 did not bind purified CaMKII. As Ca^{2+} can regulate interaction of α-actinin-1 with F-actin, we tested if inclusion of Ca^{2+} could potentiate the interaction with CaMKII. We used full-length GST-α-actinin-1, -2, and also the isolated CTD of α-actinin-2. The CTD of α-actinin-2 bound purified CaMKII, and inclusion of Ca^{2+} or

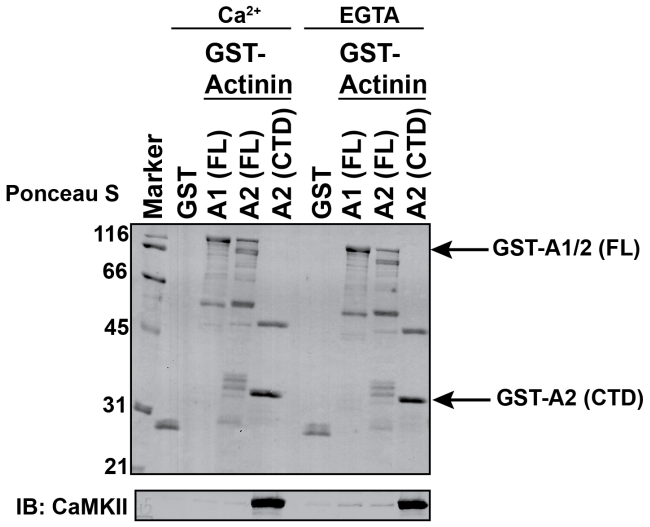

Figure 5.1 Effect of Ca^{2+} and EGTA on CaMKII binding to α-actinin
Full-length (FL) GST-α-actinin-1, or -2 (A1 or A2), or GST-A2(CTD, 819-894) were incubated with purified CaMKIIα at 1:1 molar ratio in buffer containing either 2 mm Ca^{2+} or 2 mM EGTA. Protein complexes isolated on glutathione-agarose beads were analyzed by protein staining (Ponceau S) and immunoblotting (IB) for CaMKII. n=3

EGTA in the binding buffer had no effect on the interaction (Fig 5.1). No binding of CaMKII could be detected to either full-length GST-α-actinin-1 and -2, irrespective of including Ca^{2+} in the binding buffer (Fig. 5.1).

Next, we hypothesized that pseudoligand mechanism proposed for titin binding to α-actinin could be applicable to CaMKII. Starting from GST-A2(FL), an N-terminal deletion to delete the ABD (ΔABD) had no effect in either α-actinin-1 or -2 (Fig. 5.2A). However, deletion of the ABD plus the neck region (SR1)

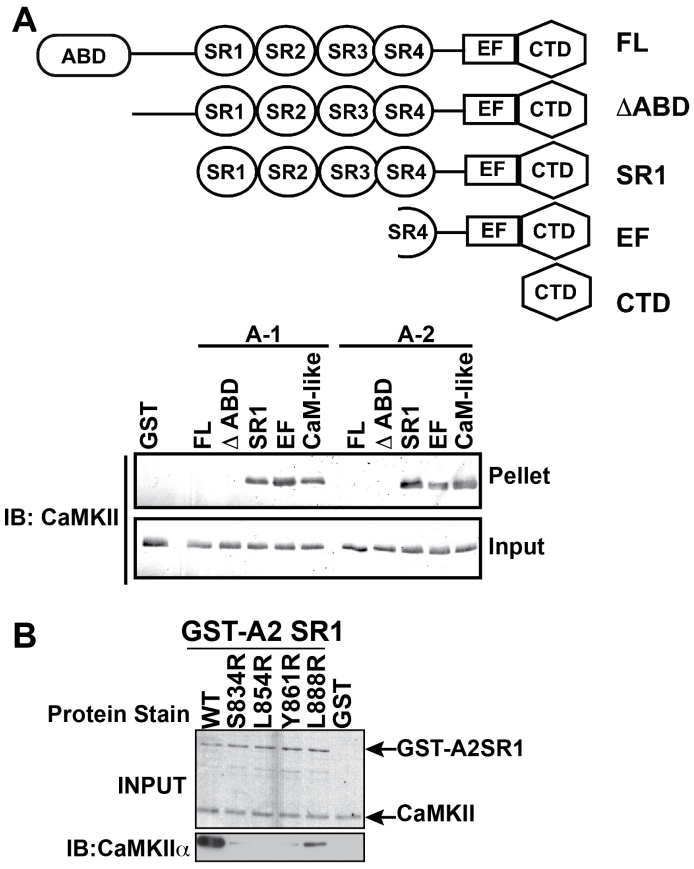

Figure 5.2 CaMKII interactions with α-actinin dimers *in vitro*

A. *Top:* Domain organization of α-actinin along with the N-terminal deletion constructs and nomenclature used in the cosedimentation assay. *Below:* CaMKII was incubated with GST alone or indicated GST-α-actinin truncation proteins. Complexes isolated on glutathione-agarose beads were analyzed by immunoblotting for CaMKII. **B.** WT or mutant GST-A2 (SR1) or GST alone incubated with purified CaMKII were analyzed by protein stain for input and immunoblotting (IB) for CaMKIIα. n=3

markedly enhanced binding of CaMKII to α-actinin-1 and -2. Since the SR1 protein dimerizes (Flood et al., 1995; Ylanne et al., 2001a, b), lack of binding to full-length or ΔABD proteins does not appear to directly result from anti-parallel dimerization. Furthermore, mutations in α-actinin that disrupt binding of the CTD to CaMKII, similarly affect CaMKII interaction with the dimeric SR1 protein (Fig. 5.2B).

CaMKII activation by SR1 α-actinin

Studies with the CTD showed that α-actinin can activate CaMKII phosphorylation of T-site substrates such as GluN2B independent of Ca^{2+} (Chapter IV). Hence we tested the ability of the SR1 protein to activate CaMKII *in vitro*. Also, even though Ca^{2+} appeared to have no effect on binding of full-length α-actinin to CaMKII, we tested whether Ca^{2+} can modulate activation of CaMKII by α-actinin SR1. Both α-actinin-1 and -2 SR1 proteins activated CaMKII phosphorylation of GluN2B. The

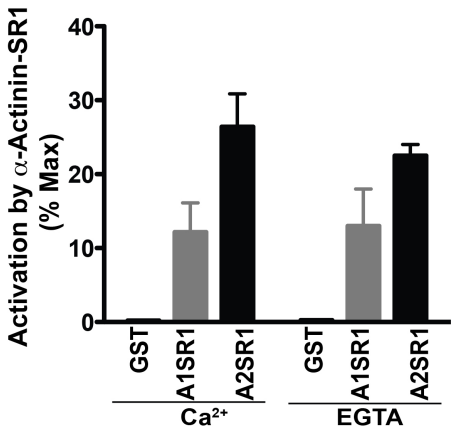

Figure 5.3 CaMKII activation by α-actinin SR1
CaMKII activity with GluN2B peptide as the substrate and indicated GST proteins as activator at 14μM in presence of Ca^{2+} (n=3) or EGTA (n=2).

activation was ~15-25% of the maximum activation by Ca^{2+}/CaM (Fig. 5.3). The EF hand domain of α-actinin-1, but not α-actinin-2, can bind Ca^{2+}. However, CaMKII was activated to a similar extent by dimeric SR1 fragments of both α-actinin isoforms, and this activation was essentially unaffected by Ca^{2+} (Fig. 5.3). In combination, these data establish that dimeric forms of multiple α-actinin isoforms can bind to and activate CaMKII in a Ca^{2+}-independent manner, but show that the N-terminal actin-binding and neck domains interfere with CaMKII binding with the CTD *in vitro*.

Interaction of CaMKII with α-actinin dimers in HEK293 cells

To investigate the effect of intramolecular interactions within α-actinin dimer on CaMKII association, we expressed Thr305/6AA CaMKIIα and either HA-tagged α-actinin-2 full-length (HA-A2FL) or HA-α-actinin-2 SR1 (HA-A2SR1) in HEK293 cells. Although both α-actinin proteins were expressed at similar levels, we observed ~10-fold more HA-A2SR1 than HA-A2FL in CaMKII immunoprecipitate

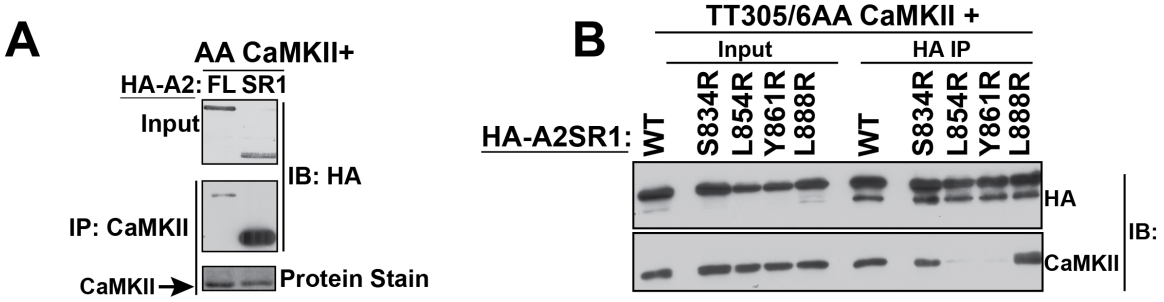

Figure 5.4 CaMKII-α-actinin interactions in HEK293 cells

A. HEK293 cell lysates expressing TT305/6AA CaMKIIα (AA CaMKII) and full-length HA-α-actinin-2 (FL) or HA-A2 SR1 (SR1) were immunoprecipitated with CaMKII antibody (IP: CaMKII). Input and immune complexes were analyzed by immunoblotting (IB) for HA and protein staining for CaMKII. **B.** HEK293 cells transfected with TT305/6AA CaMKII and WT or mutant HA-A2SR1 were lysed and immunoprecipitated with HA antibody. Input and protein complexes isolated on protein G beads were analyzed by immunoblotting (IB) for HA and CaMKII. n=3

(Fig. 5.4A). This result is consistent with the idea that head-to-tail interactions within an α-actinin dimer modulate CaMKII binding in cells. Moreover L854R and Y861R mutations in the CTD of HA-A2SR1 (Fig. 5.4B) abrogated the association of CaMKII, confirming that the interaction of CaMKII with α-actinin dimers is mediated through the CTD in cells.

Localization of CaMKII in HEK293 cells

In Chapter IV, I showed that full-length α-actinin-2 targets CaMKII to the F-actin rich regions. To test the effect of head-to-tail interactions within α-actinin on CaMKII localization I co-expressed TT305/6AA-CaMKIIα and HA-A2SR1 in HEK293 cells. I predicted that deletion of the ABD would confer a cytosolic localization for both HA-A2SR1 and CaMKII. Surprisingly, co-expression of CaMKII and WT HA-A2SR1 resulted in localization of both proteins to certain circular structures, which appear perinuclear (Fig. 5.5). Under these conditions,

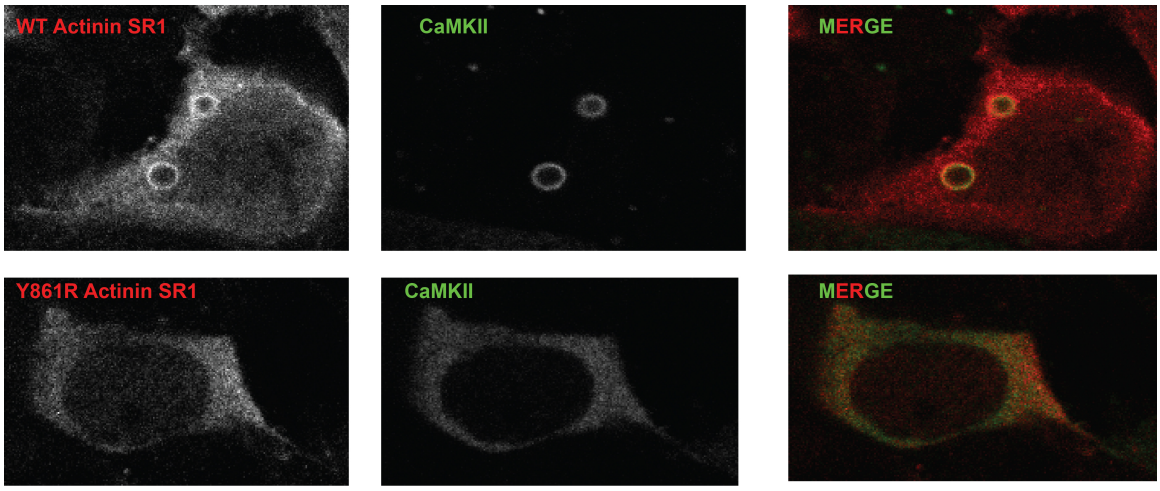

Figure 5.5 Localization of CaMKII in HEK293 cells
HEK293 cells transfected with WT or mutant (Y861R) HA-A2SR1 and TT305/6AA CaMKIIα were fixed, labeled with HA and CaMKII antibody and then imaged with confocal microscopy. Red: HA-A2SR1, Green: CaMKIIα

HA-A2SR1, although localized to the circular structures also showed some cytosolic distribution. However, CaMKII seemed to be exclusively localized to these structures. This localization of CaMKII and appearance of the structure appears to be dependent on the interaction of WT HA-A2SR1 and CaMKII, as the circular structures were not detected in cells expressing CaMKII-binding deficient mutant of HA-A2SR1(Y861R) (Fig 5.5). Similar observations were made in another cell-line, the STHdh^{+}/Hdh^{+} striatal cells (not shown).

Discussion:

As an F-actin binding protein, α-actinin plays a significant role in organizing filamentous actin in different cells by crosslinking antiparallel actin filaments giving them great mechanical stability. However, the actin cytoskeleton is also plastic in nature, thus, the protein-protein interactions at the membrane junction need to be dynamically controlled. Hence, the cell has evolved multiple mechanisms to regulate the dynamic changes the actin cytoskeleton undergoes. For example, the interactions of actin cytoskeleton or other proteins with several actin binding proteins is regulated either by divalent cations like Ca^{2+} or by membrane lipids like PIP2 (Fukami et al., 1994; Hartwig and Yin, 1988); (Fraley et al., 2005; Fukami et al., 1992). In hippocampal neurons, synaptic activation causes the remodeling of actin cytoskeleton and CaMKII is implicated in the structural plasticity of the synapse (Okamoto et al., 2009).

Although CaMKIIα does not directly bind F-actin, prior studies have shown that CaMKII associates with F-actin binding protein, α-actinin in cells, in neurons, or *ex vivo* using colocalization and/or co-immunoprecipitation (Dhavan et al.,

2002; Walikonis et al., 2001). However, these studies did not address the regulation of direct interaction of CaMKII with α-actinin.

Addition of Ca^{2+} to brain mouse lysates was shown to increase the association of CaMKII with α-actinin-1 (Dhavan et al., 2002). However, this could be an indirect effect through other interacting proteins, like Cdk5 activators, which also show increased association with Ca^{2+}. In our *in vitro* binding assay with purified proteins, Ca^{2+} showed no direct effect on CaMKII binding to α-actinin-1 or -2 *in vitro* (Fig. 5.1). Also, the activation of CaMKII by α-actinin was not affected in presence of Ca^{2+} (Fig. 5.3). However, we cannot rule out the possibility that Ca^{2+}-mediated regulation of F-actin bundling by α-actinin may modulate CaMKII binding to α-actinin.

Our initial characterization of CaMKII-α-actinin interaction used the structure of titin-α-actinin complex (Atkinson et al., 2001) as a basis to create a structural overlay of CaMKII interaction with CTD of α-actinin-2 (Chapter IV). PIP2 was shown to regulate titin-α-actinin interaction by inducing conformational change in α-actinin, which potentiated the interaction with titin (Young and Gautel, 2000). Hence we propose that PIP2 may regulate CaMKII-α-actinin interaction in a manner analogous to titin-α-actinin interaction (see chapter VI). Moreover, PIP2 binding to α-actinin is required for open conformation of the NMDAR, whereby PIP2 hydrolysis inhibited currents and PIP2 resynthesis allowed for current recovery (Michailidis et al., 2007). This PIP2 effect was mediated through α-actinin binding to the GluN1 and/or GluN2B subunit of the NMDAR. A model

was proposed whereby α-actinin interacts with PIP2 in the plasma membrane and tethers the C-termini of the NMDAR subunits to the actin cytoskeleton in an open conformation, enhancing current flow. Hydrolysis of PIP2 would somehow release α-actinin from NMDAR or the membrane, thereby inhibiting ion flow through the receptors (Michailidis et al., 2007). Hence, the regulatory mechanism proposed here would provide an elegant way to coordinate the crosslinking of actin filaments with the targeting of CaMKII to functional NMDAR at the PSD by α-actinin. The targeted CaMKII can thereby phosphorylate GluN2B at Ser1303 to desensitize the channel (Sessoms-Sikes et al., 2005) and also becomes localized closer to the receptor for a rapid response to the next Ca^{2+}-influx.

I showed that full-length α-actinin targets CaMKIIα to F-actin rich regions (Chapter IV). I predicted that the ABD domain of α-actinin would be required for this effect, and thus with the co-expression of SR1-α-actinin, CaMKII should be cytosolic. However, HA-A2SR1 targets CaMKIIα to circular structures, which appear perinuclear (Fig. 5.5). Under the imaging conditions, CaMKII seemed to be exclusively localized to these circular structures. This localization of CaMKII in the presence of A2SR1 protein was evident not only in HEK293 cells but also in the $STHdh^{+}$/Hdh^{+} striatal cells. This suggested that it is not a cell-specific mechanism. When the CaMKII-binding deficient mutant (Y861R HA-A2SR1) was used, both CaMKIIα and actinin were cytosolic and the circular structures were not observed (in both cells). These data imply the formation of these structures

may depend on CaMKII interaction with α-actinin SR1; however, more experiments are needed to understand the underlying mechanisms.

The identity of these structures is currently unknown, but they resemble pinosomes. α-Actinin is implicated in macropinocytosis, a form of endocytosis (Araki et al., 2000; Washington and Knecht, 2008). Future studies need to label cells with rhodamine-dextran to see if these structures are indeed pinosomes, and determine whether CaMKII-α-actinin interaction plays a role in formation of these structures and are these important in endocytosis. It will be interesting to co-express NMDAR with the SR1-α-actinin protein in presence or absence of CaMKII. If CaMKII-SR1 interaction is playing a role in protein endocytosis, it could provide a mechanism for NMDAR endocytosis. α-Actinin can interact with GluN2B and not the GluN2A subunit of the NMDAR; we can also investigate if only GluN2B-containing receptors are endocytosed by this mechanism. Cell-surface biotinylation experiments can provide insight into the role of α-actinin in regulating NMDAR surface expression (see Chapter VI). In the rat brain a developmental switch in the NMDAR subunit composition from GluN2B to GluN2A plays an important role in synaptogenesis and synapse stabilization (Gambrill and Barria, 2011). One can hypothesize that CaMKII-α-actinin could play a role in GluN2B-NMDAR endocytosis, thereby allowing the assembly of GluN2A-NMDAR during the developmental switch. These possibilities provide an exciting avenue to explore the role of CaMKII-α-actinin interaction in regulation of different cellular processes that modulate synaptic development and/or plasticity.

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

Summary:

The work presented in this dissertation provides new insights into the complex regulation of CaMKII by interacting/associated proteins. CaMKII can bind multiple CaMKAPs through diverse mechanisms (see chapter I), allowing CaMKII holoenzymes to form a scaffold for assembly of multiprotein complexes. These features may allow CaMKII to have synaptic roles independent of its kinase activity (Bingol et al., 2010; Hojjati et al., 2007; Pi et al., 2010a), although autophosphorylation at Thr286 may modulate these non-enzymatic roles.

The GluN2B subunit of the NMDAR has received much attention over the years as a major CaMKII binding protein that regulates CaMKII functions; however, specific functions of other CaMKAPs are less well understood. This dissertation explores the role of densin (chapter III) and α-actinin (chapter IV) in modulating CaMKII activity and/or interactions with GluN2B. I have found that both α-actinin and densin are substrate-specific modulators of CaMKII activity. These studies also identify potentially novel mechanisms for localizing CaMKII at PSDs.

Previous work suggested that densin is a CaMKIIα-specific CaMKAP, however, using CaMKIIα knockout mice, we showed that densin also interacts with CaMKIIβ. Moreover using newly developed tools for *in vitro* and cellular

studies we identified a new domain, termed densin-IN, which plays a dominant role in interacting with CaMKII. Also with an optimized protocol for looking at changes in glutamate receptor phosphorylation by CaMKII in intact cells, I found that densin-IN domain selectively inhibits GluA1 phosphorylation by CaMKII without any significant effect on GluN2B phosphorylation (chapter III) (Fig. 6.1).

Although α-actinin was identified as a CaMKAP several years ago, not much was known about the functional significance of this interaction. I found that α-actinin can localize CaMKIIα to F-actin rich structures, can activate CaMKII

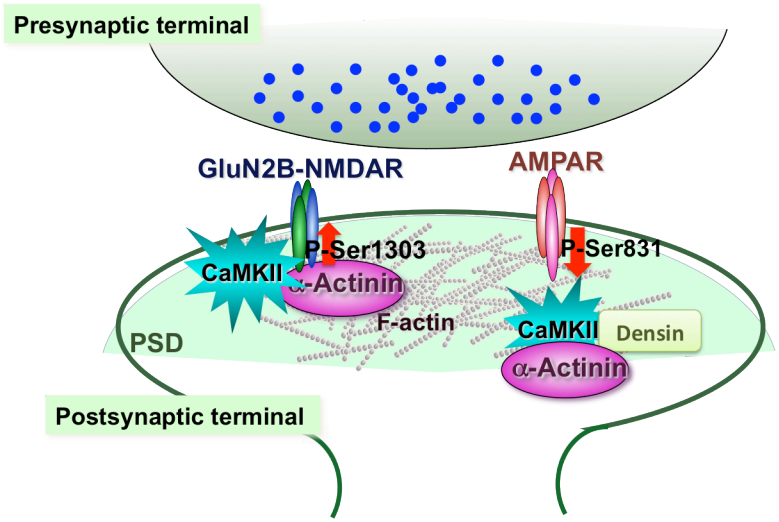

Figure 6.1 Modulation of CaMKII-phosphorylation of glutamate receptors by α-actinin and densin

α-Actinin can target CaMKII activity specifically to GluN2B-NMDAR thereby increasing Ser1303 phosphorylation. Densin and α-actinin can independently inhibit CaMKII-dependent phosphorylation of GluA1 subunit of the AMPAR at Ser831

independent of Ca^{2+}, and selectively directs CaMKII association and activity towards GluN2B (chapter IV) (Figs. 6.1 and 6.2).

These findings underscore the complexity of CaMKII targeting and regulation. In order to gain a more complete understanding of CaMKII targeting and regulation by CaMKAPs in synaptic plasticity, many more experiments will

be necessary. In this chapter I will discuss my preliminary findings and suggest some interesting avenues for future investigations.

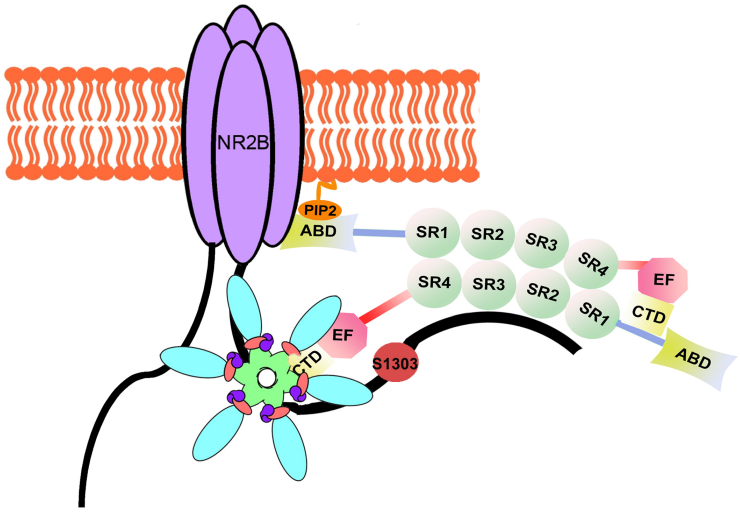

Figure 6.2 Model of α-actinin-dependent targeting of CaMKII to GluN2B-NMDAR
α-Actinin in the PIP2 bound form can interact with CaMKII via the CTD. The spectrin repeats interact with the C-tail of the receptor. This way α-actinin can target CaMKII to the NMDAR thereby increasing CaMKII-dependent phosphorylation at Ser1303 to modulate NMDAR activity.

CaMKII-densin interaction:

We found in a typical intracellular milieu that the newly identified CaMKII binding domain in context of full-length densin plays a major role in modulating CaMKII activity towards glutamate receptors (Chapter III and (Jiao Jalan-Sakrikar et al., 2011). Also it was previously shown that deletion of the central domain in densin containing the densin-IN domain disrupts densin and CaMKIIα-dependent facilitation of $Ca_V1.3$ Ca^{2+} channels in heterologous cells (Jenkins et al., 2010). These investigations of densin modulating CaMKII signaling need to be

expanded to neurons and potentially the whole animal. Several strategies can be employed with the tools already available:

1) *Role of densin-IN domain in modulating $Ca_V1.3$ L-type Ca^{2+} channels:* The natural variant of densin that lacks the densin-IN domain is deficient in supporting CaMKIIα-dependent facilitation of Ca^{2+} channels (Jenkins et al., 2010), even though it contains of the C-terminal domain that binds CaMKII. This suggests that the densin-IN domain is required for the effect. As done previously, one can transfect HEK293T cells with $Ca_V1.3$ along with CaMKIIα in presence or absence of densin-FLA, WT or the L815E mutant. Upon high-frequency stimulation, in the presence of WT densin, CaMKIIα should be able to facilitate Ca^{2+}-influx through the channels (increase in current amplitude). Co-expression of the CaMKII-binding deficient densin (L815E) mutant should occlude the effect of CaMKII.
2) *Design peptides to specifically interfere with CaMKII-densin interaction:* Although the densin-IN domain, CaMKIIN, and GluN2B appear to bind in the catalytic T-site, there are subtle differences in the binding mechanism as illuminated by the peptide competition and mutagenesis studies (chapter III and (Jiao, Jalan-Sakrikar et al., 2011). The CaMKIIN peptide (N-tide), but not GluN2B peptide (N2B-tide) efficiently compete with the densin-IN domain to bind CaMKII, but CaMKIIN and GluN2B can compete with each other. Additional mutagenesis studies in the catalytic T-site guided by an available X-ray crystal structure of CaMKIIN bound to the CaMKII catalytic domain (Chao et al., 2010a) can help shed light on the

reasons for these differences. These peptides can then be used first *in vitro* to test the effects on interaction of CaMKII with the regulatory domain and the CaMKII-binding domains in different CaMKAPs (e.g. CaMKIIN, GluN2B, SAP-97, β2a subunits of LTCC, and densin). Based on the results, the peptides can be further optimized for the specificity and potency in interfering with one interaction over the other.

The specificity of the peptides can then be tested in HEK293 cells with a TAT-sequence (GRKKRRQRRRPQ) to make the peptide cell permeable. Cells expressing CaMKII and a CaMKAP can be treated with the peptide before performing co-immunoprecipitation to test the association of the proteins.

Additionally, one can design peptides corresponding specifically to the densin-IN domain, WT or with the L815E mutation. This domain shares sequence similarity with the CaMKIIN peptide, and hence may not specifically inhibit CaMKII-densin interaction. However, either using Ala scan mutagenesis or mutagenesis based on the crystal structure of CaMKIIN-CaMKII complex, one can optimize the peptide for the specificity.

3) *Use the designed peptides to study the role of densin in regulating CaMKII functions in neurons:* The peptides designed above and tested for their specificity in HEK293 cells, can be used for studies in hippocampal neurons. These peptides can be used to disrupt the endogenous densin-CaMKII interaction. These studies can then inform the role of densin in localizing CaMKII to a specific subcellular compartment (e.g. at the PSD)

and also the phosphorylation state of CaMKII substrates (GluA1 and GluN2B). However, one should be cautious in the interpretation of the results. The observed phenotype with the peptides could be a result of disrupting densin interaction with CaMKII, direct effect on CaMKII activity, or association of CaMKII with other CaMKAPs.

4) *Role of densin in CaMKII-dependent regulation of glutamate receptors:* I showed that densin can inhibit CaMKII-dependent phosphorylation of the AMPAR subunit GluA1 at Ser831 without affecting Ser1303 phosphorylation in GluN2B of NMDAR (Jiao Jalan-Sakrikar et al., 2011). We can use electrophysiology to study the impact of these phosphorylation changes on AMPAR and NMDAR activity. Ser831 phosphorylation of the GluA1 subunit of the AMPAR by CaMKII increases channel conductance (Barria et al., 1997a). Initially in a simpler model system like HEK293 cells, we can transfect WT or L815E mutant densin along with GluA1 and CaMKII. The presence of WT densin should occlude the CaMKII-dependent increase in conductance of the AMPAR which is observed in the absence of densin or with the L815E mutant densin. To test if the observed changes are indeed due to changes in phosphorylation, we can blot the cell lysates from the transfection with phospho-specific antibodies. We can also use the phosphorylation mimic mutant (S831D) or the non-phosphorylatable mutant (S831A) as controls in parallel experiments.

CaMKII-α-actinin interaction:

The work presented in chapter IV of this dissertation investigated the role of α-actinin in CaMKIIα targeting and activity regulation. Certain CaMKIIβ splice variants can bind F-actin directly, which targets it to the F-actin cytoskeleton (O'Leary et al., 2006). I found that α-actinin-2 also interacts with CaMKIIβ *in vitro* and in HEK cells (Figs. 6.3 and 6.4). As both the isoforms (α and β) are expressed in brain, and they can form heteromers, it is important to understand how α-actinin would modulate CaMKIIβ function as a homomer and also as a heteromer with the α isoform.

Most of my experiments were performed with α-actinin-2 isoform. Others and myself have shown that α-actinin-1 (Chapter V and (Dhavan et al., 2002)), and -4 (Walikonis et al., 2001) also interacts with CaMKII. Also I show in chapter II of the thesis that α-actinin-1 and -2 can heterodimerize in HEK293 cells. As mentioned in the introduction, these isoforms differ in their ability to bind Ca^{2+} in the EF hands, which dynamically regulates F-actin bundling. Although *in vitro* studies showed no effect of Ca^{2+} on the CaMKII interaction with either α-actinin-1 or -2 (Fig. 5.1 chapter V), preliminary studies with ionophore treatment in HEK293 cells indicate that Ca^{2+} has the potential to regulate the CaMKII interaction with α-actinin (Fig. 6.5). These and other observations raise some questions:

1) *Does α-actinin regulate CaMKIIβ similar to CaMKIIα?* CaMKIIβ has a 10-fold higher affinity for Ca^{2+}/CaM than CaMKIIα. It will be interesting to test if there is an affinity difference between CaMKII α and β for α-actinin. We

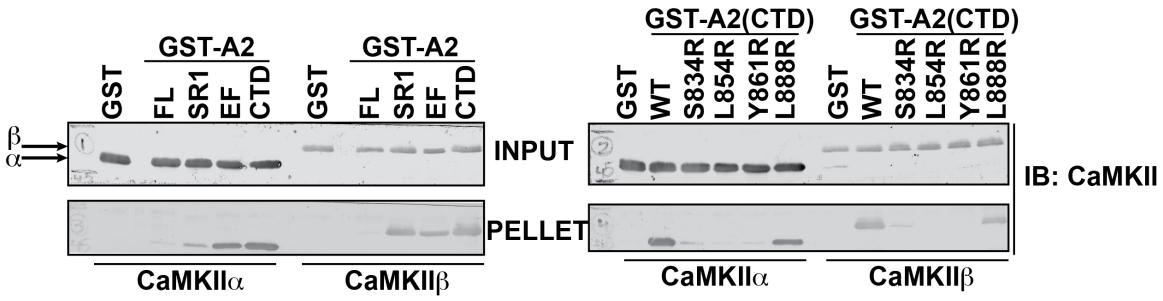

Figure 6.3 α-Actinin interaction with CaMKIIβ

Left: GST or GST-α-actinin-2 (A2) (FL or truncation mutants) were incubated with purified CaMKIIα or β. Protein complexes isolated on glutathione-agarose beads were analyzed by immunoblotting (IB) for CaMKII. *Right:* GST or GST-A2 (CTD) (WT or mutants) incubated with CaMKIIα or β were also analyzed by blotting for CaMKII. N=1

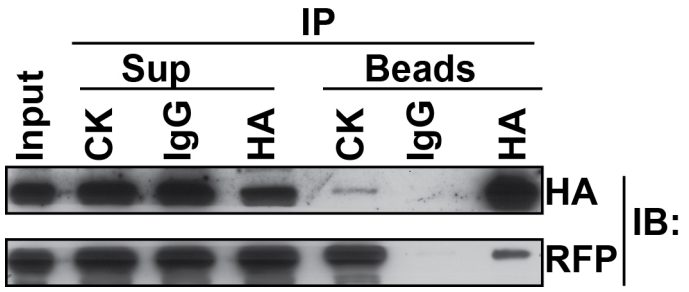

Figure 6.4. CaMKIIβ and α-Actinin interaction in HEK293 cells

HEK293 cells expressing HA-α-actinin-2 and mcherry-CaMKIIβ were lysed and immunoprecipitated (IP) with CaMKIIβ (CK), control (IgG), or HA antibody. Input, depleted IP sup and IP beads were immunoblotted with HA for α-actinin and RFP antibody for CaMKIIβ. N=2

can use fluorescence anisotropy or Isothermal Titration Calorimetry to obtain the affinity of CaMKII for α-actinin. I found that α-actinin (A1SR1 and A2SR1) activates CaMKIIβ *in vitro* towards GluN2B peptide independent of Ca^{2+}. The α-actinin-stimulated activity is 20±5% (n=2) of maximum activity obtained by Ca^{2+}/CaM. However, it remains unknown if this can be recapitulated in a cellular environment. Also a comparison of

the α-actinin effect on activity of CaMKIIα and β can provide information on the affinity of these proteins for α-actinin. We can do a dose-response activation of both the isoforms by α-actinin, and then fit the curves to one-site binding equation in Graphpad Prism to estimate K_{act} and V_{max} values. On the same lines as CaMKIIα, we can investigate if α-actinin similarly modulates CaMKIIβ localization and function in a cellular setting. To do this we can use a CaMKIIβ splice variant that does not bind F-actin.

2) *Does binding to F-actin modulate the CaMKII interaction with α-actinin-1 and -2?* F-actin co-sedimentation assays can be performed *in vitro* to examine if α-actinin binding to F-actin can modulate CaMKII interaction with α-actinin. α-Actinin construct lacking the ABD can be used as a negative control for the assay. Then, transfected HEK293 cells and/or neurons can be treated with actin depolymerizing agent, latranculin B or with actin polymerizing agent, phalloidin, before immunoprecipitation with either CaMKII or α-actinin antibody. Confocal microscopy can also be employed to track changes in localization of the two proteins with the same treatments. Downstream effects of the above mentioned treatments on CaMKII/α-actinin-mediated NMDAR localization and/or activity can also be monitored.

3) *Whether heterodimerization of α-actinin isoforms affect the protein function/localization and its impact on CaMKII?* One can approach this by co-transfecting GFP-α-actinin-1 and HA-α-actinin-2 along with CaMKII and/or NMDARs for immunoprecipitation. The amount of CaMKII and

NMDAR in the complex can then be compared to that in α-actinin-1 or -2 alone complex. Further, as Ca^{2+} enhances α-actinin-1 interaction with CaMKII (Dhavan et al., 2002), transfected cells can be treated with ionophore before immunoprecipitation under various transfection conditions. Again immunofluorescence microscopy can be employed to see changes in CaMKII localization under different conditions. Using CaMKII-binding deficient α-actinin-2 (L854R or Y861R) along with WT α-actinin-1, we can test if heterodimerization of these proteins can still allow for CaMKII binding to WT α-actinin-1. This can be informative if in future, we plan to create a mouse model of mutant α-actinin.

4) *What cellular factors regulate the CaMKII-α-actinin interaction?* Addition of Ca^{2+} to brain lysates increases the co-immunoprecipitation of α-actinin with CaMKII (Dhavan et al., 2002). However, inclusion of Ca^{2+} in an *in vitro* binding assay has no effect on CaMKII-α-actinin interaction (chapter V). Also Ca^{2+}/CaM can compete with α-actinin for binding to CaMKII *in vitro*. I have begun to address the effects of Ca^{2+} on CaMKII association with α-actinin in a cellular setting, I treated HEK293 cells co-expressing CaMKII and α-actinin-1 or -2. Preliminary experiments reveal increased α-actinin association with ionophore treatment in CaMKII immune complex (Fig. 6.5). However, there appears to be a slight decrease in the association of the two proteins in α-actinin immune complex (immunoprecipitation with HA antibody). The differences observed could be due to isolation of different pools of the protein complex. More

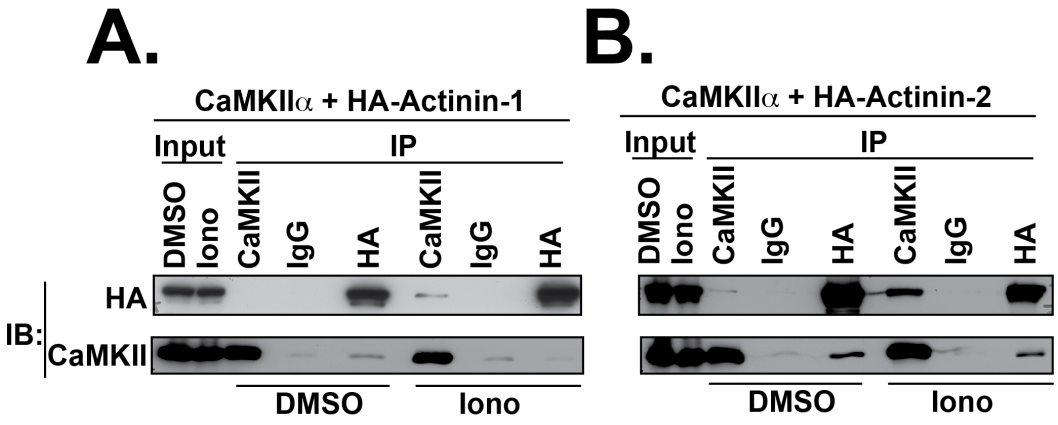

Figure 6.5 Effect of ionophore on CaMKII-α-actinin interaction
HEK293 cells transfected with HA-actinin-1 (A), or HA-actinin-2 (A) and CaMKIIα were treated with either DMSO or ionophore (Iono) in KRH buffer. After lysis, the cell lysates (input) were immunoprecipitated (IP) with either CaMKII, control (IgG), or HA antibody. Immune complexes were then analyzed by immunoblotting (IB) for CaMKII and HA. N=2

experiments with time course of ionophore treatment and truncated α-actinin (SR1) need to be done to address the effects of Ca^{2+}. If the effect of Ca^{2+} is due to its effect on F-actin bundling activity of α-actinin, then I expect not to see these effects with the α-actinin-SR1 protein.

In chapter V, I describe how intramolecular interactions within α-actinin dimer regulate binding to CaMKII. My preliminary data suggest that similar to titin binding to full-length α-actinin, PIP2 might modulate CaMKII binding to full-length α-actinin. As an initial step, I incubated purified GST-α-actinin-2 (FL) with purified CaMKII in the presence or absence of PIP2 vesicles (see Appendix for protocol). The complex was then sedimented on glutathione-agarose beads before analysis by SDS-PAGE and

immunoblotting for CaMKII. Initial studies suggested that PIP2 could potentiate CaMKII interaction with full-length α-actinin (Fig. 6.6). However, further optimization (dose-response of PIP2 and/or other lipids) and controls (lipid specificity) are needed to confirm these findings. PIP-strip assays (Mustafa et al., 2009) can be employed first to test the phospholipids to which the purified GST-α-actinin-1 and -2 bind. Then either phospholipid vesicles or liposomes can be made and used in the binding assay. The protein complex can then be sedimented either using glutathione-agarose or by ultracentrifugation of the liposomes. Lipids that do not bind α-actinin in the PIP strip assay can be used as a negative control for the experiment. PIP2-binding deficient α-actinin can also be used as a negative control.

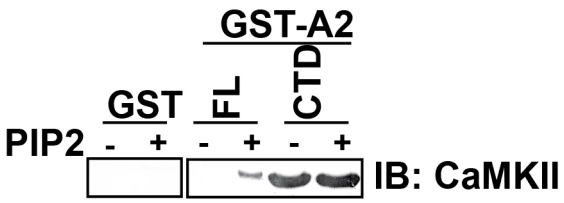

Figure 6.6 PIP2 potentiates CaMKII interaction with full-length α-actinin-2
GST or GST- actinin-2 (A2) (FL or CTD) were incubated with purified CaMKII in presence or absence of PIP2 vesicles. Protein complexes were isolated on glutathione-agarose beads were analyzed by immunoblotting (IB) for CaMKII. N=2

One can study the role of PIP2 in modulating CaMKII-α-actinin interaction in HEK293 cells or hippocampal neurons by either blocking PIP2 synthesis (by inhibiting phosphoinositide-4 kinase) or stimulating PIP2 hydrolysis (activating phospholipase C) before immunoprecipitation or imaging. Similarly inhibition of phospholipase C to increase PIP2 levels

can be employed to test if PIP2 potentiates CaMKII association with α-actinin. Similar treatments have been previously used to dissect the role of PIP2 in regulating NMDAR function (Mandal and Yan, 2009; Michailidis et al., 2007).

5) *Are there changes in CaMKII-α-actinin association in various CaMKII mutant mouse models?* Previous studies (Robison et al., 2005a) and data presented in chapter IV show that TT305/6, but not T286, autophosphorylation in CaMKII interferes with α-actinin binding to CaMKII. Further, I show that although *in vitro* autophosphorylation at Thr306, but not Thr305 interfere with α-actinin binding (chapter IV), either T305D or T306D mutations to mimic phosphorylation in CaMKII disrupt α-actinin binding (Fig. 6.7).

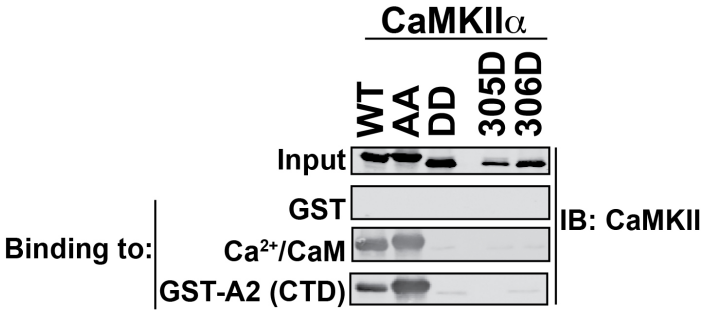

Figure 6.7 Effects of T305D and T306D mutation in CaMKII on Ca2+/CaM and α-actinin binding

HEK293 cell lysates (Input) expressing WT, TT305/6AA (AA), TT305/6DD (DD), T305D, or T306D CaMKIIα were incubated with either GST, GST-A2(CTD), or Ca^{2+}/CaM agarose. Input and protein complexes isolated on agarose beads were analyzed by immunoblotting (IB) for CaMKII.

In order to begin to understand the role of these autophosphorylation sites *in vivo*, we obtained different CaMKII

mutant genotype mouse forebrains (WT, T305D, and TT305/6VA) from Dr. Ype Elgersma (Erasmus University Rotterdam, The Netherlands). These mice have knock-in mutations at the phosphorylation sites, the T305D mutation mimics phosphorylation, and the TT305/6VA mutation prevents phosphorylation at these sites. Based on my *in vitro* characterization, I expected to see decreased association of α-actinin with CaMKII in T305D compared to WT, and an increased interaction in the TT305/6VA mice forebrain. However, initial studies with low-ionic lysis conditions revealed some unexpected results. Although CaMKII and α-actinin co-immunoprecipitated from WT mouse brain, we saw a decrease in the association of the two proteins in the TT305/6VA mouse brain (Fig. 6.8).

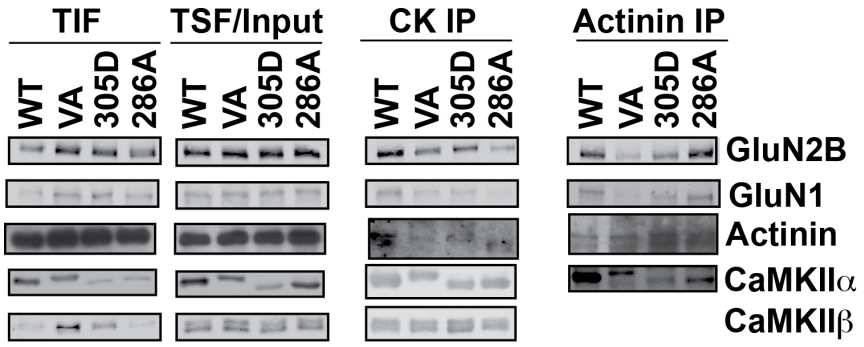

Figure 6.8 CaMKII-α-actinin association in various CaMKII mutant mice forebrain

Forebrains from WT, T305/6VA (VA), T305D, and T286A transgenic mice were homogenized in low-ionic 1% Triton X-100 buffer. The triton-soluble fraction (TSF) was immunoprecipitated (IP) with CaMKII (CK) or α-actinin antibody. Protein complexes isolated on protein G beads and triton-insoluble fraction (TIF) were analyzed by immunoblotting for proteins indicated on right. N=2

As expected, T305D showed reduced co-immunoprecipitation of α-actinin with CaMKII and vice-versa. Furthermore, in the T286A mutant CaMKII mouse brain, there appears to be a reduction in the association of two

proteins (Fig. 6.8). This experiment looks at the global change and does not provide insight into any changes in a particular subcellular

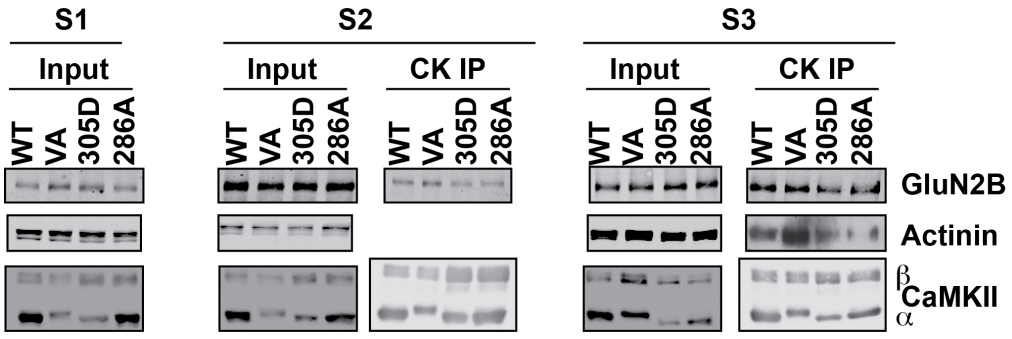

Figure 6.9 Subcellular fractionation from CaMKII mutant mice forebrains Forebrains from WT, T305/6VA (VA), T305D, and T286A mice were fractionated to isolate S1 (cytosolic), S2 (membrane-associated), and S3 (PSD-associated) proteins. The S2 and S3 fractions were then immunoprecipitated with CaMKII (CK) antibody. CaMKII immune complexes isolated on Protein G beads and the S1 fraction were analyzed by immunoblotting for proteins indicated on right. N=1

region. We know autophosphorylation of CaMKII affects CaMKII localization in the cells and these mutant mice show disrupted CaMKII localization in the brain (Elgersma et al., 2002; Gustin et al., 2011).

Using a fractionation protocol developed in our lab (chapter II and (Gustin et al., 2010), we detect differential distribution of CaMKII in different genotypes (Fig. 6.9). Moreover, in the S3/P3 fraction, which represents the synaptic and PSD fraction, we see increased association of α-actinin with CaMKII in the TT305/6VA mice compared to WT (Fig. 6.9). In T305D and T286A mice, there appears to be a slight reduction in amount of α-actinin co-precipitating with CaMKII. However, these are very preliminary findings and need to be repeated with more animals. Also at least two α-actinin bands are evident from the blots, which seem to show

differential localization (between S1, S2, and S3), and likely represent different α-actinin isoforms. Using different antibodies available (chapter II), we can investigate any isoform differences in their association with CaMKII in these mutant mice.

In the Angelman Syndrome (AS) mouse model, hyper-phosphorylation at TT305/6, which results in reduced CaMKII activity and PSD localization is believed to contribute to the phenotype as the mutation of these sites to Val/Ala rescued the deficits in the AS mice (van Woerden et al., 2007; Weeber et al., 2003). Given the effects of TT305/6 autophosphorylation on α-actinin binding, I hypothesize that a decreased α-actinin-CaMKII association in the AS mouse model may contribute to mechanisms underlying this disorder. However, we need to test this hypothesis. We can use the subcellular fractionation protocol to specifically test the association of CaMKII and α-actinin in the synaptic/PSD fraction of AS mice.

Role of α-actinin in regulating glutamate receptor function

Surface Expression of NMDAR:

α-Actinin directly interacts with the GluN1 and GluN2B subunits of the NMDAR (Wyszynski et al., 1997). Binding to the GluN1 subunit is competitive with Ca^{2+}/CaM, and is functionally implicated in Ca^{2+}-dependent inactivation (CDI) of the NMDAR (Zhang et al., 1998). α-Actinin is also implicated in supporting NMDAR activity through its interaction with PIP2 and NMDAR (Michailidis et al.,

2007). The role of α-actinin interaction with GluN2B is unknown, but my work shows that it could be important in enhancing CaMKII targeting to GluN2B-containing NMDARs (chapter IV). In many cases, α-actinin has been shown to regulate the cell-surface expression of ion channels (see Introduction). Although

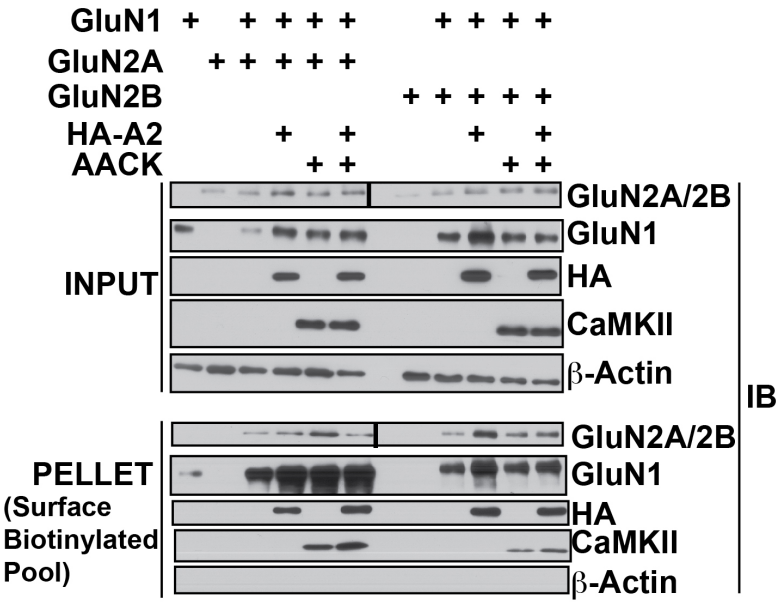

Figure 6.10 Cell-surface biotinylation of NMDAR expressed in HEK293 cells
HEK293 cells were transfected with GluN1 alone, GluN1+GluN2A/GluN2B (alone, with HA-α-actinin-2, with 305/6AA CaMKIIα, or HA-A2+ AACaMKIIα). Biotin treated cells were lysed and isolated on streptavidin beads and then analyzed by immunoblotting (IB) for indicated proteins. N=1

α-actinin has been postulated to be an anchoring protein for the NMDAR (Wyszynski et al., 1998), the role of α-actinin in regulating NMDAR expression is unknown. Cell-surface biotinylation is a common methodology employed to test the changes in surface expression of proteins. An initial experiment comparing GluN2A- and GluN2B-NMDARs, α-actinin appeared to increase the cell-surface expression of GluN2B-containing, but not GluN2A-containing NMDAR (Fig. 6.10). Moreover, additional expression of CaMKII seemed to block these effects of α-

actinin. More experiments are needed to confirm these findings (see protocol in Appendix). Furthermore, we can use the α-actinin-SR1 protein to test if the ABD of α-actinin is required for this effect.

NMDAR-activity:

As a follow-up to the biotinylation experiments, electrophysiology (whole cell patch clamp) could be employed to record NMDAR currents from HEK293 cells with or without α-actinin co-expression. If the biotinylation findings are confirmed, we should see enhanced current density in the presence of α-actinin (full-length) and reduced current density with α-actinin-SR1.

Previous studies from our lab showed that CaMKII enhances the desensitization of GluN2B-containing NMDARs (Sessoms-Sikes et al., 2005). Cells expressing NMDARs are cultured in the presence of the NMDAR antagonist, APV, to prevent excitotoxicity. One interesting aspect of this study was that the effect of CaMKII was evident and maximal when the first puff of NMDA/glycine was applied, suggesting that CaMKII was primed independent of prior NMDAR activation to modulate NMDARs. Also, CaMKII activity was required for the enhanced desensitization of the NMDARs. Furthermore Ser1303 phosphorylation of GluN2B was required for this effect (unpublished observations). These studies suggest that either the basal level of Ca^{2+} was enough to activate CaMKII, or endogenous α-actinin might have targeted CaMKII to the NMDAR for Ser1303 phosphorylation and hence desensitization. To investigate the role of α-actinin in modulating CaMKII-dependent NMDAR desensitization, either a siRNA and rescue expression or overexpression of WT

and mutant α-actinin can be used. As the tools are available, one can overexpress WT or mutant α-actinin (L854R or Y861R) deficient in CaMKII binding along with NMDAR (GluN2B- or GluN2A-containing) with or without CaMKIIα. NMDA/glycine challenge with the patch clamp can be performed. In the presence of WT α-actinin, one would expect to see faster desensitization, which will be occluded in presence of the mutant α-actinin (acting as dominant-negative).

Using siRNA designed to suppress endogenous α-actinin (isoform 1 or 4, see chapter II), α-actinin expression can be reduced. In these cells, then NMDAR (GluN2B- or GluN2A-containing) along with CaMKIIα can be expressed for the patch clamp studies with a NMDA/glycine challenge. If α-actinin is playing a role in CaMKII-dependent desensitization, I predict that there will be no changes in NMDAR kinetics with or without CaMKII. Then upon rescue with transfected WT α-actinin in the siRNA-treated cells with NMDAR and CaMKII, we should see CaMKII-mediated NMDAR desensitization. Similar experiments can then be performed in hippocampal neurons to investigate the effects of α-actinin on CaMKII-mediated regulation of NMDAR.

Regulation of AMPAR function:

Similar to the densin-IN domain, I found that α-actinin inhibits Ser831 phosphorylation of the GluA1 subunit in the AMPAR. As mentioned earlier in the future directions for densin, we can transfect HEK293 cells and/or neurons with CaMKII and GluA1 in presence or absence of α-actinin. Whole-cell patch clamp to measure the AMPAR current can provide information regarding the role of α-actinin in modulating CaMKII-mediated AMPAR regulation.

As α-actinin and densin interact with each other and CaMKII, they may collaborate to inhibit CaMKII-mediated enhancement in AMPAR conductance. If co-transfection of densin and α-actinin additively inhibit Ser831 phosphorylation, CaMKII-mediated enhancement of AMPAR conductance will be occluded. These studies will aid in our understanding how in a ternary complex, CaMKII activity is modulated to inhibit signaling through AMPAR.

Structure of the CaMKII complex

Previous studies showed that CaMKII can simultaneously bind multiple CaMKAPs. CaMKII immunoprecipitation from the brain also shows multiple associated proteins. I also showed a tripartite complex of CaMKII-α-actinin-GluN2B in transfected HEK293 cells (Chapter IV). However, it is unclear how many CaMKAPs can interact with a single holoenzyme of CaMKII and how steric constraints may effect these interactions. Such information is crucial to development of models of the PSD. One approach to answer such question is to use cryo-electron microscopy (cryo-EM). This method has been employed to get structures of CaMKII. We can form CaMKII complex with one or multiple

CaMKAPs and use cryo-EM to determine the structures and assembly of these complexes. This will provide insight into how different CaMKAPs may bind to different kinase domains simultaneously (e.g. α-actinin, densin, and GluN2B), or how they bind to same domains differentially (e.g. densin, CaMKIIN, and GluN2B). It may also provide information on how and whether the CaMKAPs also interact with each other when bound to CaMKII.

Final Summary:

Overall, the data in this dissertation present evidence that multiple PSD-enriched proteins can regulate CaMKII localization and activity. Specifically, I show how α-actinin and densin can regulate CaMKII signaling to fine-tune CaMKII actions upon changes in synaptic activity. These findings will allow us to form a model of CaMKII regulation and functions at the PSD.

The ultimate goal of this project is to assess the importance of densin and α-actinin in the molecular process of learning and memory. My hope is that the work in this dissertation has laid the groundwork for further exploration of the role of the PSD proteins in regulating CaMKII in synaptic plasticity, and contributes to our understanding of learning and memory.

Recently a paper describing the effects of deleting densin was published (Carlisle et al., 2011). Deletion of densin resulted in schizophrenia-like phenotype, concurrent with deficits in synaptic plasticity and spine morphology. Furthermore, CaMKII regulation by NMDAR activation was disrupted along with reduced total and PSD levels of α-actinin. These results illuminate the role of densin in influencing CaMKII function. It is not clear however, whether the phenotypes are

a direct effect of disrupting densin-CaMKII interaction or a secondary effect of reducing α-actinin which may alter CaMKII dynamics. Hence, one can create a knock-in transgenic mouse line containing the L815E densin mutant, which based on the results presented in chapter III, should specifically disrupt CaMKII binding. Similarly, a transgenic mouse line with the L854R or Y861R α-actinin mutant could offer an opportunity to discover the physiological effects of disrupting CaMKII-α-actinin interaction without affecting association with densin. Also a double transgenic line with the densin and α-actinin mutant may provide an avenue for uncovering the role of these CaMKAPs in CaMKII association with PSD and thereby its role in neuronal function of learning and memory.

APPENDIX

SUPPLEMENTARY METHODS

Ionophore treatment: HEK293 cells transfected with CaMKIIα and HA-α-actinin-2 (24 hrs after transfection) were incubated in 5ml of warm KRH buffer (in mM: 125 NaCl, 5 KCl, 1.2 KH_2PO_4, 25 HEPES, 2.2 $CaCl_2$, 1.2 $MgSO_4$). 10 µM Ca^{2+}-ionophore (A23187, Sigma) or DMSO was added to the cells in KRH buffer and incubated for 15 mins @ 37^0C. Then the cells were washed with ice-cold KRH buffer and lysed in low-ionic strength lysis buffer for immunoprecipitation as before (see chapter II).

PIP2 vesicles: Phosphatidylethanolamine (PE), cholesterol, and phosphatidyl choline (PC) from Avanti Polar Lipids were used from Dr. Alex Brown's laboratory. Phosphatidylinositol-4,5-bisphosphate (PIP2) was ordered from Avanti Polar Lipids. The lipids in chloroform were mixed at following concentrations in a test tube: 10mg/mL PE, 0.4mg/mL cholesterol, 10mg/mL PC, and 1 mg/ml PIP2. The mixture was then dried under nitrogen for 15 minutes or until a film of dried lipids was formed at the bottom of the tube. Reaction buffer (100 mM HEPES, 6 mM EGTA, 160 mM KCl, 2 mM DTT) was added to the dried lipids at required volume and bath sonicated for 10 minutes or till a translucent solution with no floating particles is formed. The vesicles are stable for 4 hrs at R.T.

For the GST-PD assay, GST-α-actinin-1 or -2 (FL, or CTD) is initially incubated with PIP2 vesicles for 1 hr at R.T. Then purified CaMKIIα is added and incubated further for 30 mins. After adding 40µl of glutathione-agarode beads slurry, the mix is incubated for another 1 hr. The beads are then washed 4-5 times with PD buffer and protein complex analyzed as before (chapter II).

Cell-surface biotinylation: Transfected HEK293 cells (24 hrs after transfection) were washed twice in ice-cold PBS containing 0.1mM Ca^{2+} and 1mM Mg^{2+} for 5 mins each. Cells were then incubated with Sulphosuccinimidyl1-2-(biotinamido)ethyl-1,3-dithioproprionate-biotin (Sulpho-NHS-SS-Biotin, Thermo Scientific) for 30 min at 4^0C. Excess biotin was quenched by 2 washes with 0.1 M glycine in PBS and cells were solubilized using radioimmunopreciptation assay (RIPA) buffer (100 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1 % SDS, 1 % Triton X-100, 1 % sodium deoxycholate, 5 mg/L leupeptin, 50 mM NaF, and 200 µM microcystin) buffer for 30 min at 4^0C. After centrifugation at 10,000xg for 10 minutes, the supernatants were incubated with pre-washed streptavidin-agarose beads (40 µl, 1:1 slurry, Thermo Scientific) overnight at 4^0C. The beads were then washed 3-4 times with RIPA buffer and proteins were eluted in SDS-PAGE sample buffer. Proteins separated by SDS-PAGE were then analyzed by immunoblotting as explained in chapter II.

Binding of α-actinin and Ca^{2+}/CaM to CaMKII regulatory domain mutants

In order to find the determinants in CaMKII regulatory domain for binding to CaM and α-actinin, I systematically mutated residues in the regulatory domain of CaMKIIα. Then I expressed the WT and mutant CaMKII in HEK293 cells. Binding to α-actinin and CaM was analyzed as described in Chapter II of the dissertation. The data summarized below suggests that α-actinin interacts with one face of the regulatory helix of CaMKII unlike CaM, which interacts with both sides of the regulatory domain (also see Chapter IV). These studies were mainly done with GST-A2CTD construct of α-actinin. Hence it is important to test if the findings hold true with longer constructs of α-actinin, FL and SR1.

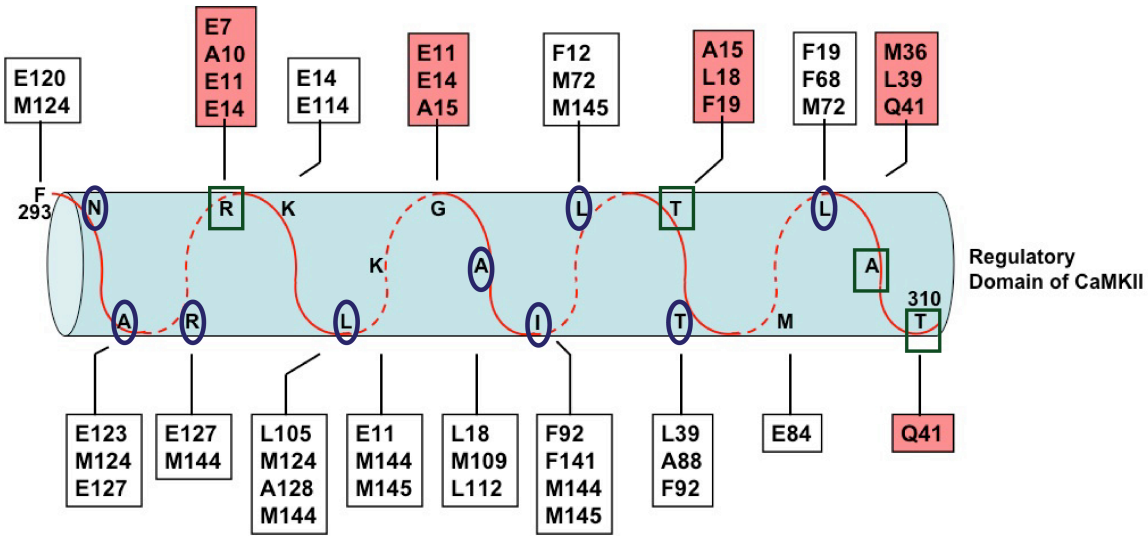

Fig A.1 Determinants in CaMKII for binding to CaM and α-actinin

A schematic of CaMKII regulatory domain showing contacts with residues in N-lobe of CaM (red boxes) and and C-lobe of CaM (white boxes). The regulatory domain residues that are important for engaging both CaM and α-actinin are highlighted in blue circles and those important only for CaM are highlighted in green rectangles.

Regulatory domain mutation	Binding to		n
	CaM	Actinin	
F293R	✗	✗	2
A295R	✗	✗	3
R296E	✗	✗	3
R297E	↓	✓	2
K298E	↓	↓	1
L299R	↓	↓	2
A302R	✗	↓	5
I303R	✗	✗	5
L304R	✗	✗	2
T305D	✗	✓	3
T306D	✗	✗	3
M307R	↓	↓	1
L308R	↓	↓	1
A309R	↓	✓	1
T310D	✗	✓	3
S314D	✗	✓	3
S314A	✓	✓	3

Table A.1 Binding of CaM and α-actinin to regulatory domain mutants of CaMKII

Regulatory domain mutants in the TT305/6AA CaMKIIα background were tested for binding to CaM and α-actinin. Data is summarized as follows compared to TT305/6AA CaMKIIα: ✗= no binding, ✓= no effect, ↓= reduced binding. N stands for number of experiments.

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