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Meagan A. Jenkins 4/1/2011

Moduation of post-synaptic ion channels by CaMKII

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An abstract of a dissertation submitted to the Faculty of the James T Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate Division of Biological and Biomedical Science Neuroscience in 2011

Abstract

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By Meagan A Jenkins

The function, trafficking and synaptic signaling of post-synaptic ion channels are tightly regulated by phosphorylation. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a key regulator of Ca^{2+}-signaling in neurons, known to bind and phosphorylate many ligand- and voltage-gated ion channels, including AMPA receptors and Ca_V1 voltage-gated Ca^{2+} channels. CaMKII phosphorylates residue Ser831 within the C-terminus of recombinant GluA1 AMPA receptors to increase single channel conductance, and these actions can be mimicked with substitution of GluA1-Ser831 with negatively charged glutamate or aspartate residues. CaMKII increases the conductance of native heteromeric AMPA receptors in hippocampal neurons via phosphorylation at Ser831. CaMKII acts to increase the coupling efficiency, or the efficiency by which the AMPA receptor translates binding of agonist into gating of the receptor. Furthermore, coexpression of TARPs with recombinant receptors is required for phosphoSer831 to increase conductance of heteromeric GluA1/GluA2 receptors. Finally, the membrane proximal region of the GluA1 C-terminal domain within which the CaMKII phosphorylation site is contained, acts as a hyper-regulatory region of AMPA receptor function. Phosphomimic mutations inserted at each of 3 different phosphorylation sites within this region, GluA1-Ser818, GluA1-Ser831, and GluA1-Thr840, lead to an increase in GluA1 receptor conductance. These residues likely couple strongly to AMPA receptor gating and permeation elements to dynamically regulate receptor conductance. Clearly there are overlapping, complex and tightly coordinated mechanisms of phosphorylation-dependent GluA1 AMPA receptor regulation, which are likely implicated in synaptic plasticity since changes in GluA1 activity and trafficking underpin changes in LTP and LTD. Specifically, CaMKII-dependent phosphorylation of GluA1-Ser831 has been a focus of studies on synaptic scaling and plasticity, thus, understanding the functional and structural mechanisms of GluA1-Ser831 phosphorylation-induced increases in AMPA receptor activity is crucial for understanding the molecular changes that occur during activity-dependent synaptic plasticity, a leading model of learning and memory.

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Chapter 1
Introduction

I. Preamble

The structure and function of neuronal ion channels has been a major focus of neurobiological research since Hodgkin and Huxley first described the initiation and propagation of the squid axon action potential and the ionic currents that underlie it (Hodgkin and Huxley, 1952). Since this seminal work, studies acheived in the past 60 years have lead to the classification and characterization of both voltage- and ligand-gated ion channels that conduct ionic current in response to changing membrane potential or neurotransmitter binding, respectively. Glutamate was originally found to excite neurons by Watkins and colleagues when they showed that L-glutamic acid dramatically increased the firing rate of a spinal cord dorsal horn interneuron (Curtis et al., 1959). Studies that were undertaken in subsequent years helped to identify three different receptor populations that were activated by glutamate and were specifically sensitive to different drug classes (Curtis and Johnston, 1974; Johnston et al., 1974; Biscoe et al., 1975; Anderson et al., 1976; Cull-Candy, 1976; MacDonald and Nistri, 1978; Krogsgaard-Larsen et al., 1980). The cloning of the glutamate receptor subunits advanced the field further, bringing the study of glutamate receptor pharmacology, structure and function to the molecular level (Hollmann et al., 1989; Hollmann and Heinemann, 1994). Finally, the recently described crystal structure of a membrane-spanning, tetrameric ligand-gated homomeric GluA2 AMPA receptor has revealed many features of channel structure including

high resolution descriptions of the channel pore, gate, and subunit symmetry (Sobolevsky et al., 2009).

While this structure has provided great advances in the understanding of ion channel function, it unfortunately lacks the C-terminal domain. This intracellular portion of each subunit contains sites of interaction with a vast array of intracellular proteins such as kinases, phosphatases, enzymes, nucleotides and structural scaffolds. Despite large macromolecular distances from the channel pore, interactions at the C-terminus of an ion channel subunit can lead to significant effects on channel gating and kinetics. Modulation of ligand- and voltage-gated ion channels via their C-termini enables greater diversity in channel function and, therefore, cell physiology.

This dissertation addresses the localized and direct modulation of Ca^{2+}-permeable, post-synaptic voltage- and ligand-gated ion channels by CaMKII. CaMKII, or Ca^{2+}-calmodulin dependent protein kinase II, is a Serine-Threonine protein kinase that is activated by Ca^{2+}-bound calmodulin (Ca^{2+}/calmodulin or CaM). CaMKII adds a phosphate group to a variety of substrates to change their activity, structure and thereby function. The ways in which phosphorylation by CaMKII can change ion channel function are extensive. I have investigated the manner by which CaMKII increases activity of both a voltage-gated Ca^{2+} channel, $Ca_v1.3$, and a ligand-gated GluA1 subunit-containing AMPA receptor, both Ca^{2+} permeable ion channels localized to excitatory post-synaptic membranes. GluA1 AMPA receptors and $Ca_v1.3$ Ca^{2+} channels mediate changes in post-synaptic membrane potential and provide a source for increases in cytosolic Ca^{2+} that

contributes to intracellular signaling pathways. My results show that CaMKII phosphorylation of the C-terminus of the pore-forming α subunit of $Ca_v1.3$ Ca^{2+} channels ($\alpha_1$1.3) enhances channel function in a frequency and Ca^{2+}-dependent manner. CaMKII phosphorylation of the C-terminus of the GluA1 AMPA receptor subunit potentiates receptor function through an increase in single channel conductance.

In the following pages, I will show the widespread effects that a single protein, CaMKII, can exert on two exemplary ion channels. First, in the current chapter, I will provide the necessary background information on CaMKII and AMPA receptors. I will then present data showing how CaMKII increases conductance of GluA1-containing AMPA receptors, and the important role that association proteins play in this functional effect in Chapters 2 and 3. In Chapter 4, I will switch gears and summarize the relevant background information on voltage-gated Ca^{2+} channels. I will then present the results from experiments showing a CaMKII-dependent increase in $Ca_v1.3$ Ca^{2+} channel activity in Chapter 5. I will finally discuss and synthesize all of my findings in Chapter 6.

II. CaMKII

In the following section, I will begin with an overview of the structure of Ca^{2+}/calmodulin-dependent protein kinase II, its isoforms, how this enzyme is activated and finally, its neuronal localization and function in the nervous system. I will then proceed by providing an extensive overview of AMPA receptor structure and function, before laying out the results of this study in the following chapters.

A. Post-synaptic density

Neurons in the CNS communicate with one another via release of neurotransmitter from one cell onto another via synapses. Pre-synaptic terminals release neurotransmitter into the synaptic active zone. The post-synaptic density (PSD) is a tightly organized biochemical structure on the cytosolic surface of the post-synaptic cell, directly oppisite the active zone. The PSD is an electron-dense region of the synapse that contains dozens of signaling molecules, ion channels, G-protein coupled receptors, scaffolding proteins and regulatory elements (Ziff, 1997; Kennedy, 2000). This area appears as a thick, dark region approximately 30 to 50 nm thick and up to 500 nm wide in electron micrographs of central nervous system tissue (Palay, 1958; Gray, 1959). There are two major types of PSDs, those at type I excitatory glutmatergic synapses, and type 2 inhibitory synapses (Gray, 1959). This region can be biochemically isolated from the plasma membrane with detergents to allow direct chemical characterization of the protein composition (Cotman et al., 1974). Early studies isolated a major

polypeptide fraction present in the PSD with a molecular weight of 53,000 daltons, which was later identified to be CaMKII (Banker et al., 1974; Kennedy et al., 1983).

B. General structure

CaMKII is a widely-distributed, multifunctional oligomeric protein kinase that mediates a vast array of physiological responses to elevated intracellular Ca^{2+} levels. CaMKII, most highly expressed in neurons, is an integral feature of many cell signaling pathways and mediates a host of neuronal functions through broad substrate specificity (Wayman et al., 2008). Ca^{2+} influx via neuronal voltage- and ligand-gated ion channels is critical to many cell functions including synaptic plasticity and maintaining the balance between normal and pathological brain states. The spatial localization, amplitude, duration and frequency of complex Ca^{2+} transients convey information and modulate cell function via a range of Ca^{2+} sensitive processes. CaMKII is prominent among Ca^{2+} sensitive proteins that translate these important signals into physiological responses. As depicted in Figure 1.1, CaMKII holoenzymes are 500-600kDa multimeric complexes comprised of 10-12 subunits of 50-60kDa each, shown initially with high magnification electron microscopy of purified CaMKII from rat cerebellum and forebrain (Hanley et al., 1987; Kanaseki et al., 1991). These individual subunits come together as stacked hexameric rings, in which association domains are at the center, and the catalytic domains project out in a flower petal-

like arrangement (Kanaseki et al., 1991; Rosenberg et al., 2005; Rosenberg et al., 2006).

C. Isoforms

There are four CaMKII isoforms, encoded by distinct genes, CaMKIIα, $-\beta$, $-\gamma$, and $-\delta$, that each play distinct roles in the CNS (Bennett and Kennedy, 1987; Lin et al., 1987; Tobimatsu et al., 1988; Tobimatsu and Fujisawa, 1989). Each of these individual isoforms possess a highly conserved N-terminal catalytic region (Figure 1.1), which contains an ATP-binding motif and is comprised mostly of β-sheets. Immediately downstream of the catalytic region lies the regulatory domain, a stretch of 40 amino acids that contains an autoinhibitory domain, a calmodulin-binding domain and the autophosphorylation sites (Thr286, Thr305). The less conserved C-terminus of CaMKII, the association domain, differs between the α, $-\beta$, $-\gamma$, and $-\delta$ isoforms due to unique mRNA splicing. The association domain is comprised mostly of α-helices and is responsible for binding the substrate to be targeted for phosphorylation (Kuret and Schulman, 1984; Hanks et al., 1988; Tobimatsu and Fujisawa, 1989). The N-terminal ATP-binding region forms a bi-lobed structure with the catalytic domain that prevents autophosphorylation at Thr286 in the absence of bound Ca^{2+}/CaM (Gaertner et al., 2004; Rosenberg et al., 2006). Autophosphorylation is examined further in the next section.

D. Mechanisms of autophosphorylation

In the absence of bound Ca^{2+}/CaM, the autoinhibitory domain blocks the ATP-binding motif in the catalytic domain in each subunit. Once saturated with Ca^{2+}, CaM binds to CaMKII and relieves this blockade. When Ca^{2+}/CaM binds CaMKII, ATP gains access to the catalytic site, providing the energy required for CaMKII activation (Cruzalegui et al., 1992; Smith et al., 1992; Brickey et al., 1994; Soderling, 1996; Waxham et al., 1998; Yang and Schulman, 1999). Autophosphorylation at Thr286 occurs when 2 Ca^{2+}-bound calmodulin proteins simultaneously bind to neighboring CaMKII subunits within the CaMKII holoenzyme, where one subunit acts as the catalytic subunit while its neighbor acts as the substrate (Hanson et al., 1994; Mukherji and Soderling, 1994; Bradshaw et al., 2002). Autophosphorylation at Thr286 leads to a 1,000-fold increased affinity of CaMKII for Ca^{2+}/ CaM but also enables autonomous CaMKII activity after CaM dissociates (Lai et al., 1986; Lou et al., 1986; Miller and Kennedy, 1986; Schworer et al., 1986; Meyer et al., 1992). CaMKII returns to the inactivated state upon Thr286 dephosphorylation mediated by phosphatases such as PP1, PP2A, and PP2C that catalyze the removal of a phosphate group by hydrolysis (Shields et al., 1985; Dosemeci and Reese, 1993; Fukunaga et al., 1993; Strack et al., 1997a).

In contrast, another CaMKII residue, Thr305, can be autophosphorylated by an “intrasubunit” reaction, in which the catalytic domain adds a phosphate to Thr305 within the regulatory domain of the same subunit. Autophosphorylation at this site blocks Ca^{2+}/CaM binding. When protein phosphatases dephosphorylate Thr305 Ca^{2+}/CaM is able to once again bind the regulatory

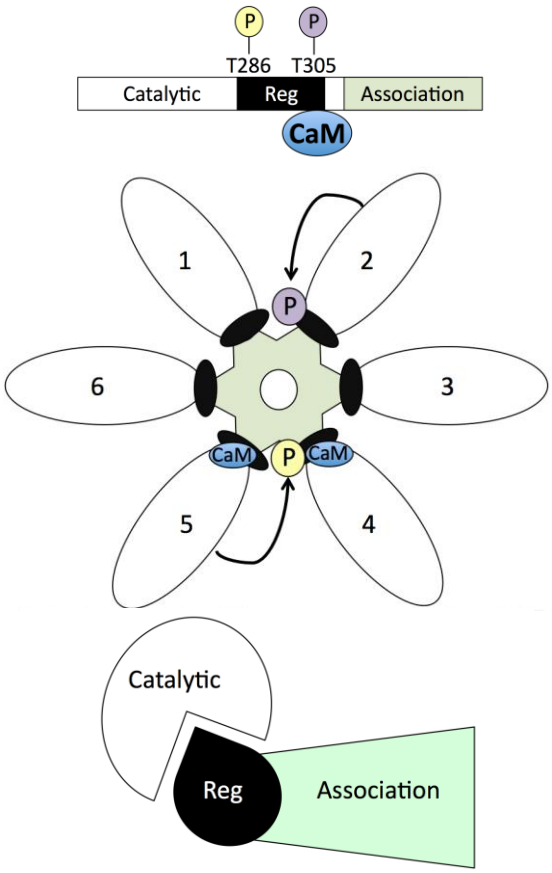

Figure 1.1. CaMKII structure and autophosphorylation. Schematic of a CaMKII subunit structure including the highly conserved catalytic (white) and regulatory (black) domains with the less conserved association domain (green). Autophosphorylation of Thr286 is depicted with a yellow "P," autophosphorylation at Thr305 is shown with a purple "P." The color scheme is maintained throughout each diagram in this figure. The C-terminal association domains assemble subunits into hexameric rings that stack on top of one another to form a dodecameric holoenzyme. The catalytic domains (white) project outward from the association domains (green). The autoinhibitory domain, the calmodulin-binding domain and autophosphorylation sites are all located within the regulatory domain (black). Intrasubunit autophosphorylation at Thr305 under basal conditions blocks Ca^{2+}/CaM binding, thereby preventing autophosphorylation of Thr286 (as shown in subunit 2). When CaM binds, the regulatory domain is displaced from the

catalytic site. When two adjacent subunits have bound CaM, “trans-autophosphorylation” of Thr286 occurs, inducing autonomous CaMKII activity (subunits 4, 5). The catalytic and regulatory domains form a bi-lobed structure to block the activation of the enzyme in the absence of bound Ca^{2+}/CaM.

domain. Thr305 can not be “re-phosphorylated” until Ca^{2+}/CaM uncouples from CaMKII (Hashimoto et al., 1987; Patton et al., 1990; Hanson and Schulman, 1992). These mechanisms of activation create many levels of control over CaMKII activity allowing this enzyme to be a robust detector of the frequency, magnitude and duration of elevated Ca^{2+} levels (De Koninck and Schulman, 1998). The intricacies underlying *in vivo* CaMKII activation and inhibition by intra- and intersubunit autophosphorylation and a range of dephosphatases are likely far more complex than is currently understood.

E. Control of subcellular localization

Subcellular localization of CaMKII is mediated by a variety of association proteins that typically localize CaMKII to the post-synaptic density (PSD), an electron-dense, cytoskeletal protein-rich region of the dendritic spine (Ziff, 1997). Early studies suggested CaMKII comprised 20-50% of purified forebrain PSD total protein (Kennedy et al 1983, Kelly et al 1984, Goldenring et al 1984). However, it has since been discovered that there is a post-mortem accumulation of CaMKII in the PSD, likely associated with pathological responses of CaMKII (Suzuki et al., 1994). While the concentration of CaMKII within the PSD is variable, in tissue that is rapidly dissected and homogenized to reduce pathophysiological conditions, CaMKII still comprises approximately 2-10% of PSD protein (Ouimet et al., 1984; Fukunaga et al., 1988; Suzuki et al., 1994; Strack et al., 1997b).

Interactions between CaMKII and the cytoskeleton aids in anchoring

CaMKII at the synapse. Both microtubules and actin filaments bind CaMKII, and association with Ca^{2+}/CaM triggers dissociation of CaMKII from actin (Ohta et al., 1986; Vallano et al., 1986). The CaMKIIβ isoform primarily associates with F-actin while CaMKIIα is largely found in the cytoplasm under basal conditions (Walikonis et al., 2001; Dhavan et al., 2002). High synaptic activity within the hippocampus leads to translocation of CaMKIIα to the PSD shown using immunofluorescence confocal microscopy in cultured hippocampal neurons (Thiagarajan et al., 2002; Ehlers, 2003). CaMKIIβ, on the other hand is decreased in hippocampal PSD fractions after increased synaptic activity (Thiagarajan et al., 2002). However, both α and β isoforms are increased in the PSD of cortical neurons in response to high synaptic activity and both are decreased in the PSD with lower levels of activity (Ehlers, 2003). Densin-180 is a PDZ domain containing protein that aids in localizing CaMKIIa to the synaptic membrane via interactions with a-actinin and ion channels, specificially the NR2B NMDA receptor subunit (Strack et al., 2000a; Strack et al., 2000b; Walikonis et al., 2001; Robison et al., 2005a; Robison et al., 2005b). The multivariate functions of densin are explored upon further in Chapter 5. Clearly, subcellular localization of CaMKII is dynamically regulated within different neuronal populations during different levels of neuronal activity. This vastly diversifies the physiological roles of this ubiquitous kinase.

F. Functional role : Plasticity

The 50kDa CaMKIIα subunit isoform represents 2% of all hippocampal

protein, an immense total for a regulatory protein, consistent with its hypothesized importance in neural function. Early studies showed that brain areas that control memory formation and exhibit high glutamatergic activity, namely the hippocampus, showed the highest levels of CaMKII (Ouimet et al., 1984; Erondu and Kennedy, 1985). This provided early evidence for the role of CaMKII in memory and learning. More recent studies show further evidence for the role of CaMKII in these important brain functions. For example, there is an increase in dendritic CaMKIIα protein levels in the hippocampus after induction of long-term potentiation (LTP), a cellular model of learning (Ouyang et al., 1999; Giovannini et al., 2001). Hippocampal NMDA receptor-dependent LTP and hippocampal-dependent behaviors (such as spatial learning) require CaMKII activity and Thr286 autophosphorylation (Pinkstaff et al., 2001; Lisman et al., 2002; Matynia et al., 2002). In addition, CaMKII inhibitors block development of LTP in the hippocampus, and perhaps the most convincing evidence for the critical roll of CaMKII in LTP may be that CaMKIIa knock-out mice are deficient in LTP (Silva et al., 1992; Barria et al., 1997b). However, the role of CaMKII in LTP is primarily in LTP induction, rather than maintenance. CaMKII inhibitors, for example, cannot reverse LTP that is already established, but can only prevent LTP before it is induced (Chen et al., 2001). CaMKII is essential for other plastic processes in the brain as well, such as reflex-sensitization in the dorsal horn of the spinal cord, which increases CaMKII expression. During this process, Thr286 autophosphorylation and CaMKII co-immunoprecipitation with NMDA receptor subunits are both increased (Fang et al., 2002; Taha et al., 2002; Garry et al.,

2003; Hardingham et al., 2003). In addition, CaMKII inhibitors block this behavioral reflex (Garry et al., 2003).

Hippocampal LTP is associated specifically with an increase in synaptic AMPA receptor conductance, likely induced via one or a combination of two distinct mechanisms. CaMKII increases synaptic incorporation of AMPA receptors (Poncer et al., 2002; Lu et al., 2010) but also directly increases the unitary conductance of AMPA receptors via phosphorylation of Ser831 in the GluA1 subunit C-terminus (Derkach et al., 1999; Kristensen et al., 2011). Evidence for GluA1-Ser831 phosphorylation-induced mechanisms for LTP is supported by the finding that when GluA1-Ser831 is mutated to block addition of a phosphate group at that site in knock-in mutant mice, LTP and LTD are both disrupted (Lee et al., 2000; Lee et al., 2003). There are an array of studies that support both hypotheses, that CaMKII increases synaptic AMPA receptors or increases AMPA receptor conductance via phosphorylation of GluA1-Ser831 to induce LTP. It is still unclear which mechanism dominates in LTP, but is likely there is a combination of both that occurs *in vivo* (Benke et al., 1998; Poncer et al., 2002; Palmer et al., 2004; Holmes and Grover, 2006).

CaMKII may also act to strengthen active synaptic connections by inducing structural synaptic rearrangements in cultured cortical neurons. Expression of a constitutively active form of CaMKII enhances connectivity between specific neuron pairs, allowing neurons to retain critical presynaptic partners while eliminating others (Pratt et al., 2003). In addition, activated purified CaMKII triggers filopodial growth and formation of new dendritic spines,

while CaMKII inhibitors block both this growth and LTP induction (Jourdain et al., 2003). The CaMKIIβ isoform interacts with F-actin to induce motility and branching of neurites in the developing brain, while later in development, CaMKIIα helps to stabilize dendritic architecture by maturation of synapse formation, and decreasing rates of branch retractions and additions (Wu and Cline, 1998; Fink et al., 2003).

Clearly the functional consequences of CaMKII interactions are extensive. This dissertation focuses on CaMKII-mediated increases in the activity of two archetypal ion channels, $Ca_V1.3$ voltage gated Ca^{2+} channels and ligand-gated GluA1 AMPA receptors. In the following pages I will provide the relevant background regarding AMPA receptors, while the necessary background regarding Ca^{2+} channels will be presented in Chapter 4.

AMPA Receptors

Ligand-gated ionotropic glutamate receptors are a family of excitatory integral membrane proteins encoded by 18 different gene products. The proteins these genes encode share a similar amino acid sequence and macromolecular architecture. Each receptor is composed of four large subunits that form a pore or channel in the cellular membrane to conduct ionic current. These receptors are divided into three main classes based on their unique pharmacology and genetics: the α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, or AMPA receptors (GluA1-4), N-methyl-D-aspartate, or NMDA receptors (GluN1, GluN2A-D, GluN3A-B) and kainate (GluK1-5) receptors (Boulter et al., 1990; Keinanen et al., 1990; Moriyoshi et al., 1991; Sommer et al., 1992; Traynelis et al., 2010). All three classes of ionotropic neurotransmitter-gated receptors bind glutamate released from presynaptic terminals to conduct a depolarizing cationic current. However, each class has distinct roles in the central nervous system (CNS). The function of kainate receptors is two-fold, to mediate post-synaptic neurotransmitter gated currents and to regulate presynaptic neurotransmitter release (Frerking and Nicoll, 2000; Lerma et al., 2001). NMDA receptors are also found at excitatory post-synaptic sites and mediate a slow component of the excitatory post-synaptic potential (EPSP). These receptors exhibit voltage-dependent activation resulting from channel block by Mg^{2+} that must be relieved by depolarizing voltages before NMDA receptors can activate (Nowak et al., 1984). NMDA receptors are also critical for synaptic plasticity and are

dysfunctional in a variety of neurological disorders (Malenka and Nicoll, 1999). During NMDA receptor-dependent synaptic plasticity, Ca^{2+} influx via these receptors activates Ca^{2+} activated kinases and phosphatases that enhance or reduce synaptic strength. One major substrate of these Ca^{2+} activated proteins is the third and final class of glutamate receptor, AMPA receptors, and is the main focus of this dissertation.

To fully review the structure and function of this class of ligand-gated receptor, I will begin by describing the subunit symmetry within the tetrameric receptor. I will then continue with an overview of the structure and function of the individual AMPA receptor subunits from the N-terminus to the C-terminus, providing descriptions of the macromolecular architecture as well as the known functional roles of each region of the protein.

I. Structure and Function of AMPA receptors

The central pore of the AMPA-selective glutamate receptor is formed by four large independent subunits, of approximately 900 amino acid residues each, that likely come together as a homomeric or heteromeric dimer-of-dimers (Sobolevsky et al., 2009; Traynelis et al., 2010). As shown in Figure 1.2, each individual subunit possesses an extracellular amino terminal domain (ATD), an extracellular ligand-binding domain (LBD), four transmembrane domains (TMD), and an intracellular carboxy terminal domain (CTD; (O'Hara et al., 1993; Paas, 1998; Sobolevsky et al., 2009). The ATD is critical for subunit trafficking,

assembly of subunits within the receptor and likely mediates initial dimer formation (Hansen et al., 2010). Pharmacological interactions at this site can modulate receptor properties. The LBD binds agonists that open the receptor pore, which is formed by the TMDs. Finally, the CTD is the site of protein-protein interactions that allow dynamic regulation of receptor function and localization. I will provide additional details regarding both the structure and function of each of these individual AMPA receptor subunit domains, after I describe the hypothesized overall structure and symmetry in the tetrameric functional receptor.

1. Structural symmetry

X-ray crystallography studies have recently lead to the first tetrameric, membrane-spanning AMPA receptor structure. Sobelesvsky et al. (2009) provided the structure of an antagonist-bound, homomeric GluA2 receptor with 3.6Å resolution. This structure confirmed the overall 2-fold rotational symmetry of the extracellular portion of the receptor perpendicular to the membrane, in which two of the four ATDs within the receptor are symmetrically paired, but the paired subunits switch at the LBD. In other words, if we label each subunit A, B, C or D, the ATD of subunits B and D are symmetrically paired, but the LBD of subunits A and C are paired (Figure 1.2*B*). The linker that connects the ATD to the LBD mediates this 2-fold rotational symmetry switch, by taking on an extended conformation in one subunit pair, but is compacted in the other pair (Sobolevsky et al., 2009). The symmetry switches yet again within the TMD to four-fold, in

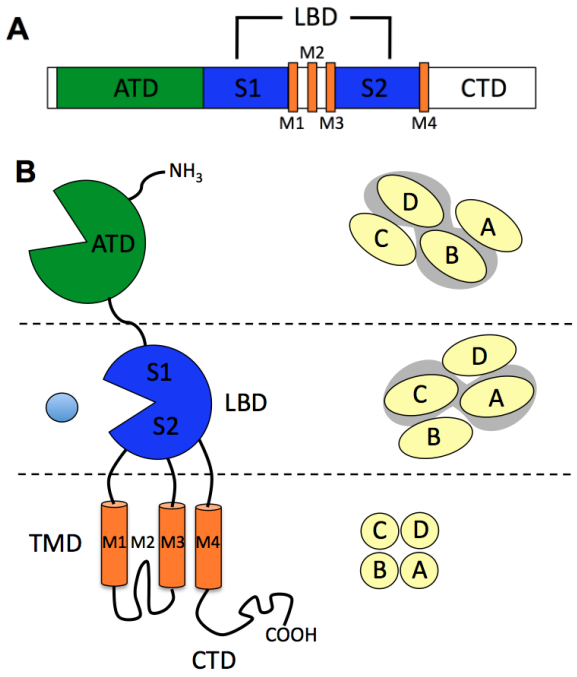

Figure 1.2. Structure of glutamate receptor subunits. ***A,*** A schematic representing the general subunit structure and membrane topology of an AMPA receptor subunit. ***B,*** Within each receptor subunit, there are two extracellular domains, the ATD and the LBD, which each take on a "clamshell" shape, formed by two segments. S1 and S2 represent the top and bottom lobe of the LBD clamshell, respectively. The TMD forms the channel pore, by M1, M3 and M4 and a re-entrant pore loop, M2. Four of these subunits come together to form a functional, tetrameric receptor. To the right of the monomeric subunit cartoon is a top-down, cross-sectional representation of the A/C, B/D subunit symmetry. This top-down view of subunit matching shows the symmetry switches at each domain of the receptor, showing how the tetrameric receptor 2-fold axis of symmetry changes at the junction between the ATD to LBD. Finally, the overall symmetry switches to a 4-fold axis of symmetry within the TMD. (Adapted from (Traynelis et al., 2010).

which half of the pore-forming TMD is related to the other half. This symmetry switch is mediated by the different conformation of the linkers that connect these two regions, denoted S1-M1, M3-S2 and S2-M4 (Figure 1.2). This creates two conformationally distinct sets of subunits, which couple to the receptor gate differently. The linker regions that mediate the symmetry mismatch are known to regulate glutamate receptor pharmacology, gating, desensitization, and open probability (Krupp et al., 1998; Yelshansky et al., 2004; Balannik et al., 2005; Schmid et al., 2007; Yuan et al., 2009). The overall symmetry rearrangement is particularly intriguing in that subunits with the same polypeptide sequence can adopt two structurally distinct conformations.

2. ATD

a. Architecture

The 400 residues that comprise the ATD fold into a semiautonomous domain with two lobes arranged in a clamshell-like formation (Clayton et al., 2009; Jin et al., 2009; Sobolevsky et al., 2009). The N-terminus is connected to the top lobe, and the linker to the ligand-binding domain is found on the bottom lobe (Figure 1.2*B*). The structure of the ligand-gated glutamate receptor ATD resembles that of the ligand-binding region of G-protein coupled metabotropic glutamate receptors, leading to the hypothesis that endogenous ligands can bind to the putative pocket between the two ATD lobes to modulate receptor activity (O'Hara et al., 1993; Paas et al., 1996; Paas, 1998; Jin et al., 2009). For example, Zn^{2+} induces voltage-dependent channel block and decreases

probability of receptor opening via interactions with the ATD of glutamate receptors. Since Zn^{2+} is found at many excitatory synapses, it is a potentially endogenous ligand at the ATD (Williams, 1996; Paoletti et al., 2000; Karakas et al., 2009; Paoletti et al., 2009).

b. Receptor formation/dimerization

A short targeting sequence found at the beginning of the ATD region (residues 14-33) is responsible for targeting the receptor subunits to the cell surface, but is cleaved from the rest of the protein by proteolysis after membrane insertion and endoplasmic reticulum (ER) processing. Subunit folding and tetrameric receptor assembly takes place in the ER, where subunit dimer formation is initiated by the ATD and tetrameric receptor assembly likely occurs via interactions at the LBD and TMD regions (Kuusinen et al., 1999; Leuschner and Hoch, 1999; Ayalon and Stern-Bach, 2001; Mansour et al., 2001; Ayalon et al., 2005). Initiation of subunit dimerization is thought to occur at the ATD since isolated GluA1, GluA2 and GluA4 ATDs can dimerize in solution (Kuusinen et al., 1999; Wells et al., 2001; Clayton et al., 2009; Jin et al., 2009; Kumar et al., 2009). AMPA receptor subunits can homo- and heterodimerize, in that these receptors can be formed by all of the same subunit or a mixture of different subunits. The subunit stoichiometry is tightly controlled by RNA-editing within the re-entrant pore loop of the GluA2 subunit, which promotes heterodimerization of GluA2-containing subunits (Mansour et al., 2001; Greger et al., 2002; Greger et al., 2003; Greger et al., 2006).

Complete removal of the ATD disrupts subunit assembly, providing further

support that this domain plays an important role in receptor formation (Kuusinen et al., 1999; Leuschner and Hoch, 1999; Ayalon and Stern-Bach, 2001; Ayalon et al., 2005). However, since mutant subunits in which the entire ATD has been removed can still form functional receptors, the ATD is therefore thought to play a regulatory role in glutamate receptor assembly (Fayyazuddin et al., 2000; Pasternack et al., 2002; Horning and Mayer, 2004; Hansen et al., 2010). Finally, glutamate receptor subunits are also glycosylated at the ATD (Hollmann et al., 1994; Standley and Baudry, 2000). Differences in glycosylation can lead to changes in ligand affinities, subunit trafficking, densensitization and maximal AMPA receptor current (Hollmann et al., 1994; Standley et al., 1998; Standley and Baudry, 2000; Clayton et al., 2009).

4. LBD

a. Agonist binding and receptor activation

The remaining extracellular portion of the AMPA receptor subunit forms the LBD, which also takes on a clamshell structure formed by two lobes (termed S1 and S2). The primary function of the ligand-binding domain is to bind agonists that activate receptor gating. Glutamate binds to the pocket within the cleft between the two lobes of the clamshell, and induces a conformational change in the LBD structure (Armstrong et al., 1998; Armstrong and Gouaux, 2000; Sobolevsky et al., 2009). Crystal structures of isolated LBDs show that the two lobes are separated and adopt an open conformation in the absence of agonist or in the presence of antagonist. However, agonist binding induces a

closed confirmation (Armstrong and Gouaux, 2000; Gonzalez et al., 2008; Sobolevsky et al., 2009). The agonist initially makes contact with the upper lobe, S1, which induces the lower lobe, S2, to transition, thus closing the clamshell. Closure of the cleft locks in, and prevents dissociation of the agonist during subsequent gating steps (Armstrong et al., 1998; Abele et al., 2000; Armstrong and Gouaux, 2000; Cheng et al., 2005).

The LBDs are arranged back-to-back with 2-fold symmetry within each subunit dimer, and this dimerization of adjacent subunits underlies coupling of LBD clamshell closing to opening of the channel pore (Armstrong and Gouaux, 2000; Sun et al., 2002; Sobolevsky et al., 2009). The interface between the S1 regions constrains the movement of these lobes (Horning and Mayer, 2004; Furukawa et al., 2005; Sobolevsky et al., 2009). Movement of the lower lobe, S2 towards S1 to close the clamshell, shifts three short linkers that connect the LBD to the TMD. Isolated LBD crystal structures show that the length of these linkers is increased in the presence of agonist (Sun et al., 2002; Jin et al., 2003; Erreger et al., 2004; Jin et al., 2005b; Armstrong et al., 2006; Zhang et al., 2008; Traynelis et al., 2010). This movement likely causes rearrangement of the TMD and provides the energy to induce opening of the ion channel pore. Further evidence for the role of the LBD-TMD linkers in determining receptor gating is found in mutagenesis studies in which point mutations in both the TMD and the linkers connecting to the LBD alter glutamate receptor gating (Schneggenburger and Ascher, 1997; Zuo et al., 1997; Krupp et al., 1998; Villarroel et al., 1998).

b. Desensitization

Glutamate receptors are subject to desensitization, or a decrease in current influx during sustained receptor activation. AMPA receptors desensitize almost completely, exhibiting little or no steady state current, and rapidly, within milliseconds of receptor activation (Mosbacher et al., 1994; Partin et al., 1995; Partin et al., 1996). Structural studies of the isolated LBD, the desensitization blocker cyclothiazide and a non-desensitizing mutant AMPA receptor have helped to reveal the mechanisms that likely underlie AMPA receptor desensitization. During desensitization the interface between dimerized subunits is destabilized. When Leu483 within the GluA2 LBD dimer interface is mutated to a tyrosine, desensitization is blocked because the tyrosine stabilizes the dimer interface via interactions with the adjacent LBD subunit (Sun et al., 2002; Horning and Mayer, 2004; Robert et al., 2005; Armstrong et al., 2006). In the desensitized state, the upper lobes (S1) of the LBD clamshell move apart, and the lower lobe of the clamshell dimer (S2) move closer. During desensitization, the S1 dimer interface "ruptures," which induces channel closure even in the agonist bound state. A reducing agent applied to AMPA receptors expressing a cysteine mutation within the S2 LBD dimer surface forms a disulfide bond that traps the receptor in a relaxed conformation, mimicking the desensitized state (Armstrong et al., 2006; Plested and Mayer, 2009). The separation of the LBD dimer S1-S1 interface keeps the channel in an agonist-bound but non-conducting conformation (Du et al., 2005).

In addition, mutations that block desensitization within glutamate receptor subunits promote ER retention. While the precise mechanisms of how

desensitization machinery affects subunit trafficking from the ER is not well-defined, it is thought that these mutations might disrupt interactions with potential chaperone proteins that help traffic the receptor from the ER (Greger et al., 2006; Priel et al., 2006).

c. *Flip and flop*

There are two splice variants of the AMPA receptor subunits that differ within the LBD, flip and flop. These two variants are generated by alternative splicing of two exons encoding 38 amino acids. (Sommer et al., 1990; Mosbacher et al., 1994). Notably, the flip isoform has greater sensitivity to different allosteric modulators, such as the desensitization blocker cyclothiazide, than flop (Partin et al., 1994; Partin et al., 1995). In addition, desensitization of the flip splice variant is approximately four times slower than flop variants (Mosbacher et al., 1994).

4. TMD

a. Ion permeation

The membrane-spanning segments of AMPA receptors arrange in a four-fold axis of symmetry, with 3 regions (M1, M3, M4) that completely traverse the membrane and one re-entrant loop (M2). These regions from each of the four subunits come together to form the pore of the ion channel. The main function of the TMDs is to enable ion flux across the cell membrane. Selectivity for the ions to which the receptor is permeant is determined largely from the steric and electrical interactions between the ions and the residues within a narrow restriction of the pore wall.

The re-entrant pore loop, or M2, contains an RNA editing site, the Q/R site, that also determines ion permeability of the glutamate receptor (Kuner et al., 2001). At this site, glutamine is post-transcriptionally converted to an arginine by adenosine deaminase which renders the receptor impermeable to Ca^{2+} (Hume et al., 1991; Sommer et al., 1991; Bass, 2002). The majority of GluA2-containing AMPA receptors in the nervous system are edited at this site, while other AMPA receptor subunits are not typically edited *in vivo*. Arginines are slightly larger residues than glutamines, so in theory editing at the Q/R site should change ion pore dimensions. However, RNA editing at the Q/R site does not change permeability of AMPA receptors, suggesting that the side chain of the Q/R site is not the major determinant of the dimensions of the narrow restriction within the pore, and that electrostatic interactions are more important in establishing ion selectivity (Kuner et al., 2001). This and the fact that under certain conditions some glutamate receptors are permeable to anions as well as cations suggests other electrostatic interactions, such as the M2 loop dipole, alter the inner cavity environment to help establish ion selectivity, similar to K^+ channels (Morais-Cabral et al., 2001).

The M2 loop Q/R site is also a major determinant of other glutamate receptor functions. For example, outward current through AMPA receptors is blocked by endogenous polyamines resulting in an inwardly-rectifying current-voltage (IV) relationship. Outward current through AMPA receptors edited at the Q/R site are not subject to polyamine block. In vivo, GluA1, 3 and 4 subunits are not typically edited, but most GluA2 subunits are edited at this site. Thus, GluA2-

containing receptors have a linear IV curve (Figure 2.5), while GluR2-lacking receptors are inwardly rectifying (Verdoorn et al., 1991; Donevan and Rogawski, 1995). Less is understood about the functional role of the M4 transmembrane region. The GluA2 crystal structure revealed that it lies on the exterior of the ion channel pore, and interacts with the ion channel core (M1-3) of adjacent subunits, to modulate receptor assembly and function (Ren et al., 2003; Sobolevsky et al., 2009). M4 is also speculated to limit interactions of the putative activation gate (M3) with the surrounding lipid bilayer and, in conjunction with M1, anchors the LBD in its place.

b. Activation gate

All ion channels have either an electrostatic or physical steric hindrance that occludes the flux of ions through the channel pore until activation. The central pore of glutamate receptors is formed by transmembrane helices that control ion flux and likely work together to form this “activation gate.” A large amount of evidence supports the claim that the activation gate of glutamate receptors resides within the M3 transmembrane helix. (Wo and Oswald, 1995; Chen et al., 1999; Kuner et al., 2003; Sobolevsky et al., 2009). This region possesses the most highly conserved motif among all of the mammalian glutamate receptor subunits, and shows a similar structural homology with the gating region in voltage-gated K^+ channels (Doyle et al., 1998; Jiang et al., 2002; Kuner et al., 2003). Point mutations within the M3 region of NMDA receptors create constitutively open receptors, as do mutations in the homologous region of Gluδ2 glutamate receptors, another member of the ionotropic glutamate receptor

family with important roles in cerebellar synaptic plasticity (Zuo et al., 1997; Chang and Kuo, 2008).

The GluA2 crystal structure shows that C-terminal ends of M3 regions from the different subunits cross to form the apex of the ion channel pore. This region forms a “collar” around the extracellular side of the TMD that occludes the ion permeation pathway (Chang and Kuo, 2008; Sobolevsky et al., 2009). At the extracellular entrance to the channel pore, the pre-M1 helices are oriented parallel to the membrane plane and make contacts with both M3 and M4. These contacts are hypothesized to restrict mobility of the M3 domain in the closed state, but help to promote opening of the receptor upon agonist binding (Beck et al., 1999; Sobolevsky et al., 2002; Sobolevsky et al., 2003). AMPA receptors open to four individual conductance states depending on how many of the four subunits within a tetrameric receptor are in the ligand-bound state (see Figure 2.4); the pre-M1 linker contacts the M3 helix within each individual subunit to influence the rearrangement that likely gives rise to the four detectable subconductance levels (Rosenmund et al., 1998; Smith and Howe, 2000; Jin et al., 2003; Prieto and Wollmuth, 2010).

Further support of M3 as the activation gate is found in the Lurcher mutation of Gluδ2 gluatmate receptors, in which substitution of an alanine residue that maintains close contacts with M3, with a more bulky residue (threonine) destabilizes the helical crossing that establishes the closed receptor confirmation. Gluδ2 receptors in these mutant mice are constitutively open as a result (Kashiwabuchi et al., 1995). The structure of an open K+ channel, with

strong structural homology to GluA1, and the isolated LBD structures in the agonist and antagonist bound states have lead to the hypothesis that the M3 helix rotates away from the central axis of the pore (Doyle et al., 1998; Jiang et al., 2002; Sobolevsky et al., 2004).

While these data and the crystal structure of the GluA2 AMPA receptor provide considerable evidence for the role of the LBD-TMD linkers and M3 in receptor gating, the detailed structural mechanisms of gating still remain elusive. The structure published by Sobolevsky and colleagues is in the antagonist-bound, closed state; however, the structure of an active receptor in the presence of agonist and absence of antagonist would be more helpful in elucidating some of the structural changes that occur during gating.

5. CTD

No structural details exist for the cytoplasmic residues C-terminal to the fourth transmembrane region. The intracellular CTD is the most structurally diverse region among glutamate receptors, both in amino acid sequence and length, and is likely responsible for much of the functional and localization differences between the different AMPA receptor subunits. The CTD sequence of some glutamate receptors contains ER retention signals that affect trafficking in alternatively spliced subunits (Horak and Wenthold, 2009). Mutant receptors lacking this region are typically still functional but have altered channel behaviors and trafficking, suggesting that this region plays a more regulatory role in receptor localization, membrane trafficking, and gating (Yan et al., 2004; Suzuki

et al., 2005; Milstein and Nicoll, 2009).

a. Protein-Protein Interactions : Trafficking

While this region of the glutamate receptor bears no sequence homology with proteins for which structural information is available, it does possess binding motifs for many interacting proteins. Glutamate receptors interact with PDZ (post-synaptic density-95, discs large, zona ocludens1) domain-containing proteins, adaptor proteins, kinases, phosphatases, cytoskeletal proteins, and a host of other signaling proteins. These interactions create an opportunity for localized signaling to influence AMPA receptor function. PICK1, a PDZ-domain containing protein known to interact with protein kinase C, binds to the short GluA2 AMPA subunit C-terminus and may underlie specific targeting of alternatively spliced glutamate receptor subunits to synaptic membranes (Dev et al., 1999; Xia et al., 1999). GRIP is an adaptor protein also shown to interact with AMPA receptor subunit C-termini to aid in synaptic clustering of the receptor (Dong et al., 1997; Wyszynski et al., 1999). SAP97, a synaptic membrane-associated guanylyl kinase, binds specifically to the distal GluA1 C-terminus also to direct AMPA receptor synaptic localization. In addition, SAP97 can target CaMKII to GluA1 subunits, and when it itself is phosphorylated, disrupts an interaction between GluA1 and AKAP79/150 to prevent phosphorylation of GluA1 by another kinase, cAMP-dependent protein kinase (Leonard et al., 1998; Nikandrova et al., 2010). A clathrin adaptor complex, AP-2, binds to the middle section of the GluA2 C-terminus to control receptor internalization during LTD (Lee et al., 2002; Kastning et al., 2007). The AP-2 binding site overlaps with the

binding site for NSF, an ATP-ase that regulates membrane fusion events (Hay and Scheller, 1997; Osten et al., 1998; Lee et al., 2002). When NSF binds the GluA2 C-terminus, AMPA-R synaptic transmission is decreased (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998a). These are some examples of the many molecular chaperones and scaffolding proteins that bind and localize AMPA receptors.

b. Protein-Protein Interactions : Phosphorylation

Interaction with a wide-array of protein kinases has particularly far-reaching consequences for AMPA receptor function. GluA1 binds cGMP-dependent protein kinase II and is phosphorylated at Ser845 to increase receptor surface expression (Serulle et al., 2007), while phosphorylation by PKA at this site causes an increase in GluA1 receptor probability of opening, or P_O (Banke et al., 2000). PKA phosphorylation of both GluA1 and GluA4 can also drive AMPA receptors into synaptic membranes (Esteban et al., 2003; Man et al., 2007). PKC phosphorylation of GluA1 at residue Ser818, on the other hand, enhances interactions with other proteins that facilitate membrane insertion of the receptor and enhances synaptic strength (Boehm et al., 2006; Lin et al., 2009). Thr840 within the GluA1 CTD, for example, is phosphorylated by PKC to enhance synaptic transmission and LTP (Lee et al., 2007b), while dephosphorylation at this site occurs during LTD (Delgado et al., 2007). Recently, a CaMKII phosphorylation site has been identified at Ser567 within the first intracellular loop of GluA1 subunits that appears to be critical for synaptic targeting of GluA1 subunits, but not necessarily for insertion in the plasma membrane (Lu et al.,

2010). Finally, CaMKII phosphorylation of GluA1-Ser831 increases single channel conductance of AMPA receptors (Derkach et al., 1999). CaMKII phosphorylation at GluA1-Ser831 is a major focus of this dissertation and will be elaborated upon in the next Chapter. The specificity and selectivity of these interactions coordinates very tight spatial and temporal regulation of receptor function.

GluA2 subunits are similarly targeted for phosphorylation to regulate synaptic strength. Phosphorylation at GluA2-Ser880 decreases LTD, likely by weakening the interaction of the GluA2 C-terminus with GRIP to slow its insertion into the synaptic membrane (Steinberg et al., 2006; Lin and Huganir, 2007). There are two major GluA2 splice variants that differ in the length of their C-termini. The long splice variant can be phosphorylated by a Jun kinase at Thr874, and when dephosphorylated promotes synaptic incorporation of GluA2 subunits (Thomas et al., 2008). A similar mechanism exists for GluA4 subunits, where synaptic activity induces dephosphorylation of GluA4-Ser855 to facilitate synaptic incorporation of this glutamate receptor subunit. Long GluA4 AMPA receptor subunit splice variants also bind and are phosphorylated by PKC at Ser842 to facilitate receptor membrane insertion and association with α-actinin (Correia et al., 2003; Nuriya et al., 2005).

B. Auxiliary AMPA receptor subunits

Recent work has lead to the discovery of a group of AMPA receptor interaction proteins that are critical for the trafficking and functioning of neuronal

AMPA receptors. The following section focuses on the functional changes these proteins exert on AMPA receptors.

1. TARPs

The transmembrane AMPA receptor regulatory proteins (TARPs) bind AMPA receptors and are thought to act as an auxiliary receptor subunit. So far, six different TARPs have been identified, γ2 (stargazin), γ3, γ4, γ5, γ7 and γ8 (Milstein and Nicoll, 2008). TARPs are integral membrane proteins found in the majority of neuronal AMPA receptor complexes and their discovery has transformed the glutamate receptor field (Fukata et al., 2005; Nakagawa et al., 2005; Vandenberghe et al., 2005; Nakagawa et al., 2006). TARPs bind early in the synthesis of AMPA receptor subunits to aid in trafficking of the receptor to the synapse (Hashimoto et al., 1999; Chen et al., 2000; Schnell et al., 2002; Tomita et al., 2004), but also increase AMPA receptor activity in a variety of ways once inserted into the synaptic membrane (Tomita et al., 2005b; Milstein and Nicoll, 2009). AMPA receptors in complex with TARPs have increased conductance, probability of opening, rate of activation and slowed deactivation and desensitization compared to recombinant receptors expressed in the absence of TARPs (Yamazaki et al., 2004; Tomita et al., 2005b; Soto et al., 2007; Soto et al., 2009). TARPs also affect the pharmacology of AMPA receptors. For example, GluA2-lacking receptors are subject to extracellular polyamine block and TARPs decrease the extent of this effect. AMPA receptors in complex with TARPs exhibit less inward rectification compared to recombinant “TARPless” AMPA

receptors (Soto et al., 2007). TARPs also change the AMPA receptor antagonist CNQX into a partial agonist (Cokic and Stein, 2008). Finally, AMPA receptors in the presence of TARPs are more sensitive to kainate than glutamate compared to AMPA receptors not in the presence of TARPs. In other words, TARPs increase kainate sensitivity more than they increase glutamate sensitivity (Tomita et al., 2005b; Turetsky et al., 2005). These diverse effects are generated via multiple interaction sites between TARPs and GluA subunits, at extracellular, intracellular and membranous sites on both proteins (Tomita et al., 2005b; Bedoukian et al., 2006; Tomita et al., 2007; Milstein and Nicoll, 2009; Sager et al., 2009). In addition, more than one TARP can simultaneously bind a tetrameric AMPA receptor allowing for many levels at which a receptor can be subject to dynamic regulation by TARPs (Vandenberghe et al., 2005; Milstein et al., 2007; Shi et al., 2009).

a. Prototypical TARPS

The first TARP to be identified was stargazin, or γ2, because of a naturally occurring mutation that arose in the gene that encodes for stargazin. These so-called stargazer mice have severely reduced expression of stargazin, causing altered AMPA receptor trafficking in the cerebellum (Letts et al., 1998). Stargazin, along with γ3, γ4, and γ8, are the “prototypical TARPs” and interact with all four AMPA receptor subunits to increase single channel conductance and P_O. These TARPs decrease desensitization, deactivation and polyamine block of AMPA receptors. Finally, they all help traffic AMPA receptors to the cell surface (Priel et al., 2005; Tomita et al., 2005b; Turetsky et al., 2005; Milstein et al., 2007).

Effects on gating are largely controlled by interactions between the extracellular portions of TARPs and the AMPA receptor pore region, while trafficking of the AMPA receptor is mediated primarily by interactions between the M1-pore loop region of the channel and the TARP C-terminus (Tomita et al., 2005b; Turetsky et al., 2005; Bedoukian et al., 2006; Cho et al., 2007; Korber et al., 2007b). In addition, there is evidence that interactions between AMPA receptors and the stargazin C-terminus not only help traffic AMPA receptors to the cell surface, but can also change gating properties of the receptor (Sager et al., 2009). Finally, the C-terminal domain of AMPA receptors is required for stargazin dependent effects. When the GluA1 C-terminus is removed and replaced with yellow fluorescent protein, stargazin-dependent AMPA trafficking is disrupted (Bedoukian et al., 2006). There are clearly many opportunities for interactions between TARPs and AMPA receptors to diversify the ways in which the receptor functions.

Overall, TARPs increase total charge transfer through AMPA receptors once activated by glutamate, but there is some inherent diversity in the specific functional effects of the individual TARPs. For example, γ4 and γ2 both slow activation rise times of GluA1 AMPA receptors but γ4 does so to a greater extent. AMPA receptor desensitization is decreased and slowed by association with γ4 (Cho et al., 2007; Korber et al., 2007a; Milstein et al., 2007). Both γ4 and γ8 induce a greater reduction in rates of deactivation than either γ2 or γ3 (Cho et al., 2007; Milstein et al., 2007). TARPs also differentially affect actions of different agonists. For example, γ2, γ3, γ4 and γ8 all reduce the EC_{50} of glutamate, but γ2

and γ3 induce a greater reduction than the others, and all potentiate GluA1 flop isoforms more so than flip (Priel et al., 2005; Tomita et al., 2005b; Kato et al., 2007; Kott et al., 2007). The differential effects on AMPA receptor properties by the different TARPs represent an opportunity for the nervous system to maintain tight control over AMPA receptor currents and fine-tune the resulting post-synaptic potentials.

b. Atypical TARPs

Less is known about the functional effects of the atypical TARPs, γ5 and γ7. These TARPs are both highly expressed in the cerebellum, and there are similarly conflicting reports on the specific effects of both of these TARPs on AMPA receptors. γ7 increases sensitivity of GluA1 to glutamate, and slows deactivation and desensitization of homomeric GluA1 receptors (Kato et al., 2007). γ7 also causes increased sensitivity to cyclothiazide in GluA2 receptors (Kato et al., 2008). However, Soto and colleagues report that γ7 does not change desensitization of GluA4 receptors (Soto et al., 2009). It is unclear if these conflicting reports result from differential effects of γ7 on different AMPA receptors, or if different conditions in the two labs lead to the contrasting findings.

There are also some discrepancies in reports on the effects of γ5 on AMPA receptors. γ5 had been shown to selectively increase peak current, decrease steady-state currents, and increase rate of activation of GluA2-containing AMPA receptors (Kato et al., 2008). However, another group had shown that γ5 regulates all long-form AMPA receptor subunit splice variants, GluA2L, GluA1, GluA4. They found only a small increase in channel

conductance, a decrease in glutamate potency, decreased P_O and an increased current density of these AMPA receptors (Soto et al., 2009). While it is unclear what underlies these discrepancies, it is possible that the phosphorylation state of the TARP itself, or interactions with scaffolding proteins in different proteins change how AMPA receptors are affected by TARPs. While many functional effects of and specific binding sites for TARPs on AMPA receptors have been described, the precise mechanisms describing how TARPs exert their effects on AMPA receptors remain ill-defined. The discrepancies in the role of the different TARPs in the literature are likely the result of different cell types being examined in different labs under different conditions. It is also plausible that different subunit combinations and varying numbers of TARPs binding to a single receptor contribute to the variability in results. There are likely complex interactions between the different post-synaptic density proteins, AMPA receptors and TARPs that specify functional effects on channels.

2. Cornichons

Another distinct class of AMPA receptor interacting proteins that has only recently been described is the cornichon proteins, or CNIH-2 and CNIH-3 (Schwenk et al., 2009). These proteins are small transmembrane proteins that were first identified in drosophila to control dorsal-ventral signaling of the fly egg chamber (Roth et al., 1995). When in complex with AMPA receptor subunits, however, these proteins can increase surface expression, slow deactivation and slow desensitization kinetics of AMPA receptors (Schwenk et al., 2009).

Interestingly, there is also evidence that CNIH-2 can compete functionally with γ8, in that coexpression of CNIH-2 abolishes some γ8-mediated effects on AMPA receptors (Kato et al., 2010). Our understanding of cornichon proteins is still rudimentary with respect to glutamate receptor modulation. However, these proteins may prove to be a valuable tool in dissecting some of the discrepancies between recombinant and native receptor properties.

C. Synaptic function

1. Synaptic subunit composition

AMPA receptors are widely expressed throughout the central nervous system, but are primarily localized within the PSD of excitatory synapses. Glutamate released from pre-synaptic terminals traverses the synaptic cleft to bind post-synaptic AMPA receptors. Binding of agonist induces a rapidly activating inward current that decays within 1-2 ms. The vast majority of synaptic AMPA receptors are comprised of heteromeric, or mixed, subunit populations rather than homomeric receptors (Lu et al., 2009). This was determined using a conditional single-cell genetic approach that selectively knocked-down each AMPA receptor subunit in individual CA1 hippocampal neurons in order to investigate the specific roles of each subunit in synaptic activity. This was accomplished by postnatal expression of Cre recombinase in CA1 pyramidal neurons from floxed GluA mice. Whole-cell patch-clamp electrophysiology then was used to directly compare neighboring cells in which one had been subject to gene deletion and the other had not. These studies revealed that all

membraneous hippocampal AMPA receptors contain GluA2 subunits, and 80% of synaptic AMPA-Rs are GluA1/2 heteromers, suggesting a subunit hierarchy in which GluA2 complexes are favored over other combinations (Lu et al., 2009).

2. Shaping post-synaptic potentials

Biophysical properties of synaptic AMPA receptors can vary depending on the subunits that comprise the tetrameric receptor. Therefore, different subunit combinations are tailored to the function of both the cell and even the synapse itself. For example, fast-acting homomeric GluA1 receptors dominate in principal neurons compared to inhibitory cortical interneuron synapses, creating large, rapidly activating and rapidly decaying EPSPs in a brain region that is responsible for detecting synchronous principal neuron spiking within a narrow time window (Geiger et al., 1997).

Incorporation of the GluA2 subunit into AMPA receptors introduces differences in tetrameric receptor function. Changes in expression and trafficking of this subunit exert significant effects on synaptic currents. In addition to their inherent low conductance, AMPA receptors that contain edited GluA2 subunits are Ca^{2+}-impermeable and not inwardly-rectifying (Swanson et al., 1997; Liu and Cull-Candy, 2000). Expression of this subunit is tightly controlled during critical neuronal processes such as development and induction of synaptic plasticity. There are low levels of GluA2 in many neuron populations during the early stages of development, therefore a large majority of these AMPA receptors are permeable to Ca^{2+} to allow Ca^{2+} influx into the cell to direct synaptic development

and neurite outgrowth (Pickard et al., 2000; Kumar et al., 2002; Eybalin et al., 2004; Ho et al., 2007; Migues et al., 2007). High neuronal activity itself can also stimulate increases in synaptic AMPA receptor expression. For example, GluA2 subunit expression undergoes a stress-induced increase in cerebellar stellate cells which causes a selective slowing of the EPSC decay time constant, increasing the probability of action potential firing (Liu et al., 2010; Savtchouk and Liu, 2011). Clearly, AMPA receptors are important to synaptic function, and these currents are critical in shaping excitatory post-synaptic currents.

3. *Synaptic Plasticity*

Long-lasting changes in the strength of glutamatergic synapses, or synaptic plasticity, is associated with changes in the function and trafficking of AMPA receptors (Derkach et al., 2007). These activity-dependent changes are widely recognized as cellular mechanisms that underpin cognitive behaviors such as learning and memory (Whitlock et al., 2006). The phenomenon known as LTP has been extensively studied within the hippocampus, and also in the cortex and cerebellum, in which a synapse is strengthened over time by specifically patterned input (Bliss and Lomo, 1970; Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). Synaptic plasticity is a bi-directional phenomenon. Brief high frequency stimulation typically induces LTP, while LTD, a prolonged weakening of synapses, is induced by low-frequency stimulation over longer periods (Bear and Abraham, 1996). Hippocampal LTP is a Ca^{2+}-dependent process that requires NMDA receptor activation, and is expressed via recruitment of AMPA

receptors to active synapses (Bliss and Collingridge, 1993; Shi et al., 1999). LTP is similarly associated with an increased AMPA receptor conductance (Benke et al., 1998; Poncer et al., 2002; Luthi et al., 2004). These and other mechanisms lead to an increase in the strength of the incoming synaptic signal. AMPA receptors are critically involved in this synaptic strengthening process, and the following paragraphs outline some of the ways in which AMPA receptors are targeting during synaptic plasticity.

a. Phosphorylation

Given that AMPA receptors mediate the majority of excitatory post-synaptic signaling in the central nervous system, and differential regulation of these receptors is evident during development and learning, it is logical that these receptors are an important target of phosphorylation during synaptic plasticity. Phosphorylation of AMPA receptors regulates both intrinsic channel properties as well as membrane trafficking and synaptic targeting of receptor subunits during plastic events. Early evidence for regulation of AMPA receptors by phosphorylation was found in studies in which intracellular application of cAMP-dependent protein kinase A (PKA) activators up-regulated AMPA receptor activity in hippocampal neurons (Greengard et al., 1991). Similar experiments using constitutively active CaMKII increased synaptic AMPA receptor currents (McGlade-McCulloh et al., 1993). C-terminal GluA1 subunit residues Ser831 and Ser845 have since been identified as the primary CaMKII and PKA substrates that lead to an increase in AMPA receptor activity (Roche et al., 1996; Barria et al., 1997a; Mammen et al., 1997; Banke et al., 2000). More specifically,

phosphorylation of Ser845 increases the probability of GluA1 receptor opening (P_O), while phosphorylation of Ser831 increases GluA1 receptor conductance (Derkach et al., 1999). GluA1 phosphorylation is increased with LTP induction and is decreased with LTD (Barria et al., 1997b; Lee et al., 1998; Lee et al., 2000). Mutation of both GluA1-Ser831 and GluA1-Ser845 to alanine not only blocks PKA- or CaMKII-mediated effects on channel function, but knock-in mice bearing alanine mutations at both sites exhibit diminished LTP and LTD is completely abolished (Lee et al., 2003). However, knock-in mice in which only one of these two residues is mutated to alanine exhibit nearly normal LTP and LTD (Lee et al., 2010). Also, GluA1-S831A mutant receptors still exhibit synaptic insertion with CaMKII activation or after LTP (Esteban et al., 2003). These findings suggest that these two phosphorylation sites in GluA1 receptors likely cooperatively control both LTP and LTD.

b. GluA2 trafficking

The unique properties of GluA2-containing receptors, namely their much reduced conductance, makes trafficking of GluA2 AMPA receptor subunits in and out of the synapse an important target for induction of synaptic plasticity. GluA2 AMPA receptor subunits are also subject to phosphorylation at Ser863 and Ser880 by PKC (Matsuda et al., 1999; McDonald et al., 2001). Ser880 is located within the PDZ binding domain of the GluA2 C-terminus, known to be an important binding site for synaptic anchoring proteins, suggesting that this phosphorylation target could potentially change localization and synaptic targeting of this particular AMPA receptor subunit (Chung et al., 2000). A

phosphodeficient mutant GluA2-S880A subunit results in reduced LTD, while mimicking phosphorylation at this residue (GluA1-S880E) attenuates synaptic transmission and occludes LTD, suggesting these processes share a common mechanism (Seidenman et al., 2003). It is hypothesized that phosphorylation at this site impedes the interaction between GluA2 and GRIP to destabilize synaptic localization of this receptor subunit.

c. TARPs

A final important mechanism for control of synaptic plasticity is found in the interaction between AMPA receptors and TARPs (Tomita et al., 2005a). TARPs associate with AMPA receptors within the ER to help export to the Golgi and ultimately to the cell membrane. Subsequently TARPs, in conjunction with PSD-95, help target perisynaptically localized AMPA receptors to the post-synaptic density (Chen et al., 2000; Chen et al., 2003). However, in order to do this TARPs must be phosphorylated by CaMKII at the C-terminus, making it difficult to tease out the exact role of CaMKII in AMPA receptor function and plasticity. Finally, phosphorylation of AMPA associated TARPs also mediates bi-directional synaptic plasticity (Tomita et al., 2005a). Phosphorylation of stargazin by CaMKII or PKC is increased with NMDA receptor stimulation and during LTP, while stargazin is dephosphorylated by PP1 during LTD. With so many synaptic substrates, it is important to identify the exact targets of CaMKII before making far-reaching conclusions on its role in plasticity and AMPA receptor gating.

D. Concluding remarks

Over the years countless studies have revealed the intricate mechanisms that define the functioning and physiological roles of AMPA receptors in the brain. There is much that is known regarding gating and modulation of AMPA receptors, but many mysteries remain. I set out to clarify some ill-defined mechanisms that underlie phosphorylation of GluA1 AMPA receptor subunits. For example, CaMKII phosphorylates the Ser831 residue within the GluA1 C-terminus to increase single channel conductance of homomeric AMPA receptors, but this effect has never been shown to occur in native brain cells before. Furthermore, it is unclear what role, if any, TARPs play in CaMKII-mediated effects on AMPA receptors. Finally, while phosphorylation at GluA1-Ser831 has been shown to directly increase the function of GluA1 AMPA receptors, there are other phosphorylation sites within the membrane proximal GluA1 C-terminus that have not been studied for direct effects on channel behavior. In Chapters 2 and 3, I will present my results in pursuit of answers to these ambiguities regarding phosphorylation-induced changes in AMPA receptor function. The same techniques were used in both sets of studies, so the methods section of Chapter 2 is applicable to the experiments presented in Chapter 3 as well.

In the subsequent chapters, I present a separate set of experiments using a similar but distinct set of methods that describe CaMKII-mediated increases in activity of a voltage-gated Ca^{2+} channel. In Chapter 4 I will present the relevant background information to the study of Ca^{2+}-channels, and then present the results of these studies in Chapter 5. Finally, in Chapter 6 I will discuss and synthesize the results of these various studies and draw parallels between the

phosphorylation of these different classes of post-synaptic ion channels by CaMKII.

Chapter 2

Mechanisms of CaMKII-dependent increase in AMPA receptor conductance

I. ABSTRACT

The function, trafficking and synaptic signalling of AMPA receptors are tightly regulated by phosphorylation. CaMKII phosphorylates residue Ser831 within the C-terminus of recombinant GluA1 AMPA receptors to increase single channel conductance, and these actions can be mimicked with substitution of GluA1-Ser831 with negatively charged glutamate or aspartate residues. For the first time, I show here that CaMKII increases the conductance of native heteromeric AMPA receptors in hippocampal neurons via phosphorylation at Ser831. In addition, coexpression of TARPs with recombinant receptors is required for phospho-Ser831 to increase conductance of heteromeric GluA1/GluA2 receptors.

II. INTRODUCTION

AMPA-selective glutamate receptors are ligand-gated cation channels that mediate fast excitatory neurotransmission in the brain (Traynelis et al., 2010). AMPA receptors are present at most excitatory synapses and thus play a role in many aspects of brain function including cognition, movement, learning, and memory. The function and number of postsynaptic AMPA receptors is dynamically regulated by phosphorylation to control synaptic strength, a key feature of cellular models of learning and memory (Malinow and Malenka, 2002; Boehm and Malinow, 2005).

AMPA receptors are tetrameric assemblies of four different subunits (GluA1-4), each containing two semiautonomous extracellular domains, an amino terminal domain and a ligand-binding domain. The ligand-binding domain is linked to three membrane-spanning α-helices and a pore-forming re-entrant loop (Sobolevsky et al., 2009; Traynelis et al., 2010). The intracellular domain of each subunit is predominantly composed of a C-terminal segment immediately downstream of the third membrane spanning helix (Figure 2.1), and contains distinct phosphorylation sites that regulate receptor gating, trafficking, and localization (Barria et al., 1997a; Derkach et al., 1999; Banke et al., 2000; Tomita et al., 2005b; Shepherd and Huganir, 2007).

Cellular models of synaptic plasticity, such as long-term potentiation (LTP) at hippocampal CA1 pyramidal cells, is characterized by an AMPA receptor-dependent increase in excitatory post-synaptic current (EPSC) amplitude

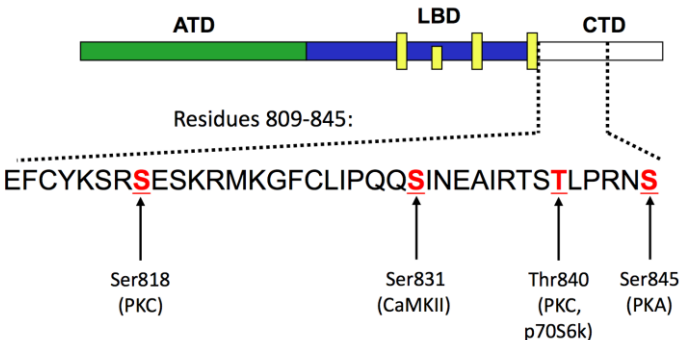

Figure 2.1. Schematic representation of the AMPA receptor C-terminal domain (CTD) highlighting the four known serine/threonine phosphorylation sites (residues highlighted in red). PKA phosphorylates Ser845, PKC phosphorylates at residues Ser818 and Thr840. Thr840 is also phosphorylated by p70S6 kinase, and CaMKII adds a phosphate group to Ser831.

(Holmes and Grover, 2006). This likely results either from enhanced AMPA receptor function or an increased delivery of AMPA receptors to the synapse (Benke et al., 1998; Malinow and Malenka, 2002; Song and Huganir, 2002; Holmes and Grover, 2006; Shepherd and Huganir, 2007). However, the relative contribution of these various mechanisms to LTP is not fully understood. In addition, both the AMPA receptor GluA1 subunit and CaMKII are required for expression of LTP at mature hippocampal CA1 pyramidal cells (Malenka et al., 1988; Silva et al., 1992; Lee et al., 2000; Lee et al., 2003; Lamsa et al., 2007; Lee et al., 2010). Biochemical studies have identified a CaMKII phosphorylation site at Ser831 on the C-terminal domain of the GluA1 subunit (Barria et al., 1997a; Barria et al., 1997b; Mammen et al., 1997). Whereas GluA1-Ser831 phosphorylation does not appear to increase synaptic localization of GluA1-containing receptors (Hayashi et al., 2000; Esteban et al., 2003; Boehm and Malinow, 2005), it does increase recombinant homomeric GluA1 receptors current response to glutamate (Derkach et al., 1999). This potentiation is similar to the increased conductance of native AMPA receptors observed following LTP (Benke et al., 1998; Poncer et al., 2002; Luthi et al., 2004; Palmer et al., 2004; Holmes and Grover, 2006).

Here, I investigate the mechanism by which phosphorylation of native and recombinant GluA1 receptors increases the unitary conductance of AMPA receptors. AMPA receptors exist in multiple open states with distinct conductance levels (Figure 2.4), determined by agonist binding and the gating of individual subunits (Rosenmund et al., 1998; Smith and Howe, 2000; Jin et al., 2003; Poon

et al., 2010; Prieto and Wollmuth, 2010). The AMPA receptor single channel conductance is a function of the number of agonist-bound subunits per receptor complex that are activated at any given moment (Rosenmund et al., 1998; Smith and Howe, 2000; Jin et al., 2003; Poon et al., 2010; Prieto and Wollmuth, 2010). Conductance increases incrementally with each additional subunit that has bound glutamate and contributes to gating. The unitary conductance of AMPA receptors is very small, making it challenging to study these receptors on a single channel level. To measure phosphorylation-induced changes in conductance, I utilized stationary variance analysis, a technique in which the weighted mean of these four conductance levels can be estimated using macroscopic currents elicited from excised-outside out membrane patches expressing non-desensitizing receptors. The opening and closing of many channels within the patch is reflected as an increase in the noise of the current trace. During the slow washout of a maximally effective concentration of glutamate the noise of the current trace varies with response amplitude. Plotting the noise (quantified as variance) versus the current amplitude yields a parabolic function, the initial slope of which is an estimation of the weighted mean conductance (γ_{MEAN}) of the receptors contained within that patch (Cull-Candy et al., 1988; Traynelis and Jaramillo, 1998).

Results obtained with this approach show that the effects of CaMKII on conductance are evident both in recombinant and native AMPA receptors, and result from phosphorylation of GluA1-Ser831. Phosphorylation of GluA1 at Ser831 can increase the efficiency by which each subunit translates ligand-

induced conformational changes within the ligand-binding domain to channel activation. CaMKII also enhances conductance of heteromeric GluA1/GluA2 receptors when the transmembrane AMPA receptor regulatory proteins (TARPs), stargazin or $\gamma 8$, are present. These data show that CaMKII-mediated phosphorylation of Ser831 is relevant to neuronal AMPA receptors, and is likely a common mechanism for enhancing AMPA receptor function during neuronal processes like synaptic plasticity.

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III. METHODS

A. Materials

Ca^{2+}/calmodulin-dependent protein kinase II and calmodulin were obtained from New England Biolabs (Ipswich, USA). All micropipettes were from World Precision Instruments (Sarasota, USA). NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione, a non-selective AMPA receptor antagonist), DL-AP5 (an NMDA receptor antagonist) and cyclothiazide (to block AMPA receptor desensitization) were obtained from Tocris (Ellisville, USA). Spermine was obtained from Sigma-Aldrich (cat #S2876, St. Louis, Missouri, USA). Poly-D-lysine was obtained from Millipore (cat #A-003-E). All cell biology reagents were from Gibco (Invitrogen, Carlsbad, CA, USA) unless otherwise stated.

B. Molecular biology

A CMV-based mammalian expression vector, pRK5 (BD Pharmingen, San Diego, USA), harbouring the coding sequences of the flip splice variants of the rat GRIA1 and GRIA2 genes was used for transient expression of GluA1 and GluA2 (short isoform) in mammalian cells. The non-desensitizing mutations, Leu-483-Tyr in GluA2 (L483Y), Leu-497-Tyr in GluA1 (L497Y), and the mutations of Ser818, Ser831, Thr840 and Ser845 in GluA1 to either alanine, glutamate, or aspartate were introduced by the QuikChange mutagenesis method according to the instructions provided by Stratagene (San Diego, USA). Post-mutational integrity of the cDNA sequences were verified by DNA sequencing (SeqWright,

Fisher Scientific. Houston, TX). Stargazin (rat) was contained within a pCI-neo vector, whereas γ8 (mouse, rat) was in an IRES-EGFP vector. GluA1 cDNA was provided by Peter Seeburg (Max Planck Institute, Heidelberg, Germany) and γ8 cDNA from Roger Nicoll (UCSF, San Francisco, CA. USA).

C. Maintenance and transfection of HEK cells

Human embryonic kidney cells (HEK293; ATCC 1573, hereafter HEK cells) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco #10569) supplemented with 10% (v/v) fetal calf serum (Gibco, cat #26400) and 1% penicillin-streptomycin (Gibco, cat #15140-122) on polystyrene culture dishes in a humidified atmosphere of 5% CO_2, 95% O_2, at 37°C. Growth media for transiently transfected GluA1 cells were furthermore supplemented with 200 μM NBQX to protect transfected cells against excitotoxicity induced by endogenous glutamate in the growth media. Cells were split using 0.05% trypsin-EDTA (Gibco, cat #25300). Cells plated on 8 mm glass coverslips coated with 100 μg/ml poly-D-lysine contained in 24-well tissue culture plates were transfected 24 hours prior to experimentation using the non-lipid, cationic transfection reagent FuGENE6 (Invitrogen, Carlsbad, USA). Briefly, plasmid DNA was diluted in pure DMEM to a concentration of 10 μg/ml and supplemented with 30 μl/ml FuGENE6 and incubated for 15 min at 37º C, after which, the transfection complex was added to cells using 20 μl mix per cm^2 growth area. Plasmid DNA harbouring a reporter cDNA encoding Green Fluorescent Protein (pMAXGFP, Amaxa

Biosystems, Germany) was added to receptor DNA at a 1:4 ratio to aid in identification of individually transfected cells.

D. Preparation of primary cultures of hippocampal neurons

All procedures involving the use of animals were reviewed and approved by the Emory University IACUC. Knock-in mice were generous gifts from Rick Huganir's laboratory (Johns Hopkins University, Baltimore, MD. USA). The hippocampal formation was dissected from the brains of P0 wild-type C57Bl/6, GluA1-S831A, GluA1-S831A,S845A, or GluA1-S831D,S845D knock-in mice, as previously described (Lee et al., 2010; Kristensen et al., 2011). Tissue was incubated in a dissociation medium comprised of (in mM): 82 Na_2SO_4, 30 K_2SO_4, 5.8 $MgCl_2$, 0.25 $CaCl_2$, 1 HEPES, 3.6% glucose, 0.1 kynurenic acid, and phenol red, pH to 7.4 with NaOH. Hippocampal tissue was digested in a 20 μg/ml papain suspension (Worthington Biochemical, cat #LS003124, Lakewood, NJ) for 15 minutes then transferred to a 30 mg/ml ovomucoid trypsin inhibitor solution (Sigma-Aldrich, cat #T92531) for an additional 15 minutes. The tissue was then triturated in OPTI-MEM solution (Gibco, cat #31985) supplemented with 2.5% glucose using two Pasteur pipettes of varying bore sizes. Cells were diluted to a concentration of 0.2 x 10^6 cells/ml in OPTI-MEM and 1 ml was plated onto 50 μg/ml poly-D-lysine-coated glass coverslips (diameter 8 mm) in 24 well plates. After settling for one hour, OPTI-MEM was exchanged for neurobasal medium (Gibco, cat #21103). A 500 ml bottle of neurobasal was supplemented with 1 ml 100X Glutamax (Gibco, cat #35050), 10 ml 50X B-27 supplement, and 5 ml of

10,000 U/ml penicillin/streptomycin. Cells were cultured for up to 25 days in 95% O_2 / 5% CO_2 at 37^{o}C and neurobasal media was exchanged every 7 days.

E. Recording and analysis of macroscopic currents from excised membrane patches

For experiments on recombinant or native AMPA receptors, outside-out membrane patches were excised from transiently-transfected HEK cells or cultured hippocampal neurons using thick-walled borosilicate micropipettes (1.5 mm OD, 0.86 mm ID) filled with internal solutions comprised of (in mM) 110 gluconic acid, 110 CsOH, 30 CsCl, 4 NaCl, 5 HEPES, 4.37 EGTA, 2.1 $CaCl_2$, 2.27 $MgCl_2$, 0.1 Spermine, 4 ATP, 0.3 GTP. The pH was adjusted to 7.3 with CsOH. For some experiments the intracellular solution was supplemented with 10 mg/ml purified calmodulin and 2 U/ml CaMKII. Pipettes had a tip resistance of 4-6 MΩ. External recording solution for all experiments were comprised of (in mM) : 150 NaCl, 10 HEPES, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, pH 7.4; 310-330 mOsm. Currents were recorded at room temperature (V_{HOLD} = -60 or +40 mV) with an HEKA EPC9 amplifier (Lambrecht/Pfalz, Germany), filtered at 5 kHz, and digitized with a sampling rate of 20 kHz. For measurement of non-desensitizing current responses, drugs dissolved in external recording solution were added to the excised patch by bath application. For measurement of rapidly desensitizing current responses, a piezo-driven double-barrelled perfusion system (Berleigh) was used to rapidly apply saturating concentrations of agonist onto excised membrane patches for 100 ms, as previously described (Traynelis and Wahl,

1997). The time course of solution exchange across the laminar flow interface was estimated by liquid junction potential measurements to be 0.1-0.4 ms (10-90% rise time) for a 10-fold difference in ionic strength; the time course of the junction potential change for our perfusion system was measured at the end of most experiments. For cultured neurons, NMDA receptor responses were blocked by the addition of 100 μM DL-AP5 and 1 mM Mg^{2+}. During experiments, internal pipette solutions that contained CaMKII and/or calmodulin were stored in a glass syringe on ice.

Stationary variance analysis of macroscopic currents was carried out as previously described (Traynelis and Jaramillo, 1998; Banke et al., 2000; Jin et al., 2003). Variance is related to the unitary current amplitude with the binomial theorem, which assumes that the four AMPA receptor subunits function independent of one another. This can be calculated using the following equations:

$$I = i\,N\,p \qquad (1)$$

where, *N* is the number of channels in a cell membrane patch, that each have a current amplitude of *i* that pass a mean current *I.* Combining equation 1 (by substituting for p) with:

$$\sigma^2 = i^2\,N\,p\,(1-p) \qquad (2)$$

where σ^2 is variance and p is the probability that the channel is open, yields :

$$\sigma^2 = i\,I - (I^2 / N) \qquad (3)$$

This quadratic equation shows that the plot of current versus variance is parabolic. Taking the slope, or derivative, of this equation yields

$$d\sigma^2/dI = i - 2I / N \quad (4)$$

where, I = 0 at the initial part of the parabola. The slope then is i, or the single channel current amplitude. Ohm's law (V=IR) yields the conductance, or γ_{NOISE}. Differences in unitary current amplitudes that arise from the subconductance levels are lost in the composite conductance value, γ_{NOISE}, obtained from this analysis. The contribution of each sublevel conductance to the mean conductance is weighted by its frequency and amplitude, and when the subconductance values are known from single channel analysis, the variance of receptors with *j* sublevels can be estimated with the following equation (Cull-Candy et al., 1988):

$$\gamma_{MEAN} = \Sigma\ N\ p_j\ \gamma_j^2 / \Sigma\ N\ p_j\ \gamma_j \quad (5)$$

where *N* is the number of channels, γ_j is conductance of the j^{th} sublevel, and p_j is the open probability for each of the conductance levels. Fitting the data to this function yields γ_{MEAN} or the weighted average of all subconductance levels.

For determination of coupling efficiency (ε) p_j was determined from binomial expansion for values of ε between 0 and 1. A theoretical curve relating coupling efficiency to weighted γ_{MEAN} was generated from equation 1 using measured conductance levels and the binomial equation (Figure 2.4). From this curve one can estimate the relative sublevel occupancy for values of ε between 0 and 1 needed to obtain the expected weighted conductance. Coupling efficiency can then be determined by comparing this theoretical relationship to the experimental values for the weighted γ_{MEAN} from variance or single channel

analysis of the GluA1 responses under various experimental conditions. The response amplitude to a maximally effective concentration of agonist can similarly be predicted from the probability of openings to each conductance level by

$$\textit{Response Amplitude} = V \Sigma N \; p_j \; \gamma_j \qquad (6)$$

where V is the membrane holding potential and N, γ, p are as defined above.

F. Statistical methods.

Unless otherwise noted, results are expressed as mean ± SEM. Statistical analysis of pairwise or multiple comparisons were performed using ANOVA, or Student's t test as appropriate. $p < 0.05$ was considered to be statistically significant. Power of all statistical tests was at least 0.8.

IV. RESULTS

A. Control of neuronal AMPA receptor function by CaMKII

CaMKII phosphorylation of GluA1 subunits enhances the single channel conductance of recombinant homomeric receptors (Derkach et al., 1999). Previous work has identified residue Ser831 on the GluA1 subunit (Figure 2.1) as the primary site for CaMKII phosphorylation (Barria et al., 1997a; Barria et al., 1997b; Mammen et al., 1997; Lee et al., 2000). I first set out to examine whether purified CaMKII increases the weighted mean unitary conductance (γ_{MEAN}) of native hippocampal AMPA receptors, which primarily express GluA1, GluA2, and GluA3 subunits (Boulter et al., 1990; Sommer et al., 1990; Wenthold et al., 1996; Lu et al., 2009). AMPA receptors recorded in outside-out patches obtained from these neurons express heterogeneous subunit compositions. Macroscopic currents, induced by bath application of 1 mM glutamate to outside-out patches isolated from cultured hippocampal neurons (Figure 2.2*A,C*), were recorded either with or without purified rat brain CaMKII (2 U/ml) in the patch pipette (plus 0.1 μM buffered Ca^{2+} and 10 μg/ml calmodulin; see *Methods*). DL-AP5 (100 μM) and Mg^{2+} (1 mM) were added to the external solution to reduce the contribution of NMDA receptor activation, and cyclothiazide (100 μM) was added to block desensitization. A current response with a graded waveform (Figure 2.2*C*) was generated by slowly washing glutamate from the bath. The γ_{MEAN} of the receptor population in the patch can be estimated using variance analysis (Figure 2*D*) of the current response during washout of agonist (Traynelis and Jaramillo, 1998; Jin et al., 2003). Inclusion of purified CaMKII, Ca^{2+} and calmodulin in the patch

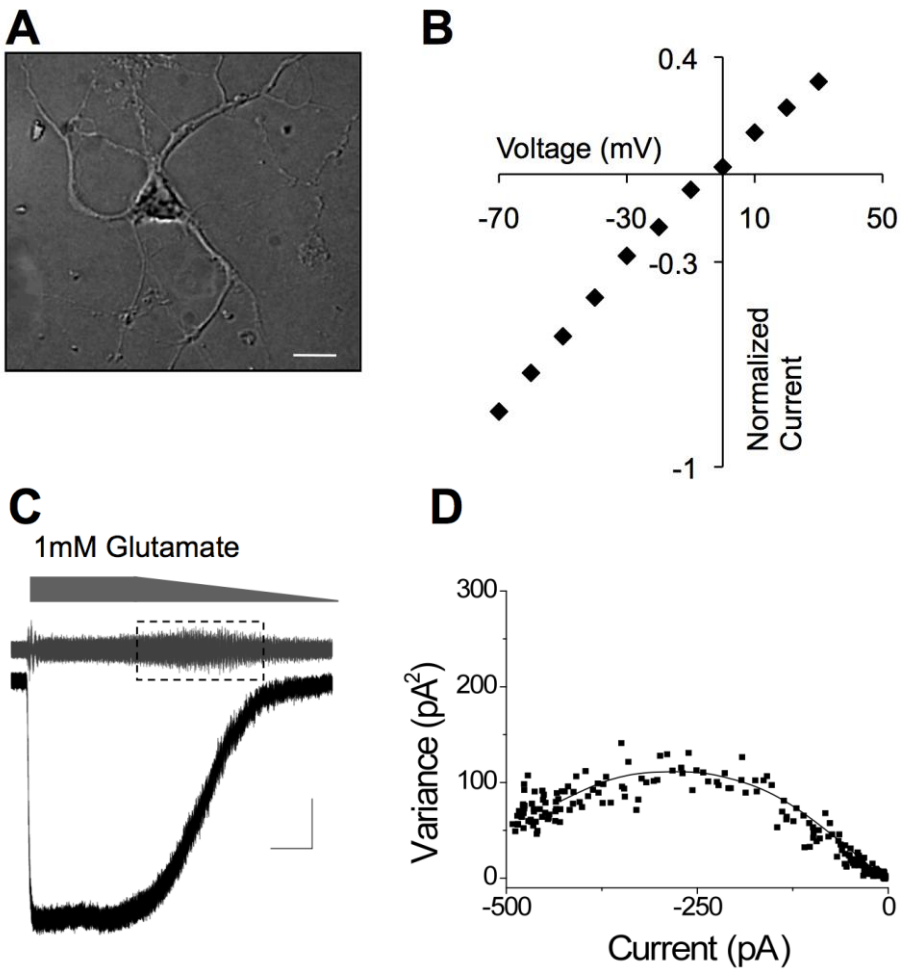


Figure 2.2. Stationary variance analysis of hippocampal AMPA receptor currents. ***A,*** Photomicrograph of a cultured hippocampal neuron, 21DIV. Scale bar, 20μM ***B,*** Plot of current-voltage relationship elicited from hippocampal macroscopic AMPA receptor currents. Points represent mean±SEM. Where error bars are not visible, they are smaller than the size of the symbol. ***C*****,** Representative macroscopic current response to 1 mM glutamate in excised outside-out patches with 100 mM DL-AP5 and 1 mM Mg^{2+} present to block NMDA receptors, and cyclothiazide to block AMPA receptor desensitization. The upper trace shows the response after high pass filtering, illustrating the increase in membrane current noise (dashed box) during channel deactivation. Scale bars represent 10 pA and 5 s. ***D,*** Representative current-variance relationship used to determine γ_{MEAN}.

pipette significantly increased the γ_{MEAN} from 4.3 ± 0.4 pS (n=11; Figure 2.3) to 9.3 ± 0.4 pS (n=12; p<0.001; Student's t test). These results show that CaMKII controls the conductance of native hippocampal AMPA receptors, consistent with previous observations on recombinant homomeric GluA1 receptors expressed in heterologous cells (Derkach et al., 1999; Derkach, 2003). The reduced conductance observed in neurons compared to homomeric GluR1 (Table 1) suggests inclusion of edited GluA2 in native AMPA receptors, which lowers unitary conductance when co-assembled with GluA1. The presence of a large portion of GluA2-containing AMPA receptors is also consistent with the linear IV relationship (Figure 2.2*B*) that these cells possess (Swanson et al., 1997).

TARPs such as stargazin associate with the membrane spanning helices of AMPA receptors to control receptor trafficking and function (Tomita et al., 2005b). CaMKII phosphorylation of a specific TARP, stargazin, appears to influence both trafficking of AMPA receptors as well as long-term potentiation of hippocampal synapses (Tomita et al., 2005a). In addition, there are numerous protein kinase targets in intracellular regions of GluA1 and other AMPA receptor subunits that could show cross-reactivity with purified CaMKII and complicate our results (Banke et al., 2000; Boehm et al., 2006; Lee et al., 2007b; Lu et al., 2010). It was therefore necessary to determine whether the effects observed in hippocampal neurons resulted directly from CaMKII phosphorylation of GluA1-Ser831 or of other GluA1 phosphorylation sites, other AMPA receptor subunits or indirectly via phosphorylation of associated proteins such as stargazin. To do this, I measured γ_{MEAN} in hippocampal neurons cultured from phosphodeficient

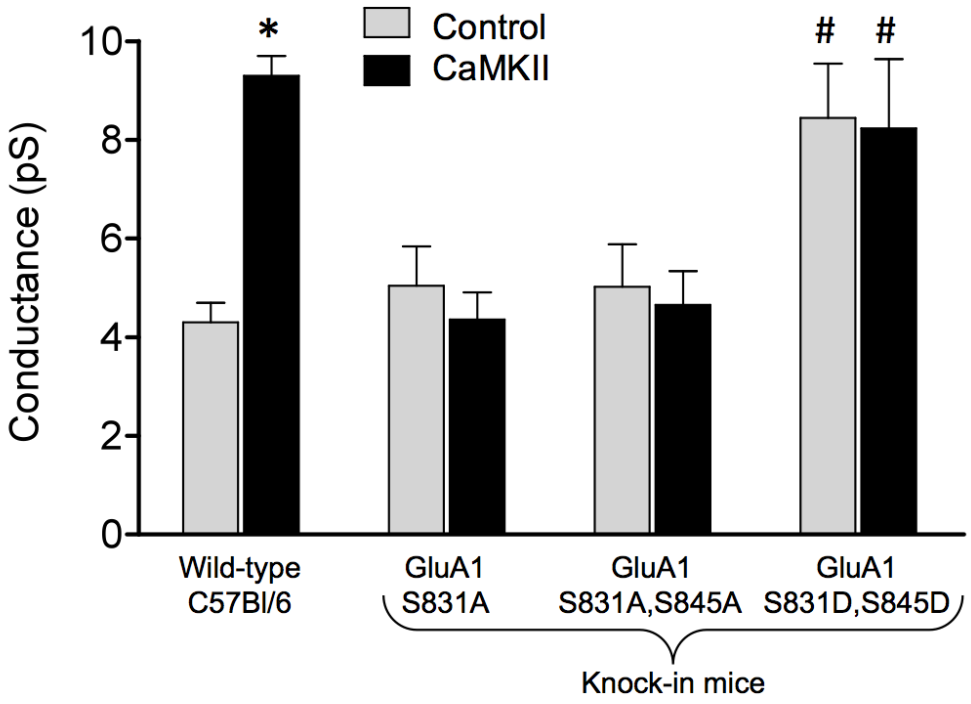


Figure 2.3. CaMKII increases γ_{MEAN} of hippocampal AMPA receptors via phosphorylation at Ser831. Summary of CaMKII effects on γ_{MEAN} of native AMPA receptors in cultured hippocampal neurons from either wild-type or knock-in mutant mice (mean±SEM). * p<0.001 for CaMKII compared to control in wild type neurons by t-test. # p<0.001 compared to GluA1-S831A-S845A and GluA1-S831A by two-way ANOVA with Bonferroni's post hoc test. Data are from 8-15 neurons for each condition.

gene knock-in mice expressing alanine substitutions at both GluA1-Ser831 and GluA1-Ser845 (GluA1-S831A,S845A) as well as at the Ser831 phosphorylation site alone (GluA1-S831A; (Lee et al., 2003; Lee et al., 2010). As shown in Figure 2.3, inclusion of CaMKII, Ca^{2+} and calmodulin in the patch pipette did not increase the γ_{MEAN} of AMPA receptors from neurons expressing GluA1-S831A,S845A (4.7 ± 0.7 pS; n=9) compared to control recordings obtained with Ca^{2+} and calmodulin alone (5.0 ± 0.8 pS; n=11; *p*=0.75). CaMKII similarly failed to increase γ_{MEAN} in neurons expressing GluA1-S831A (4.4 ± 0.5 pS; n=15) compared to control recordings (*p*=0.54). Introduction of the phosphomimic aspartate in place of GluA1-Ser831 and Ser845 (GluA1-S831D,S845D) significantly increased native AMPA receptor γ_{MEAN} (8.4 ± 1.1 pS; n=12) compared to GluA1-S831A,S845A and GluA1-S831A ($p<0.001$, Figure 2.3). Moreover, the conductance of these phosphomimic mutant native AMPA receptors was not further increased by CaMKII (8.2 ± 1.4 pS; n=8; p=0.90; Figure 2.3). Together, these data show that the increase in weighted mean conductance, $\gamma_{MEAN,}$ by CaMKII in native receptors reflects phosphorylation of GluA1-Ser831, and is not an indirect result of phosphorylation of other GluA1 residues, other AMPA receptor subunits or associated regulatory proteins.

B. Control of recombinant AMPA receptor function by CaMKII

I next explored the subunit selectivity and the mechanistic basis for the CaMKII-dependent increase in AMPA receptor γ_{MEAN} seen in neurons. To do this, I used patch-clamp recordings of HEK cells transfected with phosphomutant

GluA1 receptors, in which Ser831 was also mutated to an alanine to mimic the unphosphorylated state (S831A) or to a glutamate to mimic the phosphorylated state (S831E). Because HEK cells endogenously express PKA, these GluA1 mutant receptors also included a S845A mutation to prevent phosphorylation by endogenous PKA, as well as the L497Y (Stern-Bach et al., 1998) mutation to block macroscopically observed desensitization: GluA1-L497Y,S831A,S845A (GluA1-AA), GluA1-L497Y,S831E,S845A (GluA1-EA; Table 1). The γ_{MEAN} determined by variance analysis for GluA1 with the phosphomimic S831E mutation was significantly higher (γ_{MEAN} 14.2 ± 0.6 pS; n=19) than GluA1 with the phosphodeficient S831A mutation (γ_{MEAN} 9.4 ± 0.7 pS; n=18; Table 1). Similar results were obtained for the double phosphomimic mutant GluA1-L497Y,S831D,S845D (GluA1-DD; γ_{MEAN} 13.1 ± 1.0 pS; n=12; *p* <0.001; Table 1).

Previous work has shown that PKA phosphorylation of Ser845 increases the probability of opening of GluA1 AMPA receptors (Banke et al., 2000). To ascertain whether there was any functional overlap between the GluA1-Ser831 and GluA1-Ser845 phosphorylation sites, stationary variance analysis was again used to calculate any GluA1-S831E-induced changes in open probability (P_O). P_O of non-desensitizing receptors (L497Y mutation) was unaffected by the phosphomimic mutation determined by stationary variance analysis (Table 1), suggesting that this residue couples specifically to gating machinery that controls conductance, not P_O.

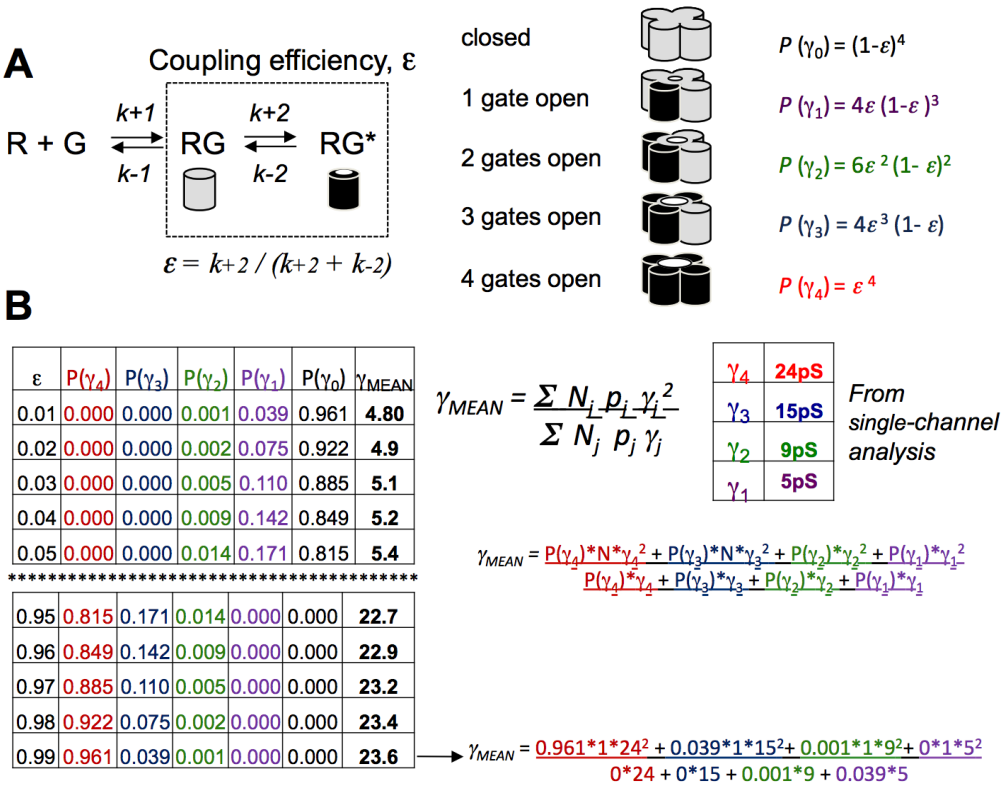
Table 2.1

Receptor	Mutation	TARPs	γ_{MEAN} (pS)	P_O	n
GluA1	S831A	--	9.4 ± 0.7	0.79 ± 0.03	18
GluA1	S831E	--	14.2 ± 0.6 *	0.73 ± 0.03	19
GluA1	S831D	--	13.1 ± 1.0 *	0.75 ± 0.02	12
GluA1	S831A	Stargazin	12.3 ± 1.1	0.96 ± 0.03 †	13
GluA1	S831E	Stargazin	16.9 ± 1.0 **	0.95 ± 0.02 †	8
GluA1/GluA2	GluA1-S831A	--	2.6 ± 0.5	0.78 ± 0.03	8
GluA1/GluA2	GluA1-S831E	--	3.0 ± 0.2	0.82 ± 0.02	11
GluA1/GluA2	GluA1-S831A	Stargazin	3.8 ± 0.4	0.71 ± 0.02	10
GluA1/GluA2	GluA1-S831E	Stargazin	6.2 ± 0.9 ***	0.79 ± 0.05	12
GluA1/GluA2	GluA1-S831A	γ8	3.0 ± 0.4	0.68 ± 0.07	10
GluA1/GluA2	GluA1-S831E	γ8	6.4 ± 1.4 ***	0.78 ± 0.07	11

Effect of CaMKII and Ser831 phosphomimic and phosphodeficient mutations on γ_{MEAN} of homomeric and heteromeric GluA1-containing receptors expressed in HEK cells. Weighted mean unitary conductance, γ_{MEAN}, was determined using stationary variance analysis of current responses obtained from transfected HEK cells. All mutant GluA1 subunits contained the L497Y mutation to block desensitization, and the S845A mutation to block endogenous PKA phosphorylation at this site. Values are mean ± SEM; n is the number of outside-out patches studied at a holding potential of -60 mV. * p<0.01 significantly different from GluA1-S831A (One-way ANOVA, with Tukey's post hoc test). † p<0.01 versus GLuA1 without stargazin (One-way ANOVA with Tukey's posthoc test). ** p<0.01 versus GluA1-AA+stargazin (One-way ANOVA, with Tukey's post hoc test). Coexpression with stargazin is described in the *Methods*. *** p<0.05 significantly different from heteromeric receptors containing GluA1-AA mutant subunits (One-way ANOVA, with Tukey's post hoc test). Open probability (P_O) was calculated as the ratio of the maximal macroscopic current to the product of the fitted unitary current and number of channels. One-way ANOVA was used to compare P_O values for homomeric GluA1 L497Y mutant and GluA1/GluA2 receptor responses.

C. Ser831 phosphorylation increases the coupling efficiency between agonist binding and gating

Coupling efficiency (denoted ε), defined as a value between 0 and 1, describes the efficiency with which a receptor subunit translates agonist binding into gating (Jin et al., 2003; Poon et al., 2010; Prieto and Wollmuth, 2010). The value ε describes the probability that an agonist-bound subunit will contribute to ion permeation, assuming all subunits function independently. The binomial theorem can be used to calculate the probability of opening to each individual conductance level by the four subunits ($P\gamma_1$, is the probability of opening to conductance level 1, $P\gamma_2$ is the probability of opening to conductance level 2, etc), using their coupling efficiency (Figure 2.4). From these equations the relationship between ε and γ_{MEAN} can be calculated using equations 1 and 2 (see *Methods*). The individual "real" conductance values at each of the four subconductance states, not the weighted mean, were required for these calculations. These values were obtained from single-channel recordings of phosphomutant GluA1 receptors previously performed in our laboratory (Kristensen et al., 2011). A table can be generated using a range of coupling efficiencies from 0-1, the four individual $P(\gamma_j)$ levels calculated using those coupling efficiency values, and the γ_{MEAN} values (calculated from the $P(\gamma_j)$ values plugged into equation 1). I was then able to identify the coupling efficiency of each GluA1 receptor mutant by looking up the corresponding γ_{MEAN} in the table (Figure 2.4*B*). The GluA1-EA receptor has a coupling efficiency of 0.54 versus the GluA1-AA receptor with a coupling efficiency of 0.29. Previous work in our



ε	P(γ4)	P(γ3)	P(γ2)	P(γ1)	P(γ0)	γMEAN
0.01	0.000	0.000	0.001	0.039	0.961	**4.80**
0.02	0.000	0.000	0.002	0.075	0.922	**4.9**
0.03	0.000	0.000	0.005	0.110	0.885	**5.1**
0.04	0.000	0.000	0.009	0.142	0.849	**5.2**
0.05	0.000	0.000	0.014	0.171	0.815	**5.4**
**********						
0.95	0.815	0.171	0.014	0.000	0.000	**22.7**
0.96	0.849	0.142	0.009	0.000	0.000	**22.9**
0.97	0.885	0.110	0.005	0.000	0.000	**23.2**
0.98	0.922	0.075	0.002	0.000	0.000	**23.4**
0.99	0.961	0.039	0.001	0.000	0.000	**23.6**

Figure 2.4. GluA1 Ser831 phosphorylation increases the coupling efficiency between agonist binding and gating. AMPA receptor subunits contribute to gating once each subunit binds glutamate and becomes activated (RG*). The efficiency with which each subunit couples to gating (ε) can be calculated from rates of activation as shown. P(γ_j) can be calculated based on coupling efficiencies. These values can be plugged into a lookup table to estimate coupling efficiency from the γ_{MEAN} values obtained with stationary variance analysis. The assumption is that the distribution of channel conductance levels (γ) reflects binomial statistics, which allows calculation of the probability of each conductance level, or *P*(γ).

laboratory shows that phosphorylation of GluA1-Ser831 increases coupling efficiency of the GluA1 receptor equally across a range of partial agonists that induce different degrees of ligand-binding domain closure (Kristensen et al., 2011). These data strongly support the hypothesis that phosphorylation of GluA1-Ser831 enhances the coupling efficiency between agonist binding and channel gating. Since the increase in coupling efficiency occurs independently of LBD closure (Kristensen et al., 2011), Ser831 phosphorylation is therefore potentiating GluA1 receptor function at the level of receptor gating.

Previous studies of the effects of CaMKII on recombinant AMPA receptors in the absence of stargazin or other accessory proteins showed that CaMKII can increase γ_{MEAN} in homomeric GluA1 but not GluA1/GluA2 heteromeric receptors (Oh and Derkach, 2005). Consistent with this report, my data show that the phosphomimic S831E mutation in GluA1 does not increase γ_{MEAN} of heteromeric GluA1/GluA2 AMPA receptor responses recorded at -60 mV (Table 1). Incorporation of GluA2 (carrying the non-desensitizing mutation L483Y) into the receptor complex lowered the conductance and yielded a linear IV curve (Table 1, Figure 2.5*A*), suggesting that responses were from heteromeric receptors. However, the γ_{MEAN} values were not significantly different between heteromeric receptors carrying either the phosphodeficient GluA1-S831A mutation or the phosphomimic GluA1-S831E mutation (p=0.65; Table 1). These results suggest that the effects of phosphorylation on GluA1 Ser831 are strongly influenced by the AMPA receptor subunit composition.

HEK cells do not appear to express TARPs, including stargazin (Deng et

al., 2006). To examine the potential role of stargazin in CaMKII regulation of AMPA receptor function, I repeated experiments with homomeric and heteromeric receptors in cells co-expressing stargazin. Stargazin increased γ_{MEAN} in homomeric GluA1/GluA2 receptors compared to the corresponding receptor not coexpressed with stargazin (Table 1), as expected from previous work, confirming association of this auxiliary subunit with the recombinant receptors (Tomita et al., 2005b; Soto et al., 2007). The presence of stargazin did not interrupt the effect of the phosphomimic mutation on homomeric GluA1 receptors, the γ_{MEAN} of phosphomimic GluA1-EA mutant receptors in cells expressing stargazin (γ_{MEAN} 16.9 ± 1.0 pS; n=8; Table 1) was significantly increased relative to phosphodeficient GluA1-AA receptors coexpressed with stargazin (γ_{MEAN} 12.3 ± 1.1 pS; n=13; p<0.01; Table 1). Importantly, the γ_{MEAN} of GluA1/GluA2 heteromeric receptors containing the GluA1 phosphomimic mutation was significantly increased (γ_{MEAN} 6.2 ± 0.9 pS; n=12; Table 1) compared to heteromeric receptors with the GluA1 phosphodeficient mutation in cells co-expressing stargazin (γ_{MEAN} 3.8 ± 0.4 pS; n=10; p<0.05; Table 1). That is, coexpression of stargazin with GluA1/GluA2 heteromeric receptors restored the effects of the GluA1-Ser831 phosphomimic mutation on γ_{MEAN} for inward currents. Open probability was not significantly altered by the phosphomimic mutation in all recordings from non-desensitizing receptors coexpressed with stargazin (Table 1). The overall conductance of GluA1/GluA2 receptors was lower than homomeric GluA1 receptors and was enhanced by coexpression of stargazin, confirming the interaction of functional stargazin and the presence of

heteromeric GluA1/GluA2 receptors.

One potential confound of this interpretation is the possible presence of a subpopulation of GluA1-EA homomeric receptors in GluA1/GluA2/stargazin-transfected cells, which in theory could account for the observed conductance increase from the GluA1-S831E mutation. To determine whether the different conductance values for phosphomimic and phosphodeficient mutations might reflect a stargazin-enhanced subpopulation of homomeric GluA1 receptors, I repeated this experiment on outward currents recorded at a positive holding potential. RNA editing at the Q/R site renders GluA2-containing receptors Ca^{2+}-impermeable, and insensitive to polyamine current block. GluA2-lacking receptors are inwardly-rectifying while GluA2-containing receptors have a linear IV relationship (Hume et al., 1991; Verdoorn et al., 1991; Donevan and Rogawski, 1995). Thereby, at +40 mV, the current contributed by GluA1 homomers should be negligible (Figure 2.5), allowing us to measure γ_{MEAN} from heteromeric channel currents alone at this membrane potential. At this holding potential, γ_{MEAN} of heteromeric receptors in the presence of the TARP stargazin was still increased by the GluA1-S831E mutation compared to GluA1-S831A from 2.9 ± 0.7 pS to 5.9 ± 0.6 pS ($p<0.01$). In homomeric GluA1 receptors, stargazin reduced the extent of inward rectification and reduced the ratio of current recorded at +40 mV to -60 mV from 0.26 to 0.43 as expected (Soto et al., 2007). By contrast, the corresponding ratio for cells expressing heteromeric GluA1/GluA2 (0.66) was not significantly changed upon coexpression of stargazin (Figure 2.5*A,B*). Using these values we can calculate the percentage of

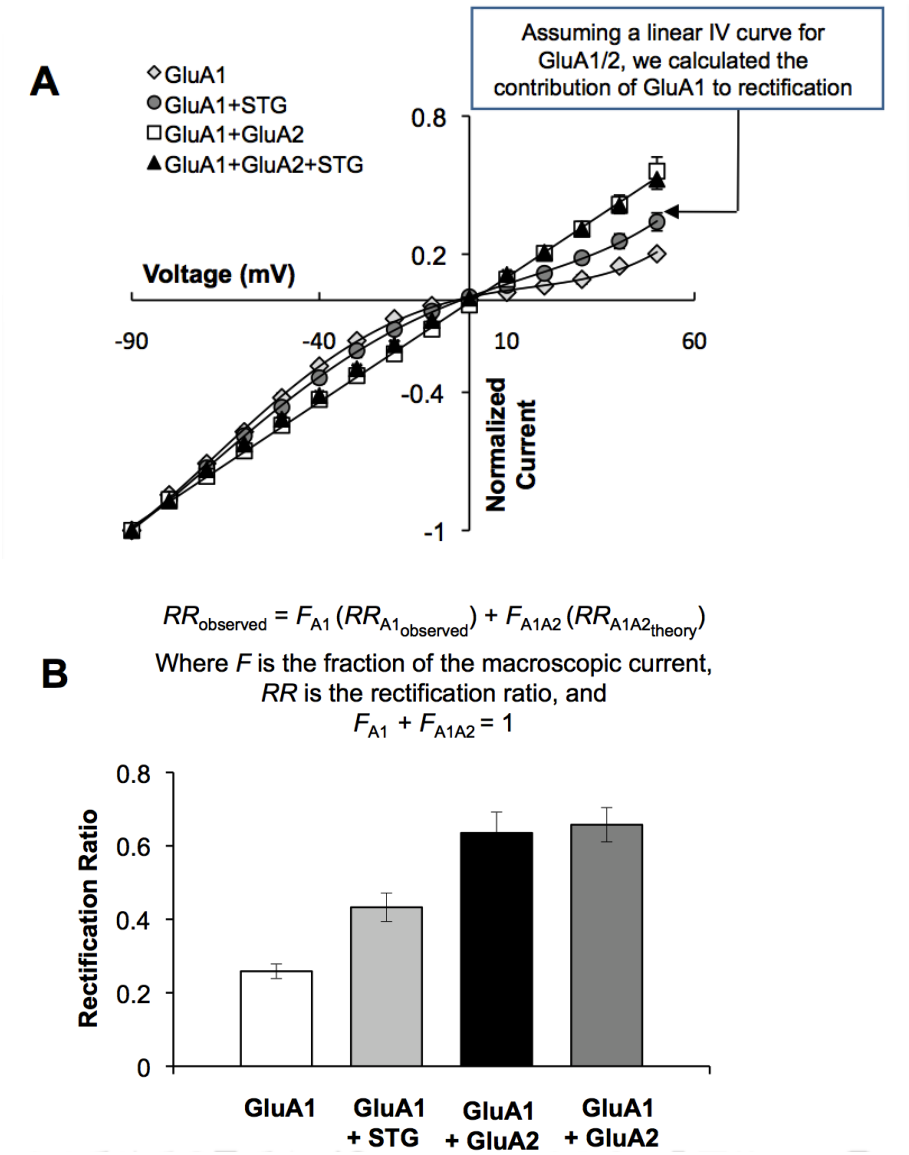


Figure 2.5. A linear IV relationship suggests a heteromeric receptor population. ***A,*** Normalized current-voltage (IV) relationship for GluA1±stargazin and GluA1/GluA2±stargazin (mean±SEM). Homomeric GluA1 receptors show inward rectification, which is reduced in the presence of stargazin. GluA2-containing receptors show a linear IV relationship that is only slightly altered by stargazin. ***B,*** Rectification ratios (RR) were calculated as the ratio of current amplitude at +40 mV, which reflects primarily GluA1/GluA2 heteromeric receptor current, to that

at -60 mV (mean±SEM; n= 8-18 cells for each condition)**.** From the stargazin-induced changes in the rectification ratio, 96% of the total current was calculated to have been contributed by GluA1/GluA2 receptors and 4% from homomeric GluA1, according to the equation :

$$RR_{observed} = F_{A1}\,(RR_{A1observed}) + F_{A1A2}\,(RR_{A1A2theory})$$

where F_{A1} is the fraction of the macroscopic current carried by homomeric GluA1, F_{A1A2} is the fraction of macroscopic current carried by heteromeric GluA1/GluA2, and $F_{A1} + F_{A1A2} = 1$. For GluA1/stargazin transfected cells $RR_{A1observed}$ was 0.43 and for GluA1/GluA2/stargazin transfected cells $RR_{observed}$ was 0.66; a linear IV yields $RR_{A1A2theory}$ of 0.67.

net AMPA receptor current contributed by homo- or heteromeric receptors. These calculations show that 96% of the current recorded from GluA1/GluA2 transfected cells in the presence of stargazin at +40 mV was from heteromeric receptors and 4% of the current was contributed by a subpopulation of homomeric receptors, calculated using the rectification ratios of homomeric GluA1 receptors (see Figure 2.5 legend for calculation). I used this relative contribution of GluA1 homomers to the macroscopic current, the weighted γ_{MEAN} values for homomeric receptors, and the weighted γ_{MEAN} for multiple subconductance levels described in equation 1 (*Methods*) to plot the γ_{MEAN} for a mixture of phosphomimic and phosphodeficient GluA1 and GluA1/GluA2 receptors over a range of hypothetical GluA1/GluA2 conductance values (Figure 2.6 legend). From this calculation, the maximal possible difference in the γ_{MEAN} caused solely by the 4% of GluA1 homomeric receptors can be plotted against GluA2 conductance values between 1 and 3 pS (Figure 2.6*A*). When the change in γ_{MEAN} caused by the S831E mutation is plotted over a range of hypothetical GluA1/GluA2 receptor γ_{MEAN} values, it is clear that the potential conductance increase caused by the S831E mutation in the 4% of homomeric GluA1 receptors cannot account for the 213% increase in γ_{MEAN} observed for GluA1/GluA2 receptors in the presence of stargazin (Figure 2.6*B*). These results further support the idea that CaMKII phosphorylation of GluA1-Ser831 in an AMPA receptor complex that contains the GluA2 subunit and stargazin can enhance the weighted mean conductance.

While stargazin is the most extensively studied TARP, it is not the

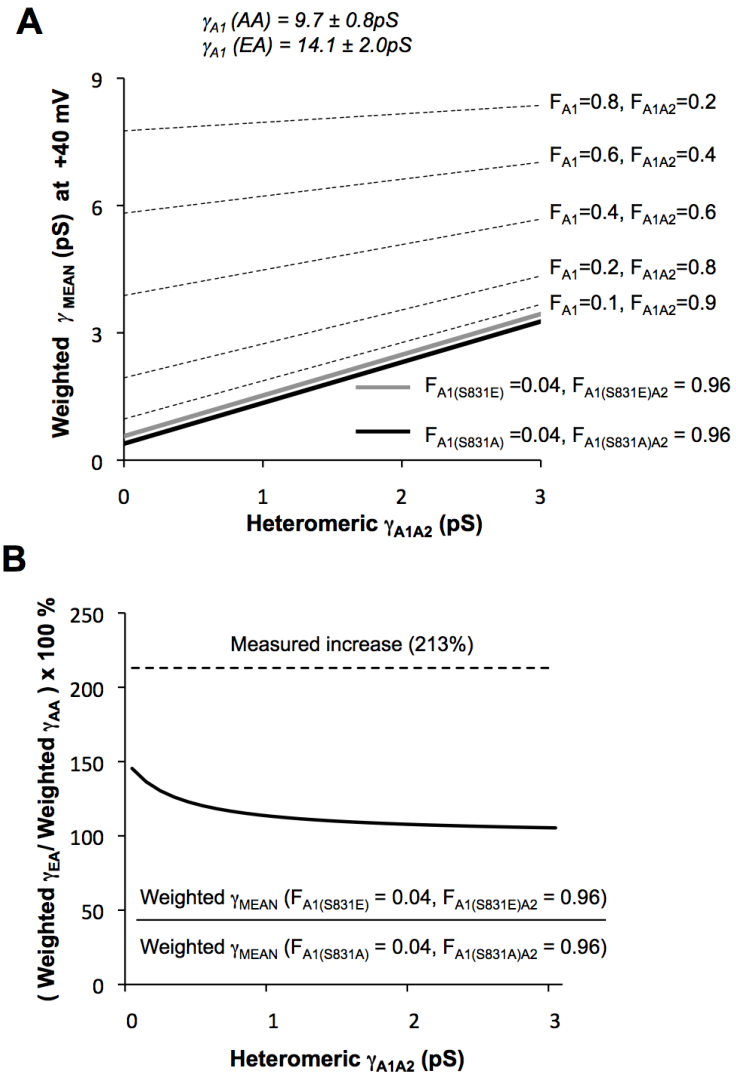


Figure 2.6. The increase in γ_{MEAN} from GluA1/GluA2 receptors in the presence of stargazin is not caused by a stargazin-induced increase in a subpopulation of homomeric GluA1-EA receptors. ***A,*** The predicted weighted mean conductance (γ_{MEAN}) is plotted as a function of an unknown "true" GluA1/GluA2 conductance according to equation 1 (*Methods*). The predicted weighted γ_{MEAN} was obtained by using the following expression:

$$F_{A1} = \frac{NP_{A1}\gamma_{A1})}{NP_{A1}\gamma_{A1}\ NP_{A1A2}\gamma_{A1A2}}$$

$$F_{A1A2} = \frac{NP_{A1A2}\gamma_{A1A2}}{NP_{A1}\gamma_{A1}\ NP_{A1A2}\gamma_{A1A2}}$$

into equation 1 (*Methods)*, yielding:

$$\gamma_{MEAN} = F_{A1}\,\gamma_{A1} F_{A1A2}\,\gamma_{A1A2}$$

where *N* is the number of channels and *P* is the open probability. The broken lines show how the weighted γ_{MEAN} changes with progressively increasing F_{A1} using the conductance of GluA1 and GluA1/GluA2. The thick black line is the weighted γ_{MEAN} when F_{A1} is the 4% of homomeric GluA1(S831A) receptors with γ of 9.7 pS determined at +40 mV, and 96% GluA1(S831A)/GluA2. The thick gray line shows weighted γ_{MEAN} for GluA1(S831E) with γ of 14.1pS determined at +40 mV and 96% GluA1(S831E)/GluA2. ***B,*** The relationship between the change in weighted γ_{MEAN} induced by the GluA1(S831E) alone in a mixed population of GluA1 (4%) and GluA1/GluA2 (96%) receptors is shown (solid curve) over a range of GluA1/GluA2 conductance values. The dotted line shows that the experimentally observed γ_{MEAN} (213%) is substantially more than that predicted from a conductance increase in only GluA1 subpopulation alone over a range of potential GluA1/GluA2 conductances.

predominant TARP within the hippocampus. I therefore chose to examine if phospho-Ser831 increases the conductance of heteromeric AMPA receptors coexpressed with another TARP, γ8. γ8 is highly expressed in the hippocampus, slows deactivation, slows desensitization, and prolongs the activation rise time of AMPA receptors (Nicoll et al., 2006; Milstein et al., 2007; Milstein and Nicoll, 2009). Experiments were carried out in the same way as with stargazin, with the exception that expression of γ8 was confirmed via fluorescent imaging because the γ8 cDNA is in a vector that co-expresses GFP. The conductance of cells expressing GluA1-EA/GluA2/γ8 was increased to 6.4 ± 1.4 pS (n=10) from 3.0 ± 0.4 pS from GluA1-AA/GluA2/γ8 (n=11; $p<0.05$; Student's t test; Table 1). These data suggest that there is a common property between TARPs that when in complex with GluA1-containing AMPA receptors enables a phospho-Ser831-dependent increase in conductance.

V. Conclusion

The data presented here show for the first time that CaMKII increases conductance of native hippocampal AMPA receptors specifically via phosphorylation at GluA1-Ser831, by evaluation of AMPA receptor response properties in the GluA1 phosphomutant knock-in mice. Moreover, previous studies in recombinant systems in the absence of TARPs established that CaMKII phosphorylation of GluA1 Ser831 increases the single channel conductance of homomeric GluA1 receptors, but not of heteromeric GluA1/GluA2 receptors(Derkach et al., 1999; Oh and Derkach, 2005). We have confirmed this

result, but further show that phosphomimic mutations of Ser831 can increase conductance of heteromeric GluA1/GluA2 recombinant channels when either stargazin or γ8 are present.

Chapter 3

Identification of a phosphorylation-dependent hyper-regulatory region within the GluA1 C-terminal domain

I. Abstract

The membrane proximal region of the GluA1 C-terminal domain acts as a hyperregulatory region of AMPA receptor function. Phosphomimic mutations inserted at each of 3 different phosphorylation sites within this region, GluA1-Ser818, GluA1-Ser831, and GluA1-Thr840, lead to an increase in GluA1 receptor conductance. In addition, the distal CTD can be removed entirely from GluA1 and a phosphomimic S831E mutation still increases recombinant homomeric GluA1 AMPA receptor conductance. These residues likely couple strongly to AMPA receptor gating and permeation elements to dynamically regulate receptor conductance.

II. Introduction

There are 4 known serine/threonine phosphorylation sites within a stretch of 27 residues in the C-terminal domain of the GluA1 AMPA receptor subunit (Barria et al., 1997a; Lee et al., 2003; Boehm et al., 2006; Delgado et al., 2007; Lee et al., 2010). Phosphorylation at these sites maintains tight control over receptor gating properties, which can exert dramatic effects on synaptic signaling. Phosphorylation of residue Ser845 in the GluA1 AMPA subunit by protein kinase A, increases the probability of opening of the receptor (Banke et al., 2000), and CaMKII increases AMPA receptor conductance by phosphorylating GluA1-Ser831 (Derkach et al., 1999).

In this chapter, I investigate how phosphorylation of GluA1-Ser818 and GluA1-Thr840 affect AMPA receptor function. Phosphorylation of GluA1-Ser818 by Ca^{2+}/phospholipid-dependent protein kinase (PKC) is increased during LTP and promotes synaptic incorporation of AMPA receptors (Boehm et al., 2006). PKC-dependent phosphorylation of GluA1-Ser818 can also enhance the interaction between GluA1 and the actin-binding protein 4.1N to facilitate insertion of this subunit into the post-synaptic membrane (Lin et al., 2009). Disrupting PKC and 4.1N-dependent synaptic insertion of GluA1 also decreases expression of LTP. Similarly, Thr840 can be phosphorylated by PKC to regulate synaptic plasticity in an age-dependent manner (Lee et al., 2007b). Finally, p70S6 kinase maintains a high basal level of phosphorylation at GluA1-Thr840, that is dephosphorylated by PP1/2A to induce LTD (Delgado et al., 2007). Clearly phosphorylation at both of these sites, Ser818 and Thr840, is critical for synaptic

plasticity and receptor trafficking; however, no information exists about whether addition of a phosphate group to these residues can regulate function of the AMPA receptor.

The close-proximity of Ser818 and Thr840 to Ser831 and Ser845 in the GluA1 C-terminus, residues known to change receptor function when phosphorylated, suggests that this region of the GluA1 C-terminus serves as a hyper-regulatory domain of receptor function. Phosphomimic mutations at either GluA1-Ser818 or GluA1-Thr840 cause an increase in homomeric recombinant GluA1 receptor conductance. Finally, removing any part of this hyper-regulatory region abrogates the phospho-dependent increase in conductance. These findings suggest that the stretch of 27 residues between Ser818 and Ser845 couple strongly to the GluA1 receptor gating machinery and serve as a hyper-regulatory domain in which phosphorylation increases AMPA receptor conductance.

The data included in this Chapter has not yet been published.

Methods

See Chapter 2

III. Results

A. The membrane proximal region of the GluA1 CTD is a "hyper-regulatory region" of AMPA receptor conductance

How gating and permeation elements are affected by addition of a charged residue or phosphate group to a distant site within the GluA1 C-terminus, is unclear. A likely explanation is that intra- or intermolecular interactions are either formed or disrupted upon phosphorylation of the GluA1 CTD that affect the AMPA receptor gating machinery to change receptor conductance. To investigate the structural mechanisms that underpin a phospho-Ser831 dependent GluA1 conductance increase, I examined the functional effects of removing portions of the distal C-terminus. If a PDZ-domain containing protein binds to the distal GluA1 C-terminus to induce a conformational change in channel structure associated with an increase in γ_{MEAN}, for example, then removing this domain by inserting a stop codon at GluA1-Ala886 should abrogate the effects of the GluA1-S831E mutation. However, compared to GluA1-AA full-length receptors, there was still an increase in γ_{MEAN} in a GluA1 construct expressing the L497Y, S831E, and S845A mutations, that also lack the final three residues required for binding a PDZ protein (Figure 3.1; $p<0.01$ by One-Way ANOVA, with Bonferroni's post hoc test). Inserting stop

codons at incremental intervals upstream of the PDZ-binding region of GluA1, at Ser877, Pro867, Glu857, and Ser850 similarly did not interrupt the increase in γ_{MEAN} caused by the GluA1-S831E mutation (Figure 3.1; $p<0.01$ by One-Way ANOVA, with Bonferroni's post hoc test). However, a GluA1 mutant receptor with a stop codon inserted in place of Ser845 did not show a GluA1-S831E dependent conductance increase compared to full-length GluA1-AA receptors ($p=1.0$, by One-Way ANOVA with Bonferroni's post hoc test). This finding suggests that residues upstream of Ser850 are required to induce the conformational changes necessary to increase conductance in GluA1-EA receptors.

I then sought out to examine the structural and biophysical significance of four phosphorylation sites existing within 27 residues of one another. In addition to GluA1-Ser831 and GluA1-Ser845, GluA1-Ser818 and GluA1-Thr840 are also known phosphorylation targets (Boehm et al., 2006; Delgado et al., 2007; Lin et al., 2009). Both of these phosphorylation sites are important for receptor trafficking and plasticity, however, the direct actions of phosphorylation at GluA1-Ser818 and GluA1-Thr840 on AMPA receptor function have not been explored. I expanded the phosphomutant substitutions within the GluA1 C-terminus to include these phosphorylation sites, and applied the same stationary variance analysis to macroscopic currents obtained from HEK cells transfected with these mutant subunits. When a glutamate substitution is inserted into GluA1-Ser818, with alanine substitutions at Ser831, Thr840 and Ser845 (Table 1; GluA1-S818E,S831A,T840A,S845A, or GluA1-EAAA), homomeric GluA1 receptors

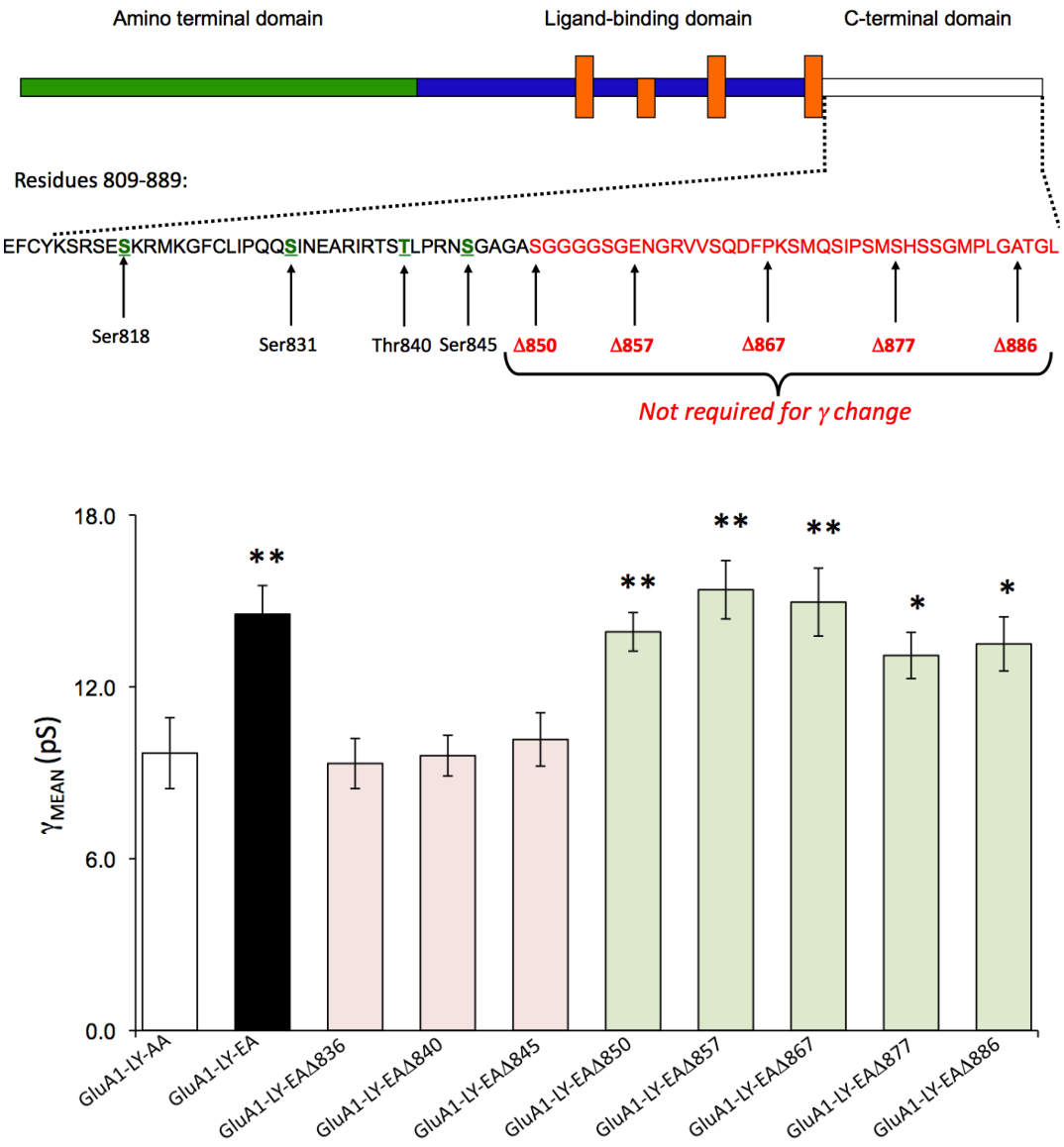


Figure 3.1. Residues downstream of GluA1-Ser850 are not required for a phosphoSer831-dependent GluA1 receptor conductance increase. A schematic representation of the GluA1 subunit, indicating C-terminal residues at which sites stop codons were inserted to create truncation GluA1 subunit mutants. Below is a summary of γ_{MEAN} values from a series of GluA1-EA truncation mutants (mean±SEM) with stop codons inserted in place of the indicated amino acid codons. Removing the distal C-terminus does not block a conductance increase caused by the S831E mutation. * p<0.01, ** p<0.001 compared to full-length GluA1-AA by one-way ANOVA with Bonferroni's post hoc test. Data are from 10-18 HEK cell patches for each mutant receptor.

exhibit an increased γ_{MEAN} compared to receptors with alanine substitutions at all four sites (GluA1-S818A,S831A,T840A,S845A, or GluA1-AAAA; $p<0.01$ by One-Way ANOVA with Bonferroni's post hoc test; Table 1). The GluA1-T840E mutation similarly increases GluA1 γ_{MEAN} in phosphomutant receptors in which the other three phosphorylation sites are mutated to alanine (GluA1-S818A,S831A,T840E,S845A or GluA1-AAEA; $p<0.01$ by One-Way ANOVA with Bonferroni's post hoc test). These data suggest that a charged residue at any of these three phosphorylation sites, Ser818, Ser831 or Thr840, increases homomeric GluA1 receptor conductance. This result shows that conductance can be controlled by phosphorylation throughout this membrane proximal C-terminal region.

To determine if an increase in conductance is the only functional consequence of phosphomimic mutations at Ser818, Ser831 and Thr840, concentration-response relationships were determined for mutant receptors with a glutamate substitution at each of these sites, and alanine substitutions at the three other sites. The concentration response was unaffected by phosphomimic mutations. Neither the EC_{50} value ($p=0.222$) nor or the Hill slope ($p = 0.93$) were significantly affected in GluA1-EAAA, GluA1-AEAA or GluA1-AAEA receptors compared to both GluA1-AAAA and wild-type (GluA1-SSTS) receptors (Figure 3.2). The time course of desensitization and deactivation were measured during rapid applications of a maximally effective concentration of glutamate (10mM) to address whether phosphorylation at these sites could influence GluA1 receptor response characteristics (Figure 3.3). Analysis of the response time

Table 3.1.

	γ_{MEAN} (pS)	P_O	n
GluR1-LY-AAAA	10.14 ± 0.74	0.67 ± 0.05	17
GluR1-LY-EAAA	13.87 ± 0.73 *	0.73 ± 0.04	15
GluR1-LY-AEAA	14.87 ± 0.99 *	0.78 ± 0.04	15
GluR1-LY-AAEA	13.87 ± 0.86 *	0.72 ± 0.05	17
GluR1-LY-EEAA	13.28 ± 0.87	0.64 ± 0.03	15
GluR1-LY-EAEA	14.26 ± 1.40	0.67 ± 0.02	13
GluR1-LY-AEEA	13.05 ± 0.58	0.67 ± 0.03	14

Effect of Ser818, Ser831, Thr840 phosphomimic mutations on γ_{MEAN} of homomeric GluA1 receptors. Weighted mean unitary conductance, γ_{MEAN}, was determined using stationary variance analysis of current responses obtained from transfected HEK cells. Values are mean ± SEM; n is the number of outside-out patches studied at a holding potential of -60 mV. * p<0.01 significantly different from GluA1-LY-AAAA by one-way ANOVA and Tukey's post hoc test. There was no significant difference between GluA1-EAAA, GluA1-EEAA and GluA1-EAEA (ANOVA). GluA1-AEAA was not significantly different from GluA1-EEAA and GluA1-AEEA (ANOVA). There was also no difference between GluA1-AAEA, GluA1-EAEA and GluA1-AEEA (ANOVA). Open probability (P_O) was not changed by any of the phosphomutations (One-way ANOVA).

course in non-desensitizing homomeric GluA1 receptors containing the L497Y mutation and one of three different phosphomimic mutations (GluA1-EAAA, GluA1-AEAA, GluA1-AAEA), showed no significant effect in deactivation compared to GluA1-AAAA ($p=0.47$; Figure 3.3, Table 3.3). Similarly, these same mutations do not change the time constant or extent of desensitization in Leu497 (WT) desensitizing receptors ($p=1.0$) compared to GluA1-AAAA receptors (Figure 3.3; Table 3.3).

Finally, I chose to examine any synergistic or additive effects of multiple sites being simultaneously phosphorylated. If the presence of only one charged group within this site is required to increase conductance, then multiple glutamate substitutions within this region may be additive or synergistic to exert an even larger increase in γ_{MEAN}. Pairs of glutamate substitutions at Ser818, Ser831 and/or Thr840 in differing combinations were introduced in to the GluA1 subunit, again containing the L497Y non-desensitizing mutation. When more than one residue was simultaneously mutated to a glutamate, γ_{MEAN} was not significantly changed compared to the corresponding single glutamate mutant receptor determined with stationary variance analysis. In other words, the conductance of GluA1-EAAA was not significantly different from GluA1-EEAA or GluA1-EAEA ($p=0.77$). GluA1-AEAA was not significantly different from GluA1-EEAA or GluA1-AEEA ($p=0.27$). And finally, γ_{MEAN} was not different between GluA1-AAEA receptors and GluA1-EAEA and GluA1-AEEA receptors ($p=0.76$, all by one-way ANOVA, Power = 0.8). These findings suggest that a single glutamate substitution at any of these known phosphorylation sites within this

Table 3.2

Receptor	EC50 (μM)	Hill slope	n
GluA1-LY-AAAA	7.6	1.86	5
GluA1-LY-EAAA	7.1	1.89	4
GluA1-LY-AEAA	9.3	1.94	4
GluA1-LY-AAEA	6.4	1.84	5

Phosphomutations at Ser818, Ser831 and Thr840 do not change parameters measuring concentration-response relationship of recombinant homomeric GluA1 receptors to glutamate. Measurements were made from whole-cell patch-clamp recordings from HEK cells transfected with indicated non-desensitizing phosphomutant GluA1 cDNA, held at -60 mV. EC_{50} is the glutamate concentration required for half maximal response. Composite data were plotted on a logarithmic scale and fitted with the Hill equation to calculate the Hill slope value. *n* is the number of patches.

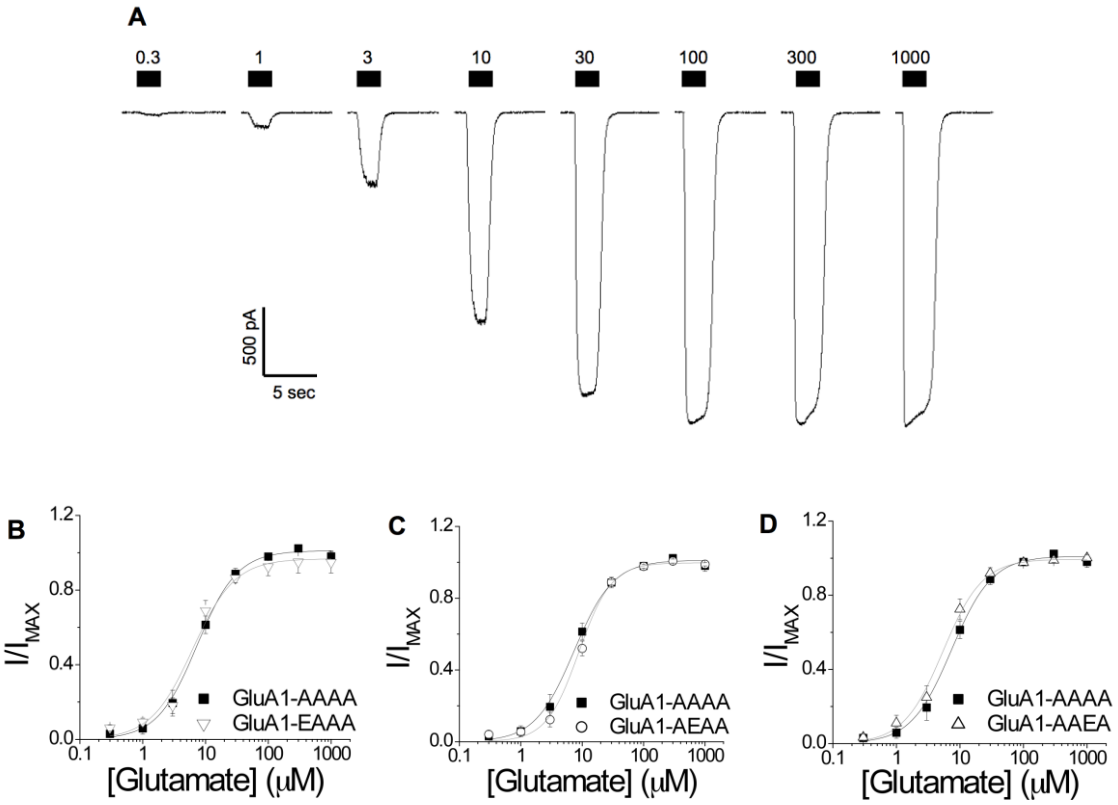


Figure 3.2. Phosphomimic mutations do not affect the glutamate EC_{50} at GluA1. ***A,*** Representative whole-cell current trace from a HEK cell expressing GluA1-LY-AAAA receptors. Black boxes above trace represent agonist application time, with concentrations indicated above (in μM). ***B,*** Concentration-response relationships were determined with glutamate concentrations ranging from 0.3μM to 1000μM for each GluA1 mutant receptor expressed in HEK cells. Composite data were plotted on a logarithmic scale and fitted with the Hill equation.

hyper-regulatory region of the GluA1 C-terminus is sufficient to increase receptor conductance, but multiple glutamate substitutions but are neither additive nor synergistic.

IV. Conclusions

These data show that the membrane proximal GluA1 C-terminal domain is a hyper-regulatory region of AMPA receptor conductance. The distal GluA1 C-terminal residues are not required for a phosphorylation-dependent conductance increase, and phosphomimic mutations at three different sites in the membrane proximal region increase recombinant homomeric AMPA receptor conductance. These observations are critical to understanding the structure and function of the GluA1 C-terminal domain, and given the role of this important AMPA receptor subunit in synaptic plasticity, could be used to elucidate mechanisms of LTP and LTD. These ideas are explored further in the discussion provided in Chapter 6.

Future work will evaluate whether or not a glutamate substitution at any site within this region, or only glutamate substitutions at the known phosphorylation sites, increase conductance. Alternatively, could substituting this region into the GluA4 subunit, which bears strong sequence homology to GluA1 but does not possess these same phosphorylation targets at homologous residues, also transfer an increase in conductance? These ideas may provide a structural framework around which to further explore the mechanism by which GluA1 phosphorylation influences AMPA receptor function.

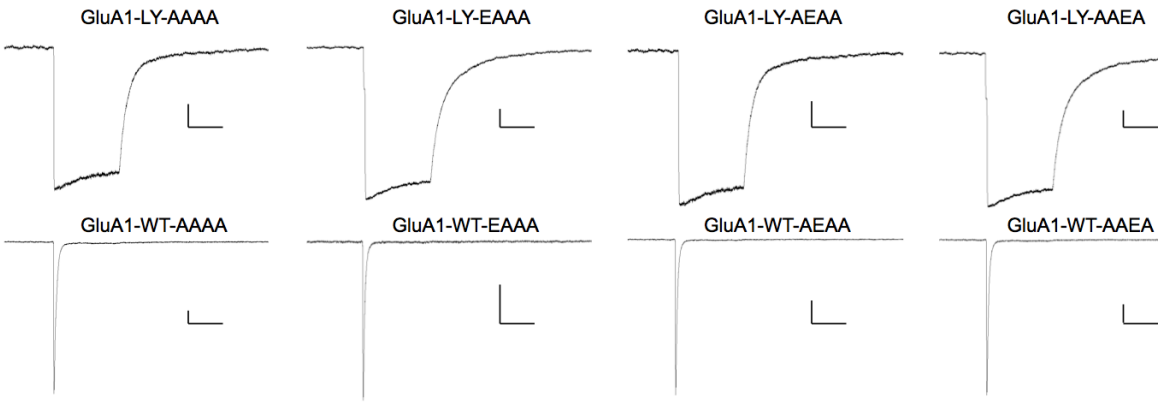


Figure 3.3. Phosphomutations at Ser818, Ser831 and Thr840 do not alter time course of GluA1 phosphomutant receptor currents. Representative current traces from excised outside-out membrane patches from HEK cells transfected with indicated non-desensitizing or desensitizing phosphomutant GluA1 cDNA, held at -60 mV. Scale bars represent 50 pA and 50 ms.

Table 3.3

DEACTIVATION

Receptor	10-90% Rise (ms)	τ_s (ms)	τ_f (ms)	τ_w (ms)	% τ_f	SS/PK	n
GluA1-LY-AAAA	1.04	15.7	198.0	31.6	86.5%	0.92	6
GluA1-LY-EAAA	0.95	15.2	129.1	28.4	86.4%	0.9	5
GluA1-LY-AEAA	1.06	18.0	136.9	36.6	76.5%	0.89	5
GluA1-LY-AAEA	0.67	17.3	157.9	32.1	84.9%	0.92	5

DESENSITIZATION

Receptor	10-90% Rise (ms)	τ_s (ms)	τ_f (ms)	τ_w (ms)	% τ_f	SS/PK	n
GluA1-WT-AAAA	0.41	2.2	48.7	3.6	88.3%	0.023	5
GluA1-WT-EAAA	0.42	3.0	47.9	3.7	84.9%	0.025	6
GluA1-WT-AEAA	0.42	2.9	54.7	3.8	89.3%	0.024	5
GluA1-WT-AAEA	0.61	2.5	41.9	3.9	82.5%	0.019	5

Phosphomutations at Ser818, Ser831 and Thr840 did not change deactivation or desensitization of recombinant homomeric GluA1 receptors. Measurements were made from excised outside-out membrane patches from HEK cells transfected with indicated non-desensitizing or desensitizing phosphomutant GluA1 cDNA, held at -60 mV. Rise time is measured as the time it takes for the response to increase from 10 to 90% of the maximal amplitude. τ_f is the fast and τ_s is the slow time constant component of the decay. SS/PK represents the ratio of the peak (PK) to steady state (SS) current amplitude. Measurements made for each mutant were not significantly different by one-way ANOVA. Responses were fit to:

$$\text{Response} = Amp_{\text{FAST}}\ (\text{exp}-\text{time}/\tau_{FAST}) + Amp_{\text{SLOW}}\ (\text{exp}-\text{time}/\tau_{SLOW})$$

where τ_{FAST} is the fast deactivation time constant, τ_{SLOW} is the slow deactivation time constant, Amp_{FAST} is the amplitude of the fast deactivation component, and Amp_{SLOW} is the amplitude of the slow deactivation component. In order to simplify some comparisons, weighted deactivation time constants were calculated using the following equation:

$$\tau_W = \{ [\ Amp_{\text{FAST}} / (Amp_{\text{FAST}} + Amp_{\text{SLOW}})\ ] \times \tau_{FAST} \} + \{ [\ Amp_{\text{SLOW}} / (Amp_{\text{FAST}} + Amp_{\text{SLOW}})\ ] \times \tau_{SLOW} \}$$

where τ_W is the weighted deactivation time constant.

Chapter 4

Introduction : Voltage-gated Ca^{2+} Channels

Voltage-gated Ca^{2+} channels (VGCCs) are cationic channels that couple changes in membrane potential to gating of the ion channel pore and the influx of Ca^{2+}. VGCCs are a diverse family of ion channels that regulate a wide array of cell functions including, but not limited to, cell excitability and firing, gene expression, excitation contraction coupling, and synaptic release (Catterall, 2000). VGCCs are grouped into the following three major groups based on current, genetic similarity, and structure of the pore-forming α1 subunit: Ca_v1 (L-type current), Ca_v2 (N, P/Q, and R-type current) and Ca_v3 (T-type current). The Ca_v nomenclature identifies the genetic identity of the channel itself, while the L-, N-, and T-type nomenclature refers to the type of current these channels conduct.

Of the four L-type conducting channels ($Ca_v1.1$-1.4), $Ca_v1.2$ and $Ca_v1.3$ are the predominant Ca_v1 channels in the central nervous system (CNS). As a result of their comparatively low expression in the brain and their limited pharmacology, relatively little is known of the mechanisms that confer the specific roles of $Ca_v1.3$ channels and how they selectively control certain brain functions under specific conditions. The goal of this portion of this dissertation is to fill this void in our understanding by analyzing how CaMKII selectively modulates $Ca_v1.3$ Ca^{2+} signals during cell specific behaviors.

I. Voltage-gated Ca^{2+} channel structure and function

A. General structure

Biochemical purification experiments revealed that voltage-gated Ca^{2+} channels consist of a pore-forming $\alpha 1$ subunit, and the auxiliary intracellular β and the transmembrane disulfide linked $\alpha 2\delta$ subunits (Figure 4.1; (Curtis and Catterall, 1984; Hosey et al., 1987; Leung et al., 1987; Striessnig et al., 1987; Takahashi et al., 1987). Similar to voltage-gated Na^{+} channels, the $\alpha 1$ subunit is comprised of four domains, each consisting of six transmembrane spanning α-helical segments, demonstrated through cloning and sequencing (Tanabe et al., 1987). The structure of this ion channel differs from glutamatergic receptors in that one DNA sequence encodes the entire functional receptor. The four domains within this single ion channel protein are akin to the four distinct subunits encoded by different gene products in glutamate receptors. The N-terminal of VGCCs is located intracellularly, while glutamate receptors have an extracellularly localized N-terminus. Also, the four domains of VGCCs each have 6 transmembrane domains and one reentrant loop, while a glutamate receptor subunit has only 3 transmembrane domains and 1 reentrant loop. However, there remain some interesting structural similarities between these two distinct ion channels. Namely, both glutamate receptors and VGCCs have a large intracellular C-terminus that has

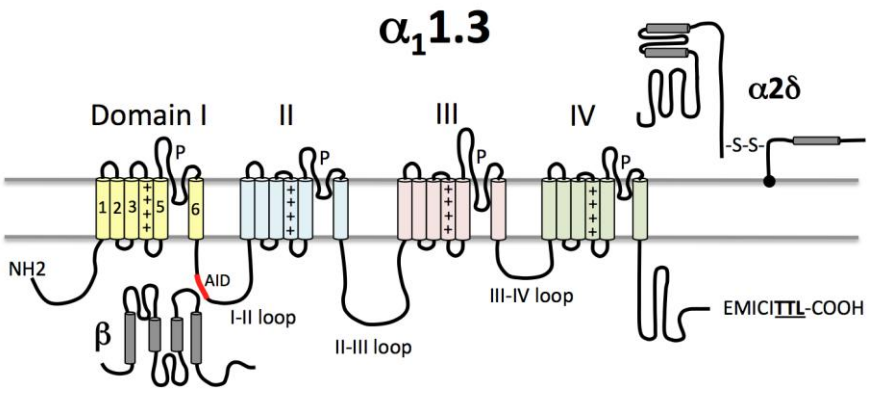


Figure 4.1. $Ca_v1.3$ voltage-gated Ca^{2+} channel general structure. Membrane topology diagram indicating four $\alpha_1$1.3 domains, transmembrane segments, and intracellular regions, including the PDZ-domain binding sequence as the distal C-terminus. Also shown are auxiliary β and $\alpha 2\delta$ subunits and relative localization in relation to $\alpha_1$1.3. Adapted from (Catterall, 2000).

extensive protein-protein interactions that modulate receptor behaviors as well as subcellular localization.

B. Voltage-sensor

The fourth α-helical transmembrane segment within the fourth voltage-gated channel domain (IV-S4) acts as the activation voltage-sensor (Catterall, 2010). While there is some dispute regarding the mechanisms that underlie how S4 actually responds to and induces the conformational changes that open the channel pore, the “sliding helix” model is the most widely accepted (Figure 4.2). The residues in the S4 region of voltage-gated channels are arranged in an α-helical arrangement in which every third or fourth residue is positively charged (Tanabe et al., 1987; Stuhmer et al., 1989; Papazian et al., 1991). These positively charged amino acids pair with negatively charged residues within the S1- S3 segments. These four to seven basic residues are drawn down into the membrane by the negative internal resting potential. When the membrane is depolarized, this electromechanical force is relieved and the S4 segment rotates in an outward spiral away from the positive internal membrane potential (Guy and Seetharamulu, 1986; Catterall, 2010). This outward movement, depicted in Figure 4.2, likely pulls on the S4-S5 linker, bending the helical bundle at the intracellular end of the S6 segment to pull open the ion channel pore (Doyle et al., 1998; Long et al., 2005).

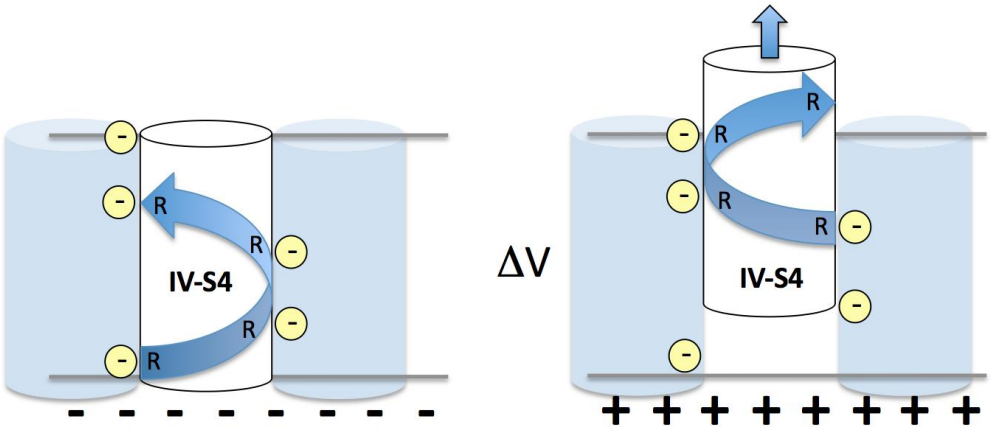


Figure 4.2. Sliding-helix model of voltage-gated channel gating. The final S4 transmembrane segment, within domain IV (IV-S4) is drawn as a cylinder to represent the α helix. The four potential gating charges, arginines (R), are neutralized from negative residues in surrounding transmembrane regions. Negative internal membrane potential draws the positive arginine residues towards the cytoplasm. Upon membrane depolarization, IV-S4 rotates outward in a spiral, exchanging negative residue partners from adjacent segments along the way. This action likely pulls on the S5-S6 linker to open the channel pore and allow ion flux. Adapted from (Catterall, 2010)

C. Ion permeation

Most voltage-gated channels have specific ion permeability, this holds true for voltage-gated Ca^{2+} channels that are permeant to divalent cations. These channels preferentially conduct Ca^{2+} ions, but other less physiologically relevant charge carriers, such as Ba^{2+} and Sr^{2+}, can be utilized under experimental conditions to differentiate Ca^{2+}- versus voltage-dependent effects on channel function. The pore of the channel is formed by the highly conserved glutamate residue-rich membrane-associated loop between S5 and S6 and confers Ca^{2+} selectivity (Yang et al., 1993; Ellinor et al., 1995). Specifically, there are four negatively charged glutamate residues in this S5-6 linker that bind Ca^{2+} ions within the pore to enable ion permeation and flux (Kim et al., 1993; Ellinor et al., 1995). These glutamate residues may allow simultaneous interactions with multiple Ca^{2+} ions moving single-file within the pore. These Ca^{2+} ions compete with one another for binding to individual gluatmates and electromechanically repel one another, each ion helping to push the others through the channel, resulting in rapid flow rates through the pore (Yang et al., 1993).

The S5-6 region, composed of the entire transmembrane domains 5 and 6 as well as the linker, has also been suggested to be the sole determinant of single channel conductance in Ca^{2+} channels and other voltage-gated channels (Hartmann et al., 1991; Yatani et al., 1994; Dirksen et al., 1997). Structural differences between this region of the various VGCCs likely underlie the differences in unitary conductance (Cibulsky and Sather, 2003). The S5-S6

linker is also where dihydropyridines, Ca^{2+} channel antagonists, bind (Hockerman et al., 1995; Peterson et al., 1996; Hockerman et al., 1997).

D. Auxiliary subunits

1. $Ca_v\beta$ subunits

The pore-forming α subunit of VGCCs is responsible for channel activation and ion permeation, but they are also associated with a variety of auxiliary subunits that are equally critical to channel function. The intraceulluar $Ca_v\beta$ subunit has five regions, the second region is homologous to a Src homology 3 (SH3) domain and the fourth region is homologous to the guanylate kinase domain (GK). These two regions are highly conserved among the four different genes that encode β subunits. The other domains, however, are highly variable in sequence and length and are likely responsible for the functional differences between the different genes and splice variants (Hanlon et al., 1999; Opatowsky et al., 2003; Buraei and Yang, 2010). The intracellular loop connecting domains I and II of the α subunit (referred to as the AID; Figure 4.1) interacts with the hydrophobic GK region of the β subunit; all four β subunit genes bind to the AID (Pragnell et al., 1994; Chen et al., 2004).

The β subunits also enhance VGCC current via an increase in channel P_O (Colecraft et al., 2002; Herzig et al., 2007), but the dramatic increase in current size primarily reflects increased surface expression of the channel. The pore-forming α subunit can not translocate to the cell surface independently in heterologous expression systems, β subunits are required for surface expression

of VGCCs (Lacerda et al., 1991; Mori et al., 1991; Williams et al., 1992; Shistik et al., 1995). Association of Ca_v channels with β subunits modulates other forms of channel gating, in addition to P_O (Colecraft et al., 2002). The β2α subunit, for example, when assembled with the a pore-forming subunit, slows inactivation compared to β1b (Stephens et al., 2000). The interaction between the Ca_v channel I-II linker and $Ca_vβ$ is also hypothesized to dictate voltage-dependent inactivation, a reduction in the amount of Ca^{2+} allowed to flow through VGCCs in response to repetitive depolarization (Patil et al., 1998). Most β subunits shift the voltage-dependence of inactivation to ~10-20 mV more hyperpolarized voltages, meaning weaker depolarizations induce inactivation of VGCCs in complex with $Ca_vβ$ subunits (Neely et al., 1993; Stephens et al., 2000; Stotz et al., 2000; Cens et al., 2006).

2. α2δ subunits

The heavily glycosylated α2 subunit is located extracellularly and is attached to the δ subunit via a disulfide linkage (Figure 4.1), and these disulfide linked peptides are attached to the membrane through a glycosylphosphatidylinositol linker (Ellis et al., 1988; Gurnett and Campbell, 1996; Gurnett et al., 1996; Davies et al., 2010). There are four unique genes that encode α2δ subunits, each with splice variants (Klugbauer et al., 1999; Qin et al., 2002). The α2δ subunits also modify biophysical properties of VGCCs, but their main role is to aid in trafficking and anchoring of the α1 subunit to the plasma membrane (Lacerda et al., 1991; Singer et al., 1991; Gurnett et al., 1997; Klugbauer et al., 1999; Davies et al., 2006). Loss of α2δ in mice is associated

with short lifespan, epilepsy, cerebellar ataxia, slower spike firing and decreased dendritic arborization (Davies et al., 2007; Buraei and Yang, 2010).

II. Voltage-Gated Ca^{2+} Channel Diversity and Characterization

A. Ca_v3 channels

Ca^{2+} currents were originally described both pharmacologically and physiologically. Cloning of VGCCs and homology screening lead to a nomenclature and classification system that divides the 10 different α subunits into three families based on sequence similarity, Ca_v1, Ca_v2 and Ca_v3. The properties of Ca_v3 channels, or T-type current, were first described using voltage-clamp recordings of starfish eggs (Hagiwara et al., 1975) and subsequently of cerebellar Purkinje neurons (Llinas and Yarom, 1981). Voltage-clamp recordings from dorsal root ganglion neurons demonstrated that these currents activate at hyperpolarized membrane potentials, exhibit small single channel conductance, rapid inactivation, and slow deactivation (Carbone and Lux, 1984; Fedulova et al., 1985; Nowycky et al., 1985; Swandulla and Armstrong, 1988). There are three genes that encode the proteins that conduct T-type current, $Ca_v3.1$-3.3. All three are found in neurons, while $Ca_v3.1$ and 3.2 are also found in cardiac muscle, and $Ca_v3.1$ is also found in skeletal muscle (Perez-Reyes et al., 1998).

B. Ca_v2 channels

Dorsal root ganglion recordings also helped to define the Ca_v2 family of VGCCs. The Ca_v2 channels are found in presynaptic neuronal nerve terminals and consists of 3 genes, $Ca_v2.1$-2.3. Ca^{2+} influx through presynaptic Ca_v2 channels triggers vesicle fusion and subsequent synaptic transmitter release. These channels are subject to regulation by SNARE proteins, G-proteins and kinases (Hille, 1994; Jones and Elmslie, 1997; Ikeda and Dunlap, 1999). N-type currents via $Ca_v2.2$ channels, activate at slightly more positive potentials than T-type currents, with slightly slower rates of inactivation (Nowycky et al., 1985). $Ca_v2.2$ channels are blocked by the spidertoxin, ω-conotoxin GVIA (McCleskey et al., 1987; Tsien et al., 1988), which has largely helped to define the properties and function of $Ca_v2.2$. Pharmacological tools have similarly helped to define the other Ca_v2 family currents; P/Q-type currents via $Ca_v2.1$ channels, shown through patch-clamp recordings from both cerebellar Purkinje and granule cells, are blocked by ω -agatoxin IVA (Mintz et al., 1992; Randall and Tsien, 1995). Finally, the R-type currents are “resistant” to these subtype-specific Ca^{2+} channel blockers (Randall and Tsien, 1995).

C. Ca_v1 channels

Ca_v1 Ca^{2+} channels conduct L-type current and consist of four distinct $\alpha 1$ subunit genes, $Ca_v1.1$-$Ca_v1.4$. The N-, P/Q-, and R-type currents are found predominantly in the brain, but this final family of VGCCs is widely expressed throughout many tissues. These receptors were originally shown to be responsible for excitation-contraction coupling in cardiac, smooth and skeletal

muscle cells (Schneider and Chandler, 1973; Reuter, 1985; Beam et al., 1989; Tanabe et al., 1990; Franzini-Armstrong and Protasi, 1997). L-type currents also initiate hormone secretion in endocrine cells, (Milani et al., 1990; Ashcroft et al., 1994) and regulate gene expression, (Bading et al., 1993; Deisseroth et al., 1998) mRNA stability, (Galli et al., 1995; Schorge et al., 1999) cell survival, (Marshall et al., 2003) axonal injury (Ouardouz et al., 2003) and synaptic efficacy (Christie et al., 1997; Lei et al., 2003; Adermark and Lovinger, 2007) in neurons. L-type currents are uniquely sensitive to dihydropyridines (DHP) so early pharmacological studies had grouped all DHP-sensitive channels together and all were assumed to exhibit similar properties (Peterson et al., 1996). Originally, all L-type channels were thought to exhibit a more depolarized voltage-dependence of activation, large single-channel conductances, and slow inactivation (Catterall, 2000). $Ca_v1.1$ channels open with slow kinetics, are expressed in skeletal muscle, and couple to ryanodine receptors to release intracellular Ca^{2+} stores in response to membrane depolarizations (Rios and Brum, 1987; Flucher and Franzini-Armstrong, 1996). $Ca_v1.4$ channels are located in synaptic terminals of bipolar cells of the retina to regulate tonic neurotransmitter release. These channels do not exhibit Ca^{2+}-dependent inactivation, are less sensitive to dihydropyridines and are activated at slightly more hyperpolarized potentials than $Ca_v1.2$ (Murakami et al., 2001; Berntson et al., 2003). The final two L-type conducting channels, $Ca_v1.2$ and $Ca_v1.3$, are examined in detail in the next section.

III. $Ca_v1.2$ and $Ca_v1.3$ Channels

A. $Ca_v1.2$ expression

Of the four L-type conducting channels, $Ca_v1.2$ and $Ca_v1.3$ are predominantly found in the CNS. $Ca_v1.2$ is expressed in neurons, ventricular cardiac and smooth muscle, pancreatic cells and fibroblasts (Koch et al., 1990; Perez-Reyes et al., 1990; Diebold et al., 1992; Soldatov, 1992; Mori et al., 1993; Schultz et al., 1993; Takimoto et al., 1997; Welling et al., 1997). Within neurons, $Ca_v1.2$ is located post-synaptically in dendrites and spines, but is also found in the cell bodies of cortical and hippocampal neurons (Westenbroek et al., 1990; Hell et al., 1993b). Autoradiography and immunohistochemical studies suggest that $Ca_v1.2$ channels make up approximately 80% of the Ca_v1 channels in the brain (Hell et al., 1993b; Clark et al., 2003; Tippens and Lee, 2007).

B. $Ca_v1.2$ physiology

No specific antagonists have been developed for $Ca_v1.2$ over $Ca_v1.3$ so, historically, isolating these currents from one another has been virtually impossible. Some recent studies have employed the slightly different sensitivity to DHPs. $Ca_v1.2$ channels have an IC_{50} for dihydropiridine block that is approximately 8 to 20-fold lower than that of $Ca_v1.3$ channels in heterologous expression systems (Koschak et al., 2001; Xu and Lipscombe, 2001). Although, inhibition of $Ca_v1.3$ channels by DHPs is enhanced when the channel is opened by depolarized membrane potentials, resulting in a narrow window within which $Ca_v1.2$ channels are blocked and $Ca_v1.3$ channels are active. Utilizing this

pharmacological phenomenon to isolate these currents is fractious and likely unreliable (Berjukow et al., 2000; Xu and Lipscombe, 2001).

Development of cloning has been the most valuable innovation in defining the unique properties of $Ca_v1.2$ versus $Ca_v1.3$ channels. While L-type Ca^{2+} current in native cells was originally described to exhibit slow activation kinetics using pharmacological subtraction methods (Mermelstein et al., 2000; Yasuda et al., 2003), studies using cloned $Ca_v1.2$ and $Ca_v1.3$ channels revealed that they are, in fact, fast activating (Xu and Lipscombe, 2001). VGCCs likely have many interaction partners that could change gating properties in neurons that are absent in recombinant systems, making it difficult to define properties of channels that would exist *in vivo* in the presence of such factors. Transgenic mice bred with a mutation that abrogates DHP sensitivity in the $\alpha_1$1.2 subunit, allow pharmacological isolation of the two receptor populations and have helped to address this problem. DHP applied to tissue isolated from these mice blocks only $Ca_v1.3$ currents, thereby isolating $Ca_v1.2$ L-type current (Sinnegger-Brauns et al., 2004). Studies using these mice have revealed that $Ca_v1.2$ channels exhibit long re-openings after recovery from strong depolarizations (Koschak et al., 2007). Chimera experiments indicate that gating differences in the $\alpha_1$1.2 domain I S3-S4 linker determine unique gating properties of $Ca_v1.2$ channels, while sequence differences in the domain II-III linker impart cardiac muscle specific excitation contraction coupling versus that seen in skeletal muscle (Adams et al., 1990; Tanabe et al., 1990; Nakai et al., 1994). $Ca_v1.2$ is also activated by depolarizing potentials beyond -30mV, helps to shape cardiac action

potentials, and couples membrane depolarization in neurons to gene expression (Reuter et al., 1988; Tanabe et al., 1990; Dolmetsch et al., 2001; Weick et al., 2003). Other studies showing that these channels contribute to Ca^{2+} influx in response to brief action potential stimuli support the idea that these are fast activating channels (Liu et al., 2003). Finally, studies using mice in which $Ca_v1.2$ expression in the hippocampal formation specifically is suppressed have helped to show the importance of this channel in NMDA receptor independent synaptic plasticity and spatial memory (Moosmang et al., 2005b; Moosmang et al., 2005a).

C. $Ca_v1.3$ physiology

Unique $Ca_v1.3$ properties were not identified in early studies. All L-type current channels were originally described as slow, high voltage-activating channels with high sensitivity to DHPs (Bourinet et al., 1994; Ertel et al., 2000; Altier et al., 2001). Many early studies were performed with high charge carrier concentrations, thereby shifting voltage-dependent activation and exhibiting a more depolarized activation level of $Ca_v1.3$ (Frankenhaeuser and Hodgkin, 1957; Williams et al., 1992; Ihara et al., 1995; Bell et al., 2001). Cloning of the $\alpha_1 1.3$ subunit and generation of $Ca_v1.3$ knock-out ($Ca_v1.3$-/-) mice supported the effort to classify the unique properties of $Ca_v1.3$ (Platzer et al., 2000; Zhang et al., 2002; Mangoni et al., 2003). These channels have faster activation kinetics, are activated at slightly more hyperpolarized potentials and are less sensitive to dihydropyridines than $Ca_v1.2$ (Seino et al., 1992; Ihara et al., 1995; Avery et al.,

1996; Kollmar et al., 1997a; Kollmar et al., 1997b; Morgans et al., 1998; Taylor and Morgans, 1998; Morgans, 1999; Platzer et al., 2000; Koschak et al., 2001; Safa et al., 2001; Scholze et al., 2001; Xu and Lipscombe, 2001; Habermann et al., 2003; Russo et al., 2003; Liu et al., 2004).

D. $Ca_v1.3$ expression

While it has been difficult to identify the precise physiological roles of $Ca_v1.3$ channels, and a poor selection of $Ca_v1.3$-specific antibodies makes it difficult to determine their subcellular localization, recent advancements have revealed their importance in a variety of cell types. $Ca_v1.3$ comprises about 20% of the Ca_v1 channels in the brain and is also expressed in pancreatic β cells, neuroendocrine cells, photoreceptors, amacrine cells, cochlear inner hair cells and atrial cardiac muscle where it contributes to pacemaking (Hell et al., 1993b; Clark et al., 2003). $Ca_v1.3$ is also critical to the function of the striatum, it is required for LTD (Adermark and Lovinger, 2007) and mechanisms of fear conditioning (McKinney and Murphy, 2006). Pharmacologically blocking L-type Ca^{2+} channels improves age-related working memory deficits and reduces $Ca_v1.3$ expression in the hippocampus (Veng et al., 2003). $Ca_v1.3$ is well-suited to mediate subthreshold Ca^{2+} signaling since it activates at -55mV, 25mV more hyperpolarized than $Ca_v1.2$ (Koschak et al., 2001; Safa et al., 2001; Scholze et al., 2001; Xu and Lipscombe, 2001). For example, $Ca_v1.3$ L-type Ca^{2+} current contributes to low-threshold Ca^{2+} current in the cochlea, sinoatrial node, (Platzer et al., 2000; Zhang et al., 2002; Mangoni et al., 2003) and endogenous

pacemaking in substantia nigra *pars compacta* dopaminergic neurons (Chan et al., 2007). This hyperpolarized voltage-dependent activation allows $Ca_v1.3$ to underlie oscillatory activity in a variety of excitable cells as well. DHP block of $Ca_v1.3$ inhibits endogenous Ca^{2+} oscillations in the suprachiasmatic nucleus, corticostriatal neurons and in early postnatal Purkinje neurons (Liljelund et al., 2000; Platzer et al., 2000; Pennartz et al., 2002; Vergara et al., 2003). Ca^{2+} mediated oscillations may also lead to excessive Ca^{2+} influx and pathologic states, such as has been suggested to occur in the medium spiny neurons of the striatum in response to Parkinson's Disease (Day et al., 2006). Development of specific blockers for $Ca_v1.2$ over $Ca_v1.3$ will improve the study of endogenous channels and the specific properties and roles of these two channels in the CNS.

IV. Regulation of L-type VGCCs

A. Calmodulin

L-type Ca^{2+} current is subject to dynamic regulation by protein-protein interactions at the large cytoplasmic C-terminal region. Calmodulin (CaM), a ubiquitous Ca^{2+}-binding protein, for example, aids in positive and negative feedback regulation of Ca_v1 channels. Ca^{2+} ions bind to the N- or C-terminal lobe of CaM to trigger a conformational change in its structure (Peterson et al., 1999; Fallon et al., 2005). Ca^{2+}-bound CaM binds to a region about 200 residues C-terminal to the final transmembrane segment, the isoleucine glutamine (IQ) domain, to either enhance the strength of the Ca^{2+} signal or prevent Ca^{2+} overload (Zuhlke and Reuter, 1998; Peterson et al., 1999; Qin et al., 1999;

Zuhlke et al., 1999; Budde et al., 2002). These phenomena, referred to as Ca^{2+}-dependent facilitation and Ca^{2+}-dependent inactivation, are both regulated by CaM interactions with VGCCs but it remains unclear how a single protein binding to a single site within the Ca_v1 channel can result in two opposing functional effects. Facilitation is evident as an increase in Ca^{2+} currents during repetitive trains of depolarizations, and inactivation is a pronounced decay in Ca^{2+} current compared to baseline during sustained depolarizing stimuli. Mutations in the IQ domain of Ca_v1 channels or CaM mutations that prevent binding to the Ca_v1 C-terminus, suppress inactivation (Peterson et al., 1999; Qin et al., 1999; Zuhlke et al., 1999; Zuhlke et al., 2000). Both Ca2+-dependent inactivation and facilitation also require an intact AID domain within the domain I S6 linker, so interactions at this site likely transmit conformational changes into the pore to change gating (Findeisen and Minor, 2009).

B. Ca^{2+}-binding proteins

Another set of proteins that interact with and modulate Ca_v1 channels are the largely brain-specific Ca^{2+} binding proteins (CaBP1-8). CaBP1 for example, is similar in structure to CaM and suppresses inactivation by displacing CaM from the IQ domain (Zhou et al., 2004; Zhou et al., 2005; Yang et al., 2006). A CaBP1 splice variant (caldendrin), also binds with the IQ domain to suppress CDI (Tippens and Lee, 2007). There is also evidence that CaBP1 suppresses inactivation via interactions at the N-terminus, however the precise mechanisms that underlie this effect are not completely understood. CaBP4, an additional

Ca^{2+} binding protein, binds to the C-terminus of $\alpha_1$1.4 to enhance voltage-dependence of activation and is required for synaptic functioning in retinal photoreceptors (Haeseleer et al., 2004). CaBP4 suppresses inactivation of Ca_v1.3 channels in both photoreceptors and inner hair cells, possibly to amplify presynaptic Ca^{2+} influx to maintain neurotransmitter release (Yang et al., 2006; Lee et al., 2007a). The detailed mechanisms by which CaBPs modulate Ca^{2+} channels remain to be fully explained; however, these interactions promise to be relevant for mechanisms that underlie Ca^{2+}-dependent on VGCC properties.

C. Protein Kinases

Protein kinases can couple activation of G-protein coupled receptors, Ca^{2+} influx and intracellular Ca^{2+} release to Ca_v1 channels to change their activity. The modulation of Ca^{2+} channel activity by protein kinases contributes to the dynamic regulation of cell physiology. For example, activation of α1-adrenergic receptors in ventricular myocytes and subsequent activation of PKC increases L-type current and contractile force in these cells (Woo and Lee, 1999). In addition, insulin-growth factor 1 (IGF-1) enhances L-type Ca^{2+} current in cardiac myocytes by causing a negative shift in voltage-dependent activation (Sun et al., 2006).

Regulation of Ca_v1.2 by phosphorylation has been studied extensively due to the fact that this interaction underlies the cardiac fight-or-flight response. G_s coupled β adrenergic receptors induce phosphorylation of the Ca_v1.2 channel α subunit C-terminus by PKA to potentiate receptor activity (Gao et al., 1997).

Both protein phosphatase 2A and calcineurin, on the other hand, dephosphorylate this site to reverse the actions of PKA (Davare et al., 2000; Oliveria et al., 2003). A-kinase anchoring proteins (AKAPs) tether both PKA and calcineurin to $Ca_v1.2$ allowing PKA to directly bind to and enhance channel function (Davare et al., 2000). AKAPs not only help kinases target Ca_v channels, but also help to couple $Ca_v1.2$ channels to transcription factors, such as NFATc4, allowing Ca^{2+} signals via Ca_v1 channels to change gene expression (Oliveria et al., 2007).

PKA phosphorylation of the $\alpha_1$1.3 C-terminus increases expression of $Ca_v1.3$ channels in tsA201 cells and potentiates $Ca_v1.3$ channels in the atrium of the heart after activation of β-adrenergic and 5HT4 receptor (Ouadid et al., 1992; Qu et al., 2005). There are multiple consensus sites for PKA phosphorylation in the $\alpha_1$1.3 subunit as well as the β2a subunits so it can be difficult to define exactly where and how PKA is exerting its actions on the channel (Bunemann et al., 1999; Nattel and Li, 2000; Kurokawa et al., 2003). In addition, PKA-mediated channel regulation differs in native and heterologous cells, suggesting that critical factors are absent in recombinant systems, such as AKAPs, that are required to facilitate phosphorylation of Ca_v1 channels.

Finally, CaMKII can potentiate L-type Ca^{2+} current in ventricular myocytes after α1 adrenergic receptor stimulation (Jin et al., 2005a). Ultrastructural immunolabeling data showing active CaMKII in close proximity to L-type channels within T-tubules of myocytes suggest this facilitation may be caused by direct phosphorylation of the channel. A study performed in an heterologous

expression system showed that CaMKII interacts with $Ca_v1.2$ by immunoprecipitation, and that CaMKII facilitates $Ca_v1.2$ currents in a Ca^{2+} dependent manner (Lee et al., 2006). This phenomenon occurs in response to CaMKII phosphorylation of two serines flanking the EF hand domain in the $Ca_v1.2$ C-terminus. The EF hand is a helix-loop-helix domain consisting of two α helices linked by a short loop that binds Ca^{2+} (Cens et al., 2006). Deletion of this region of the $Ca_v1.2$ C-terminus exerts dramatic effects on both Ca^{2+}- and voltage-dependent facilitation suggesting that the EF hand acts as a signal transducer for Ca_v1channel facilitation (Hudmon et al., 2005; Erxleben et al., 2006; Lee et al., 2006). While the specific role of the EF hand and facilitation machinery in Ca_v1 channels remains ill-defined, it is clearly important for channel function and changes in the structure of the domain surrounding this motif are important for channel regulation.

D. PDZ Proteins

Post-synaptic density-95 (PSD-95), discs-large, zona occludens 1 (PDZ) domain-containing proteins, one of the most abundant scaffolding protein classes in biological systems, can also directly modulate Ca_v1 channel properties (Feng and Zhang, 2009). PDZ domains consist of 80-90 amino acids arranged in a globular structure with six β sheets and two α helices. Antiparallel β sheets interact to create a groove into which a peptide fragment at the C-terminus of a ligand can fit with relatively weak binding affinity (Zhang and Wang, 2003). This allows PDZ domains to bind and assemble macromolecular signaling complexes

(Craven and Bredt, 1998; Robison et al., 2005a). There are different classes of PDZ domains, Class I, for example, bind to the consensus sequence S/T-X-ϕ-COOH, where X is any amino acid and ϕ is a hydrophobic amino acid (Songyang et al., 1997). Both $Ca_v1.2$ and $Ca_v1.3$ contain this sequence at the distal C-terminal region of the $\alpha 1$ subunit, and interactions with class I PDZ domains have been demonstrated to change channel localization, coupling to gene transcription machinery and direct channel modulation. $Ca_v1.2$ binds both neuronal interleukin-16 (NIL-16) and channel interacting PDZ protein (CIPP), for example. NIL-16 is a cytosolic protein that is detected only in the immune system and in neurons of the cerebellum and the hippocampus. CIPP is expressed exclusively in brain and kidney, and the highest CIPP mRNA levels are in neurons of the cerebellum, inferior colliculus, vestibular nucleus, facial nucleus, and thalamus (Kurschner et al., 1998; Kurschner and Yuzaki, 1999).

Neuronal $Ca_v1.3$ channels are localized to the PSD and couple to dopamine D2 and muscarinic M1 receptors via interactions with the PDZ protein shank (Olson et al., 2005; Zhang et al., 2005). In addition, the distal $\alpha_1 1.3$ C-terminus binds a PDZ protein erbin that directly enhances voltage-dependent facilitation of $Ca_v1.3$ currents. Erbin is believed to relieve an autoinhibitory domain of the distal $\alpha_1 1.3$ C-terminus, since the short splice variant of the channel exhibits robust facilitation that can not be enhanced further by erbin (Calin-Jageman et al., 2007). Finally, interactions with PDZ domains allow $Ca_v1.2$ and $Ca_v1.3$ channels to couple to gene expression via second messenger mediated CREB phosphorylation (Weick et al., 2003). The PDZ protein densin is

known to scaffold macromolecular complexes, and binds to and is phophorylated by CaMKII (Strack et al., 2000a; Walikonis et al., 2001); therefore, I hypothesized that densin may target CaMKII to potentiate $Ca_v1.3$ voltage-gated Ca^{2+} channels.

Chapter 5

Densin and Ca^{2+}/Calmodulin-Dependent Protein Kinase II Potentiate $Ca_v1.3$ Ca^{2+} Channels

I. Abstract

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a key regulator of Ca^{2+}-signaling in neurons, known to bind and phosphorylate many ion channels, including Ca_v1 voltage-gated Ca^{2+} channels. The role of this kinase in direct feedback regulation of the $Ca_v1.3$ channel is unknown. Here I report that CaMKII facilitates $Ca_v1.3$ Ca^{2+} currents during high frequency voltage-stimuli when coexpressed with the PDZ domain-containing protein densin. Neither densin nor CaMKII independently enhance $Ca_v1.3$ currents, and densin must bind both CaMKII and the channel to facilitate $Ca_v1.3$ Ca^{2+} currents. $Ca_v1.3$, CaMKII and densin colocalize and bind one another in hippocampal tissue, consistent with the idea that this form of channel regulation may be operational in hippocampal physiology. I propose that densin functionally recruits CaMKII to $Ca_v1.3$ channels which leads to a frequency- and Ca^{2+}-dependent enhancement of $Ca_v1.3$ channel currents. These findings help to elucidate the conditions that underlie direct CaMKII-mediated modulation of $Ca_v1.3$ currents, and may yield insights into how $Ca_v1.3$ channels selectively contribute to normal and pathological Ca^{2+} signals in neurons.

II. Introduction

Neuronal Ca_v1 channels couple changes in membrane potential to changes in intracellular Ca^{2+} concentrations to influence cell excitability (Marrion and Tavalin, 1998), gene expression (Bading et al., 1993; Weick et al., 2003) and synaptic plasticity (Johnston et al., 1992). $Ca_v1.3$ channels are expressed throughout the central nervous system (CNS) (Hell et al., 1993b), notably, in the substantia nigra where they maintain pacemaking activity and underlie spine degeneration in Parkinson's Disease (Day et al., 2006; Chan et al., 2007; Puopolo et al., 2007). These channels are subject to dynamic regulation by kinases and other intracellular factors that likely evolved to meet the intense signaling demands of this channel in neurophysiological processes. Maintaining the proper balance of excitation of this channel is critical to prevent pathophysiological conditions in the CNS.

Ca_v1 channels are modulated by protein kinases acting at the pore-forming α_1 subunit C-terminus (Kanaseki et al., 1991; Silva et al., 1992; Soderling, 1996; Hudmon and Schulman, 2002). cAMP-dependent protein kinase (PKA) phosphorylates the C-terminal region of the pore forming $\alpha_1 1.2$ channel subunit, for example, to potentiate $Ca_v1.2$ currents (Gao et al., 1997). AKAPs tether PKA to the $\alpha_1 1.2$ C-terminus domain allowing it to directly bind to and increase channel activity (Davare et al., 2000). CaMKII similarly binds to and phosphorylates both the $\alpha_1 1.2$ subunit and the auxiliary $Ca_v\beta$ subunit (Grueter et al., 2006; Grueter et al., 2008) to facilitate $Ca_v1.2$ currents in a Ca^{2+}-dependent manner, a feedback process known as Ca^{2+}-dependent facilitation (Hudmon et

al., 2005). Similarly, the interaction between CaMKII and the $\alpha_1$1.2 subunit is also required for voltage-dependent facilitation of Ca_v1.2 Ca^{2+} currents in response to strong depolarization (Lee et al., 2006). Ca_v1.3 currents can be subject to similar forms of regulation. Insulin-like growth factor-1 (IGF-1) potentiates Ca_v1.3 currents via a signaling pathway that includes CaMKII (Gao et al., 2006). Evidence for the direct modulation of Ca_v1.3 by CaMKII has been incomplete, but the functional interactions between them may require tethering of CaMKII to the Ca_v1 channel complex.

PDZ domain-containing proteins are an important class of synaptic scaffolding proteins, several of which are known to bind both $\alpha_1$1.3 and $\alpha_1$1.2. The PDZ domain binds the C-terminus of their ligands to localize proteins to specific subcellular targets. For example, neuronal interleukin-16 (NIL-16), a PDZ protein, acts as a Ca_v1.2 channel scaffold in the cerebellum (Kurschner and Yuzaki, 1999), while shank, a synaptic molecular scaffold that also contains a PDZ domain, directs Ca_v1.3 channels to the post-synaptic membrane (Zhang et al., 2005; Zhang et al., 2006). An additional leucine rich repeat and PDZ domain (LAP) protein, erbin, has direct effects on channel function and enhances voltage-dependent facilitation of Ca_v1.3 (Calin-Jageman et al., 2007). Densin is another member of the LAP protein family, and acts as a scaffold for many postsynaptic proteins including shank (Quitsch et al., 2005), δ-catenin (Izawa et al., 2002) and MAGUIN (Ohtakara et al., 2002). Densin is largely brain specific, is localized to the postsynaptic density (PSD) and binds to and is phophorylated by CaMKII (Apperson et al., 1996; Strack et al., 2000b; Walikonis et al., 2001).

Here I show that densin and CaMKII coordinately facilitate $Ca_v1.3$ Ca^{2+}-channels during high frequency stimulation. $Ca_v1.3$ and CaMKII coimmunoprecipitate with densin from the hippocampus, and all three colocalize in cultured hippocampal neurons. Autophosphorylation of CaMKII is required to facilitate $Ca_v1.3$ channels and densin must bind both the $\alpha_1$1.3 subunit and CaMKII. These effects may result from recruitment of CaMKII to the channel by densin, similar to recruitment of PKA to $\alpha_1$1.2 by AKAPs. Until now, evidence of the processes that underlie direct CaMKII-dependent modulation of $Ca_v1.3$ has been lacking. This study fills this gap by assaying a novel form of Ca^{2+} channel regulation via the densin-CaMKII-$Ca_v1.3$ complex, in which frequency-dependent facilitation of Ca^{2+} signals may regulate neuronal excitability.

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III. Methods

A. Constructs and molecular biology.

The $\alpha_1$1.3 of the rat Ca_v1.3 α_1 subunit (GenBank accession number AF370009; provided by Dr. D. Lipscombe, Brown University, Providence, RI) and auxiliary channel subunits β1b (GenBank accession number NM017346) and $\alpha 2\delta$ (GenBank accession number M21948) were used in this study. FLAG- $\alpha_1$1.3 was generated by PCR amplification of a FLAG-tagged fragment (nucleotides 1–660 of $\alpha_1$1.3) and cloned into *Nhe*I and *Ale*I sites of rat $\alpha_1$1.3-pcDNA6/V5-His (Xu and Lipscombe, 2001); FLAG- $\alpha_1$1.2 was described previously (Zhou et al., 2004). Murine CaMKIIα, CaMKIIαT286A, rat densin and all truncation mutants were generous gifts from the laboratory of Dr. R.J Colbran (Vanderbilt University, Nashville, TN.) and generated as described previously (Brickey et al., 1990; McNeill and Colbran, 1995; Strack et al., 2000b; Jiao et al., 2008). Plasmid DNA was extracted and purified using Qiagen Maxi-Prep kits and verified by sequencing or restriction digest. For pull-down and overlay assays, glutathione *S*-transferase (GST)-tagged constructs containing the cytoplasmic C-terminal domain of rat $\alpha_1$1.3 [GST-$\alpha_1$1.3 CT], nucleotides 5886–6465, encoding residues 1962-2155, were subcloned into *Bam*HI/*Not*I sites of pGEX4T.1 (GE Healthcare, Piscataway, NJ). Desired fragments of densin cDNAs were amplified by PCR using oligonucleotide primers containing *BamH*I and *EcoR*I restriction enzyme sites as described (Robison et al., 2005b). PCR products were ligated into the pGEX-2T (Amersham Biosciences) vectors and transformed into BL21-DE3 *Escherichia coli* bacteria. After induction of protein expression, His6 fusion

proteins (densin-1247–1542) were purified using His-Select Nickel Affinity Gel (Sigma), according to the manufacturer's protocol. Purified protein concentrations were determined using Bradford (Bio-Rad) assays.

B. Antibodies.

Rabbit polyclonal $\alpha_1$1.3 antibodies, were generated against a GST fusion protein containing amino acids 1–41 (MQHQRQQQEDHANEANYARGTRLPISGEGPTSQPNSSKQTV) of rat $\alpha_1$1.3 (GenBank accession number AF370009). For goat polyclonal $\alpha_1$1.3 antibodies, two peptides corresponding to an N-terminal sequence (amino acids 24–37, PISGEGPTSQPNSS) and a sequence in the cytoplasmic loop linking domains II and III (amino acids 810–827, DNKVTIDDYQEEAEDKD) were used as dual immunogens for antisera generated by a commercial source (ProSci, Poway, CA). The generation, purification and testing for specificity of $\alpha_1$1.3 antibodies was described previously (Calin-Jageman et al., 2007). Anti-densin polyclonal antibodies were produced and characterized previously (Robison et al., 2005b). Other antibodies used were mouse monoclonal antibodies against CaMKII (Millipore, Billerica, MA), FLAG (Sigma-Aldrich, St. Louis, MO) and GFP (Santa Cruz Biotechnology, Santa Cruz, CA).

C. Cell culture, transfection, and lysate preparation.

A human embryonic kidney cell line was maintained in DMEM with 10% fetal bovine serum (Invitrogen, Gaithersburg, MD) at 37°C in a humidified

atmosphere with 5% CO_2. Cells were grown to 85% confluence and transfected using GenePorter reagent (Gene Therapy Systems, San Diego, CA). For pull-down assays, HEK cells grown on a 150 mm cell culture dish were transfected with 6μg GFP-densin. For immunoprecipitation, HEK cells were transfected with cDNAs encoding $Ca_v1.3$ (6μg of FLAG- $\alpha_1$1.3, 2μg of β1b, and 2μg of α2δ) with or without GFP-densin (4μg). For electrophysiological experiments, HEK293T cells were transfected with 1.5 μg of FLAG-$\alpha_1$1.3 or FLAG- $\alpha_1 1.3_{L\text{-}A}$, $\alpha_1 1.3_{S1486A}$, 0.5μg of β1b, and 0.5μg of α2δ. For some experiments, 0.5μg of GFP-densin, densinΔ483-1377-GFP, or GFP-densinΔPDZ and/or either 0.5μg CaMKII, $CaMKII_{T286A}$ were also cotransfected as indicated.

D. Pull-down binding assays.

GST-fusion proteins were prepared and purified on glutathione-agarose beads as described previously (Robison et al., 2005b; Calin-Jageman et al., 2007). GST-$\alpha_1$1.3CT immobilized on beads was incubated with either purified GFP-densin or GFP-densin transfected cell lysate with binding buffer [50mM Tris-buffered saline [(TBS; 50mM Tris-HCl, pH 7.5, and 150mM NaCl)/0.1% Triton X-100/protease inhibitors (1mg/ml each of PMSF, pepstatin, aprotinin, and leupeptin)]. Binding reactions were incubated at 4°C for 90 minutes. Beads were washed three times with binding buffer (1ml) at 4°C, and bound proteins were eluted, resolved by SDS-PAGE, and transferred to nitrocellulose. Western blotting was performed with appropriate antibodies followed by HRP-conjugated secondary antibodies and enhanced chemiluminescent detection reagents (GE

Healthcare). Ponceau staining was used to verify that equal levels of immobilized GST-$\alpha_1$1.3CT proteins were used in each experimental group. Interpretations of results from pull-down assays were based on at least three independent experiments.

E. Coimmunoprecipitation assays.

Transfected HEK cells were solubilized in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, and protease inhibitors), incubated at 4°C for 30 min and subjected to centrifugation at 100,000x*g* (30 min) to remove insoluble material. Lysates were incubated with FLAG antibodies or IgG (5 μg) and 50 ml of protein A-Sepharose (50% slurry) for 4 h, rotating at 4°C. After three washes with RIPA buffer (1 ml), proteins were eluted with SDS-containing sample buffer and subjected to SDS-PAGE. Coimmunoprecipitated proteins were detected by western blotting with specific antibodies as indicated.

For coimmunoprecipitation from mouse hippocampus, a Triton X-100-soluble fraction (0.5 ml) was prepared as described previously (Abiria and Colbran, 2010) and incubated with 10 μg of either goat IgG or affinity-purified goat antibodies that densin. After 1 h, 10μl of protein-G Sepharose (GE Healthcare Bio-Sciences) was added and the incubation continued for 2 h at 4°C. The resin was rinsed three times in 1 ml of solubilization buffer and bound proteins were analyzed by SDS-PAGE and Western blotting with mouse antibodies to CaMKII or rabbit antibodies to densin and $\alpha_1$1.3 (Ab144).

Immunocytochemistry. For triple-label immunofluorescence, primary cultures of neurons were a generous gift from Gary Bassell (Emory University, Atlanta, GA). Embryonic day 18 (E18) rat hippocampi were cultured as described (Banker and Goslin, 1998; Antar et al., 2004). Cells were plated (90,000–120,000 cells per square centimeter) on poly-L-lysine-coated coverslips (1.0 mg/ml) in minimal essential medium with FBS (10%) for 2 hr, inverted onto dishes containing astroglia, and grown in defined N2-conditioned medium (Banker and Goslin, 1998; Antar et al., 2004). Coverslips with hippocampal neurons (25 d in culture) were fixed in -20˚C methanol for 20 minutes then rinsed in PBS. Samples were incubated in 10% donkey serum (DS) with 0.1% Triton X-100 in TBS. After blocking, samples were simultaneously incubated with rabbit anti-$\alpha_1$1.3 antibodies (1:2000), goat anti-densin antibodies (1:500) and mouse anti-CaMKII antibodies (1:100) overnight at 4°C. After rinsing 3 times for 5 minutes in TBS, coverslips were incubated for 1 hour with Biotin-SP-conjugated affinitypure donkey anti-rabbit IgG, donkey FITC-anti goat IgG, and donkey Cy3-anti mouse IgG (1:300, Jackson ImmunoResearch, West Grove, PA). Finally, after an additional series of rinses, coverslips were incubated with Texas Red Avidin (1:1000, Jackson ImmunoResearch). All antibodies were diluted in TBS with 2.5% DS. The coverslips were mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA) for viewing on a Zeiss (Oberkochen, Germany) LSM510 Meta confocal microscope. Image processing was with Zeiss LSM Image Browser and Adobe Photoshop (Adobe Systems, San Jose, CA) software. Linescan analysis used to determine extent of colocalization as described

previously (Di Biase et al., 2009).

F. Electrophysiological recordings.

At least 48 h after transfection, whole cell patch-clamp recordings of transfected cells were acquired with a HEKA Elektronik (Lambrecht/Pfalz, Germany) EPC-9 patch-clamp amplifier. Data acquisition and leak subtraction using a P/4 protocol were performed with Pulse software (HEKA Elektronik). Extracellular recording solutions contained (in mM): 150 Tris, 1 $MgCl_2$, and 10 $BaCl_2$ or 10 $CaCl_2$. Intracellular solutions contained (in mM): 140 *N*-methyl-D-glucamine, 10 HEPES, 2 $MgCl_2$, 2 Mg-ATP, 5 EGTA with or without either 10mM KN-93 or KN-92, pH of 7.3 adjusted with methanesulfonic acid. Electrode resistances were 4 MΩ in the bath solution compensated up to 80%. Igor Pro software (Wavemetrics, Lake Oswego, OR) was used for data analysis. All averaged data are presented as the mean +/- SEM. Statistical significance was determined with either Student's *t*- test or one-way ANOVA as indicated (SigmaPlot; Systat Software, San Jose, CA).

IV. Results

A. CaMKII does not modulate $Ca_v1.3$ properties

The cytoplasmic C-terminal domain of the pore-forming α_1 subunit of Ca_v1 channels interacts with a variety of regulatory proteins that change channel function (Ertel et al., 2000). Due to the importance of CaMKII as a regulator and transducer of Ca_v1 Ca^{2+} signals (Dzhura et al., 2000; Wheeler et al., 2008), I tested whether CaMKII might also directly influence $Ca_v1.3$ channel function. Patch-clamp recordings from HEK cells cotransfected with $Ca_v1.3$ subunits (FLAG-$\alpha_1$1.3, β1b, and $\alpha2\delta$) were compared to those obtained from cells cotransfected with the same channel subunits and CaMKII. This system allows for analysis of recombinant channels in the relative absence of the many competing influences in neurons. Similar experiments performed in neurons are complicated by the difficulty in isolating both $Ca_v1.3$ currents and specific protein interactions with these channels. GFP was cotransfected in order to identify the cells that had undergone transfection with the indicated DNA.

Ba^{2+} was used as the charge carrier in order to isolate voltage-dependent effects on $Ca_v1.3$ channels by densin and CaMKII, without the competing effects of Ca^{2+}-dependent processes. However, CaMKII did not change $Ca_v1.3$ voltage-dependence of activation. Parameters describing current-voltage (IV) curves were not different in cells transfected with $Ca_v1.3$ (Figure 5.1*A*; $V_{1/2}$=-24.88 ± 3.94 mV) and those cotransfected with CaMKII ($V_{1/2}$ =-29.95 ± 1.64 mV; p=0.31, Student's *t* test). To look for potentiation of $Ca_v1.3$ currents by CaMKII, I

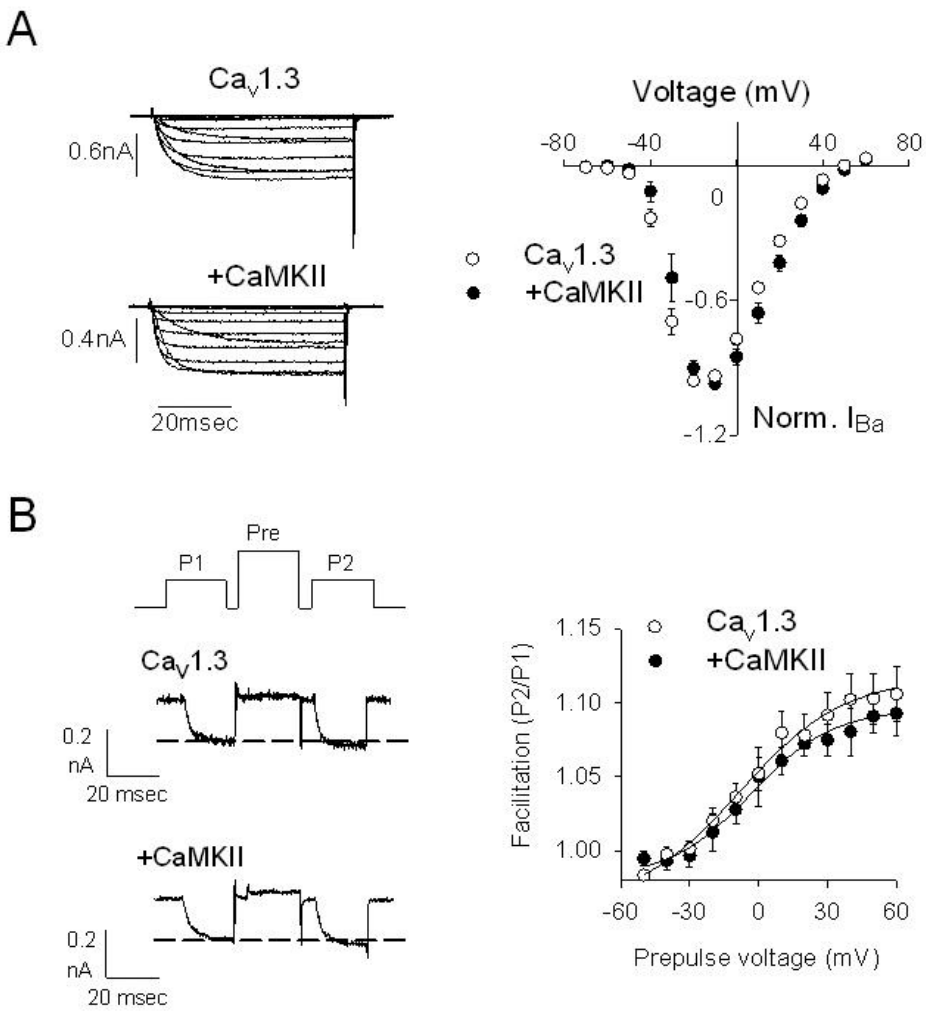

Figure 5.1. CaMKII does not modulate $Ca_v1.3$ channel properties. ***A***, Representative Ba^{2+} current traces and current-voltage curves for HEK cells transfected with $Ca_v1.3$ alone (*n*=8, open circles) or cotransfected with CaMKII (*n*=10, closed circles). Test pulses were applied from a holding voltage of -90 mV to various voltages between -70 and -60 mV. Peak current amplitudes were measured, normalized to the largest current in the protocol and plotted against test voltage. Error bars represent SEM; where error bars are not visible, they are smaller than the size of the symbol. ***B,*** Representative Ba^{2+} currents, evoked before (P1) and after (P2) a conditioning 20 ms prepulse voltage step from -90 to 20 mV. The ratio of the P2 to P1 test currents was calculated and plotted against voltage for cells transfected with $Ca_v1.3$ alone (n=7, open circles) or cotransfected with CaMKII (n=8, closed circles). Error represents SEM.

compared peak I_{Ba} amplitudes between the same two transfection conditions. Mean $Ca_v1.3$ peak current amplitudes were not changed by cotransfection of CaMKII ($Ca_v1.3$ alone 535 ± 78 pA, n=18; +CaMKII 844 ± 227 pA, n=16; p=0.18 by Student's *t* test).

$Ca_v1.3$ currents are subject to prominent voltage-dependent facilitation that can be observed in recombinant systems independent of G-proteins, kinases or other supporting proteins (Safa et al., 2001). Since CaMKII is known to enhance facilitation $Ca_v1.2$ channels (Lee et al., 2006), I then chose to investigate if CaMKII might exert the same actions on $Ca_v1.3$. To do this, a paired-pulse protocol in which the amplitude of two 10 ms test currents (P1 and P2) evoked by the same steps were compared before and after a 20msec depolarizing prepulse to various voltages (Figure 5.1*B*). This short conditioning prepulse, from -90 mV to +60 mV, induces a slight increase in $Ca_v1.3$ I_{Ba} amplitude during the second test pulse, P2 compared to the first, P1 (Figure 5.1*B*). Net facilitation, or the ratio of P2 to P1, was not significantly changed in cells cotransfected with $Ca_v1.3$ subunits and CaMKII when compared to those cotransfected with $Ca_v1.3$ alone. This was shown by plotting the ratio of P2 to P1 current amplitude (P2/P1) against prepulse voltage; the P2/P1 ratio was unchanged by CaMKII at all voltages (Figure 5.1*B*).

Since CaMKII did not modulate these $Ca_v1.3$ properties, I then chose to examine if CaMKII changed the response of $Ca_v1.3$ to sustained or repetitive voltage stimuli. I_{Ba} responses to a sustained depolarizing voltage step from cells

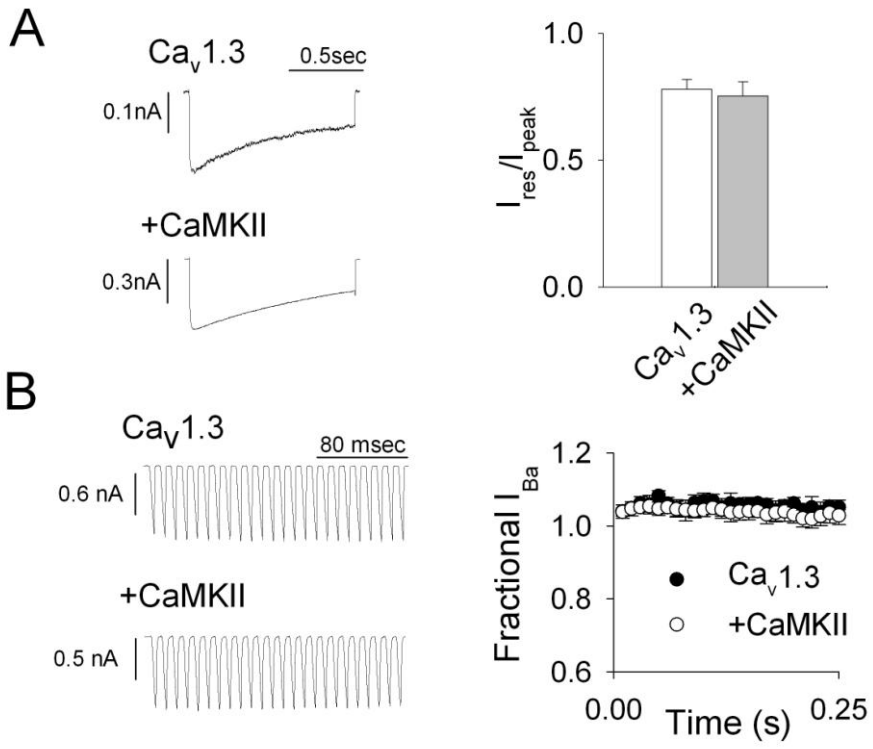

Figure 5.2. CaMKII does not change $Ca_v1.3$ current responses to sustained or repetitive stimuli. ***A,*** Current response to a 1sec voltage step from -90mV to -10mV was applied to cells transfected with either $Ca_v1.3$ (n=7) or +CaMKII (n=5). The ratio of the peak I_{Ba} amplitude to the residual current amplitude, $I_{res}/_{peak}$ is indicated in the bar graph (error bars represent SEM). ***B,*** A voltage step from -90mV to -10mV was applied at 100Hz to cells cotransfected with $Ca_v1.3$ alone (n=11, open circles) or with CaMKII (n=7, closed circles). Test current amplitudes normalized to the first current response (fractional current) were plotted against time for each transfection condition (error bars represent SEM).

transfected with $Ca_v1.3$ alone were compared to those cotransfected along with CaMKII. The amplitude of the current at the end of the test pulse normalized to the peak current, or $I_{res}/I_{peak,}$ was compared between cells transfected with $Ca_v1.3$ alone to $Ca_v1.3$ and CaMKII (Figure 5.2*A*). I_{res}/I_{peak} was not significantly changed by coexpression of CaMKII with $Ca_v1.3$ (I_{res}/I_{peak}=0.75) when compared to recordings obtained from cells transfected with $Ca_v1.3$ subunits alone (I_{res}/I_{peak}=0.79; p=0.72, Student's *t* test). To evaluate if CaMKII had an effect on $Ca_v1.3$ currents in response to a more physiological stimulus, $Ca_v1.3$ I_{Ba} was measured during trains of repetitive depolarizations in (Figure 5.2*B*). I compared the average fractional current from the last 10 current responses in the pulse train normalized to the first response from cells transfected with $Ca_v1.3$ alone to those transfected with CaMKII. CaMKII did not change $Ca_v1.3$ currents (p=0.83, by Student's *t* test) in response to a 100Hz repetitive voltage-stimulus.

B. $Ca_v1.3$, densin and CaMKII form a protein complex

Previous work has shown that CaMKII potentiates $Ca_v1.3$ currents following stimulation with insulin growth-factor (IGF-1) in SH-SY5Y neuroblastoma cells and in cortical neurons (Gao et al., 2006), but there did not appear to be any direct effects of CaMKII on $Ca_v1.3$ current in HEK cells. Feedback modulation of $Ca_v1.3$ by CaMKII may then require additional adaptor proteins present in neurons but not HEK cells, analogous to the role of AKAPs in PKA regulation of Ca_v1 channels. There is already

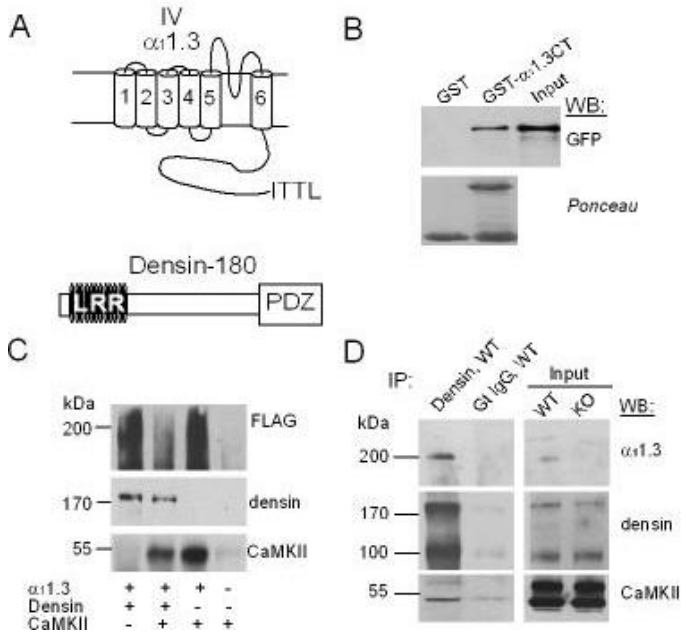

Figure 5.3. $Ca_v1.3$ binds both densin and CaMKII. ***A***, Schematic of rat $\alpha_1$1.3 showing the class I PDZ-binding consensus sequence (TTL) at the distal C-terminal domain. Amino acids 1962-2155 at the distal C-terminus were included in the GST-$\alpha_1$1.3CT construct used in pull-down assays. Schematic of rat densin indicating the LRR and PDZ domains. ***B,*** GST control (lane 1) or GST- $\alpha_1$1.3CT (lane 2) was immobilized on glutathione-agarose beads and incubated with lysates from cells transfected with GFP-densin. Bound GFP-densin was detected by western blot (WB) with anti-GFP antibodies. Lane 3 shows GFP-densin input used in the pull-down assay. Densin binds to the distal C-terminus of $\alpha_1$1.3 but not GST. Pull-down assays were repeated three times with consistent results. ***C***, HEK cells were cotransfected with different combinations of all three proteins, $Ca_v1.3$ ($\alpha_1$1.3, β1b, $\alpha2\delta$), +/- densin, +/- CaMKII as indicated, and subjected to lysis and immunoprecipitation using rabbit antibodies against FLAG (all lanes). Immunoprecipitated proteins were detected by western blotting with antibodies recognizing FLAG, densin or CaMKII. CaMKII does not have a non-specific interaction with the FLAG antibody (lane 4). Coimmunoprecipitation assays were repeated three times with consistent results. ***D***, Mouse brain hippocampal lysates were incubated with goat densin antibodies or

control goat IgG for immunoprecipitation. $\alpha_1$1.3, CaMKII and densin were detected by western blotting with rabbit $\alpha_1$1.3, rabbit CaMKII and goat densin antibodies, respectively. A 200 kDa protein corresponding to $\alpha_1$1.3, a 180kDa and a 100kDa band corresponding to densin and a 55kDa band corresponding to CaMKII selectively corimmunoprecipitated with the densin antibody (lane 1) but not the IgG control (lane 2). This experiment was repeated using Ca_v1.3+/+ (WT) or Ca_v1.3-/- (KO) mice. A 200kDa band is seen in the WT tissue but not the KO, suggesting this band does, in fact, represent $\alpha_1$1.3. Brain coimmunoprecipitation experiments were repeated five times with consistent results.

precedent for the participation of CaMKII in postsynaptic signaling complexes with the LAP protein densin (Strack et al., 2000a; Strack et al., 2000b; Walikonis et al., 2001; Robison et al., 2005a; Robison et al., 2005b), which also has a type I PDZ binding domain that could associate with the $\alpha_1$1.3 CT. We, therefore, considered densin as a potential CaMKII-Ca_v1.3 targeting protein.

The most distal C-terminal residues of $\alpha_1$1.3 (-TTL) follow the consensus sequence for binding class I PDZ domains, S/T-X-ϕ-COOH, where X is any amino acid and ϕ is an hydrophobic amino acid (Songyang et al., 1997). Therefore, the PDZ domain of densin should bind to this region of $\alpha_1$1.3 (Figure 5.3*A*). I confirmed this interaction between densin and $\alpha_1$1.3 using a pulldown assay with immobilized GST- $\alpha_1$1.3CT, which contains a portion of the distal C-terminus. GST- $\alpha_1$1.3CT, but not the GST control, precipitated GFP-densin from transfected cell lysates (Figure 5.3*B*). To confirm that densin interacts not only with the isolated C-terminal fragment of $\alpha_1$1.3 but also with the intact channel in mammalian cells, and to investigate if CaMKII can simultaneously interact with this complex, coimmunoprecipitation experiments were performed using lysates of HEK cells cotransfected with Ca_v1.3 subunits (FLAG-tagged $\alpha_1$1.3, β1b and α2δ), and GFP-tagged densin and/or CaMKII (Figure 5.3*C*). Antibodies for FLAG pulled down the FLAG-$\alpha_1$1.3 subunit with GFP-densin (lane 1), CaMKII (lane 3), and both GFP-densin and CaMKII simultaneously (lane 2). This shows that densin and CaMKII can independently and simultaneously bind Ca_v1.3.

To investigate if densin, CaMKII and Ca_v1.3 not only form a complex in a recombinant expression system but in native cells, I performed

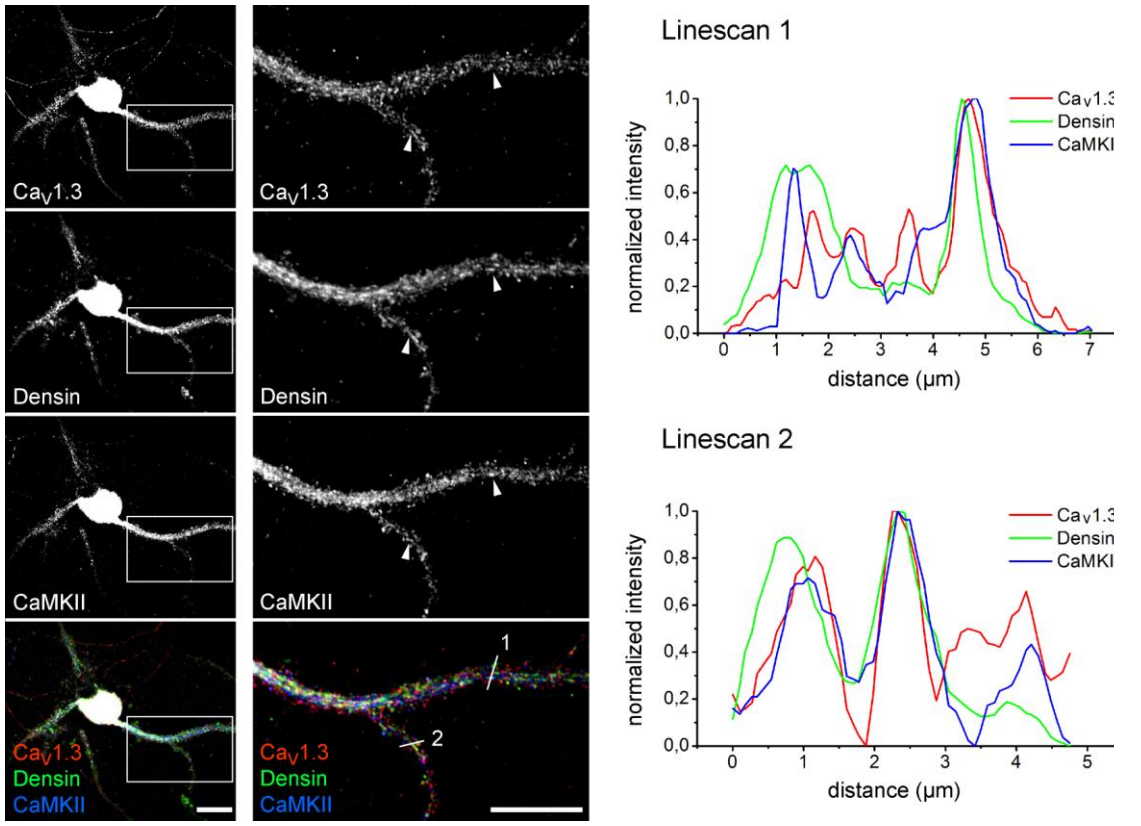

Figure 5.4. Ca$_v$1.3 and densin colocalize in somatodendritic domains of hippocampal neurons in culture. Confocal images of low-density primary hippocampal neuron cultures, 25 days *in vitro* triple labeled with antibodies against $\alpha_1$1.3, to indicate Ca$_v$1.3 expression, densin and CaMKII (leftward column shows 40X magnification, scale bar 20 µm; column to the right shows 63X magnification, scale bar 5 µm). Immunofluorescence was viewed under optics for rhodamine for Ca$_v$1.3, fluorescein for densin, Cy3 for CaMKII, and merged images are below. Normalized staining intensity for the three fluorophores was plotted against the distance across the dendrites indicated in the merged image. Areas of colocalization of densin, CaMKII and $\alpha_1$1.3 are indicated by overlapping peaks of relative intensity. Immunocytochemistry experiments were repeated five times with consistent results.

coimmunoprecipitation experiments using mouse brain. Densin was immunoprecipitated from solubilized mouse hippocampal post-synaptic density membrane fractions with densin antibodies (Figure 5.3*D*). Densin and associated $\alpha_1$1.3 and CaMKII were detected by western blot with densin antibodies, $\alpha_1$1.3 and CaMKII antibodies, respectively. Densin antibodies precipitated densin and coimmunoprecipitated $\alpha_1$1.3 and CaMKII (lane 1). There are multiple densin splice variants (Jiao et al., 2008), two of which were present here, one at 100kDa and, the main, full-length isoform, at 180 kDa. The coimmunoprecipitation was specific in that a similar result was not obtained with an equivalent concentration of control goat IgG (lane 2). I repeated this experiment with Ca_v1.3+/+ (WT) and Ca_v1.3-/- (KO) tissue. Again, densin coimmunoprecipitated with CaMKII and $\alpha_1$1.3 in WT tissue, and with CaMKII in KO tissue. There was no band for $\alpha_1$1.3 in the KO, suggesting that the band at 200 kDa does, in fact, represent the Ca_v1.3 channel. These results confirm the potential for densin/CaMKII/Ca_v1.3 interactions in the brain.

I then used confocal microscopy and triple-label immunofluorescence of hippocampal neurons in culture to assess the potential of protein colocalization. I observed densin, CaMKII and $\alpha_1$1.3 punctate immunofluorescence in hippocampal dendrites (Figure 5.4). Colocalization of densin, CaMKII and $\alpha_1$1.3 in the soma and dendritic regions was present in virtually all neurons examined. Many puncta showed only $\alpha_1$1.3, densin or CaMKII staining, as expected, in light of the fact that CaMKII plays many roles in the CNS and would be expected to interact with other proteins than only Ca_v1.3 and densin. Extent of colocalization

was analyzed using linescan analysis, in which relative staining intensity is compared between the three fluorophores across the specified area of a dendrite. Areas of strong colocalization are evident as overlapping peaks in this plot. Two representative linescans are shown. This analysis shows areas of triple colocalization in areas of the dendrite, and the staining pattern suggests this is most likely occurring in dendritic spines.

C. Densin and CaMKII enhance $Ca_v1.3$ channel function

Once I confirmed the interaction between $Ca_v1.3$, densin and CaMKII and the potential for this interaction to occur in neurons, I investigated the functional effects of this interaction. Patch-clamp recordings from HEK cells cotransfected with $Ca_v1.3$ subunits (FLAG-$\alpha_1$1.3, β1b, and $\alpha2\delta$) were compared to those obtained from cells cotransfected with the same channel subunits, CaMKII and GFP-tagged densin. GFP-tagged densin was used to identify the cells that were transfected with the indicated DNA. CaMKII and densin significantly increased the amplitude of $Ca_v1.3$ Ca^{2+} currents evoked in response to a 100Hz repetitive depolarizing stimulus (Figure 5.5*A*). With this voltage-protocol, the peak of each response is decreased compared to the previous peak (~40%, within 50msec), due to Ca^{2+}-dependent inactivation mediated by calmodulin. Amplitudes at the end of the train were ~45% greater in cells transfected with $Ca_v1.3$, CaMKII and densin than in cells with $Ca_v1.3$ alone (Figure 5.5*A*). To determine the extent of the Ca^{2+}-dependence of this effect, experiments were repeated using Ba^{2+} as the charge carrier in the extracellular solution. In contrast to the effects on I_{Ca},

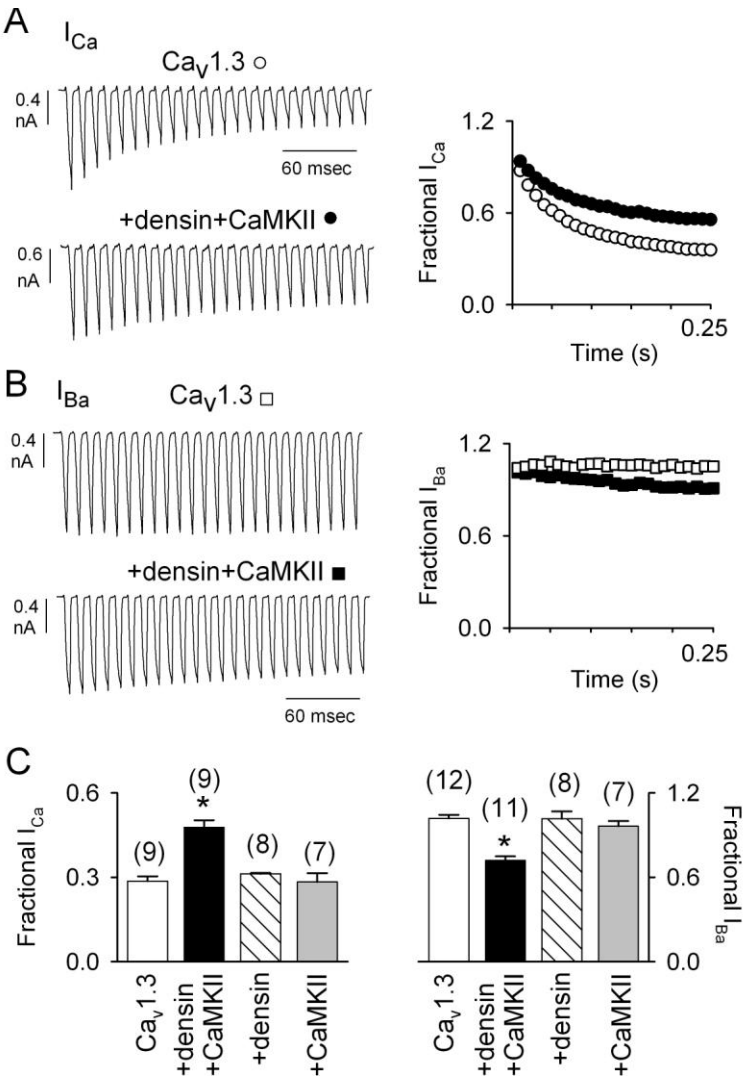

Figure 5.5. Densin and CaMKII potentiate $Ca_v1.3$ I_{Ca} in response to a repetitive voltage stimulus. I_{Ca} **(*A*)** and I_{Ba} **(*B*)** were evoked by 5msec pulses from -90 to -10 mV at 100Hz in cells transfected with $Ca_v1.3$ alone ($\alpha_1$1.3, β1b, $\alpha2\delta$) or cotransfected with densin and CaMKII. Test current amplitudes were normalized to the first in the train (Fractional I_{Ca}, Fractional I_{Ba}) and plotted against time. ***C,*** Summary of data represented in A and B. Fractional current for the final 11 pulses of the train were averaged. Number of cells is indicated in parentheses. * $p<0.05$, by one-way ANOVA and Bonferroni's *post hoc* test.

CaMKII and densin modestly increased inactivation of I_{Ba} (11.8%; Figure 5.5*B*). This effect was not seen when CaMKII or densin were singly cotransfected with Cav1.3. These results reveal that $Ca_v1.3$ currents are potentiated by CaMKII and densin during repetitive stimuli in a Ca^{2+}-dependent manner.

Parameters measuring voltage-dependence of activation in cells transfected with $Ca_v1.3$ alone (k=-12.0 ± 1.0, $V_{1/2}$ = -7.1 ± 0.3, p=0.68; n=9) were not significantly different from $Ca_v1.3$ plus densin and CaMKII (*k*=-12.5 ± 2.2; $V_{1/2}$ = -7.2 ± 0.5, p=0.34; n=10 by Student's *t* test). Current amplitudes were similarly unaffected by CaMKII and densin ($Ca_v1.3$ I_{Ca} amplitude at -10mV = 1.3 ± 0.3 nA; $Ca_v1.3$+Densin+CaMKII = 0.70 ± 0.2 nA; p=0.21 by Student's *t* test). To verify that this effect was dependent on both densin and CaMKII, and not just densin alone, cells cotransfected with $Ca_v1.3$ and densin were also examined. Like CaMKII, densin alone did not change any of the physiological properties of $Ca_v1.3$.

D. Effects of CaMKII and densin on $Ca_v1.3$ is dependent on Ca^{2+} influx frequency

High-frequency Ca^{2+} spikes limit Ca^{2+}/CaM dissociation from CaMKII thereby supporting autonomous enzymatic activity even after dissipation of Ca^{2+} (Meyer et al., 1992; Hanson et al., 1994; Soderling, 1996; De Koninck and Schulman, 1998). Because of the frequency detection capabilities of CaMKII, I wanted to investigate if the observed effects on $Ca_v1.3$ were dependent on the repetitive nature of the stimulus. If frequency-dependent modulation of CaMKII

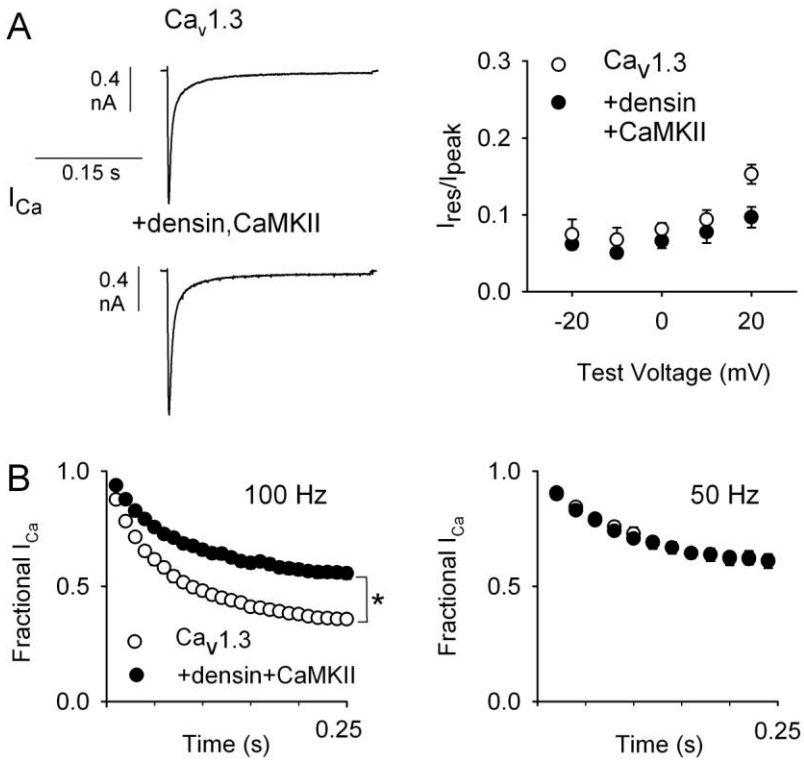

Figure 5.6. Densin and CaMKII only enhance $Ca_v1.3$ currents in response to high frequency voltage stimulation. ***A,*** Representative current responses to a 300msec voltage step from -90mV to voltages between -30mV and +30mV was applied to cells transfected with either $Ca_v1.3$ or +densin+CaMKII. The ratio of the peak current amplitude to the residual current amplitude, I_{res}/I_{peak} is plotted against test voltage (open circles, $Ca_v1.3$ n=7, closed circles+densin+CaMKII n=8). Error bars represent SEM; where error bars cannot be seen, they are smaller than the size of the symbol. ***B,*** Fractional I_{Ca} obtained from 100Hz and 50 Hz voltage stimulations (5msec pulses from -90 to -10mV) for $Ca_v1.3$ alone (open circles, n=9) versus plus densin and CaMKII (closed circles, n=9).

contributes to CaMKII-dependent effects on $Ca_v1.3$, these effects should be reduced during sustained or low-frequency depolarizations. Alternatively, if these results depend on only a single burst of Ca^{2+} through $Ca_v1.3$ channels, CaMKII and densin should still facilitate I_{Ca} evoked by sustained or low-frequency depolarizations. To examine these potential mechanisms, current responses to sustained depolarizing steps from -90mV to a series of voltages in cells cotransfected with either $Ca_v1.3$ alone or with densin and CaMKII were analyzed (Figure 5.6*A*). Similar to Figure 5.2A, the residual current (I_{res}) at the end of the pulse was plotted as a ratio of the peak current (I_{peak}) against the test voltage. I_{res}/I_{peak} for Ca^{2+} currents through $Ca_v1.3$ channels shows a strong Ca^{2+}-dependent inactivation, evident as a U-shaped dependence on membrane potential (Brehm and Eckert, 1978). I_{res}/I_{peak} was not significantly changed by densin and CaMKII at holding potentials between -20mV and +20mV, including -10mV (the holding potential at which repetitive stimuli experiments were performed), suggesting that that CaMKII and densin can not enhance $Ca_v1.3$ functioning during sustained depolarizations.

Because the effect of CaMKII and densin on $Ca_v1.3$ is dependent on repetitive Ca^{2+} influx, I then examined if the specific rate at which Ca^{2+} enters the cell is important for this effect. I_{Ca} from cells transfected with $Ca_v1.3$ alone were compared to those transfected with $Ca_v1.3$, densin and CaMKII during either a 100Hz or 50Hz depolarizing voltage stimulus (Figure 5.6*B*). The fractional current was plotted against time, and the average fractional I_{Ca} was compared between cells transfected with $Ca_v1.3$ alone or cotransfected with densin and

CaMKII at each of the different stimulation frequencies. I_{Ca} was potentiated by densin and CaMKII during the 100Hz voltage pulse ($p<0.001$ by Student's *t* test), but not during the 50Hz voltage stimulus ($p=0.62$, by Student's *t* test). These results indicate that the effects of CaMKII and densin on $Ca_v1.3$ are Ca^{2+}- and frequency-dependent.

Since densin and CaMKII only increase $Ca_v1.3$ currents during high frequency repetitive stimulation, I hypothesized that accumulation of intracellular Ca^{2+} is important for the CaMKII-dependent effects on $Ca_v1.3$ channels. If Ca^{2+} influx through and accumulation around neighboring channels allows for more widespread, macroscopic activation of CaMKII, then the enhanced function of $Ca_v1.3$ by CaMKII should be increased with larger amplitude whole-cell currents. To investigate this, recordings that yielded smaller I_{Ca} (<250pA; Figure 5.7*A*) were analyzed independently from those that resulted in larger currents (>250pA; Figure 5.7*B*). Densin and CaMKII do not enhance small $Ca_v1.3$ Ca^{2+} currents to the same extent as they do with larger I_{Ca} ($p=0.91$). When there is minimal intracellular Ca^{2+} accumulation during smaller currents, densin and CaMKII can not fully exert their effects on $Ca_v1.3$ channel function.

These data suggest that more global rises in intracellular Ca^{2+} mediate CaMKII-dependent effects on $Ca_v1.3$ channels. Ca^{2+}-dependent channel regulation can be mediated by a "global" Ca^{2+} increase that arises from the immediate environment at the channel or from spatially distant sources. These more global Ca^{2+}-mediated changes are permitted under modest intracellular Ca^{2+} buffering (0.5 mM EGTA) resulting from Ca^{2+} contributions from several

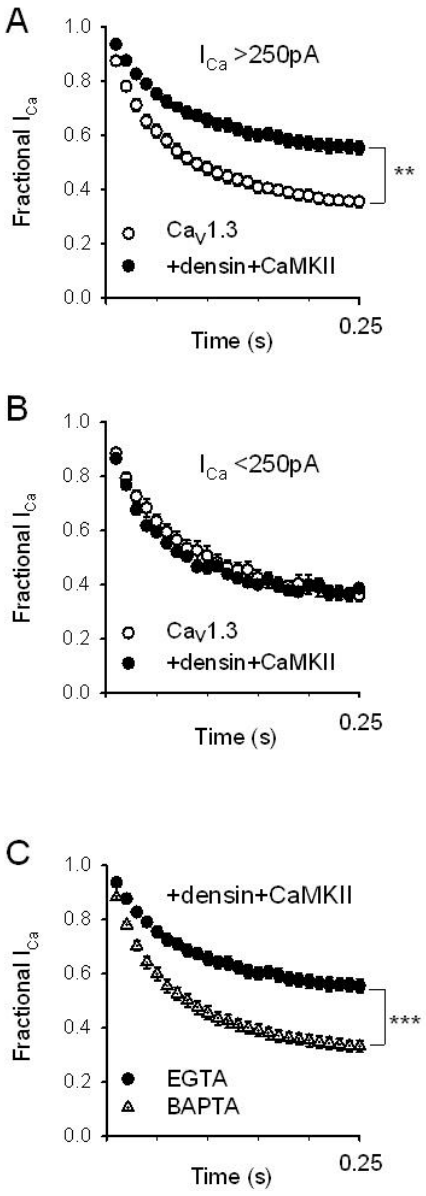

Figure 5.7: Effects of CaMKII and densin on I_{Ca} are dependent on "global" increases in Ca^{2+} in the channel microdomain. ***A***, Fractional I_{Ca} was plotted against time for cells transfected with $Ca_v1.3$ alone or cotransfected with $Ca_v1.3$, densin and CaMKII for recordings that elicited either a smaller current response (<250pA, $Ca_v1.3$ n=9, open circles; +densin+CaMKII n=9, closed circles) or ***B,*** a larger current response (>250pA, $Ca_v1.3$ n=9 open circles, +densin+CaMKII n=11 closed circles). Potentiation of $Ca_v1.3$ currents is only evident during current amplitudes greater than 250pA. Error represents SEM. ***C***, Fractional current was plotted

against time for cells transfected with $Ca_v1.3$, densin and CaMKII and recorded with either EGTA (n=11, closed circles) or BAPTA (n=11, grey triangles) in the intracellular solution. Strong Ca^{2+} buffering with BAPTA prevents the actions of CaMKII and densin. Error represents SEM.

nearby channels within hundreds of nanometers of each other (Borst and Sakmann, 1996, 1998; Song et al., 1998b). Strong buffering (5 mM BAPTA), on the other hand, localizes Ca^{2+} to channel "nanodomains" that are defined as a range of up to 20 nm from the channel (Stanley, 1993). These different Ca^{2+} buffers, EGTA and BAPTA can distinguish between modulation by Ca^{2+} from the immediate local environment, "nanodomain", or rather from a more widespread "microdomain," because BAPTA has faster Ca^{2+} binding kinetics compared to EGTA (Tsien, 1980). I therefore compared the actions of densin and CaMKII during low and high Ca^{2+} buffering with 5 mM EGTA or 10 mM BAPTA, respectively, in the intracellular solution. When BAPTA was included in the intracellular solution, CaMKII and densin no longer enhanced $Ca_v1.3$ currents in response to a repetitive voltage stimulus (Figure 5.7*C*; $p<0.01$ by Student's *t* test). This result shows that global intracellular Ca^{2+} accumulation in the channel microdomain is required for CaMKII and densin to change the properties of $Ca_v1.3$ channels.

$Ca_v\beta$ subunits potentiate peak current amplitude and influence CaMKII regulation of other ion channels, like $Ca_v1.2$. Thus, low $Ca_v\beta$ subunit expression might yield small $Ca_v1.3$ currents insensitive to densin and CaMKII. To ensure the I_{Ca} size dependence was not an artifact of decreased $Ca_v\beta$ subunit expression, I analyzed the voltage-dependence of activation in small versus large-amplitude currents. $Ca_v\beta$ subunits cause a hyperpolarized shift in Ca_v1 channel activation, therefore, lower expression levels of this auxiliary subunit would show a depolarized shift in IV parameters. However, voltage-dependence

	k	p	$V_{1/2}$	n	p
I_{Ca} <250 pA		0.14		6	0.74
Ca_v1.3	-5.54 ± 0.45		-10.51 ± 0.86	9	
+densin+CaMKII	-6.4 ± 0.59		-9.89 ± 0.51		
I_{Ca} >250pA					
Ca_v1.3	-8.05 ± 1.08		-9.90 ± 0.55	9	
+densin+CaMKII	-5.46 ± 0.88		-10.59 ± 0.48	10	

Table 5.1: Large and small currents exhibit similar voltage-dependence of activation. Step depolarizations from -90 mV to various voltages were used to generate *I-V* relationship for I_{Ca} in HEK293T cells cotransfected with Ca_v1.3 alone or with Ca_v1.3, densin, CaMKII. Results are shown for small- (<250 pA) and large- (>250 pA) currents. *I-V* curves were fit with the function: $g(V-E)/(1+\exp[(V-V_{1/2})/k] + b)$ where *g* is the maximum conductance, *V* is the test potential, *E* is the apparent reversal potential, *V*1/2 is the potential of half-activation, *k* is the slope factor, and *b* is the baseline. Relevant parameters are shown as mean ± SEM. p-values were determined by one-way ANOVA.

of activation was unchanged in any of the transfection conditions or current sizes (Table1), further supporting the finding that CaMKII and densin enhance large but not small $Ca_v1.3$ currents that is a result of changing β subunit expression levels. Together, these data show that maintenance of high-frequency, robust increases in Ca^{2+} are required for modulation of $Ca_v1.3$ properties.

E. CaMKII must be autophosphorylated to enhance $Ca_v1.3$ channel activity

Binding of Ca^{2+}-bound calmodulin (Ca^{2+}-CaM binding) to the CaM binding domain of CaMKII stimulates relief of the autoinhibitory domain and subsequent autophosphorylation of threonine286 (Thr286). This then allows CaMKII to maintain its kinase activity even after unbinding of Ca^{2+}-CaM, Ca^{2+} dissipates or is cleared by buffering (Hanson et al., 1994; Soderling, 1996; De Koninck and Schulman, 1998). Because the effects of CaMKII are Ca^{2+}-dependent, the kinase activity of CaMKII might be crucial for modulation of $Ca_v1.3$. If so, then preventing autophosphorylation should block the effects on $Ca_v1.3$. KN-93 inhibits CaMKII in a competitive fashion against Ca^{2+}-CaM binding, thus preventing autophosphorylation. As predicted, inclusion of 10 μM KN-93 in the intracellular solution prevented the effects of CaMKII and densin on $Ca_v1.3$, but these effects were still evident in the presence of KN-92 an inactive analogue of KN-93 (Figure 5.8*A*, $p<0.001$ by one-way ANOVA and Bonferroni's post hoc test).

To more specifically target CaMKII autophosphorylation, a CaMKII construct in which alanine was substituted for threonine at residue 286

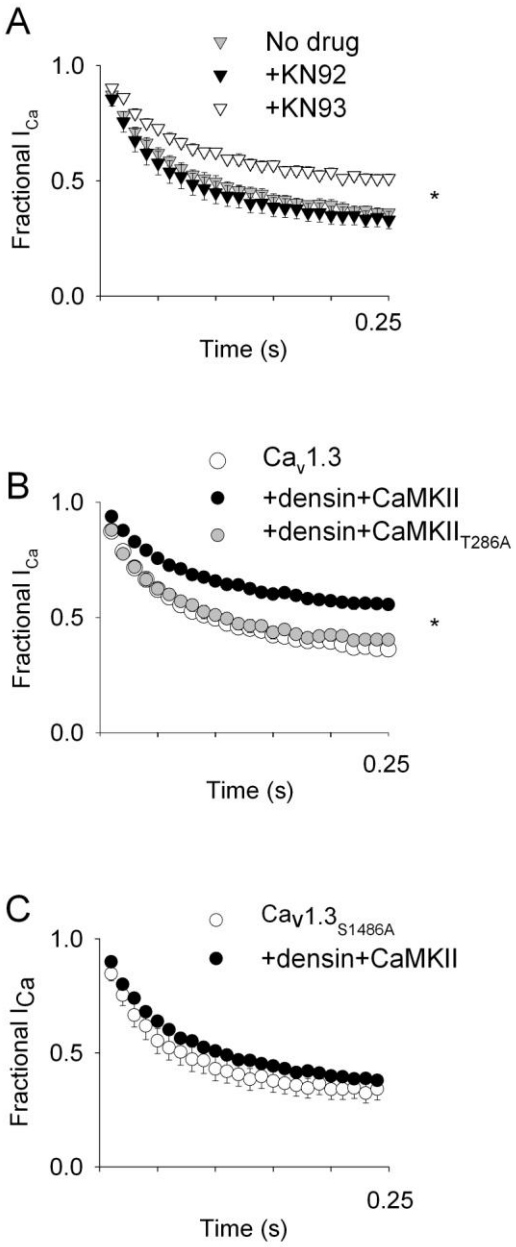

Figure 5.8. Autophosphorylation of CaMKII is required to potentiate $Ca_v1.3$ channels. A, Fractional current is plotted against time for cells transfected with $Ca_v1.3$, densin and CaMKII and recorded with either no drug (n=11, gray triangles), 10uM KN-93 (n=7, closed triangles) or KN-92 (n=6, open triangles) contained in the intracellular solution. KN-93 but not its inactive analogue, KN-92, blocks the effect of densin and CaMKII. Error represents SEM. **B,** Fractional I_{Ca} is plotted against time for cells transfected with $Ca_v1.3$ alone (n=9, open circles), with densin+CaMKII (n=11, closed circles) or with densin+$CaMKII_{T286A}$ (n=7, gray circles). Error represents SEM.

Blocking addition of a phosphate to Thr286 prevents facilitation of $Ca_v1.3$ currents. **C,** Fractional I_{Ca} versus time is plotted for cells transfected with $Ca_v1.3_{S1486A}$ alone (n=6, open circles) or with densin+CaMKII (n=10, closed circles). Mutation of the putative phosphorylation site in the C-terminus of the pore-forming $\alpha_1$1.3 channel subunit blocks effects on $Ca_v1.3$. Error represents SEM; where error bars cannot be seen, they are smaller than the size of the symbol. *p<0.01.

($CaMKII_{T286A}$) was used to prevent addition of a phosphate group to this residue. If CaMKII autophosphorylation was required to potentiate $Ca_v1.3$ channel activity, cotransfection of $CaMKII_{T286A}$ and densin should not increase $Ca_v1.3$ currents as it does in cells transfected with wild-type (WT) CaMKII and densin. To test this, cells transfected with $Ca_v1.3$ alone were compared to +densin+CaMKII and +densin+CaMKIIT286A. In cells transfected with $Ca_v1.3$, densin and $CaMKII_{T286A}$, $Ca_v1.3$ currents were no longer potentiated as with WT CaMKII (Figure 5.8*B*, $p<0.001$) and was unchanged compared to cells transfected with $Ca_v1.3$ alone ($p=0.98$ by one-way ANOVA with Bonferroni's post hoc test). These results confirm that CaMKII activation is required to enhance $Ca_v1.3$ channel function, suggesting that the kinase activity of CaMKII is involved in the modulation of $Ca_v1.3$ by densin and CaMKII.

In order to try to more specifically identify the target of CaMKII phosphorylation, I took advantage of a $Ca_v1.3$ channel construct in which a serine was substituted for alanine in the putative CaMKII phosphorylation site at residue 1486 (S1486A). This site was identified as a potential target of CaMKII phosphorylation in a pathway that includes IGF-1 (Gao et al., 2006). Ser1486 is found within the EF hand domain of the $\alpha_1$1.3 CT, a domain postulated to have a role in Ca_v1 channel facilitation (Hudmon et al., 2005; Erxleben et al., 2006; Lee et al., 2006), thus it is likely that phosphorylation at this site by CaMKII might influence $Ca_v1.3$ channel function. The S1486A mutation would prevent addition of a phosphate group at this residue, thus preventing effects on $Ca_v1.3$ channels if phosphorylation of the channel is required. To test this, I compared cells

transfected with $Ca_v1.3$ channels containing the S1486A mutation ($Ca_v1.3_{S1486A}$) to cells transfected with the mutant channel as well as densin and CaMKII. Densin and CaMKII do not significantly change $Ca_v1.3_{S1486A}$ currents (Figure 5.8*C*, p=0.26 by *t* test). These results suggest that phosphorylation of the $\alpha_1$1.3 subunit C-terminus at Ser1486 is required for the CaMKII and densin-dependent increase in $Ca_v1.3$ channel function.

F. Densin must bind the $\alpha_1$1.3 C-terminus and CaMKII to increase $Ca_v1.3$ function

Biochemical evidence supports an interaction between densin and $\alpha_1$1.3 (Figure 5.2,5.3), and previous studies have established the interaction between CaMKII and densin. Therefore, I hypothesized that these three proteins form a complex in which densin must scaffold CaMKII to the $\alpha_1$1.3 C-terminus to enhance $Ca_v1.3$ channel function. Preventing densin from binding to $\alpha_1$1.3 should block the effects of CaMKII and densin on $Ca_v1.3$ currents. This prediction was tested with a truncated densin construct that lacks the PDZ domain (densinΔPDZ). Cotransfection of CaMKII and $Ca_v1.3$ channels with densinΔPDZ did not enhance $Ca_v1.3$ currents in the way that full-length densin and CaMKII do (Figure 9*A*, p<0.001 by one-way ANOVA and Bonferroni's post hoc test). DensinΔPDZ does not significantly enhance $Ca_v1.3$ channel function (p=0.4) confirming the importance of the densin PDZ domain for this effect.

Although most PDZ domains interact with the consensus sequence for PDZ binding at the extreme C-terminus of their binding partner, some also

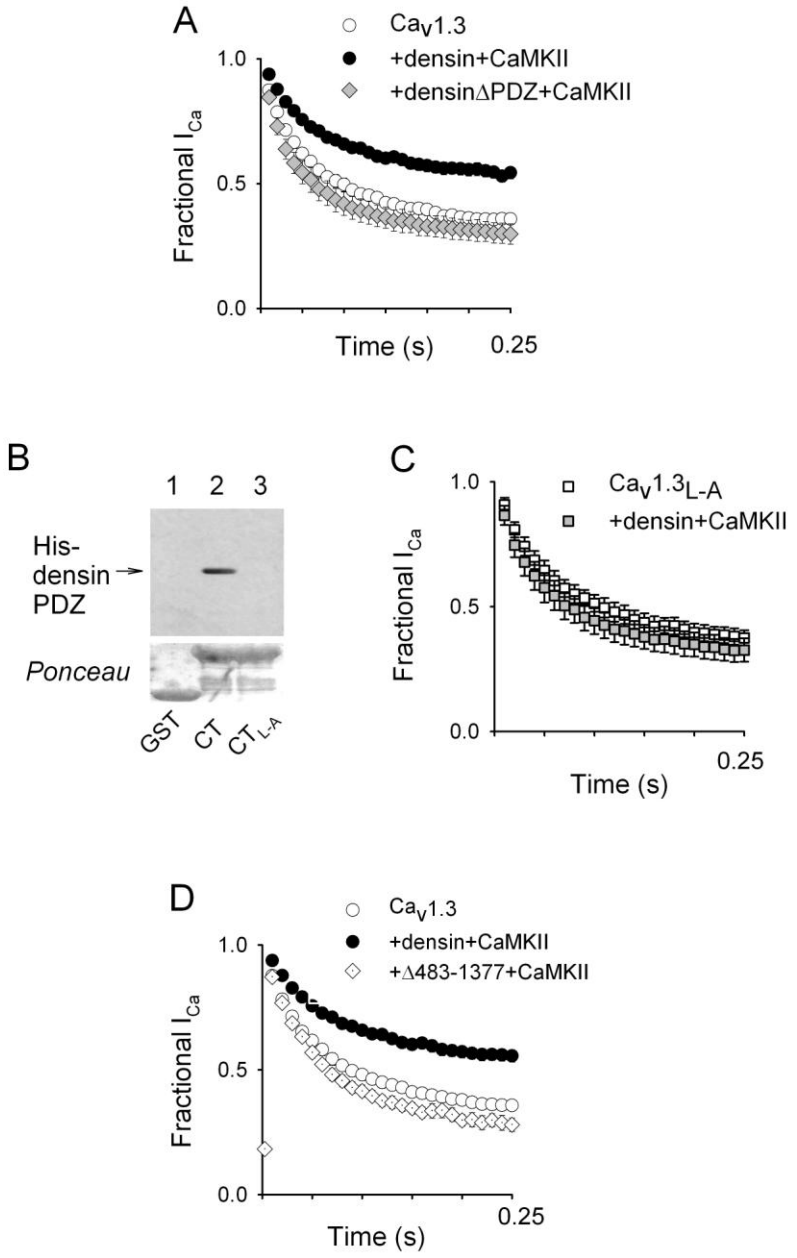

Figure 5.9. Densin binding to the $\alpha_1$1.3 C-terminus and CaMKII is required to enhance Ca$_v$1.3 channel function. **A,** Fractional I_{Ca} is plotted against time for cells transfected with Ca$_v$1.3 alone (n=9, open circles), with densin+CaMKII (n=8, closed circles), or with densinDPDZ+CaMKII (n=11, diamonds). DensinΔPDZ does not mimic the actions of full-length densin. Error represents SEM. **B,** Densin overlay assay in which GST fusion proteins containing

either the wild-type C-terminal region of $\alpha_1$1.3 or containing the L-A mutation in the PDZ binding domain were immobilized on nitrocellulose membranes and incubated with purified densin. Densin binds GST-CT (lane 2) but not GST control (lane 1) or GST-$CT_{L\text{-}A}$ (lane 3). **C,** Fractional current versus time is plotted for cells transfected with $Ca_v1.3_{L\text{-}A}$ (n=8, open squares) or with $Ca_v1.3_{L\text{-}A}$+densin+CaMKII (n=7, gray squares). Mutation of the $\alpha_1$1.3 PDZ binding domain site blocks the effect of CaMKII and densin on Ca_v1.3 inactivation. ***D,*** Fractional I_{Ca} versus time is plotted for cells transfected with Ca_v1.3 (n=9, open circles) and either densin+CaMKII (n=11, closed circles) or densinΔ483-1377-GFP+CaMKII (n=11, diamonds). DensinΔ483-1377 does not mimic the actions of full-length densin. Error represents SEM. Where error bars are not visible, they are smaller than the size of the symbol.

recognize other internal channel motifs (Penkert et al., 2004; Zhang et al., 2005). It was necessary to confirm that densin did not exert functional effects on $Ca_v1.3$ channels via interactions with other $\alpha_1$1.3 residues. The hydrophobic nature of the final residue in the $\alpha_1$1.3 C-terminal PDZ binding sequence (TTL) is critical for maintaining PDZ–ligand interactions (Songyang et al., 1997) so the substitution of alanine for the final leucine in this sequence ($\alpha_1 1.3_{L–A}$) should significantly weaken the interaction with densin. To first confirm that densin binding to the $\alpha_1$1.3 subunit C-terminus depends on the PDZ recognition site, I mutated the final residue within the PDZ binding domain (L-A), which should block binding. The isolated densin PDZ domain no longer bound to the $\alpha_1$1.3 CT_{L-A} mutant GST fusion protein (Figure 5.9*B*). These results suggest that densin does directly bind the $\alpha_1$1.3 C-terminal PDZ binding sequence. To verify if this mutation not only prevented the physical interaction between densin and the channel, but also the functional effects on $Ca_v1.3$ currents, cells transfected with $\alpha_1$1.3 subunits containing the L-A substitution in the α_1 subunit were compared to currents from cells transfected with $Ca_v1.3_{L-A}$ densin and CaMKII. Densin and CaMKII do not enhance $Ca_v1.3_{L-A}$ currents in response to the 100Hz voltage stimulus (Figure 5.9*C*; p=0.23 by Student's t test). These experiments confirm the importance of the interaction between the densin PDZ domain and the $\alpha_1$1.3 PDZ binding sequence in enhancing $Ca_v1.3$ activity, and suggest densin binds $\alpha_1$1.3 via its PDZ domain to scaffold CaMKII to the channel to exert their effects on $Ca_v1.3$ channels.

It is well-established that CaMKII binds directly to the C-terminus of densin

in vitro (Kennedy et al., 1990; Strack et al., 2000b; Walikonis et al., 2001). In ongoing studies to fully describe CaMKII-densin interactions, removal of a large segment of densin between residues 483-1377 appears to prevent CaMKII-densin binding (Jenkins et al., 2010). I utilized this construct, rather than a truncation mutant lacking the accepted CaMKII binding site, to investigate the role of densin/CaMKII binding in modulation of $Ca_v1.3$ because it retains the C-terminus that is required to bind the channel. If densin binding to CaMKII, as well as to the $\alpha_1$1.3 C-terminus, was required to enhance $Ca_v1.3$ channel function, densinΔ483-1377-GFP should be ineffective at enhancing $Ca_v1.3$ currents. Cells transfected with $Ca_v1.3$ were compared to those transfected with CaMKII and either full-length densin or densinΔ483-1377-GFP. Activity of $Ca_v1.3$ channels was not significantly increased by cotransfection with densinΔ483-1377-GFP and CaMKII (Figure 5.9*D,* $p<0.001$ by one-way ANOVA and Bonferroni's post hoc test). Together, these results support a mechanism in which the PDZ domain of densin binding to the C-terminal domain of $\alpha_1$1.3 is necessary but not sufficient to potentiate $Ca_v1.3$ currents. Both the interaction with the channel and with CaMKII are required to increase $Ca_v1.3$ currents during repetitive stimuli, suggesting densin acts as a scaffold to assemble this protein complex.

G. Role of β subunits in $Ca_v1.3$ modulation by densin and CaMKII

Because the auxiliary β subunit can influence Ca_v1 channel gating, I next examined what role this subunit may have in CaMKII and densin-mediated

$Ca_v1.3$ modulation. Association of different β subunits with Ca_v1 channels affect

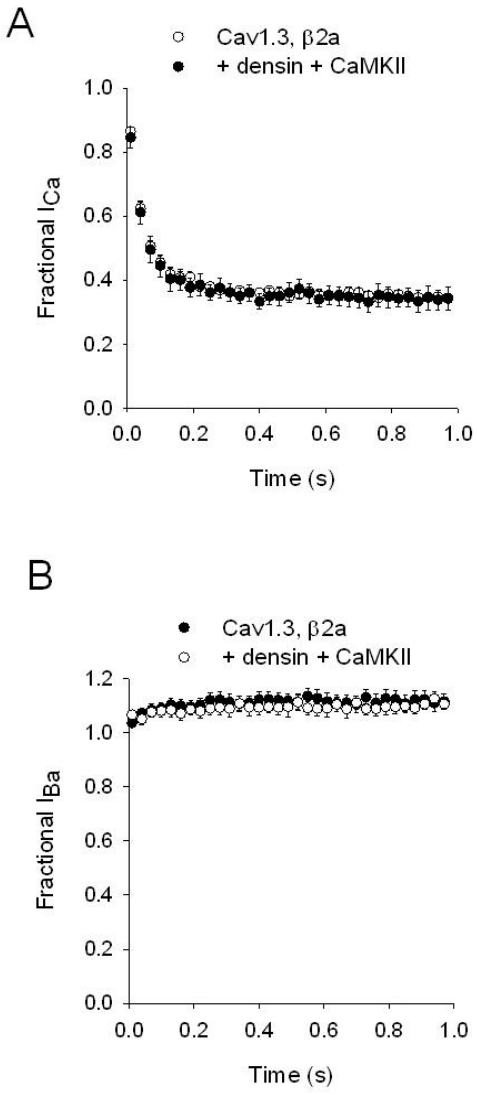

Figure 5.10: Effects of CaMKII and densin on $Ca_v1.3$ channels depend on auxiliary $Ca_v\beta$ subunit. ***A,*** Fractional I_{Ca} in response to the 100Hz voltage stimulus was plotted over time for cells cotransfected with $Ca_v1.3$, β2a and α2δ (n=11, open circles) or also with densin and CaMKII (n=6, closed circles). ***B,*** Fractional I_{Ba} in response to the 100Hz voltage stimulus was plotted over time for cells cotransfected with $Ca_v1.3$, β2a and α2δ (n=9, open circles) or also with densin and CaMKII (n=5, closed circles). Densin and CaMKII change neither I_{Ca} nor I_{Ba} of $Ca_v1.3$ channels containing the β2a subunit.

kinetic channel gating transitions (Colecraft et al., 2002). The β2a subunit, for example, exhibits slower inactivation kinetics compared to β1b (Stephens et al., 2000; Stotz et al., 2000; Cens et al., 2006). All recordings presented thus far were performed using channels containing the β1b subunit. To examine if β1b confers specific $Ca_v1.3$ channel gating properties that are important for modulation by CaMKII and densin, recordings were repeated using the β2a subunit. Densin and CaMKII did not change the $Ca_v1.3$ I_{Ca} or I_{Ba} response to the 100Hz voltage stimulus when the β2a subunit was used (Figure 5.10; p=86 by Student's t test). $Ca_v1.3$ currents through channels containing β2a were unchanged by densin and CaMKII. This supports the idea that CaMKII and densin are only able to target $Ca_v1.3$ machinery to induce conformational changes required to facilitate $Ca_v1.3$ currents in certain gating modes set by the auxiliary β subunit This adds another level at which a cell can maintain control of $Ca_v1.3$ channel properties, not only can densin help to target CaMKII to $Ca_v1.3$ channels to increase their activity, but this mechanism will only occur specifically in $Ca_v1.3$ channels that are in complex with β1b subunits.

V. Discussion

These results reveal a novel mechanism for targeting $Ca_v1.3$ channels. Densin and CaMKII work together to enhance $Ca_v1.3$ currents during high frequency repetitive stimuli. This effect is not observed during low frequency or sustained stimuli, nor is it is seen during high Ca^{2+} buffering or during low amplitude Ca^{2+} influx. Thus, the extent to which activity of neuronal $Ca_v1.3$

channels can be increasd by CaMKII and densin in response to membrane depolarization *in vivo* may depend on cell-type specific firing frequency and Ca^{2+} buffering capabilities.

Evidence of direct modulation of $Ca_v1.3$ channels by CaMKII has been incomplete until now. The data within this chapter show for the first time the direct regulation of $Ca_v1.3$ channels by CaMKII in the presence of a scaffolding protein, densin. Densin binding to $Ca_v1.3$ is not competitive with binding to CaMKII; therefore, densin may be responsible for linking this protein complex. Densin is responsible for anchoring CaMKII and NMDA-Rs to the PSD, allowing the kinase to respond to localized Ca^{2+} influx (Strack et al., 2000b). Densin may be recruiting CaMKII to $Ca_v1.3$ in a similar way that AKAPs anchor PKA to specific subcellular compartments (Faux and Scott, 1996; Gray et al., 1998). Skeletal muscle Ca_v1 channels undergo prominent PKA- and voltage-dependent potentiation during high frequency stimulation of muscle fibers that may mediate an increased force of muscle contraction during tetanus (Sculptoreanu et al., 1993). I describe here a similar requirement for the anchoring of CaMKII to $\alpha_1 1.3$ channel C-terminus by densin for direct Ca^{2+}- and frequency-dependent potentiation of $Ca_v1.3$ currents. I will elaborate on this idea further in the discussion in Chapter 6.

This densin/CaMKII/$\alpha_1 1.3$ complex potentially enables $Ca_v1.3$ to behave differently under different neuronal firing frequencies. Under certain conditions, it would be favorable for a neuron to increase Ca^{2+} influx in order to maintain a certain firing frequency. These findings may highlight how $Ca_v1.3$ channels can

selectively contribute to normal and pathological Ca^{2+} signals in neurons. High-frequency stimulation (HFS) and direct activation of $Ca_v1.3$ are well-documented mechanisms for induction of LTD in the striatum (Choi and Lovinger, 1997a, b; Gerdeman and Lovinger, 2003). A CaMKII-dependent increase in Ca^{2+} influx via $Ca_v1.3$ channels during high frequency stimulation might enable more efficient coupling to the gene-expression machinery that underlies LTD. Alternatively, this protein complex and enhanced $Ca_v1.3$ Ca^{2+} influx may be linked to pathological conditions. Parkinson's Disease is known to cause spine degeneration of MSNs in the striatum, and this process is postulated to be the result of disinhibition of $Ca_v1.3$ channels (Day et al., 2006). It is possible that excessive enhancement of $Ca_v1.3$ currents during high frequency stimulation in these neurons could lead to over activation of certain signaling cascades which may cause cytoskeletal disassembly in MSN spines (Rajadhyaksha et al., 1999; Dolmetsch et al., 2001; Oertner and Matus, 2005; Olson et al., 2005). While further studies need be carried out to investigate these possible connections, it is clear that there are many neuronal processes and functions in which this particular form of channel regulation is likely essential.

Chapter 6

DISCUSSION

In this final chapter, I will discuss the key conclusions and implications of the CaMKII-dependent facilitation of voltage-gated Ca^{2+} channels, and the role of phosphorylation in the control of AMPA receptor gating. I will primarily focus on the CaMKII-dependent increase in GluA1 AMPA receptor conductance, address some potential caveats in my results, and discuss the implications of my conclusions for synaptic communication and plasticity. In addition, I will propose potential structural mechanisms that may describe the function of the GluA1 C-terminal hyper-regulatory region. I will finish by providing a synthesis of my work on Ca^{2+} channels by drawing parallels with the scaffolding mechanisms known to coordinate functional changes in GluA1 receptors.

I. CaMKII increases AMPA receptor conductance in neurons

The data presented in Chapter 2 provide evidence that CaMKII increases the conductance of native hippocampal AMPA receptors specifically via phosphorylation at GluA1-Ser831. In phosphomutant knock-in mice, in which GluA1-Ser831 was mutated to alanine to prevent phosphorylation, purified CaMKII no longer increased AMPA receptor conductance as in WT mice. Furthermore, in knock-in mice expressing a phosphomimic aspartate at GluA1-Ser831 resulted in a constitutive increase in AMPA receptor conductance that was not further increased by CaMKII. This is a notable finding for a number of

reasons. While the CaMKII-dependent increase in AMPA receptor conductance had first been described over a decade ago, it has never been shown to occur in neurons (Derkach et al., 1999; Derkach, 2003). Previous studies have demonstrated that CaMKII increases EPSC amplitude, potentiates neuronal AMPA receptor currents and enhances LTP (Poncer et al., 2002; Lee et al., 2003; Lee et al., 2010). However, data presented in this dissertation provide the first evidence that CaMKII specifically increases conductance of AMPA receptors in neurons by phosphorylating GluA1-Ser831.

Oh and Derkach have shown that phosphorylation of GluA1-Ser831 by CaMKII increases the unitary conductance of recombinant homomeric GluA1 receptors, but not of recombinant heteromeric GluA1/GluA2 receptors (Derkach et al., 1999; Oh and Derkach, 2005). Since the majority of neuronal AMPA receptors are heteromeric assemblies that include GluA2 (Lu et al., 2009), the findings presented by Oh and Derkach suggest that CaMKII could only increase the conductance of a small population of GluA2-lacking AMPA receptors in the CNS. In contrast to this result, I observed a CaMKII-dependent AMPA receptor conductance increase in hippocampal neurons, in which homomeric GluA1 current contributes only a small percentage of the net AMPA current, as judged by the linear current-voltage relationship (Figure 2.5). When the conductance of GluA1/GluA2 receptors were shown to be unaffected by purified, active CaMKII or GluA1 phosphomimic mutations, the study of TARPs and their widespread association with native AMPA receptors was in its infancy. However, we now know the importance of TARPs in all facets of AMPA receptor function. Given the

pre-eminent role of TARPs as accessory subunits that control AMPA receptor function, I hypothesized that association with TARPs enabled the CaMKII-dependent conductance increase observed in hippocampal AMPA receptors. To test this hypothesis, I first confirmed the findings of Oh and Derkach, but further showed that a GluA1-S831E phosphomimic mutation can increase conductance of heteromeric GluA1/GluA2 recombinant receptors when the TARPs stargazin or γ8 are coexpressed. Most neuronal AMPA receptors are in complex with one or more TARPs (Chen et al., 2000; Shi et al., 2009; Kim et al., 2010), which likely explains why AMPA receptor conductance is increased by CaMKII in hippocampal neurons, and suggest that the results and mechanisms described here are relevant to the large number of heteromeric GluA1/GluA2 receptors in the central nervous system.

The implications of these findings for AMPA receptor biology are significant. The results showing that conductance of GluA1/GluA2 AMPA receptors can be increased by GluA1 phosphomimimc mutations suggests that all four subunits within a tetrameric AMPA receptor do not need to be phosphorylated to increase conductance. The logical explanation to Oh and Derkach's data was that all four receptors in a tetrameric receptor must have a phosphate group attached to GluA1-Ser831 for the AMPA receptor conductance increase to occur. My data suggest this is not the case, as long as the receptor is part of a complex with TARPs. It would be useful to know exactly how many subunits within a tetrameric AMPA receptor complex need to be phosphorylated at GluA1-Ser831 to increase AMPA receptor conductance. Complete control

would need to be maintained over subunit composition to compare the conductance values obtained from receptors with one or two GluA1-S831E mutant subunits in complex with the GluA2 subunit and unphosphorylated GluA1-Ser831A subunits.

The finding that CaMKII increases the conductance of neuronal AMPA receptors by phosphorylation of GluA1-Ser831 is equally important for synaptic transmission and plasticity. The conductance of AMPA receptors is increased with LTP (Benke et al., 1998; Poncer et al., 2002; Luthi et al., 2004; Palmer et al., 2004; Holmes and Grover, 2006); however, the precise mechanisms that lead to this increase are up for debate. Insertion of GluA2-lacking receptors into the synapse or CaMKII phosphorylation of GluA1-Ser831 could increase synaptic AMPA receptor conductance during LTP induction (Palmer et al., 2004; Holmes and Grover, 2006; Plant et al., 2006; Gray et al., 2007; Lu et al., 2007). My results show that CaMKII directly increases conductance of the heterogenous AMPA receptor population in the hippocampus via phosphorylation at GluA1-Ser831. These findings support the hypothesis that the conductance of a large fraction of synaptic AMPA receptors could potentially be increased by phosphorylation of GluA1-Ser831 by CaMKII, which could fully account for the increased AMPA receptor conductance observed during LTP. However, it is likely that both mechanisms, a direct conductance increase of GluA1-containing receptors by CaMKII and increased insertion of GluA2-lacking receptors, must occur in neurons to change synaptic strength (Palmer et al., 2004; Holmes and Grover, 2006).

CaMKII enhances gating of GluA1 AMPA receptors

Two potential hypotheses exist to describe the molecular mechanisms that underlie the increase in AMPA receptor conductance brought about by phosphorylation of GluA1-Ser831. Addition of a charged phosphate to Ser831 within the GluA1 C-terminus either alters gating to allow larger conductance levels to open more frequently, or increases ion flux through the pore for some or all AMPA receptor conductance levels. Previous work supports the first hypothesis, since individual conductance level amplitudes are not increased by phosphoSer831 in recombinant homomeric GluA1 receptors, but show an increase in the relative proportion of larger conductance level openings with the phosphomimic mutation GluA1-S831E (Derkach et al., 1999; Kristensen et al., 2011). The increased relative frequency of larger conductance level openings suggests that Ser831 phosphorylation influences GluA1 subunit gating.

Previous studies in our laboratory showed that CaMKII increases GluA1 receptor coupling efficiency to a similar extent whether the receptors are activated by a high or low efficacy agonist (Kristensen et al., 2011). It has been suggested that different agonist efficacies induce different degrees of ligand-binding domain closure (Jin et al., 2003). There is no significant correlation between the extent or frequency of domain closure induced by different agonists and changes in coupling efficiency induced by CaMKII (Kristensen et al., 2011). This finding suggests that GluA1-Ser831 phosphorylation by CaMKII does not affect any conformational changes associated with agonist binding, but rather

targets the subsequent receptor gating steps. GluA1-Ser831 phosphorylation likely reduces the energy needed for a subunit to undergo transition to an active state that allows ion permeation.

My data showing an increase in coupling efficiency with the GluA1-S831E mutation are consistent with this finding. An important caveat to this conclusion is that substitution of a phosphomimic glutamate or aspartate for GluA1-Ser831 merely mimics addition of a negatively charged phosphate group to that serine. Clearly, the structure of a serine bound to a phosphate ion and the glutamate amino acid are not one and the same. However, the glutamate substitution at GluA1-Ser831 does provide the negative charge that mimics the increase in conductance caused by phosphorylation of GluA1 by CaMKII. For the purposes of this study, the glutamate phosphorylation mimic appears to be sufficient for these effects, and allows better control over the system than inclusion of purified CaMKII in the intracellular solution.

The role of TARPs in the CaMKII-mediated AMPA receptor conductance increase

Stargazin and γ8 both promote the increase in heteromeric AMPA receptor conductance caused by the GluA1-S831E phosphomimic mutation. What structural mechanisms might explain this result? TARPs may be triggering a structural change within the GluA1/GluA2 receptor subunit C-terminus that allows for a phosphorylation-dependent increase in conductance. It is possible that stargazin or γ8 relieve a steric hindrance near the channel pore that, in

"TARPless" AMPA receptors, physically prevents heteromeric receptors from transitioning into the high subconductance levels. The crystal structure of a homomeric GluA2 AMPA receptor reveals one potential interaction site that might underlie this effect. A cavity exists within the central transmembrane ion channel domain and gaps between the individual transmembrane domains create a portal between this central cavity and the membraneous environment surrounding the ion channel (Sobolevsky et al., 2009). It has been speculated that this portal is filled by interactions with TARPs, which may explain how TARPs enhance AMPA receptor gating and decrease polyamine block in edited GluA2-containing receptors. Stargazin or γ8 may be interacting with this portal in heteromeric GluA2-containing AMPA receptors in such a way as to enable the conformational changes to occur that are required for the increase in conductance caused by phosphorylation of GluA1-Ser831. Perhaps in the absence of TARPs when this portal is not filled, GluA2-containing AMPA receptors are constrained, thereby preventing the changes in channel structure that are necessary to enhance gating caused by GluA1-Ser831 phosphorylation.

Alternatively, interactions between TARPs and the GluA1 C-terminus could potentially explain how GluA1-Ser831 phosphorylation increases conductance of heteromeric receptors in the presence of stargazin or γ8. While stargazin does not bind the C-terminus of AMPA receptor subunits directly, the GluA1 C-terminus is necessary for the stargazin-dependent trafficking of AMPA receptors to the plasma membrane (Bedoukian et al., 2006). When the GluA1 C-terminus is replaced with yellow fluorescent protein, stargazin-dependent AMPA

receptor trafficking is interrupted and AMPA receptors are held in the cytoplasm shown through fluorescent confocal imaging of transfected HEK cells (Bedoukian et al., 2006). This finding suggests there are likely some important positive interactions that exist between stargazin and the C-terminus of AMPA receptor subunits, or even a third scaffolding protein that binds both stargazin and the GluA1 C-terminus, that may be critical to the phospho-dependent gating changes. To identify these structural mechanisms, GluA1 receptors mutated at the potential stargazin interaction sites could be examined to see if preventing these interactions blocks the TARP- and phosphorylation-dependent increase in heteromeric AMPA receptor conductance.

Physiological relevance of non-desensitizing AMPA receptors.

One potential caveat in my conclusions regarding the importance of TARPs is that all of the experiments that show the phosphoSer831-dependent conductance increase were performed in non-desensitized receptors. This was necessary in order to perform stationary variance analysis on macroscopic currents elicited during slow washout of agonist to estimate γ_{MEAN} (Traynelis and Jaramillo, 1998). However, recent evidence suggests that stargazin dissociates from desensitized receptors (Morimoto-Tomita et al., 2009). The primary evidence in support of this hypothesis was found in the bell-shaped concentration-response curve observed in cerebellar granule cells and cochlea nucleus neuronal populations. Recombinant “TARPless” AMPA receptors exhibit a traditional sigmoidal concentration-response curve. The bell-shaped

concentration-response curve is the result of a decrease in the amplitude of steady-state AMPA receptor current at glutamate concentrations above 100 μM due to the loss of allosteric modulation of channel gating by stargazin in desensitized receptors. In addition, when lysates from *Xenopus* oocytes expressing GluA1 and stargazin were incubated in glutamate concentrations of 100 μM or greater for 20 minutes, stargazin no longer coiummunoprecipitates with GluA1. While this is not a physiologically relevant time course for glutamate in a synapse, if stargazin does dissociate from desensitized AMPA receptors *in vivo*, then GluA1-Ser831 phosphorylation may not increase conductance of desensitized heteromeric AMPA receptors.

While the activity-dependence of stargazin is intriguing, this phenomenon would likely not impact the results presented here for the following reasons. Firstly, stargazin dissociation from desensitized AMPA receptors was reported to be less robust for AMPA receptors containing GluA1 flip isoforms, which are used in the recombinant experiments included in this study. My data show that phosphorylation of Ser831 in GluA1 flip isoforms increases conductance of desensitized heteromeric receptors in complex with stargazin. Although, GluA1-Ser831 phosphorylation may not increase conductance of heteromeric receptors containing GluA1 flop isoforms to the same extent as I show, since stargazin dissociation from flop is more robust. This may even be a cellular mechanism for controlling which heteromeric receptor populations, those containing flip versus flop, are subject to a CaMKII-dependent conductance increase. Secondly, the bell-shaped glutamate concentration-response curve that shows the loss of

stargazin-mediated modulation of desensitized AMPA receptors has been reported in cerebellar granule cells and cochlear nucleus neurons. Hippocampal CA1 pyramidal neurons, on the other hand, exhibit a traditional sigmoidal concentration-response relationship. Thio et al show a sigmoidal concentration-response in hippocampal neurons using quisqualate (Thio et al., 1991). Similarly, Patneau et al. also showed a sigmoidal concentration-response relationship for AMPA receptors using both willardiine analogues (Patneau et al., 1992) and glutamate (Patneau and Mayer, 1990) in hippocampal pyramidal neurons. This suggests that in hippocampal pyramidal cells stargazin does not dissociate from desensitized AMPA receptors as it does in cerebellar granule cells. Thus, if TARPs do not dissociate from AMPA receptors in hippocampal neurons, then my neuronal data would likely not be different in recordings lacking cyclothiazide. It remains to be seen if CaMKII increases conductance of AMPA receptors in these other brain regions that do show stargazin dissociation from desensitized AMPA receptors. Activity-dependent TARP-GluA1 interactions could be yet another important mechanism for differentially targeting CaMKII-mediated effects to certain neuronal populations.

Finally, I attempted to experimentally control for these potential pitfalls related to my conclusions. I used fast-application of glutamate (0.1-0.4 ms solution exchange time) on excised outside-out patches from HEK cells cotransfected with stargazin and the desensitizing GluA1 and GluA2 subunits that lacked the L497Y/L483Y mutations, respectively. I then applied non-stationary variance analysis to the GluA1-EA or -AA/GluA2/stargazin receptor

desensitizing current responses. γ_{MEAN} can be estimated using non-stationary variance analysis on desensitizing current responses, where many desensitized current responses are averaged and then subtracted from individual responses (Traynelis and Jaramillo, 1998). The random current fluxuations that arise from stochastic ion channel properties can be analyzed to estimate γ_{MEAN} using equation 1 (*Methods*) modified to control for time dependent changes in variance and current. I selected only recordings that exhibited a linear IV relationship, in order to limit analysis to currents elicited exclusively from heteromeric receptors.

When coexpressed with stargazin, GluA1-EA/GluA2 receptors did, in fact, show an increased conductance compared to GluA1-AA/GluA2 receptors. However, these data were not included in Chapter 2 because the conductance values obtained with non-stationary variance analysis were approximately 2-fold higher and more variable than those obtained with stationary variance analysis. These higher conductance values may reflect the fact that when using non-stationary variance analysis desensitizing receptors transition directly to the closed state compared to stationary variance analysis, for which individual sublevel transitions of non-desensitizing receptors make a more significant contribution. Moreover, desensitization dramatically reduces the signal to noise ratio at steady state, making non-stationary variance analysis of heteromeric AMPA receptor currents challenging.

A final reason I chose to not include these data in Chapter 2 is that the GluA1/GluA2/stargazin macroscopic currents also exhibited a large variability in the peak to steady-state ratio. This is likely a result of a mixed subunit

stoichiometry in the receptor population of these patches. There was no reliable way to force tetrameric AMPA receptors to consist of 2 GluA1 subunits and 2 GluA2 subunits in these experiments, so there is likely a receptor population that consists of some GluA1 homomeric receptors, and varying combinations of GluA1 to GluA2 subunit ratios within a single receptor. Further complexity and variability results from more than one TARP simultaneously interacting with a single AMPA receptor (Shi et al., 2009; Kim et al., 2010). There may be differing degrees of stargazin-dependent effects on the heterologously expressed receptors contained within these patches. These factors will also contribute to the variability seen in non-stationary variance analysis. In the future, it will be necessary to maintain control over receptor subunit composition and TARP stoichiometry in order to carry out these types of experiments in a manner that allows for interpretable results. For example, tandem contructs, in which the cDNAs encoding GluA1 and GluA2 are linked by a short connector sequence, could be used to achieve this result.

GluA1 C-terminal hyper-regulatory region

My data showing that phosphomimic mutations at GluA1-Ser818, GluA1-Ser831 or GluA1-Thr840 increase AMPA receptor conductance help to identify the structural basis by which phosphorylation couples to AMPA receptor gating. The presence of four phosphorylation sites within only 27 residues of the GluA1 C-terminal domain, phosphorylation of three of which increase AMPA receptor conductance, suggests that this highly regulated region of the GluA1 C-terminus plays important structural or biophysical roles in GluA1 receptor function.

Functional interactions between these phosphorylation sites and the AMPA receptor channel pore or between this region and intracellular association proteins could underlie the conductance increase. Identifying these inter- or intraprotein interactions will provide a structural framework for the mechanism by which this hyper-regulatory region of GluA1 controls AMPA receptor gating.

When residues C-terminal to GluA1-Ser844 are removed, the GluA1-S831E phosphomimic mutation no longer increases conductance of recombinant GluA1 receptors. The logical conclusion would be that GluA1-Ser845 is required for the phosphoSer831-mediated GluA1 receptor conductance increase, since serine residues help to establish secondary structure through hydrogen bonding and can be phosphorylated by a variety of kinases. This serine, GluA1-Ser845, is phosphorylated by PKA and phosphorylation of both GluA1-831 and GluA1-Ser845 are required to induce LTP and LTD in the hippocampus (Lee et al., 2010). There may be cross-talk or functional overlap or between these two residues that maintain the effects of phosphorylation on the receptor and synaptic transmission. These findings all support the conclusion that GluA1-Ser845 must be required for the increase in conductance caused by GluA1-Ser831. However, the full-length GluA1 receptors used throughout this study, in which a phosphomutation is inserted at GluA1-Ser831, all also posses a phosphodeficient GluA1-S845A mutation intended to block phosphorylation by endogenous PKA in HEK cells (Methods, Chapter 2). This suggests then that a serine residue at that site is not required for the increased homomeric GluA1 receptor conductance observed with the GluA1-S831E mutation. If it were, there

would not be an increase in conductance of full length GluA1-Ser831E-S845A receptors.

This suggests then that the Gly-Ala-Gly-Ala stretch of amino acids in the GluA1 C-terminus, downstream of GluA1-Ser845 are required for a phosphorylation-dependent increase in AMPA receptor conductance. Perhaps phosphorylation within the hyper-regulatory region stimulates a change in secondary structure that is directed by the Gly-Ala-Gly-Ala stetch. This conformational change in the GluA1 C-terminus may induce the necessary changes within the channel that lead to an to increase in AMPA receptor conductance. A final possibility is that a scaffolding protein binds to this Gly-Ala-Gly-Ala stretch that promotes the phosphorylation-dependent conductance increase. It might be seem unlikely that a protein would interact with such a simple amino acid sequence, however, there are other examples of seemingly inoccuous sequences that establish binding with important structural scaffolds. For example, the PDZ binding domain is only 3 amino acids long, a Ser/Thr, any amino acid and a hydrophobic residue.

Of course, to unequivocally identify the structural mechanisms that underlie the role of the glycine/alanine-rich region, further studies would need to be carried out. Individual stop codons could be inserted at GluA1-Gly846, Ala847, Gly848, and Ala849 to identify if there is one of these residues in particular that is important, or if the entire stretch of amino acids is important for maintaining a phosphorylation-dependent GluA1 conductance increase. Yeast-two hybrid assays could be used to screen for any scaffolding proteins that bind

to this specific stretch of amino acids within the GluA1 C-terminus. While many studies have already attempted to identify all the protein-protein interactions that occur in the GluA1 C-terminus, the GluA1 C-terminus is a dynamic locus of protein-protein interactions that dictate if and how phosphorylation changes receptor function. There may be a yet unidentified association or scaffolding protein that could be essential to a phospho-dependent conductance increase. One potential candidate that might be important for the function of the hyper-regulatory region is 14-3-3, a family of conserved regulatory proteins first described to be abundant in brain tissue (Fu et al., 2000). There are seven 14-3-3 isoforms encoded by distinct genes (Ichimura et al., 1988; Martin et al., 1993). These proteins are now known to be present in all eukaryotic cells and interact with more than 50 ligands such as kinases, phosphatases and ion channels (Celis et al., 1990; Wang and Shakes, 1996). These interactions allow 14-3-3 to regulate such diverse processes as neuronal development, cell death, cell growth, and the cell cycle. A consensus site for binding of this ubiquitous protein is localized within this GluA1 C-terminal hyper-regulatory region (Arg-X-X-pSer/Thr-X-Pro, where X is any amino acid, and pSer/Thr is a phosphorylated serine or threonine residue), specifically around Thr840 (Muslin et al., 1996; Peng et al., 1997). p70S6 kinase and PKC both phosphorylate Thr840 (Delgado et al., 2007; Lee et al., 2007b), and 14-3-3 may trigger this reaction and help to establish conformational changes within the channel to promote the phosphorylation-induced increase in conductance. To investigate if 14-3-3 is critical for this effect, binding or coimmunoprecipitation assays would first need to

be performed to determine if GluA1 binds any 14-3-3 isoforms. If disrupting this interaction, either by siRNA knock-down of endogenously expressed 14-3-3 or transfecting a dominant negative form of 14-3-3 in HEK cells, prevented a phosphorylation-mediated GluA1 conductance increase, then this protein might perform a structural role in an AMPA receptor conductance increase.

Synthesis of ideas

The most important result from my experiments involving $Ca_v1.3$ Ca^{2+}-channels is the novel feedback regulation by CaMKII that requires association with the scaffolding protein densin. Densin binding to both CaMKII and the distal C-terminus of the pore-forming $\alpha_1$1.3 subunit promotes CaMKII-mediated facilitation of $Ca_v1.3$ Ca^{2+} currents during high frequency voltage stimuli. This effect is dependent on both activation of CaMKII by autophosphorylation and a more widespread accumulation of Ca^{2+} ions within hundreds of nanometers of the Ca^{2+} channel, or "microdomain" (See Results, Chapter 5). My data also suggest that CaMKII phosphorylates Ser1486 within the $\alpha_1$1.3 subunit C-terminus to potentiate $Ca_v1.3$ channels in a Ca^{2+}- and frequency-dependent manner.

CaMKII-mediated phosphorylation of both GluA1 AMPA receptors and $Ca_v1.3$ Ca^{2+} channels upregulates activity of these two ion channels in different ways. The net effect of the addition of a phosphate group to the C-termini of these two channels is to enhance the amount of current that flows through them once they are opened. In a similar way, CaMKII enhances Ca^{2+}-dependent

facilitation of $Ca_v1.2$ Ca^{2+} channels by phosphorylating the $\alpha_1$1.2 C-terminus, but also at least one β subunit isoform (β2a) (Jahn et al., 1988; Hell et al., 1993a; Abiria and Colbran, 2010). Other voltage-gated Ca^{2+} channels are also regulated by CaMKII, not only L-type channels. For example, CaMKII binds to the $Ca_v2.1$ Ca^{2+} channel to slow its voltage-dependence of inactivation (Jiang et al., 2008). Phosphorylation of $Ca_v3.1$ by CaMKII induces a hyperpolarizing shift in the voltage-dependence of activation (Wolfe et al., 2002; Welsby et al., 2003). dEag is a drosophila voltage-gated K^+ channel that is homologous to the mammalian K_v10 channel. CaMKII phosphorylates the C-terminus of dEag to enhance current amplitude and slow inactivation (Wang et al., 2002). CaMKII also phosphorylates $K_v4.2$ K^+ channels to aument A-type K^+ currents, and inactivation of $K_v1.4$ channels is slowed by CaMKII (Roeper et al., 1997; Varga et al., 2004). NR2B NMDA receptor subunits are also phosphorylated by CaMKII to enhance receptor desensitization (Sessoms-Sikes et al., 2005). CaMKII also binds to the NR2A NMDA receptor subunit, and this binding can be reduced by PKC phosphorylation of the NR2A subunit C-terminus (Gardoni et al., 1998; Gardoni et al., 2001). The interaction of CaMKII with NMDA receptors not only modulates NMDA receptor-mediated currents, but is hypothesized to be a molecular mechanism for targeting CaMKII to the PSD (Bayer et al., 2001; Bayer et al., 2006).

CaMKII not only phosphorylates ion channels within the PSD, but also a host of other signalling molecules. For example, CaMKII can phosphorylate densin, which as outlined in Chapters 4 and 5 is a post-synaptic scaffolding

protein. This interaction is thought to underlie constitutive association of CaMKII with the PSD (Strack et al., 2000a; Strack et al., 2000b; Walikonis et al., 2001; Robison et al., 2005a; Robison et al., 2005b). SynGAP, a Ras GTP-ase activating protein, is phosphorylated by CaMKII to inhibit its GAP activity (Chen et al., 1998). CaMKII also phosphorylates cdk5, a cyclin-dependent protein kinase that binds cytoskeletal elements and is found in post-mitotic cells. cdk5 associates with CaMKII and α-actinin in a Ca^{2+}-dependent manner, and is enhanced by NMDA receptor activation. It is thought that this protein complex helps to target cdk5 to the PSD (Dhavan et al., 2002). CaMKII, while a primarily post-synaptic density protein also phosphorylates some pre-synaptic proteins such as synapsin 1 and syntaxin1A which may promote exocytosis of synaptic vesicles (Benfenati et al., 1992; Ohyama et al., 2002). Clearly, the roles of this one protein, CaMKII, in the PSD are vast. The CNS must be able to direct the effects of CaMKII to specific targets, in order to properly maintain the balance of activity of the many substrates this protein has. This is achieved in many cases through association proteins.

The data included in Chapters 2 and 5 provide evidence that CaMKII-dependent changes in both $Ca_v1.3$ channels and heteromeric AMPA receptors are dependent on expression of a third association protein. Densin is required for Ca^{2+}-dependent potentiation of $Ca_v1.3$ Ca^{2+} currents by CaMKII, and stargazin is necessary for the CaMKII conductance increase in heteromeric GluA1/GluA2 AMPA receptors. While I do not suggest that the structural mechanisms by which densin and stargazin are facilitating CaMKII-mediated effects on ion

channels are similar, a common theme is evident: the post-synaptic membrane is a complex milieu of protein-protein interactions that enable selective targeting of specific effects that enhance the diversity of post-synaptic potentials. The GluA1 C-terminus provides a model of how complex protein-protein interactions dictate the phosphorylation state of, and resulting activity changes that occur in, AMPA receptors. Many association proteins target kinases to their substrates to allow for fast and specific responses to changes in neuronal activity. For example, SAP97 and AKAP79, two kinase scaffolding proteins, reduce the concentration of PKC necessary to phosphorylate GluA1 Ser831 by positioning it close to its substrate (Tavalin, 2008). Coexpression of AKAP79 increases the extent of GluA1-Ser831 phosphorylation induced by the PKC activator, PMA, and only upon AKAP coexpression can the purified catalytic fragment of PKC, PKM, augment recombinant GluA1 currents (Tavalin, 2008). This mechanism promotes PKC-mediated phosphorylation of GluA1-Ser831 over phosphorylation of this residue by CaMKII. When CaMKII is activated, it can bind and phosphorylate SAP97 to induce uncoupling of AKAPs from GluA1 subunits (Nikandrova et al., 2010). This promotes CaMKII phosphorylation of GluA1-Ser831 rather than PKC phosphorylation at this site (Figure 6.1). This was shown through a series of complex experiments in which an AKAP-induced rundown of GluA1 currents was blocked by purified CaMKII and SAP97 phosphorylation. In addition, AKAPs also target the dephosphatase PP2B to GluA1 to remove the phosphate group from GluA1-Ser845. This has been proposed to underlie LTD (Dell'Acqua et al., 2002; Tavalin et al., 2002). Ser845 can no longer be

dephosphorylated after dissociation of AKAP79, thereby maintaining phosphorylation at this site and preventing LTD. Moreover, the dual phosphorylation of GluA1-Ser831 by CaMKII and phosphorylation of GluA1-Ser845 by PKA also favors LTP (Lee et al., 2003; Lee et al., 2010). These mechanisms that are responsible for modulating synaptic strength are clearly tightly coordinated by a host of association proteins in order to maintain appropriate levels of AMPA receptor activity during periods of synaptic plasticity.

In a similar way, densin parallels the actions of AKAPs on AMPA receptors but with $Ca_v1.3$ channels, as I show in Chapter 4. Densin targets CaMKII to the $Ca_v1.3$ voltage-gated Ca^{2+} channel, as AKAPs target PKA to AMPA receptors. Data that support this comparison are also found in examples of AKAP-directed influences on other voltage-gated Ca^{2+} channels. Skeletal muscle Ca_v1 channels were first shown to undergo prominent PKA-dependent potentiation during high frequency stimulation of muscle fibers (Sculptoreanu et al., 1993). The spatiotemporal resolution required for this type of regulation suggested PKA was kept in close proximity to Ca_v1 channels via AKAPs (Faux and Scott, 1996; Johnson et al., 1997). PKA and AKAP-15 also coimmunoprecipitate with $\alpha_1 1.2$ subunits, and both proteins are required to increase

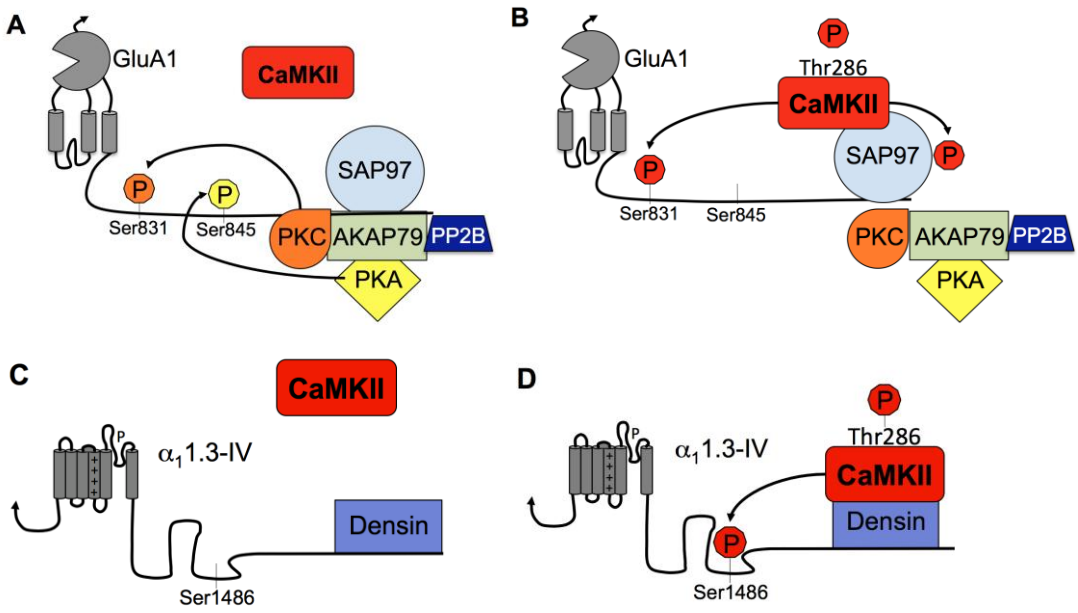

Figure 6.1. Phosphorylation of post-synaptic ion channels is coordinated by association proteins. ***A,*** AKAP79 localizes PKA to the GluA1 C-terminus to allow phosphorylation and dephosphorylation of Ser845 by PKA and PP2B, respectively (Tavalin et al., 2002). AKAP79, with SAP97, promote phosphorylation of GluA1 at Ser831 by PKC. ***B,*** When CaMKII is activated by autophosphorylation, it binds and phosphorylates SAP97, which disrupts phosphorylation of GluA1 by PKC and dephosphorylation of GluA1-Ser845 by PP2B. Phosphorylation at Ser831 is then mediated primarily by CaMKII rather than PKC (Tavalin, 2008; Nikandrova et al., 2010). ***C,D,*** In a similar fashion, densin binds and targets CaMKII to the C-terminus of $\alpha_1$1.3 subunits to enhance the function of Ca_v1.3 voltage-gated Ca^{2+} channels.

cardiac $Ca_v1.2$ channel activity (Gao et al., 1997). In much the same way, I show that CaMKII and densin coimmunoprecipitate with the $\alpha_1$1.3 C-terminus and binding of densin to CaMKII is required to increase the activity of $Ca_v1.3$. My proposed model is that densin anchors CaMKII at the $\alpha_1$1.3 C-terminus in a similar way as AKAPs target PKA, allowing CaMKII to quickly and directly increase $Ca_v1.3$ currents during high frequency voltage stimulation. Densin has already been shown to exhibit similar behaviors as AKAPs in that they can both target their associated kinases to either voltage- or ligand-gated ion channels (Hoshi et al., 2005). Densin also directs CaMKII to bind and phosphorylate NR2B NMDA receptor subunits in the post-synaptic membrane via a complex that includes α-actinin (Strack et al., 2000b; Robison et al., 2005a; Robison et al., 2005b). Densin and its many splice variants (Jiao et al., 2008) are likely a functionally similar class of proteins as the AKAPs, in that they localize kinases to both ligand- and voltage-gated ion channels. It would be interesting to investigate how the phosphorylation state of densin changes $Ca_v1.3$ regulation. Binding of densin to CaMKII is increased when phosphorylated by CaMKII (Strack et al., 2000a; Walikonis et al., 2001; Robison et al., 2005a; Robison et al., 2005b). Perhaps when dephosphorylated, densin uncouples from CaMKII and targets $Ca_v1.3$ for phosphorylation by another kinase, in the same way SAP97 does with GluA1 receptors. In any event, there is likely a similar arrangement in $Ca_v1.3$ receptor complexes as GluA1, where phosphorylation at different sites by different kinases converges and diverges based on specific association or scaffolding proteins.

Relevance to neuroscience

The implications of this work to CNS function and disease are extensive. Influx of Ca^{2+} into neurons via AMPA receptors or voltage-gated Ca^{2+} channels depolarizes the post-synaptic membrane thereby enhancing cell activity, but also activates a host of cellular signaling cascades. A sensitive balance must be maintained with negative- and positive-feedback mechanisms to regulate Ca^{2+} permeable ion channels. The work I will present in the subsequent chapters of this dissertation show how CaMKII can upregulate activity of both AMPA receptors and voltage-gated Ca^{2+} channels. AMPA receptors control processes as diverse as neuronal development, cognition, movement, synaptic plasticity, and learning and memory in the mammalian CNS. Overactivity of these receptors may contribute to pathological states such as epilepsy, ischemia and neurodegenerative diseases (Choi, 1992). Glutamate-induced neurotoxicity is associated with cerebrovascular insult and traumatic brain injury, and drugs that decrease activity of AMPA receptors, such as the AMPA receptor antagonist NBQX, has been shown to be neuroprotective (Catarzi et al., 2007). Similar analogues have shown to have protective effects on white matter injury (Follett et al., 2000). However, these drugs have not been useful for neuroprotection in clinical trials due to their low solubility and high incidence of side effects. Recently, two non-competitive AMPA receptor antagonists have been investigated for neuroprotective effects in ALS and neuropathic pain (Swanson, 2009). Clearly, AMPA receptors could be a potentially effective target for preventing glutamate-induced neurotoxicity, but more specific effective ways of

reducing their activity must be developed.

Voltage-gated Ca^{2+} channels may similarly be targeted in the treatment of various neurological diseases. $Ca_v1.3$ channels maintain spontaneous firing in neurons in the substantia nigra *pars compacta* that die in Parkinson's Disease (PD). These channels also mediate spine degeneration in medium spiny neurons of the striatum caused by PD (Day et al., 2006; Chan et al., 2007). Overactivity of these ion channels, as well as excessive reduction of their activity, could potentially lead to abnormal neurological function and disease. Highlighting ways in which homeostasis of these channels is maintained by the CNS with proteins such as CaMKII might eventually guide the development of drugs that could exert the same functions. This may allow us to someday treat a host of neurological diseases that are manifest by altered AMPA or Ca^{2+} channel activity.

Concluding remarks

Phosphorylation plays a critical role in many facets of glutamate receptor function. PKA phosphorylates GluA1-Ser845 to increase the AMPA receptor open probability (Banke et al., 2000), and phosphorylation of GluA1 at Ser818 by PKC induces LTP expression and incorporation of GluA1 subunits into the synapse (Boehm et al., 2006; Lin et al., 2009). PKC has also been shown to phosphorylate GluA1-Ser831, the CaMKII phosphorylation site, and GluA1-Thr840 (Roche et al., 1996; Lee et al., 2007b). Finally p70S6 kinase maintains phosphorylation at Thr840 during normal levels of cell activity that is

dephosphorylated during LTD (Delgado et al., 2007). In addition, my data suggest the existence of a “hyper-regulatory” domain in the membrane proximal GluA1 C-terminus, in which phosphorylation of three different residues increases AMPA receptor conductance. Clearly, there are overlapping, complex and tightly coordinated mechanisms of phosphorylation-dependent GluA1 AMPA receptor regulation, which are likely implicated in synaptic plasticity since changes in GluA1 activity and trafficking underpin changes in LTP and LTD (Roche et al., 1996; Benke et al., 1998; Hayashi et al., 2000; Lee et al., 2000; Malinow and Malenka, 2002; Lee et al., 2003; Boehm and Malinow, 2005; Holmes and Grover, 2006; Lisman and Raghavachari, 2006; Shepherd and Huganir, 2007; Lee et al., 2010). Specifically, CaMKII-dependent phosphorylation of GluA1-Ser831 has been a focus of studies on synaptic scaling and plasticity (Benke et al., 1998; Lee et al., 2010), thus, understanding the functional and structural mechanisms of GluA1-Ser831 phosphorylation-induced increases in AMPA receptor activity is crucial for understanding the molecular changes that occur during activity-dependent synaptic plasticity, a leading model of learning and memory.

While we do not have a C-terminal structure determined with X-ray crystallography, the work I have presented in this dissertation clearly establish the existence of a hyper-regulatory region that strongly couples to gating machinery and determines AMPA receptor conductance properties. In addition, my data suggest that AMPA receptor populations in the brain are likely subject to CaMKII-mediated increases in conductance. The studies presented here further provide an important advance in our understanding of the role of TARPs and the

functional effects of AMPA receptor phosphorylation. The glutamate receptor field is only beginning to understand the intricate roles TARPs play in all facets of AMPA receptor behavior, and the results within this dissertation have provided a major development in this area. In addition, because phosphorylation of this region of GluA1 is relevant to synaptic plasticity, including LTD and LTP, understanding the molecular mechanisms that regulate GluA1 AMPA receptor activity will directly yield insight into plastic events that occur within the central nervous system. This work is fundamental to establishing the critical role of the GluA1 receptor C-terminus in potentiating AMPA receptor activity, and thereby, can be utilized to elucidate mechanisms of synaptic communication and plasticity.

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