Role of Ca^{2+}/Calmodulin-Dependent Protein Kinase II in Regulating the Metabotropic Glutamate Receptor 5

By

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To my husband, Richard Kershaw,

for the love, support, and laughs through this process.

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CHAPTER I

Introduction

Experiences, like learning, stress, and even physical practice change the brain by altering the pathways and strengths of connection between neurons. These experiences modify our brain function by changing synaptic transmission between cells, referred to as synaptic plasticity. Neurons are connected through structures that protrude from the cell called synapses (Kandel, 2013). Synapses create a point of contact between two cells where an activated cell can release neurotransmitters to induce signaling to a synaptic partner cell. Connections in the brain can be strengthened (potentiated) or the connections can be made weaker (depressed), meaning that synaptic strength at excitatory synapses is modified bidirectionally (Citri and Malenka, 2008). These modifications are caused by different patterns of synaptic activity that are translated by the cell. This process of strengthening and weakening different synapses is important to the proper function of the brain. Too much in either direction can be a cause for neurological disorders. Understanding the mechanisms involved in plasticity are important to understanding basic brain function, connections between brain areas, and overall impact of the brain on health and behavior (Bliss et al., 2014). The molecular mechanisms that underlie proper regulation of this process are important to understand so that we can determine the processes underlying both normal and pathological brain function.

One classically studied process in plasticity is that of learning and memory. Memories are thought to form based on potentiation of synapses in the hippocampus, and so this brain region has been

well studied (Bliss and Collingridge, 1993). Potentiation of synaptic signaling can occur on different time scales. Different types of stimulation can lead to a long-term persistence of the enhancement or reduction of synaptic strength (Citri and Malenka, 2008). When these changes are long-lasting they can last for hours or presumably even days and this cellular response is referred to as long-term potentiation (LTP) or long-term depression (LTD) (Malenka and Bear, 2004). In addition to long lasting forms of neuronal plasticity, short-term plasticity is also being investigated by various groups. These short-term changes are thought to play a role in adapting short-term inputs such as changes in sensory inputs (Zucker and Regehr, 2002). Because glutamatergic signaling has been well studied, we will begin with an overview of ionotropic glutamate receptors and their roles in plasticity.

Glutamate Receptors

Synaptic plasticity has been most intensively studied at synapses that release the major excitatory neurotransmitter in the brain, glutamate. Glutamatergic synaptic terminals connect to a post-synaptic dendritic spine where they can release glutamate to induce signaling in the post-synaptic cell through chemical and electrical gradients. These receptors are located in and around the post-synaptic density (PSD) of spines. The PSD is an electron dense area of the synapse where synaptic proteins are concentrated and ready to mediate cellular changes due to synaptic activity. Many protein-protein interactions and signaling regulators spatially and temporally decode messages from presynaptic signals (Sheng and Hoogenraad, 2007). A number of different ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGlu receptors) mediate normal glutamatergic transmission and affect the intracellular signaling and downstream effects in different ways. These signals are major translators of the chemical neurotransmitter signal that

results in prolonged changes in synaptic strength defined as LTP and LTD. We will first discuss the receptors important to these processes. Differences in presynaptic release or postsynaptic interpretation of these signals by receptors such as ionotropic and metabotropic glutamate receptors can lead to disorders in normal brain function. I will review both of these types of glutamate receptors, but mGlu receptors will be the main focus of this thesis.

Ionotropic Glutamate Receptors

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

AMPARs have been shown to play a large role in glutamatergic synaptic plasticity. These receptors are responsible for the fast excitatory synaptic transmission in the brain. These receptors are concentrated in the protein-rich PSD. AMPARs quickly relay pre-synaptic glutamate release into a post-synaptic signal by allowing the influx of Na^+ ions to flow into the postsynaptic neuron leading to a depolarization of the cell. The function, surface expression, and subcellular localization of AMPARs are important because AMPARs are largely responsible for the strength of a synapse. Cellular activity can lead to the insertion of AMPARs at the post-synaptic density, resulting in an increase in synaptic strength, and under the right conditions can lead to LTP. Alternative signaling that leads to a reduction in synaptic strength causes LTD by the internalization of AMPARs.

These receptors are made up of four subunits that can form combinations of four separate genes encoding GluA1-GluA4. These AMPAR subunits differ in their intracellular C-tails which can be modified by alternative splicing and in protein-protein interactions. AMPAR-interacting proteins can affect cellular strength by altering AMPAR permeability to ions, cellular localization, and

trafficking. Kinases have also been shown to be important regulators of AMPAR function by altering synaptic localization and trafficking, open probability, and channel conductance. Cellular depolarization by AMPARs is a precondition for opening of another ionotropic glutamate receptor, N-methyl-D-aspartate receptor (NMDARs).

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

NMDARs are ligand and voltage-gated cation channels that play important roles in synaptic plasticity and learning and memory. Endogenously, NMDARs are activated by glutamate but are named because they are activated by the ligand N-methyl-D-aspartate, which is inactive at other glutamate receptors. Under low levels of cellular activity, NMDARs are inactive because of the voltage-dependence of the NMDAR. At resting membrane potentials, extracellular Mg^{2+} can block the pore of the NMDAR and inhibit cation flow through these channels. Excitatory synaptic transmission stimulated by glutamate is initially mediated by the activation of AMPARs in a fast and transient manner. Basal excitatory events that are not strong enough to remove the Mg^{2+} block do not cause ion flux through NMDARs. Stronger cellular depolarizations release the NMDAR Mg^{2+} block and allow for a flow of Na^{+} and Ca^{2+} into the cells as well as K^{+} out of the cell. In this way the NMDAR works as a molecular coincidence detector because activation requires a sufficient cellular depolarization and presynaptic glutamate release. Detection of this type of event by the NMDAR has important implications for the function of the synapse because of the ability to activate Ca^{2+} dependent signaling cascades. In addition, NMDARs have a slow activation and deactivation that allows for the complex Ca^{2+} signals. These signals are decoded by the cells to alter synaptic activity. Activation of NMDARs has been shown to play a role in synaptic potentiation as well as depression in many different brain areas.

Metabotropic glutamate receptors (mGlus)

The iGluRs including AMPA and NMDARs function as ion channels, opening in response to glutamate activation. Metabotropic glutamate receptors, on the other hand, are G-protein coupled receptors. G-protein coupled receptors (GPCRs) are the most abundant receptor gene family in the human genome (Niswender and Conn, 2010). These membrane-bound proteins respond to extracellular ligands such as hormones and neurotransmitters to transduce intracellular signals through second messenger systems. GPCRs couple to heterotrimeric G proteins composed of α, β, and γ subunits to induce signal transduction. Activation of a GPCR by a ligand results in a conformational change that causes an exchange from inactive, GDP-bound α subunit of the G protein to the active GTP-bound form. Activated G protein subunits can have a number of intracellular effects such as modulation of enzymatic activity and cellular transcriptional regulation. When the bound GTP is hydrolyzed to GDP, the G protein is then inactive and the heterotrimeric form of the G protein can reassemble. About 35% of approved drugs target GPCRs highlighting the importance of understanding these receptors (Sriram and Insel, 2018).

mGlu receptors belong to class C GPCRs, which form constitutive dimers. These receptors are distinguished from other GPCR classes by the presence of a large extracellular N-terminal domain termed the Venus flytrap domain (VFD), which contains the glutamate-binding site. Crystal structures of VFDs of mGlus shows that each VFD has two lobes. Evidence suggests that two VFDs dimerize together, back to back, and large conformational changes are induced when agonists bind to one or both VFDs. Different VFD states exist. The inactive conformation is stabilized by antagonists, and ligands induce the active conformation of these receptors (reviewed by Niswender and Conn, 2010), (Figure 1.1).

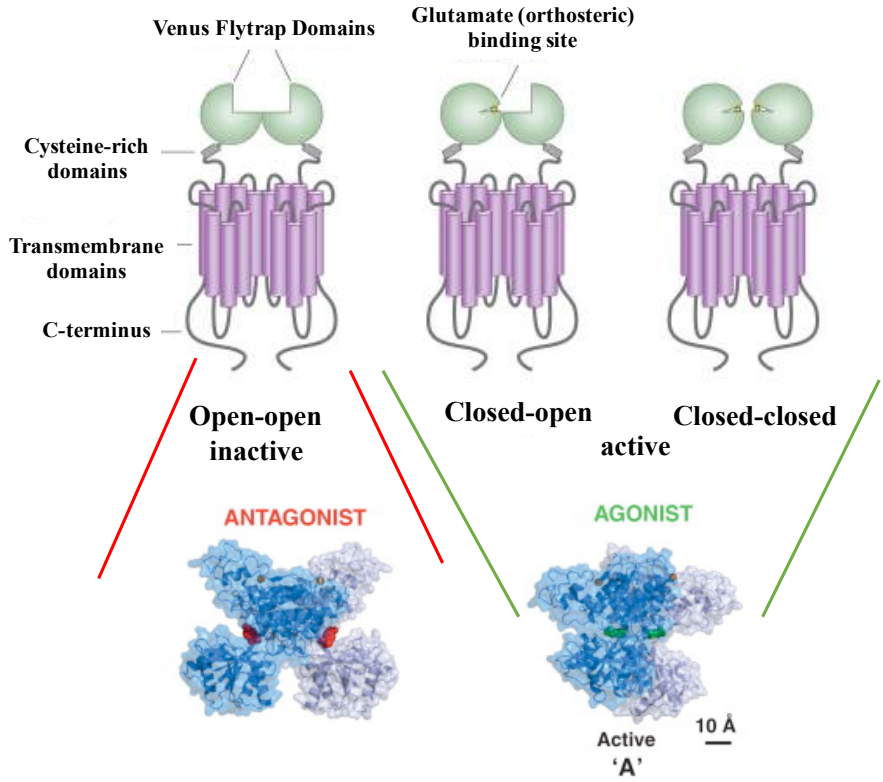

Figure 1.1 *General mGlu receptor structure and activation states.* mGlu receptors form dimers that contain two large extracellular domains called the Venus flytrap domains (VFDs), which bind glutamate and other orthosteric ligands. The VFD is linked to the cysteine-rich domain to connect the extracellular domain to the transmembrane domains of the receptor and the intracellular C-terminus. The open-open state (*left*) is the inactive state. This state can be stabilized by antagonists as shown in the crystal structure below of the VFDs. In the structure, the antagonist is shown in red. Either one or two VFDs can bind an orthosteric agonist resulting in active receptor conformations that lead to VFD closure. In the structural depiction the agonist is shown in green Adapted from Niswender & Conn, 2010 and Ronard & Pin, 2015.

There are eight mGlus ($mGlu_1$-8) subclassified into three groups based on sequence homology, G-protein coupling, and ligand selectivity (Figure 1.2). Group I includes mGlu receptors 1 and 5, Group II includes mGlu receptors 2 and 3, and Group III includes mGlu receptors 4, 6, 7, and 8. This dissertation will largely focus on the Group I mGlu receptors discussed in more detail below.

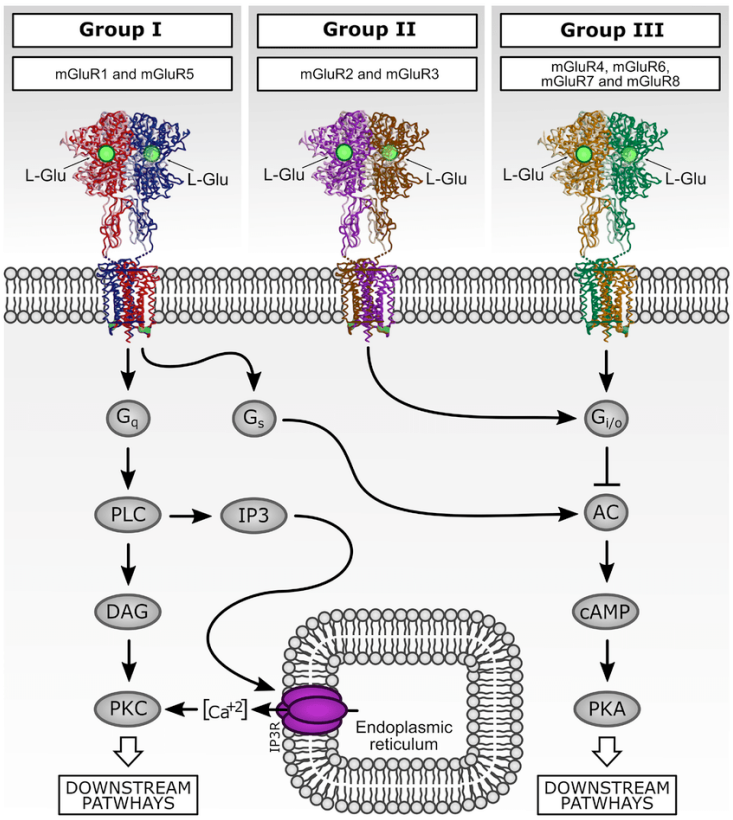

Figure 1.2 *mGlu receptor families I, II, and III.* mGlu receptors are comprised of eight receptor subtypes divided into three groups based on their sequence homology, pharmacology, and second messenger signaling pathway association. Group I mGlu receptors are the main focus of this dissertation and are coupled primarily to G_q (but also G_s) proteins and their activation stimulates PLC and production of IP_3 and diacylglycerol, stimulating intracellular Ca^{2+} release from the endoplasmic reticulum and PKC activation. mGlu receptors of groups II and III are coupled predominantly to $G_{i/o}$ proteins and inhibit adenylyl cyclase (AC). Activation of mGlu receptors modulates downstream signaling through these pathways. Figure from Pereira et al., 2017.

Group I mGlu receptors ($mGlu_{1/5}$)

$mGlu_1$ and $mGlu_5$ and constitute the Group I subclass of mGlu receptors that are canonically linked to the $G\alpha_{q/11}$ heterotrimeric G proteins. Group I mGlu receptors function as modulators of neuronal physiology and synaptic transmission and have been the target of therapeutic drug development for pathologies including Fragile X syndrome, schizophrenia, addiction and obsessive compulsive

disorder (Ade et al., 2016; Ayala et al., 2009; Ayoub et al., 2012; Ghoshal et al., 2017; Kinney et al., 2003; Michalon et al., 2012; Niswender and Conn, 2010; Pomierny-Chamiolo et al., 2014; Pop et al., 2014; Scharf et al., 2015; Vinson and Conn, 2012). A better understanding of the signaling of these receptors will lead to greater therapeutic potential in the future.

Distribution and Downstream Signaling

Group I mGlus are broadly expressed in the brain, but do show different patterns of expression. Staining for $mGlu_1$ is found in the olfactory bulb, cerebellar Purkinje cells, neurons of the lateral septum, the pallidum and in the thalamus (Ferraguti and Shigemoto, 2006) (Figure 1.3A). Expression of $mGlu_5$ is most highly expressed in the cerebral cortex, hippocampus, subiculum, olfactory bulb, striatum, nucleus accumbens and lateral septal nucleus (Figure 1.3B). Although $mGlu_1$ and $mGlu_5$ are both expressed in the hippocampus, their expression is complementary (Figure 1.3 B,C from Ljuan et al., 1996). $mGlu_1$ is most prominent on interneurons in the alveus and stratum oriens of the CA1 regions and throughout CA3 and the molecular layer of the dentate gyrus (Figure 1.3C). $mGlu_5$ expression is strongest in the CA1 region while expression is less strong in the CA3 region and the molecular layer of the dentate gyrus (Figure 1.2D). Both Group I receptors are typically post-synaptically located in the perisynaptic space of dendritic spines (Lujan et al., 1996). This allows for these receptors to rapidly regulate excitatory synaptic strength.

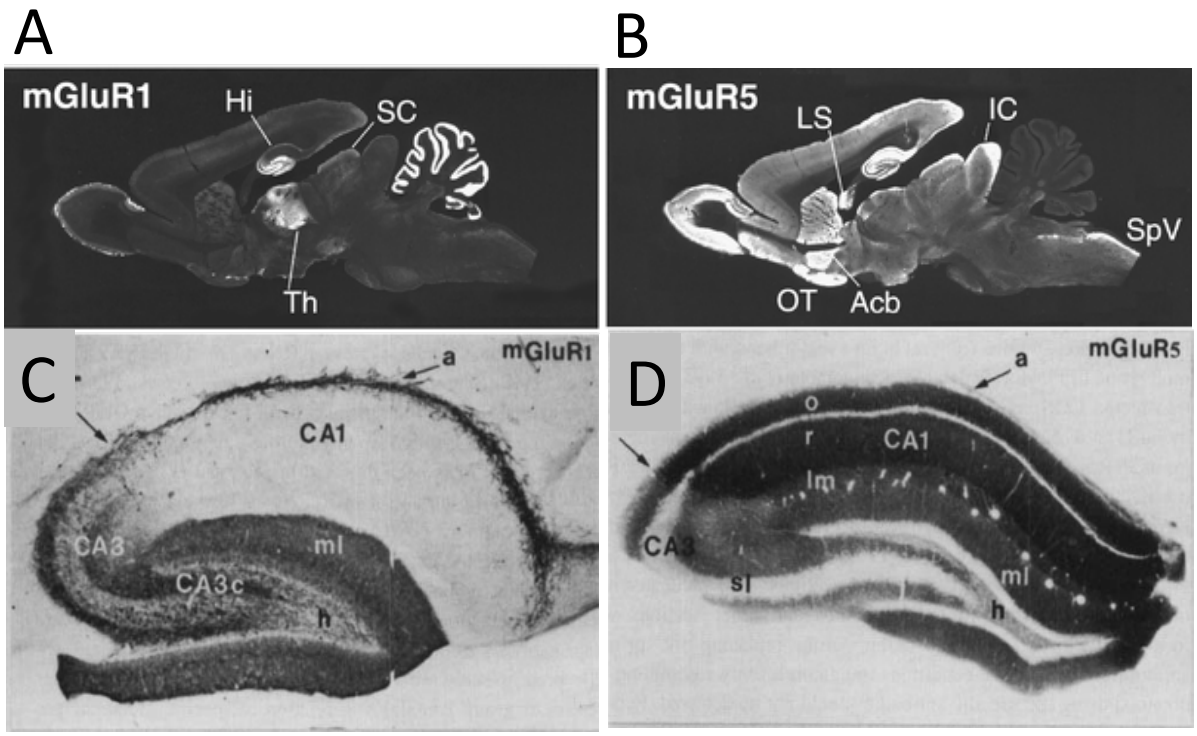

Figure 1.3. *Group I mGlu receptor distribution* Staining for A. mGlu1 and B. $mGlu_5$ distribution across the brain. A closer look at the hippocampus shows the differential distribution of C. $mGlu_1$ and D. $mGlu_5$. (Adapted from Ferraguti and Shigemoto, 2006 and Lujan et al., 1996)

The extracellular VFD of these receptors contains the orthosteric binding site and a cysteine-rich domain (Cao et al., 2009). Covalent receptor dimerization occurs in the extracellular domain (Kunishima et al., 2000; Romano et al., 1996). Binding of agonists to the VFD transduces information from the extracellular domain to intracellular signaling systems. These receptors couple to the G_q /G_{11} heterotrimeric G-protein. This coupling activates phospholipase C_β, causing the hydrolysis of phosphoinositides and generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) resulting in the mobilization of intracellular Ca^{2+} and activation of protein kinase C (PKC). Response to $mGlu_{1/5}$ activation can differ between neuronal cell types, but activation of $mGlu_{1/5}$ has also been shown to activate the mitogen-activated protein

kinase/extracellular receptor kinase (MAPK/ERK) and the mammalian target of rapamycin (mTOR) pathways and these have been shown to be important to changes in synaptic plasticity (Hou and Klann, 2004; Page et al., 2006).

Alternative splicing allows for translation of different proteins from a single gene. The production of spliced mRNAs results in proteins that differ in their amino acid sequence that can affect protein function. Splice variants of these two receptors exist and can alter the receptor signaling. Four C-terminal splice variants exist of $mGlu_1$ ($mGlu_1$a, b, c, and d), and $mGlu_5$ exists as two main splice variants: $mGlu_{5a}$ and $mGlu_{5b}$. The $mGlu_5$ splice variants have been shown to be developmentally regulated, but no major differences in signaling between the two $mGlu_5$ splice variants have been reported (Joly et al., 1995; Minakami et al., 1995; Romano et al., 1996). There is at least one allosteric binding site within the seven-transmembrane domain. Allosteric modulators at this site can modulate $mGlu_{1/5}$ activity specifically and have been useful in a number of pharmacological studies to determine differences between $mGlu_1$ and $mGlu_5$ signaling in neurons (Niswender and Conn, 2010), discussed later in this chapter.

$mGlu_5$ expression is highly regulated, with higher levels found in developing animals (Romano et al., 1996). An antibody that specifically reacts with the $mGlu_{5b}$ insert was used to measure the distribution of $mGlu_{5b}$ in the mouse and rat brain. It has been shown that $mGlu_{5b}$ is more highly expressed in adult animals and $mGlu_{5a}$ is more of a neonatal variant. It seems that most of the developmental alteration in total $mGlu_5$ is due to a reduction in the expression of $mGlu_{5a}$ through development (Figure 1.4). Comparison of $mGlu_5$ protein and mRNA levels indicates that post-transcriptional regulation is different across brain regions (Romano et al., 2002).

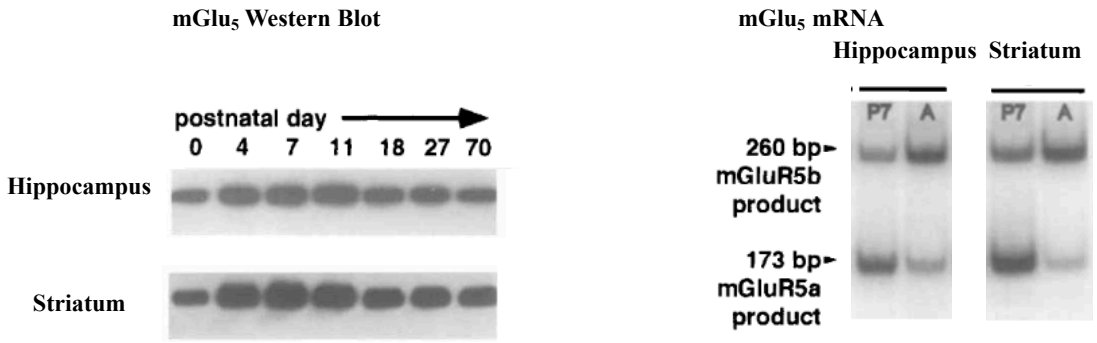

Figure 1.4 *$mGlu_5$ expression through development* A. Western blot analysis of $mGlu_5$ from rat hippocampal and striatal lysates shows that $mGlu_5$ is developmentally regulated with the highest protein levels occurring around P7-P11 and stabilizing in adulthood. B. Expression of mGlu5 mRNA splice variants. RNA was prepared from adult and P7 rat brain regions. All brain regions showed enhanced early expression of $mGlu_{5a}$ at P7 which undergoes a marked decrease in expression with maturation. In contrast, $mGlu_{5b}$ does not decline between P7 and adult stages making it the predominant form in adulthood. Shown are hippocampus and striatum. (adapted from Romano et al., 1996)

Cellular Ca^{2+} signaling

Both excitable and non-excitable eukaryotic cells rely on intracellular signaling and the ubiquitous cation Ca^{2+} supports many of these processes. Ca^{2+} controls many aspects of cellular function by acting as a diffusible second messenger that allows for communication across the cell. Rises in intracellular Ca^{2+} occurs in specific spatial and temporal patterns that must be received and decoded by cells. Although Ca^{2+} signaling is important in many cell types, the properties controlled by Ca^{2+} are cell-specific, such as the contraction and relaxation of muscles (Carafoli, 2002). To generate the appropriate response to a Ca^{2+} signals, cells are equipped with specific proteins that allow for proper translation and transduction of the Ca^{2+} stimulus.

Cells are incredibly well-equipped to handle calcium and intracellular Ca^{2+} concentrations under basal conditions falls in the 10-50 nM range, but activation of signaling can lead to transient rises

to .5–1 μM or more (Berridge, 2004). These increases can be much higher in the microdomains surrounding points of Ca^{2+} entry (Parekh, 2011). Increases caused by cellular stimulation result in Ca^{2+} entry from extracellular sources or intracellular stores like the endoplasmic or sarcoplasmic reticulum. This entry is balanced and opposed by removal of cytosolic Ca^{2+} by Ca^{2+} -ATPases (transporters), Na^{+}/Ca^{2+} exchangers, and fast-acting Ca^{2+} buffering proteins such as calbindin, calretinin, and parvalbumin (Berridge et al., 2003; Fedrizzi et al., 2008) (Figure 1.5). Abnormal Ca^{2+} regulation and signaling can also lead to pathophysiology (Berridge, 2014; Berridge, 2017) Many tissues in the body rely heavily on Ca^{2+} signaling but this dissertation will focus on neuronal calcium signaling.

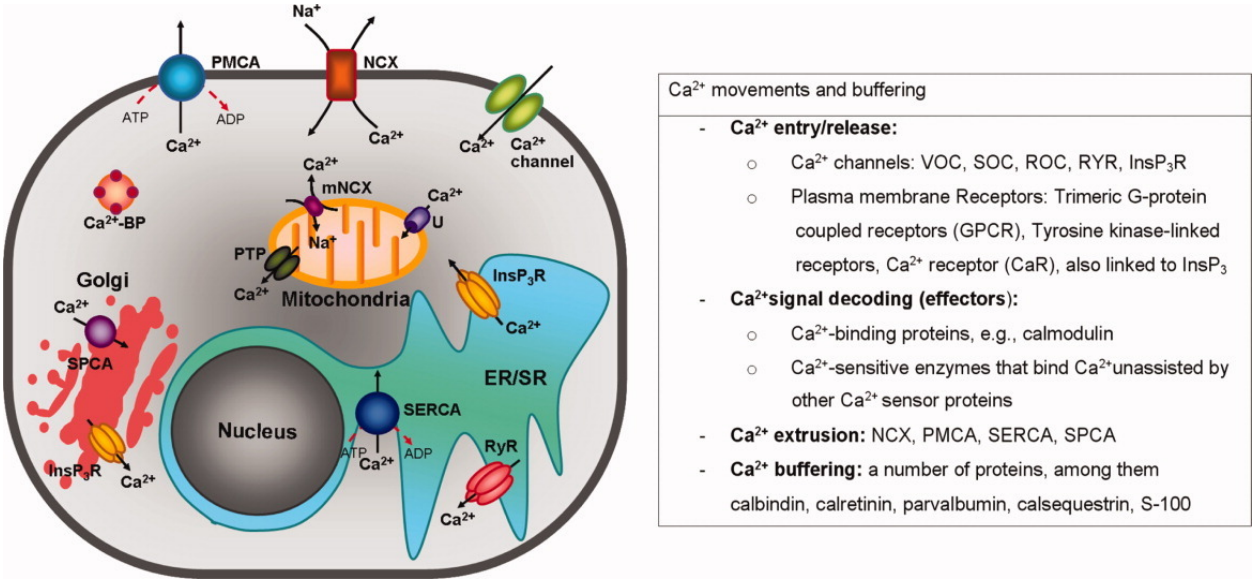

Figure 1.5 *Mechanisms of Ca^{2+} regulation in cells.* Cells display complex mechanisms to control levels of intracellular Ca^{2+}. These include plasma membrane Ca^{2+} channels gated by voltage, ligands or by the emptying of intracellular Ca^{2+} stores on the endoplasmic and sarcoplasmic reticulum, the nuclear membrane, mitochondria, and the Golgi apparatus. Ca^{2+}-binding proteins (Ca^{2+}-BP) such as calmodulin can process the Ca^{2+} signal. (adapted from Fedrizzi et al., 2008).

Calmodulin

Some of the effects of Ca^{2+} are due to Ca^{2+} binding directly to different proteins to cause changes in function, but cells also express a large number of Ca^{2+} sensor proteins. The ubiquitous Ca^{2+} sensor calmodulin (CaM) is present in all eukaryotic cells. CaM is a small, acidic, 17 kDa protein. The monomeric protein is part of the EF-hand family of Ca^{2+}-binding proteins. The structure of CaM is made up of two nearly symmetrical lobes that resemble a dumbbell, but with a flexible joint in the middle (Meador et al., 1992). The Ca^{2+} binding sites are largely unoccupied at the resting cell Ca^{2+} concentrations, but become highly occupied at the higher levels after a stimulus. The four Ca^{2+}-binding EF hands of calmodulin have distinct affinities for Ca^{2+} , but binding of Ca^{2+} to each lobe increases the affinity of other sites and make CaM a unique decoder of Ca^{2+}

signals (Zhang et al., 2012). Binding of Ca^{2+} is associated with a large change in the CaM conformation that leads to the exposure of hydrophobic surfaces within each domain. This conformational change allows CaM's Ca^{2+} sensor activity by allowing it to interact with target proteins (Hoeflich and Ikura, 2002). A number of CaM-binding proteins have been described that regulate different cellular functions such as activation of kinases and modulation of gene expression. In the brain, CaM is important for the synthesis of neurotransmitters and activation of kinases important for synaptic plasticity and it is also and is found enriched in postsynaptic membranes (Sola et al., 2001). The role of CaM in different neuronal cell types can be controlled by variables such as the presence of specific binding partners, expression level, and subcellular localization (Teruel et al., 2000).

Calmodulin-dependent kinases

While Ca^{2+} can directly bind to some kinases like PKC to regulate kinase activation, others are regulated by Ca^{2+} sensors or "transducers". CaM-dependent kinases (CaMKs) are a class of kinase affected by CaM. These kinases alter cellular function by phosphorylation of other proteins at serine or threonine residues in a specific, rapid, and reversible manner. Phosphorylation by CaMKs acts as a signal to control a number of different cellular processes including synaptic plasticity, gene transcription, translation, and apoptosis in response to ligand- or voltage-dependent increases in intracellular Ca^{2+} (Swulius and Waxham, 2008). As the name implies, activation of these kinases is initially dependent on the binding of (Ca^{2+} bound) CaM to the kinases. Some of the CaMKs are capable of becoming Ca^{2+}/CaM-independent after activation or require further modification to become full activated (Soderling and Stull, 2001). These differences in regulation and activation allow for a broad range of cellular functions from these kinases.

Structurally, CaMKs include a catalytic domain that is followed by a regulatory component that harbors both an autoinhibitory domain and a CaM-binding domain. At resting levels of Ca^{2+}, the autoinhibitory domain renders the kinases inactive by inhibiting substrate binding to the kinase, making the catalytic domain inoperative. Increased intracellular Ca^{2+} results in CaM binding to Ca^{2+} allowing for binding to the CaM-binding domain of CaMKs (Hook and Means, 2001). CaM binding releases autoinhibition by disrupting the interaction between the autoinhinitory domain and the catalytic domain, allowing CaMKs to bind to substrates and phosphorylate their substrates.

There are two classes of CaMKs. These are substrate specific CaMKs (CaMKIII, phosphorylase kinase, and the myosin light chain kinases) that only have one known downstream target, and CaMKs (CaMKK, CaMKI, CaMKII and CaMKIV) with multiple downstream targets including other CaMKs. Some CaMKs can phosphorylate themselves in a regulatory event called autophosphorylation. The general consensus sequence for CaMKs is (R-X-X-S/T), but this is not always strictly followed(White et al., 1998). It is clear that the CaMKs have evolved as important regulators of Ca^{2+} signaling within cells. We will focus on the multifunctional CaMKII in this dissertation.

CaMKII

CaMKII is the best-studied member of the CaMK protein family and is the experimental focus of this dissertation. CaMKII phosphorylates Ser/Thr residues in many substrates involved in processes including cardiac function and learning and memory (Erickson, 2014; Shonesy et al., 2014b). Similar to other CaMKs, all of the CaMKII isoforms contain a catalytic and autoregulatory domain. Unlike the other CaMKs, CaMKII forms a holoenzyme because of the presence of an association domain. The native CaMKII holoenzyme is dodecameric, containing two rings of six

subunits. CaMKII is derived from four distinct genes (α, β, γ and δ). CaMKIIα and β are highly expressed brain-specific isoforms that constitute about 1% of total protein in the forebrain (Erondu and Kennedy, 1985).

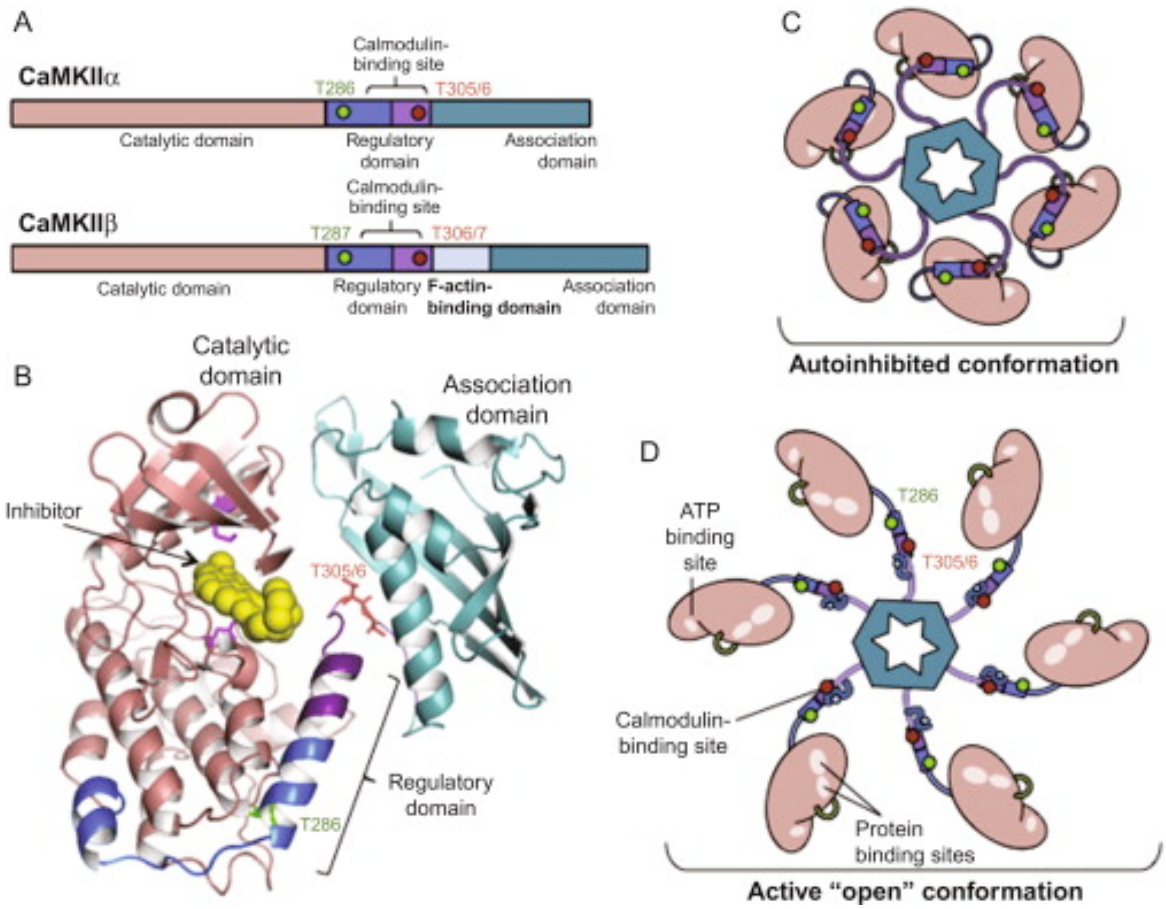

Figure 1.6 *Domain organization and structure of CaMKII.* A. The two major neuronal isoforms of CaMKII, CaMKIIα and CaMKIIβ, have a similar overall domain organization with the exception of an F-actin-binding domain inserted in CaMKIIβ. B. Structure of an individual "autoinhibited" CaMKII subunit. This individual subunit structure was "excised" from a structure (PDB:3SOA) of an intact inactive CaMKIIα holoenzyme structure using PyMol (DeLano Scientific). Domains and key residues in CaMKII were colored as in A. The yellow structure in the center is a CaMKII inhibitor bound in the kinase active site C. Schematic of the compact inactive CaMKII holoenzyme structure. The kinase catalytic domains (pink) decorate the outside of a central hub formed by the association domains (teal), linked by the regulatory domains. Autoinhibitory interactions of the regulatory and catalytic domains hold the kinase domains in a compact closed conformation. For clarity, the illustration includes a single ring, whereas the intact dodecameric holoenzyme consists of a stacked pair of rings. D. Cartoon illustrating the conformation associated with CaMKII activation. Binding of Ca^{2+}/CaM disrupts interactions of the regulatory (blue) and catalytic (pink) domains; the kinase domains swing away from the hub of the holoenzyme in an open conformation such that the active site is accessible to ATP and protein substrates. (From Shonesy et al., 2014).

CaMKII works to decode Ca^{2+} signals through its unique activation properties. During basal conditions, when intracellular Ca^{2+} concentrations are low, CaMKII is inhibited by the interaction of the catalytic domain to the regulatory domain in a state of auto-inhibition that holds the kinase in a compact closed conformation. Binding of Ca^{2+}/CaM to the regulatory domain disrupts the binding interaction between the regulatory and catalytic domains, allowing for the kinase domain to hinge open away from the central hub of the dodecameric structure (Figure 1.6). In this conformation, the CaMKII active site is accessible to ATP and CaMKII binding partners and substrates. The regulatory domain of CaMKII allows for a form of Ca^{2+}/CaM-independent activation that allows prolonged transduction of a Ca^{2+} signal even after levels of intracellular Ca^{2+} have returned to a resting state. To produce Ca^{2+}/CaM-independent activity, CaMKII undergoes an autophosphorylation at Thr286 in CaMKII α (Thr287 in β) (Schworer et al., 1988). This autophosphorylation requires coincident Ca^{2+}/CaM binding between neighboring subunits within a holoenzyme. Individual neighboring subunits act as a kinase as well as a substrate when they are bound to Ca^{2+}/CaM. Autophosphorylation at the Thr286/7 (α/β) residue is both necessary and sufficient for subunits to retain autonomous activity in the absence of Ca^{2+}/CaM (Fong et al., 1989). Phosphorylation of Thr286 increases the affinity of CaMKII for Ca^{2+}/CaM. CaMKII has been referred to as a "memory molecule" because of this ability to transduce Ca^{2+} signals after intracellular Ca^{2+} levels have returned to baseline.

In addition to an autoregulatory phosphorylation site that increases its activity, CaMKII also has a mechanism to reduce its own activation. This is through autophosphorylation at a site within the CaM binding region, Thr305 and/or Thr306 (Colbran and Soderling, 1990) Autophosphorylation at these inhibitory sites block the binding of Ca^{2+}/CaM to the kinase (Colbran, 1993). CaMKII that is sequentially phosphorylated at Thr286 and Thr305 or 306 remains active until Thr286 is

dephosphorylated. Although I have cited a few sources for these findings, many laboratories have contributed to the work surrounding CaMKII autoregulation and this subject has been reviewed in more detail (Andy Hudmon and Howard, 2002; Coultrap and Bayer, 2012; Lisman et al., 2002; Shonesy et al., 2014b).

These autophosphorylation mechanisms have important physiological consequences. This was demonstrated in mice with a phosphoinhibitory mutation of Thr286 to Ala. The inability for Thr286 to be phosphorylated inhibits autonomous CaMKII activity and caused a reduction in CaMKII targeting to the PSD, suggesting the importance of this regulatory site in CaMKII targeting (Gustin et al., 2011). These mice also show a reduction hippocampal LTP (Giese et al., 1998). Behaviorally, these mice displayed cognitive impairments and showed deficiencies in learning and memory tasks (Giese et al., 1998; Gustin et al., 2011).

Autophosphorylation at Thr305/6 has also been shown to be important for synaptic regulation and learning and memory tasks. Knock-in mice expressing phosphoinhibitory mutations at Thr305/306 showed that blocking inhibitory phosphorylation increases CaMKII in the PSD and lowered the threshold for hippocampal LTD. Alternatively, mice harboring mutations that mimicked phosphorylation at these sites showed decreased CaMKII in the PSD, a reduction hippocampal LTD, and learning impairments (Elgersma et al., 2002).

These results demonstrate that activated CaMKII is sufficient to trigger LTP and that CaMKII activation is necessary for the induction of LTP and certain forms of learning. It has been shown that the extent of CaMKII activity is dependent on the frequency and amplitude of Ca^{2+} oscillations. In this way CaMKII can decode Ca^{2+} signals to finely tune synaptic signaling (Bayer et al., 2002; De Koninck and Schulman, 1998; Li et al., 2012).

Calcium signaling in neurons

Proper control of intracellular Ca^{2+} concentrations in neurons is important for a number of different neuronal functions including neurite growth and development, synaptic remodeling, transcriptional regulations, and control of synaptic strength (Brini et al., 2014). There are a number of different surface Ca^{2+} channels that determine both spontaneous and evoked neuronal activity and pre-synaptic Ca^{2+} is important for neurotransmitter release essential for chemical signal transduction at synaptic terminals. Certain induction protocols for both LTP and LTD require intracellular Ca^{2+} increases. Activity-dependent Ca^{2+} signals also create a path for communication that extends to the nucleus of neurons to regulate transcriptional regulators and gene transcription. The effects of Ca^{2+} are diverse in neurons and in many cases seem to be specific to the cell-type and source of calcium.

As discussed earlier, a main mechanism of Group I mGlu signaling occurs through intracellular Ca^{2+} release. This is particularly interesting when examining the differences between $mGlu_1$, which causes a single sustained Ca^{2+} release, and $mGlu_5$ that presents with oscillatory increases in cytosolic Ca^{2+} levels in heterologous cell systems (Dale et al., 2001; Kawabata et al., 1996). Oscillations in Ca^{2+} are observed in many different cells types, suggesting that they represent a universal signaling method. The most simple model of $mGlu_5$ oscillations suggests that $mGlu_5$ activation occurs through activation of PLC, leading to increases in IP_3 concentrations and Ca^{2+} from intracellular stores by activation of the IP3 receptor (IP3R). Byproducts of PLC activation lead to the activation of PKC which can feedback to phosphorylate mGlu5 and cause uncoupling from the G protein. During this period, IP_3 levels fall along with intracellular Ca^{2+} concentrations. Dephosphorylation of the receptor resets the cycle and allows for succeeding oscillations.

(Kawabata et al., 1996). Alterations in the frequency or amplitude of intracellular Ca^{2+} oscillations can activate distinct Ca^{2+}-responsive pathways. This is particularly interesting in the case of CaMKII because it's activity has been shown to be modulated by specific frequencies of Ca^{2+} (De Koninck and Schulman, 1998; Li et al., 2012). Modulation of this system is one way that $mGlu_5$ binding partners can impact signaling downstream of $mGlu_5$.

Mechanisms of synaptic plasticity

Understanding behavior has been a major motivator of neuroscience research. It is thought that experience modifies behavior through modifications of synaptic strength. The study of these processes allow us to progress our understanding of brain regions important for specific behaviors and how disorders can alter the normal transduction of cellular signals. The development of tools to study these mechanisms has allowed us to understand some of the requirements for induction and maintenance of changes in synaptic plasticity. Plasticity can be studied in *in vivo* and *in vitro* preparations. *In vivo* studies are performed by implanting electrodes to stimulate and record synaptic potentials in living animals. Much of our understanding of LTP comes from *in vitro* studies of the hippocampal CA1 pyramidal neurons where transverse slices of the brain are cut and maintained for hours to study synaptic responses (Figure 1.7). I will touch on several aspects of LTP and LTD, but I will largely focus on evidence suggesting the role of Group I mGlu receptors in these processes.

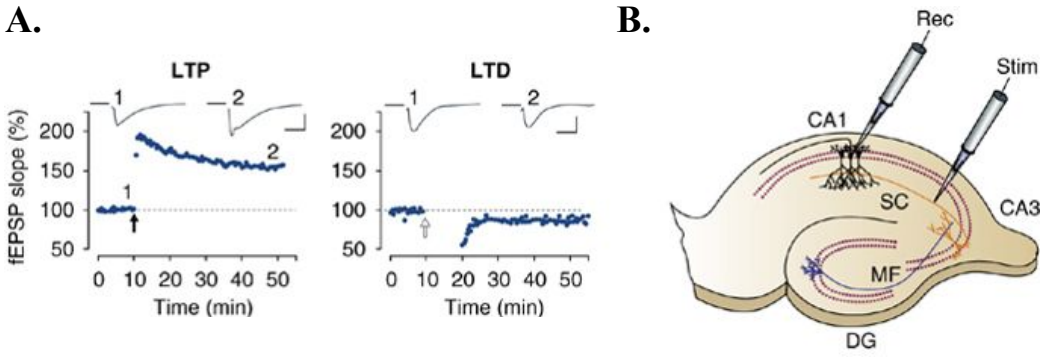

Figure 1.7. *Studying LTP and LTD in vitro.* A. Sample experiments illustrating LTP and LTD in the CA1 region of the hippocampus. Synaptic strength, defined as the initial slope of the field excitatory postsynaptic potential (fEPSP; normalized to baseline) is plotted as a function of time. Left panel demonstrates LTP elicited by high-frequency tetanic stimulation (100 Hz stimulation for 1 s; black arrowhead). Right panel illustrates LTD elicited by low-frequency stimulation (5 Hz stimulation for 3 min given twice with a 3 min interval; open arrow). B. A schematic diagram of the rodent hippocampal slice preparation, demonstrating the CA1 and CA3 regions as well as the dentate gyrus (DG). (SC=Schaffer collateral; MF=mossy fiber). Typical electrode placements for studying synaptic plasticity at Schaffer collateral synapses onto CA1 neurons are indicated (Stim=stimulating electrode; Rec=recording electrode) (Adapted from Citri and Malenka, 2008).

LTP

For the reasons expressed above, LTP has been a major focus of many research studies to understand behavioral outcomes of LTP and how these mechanisms relate to typical and disordered neuronal function. In slice studies, LTP is induced by electrical stimulation of axons and recording of postsynaptic responses in the cells body of CA1 pyramidal neurons (Figure 1.7) (Citri and Malenka, 2008). Several forms of LTP have been described in many brain regions, but the most common form of LTP is induced by applying high frequency stimulation. In CA1 pyramidal cells, this results in a long-lasting LTP that is dependent upon NMDAR postsynaptic increases in intracellular calcium, and depolarization (Collingridge et al., 1983; Dunwiddie and Lynch, 1978; Lynch, 1989).

The involvement of postsynaptic $mGlu_1$ and $mGlu_5$ receptors have also been studied in this process. One group showed a reduced CA1-LTP and impairment of context-specific learning in $mGlu_1$ KO mice (Aiba et al., 1994a), but this reduction in $mGlu_1$-dependent LTP was not observed in a separate study (Conquet et al., 1994). Additionally, a $mGlu_1$ antagonist prevented the induction but not maintenance of LTP (Neyman and Manahan-Vaughan, 2008). *In vitro*, several studies indicate that $mGlu_1$ activation increases the number of functional NMDARs on the postsynaptic membrane (Lan et al., 2001a; Lan et al., 2001b; Skeberdis et al., 2001) While $mGlu_1$ may participate in regulation of LTP, the role of $mGlu_1$ in the induction and maintenance of LTP has not been completely consistent across studies (Francesconi et al., 2004; Naie and Manahan-Vaughan, 2005; Neyman and Manahan-Vaughan, 2008).

The other Group I mGlu receptor, $mGlu_5$, has been highly implicated as a major regulator of hippocampal LTP. Similarly to $mGlu_1$, genetic knockouts of $mGlu_5$ (Jia et al., 1998; Lu et al., 1997) and $mGlu_5$-selective antagonists (Francesconi et al., 2004) impair hippocampal LTP. $mGlu_5$ plays a role in both the induction and maintenance of LTP. Positive modulation of $mGlu_5$ can enhance hippocampal LTP (Ayala et al., 2009) and antagonism of $mGlu_5$ disrupts LTP persistence (Francesconi et al., 2004). Regulation of LTP by $mGlu_5$ activation may be partly attributed to $mGlu_5$ potentiation of NMDAR currents (Fitzjohn et al., 1996; Mannaioni et al., 2001) and the $mGlu_5$ contribution to induction of *de novo* protein synthesis necessary for LTP (Deadwyler et al., 1987; Francesconi et al., 2004; Frey et al., 1988; Stanton and Sarvey, 1984). The potentiation of NMDAR currents by $mGlu_5$ activation has been well studied. For instance, in hippocampal neurons, positive modulators of $mGlu_5$ in the presence of a subthreshold agonist concentrations can potentiate NMDAR currents, phosphorylation, and LTP (Liu et al., 2006; O'Brien et al., 2004; Rosenbrock et al., 2010). The regulation of this process differs across distinct brain regions For

instance, co-activation of NMDARs and $mGlu_5$ is required for the enhancement of NMDAR currents and a long lasting potentiation of excitatory synaptic transmission in hippocampal cultures (Kotecha et al., 2003). In CA3 pyramidal cells, activation of $mGlu_5$-mediated enhancement of NMDAR currents required G-protein activation, PKC, and Src while $mGlu_1$ potentiated NMDAR currents via a G-protein-independent mechanism involving Src kinase activation (Benquet et al., 2002). In cortical neurons, activation of $mGlu_1$ induced enhanced NMDAR currents that were dependent on the activation of Src-family kinases and independent of PKC (Heidinger et al., 2002). In contrast, hypothalamic melanin-concentrating hormone neurons demonstrated a PKA and PKC activity-dependent DHPG-induced enhancement of NMDA-evoked currents (Huang and van den Pol, 2007).

CaMKII in LTP

Although several different proteins are important in LTP, CaMKII has been studied as a necessary mediator of this process. Genetic mutations of CaMKII to inhibit proper activation (Giese et al., 1998; Silva et al., 1992) or use of peptides to inhibit CaMKII (Malinow et al., 1989) can block LTP. These reductions in LTP coexist with deficits in learning and memory in these mice. Increasing CaMKII activity can also increase synaptic strength (Lledo et al., 1995; Pettit et al., 1994). These studies have strengthed the role of CaMKII as a mediator of LTP and a memory molecule.

During the induction of LTP, Ca^{2+} entry into the postsynaptic cells occurs largely through NMDARs induced by the presence of glutamate and concurrent strong postsynaptic depolarization. Once activated by calcium entry through NMDARs, CaMKII is translocated from the cytoplasm to the synapse (Leonard et al., 1999; Shen and Meyer, 1999; Strack and Colbran,

1998; Strack et al., 2000). CaMKII is capable of binding to the GluN2B subunit of the NMDAR (Leonard et al., 1999; Strack and Colbran, 1998). The CaMKII-GluN2B complex is largely increased after NMDA activation in the hippocampus and this interaction is important for CaMKII translocation, LTP, and learning and memory. A GluN2B mutant that does not bind CaMKII or overexpression of the CTD of GluN2B reduces LTP and causes learning deficits (Barria and Malinow, 2005; Zhou et al., 2007).

CaMKII can phosphorylate the GluN2B at Ser1303 (Omkumar et al., 1996) which enhances NMDAR desensitization (Sessoms-Sikes et al., 2005; Tavalin and Colbran, 2017) and causes a reduction in the CaMKII-NMDAR complex (Raveendran et al., 2009; Strack et al., 2000). CaMKII binding to GluN2B also increases CaMKII affinity for ATP, increases the catalytic parameters of CaMKII and locks the kinase in an active conformation that allows for prolonged formation of the NMDAR-CaMKII for times exceeding 60 minutes (Bayer et al., 2001; Bayer et al., 2006; Cheriyan et al., 2011; Pradeep et al., 2009). This process is thought to be important for the maintenance of LTP because it was shown that CaMKII inhibitors that block the CaMKII interaction with the NMDAR can reverse saturated LTP (Sanhueza et al., 2011). This suggests that, in addition to the catalytic action of CaMKII, there is a structural component of CaMKII in the PSD that is important for sustaining LTP (Hell, 2014). This is partly due to the many CaMKII-associated proteins (CaMKAPs) present in the PSD including some structural scaffolding proteins like Densin-180, spinophillin, SAP-97, and α -actinin (Baucum et al., 2010; Jalan-Sakrikar et al., 2012; Jiao et al., 2011; Nikandrova et al., 2010).

The role of CaMKII in LTP has also been well-studied in its relationship to AMPA receptor signaling. LTP is dependent on the number and function of AMPA receptors within the synapse.

AMPA receptor subunits GluA1 and GluA2 heteromeric complexes constitute the majority of AMPA receptors in the synapse (Lu et al., 2009). CaMKII phosphorylation of GluA1 at Ser831 enhances AMPAR conductance and has been shown to be increased after the induction of LTP (Barria et al., 1997; Benke et al., 1998; Derkach et al., 1999). Phosphoinhibitory Ser to Ala mutations at Ser831 and the PKC site Ser845 reduced LTP, LTD, and disrupted performance in learning and memory tasks (Lee et al., 2003). In agreement with this data, mice with a phosphomimetic mutation at residue 831 (Ser/Asp) showed increased channel conductance by improving channel efficiency (Kristensen et al., 2011).

Other laboratories have questioned the importance of Ser831 in LTP because it was demonstrated that normal LTP could be established in neurons with an AMPAR that lacked the Ser831 harboring C-tail (Granger et al., 2013). Further, using a method that allows for the stoichiometric analysis of phosphorylation sites, it was shown that less than 1% of GluA1 subunits were phosphorylated at Ser831 in the hippocampus after LTP induction or learning (Hosokawa et al., 2015), but this was quickly refuted by other work showing that phosphorylated AMPARs represent 12-50% of receptors (Diering et al., 2016) . CaMKII phosphorylation of the auxiliary protein Stargazin is also necessary for these effects because it causes an increase in AMPAR retention in the PSD (Opazo et al., 2010; Sumioka et al., 2010). Because GluA1 phosphorylation has been largely investigated, but mutation or deletion of these residues has failed to completely inhibit the induction of LTP, new hypotheses that incorporate other mechanisms into LTP expression are being created and tested (Herring and Nicoll, 2016).

LTD

Understanding the bidirectionality of synaptic plasticity was an important advance in the study of synaptic physiology. A form of LTD dependent on the activity of the NMDAR was reported in hippocampal CA1 pyramidal cells (Dudek and Bear, 1992). This supported the idea that memories and experiences can be encoded by the brain through the weight of synaptic strength and showed that neuronal activity is able to be downregulated in response to activity. NMDAR LTD was the first to be established but other forms of LTD exist. We will focus on the role of $mGlu_{1/5}$ in plasticity in this section.

Group I mGlu receptor LTD

Although Group I mGlu receptors have been shown to facilitate both LTP and LTD, mGlu receptor-LTD of excitatory synapses is the most well characterized form of synaptic regulation by these receptors. Group I mGlu receptors can trigger LTD in a number of different brain regions including the hippocampus, cerebellum, and dorsal and ventral striatum (Bellone and Luscher, 2005; Gladding et al., 2009; Grueter et al., 2006; Gubellini et al., 2001; Huang et al., 2011; Kano et al., 2008; Palmer et al., 1997; Sung et al., 2001). $mGlu_1$ and $mGlu_5$ have long been implicated in multiple forms of LTD that require new protein synthesis (Huber et al., 2001a; Oliet et al., 1997; Palmer et al., 1997) or increased endocannabinoid signaling (Luscher and Huber, 2010).

$mGlu_{1/5}$ receptor-mediated LTD was first characterized at parallel fiber-Purkinje cell synapses of the cerebellum (Aiba et al., 1994b; Linden et al., 1991; Shigemoto et al., 1994). $mGlu_1$ is the primary Group I receptor expressed in cerebellar purkinje cells and is responsible for cerebellar mGlu receptor-LTD, (Kano et al., 2008). However, in other brain regions, deciphering the

individual impact of $mGlu_{1/5}$ in LTD has proven difficult; however there have been some differences reported on the role of each individual receptor in synaptic plasticity. For instance, in hippocampus, $mGlu_1$ increases the frequency of spontaneous inhibitory post-synaptic currents while $mGlu_5$ potentiates NMDAR currents (Mannaioni et al., 2001). Additionally, antagonism of $mGlu_1$ in the hippocampus results in a complete blockade of LTD initiation but not maintenance (Mannaioni et al., 2001). In contrast, blocking $mGlu_5$ signaling blocked both LTD induction and the persistence of LTD. Positive modulation of $mGlu_5$ can also potentiate mGlu receptor-LTD induced chemically or electrically (Ayala et al., 2009). It is not always easy to determine the individual roles of these receptors. For example, only the combination of an $mGlu_1$and an $mGlu_5$ antagonist completely abolish LTD in the hippocampus (Volk et al., 2006).

Mice lacking $mGlu_1$ or $mGlu_5$ have also been used to investigate the roles of these receptors individually. While $mGlu_5$ KO mice show an absence of LTD, $mGlu_1$ KO mice have reduced hippocampal LTD, suggesting both $mGlu_1$ and $mGlu_5$ play a role in this process (Huber et al., 2001a; Volk et al., 2006). Because of their perisynaptic localization, $mGlu_{1/5}$ are activated when excess glutamate spills out of the synaptic cleft. Electrical induction protocols can cause a fraction of synapses to express LTD, but many laboratories have used chemical induction methods to induce a more reliable form of chemically-induced LTD. Dihydroxyphenylglycine (DHPG) activates both $mGlu_1$ and $mGlu_5$ and has been a common drug used to investigate $mGlu_{1/5}$ LTD. DHPG-LTD is thought involve similar expression mechanism to paired pulse lower frequency stimulation (PP-LFS) LTD electrically induced by 900 paired stimulations at 1 Hz ms, because the two forms of LTD occlude one another (Huber et al., 2001b). Pharmacological experiments and receptor KO mice indicate that DHPG-LTD depends principally on $mGlu_5$ receptor activation, although the $mGlu_1$ receptor may also have a role in LTD induction (Fitzjohn et al., 2001).

An important feature of hippocampal $mGlu_{1/5}$ LTD is that is independent of NMDAR activation (Oliet et al., 1997). After the induction of NMDAR LTD, further synaptic depression can be induced with DHPG, suggesting two distinct mechanisms of induction (Fitzjohn et al., 1999). However, this is not true in all brain areas or across all age ranges. For instance both NMDAR and $mGlu_{1/5}$ activation is required for LTD in the amygdala (Wang and Gean, 1999). In the hippocampus, a LFS-induced LTD that is absent in adults but is observed in animals less than postnatal day 21 (Kemp et al., 2000). PP-LFS LTD can be induced at all ages, although the role of NMDAR activation in this process only seems to be required in young animals, and unnecessary in adult animals (Kemp et al., 2000). A number of regulators including scaffolding proteins, kinases, and post-translational modifications regulate $mGlu_{1/5}$ receptor control of synaptic strength. Differences in $mGlu_{1/5}$ expression and signaling can lead to neuropathologies and effects on cognition.

Post-synaptic expression of $mGlu_{1/5}$-LTD requires rapid translation of proteins in many brain regions, including cerebellar Purkinje cells and the hippocampus (Costa-Mattioli et al., 2009; Hou and Klann, 2004; Huber et al., 2000; Kano et al., 2008; Yin et al., 2006). Translation of a number of different proteins ultimately lead to the internalization of post-synaptic AMPARs and a persistent reduction in synaptic strength (Bellone and Luscher, 2005; Snyder et al., 2001; Waung et al., 2008). This hippocampal DHPG-induced LTD has been shown to be Ca^{2+} independent (Fitzjohn et al., 2001) in some cases and to require Ca^{2+} in others (Holbro et al., 2009).

$mGlu_{1/5}$ receptor activation-dependent local mRNA translation became a major interest to many groups studying autism when it was shown that DHPG-induced LTD is enhanced in a mouse that lacks functional fragile X mental retardation protein (FMRP). The FMRP KO mouse is a genetic

model of Fragile-X syndrome (FXS), an X-linked form of mental retardation. FMRP works as a repressor of translation and has been hypothesized that the absence of FMRP leads to enhanced $mGlu_5$ signaling, overactive protein translation, and enhancement of $mGlu_5$-LTD (Dolen and Bear, 2008). Increases in LTD lead to weaker synapses and more immature dendritic spines, which may play a role in the disease phenotypes.

Understanding the production of new proteins at the synapse during LTD is key to understanding molecular mechanisms of this process in healthy individuals and during disease processes. The rapid synthesis of new proteins by the activation of $mGlu_5$ receptors occurs locally and relies on a number of downstream mechanisms that lead to the internalization of postsynaptic AMPARs (Huber et al., 2000; Huber et al., 2001b; Snyder et al., 2001).

One protein that has been shown to be important in this process is activity-regulated cytoskeleton-associated protein (Arc/Arg3.1*)*. mGlu receptor activation causes a rapid translation of Arc in dendrites and a genetic reduction of Arc blocks $mGlu_{1/5}$-dependent LTD (Park et al., 2008; Waung et al., 2008). Arc increases AMPAR endocytosis through interactions with endocytic machinery (Chowdhury et al., 2006). Other proteins that are rapidly translated following Group I mGlu receptor activation have been shown to be important in this process. It has been shown that dephosphorylation of the AMPAR GluR2 is necessary for Group I mGlu receptor LTD (Moult et al., 2006). Striatal-enriched protein tyrosine phosphatase (STEP) was identified as the protein phosphatase linked to mGlu receptor-driven AMPAR endocytosis (Zhang et al., 2008). DHPG triggers translation of STEP, and inhibition of STEP activity blocks $mGlu_{1/5}$-LTD (Zhang et al., 2008). These two examples show that translation induced by mGlu receptor activity stimulates

protein expression important for AMPAR endocytosis required for the persistence of $mGlu_{1/5}$-LTD.

The signaling mechanisms important to this process include the activation of the ERK1/2 pathway and the MAPK pathways. Activation of these pathways leads to the activation of transcription factors and the mTOR pathway (Ronesi and Huber, 2008a; Ronesi and Huber, 2008b; Waung et al., 2008). Highlighting the importance of this process, mGlu receptor-LTD in the hippocampus requires both the ERK and PI3K-mTOR pathway (Gallagher et al., 2004; Hou and Klann, 2004).

CaMKII in LTD

Although CaMKII has been well studied in LTP, there is also evidence for the role of CaMKII in LTD. Both $mGlu_{1/5}$- and NMDAR- dependent LTD induce CaMKII autophosphorylation at Thr286 (Marsden et al., 2010; Mockett et al., 2011). It was shown that autonomous CaMKII could cause both LTP or LTD depending on the phosphorylation status of the inhibitory CaMKII phosphorylation sites Thr305/306 (Pi et al., 2010). As discussed above, CaMKIIα translocates to excitatory synapses following NMDAR stimulation. Interestingly, it was shown that CaMKIIα translocates to inhibitory but not excitatory synapses in response to more moderate stimulus that enhances inhibitory transmission (Marsden et al., 2010). Stronger glutamatergic stimulation, coupled to AMPA receptor, insertion led to Thr286 autophosphorylation of CaMKII but accumulation of CaMKII at inhibitory synapses was prevented under these conditions by a phosphatase, PP2B/calcineurin (CaN). This preferential targeting of CaMKIIα directed by the strength of glutamatergic stimulation shows the dynamic capabilities of CaMKII to provide neurons with a mechanism to selectively potentiate excitation or inhibition (Marsden et al., 2010). The internalization of AMPARs necessary for some forms of LTD also seems to be a mechanism

that can be regulated by CaMKII. Phosphorylation of a CaMKII site on the first intracellular loop of GluA1 (Ser567) reduces localization of CaMKII to dendritic spines (Woolfrey et al., 2018).

The autoregulatory mechanisms of CaMKII allow for tight control of CaMKII activity in neurons. CaMKII is capable of decoding frequencies into different levels of activation (Andy Hudmon and Howard, 2002; De Koninck and Schulman, 1998; Hanson et al., 1994; Romano et al., 2017). Some stimulations can bias activation of CaN over activation of CaMKII (Li et al., 2012). This type of regulation by Ca^{2+} frequencies is also decoded into specificity of gene expression (Dolmetsch et al., 1998). This type of regulation of protein activation by differing levels of Ca^{2+} influx is likely important to how CaMKII contributes to the induction of both LTP and LTD.

Group I mGlu receptors in selected neurological disorders

Group I mGlu receptors have been a major area of focus in neuronal disorders because of the many roles that they play. There has been evidence showing that abnormal expression, signaling and function of $mGlu_{1/5}$ in the pathophysiology of a number of neuronal disorders.

Group I mGlu receptors play an important role in neurogenesis and proper synaptic neuronal development (Castiglione et al., 2008; Catania et al., 2001; Copani et al., 1998; Di Giorgi Gerevini et al., 2004) and therefore $mGlu_5$ dysregulation has been a target of study for developmental disorders such as autism spectrum disorders (D'Antoni et al., 2014). Studies have shown mutations of Group I mGlu receptors in schizophrenic patients and $mGlu_1$-KO and $mGlu_5$-KO mice exhibit schizophrenic behaviors (Ayala et al., 2009; Ayoub et al., 2012; Brody et al., 2003; Brody et al., 2004; Cho et al., 2014). Because of the role of these receptors on synaptic plasticity, aberrant signaling can also lead to synaptic adaptations that underlie pathological behavioral changes such

as addiction. It seems that Group I mGlu receptors may also play a role in neuronal disorders associated with aging such as Parkinson's disease, and Alzheimer's disease, and Huntington's disease (Kumar et al., 2015; Litim et al., 2017; Ribeiro et al., 2010).

Here, I will discuss a few neurological disorders including Fragile X Syndrome, addiction, and Parkinson's Disease in more detail. I felt that these three disorders exhibit the role of Group I mGlu receptors in mediating developmental disorders, dysregulation of synaptic plasticity, and a disease associated with the aging brain. There are many other disorders that I have not covered in detail including some that have been a major focus for many laboratories, such as schizophrenia, obsessive compulsive disorder, Alzheimer's disease, and epilepsy (Kumar et al., 2015; Ngomba and van Luijtelaar, 2018; Stansley and Conn, 2018).

Fragile X Syndrome

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability and autism spectrum disorder. FXS patients often present with severe phenotypes including hyperactivity, anxiety, and seizures. FXS is a X-linked disorder associated with trinucleotide repeat expansion in the Fragile X mental retardation 1 (*FMR1*) gene. This mutation leads to a silencing of Fragile X mental retardation protein (FMRP) (Bear, 2005). FMRP is a repressor of mRNA translation and a reduction of FMRP leads to excess protein synthesis. Many of the proteins regulated by FMRP are involved in the regulation of synaptic strength and signaling. Altered regulation of these processes are thought to cause many of the phenotypes associated with FXS.

The *Fmr1* KO mouse has been extensively used as an animal model of FXS. The role of Group I mGlu receptors in FXS was recognized because *FMR1* KO mice show enhanced mGlu receptor-

mediated LTD (DHPG-LTD) in the hippocampus (Huber et al., 2002). DHPG-LTD requires protein translation, and under normal conditions FMRP suppresses mRNAs implicated in this reduction in synaptic strength. Activation of Group I mGlu receptors typically leads to the degradation of FMRP (Nalavadi et al., 2012). This leads to increased local protein translation of proteins necessary for LTD (Niere et al., 2012). Excessive protein translation in the absence of FMRP, as in FXS, leads to higher levels of protein synthesis of $mGlu_{1/5}$ regulated proteins and enhanced LTD.

The evidence supporting the role of $mGlu_5$ in FXS models is robust. Reducing $mGlu_5$ expression or the use of $mGlu_5$ antagonists/NAMs has been shown to reverse some of the behavioral and synaptic phenotypes of FMRP KO mice including improvements in learning deficits, normalizing DHPG-induced LTD, reductions in audiogenic seizures, corrections in aberrant dendritic spine density, and overactive ERK and mTOR signaling (Dolen et al., 2007; Michalon et al., 2014; Michalon et al., 2012; Thomas et al., 2012). Because of these data, the $mGlu_5$ theory of FXS has been reviewed by multiple authors (Bear et al., 2004; Dolen and Bear, 2008; Ronesi and Huber, 2008b; Scharf et al., 2015; Waung and Huber, 2009).

Translating these findings to human trials has not been as successful as was expected considering the dramatic effects of $mGlu_5$ antagonism in animal models of FXS (Ligsay and Hagerman, 2016). The failure of drugs targeting $mGlu_5$ in clinical trials has raised questions about the validity of the *Frm1* KO mouse model of FXS as well as the outcome of the clinical trials because of small patient populations, behavioral measurement protocols, and broad age-range of patients (Gomez-Mancilla et al., 2014). The production of new $mGlu_5$ NAMs and the investigation of targets downstream of $mGlu_5$ activation is ongoing for FXS treatment.

Addiction

Addiction to different drugs of abuse is a chronic relapsing disorder that can lead to a number of deleterious effects on health. The reinforcing and rewarding effects associated with the initial onset of drug use drive further abuse of substances and chronic use despite negative consequences. Addiction research has led to the hypothesis that addictive drugs and the experience of their use induce synaptic adaptations that underlie behavioral changes. Addiction studies support that drug-evoked changes in synaptic plasticity are driven by alteration of glutamatergic signaling in the mesocorticolimbic dopamine system. Addiction circuity and drug-evoked synaptic plasticity have been well-studied and summarized by a number of other groups (Cleva et al., 2010; Grueter et al., 2012; Joffe et al., 2014; Luscher and Malenka, 2011; Nestler, 2004; Vranjkovic et al., 2017; Wolf, 2016). Because of the data on the importance of Group I mGlu receptors in the addiction process, the production of allosteric modulators for these receptors have been a focus of study (Caprioli et al., 2018).

Both $mGlu_1$ and $mGlu_5$ have been implicated in addiction and have been shown to modulate addictive behavior in a number of ways with different drugs of abuse. Studies have used both pharmacological and genetic manipulations to study the role of these receptors. The role of $mGlu_5$ in addiction was realized when it was shown that $mGlu_5$-KO mice do not self-administer cocaine and have no cocaine-induced increase in locomotor activity (Chiamulera et al., 2001). Additionally, Group I mGlu receptor-LTD is disrupted by cocaine administration (Grueter et al., 2006) and $mGlu_5$ null mice do not display cocaine-induced synaptic potentiation (Bird et al., 2010).

Behavioral assays have been developed to determine the desire for drug seeking in animal models. Conditioned place preference (CPP) is a behavioral test used to measure the reinforcing properties

of drugs where a drug of abuse or a control is paired with a conditioned stimulus (context or location). After training to associate drug with the given context, the animal will demonstrate an increased amount of time spent with the conditioned stimulus. From this behavioral test, researchers can test the acquisition, expression, and reinstatement of CPP. Antagonism of $mGlu_5$ decreases cocaine self-administration and drug seeking behavior (Amato et al., 2013; Keck et al., 2014; Kenny et al., 2003) and acquisition of a cocaine CPP in mice (McGeehan and Olive, 2003). Pharmacological inhibition of $mGlu_5$ also reduced cocaine self-administration and reinstatement of drug seeking behavior in monkeys (Lee et al., 2005).

In alcohol addiction studies, a selective $mGlu_1$ antagonist decreased alcohol CPP and seizures associated with alcohol withdrawal (Kotlinska et al., 2011). Genetic deletion of $mGlu_5$ resulted in reduced alcohol consumption and CPP, but exhibited increased sensitivity to alcohol (Bird et al., 2008). Multiple studies have looked at the effect of blocking $mGlu_5$ with specific antagonists, demonstrating that inhibition of $mGlu_5$ has many desirable effects including reducing alcohol self-administration, seeking, reinstatement, CPP to alcohol, seizures, and anxiety associated with alcohol withdrawal (Backstrom et al., 2004; Besheer et al., 2008; Blednov and Harris, 2008; Cowen et al., 2005; Hodge et al., 2006; Kotlinska and Bochenski, 2008; Kotlinska et al., 2011; Kumar et al., 2013; McMillen et al., 2005; Olive et al., 2005; Schroeder et al., 2005; Sinclair et al., 2012). By altering the timing of $mGlu_5$ antagonist administration it was reported that $mGlu_5$ was important in the expression and reinstatement of alcohol CPP but not in alcohol CPP acquisition (Lee et al., 2016). Additionally, Group I LTD is absent in the hippocampus of chronic intermittent alcohol-exposed mice (Wills et al., 2017). Blocking Group I mGlu receptors may have positive effects on the addictive properties of alcohol by normalizing changes in synaptic plasticity developed during the development of dependence. A better understanding of the molecular

mechanisms and synaptic alterations that occur provide insight into how these drugs can overcome normal learning to produce harmful behavior and pathology.

Parkinson's Disease

Parkinson's Disease (PD) is a neurodegenerative disorder that results in a loss of dopaminergic neurons in the basal ganglia (BG). The BG network is important for the proper control of voluntary movements and other cognitive processes, but the imbalance of dopaminergic signaling and excessive activity of glutamatergic neurons contribute to the symptoms that are associated with PD including the characteristic motor complications (bradykinesia, rigidity, and tremor) and cognitive deficits and neuropsychiatric disorders such as depression, anxiety, and psychosis (Amalric, 2015; Chaudhuri et al., 2011). The classical treatment of PD to alleviate symptoms is l-3,4-dihydroxyphenylalanine (l-DOPA), the precursor to dopamine, that provides beneficial treatment of motor deficits. Treatment with l-DOPA becomes less efficatious over time and leads to abnormal involuntary movements, termed l-DOPA-induced dyskinesias (LID) after 5–10 years of treatment. Because of the problems with targeting the dopaminergic network, many groups have studied treatments that could improve symptoms of PD patients by targeting the overactive glutamatergic system. The modulatory role of mGlu receptors on glutamatergic signaling and their expression in the BG made these receptors an attractive target for PD (Rouse et al., 2000). Research has focused on finding better and more long-term solutions for the treatment of PD.

Animal models of PD have been used to study the loss of mid-brain dopamine neurons seen in human patients. A common experimental model is using 6-OH-dopamine as a neurotoxin to selectively target and kill dopamine neurons. This model is used in rodents and monkeys who display many of the motor phenotypes seen in human patients. Increases in $mGlu_5$ expression are

also observed in the striatum of human PD patients and a model of PD monkeys (Morin et al., 2013; Ouattara et al., 2010; Ouattara et al., 2011). A number of recent reviews have summarized the work that has been done revolving around Group I metabotropic receptors in PD (Amalric, 2015; Dickerson and Conn, 2012; Gasparini et al., 2013; Litim et al., 2017).

The absence of Group I mGlu-LTD in the striatum of PD models also made Group I mGlu receptors an exciting target (Kreitzer and Malenka, 2007). Many different studies have found that blockade of $mGlu_5$ in models of PD are helpful for a number of different phenotypes including restoration of proper synaptic plasticity, dopaminergic degradation, working memory and motor performance (Black et al., 2010; Chen et al., 2011; De Leonibus et al., 2009; Gregoire et al., 2011; Hsieh et al., 2012; Kreitzer and Malenka, 2007; Mela et al., 2007; Morin et al., 2013). Despite the preclinical promise of $mGlu_5$ antagonists, the effectiveness of $mGlu_5$ antagonists in human patients have yielded mixed results (Wang et al., 2018). More studies on this treatment need to be conducted because of a high degree of variation in the treatments and low statistical power currently reported in the literature.

Regulation of Group I mGlu receptors by interacting partners

The intracellular C-terminal domains (CTDs) of $mGlu_1$ and $mGlu_5$ have emerged as important loci for regulation by protein binding and phosphorylation (Enz, 2012; Mao et al., 2008b; Mao and Wang, 2016). While there are two splice variants of $mGlu_5$ ($mGlu_{5a}$ and $mGlu_{5b}$), most studies have focused on $mGlu_{5a}$. The CTD of Group I mGlu receptors has been shown to be phosphorylated by kinases and bind to a number of different proteins to regulate cell surface localization, expression, and intracellular signaling of the receptor.

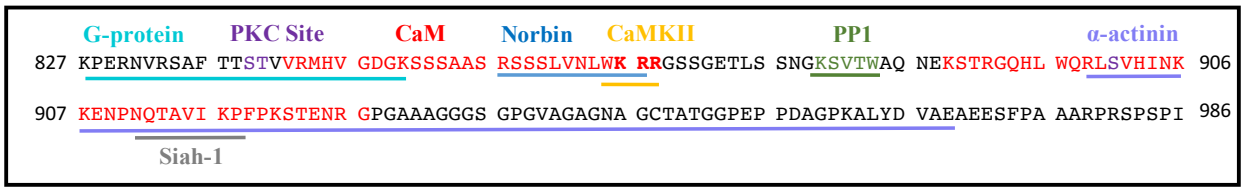

Figure 1.8 *Known mGlu$_5$-CTD binding partners.* Sequence of the N-terminal region of the mGlu$_{5a}$-CTD with marked protein binding regions. PKC phosphorylation sites are marked with purple text. CaM binding sites are marked in red. All other proteins are labeled with a bar corresponding to the color of the text above. A CaMKII binding site was identified in these studies (residues KRR 866-868). Not shown is Homer which binds to residues 1124-1127 or calcineurin (CaN) which for which a binding region has not been identified. Citations for these interactions are distributed throughout the text.

Group I mGlu-CTDs contain binding sites for heterotrimeric G-proteins, kinases, and phosphatases, that can bind directly or indirectly to ion channels, receptors, and scaffolding proteins important for receptor signaling (Figure 1.8). Interacting proteins affect mGlu$_{1/5}$ receptor characteristics including G protein-coupling, receptor localization, and modulation of multiple signaling pathways. Sometimes these proteins occupy overlapping binding domains, suggesting that there is a high degree of regulation of these binding sites. In some cases, proteins compete for binding to induce regulatory control of mGlu$_{1/5}$ signaling.

Group I mGlu receptor binding proteins

Homer

One of the first proteins that was reported to bind to Group I mGlu receptors was Homer (Brakeman et al., 1997). Homer has been a large focus of mGlu receptor regulation because of its importance in synaptic regulation (Fagni et al., 2002). Homer proteins are scaffolding proteins

present in the PSD and mainly localized at glutamatergic excitatory synapses. Synaptic scaffolding proteins like Homer are critical to proper synaptic function because they facilitate interactions by dynamically controlling the localization of important regulators of synaptic transmission (Saito et al., 2002). Improper assembly of mGlu receptor macro protein complexes can lead to impaired signal transduction and neurological disorders (Fagni, 2012).

Homer is produced by 3 genes (Homer1, 2, and 3) that give rise to a number of different splice variants (Shiraishi-Yamaguchi and Furuichi, 2007). Much of the work with Group I receptors has focused on Homer1. The Homer1 gene gives rise to 3 major isoforms Homer1a, b, and c. Homer1b and Homer1c are longer isoforms that have a C-terminal coiled-coil domain that allows for these proteins to oligomerize. Homer1a serves as a dominant negative form that is incapable of forming oligomers (Xiao et al., 1998). The N-terminus of both short and long Homer isoforms interacts with a consensus binding motif (PPxxF) present on the cytosolic C-terminus of $mGlu_1$ and $mGlu_5$ receptors. mGlu receptors bind indirectly to Shank and the IP_3 and ryanodine receptors through oligomeric Homer complexes to control intracellular Ca^{2+} release from the endoplasmic reticulum (Feng et al., 2002; Tu et al., 1999; Tu et al., 1998). Homer proteins therefore confine a number of proteins that control the release of Ca^{2+} from intracellular stores in close proximity to tightly control the intracellular Ca^{2+} release. Homer1a expression is upregulated in response to specific forms of synaptic activity, while other Homer isoforms are constitutively expressed (Brakeman et al., 1997). Because this immediate early gene form of Homer1 is unable to oligermerize, increases in expression of Homer1a disrupt the multi-protein postsynaptic complexes connected through the longer Homer isoforms (Kammermeier and Worley, 2007). Homer1a disruption of oligomerized Homer results in a reduction in $mGlu_5$ coupling to postsynaptic signaling effectors (i.e. changing Ca^{2+} responses and activation of ERK1/2) without large changes in receptor distribution

(Kammermeier and Worley, 2007).

In addition to being present on the CTD of $mGlu_{1/5}$, IP3Rs, and ryanodine receptors, the Homer binding motif (PPxxF) is also present on a number of different proteins that can control intracellular Ca^{2+} signaling including the transient receptor-channels 1 and 4 (Yuan et al., 2003), the $Ca_v2.1$ subunit of voltage-gated Ca^{2+} channels (Worley et al., 2007), adrenergic receptor subtypes, dynamin 3 (Gray et al., 2003), and Shank (Tu et al., 1999). In this way, Homer facilitates proper localization and control of the synaptic localization of many Ca^{2+} effectors . $mGlu_{1a}$, as well as both isoforms of $mGlu_5$, show constitutive activity when expressed in heterologous cells, but this is not the case in neurons (Ango et al., 2001). It is hypothesized that the macro-complexes formed with Homer bound to mGlu receptors could create physical restrictions to the mGlu receptor CTDs to prevent agonist-independent activity of the receptor during basal activity (Chung and Kim, 2017). Different forms of Homer seem to play different roles tightly controlled by this synaptic scaffolding protein. While this is a general overview of the function of Homer on Group I mGlu receptors, Homer binding to mGlu receptors does not affect mGlu receptor signaling uniformly across brain regions and cell-types.

Calmodulin

A critical distinction between $mGlu_1$ and $mGlu_5$ is that only $mGlu_5$ is capable of binding calmodulin (CaM). This additional binding site on $mGlu_5$ has revealed a molecular mechanism for functional differences in these two highly homologous receptors (Choi et al., 2011). CaM has been a very interesting $mGlu_5$ regulator because of its Ca^{2+}-dependence. CaM facilitates a number of Ca^{2+}-dependent processes because it is a calcium-binding messenger protein activated by the presence of intracellular Ca^{2+}. CaM binding to the $mGlu_5$-CTD promotes $mGlu_5$ surface expression

in heterologous cells. CaM competes with PKC phosphorylation of the receptor to regulate surface expression (Lee et al., 2008; Minakami et al., 1997). The binding site for CaM also overlaps with other regulators of $mGlu_5$ surface expression, including CaMKII and the E3 ligase Siah-1a (Jin et al., 2013b; Ko et al., 2012). It seems that tight regulation of this portion of the $mGlu_5$-CTD is important because of the multiple proteins that have been shown to bind in this region. It's possible that CaM and other Ca^{2+} sensitive proteins like CaMKII can regulate the binding of other partners to $mGlu_5$ to accurately control phosphorylation of the receptor.

The importance of CaM in $mGlu_5$ function was recently expanded when it was shown that CaM activity is required for $mGlu_5$-mediated ERK1/2 activation, Arc expression, and LTD in the hippocampus (Sethna et al., 2016). $mGlu_5$ is not the only mGlu receptor that has been shown to be regulated by CaM. A lot of work has also been done to study the role of CaM on the regulation of $mGlu_7$ (Fagni et al., 2004; Nakajima et al., 1999). These studies may also further our insight into the role of CaM in the regulation of $mGlu_5$.

Norbin/neurochondrin

Norbin/neurochondrin is another neuronal protein that was shown to bind to the $mGlu_5$-CTD and coimmunoprecipitate with $mGlu_5$ receptor from rat brain lysates. Norbin has been shown to be important for synaptic transmission because genetic deletion of the protein induced schizophrenia model-like behavior, reduced DHPG-induced LTD, and abolished the induction LTP in the hippocampus. Defects in prepulse inhibition of startle (PPI) is due to abnormalities of sensorimotor gating and a behavioral phenotype of schizophrenia exhibited by $mGlu_5$ KO and Norbin KO mice (Wang et al., 2009).

Optineurin

Hungtington (Htt) is a large cytosolic protein (3144 amino acids) associated with Huntington's disease. The function of WT Htt is unknown, but the abnormal expansion of a CAG tri-nucleotide repeat within exon 1 of the *HTT* gene causes aggregation of mutant Htt that produces dysfunction most well characterized in the medium spiny neurons of the striatum. Optineurin, is a Htt binding partner, that also directly binds the $mGlu_{1a}$-CTD to promote formation of a complex with Htt (Anborgh et al., 2005).

Optineurin competes with GRK2 for $mGlu_{1a}$ binding to desensitize receptor-induced activation of the PLC pathway and IP_3 production. This is of great interest because group I mGlu receptors function is modified during the progression of Huntinton's disease (Ribeiro et al., 2010).

Group I mGlu receptor phosphorylation

The role of kinases and phosphorylation on Group I mGlu receptors have been a major focus of early studies on Group I mGlu receptors. A number of different kinases are capable of phosphorylating Group I mGlu receptors and these have specific consequences in receptor regulation. These have been studied in heterologous cells and neurons to elucidate the role of phosphorylation on mGlu receptor signaling. My work has expanded our understanding of the role of kinases on $mGlu_{5;}$ therefore, I will discuss what is known about Group I mGlu receptor regulation by phosphorylation below.

PKC

Protein Kinase C (PKC) was a large focus of early studies on Group I mGlu receptor signaling. A PKC phosphorylation site was identified on the CTD of $mGlu_{5a}$ at Thr840 following evidence that PKC was capable of phosphorylating $mGlu_{1a}$ (Alaluf et al., 1995; Kawabata et al., 1996). It was later reported that PKC phosphorylation of $mGlu_5$ likely occurs at Ser839, but that Thr840 plays a permissive role in this phosphorylation (Kim et al., 2005). The site homologous to Thr840 in $mGlu_{1a}$ is an aspartate residue (D854). This T/D residue variation does not allow for PKC phosphorylation of the conserved serine in $mGlu_{1a}$ (Ser853) (Kim et al., 2005). This difference in PKC phosphorylation was one of the first reports to show differences between $mGlu_1$ and $mGlu_5$ intracellular signaling. While activation of $mGlu_{1a}$ produced a single intracellular Ca^{2+} peak, $mGlu_5$ activity elicited distinct oscillations in intracellular Ca^{2+}. The discovery of this PKC phosphorylation site on $mGlu_5$ provided a mechanism for the observed differences in the Ca^{2+} mobilization patterns of $mGlu_1$ and $mGlu_5$ in transfected heterologous cells (Kawabata et al., 1996). Thr840 was shown to be critical to these $mGlu_5$-specific Ca^{2+} oscillations because a Thr/Asp mutation at Thr840 (to mimic Asp854 in $mGlu_1$) abolished $mGlu_5$-dependent Ca^{2+} oscillations (Kawabata et al., 1996). While the exact mechanism of how PKC phosphorylation induces these Ca^{2+} oscillations is not understood, it is worth noting that Ser839 lies in the G protein-coupling region of the $mGlu_5$-CTD. Regulation of $mGlu_5$ Ca^{2+} oscillations by PKC was also investigated in cultured astrocytes where a PKC inhibitor turned oscillatory $mGlu_5$ Ca^{2+} signals into single peaked responses. In cultured astrocytes that solely express $mGlu_5$, oscillatory responses of intracellular Ca^{2+} were converted to non-oscillatory responses by a PKC inhibitor and rises in Ca^{2+} were completely blocked by PKC activation (Nakahara et al., 1997). This

demonstrated for the first time that PKC phosphorylation controlled oscillatory patterns of $mGlu_5$ activation in astrocytes.

PKC phosphorylation has also been shown to regulate receptor desensitization. This was initially shown pharmacologically because PKC inhibitors reduced Group I mGlu receptor desensitization in cultured neurons (Catania, 1991, Aronica, 1993). Multiple PKC consensus sites are present in $mGlu_{1/5}$ and have led groups to determine the role of these sites in receptor function (Table 1.2). Mutagenesis studies revealed five residues in the 1st and 2nd intrcellular loops as well as the $mGlu_5$-CTD that were capable of reducing $mGlu_5$ desensitization (Gereau and Heinemann, 1998) (Table 1.2). Mutation of Thr840 had no effect on $mGlu_5$ desensitization, but this result may have been misinterpreted since it is now believed that Ser839 is the main site of phosphorylation responsible for the generation of Ca^{2+} oscillations (Gereau and Heinemann, 1998; Kim et al., 2005) A single threonine residue seems to be the major PKC phosphorylation site responsible for this desensitization in $mGlu_1$ (Francesconi and Duvoisin, 2000). These PKC phosphorylation sites of Group I mGlu receptors are thought to mediate their effects by disrupting G-protein coupling to the receptor, resulting in a reduction in the effectiveness of $mGlu_{1/5}$ signaling.

PKC phosphorylation is also a regulator of mGlu binding interactions. This has been well documented in the case of the interaction of $mGlu_5$ with CaM (Lee et al., 2008; Minakami et al., 1997). Phosphorylation of Ser890 affects mGlu5 binding to CaM and enhances mGlu5 internalization in heterologous cells (Lee et al., 2008). Other GPCRs have also been shown to bind to CaM with phosphorylation-dependent regulation. This includes another mGlu receptor, mGlu7 (Nakajima et al., 1999) and the $5\text{-}HT_{1A}$ receptor (Turner et al., 2004). PKC activation selectively inhibits agonist-dependent stimulation of the IP_3-production of $mGlu_{1a}$ by phosphorylation of a

threonine residue (Thr695) located in the G protein-interacting domain of the receptor disrupting $mGlu_{1\alpha}$–$G_{q/11}$ interaction (Francesconi and Duvoisin, 2000). PKC regulation in Group I mGlu receptor signaling has been shown to be important in synaptic plasticity (Bortolotto and Collingridge, 2000; Francesconi and Duvoisin, 2000; Gass and Olive, 2009; Malinow et al., 1989; Poisik et al., 2003; Schmidt et al., 2015; Skeberdis et al., 2001).

GRK

In addition to PKC, the G protein-coupled receptor kinase (GRK) family of kinases can also regulate mGlu receptor signaling. GRKs have been studied in their role in the desensitization and endocytosis of many GPCRs and GRKs have also been demonstrated in Group I mGlu receptor desensitization (Gainetdinov et al., 2004).

The GRK family consists of GRK1-7 and it has been shown that GRK2, GRK4, and GRK5 may all phosphorylate $mGlu_{1a}$ to regulate mGlu receptor desensitization and endocytosis (Dale et al., 2000; Dhami et al., 2002; Dhami et al., 2005; Dhami et al., 2004; Iacovelli et al., 2003; Mundell et al., 2003; Sallese et al., 2000). GRK2 mediated desensitization of $mGlu_{1a}$ occurs through direct binding of GRK2 to the receptor through its regulator of G protein signaling (RGS) homology domain. The RGS homology domain of GRK2 binds to the IL2 of $mGlu_{1a}$ at Lys691/Lys692 (Lys677/678 in $mGlu_{5a}$) (Dhami et al., 2005). It is thought that binding of GRK2 to IL2 may disrupt G protein-coupling and, consequently, reduce $mGlu_1$ signaling. Although most studies have focused on $mGlu_1$ regulation by GRKs, $mGlu_5$ can also be regulated by a kinase activity dependent mechanism involving GRK2 (Sorensen and Conn, 2003). A kinase dead mutant of GRK2 abolished the effects of GRK2 on $mGlu_5$ desensitization, suggesting that, unlike $mGlu_1$, the GRK2 mediated effects on $mGlu_5$ are activity (phosphorylation) dependent. Mutation of the Thr840 site

on $mGlu_5$ reduced the effects of GRK2, so this site seems to be at least partially responsible for GRK2 regulation of $mGlu_5$. GRKs, unlike many kinases, do not have a consensus sequences that makes phosphorylation predictable, therefore the sites of GRK phosphorylation have been difficult to study and have not yet been elucidated.

PKA

Protein kinase A (PKA) is a kinase that is activated by increases in intracellular levels of cyclic AMP. In this way, PKA works as a regulator of synaptic signaling during times of increased cAMP production such as with activation of $G_{\alpha s}$-coupled GPCRs. A recent study demonstrated PKA phosphorylation of $mGlu_5$ and identified the phosphorylated residue (Ser870) (Uematsu et al., 2015). This phosphorylation was identified in vitro and in mouse striatum with a phospho-specific antibody. DHPG-stimulated levels of phosphorylated ERK1/2 levels were prevented by an Ser/Ala mutation at residue 870 suggesting that PKA phosphorylation of the receptor is necessary for $mGlu_5$-dependent ERK phosphorylation. The phospho-inhibitory Ser/Ala mutation at Ser870 also significantly reduced the number of $mGlu_5$-transfected HEK cells that responded with Ca^{2+} oscillations and reduced the total number of Ca^{2+} oscillations per cell.

Distinct roles of PKA and PKC on $mGlu_{1a}$ signaling have also been demonstrated. While PKC activation selectively inhibits agonist-dependent stimulation of the IP_3 pathway, PKA potentiated agonist-independent signaling by IP_3 (Francesconi and Duvoisin, 2000). These data show how selective regulation of mGlu receptor signaling can be fine-tuned by local signaling machinery, such as kinases. This regulation may also differ based on brain region.

The importance of PKA in mGlu receptor signaling has been demonstrated in the globus pallidus in rat brain slices where it was found that inhibition or stimulation of PKA activity or D1 or D2 dopamine receptors impacted the signaling properties of $mGlu_1$ and $mGlu_5$ (Poisik et al., 2003). It is possible that PKA regulation of Group I mGlu receptors can relay information between glutamatergic and dopaminergic signaling pathways in certain regions of the brain.

CDK5

Cyclin-dependent kinase 5 (CDK5) is a proline-directed serine/threonine kinase involved in synaptic function and plasticity. In vitro kinase assays revealed that CDK5 could phosphorylate $mGlu_5$ within the Homer binding domain of the CTD (Orlando et al., 2009). $mGlu_5$ phosphorylation by CDK5 increases the interaction between the $mGlu_5$-CTD and Homer. Homer interactions with Group I mGlu receptors have been shown to regulate receptor surface expression and signaling. Phosphorylation of this region by CDK5 may be another way that to regulate the connection between Group I mGlu receptors and postsynaptic regulators of mGlu receptor trafficking, signaling, and localization.

Kinase	Phosphorylation site	Effect	Receptor	Reference
CDK5	T1164/S1167	Increases binding to homer	$mGlu_5$	Orlando et al., 2009
CaMKII	T871	Contributes to agonist-induced desensitization.	$mGlu_1$	Jin et al., 2013a
GRK2	mGlu1a : K691/K692 mGlu5a: K677/678	Activity independent binding disrupts G protein-coupling at IL-2	$mGlu_1$	Dhami et al., 2005
GRK2	Thr840	Activity dependent desensitization of mGlu5	$mGlu_5$	Sorensen and Conn, 2003
PKA	S870	Regulates ERK activation and IP3-mediated Ca^{2+} release	$mGlu_5$	Uematsu et al., 2015
PKC	S839	S839 disrupts G protein-coupling at the CTD	$mGlu_5$	Kawabata et al., 1996, Kim et al., 2005
PKC	T606, S613, T665, S881, and S890	Mutation of all block PKC-dependent desensitization	$mGlu_5$	Gereau and Heinemann, 1998
PKC	T695	Disrupts IL-2 G protein interaction	$mGlu_1$	Francesconi and Duvoisin, 2000

Table 1.2. *Reported phosphorylation sites and the effect of phosphorylation on $mGlu_{1/5}$*

Protein Phosphatases

Protein phosphorylation is bidirectionally adjusted by the presence of both protein kinases and protein phosphatases (PPs). The regulation of Group I mGlu receptors by PPs has been supported through a number of pharmacological and biochemical studies.

Both catalytic γ-isoforms of protein phosphatase 1 (PP1γ1 and PP1γ2) bind to $mGlu_{1a}$, 5a, and 5b in yeast cells and pull-down assays at a 5 amino acid residue stretch of the CTD (residues KSV[S/T]W 891-895 in $mGlu_{5a}$ and 880-884 in $mGlu_{5b}$) that is necessary and sufficient for binding (Croci et al., 2003). This binding site on $mGlu_5$ contains a serine residue that can be phosphorylated by PKC (Ser881) that has been shown to be important for PKC dependent desensitization of the receptor.

In addition to PP1, an interaction was shown between $mGlu_{5a}$ and PP2A in neurons via a coimmunoprecipitation assay. Inhibition of PP2A mimicked the effects of an $mGlu_5$ agonist in increasing phosphorylation of ERK1/2 (Mao et al., 2005). Further, activation of $mGlu_5$ selectively inhibited PP2A and reduced $mGlu_5$-PP2A binding (Mao et al., 2005). After agonist treatment, $mGlu_5$ can be internalized and enter the recycling compartment before recycling back to the cell surface and it was reported that this process is dependent on PP2A and partially dependent on calcineurin (Mahato et al., 2015).

One well studied effect of mGlu receptor activation is an enhancement of agonist-evoked currents through the NMDAR. Pharmacological evidence suggests that PPs are important in this process. NMDA induced a robust potentiation of IP_3 production in cortical rat slices that could be blocked with inhibitors to CaN or mimicked with PKC inhibitors (Alagarsamy et al., 1999). A physical interaction and dephosphorylation of PKC sites have been demonstrated with purified CaN and the $mGlu_5$-CTD (Alagarsamy et al., 2005). While CaN potentiated IP_3 production of the WT receptor, the use of an $mGlu_5$ construct in which a PKC phosphorylation site was mutated (Ser890Gly) lacked desensitization and the potentiating effect of NMDA (Alagarsamy et al., 2005). Related to these effects, it was also shown that both $mGlu_1$ and $mGlu_5$ interact with the CaN inhibitor protein (CAIN) in heterologous cells and mouse brain lysates in the IL-2 to disrupt mGlu receptor/$G\alpha_{q/11}$ interactions (Ferreira et al., 2009). This leads to the attenuation of mGlu receptor-stimulated IP_3 production and agonist-stimulated endocytosis (Ferreira et al., 2009). This compilation of work suggests that Group I mGlu receptors may be found in a complex with PKC, CaN, CaM, and CAIN. The association of these Ca^{2+} dependent protein complexes is likely very important for spatial and temporal regulation of Group I mGlu receptor signaling in response to intracellular Ca^{2+} release.

CaMKII

Interactions of CaMKII with CaMKII associated proteins (CaMKAPs) and roles of CaMKII in modulating ionotropic glutamate receptors and voltage gated ion channels have been studied intensively, but the role of CaMKII in GPCR regulation is less well understood.

CaMKII has been shown to phosphorylate the CTD of $mGlu_1$ at a threonine site (Thr871) and CaMKII activity in the striatum contributes to agonist-induced $mGlu_1$ desensitization (Jin et al., 2013a). We have evidence that CaMKII can also phosphorylate the CTD of $mGlu_5$ (Chapter V). Because CaMKII is activated by the presence of Ca^{2+}, CaMKII works to decode synaptic activity conveyed through local Ca^{2+} release. CaMKII has a consensus sequence for phosphorylation sites (RXX(S/T), and many examples of this can be found in the CTDs of $mGlu_1$ and $mGlu_5$. Although no phosphorylation site has been reported for $mGlu_5$, groups have reported that CaMKII can bind to the CTD and IL-2 of both $mGlu_1$ and $mGlu_5$ (Jin et al., 2013b; Raka et al., 2015).

Before binding and phosphorylation sites of CaMKII were defined, pharmacological studies provided evidence that $mGlu_5$ and CaMKII are linked functionally in heterologous cells and in neurons. Internalization of $mGlu_{1a}$ was shown to be dependent on CaMKII and PKC in heterologous cells by increasing the association between $mGlu_1$ and GRK2 (Mundell et al., 2004). $mGlu_5$ agonist-induced internalization and ERK1/2 activation are also modulated by CaMKII in heterologous cells (Raka et al., 2015).

In the initial characterization of the CaMKII binding site on $mGlu_5$, it was determined that, like PKC, CaMKII competes with CaM binding to the CTD of the receptor (Jin et al., 2013b). This will be a very interesting topic of study in the future because precise control of $mGlu_5$ signaling is

likely conveyed through these three Ca^{2+} sensitive proteins competing for a small region of the $mGlu_5$-CTD. Cultured striatal neurons were used to show that $mGlu_5$ activation leads to increased CaMKII binding and phosphorylation of a CaMKII sensitive site on NMDAR GluN2B subunits (Jin et al., 2013b). As discussed above, $mGlu_5$ potentiation of NMDAR activity has been well studied. $mGlu_5$ activation was also shown to enhance GluN1 and GluN2B subunit expression on the surface of neurons in a CaMKII-dependent process (Jin et al., 2015). The phosphorylation and binding of $mGlu_5$ and CaMKII have larger implications in synaptic plasticity that I will continue to discuss in the next section.

Support for the $mGlu_5$-CaMKII interaction in synaptic signaling

The focus of this dissertation will be the tie between CaMKII and $mGlu_5$ signaling; therefore, I will now discuss linkages in CaMKII and $mGlu_5$ in synaptic plasticity. The role of CaMKII and $mGlu_5$ interaction in modulating synaptic activity has been shown in many different brain regions. For instance, acute inhibition of CaMKII with KN62 inhibited the induction of hippocampal DHPG-induced LTD through Group I mGlu receptors. In support of this role, CaMKII Thr286 autophosphorylation was increased after DHPG application and necessary for mGlu receptor-mediated protein synthesis required for LTD (Mockett et al., 2011). This finding was specific to CaMKII because this reduction in LTD and protein synthesis was not seen by blocking PKC or PLC (Mockett et al., 2011). This role of CaMKII in $mGlu_{1/5}$-LTD was later confirmed using a more specific inhibitor of CaMKII (Bernard et al., 2014).

CaMKIIα is essential for LTD at cerebellar parallel fiber–Purkinje cell synapses (Hansel et al., 2006). This is interesting because the CaMKIIα-KO mice do not have deficits in LTP at Purkinje cell synapses, suggesting a specific role of CaMKII in mGlu receptor-dependent LTD in this brain

region. Furthermore, in mouse nucleus accumbens (NAc) slices, electrically-evoked Group I mGlu receptor LTD was enhanced by antagonism of NMDAR or CaMKII during but not after electrical stimulation and increased the interaction of $mGlu_5$ with the scaffolding protein Homer1b/c. These results were not replicated by DHPG-induced LTD, suggesting that glutamate release can activate NMDAR and Group I mGlu receptors in NAc neurons and impair the interaction between $mGlu_5$ and Homer1b/c to attenuate LTD induction. mGlu and NMDARs are closely clustered in postsynaptic membranes and there is evidence that these receptors interact to control excitatory synaptic transmission. DHPG application to striatal neurons concentration-dependently increased surface expression of the NMDARs GluN1 and GluN2B, enhanced CaMKIIα activity, and elevated GluN2B phosphorylation by CaMKII at Ser1303. This effect was blocked by antagonism of $mGlu_5$ or CaMKII (Jin et al., 2015).

Our lab has demonstrated that CaMKII plays a role in endocannabinoid-mediated plasticity in the striatum where activation of Group I mGlu receptors produces endocannabinoids through activation of diacylglycerol lipase α (DGLα). Deletion of DGLα results in anxiety phenotypes and depletion of the DGLα product 2-arachidonoylglycerol increases susceptibility to stress (Bluett et al., 2017; Shonesy et al., 2014a). CaMKIIα activity attenuates DGLα synthesis of 2-AG and causes a tonic reduction of endocannabinoid short-term depression (Shonesy et al., 2013). This is a finding counter-intuitive since it seems like DHPG induces both CaMKII activation and increased endocannabinoid production. I have data that show that the genetic deletion of $mGlu_5$ results in large increases in Thr286 phosphorylated CaMKII in multiple brain regions including the hippocampus and the striatum (Chapter VII), which may lead to further insights into the bidirectional signaling between these two important regulators of synaptic transmission.

Other proteins involved in CaMKII or $mGlu_5$ localization and signaling have also shed light on the interaction between these two proteins. For example, densin-180 is a scaffolding CaMKAP in the PSD that forms a high-affinity complex with α-actinin (Jiao et al., 2011). Densin knock-out mice displayed ASD and schizophrenia-like phenotypes and a selective loss of synaptic $mGlu_5$ (Carlisle et al., 2011). This loss of $mGlu_5$ was accompanied by impairment of both Group I mGlu and NMDAR-dependent LTD. While CaMKII localization and expression seemed to be unchanged, basal levels of Thr286 autophosphorylation were reduced by 25% and activity-driven activation of Thr286 CaMKII was two-fold higher in densin-180 knock-out neurons (Carlisle et al., 2011).

It was recently shown that calcium influx via L-type voltage gated calcium channels (VGCCs) is a necessary aspect of mGlu-LTD induction (Bernard et al., 2014). This is interesting because our lab recently published work showing CaMKII regulation in the control of the LTCC $Ca_V1.3$ to initiate long-range signaling to the nucleus to control cellular excitation-transcription coupling. (Wang et al., 2017). L-type calcium channels were hypothesized by Bernard et al. to mediate calcium accumulations that stimulate CaMKII to mediate mGluR-LTD induction (Bernard et al., 2014). Their findings also suggest that persistent phosphorylation of CaMKII substrates may reduce the need for CaMKII and L-type VGCC activation at the time of mGluR-LTD induction in neurological disorders such as early life seizures and Fragile-X syndrome. This study shows the wide range of CaMKII effects on $mGlu_5$ that are likely regulated by brain region specific protein complexes and local signaling molecules.

From the studies above linking $mGlu_5$ and CaMKII signaling, it seems that both proper expression, localization, and activation levels of both of these proteins have large effects on proper control of

synaptic strength. A greater understanding of the interaction between $mGlu_5$ and CaMKII signaling is necessary to increase our understanding of these complex protein interactions and signaling pathways.

Overview of work presented in this thesis

As described above, there are a number of studies linking $mGlu_5$ and CaMKII signaling in synaptic function in normal and disease physiology. This project is conceptually innovative because little is known about the direct effect of CaMKII binding and phosphorylation on $mGlu_5$ function. Therefore, we hypothesized that CaMKII binding or phosphorylation of $mGlu_5$ could regulate receptor function and signaling. Because $mGlu_5$ activation results in intracellular Ca^{2+} release and CaMKII activation is dependent on rises in intracellular Ca^{2+}, we also hypothesized that $mGlu_5$ activity could work as a feedback regulator of CaMKII activation and synaptic function (Figure 1.9). To test this hypothesis, I generated two aims as follows:

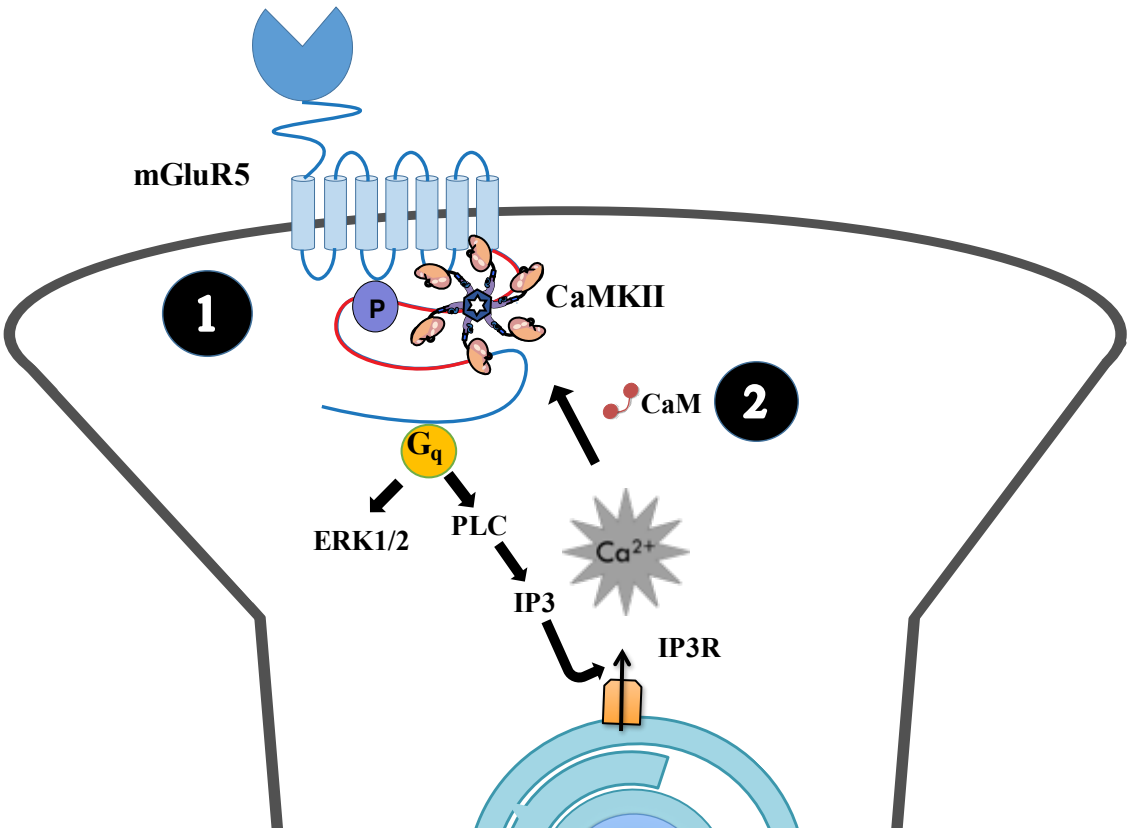

Figure 1.9. *Schematic representation of the aims outlined in this dissertation.* Aim 1 examines the role of CaMKII in modulating downstream $mGlu_5$ signaling by first characterizing the CaMKII interaction and phosphorylation of the $mGlu_5$-CTD then showing the effect of this interaction on $mGlu_5$ surface expression, intracellular Ca^{2+} release, and activation of ERK1/2. Aim 2 examines the role of the $mGlu_5$-CaMKII interaction and the feedback of $mGlu_5$-activation in regulating CaMKII activation and synaptic function.

Aim 1: To determine the role of CaMKII in modulating downstream $mGlu_5$ signaling.

I tested the hypothesis that CaMKII modulates signaling via an interaction with $mGlu_5$ through binding or phosphorylation of the receptor. First, I further characterized the CaMKII interaction with the $mGlu_{5a}$-CTD and showed that CaMKII binding to the $mGlu_{5a}$-CTD *in vitro* is largely increased by CaMKIIα. I show that the interaction between $mGlu_5$ and activated CaMKII can be disrupted by the presence of Ca^{2+}/CaM and that this interaction requires three basic residues (Lys^{866}-Arg^{867}-Arg^{868}) on the $mGlu_5$-CTD. I show the CaMKII effect on $mGlu_{5a}$ surface

expression and Ca^{2+} signaling in heterologous cells, demonstrating that CaMKIIα can increase cell surface expression of $mGlu_{5a}$ and effect $mGlu_{5a}$-dependent Ca^{2+} mobilization. All of these effects are prevented by the triple Ala substitution for Lys^{866}-Arg^{867}-Arg^{868} in the CTD.

Moreover, I provide data showing that CaMKII can phosphorylate both $mGlu_{5a}$ and $mGlu_{5b}$. Importantly, CaMKII binding to Lys^{866}-Arg^{867}-Arg^{868} in the CTD also disrupts CaMKII phosphorylation of $mGlu_5$. Using mass spectroscopy, I was able to identify a number of possible phosphorylation sites on both $mGlu_{5a}$ and $mGlu_{5b}$. Phosphorylation of both $mGlu_5$ isoforms by CaMKII can be inhibited by the presence of Ca^{2+}/CaM with different potencies.

In addition to characterizing the binding and phosphorylation of $mGlu_5$ by CaMKII, I also investigated the role of CaMKII in regulating neuronal Ca^{2+} signaling. In order to do this, we have examined the role of CaMKII in intracellular increases in Ca^{2+} through activation of different sources, including LTCCs and $mGlu_{1/5}$. We developed techniques to examine the responses from specific Ca^{2+} inputs in the presence or absence of CaMKII using shRNA-knockdown in hippocampal neuronal cultures. We expanded this work to examine the role of Shank-3, an important PSD scaffolding protein, in responses to LTCC and $mGlu_5$ activation uncovering the importance of the proper PSD composition for proper Ca^{2+}-influx from multiple sources.

Aim 2: To determine the role of $mGlu_5$ in regulating CaMKII activation and synaptic function.

To begin studying how $mGlu_5$ activity could work as a feedback regulator of CaMKII activation and synaptic function, I examined the autophosphorylation of CaMKII in $mGlu_5$ knock-out mice to show that the absence of $mGlu_5$ causes aberrant CaMKII activation. Using slice pharmacology

experiments, we also showed that activation of Group I mGlu receptors could reduce CaMKII autophosphorylation in the hippocampus and striatum of mice. I used *in vitro* binding studies with GST-$mGlu_{5a}$-CTD and purified CaMKII to show that the $mGlu_5$-CTD enhances the apparent cooperativity for CaMKII activation by Ca^{2+}/CaM *in vitro*, causing it to be activated in a more switch-like manner.

I undertook these studies because understanding the interplay between $mGlu_5$ and CaMKII will provide mechanistic insights into the modulation of synaptic transmission physiologically, and in neuropsychiatric diseases. The work done on these aims is separated into chapters characterizing the $mGlu_5$-CaMKII interaction, study of the $mGlu_5$-CaMKII interaction on $mGlu_5$ signaling, CaMKII phosphorylation of the $mGlu_5$-CTD, the role of CaMKII in neuronal Ca^{2+} signals, and finally preliminary work understanding the role of $mGlu_5$ on CaMKII activation.

Chapter II

Materials and methods

Molecular Biology

DNA constructs

The GST-mGlu$_{5a}$-CTD and the GST-mGlu$_{5b}$-CTD expression constructs were created by PCR amplification of the region encoding residues 827-964 of mGlu$_{5a}$ using primers 5'CTGGAAGTTCTGTTCCAGGGGCCCGGATCCAAACCGGAGAGAAAT 3' (Forward) and 5' GCCGCAAGCTTGTCGACGGAGCTCGAATTCTTAGGTCCCAAAGCGCTT 3' (Reverse) and inserting the product into BamHI/EcoR1 sites of pGEX6P using a sequence and ligation independent cloning (SLIC) cloning protocol (Li and Elledge, 2012).

The pCGN plasmid to express WT mGlu$_{5a}$ with an N-terminal HA-tag was made by amplifying the entire rat mGlu$_{5a}$ coding sequence (forward primer: 5'TGACGTGCCTGACTATGCCTCTAGAATGGTCCTTCTGTTGATCCT3'; reverse primer: 5' ACTCACCCTGAAGTTCTCAGGATCCTCACAACGATGAAGAACTCT3') and inserting the fragment into XbaI and BamHI restriction sites of the empty pCGN plasmid (a gift from Dr. Winship Herr, Université de Lausanne, Switzerland, Addgene plasmid ID 53308).

Primer	5' Sequence 3'
$mGlu_5$ 827-964 Forward	CTGGAAGTTCTGTTCCAGGGGCCCGGATCCAAACCGGAGAGAAAT
$mGlu_5$ 827-964 Reverse	GCCGCAAGCTTGTCGACGGAGCTCGAATTCTTAGGTCCCAAAGCGCTT
T1 $mGlu_5$ 827-873 Forward	GCCCCTGGGATCCCCGGAATTCAAACCGGAGAGAAATGTGCG
T1 mGlu5.827-873 Reverse	GTCAGTCACGATGCGGCCGCttaAGAGGAGCCCCTCCTCTTCC
T2 mGlu5.853-899 Forward	GCCCCTGGGATCCCCGGAATTCTCATCGTCCGCTGCCAGCAG
T2 mGlu5.853-899 Reverse	GTCAGTCACGATGCGGCCGCttaCCACAAATGTTGCCCCCGGG
T3 mGlu5.879-925 Forward	GCCCCTGGGATCCCCGGAATTCTCCAACGGAAAATCTGTGAC
T3 mGlu5.879-925 Reverse	GTCAGTCACGATGCGGCCGCttaTGTGCTCTTGGGAAAGGGTT
T4 mGlu5.905-964 Forward	GCCCCTGGGATCCCCGGAATTCCACATCAACAAGAAGGAGAA
T4 mGlu5.905-964 Reverse	GTCAGTCACGATGCGGCCGCttaGTCAGTCACGATGCGGCCGC
$K^{866}RR^{868}$ to AAA Forward	GGGTTTCCCCAGAGGAGCCGGCGGCGGCCCACAGGTTGACTAGGCTGCT
$K^{866}RR^{868}$ to AAA Reverse	AGCAGCCTAGTCAACCTGTGGGCCGCCGCCGGCTCCTCTGGGGAAACCC
$SS^{870/1}$ to AA Forward	GGAAGAGGAGGGGCGCCGCCGGGGAAACCCTAAG
$SS^{870/1}$ to AA Reverse	CTTAGGGTTTCCCCGGCGGCGCCCCTCCTCTTCC
S^{887} $mGlu_{5b}$ to A Forward	GTGAAACACTCTATTTCGGCCTTGTGCTGGGCCAGTC
S^{887} $mGlu_{5b}$ to A Reverse	GACTGGCCCAGCACAAGGCCGAAATAGAGTGTTTCAC

Table 2.1. Primers used in generating mGlu5 truncations and mutagenesis

Mutagenesis of $K^{866}RR^{868}$ and $SS^{870/871}$ to alanine mutation in $mGlu_{5a}$ and S^{887} to alanine in $mGlu_{5b}$ were generated by site-directed mutagenesis of the pGEX6P or pCGN constructs (see above) using a Quick Change protocol (Agilent). The $mGlu_{5a}$-truncation constructs were created from the GST-$mGlu_{5a}$-CTD construct. The mutagenesis and truncation primers are listed in Table 2.1.

We used pcDNA3.1 constructs to express untagged and mApple-tagged WT-CaMKIIα, a constitutively active T286D/T305A/T306A triple mutant of CaMKIIα (CA-CaMKIIα), and a catalytically dead kinase mutant K42R (KD-CaMKIIα) as previously described (Jalan-Sakrikar et al., 2012; Jiao et al., 2008; Stephenson et al., 2017). In the CA-CaMKIIα, the phospho-mimetic T286D mutation results in constitutive CaMKIIα activity and the phospho-null T305A/T306A mutations prevent CaMKIIα phosphorylation at these sites, which interferes with binding of Ca^{2+}/CaM and α-actinin (Jalan-Sakrikar et al., 2012).

CaMKII shRNA constructs for neuronal Ca^{2+} imaging were expressed with mApple using a pLL3.7 plasmid (a gift from Luk Van Parijs lab, Massachusetts Institute of Technology, Cambridge, Massachusetts) as described in (Wang et al., 2017). The shRNA-targeted sequence in the mouse CaMKIIIα cDNA contains two mismatches from the corresponding rat sequence, rendering it resistant to the shRNA. Knockdown and shRNA-resistance were confirmed by western blot and immunostaining. All constructs were confirmed by DNA sequencing.

Shank-3 shRNA was designed by Tyler Perfitt and targets exon 21 of mouse and rat Shank3b residues 1881-11933. The sequence of this construct is as follows:

Shank3 shRNA: 5'-GGAAGTCACCAGAGGACAAGA-3'

Shank3 shRNA control: 5'-GAAAAAGCCCGGAGGACAAGA-3'

Recombinant protein purification

Expression and purification of recombinant mouse CaMKIIα has been described before (McNeill and Colbran, 1995). To express GST-tagged proteins, pGEX6P-1 plasmids were transformed into BL21(DE3) bacteria cells. Cells were grown in LB media at 37°C to reach OD~0.6. Cells were

cooled to room temperature and IPTG (0.2 mM) was then added to induce protein expression for 12-16 hours. Inducing protein expression at room temperature substantially reduced protein degradation seen when proteins were expressed at 37°C. Expressed proteins were purified using Pierce Glutathione Agarose beads (Cat. #16101) following manufacture's instruction. Eluted proteins were then dialyzed in 10 mM HEPES pH 7.5, 25 μM PMSF, 62.5 μM Benzamidine, 62.5 μM EDTA, 0.1% Triton X-100 overnight with one buffer change.

CaMKIIα autophosphorylation

Purified mouse CaMKIIα was autophosphorylated under two different conditions. Typically, CaMKIIα was incubated with 50 mM HEPES, pH 7.5, 10 mM $Mg(CH_3\text{-}COO)_2$, 0.5 mM $CaCl_2$, 2 μM CaM, 40 μM ATP on ice for 90 s before addition of EDTA and EGTA (20 mM final) to terminate phosphorylation by chelation of Mg^{2+} and Ca^{2+}. Similar conditions were previously shown to result in the selectively autophosphorylation of Thr286 (McNeill and Colbran, 1995). Where indicated, identical autophosphorylation reactions were incubated for 10 minutes at 30°C to perform a more extensive phosphorylation at several additional sites (Baucum et al., 2015).

GST pulldown and CaM binding competition

Purified GST-$mGlu_5$-CTD (1 μM) and CaMKIIα (62.5 nM; pre-autophosphorylated as indicated in figure legends) were incubated at 4°C in GST-pulldown buffer (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% (v/v) Triton X-100) with either 2 mM EGTA or 2.5 mM $CaCl_2$ plus 10 μM calmodulin, as indicated. An aliquot (5%) of each incubation was saved as an input sample. After 1 h, pre-washed glutathione agarose beads (Pierce Cat. #16101) (15 μl of a 50:50 slurry) were added and incubation was continued at 4°C for an additional 1 h. The beads were then separated

by centrifugation (2000 x g, 30 s), and washed three times with GST-pulldown buffer containing either 2 mM EGTA or 2.5 mM $CaCl_2$, respectively. Beads were then incubated at 4°C with GST pulldown buffer containing 20 mM glutathione, adjusted to pH 8.0, for 10 min. After centrifugation, eluted proteins were transferred to a new tube, mixed with 4X SDS-PAGE buffer and heated for 10 minutes at 90°C prior to SDS-PAGE and Western Blot analysis.

In Vitro Phosphorylation Assay

CaMKII was pre-autophosphorylated by incubation with 50 mM HEPES, pH 7.5, 10 mM *Mg*$(CH_3COO)_2$, 0.5 mM $CaCl_2$, 2 μM CaM, 40 μM ATP on ice for 90 s before addition of EDTA and EGTA (20 mM final) to terminate phosphorylation by chelation of Mg^{2+} and Ca^{2+}. Phosphorylation of purified GST-tagged $mGlu_{5a}$ or $mGlu_{5b}$ CTDs (1 μg) were incubated with a final concentration of 10 nM in 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 0.5 mM $CaCl_2$, 1 mg/mL bovine serum albumin, 1 mM DTT, and 0.4 mM [γ-^{32}P] ATP (~500 cpm/pmol) with indicated concentrations of CaM at 30°C for 10 minutes before being stopped by 1x Laemmli buffer. Samples were then resolved on a SDS-PAGE gel which was dried to complete autoradiography. For cooperativity studies CaMKII was preincubated with GST-$mGlu_{5a}$-CTD or GST for 30 minutes on ice. Reactions were started by addition of indicated concentrations of CaM, along with 2mM Ca^{2+}, 0.4 mM [γ-^{32}P] ATP, and 0.2 mM syntide-2. After 10 minutes at 30°C.

To count ^{32}P incorporation the same procedure was carried out in reactions involving GST-$mGlu_5$ and CaMKII, except that 15 μl aliquots of the reactions were stopped on P82 Whatman paper. The papers were then washed and phosphorylation stoichiometries were determined by quantifying ^{32}P incorporation using a scintillation counter.

CaMKII phosphorylation for mass spectrometry was performed the same way as above except that nonradioactive ATP was used. Samples were resolved on a SDS-PAGE gel and stained with Colloidal Blue (Thermo Fisher Scientific, Cat. #LC6025) for three hours and washed with deionized water overnight. The band that corresponds to full-length proteins was then excised for mass spectrometry analysis at the proteomics core facility of Vanderbilt University.

Immunoblotting and semi-quantitative analysis

Since heating samples results in aggregation of full-length $mGlu_5$ protein, all samples that were blotted for the full-length receptor were eluted in SDS sample buffer containing 150 mM DTT for 10 minutes at room temperature before SDS-PAGE. SDS-polyacrylamide gels were transferred to nylon-backed nitrocellulose membranes in 10 mM CAPS buffer. After blocking in TTBS (50 mm Tris-HCl, pH 7.5, 0.1% (v/v) Tween 20, 150 mm NaCl) containing 5% nonfat milk, membranes were incubated for either 2 h at room temperature for purified protein studies or overnight at 4 °C in HEK293A and brain lysate samples with primary antibodies diluted in TTBS with 5% milk. Membranes were washed 5 times in TTBS and incubated for 1 h at room temperature with secondary antibodies conjugated to horseradish peroxidase (Promega or Santa Cruz Biotechnology), or infrared dyes (LiCor Biosciences, Lincoln, NE) diluted in TTBS with 5% milk. Antibody signals were visualized via enzyme-linked chemiluminescence using the Western Lightening Plus-ECL, enhanced chemiluminescent substrate (PerkinElmer) and visualized using Premium X-ray Film (Phenix Research Products). Images were quantified using ImageJ software. Secondary antibodies conjugated to infrared dyes (LI-COR Biosciences) were used for development with an Odyssey system (LI-COR Biosciences).

Antibodies

The following antibodies were used for immunoblotting at the indicated dilutions: Total CaMKIIα (Thermo Catalog # MA1-048 1:5000) and p-Thr286 CaMKIIα (Santa Cruz Biotechnology Catalog # sc-12886-R, 1:3000), $mGlu_5$–specific antibody (Millipore, Catalog # AB5675, 1:3000), rabbit anti-HA (Santa Cruz, Catalog #sc805, 5 µL for immunoprecipitation), goat-GST antibody (Abcam Catalog # ab181652, 1:10,000).

Secondary antibodies: HRP-conjugated anti-rabbit (Promega catalog #W4011, 1:3000), HRP-conjugated anti-mouse (Promega catalog #W4021, 1:3000), and HRP-conjugated anti-goat (Santa Cruz Biotechnology catalog #sc-2056, 1:3000), IR dye-conjugated donkey anti-rabbit 800CW (LI-COR Biosciences catalog #926–32213, 1:10,000), and IR dye-conjugated donkey anti-mouse 680LT (LI-COR Biosciences catalog #926–68022, 1:10,000).

Heterologous Cell Experiments

Cell culture, transfection and immunoprecipitation

HEK293A cells (Invitrogen Catalog #R70507) were maintained in complete DMEM supplemented with 5% FBS, 2 mM L-glutamine, 20 mM HEPES, 0.1 mM Non-Essential Amino Acids, 1 mM sodium pyruvate, at 37 °C in a humidified incubator containing 5% CO_2 and 95% O_2. Vectors encoding mApple-CaMKIIα (WT or CA) and $mGlu_{5a}$ (3 µg DNA each) or empty vector controls (3 µg) were co-tranfected into one 10-cm dish of 60-70% confluent HEK293A cells using 3 µl of Fugene 6 (Promega Catalog # E2691) per µg of DNA. About 48 hours later, cells were lysed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5% NP-40 (v/v), 0.5% deoxycholate (v/v), 0.2 mm PMSF, 1 mm benzamidine, 10 µg/ml

leupeptin, 10 μm pepstatin, and 1 μm microcystin. Cell lysates were cleared by centrifugation (10 min at 12,000 x *g*), and a 30 μL sample of the input was saved for SDS-PAGE. The remaining supernatant was incubated at 4°C for 1 hour with rabbit anti-HA antibodies and 20 μl prewashed Dynabeads Protein A (Thermo-Fisher, Cat. #10001D, 50% v/v). Beads were isolated magnetically, washed three times using lysis buffer and eluted using 2X Laemmli sample buffer for 10 minutes at room temperature prior to SDS-PAGE and Western Blotting.

ERK activation experiments

For ERK activation experiments we used HEK293A cells stably expressing low amounts of the rat $mGlu_{5a}$ receptor (*293A-5a*^{LOW} cells) as previously described (Gregory et al., 2012; Hammond et al., 2010; Noetzel et al., 2012). The cells were maintained at 37°C in complete DMEM supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 20 mM HEPES pH 7.5, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, antibiotic/antimycotic solution (Invitrogen), and 500 μg/ml G418, in a humidified incubator containing 5% CO_2/95% O_2. For experiments, 10 cm dishes were transfected with 3 μg of mApple control vector (mApp), 3 μg mApp-CaMKIIα (wild type, KD, or CA; see above). The following day, the media was replaced with DMEM containing 10% dialyzed FBS, 20 mM HEPES, 1 mM sodium pyruvate, and incubated overnight at 37°C in 5% CO_2. Approximately 24 hours later, medium was manually removed and replaced with fresh DMEM containing 10% dialyzed FBS, 20 mM HEPES, 1 mM sodium pyruvate for 1 hour. After incubation for 1 hour at 37°C in 5% CO_2, cells were treated with direct addition of glutamate to the plate for a final concentration of 100 μM glutamate for 5 minutes. The reaction was stopped by putting the plate on ice and addition of 2X Lamelli Sample Buffer containing 150

mM DTT directly to the cells. The cells were scraped down, transferred to 1.5 mL tubes and sonicated before immunoblotting for ERK/pERK, $mGlu_5$, and CaMKII.

Biotinylation and cell surface expression

Transfected HEK293A cells (see above) were placed on ice, the media was gently removed and the cells were immediately washed two times using ice cold PBS. Cells were then scraped into ice-cold PBS, transferred to a 1.5 mL tube, centrifuged at 4°C (500 x *g*; 3 min), and gently resuspended in 1 mL of cold PBS containing 2 mg of EZ-Link Sulfo-NHS-SS-Biotin. After gently rocking for 1 hour, excess reagent was quenched by addition of 50 mM Tris HCl pH 8.0, and cells were centrifuged and washed again in 1 mL of 50 mM Tris HCl. Cells were then suspended in 1 mL of ice-cold lysis buffer (25 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate containing 0.2 mm PMSF, 1 mm benzamidine, 10 μg/ml leupeptin, and 10 μm pepstatin), and incubated on ice for 30 min. Insoluble material was removed by centrifugation (16,000 x *g*; 10 min, 4°C) and a 30 μL aliquot of the supernatant was saved for an input sample for SDS-PAGE (Cho et al., 2014). The remaining supernatants were mixed for 1 hour at 4°C with magnetic NeutrAvidin beads (30 μL; 50% slurry). The beads were separated magnetically and washed three times with lysis buffer. Biotinylated proteins were dissociated from the beads in SDS sample buffer containing 150 mM DTT for 10 minutes at room temperature. The biotinylated and total protein samples were analyzed by Western blotting for $mGlu_5$.

Ca^{2+} Imaging Experiments

Ca^{2+} Imaging in 96-well Plates

A FlexStation II liquid handler/plate reader (Molecular Devices) was used for intracellular Ca^{2+} measurements in HEK293A cells stably expressing low amounts of the rat $mGlu_{5a}$ receptor (*293A-5a^{LOW}* cells) as previously described (Gregory et al., 2012; Hammond et al., 2010; Noetzel et al., 2012). The cells were maintained at 37°C in complete DMEM supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 20 mM HEPES pH 7.5, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, antibiotic/antimycotic solution (Invitrogen), and 500 μg/ml G418, in a humidified incubator containing 5% CO_2/95% O_2. For experiments, 10 cm dishes were transfected with 3 μg of mApple control vector (mApp) or 3 μg mApp-CaMKIIα (wild type or CA; see above). The following day, cells were transferred to clear-bottomed, black-walled, poly(d-lysine)-coated 96-well plates (BD BioCoat, Bedford, MA) (3×10^4 cells per well) in DMEM containing 10% dialyzed FBS, 20 mM HEPES, 1 mM sodium pyruvate, and incubated overnight at 37°C in 5% CO_2. Approximately 24 hours later, medium was manually removed and replaced with Hanks' balanced salt solution containing 20 mM HEPES, 2.5 mM probenecid, and 2 μM Fluo-4/acetoxymethyl ester dye, pH 7.4, and plates were incubated for 30 min (37°C, 5% CO_2). This medium was manually removed and replaced with 40 μl of calcium assay buffer (Hanks' balanced salt solution, 20 mM HEPES, and 2.5 mM probenecid, pH 7.4). Glutamate additions were performed after a 30 s baseline to construct concentration-response curves (CRCs) and plates were monitored for a total of 120 sec using an excitation wavelength of 488 nm, an emission wavelength of 525 nm, and a cutoff wavelength of 515 nm. Data were collected with SoftMax Pro (Molecular Devices) then transformed and agonist concentration-response curves were fitted to a four-

parameter logistic equation with GraphPad Prism. For area under the curve measurements, time parameters were set to measure the area under the curve between the timepoints of 10-60 seconds to capture the initial Ca^{2+} peak. Raw values were generated by SoftMax Pro and normalized to the max response of control cells.

Single Cell Ca^{2+} Imaging

HEK293A cells were transiently transfected to express full-length $mGlu_{5a}$ (WT or AAA: 0.3µg DNA) and either mApple or mApple-CA-CaMKIIα (3 µg DNA). The following day, transfected cells were plated in clear glass-bottomed, poly(D-lysine)-coated 29 mM dishes (Cellvis D29-10-1.5-N) (5×10^4 cells) in DMEM containing 10% dialyzed FBS, 20 mM HEPES, 1 mM sodium pyruvate, and 1% Pennicillin/Streptomycin (Gibco) and incubated overnight at 37°C in 5% CO_2. The day of the experiment, cells were incubated in media supplemented with 2 µM Fura-2 acetoxymethyl ester (Molecular Probes) for 20 minutes at 37°C in 5% CO_2, and then transferred to Ca^{2+} imaging solution (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES pH 7.5 (~313 mOsm)). After incubation for 20 minutes at 37°C in 5% CO_2, fluorescence imaging was performed using a Nikon Eclipse TE2000-U microscope equipped with an epifluorescence illuminator (Sutter, Inc), a CCD camera (HQ2; Photometrics Inc), and Nikon Elements software. Cells were perfused at 37°C at a flow of 2 mL/min with Ca^{2+} imaging solution. First, the field of view was imaged using 568 nm excitation to detect cells expressing either mApple-CA-CaMKII or mApp-PCDNA3.1 control. Then, ratios of emitted fluorescence (at 510 nm) from mApple positive cells were measured following excitation at 340 nm and 380 nm (F340/F380); ratios were measured every 3 seconds for a 1 min baseline period. Thn the cells were treated with 100 µM glutamate (added to the Ca^{2+} imaging solution) for 10 min during which

the Fura-2 F340/F380 ratios were collected every 3 s. Relative changes in Ca^{2+} of the mApple expressing cells were analyzed using Nikon Elements software. The F340/F380 ratios of each cell were normalized to the first F340/F380 ratio acquired for that cell during the baseline period (F/F0 = (340/380 value)/(baseline 340/380 value)) and then analyzed using Clampfit software (Molecular Devices, Sunnyvale, California). Peak Ca^{2+} responses for all cells were aligned (at 45 seconds), a ROUT test was first used to identify outliers in the maximal ΔF/F0 values for all cells within each experimental group, and then ΔF/F0 values for each time point were averaged together for each dish of cells. The maximal Ca^{2+} response (Max Peak) was defined as the average of all the peak ΔF/F0 values on an experimental day. An average trace for each day of experiments was generated to calculate the half-life of each condition. To compare half-lives of the Ca^{2+} signals the decline of the average ΔF/F0 values in each dish were normalized to the average max peak in that dish, and then fitted to a non-linear one phase exponential decay fit constrained to y0 = 1 using Graphpad Prism software (version 6.0). We determined ΔF/F0 and half lives in 5 independent experiments (transfections) on separate days (19-124 cells per condition per day) and tested for differences using a Student's T-Test. All values are presented as the mean ± SEM.

Neuronal Ca^{2+} imaging

Dissociated rat hippocampal neurons from rat E18 were prepared as previously described (Wang et al., 2017) and cultured in coated 29-mm glass bottom dishes (Cellvis, catalog no. D29-10-1.5-N) for CaMKII KD experiments, cells were transfected with a total of 2 μg of DNA/dish after 8 DIV. All neurons were imaged on DIV 13–14. CaMKII shRNA constructs lacking the CaMKII promoter and GFP were co-expressed with mApple to label transfected cells. Cells were incubated at 37 °C for 20 min in culture medium (neural basal medium with 2% B27, 0.25% glutamax, and 1% penicillin-streptomycin) supplemented with 2 μm Fura-2 acetoxymethyl ester (Fura-2AM)

(Thermo Fisher Scientific, catalog no. F1221), washed twice with 5K Tyrode's solution, and then incubated at 37 °C for 20 min in 5K Tyrode's solution with 1 μM TTX, 10 μM APV 50 μM NBQX, 10 μM VU-50, and 10 μM MPEP as indicated in each figure. For nimodipine-treated groups, this medium was replaced with 5K Tyrode's solution containing 10 μm nimodipine (in addition to TTX, APV, and NBQX) ~5 min before imaging. All except ~100 μl of the 5K Tyrode's solution was removed immediately prior to imaging. At this point a 30 sec. baseline was recorded followed by addition of 1 mL of 40K Tyrode's solution or 5K Tyrode's solution containing 100 μM DHPG in addition to the inhibitors included in the preincubation.

For experiments including Shank-3 KD and DHPG treatment, cells were transfected at ~14 DIV 2.5 μl Lipofectamine 2000 for 2-3 hours with 2 μg soluble mApple or 2 μg Shank shRNA for imaging at DIV 21. Cells were incubated at 37 °C for 20 min in culture medium supplemented with 2 μm Fura-2AM (Thermo Fisher Scientific, catalog no. F1221), washed twice with 5K Tyrode's solution, and then incubated at 37 °C for 25 min in 5K Tyrode's solution with TTX, APV, VU-50 and NBQX.

For 40K treatment conditions, cells were transfected at 8 DIV with 1.8 μg Shank3 shRNA, 0.2 μg mApple, or 1.8 μg shRNA and 0.2 μg of mApple tagged Shank3 resistant shRNA.

For all experiments, Fura-2 fluorescence images were collected using a Nikon Eclipse TE2000-U microscope equipped with an epifluorescence illuminator (Sutter Instrument Co.) and an HQ2 CCD camera (PhotoMetrics Inc.). Cell somas were selected as regions of interest using Nikon Elements software; transfected neurons were selected based on mApple fluorescence. The ratios of emitted fluorescence (505 nm) intensities at excitation wavelengths of 340 and 380 nm (F_{340}/F_{380}) were measured every 3 sec. Responses of individual cells at each time point were

quantified as the change in fluorescence ratio above baseline (ΔF = (340/380 value)/(baseline 340/380 value)). The peak change in fluorescence ratio was used to compare responses between cells within each group (ΔF = (maximum 340/380 value)/(baseline 340/380 value)), and outlier cells were excluded based on a ROUT outliers test (Q = 1%).

Mouse work

Mice

CaMKII-KO mice were generated in the Vanderbilt Transgenic Mouse Core as a by-product of published CRISPR/Cas9 mediated experiments directed at creating a knock-in E183V mutation of CaMKIIα (Stephenson et al., 2017). We selected a founder containing a deletion of 11-base pairs (TGCTGAGGAAG) from exon 8, leading to a frame shift and early translational termination. The knockout of CaMKIIα was confirmed by Western Blot. Primers used to genotype the CaMKIIα knockout mice are: (Forward) 5'GATACCTCTCCCCAGAAGGAC3', (Reverse) 5'TGCAGTGGTAAGGAGTGGTG3' for wild-type and (Forward) 5'GGACAGTACAACCCCAGCTT3' and (Reverse) 5'CCCGTACGGGTCCTTCCTCA3' for knockout generating a 206bp band for WT, 351bp band for KO, and 557bp band for all mice and the CaMKIIα-KO was confirmed by immunoblotting brain lysates.

$mGlu_5$ KO mice were a generous gift from Dr. Danny Winder and are from Jackson Laboratory stock number 003121. I developed and confirmed a new genotyping PCR for these mice that allowed for me to perform genotyping in a single reaction. The primers for this reaction are:

mGlu5 Fwd: CAC ATG CCA GGT GAC ATT AT

mGlu5 Rev: CCA TGC TAG TTG CAG AGT AA

Jax 2060: CAC GAG ACT AGT GAG ACG TG

For future reference I will also include the PCR reaction details because this is a new mouse line to our lab in Table 2.2 and Table 2.3 below.

Reagent	Volume (µL)
Std Taq Buffer	2.5
10mM dNTp	2
mG5 F	3
mG5 R	1
Jax Primer	2
Std Taq	0.3
H2O	13.2
DNA	1

Table 2.2 *mGlu5-KO genotyping PCR reaction*

Cycling			
Step #	Temp °C	Time	Note
1	95	3 min	-
2	95	30	-
3	61	30 sec	
4	68	45 sec	2-4 X 38 cycles
	-	-	
5	68	5 min	-
6	25	hold	-

Table 2.3. *mGlu5-KO genotyping PCR cycle details*

All mice were on a mixed B6D2 (C57BL/6J (B6) x DBA/2J (D2)) background and were housed (2–5 per cage) on a 12 h light-dark cycle with food and water *ad libitum*. Wild-type and knockout experimental mice (littermates) were generated using a HETXHET breeding strategy. All animal procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee in accordance with the National Institutes of Health *Guide for the care and use of laboratory animals*.

Mouse brain tissue preparation and immunoprecipitation

Both male and female mice were anesthetized with isofluorane, decapitated, and forebrains were quickly dissected. Half of a forebrain (cut along the mid-line) was homogenized using at least 20 strokes with a Dounce homogenizer in 1.5 mL of an isotonic buffer containing 150 mM KCl, 50 mM Tris-HCl, 1 mm DTT, 1% (v/v) Triton X-100, 1% sodium deoxycholate, 0.2 mM PMSF, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μM pepstatin, and 1 μM microcystin. The homogenate was rotated end-over-end at 4°C for 30 min and then centrifuged at 10,000 × *g* for 30 min to remove insoluble material. A 30μL input sample was saved before CaMKIIα (MA1-048) antibody and 20μL magnetic Protein G beads (Invitrogen Catalog #10003D) was added to 1 mL of homogenate and rotated end over end for 3-4 hours. Beads were separated magnetically and washed three times with homogenization buffer. Immunoprecipitated complexes were eluted using 2X Lamelli Sample Buffer containing 150 mM DTT for 10 minutes at room temperature. CaMKII and $mGlu_5$ immunoprecipitation was analyzed by immunoblot analysis.

Acute slice preparation and slice pharmacology

Mice were anesthetized using isofluorane and then decapitated. Brains were removed, cut into left and right hemispheres, and then 300 μm coronal slices were made at 1-4°C in oxygenated (95% v/v O_2, 5% v/v CO_2) dissecting solution (208 mM sucrose, 2.5 mM KCl, 1 mM $CaCl_2$, 4 mM $MgCl_2$, 4 mM $MgSO_4$, 1.6 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, and 3 mM Na-pyruvate) using a Vibratome 3000 (The Vibratome Company). Slices including the hippocampus and the striatum were collected for analysis. Slices were allowed to recover on a nylon mesh for 1 h at 30°C in oxygenated ACSF (113 mM NaCl, 2.5-5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, 20 mM glucose, and 3 mM Na-pyruvate) followed by addition of picrotoxin (50 μM) for 30 min. Slices were then transferred to oxygenated 30°C ACSF solutions supplemented with vehicle or 100 μM DHPG for 10 min. Punches (2 mm diameter) of hippocampus or dorsal striatum, were collected from slices on ice after incubation. Total lysates were prepared by immediately homogenizing striatal tissue punches in lysis buffer (2% SDS, 2 mM EGTA, 0.2 mM PMSF, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μM pepstatin, and 1 μM microcystin). Protein concentrations in total striatal lysates were determined by BCA assay (Thermo Scientific), using a bovine serum albumin standard.

Fractionation

Mice were decapitated and the hippocampus and striatum of mice ~P30-P60 were microdissected Tissue was homogenized in isotonic buffer (IB) without detergents (150 mM KCl, 50 mM Tris-HCl pH 7.5, 1 mM DTT, 0.2 mM PMSF, 1 mM Benzamidine, 1 μM Pepstatin, 10 mg/l Leupeptin, 1 μM microcystin). Protein concentrations were measured by BCA assay and all samples were diluted to 1 μg/μL prior to incubation at 4°C with rocking for 30 min prior to centrifugation at

100,000 × g for 1 h. The supernatant (S1 fraction, “cytosolic”) was saved, and the pellet was resuspended in IB containing 1% (v/v) Triton X-100 and then incubated at 4°C with rocking for 30 min. This lysate was then centrifuged at 18,403 × g, and the resulting supernatant (S2 fraction, “Extrasynaptic membrane”) was saved. The pellet was resuspended in 1/3 of the original volume of isotonic buffer containing 1% Triton X-100 and 1% deoxycholate and sonicated (S3 fraction, “Synaptic”). 4X Lamelli Sample Buffer containing 150 mM DTT was added to the samples before SDS-PAGE and immunoblotting. Subcellular fractionation efficiency was confirmed by evaluating the distribution of a cysolic S1 protein GAPDH, S2 protein IP3R, and a synaptic S3 proteins PSD-95.

Chapter III

The CaMKII interaction with $mGlu_5$

Introduction

The $mGlu_5$-CTD is a highly regulated portion of the receptor that is modulated by phosphorylation and protein binding. CaMKII activation is necessary for interaction with many proteins and less is known about proteins that interact with inactive kinase. After an interaction was shown between CaMKII and $mGlu_1$, the interaction between $mGlu_5$ and CaMKII was also described (Jin et al., 2013a; Jin et al., 2013b). It was reported that CaMKII activation increased the interaction between CaMKII and $mGlu_1$ (Jin et al., 2013a). The interaction between $mGlu_5$ and CaMKII was reported to be reduced by kinase autophosphorylation (Jin et al., 2013b).

After an initial confirmation that $mGlu_5$ and CaMKII bind in mouse forebrains using co-immunoprecipitation studies, I began to more closely investigate the interaction between CaMKII and $mGlu_5$ using purified GST-tagged protein constructs. In an attempt to replicate the previously reported results, I found that inactive CaMKII bound weakly to $mGlu_5$. Therefore, I more carefully characterized the interaction using different autophosphorylation protocols for CaMKII and was able to show that active CaMKII can bind more strongly to the $mGlu_5$-CTD depending on the autophosphorylation conditions. This was true for binding with purified protein constructs and in heterologous cells. I was also able to identify a tri-basic residue that disrupted CaMKII binding to

both purified CTD constructs of $mGlu_5$ and full-length receptor. This work allowed for the study of the consequences of CaMKII binding to $mGlu_5$ on receptor signaling discussed in the following chapter. The work in this chapter, with the exclusion of Figure 3.1, was published in Molecular Pharmacology (Marks et al., 2018).

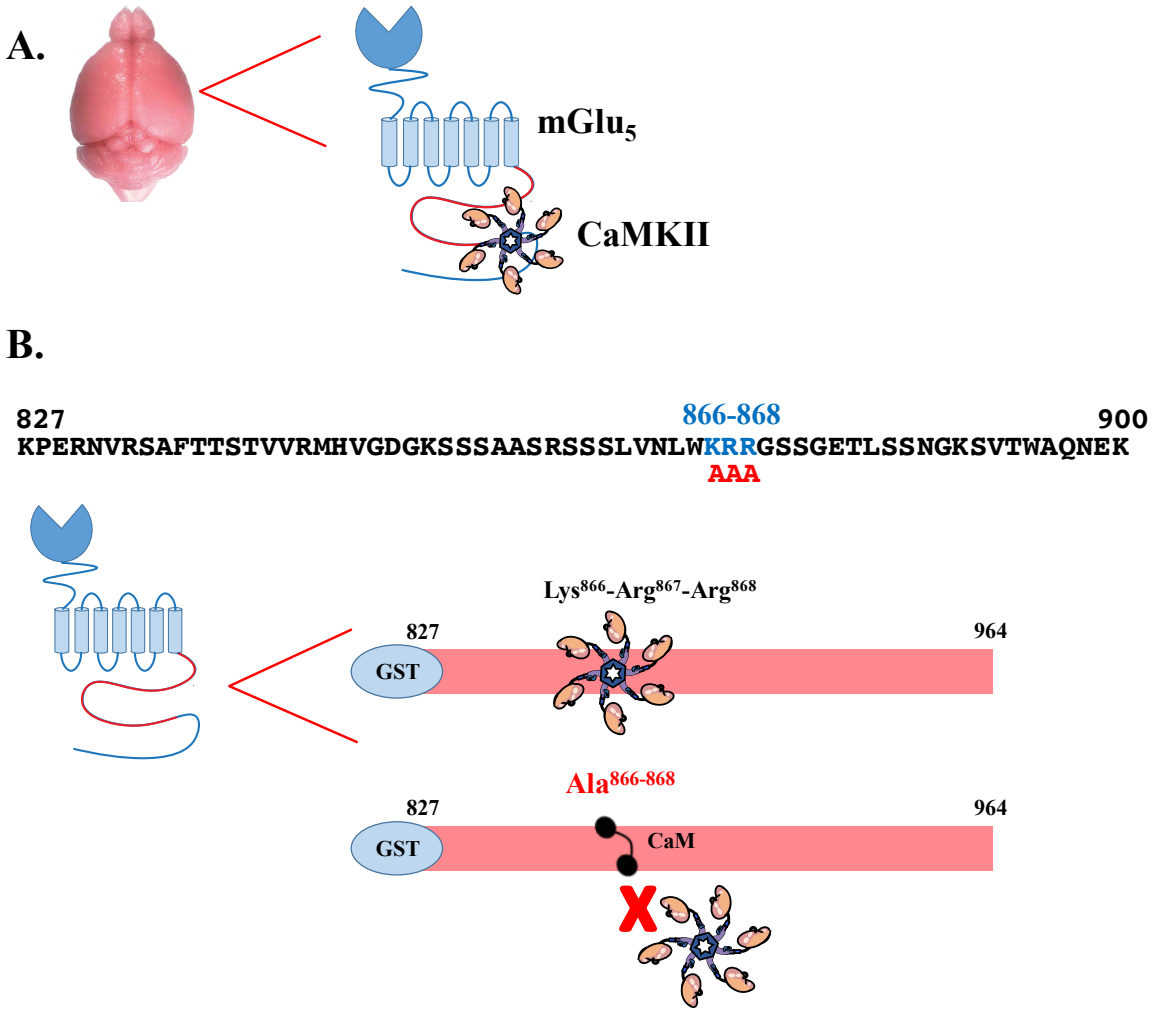

Figure 3.1 *Overview of work performed in Chapter 3.* A. Initial confirmation that $mGlu_5$ and CaMKII bind in mouse forebrains using co-immunoprecipitation studies. B. Creation of a GST-fusion protein of the membrane-proximal region of the $mGlu_5$-CTD (residues 827-964) allowed for *in vitro* binding assays to show that activation of CaMKII increases CaMKII binding to the $mGlu_5$-CTD *in vitro* and in full-length $mGlu_5$ constructs. Additional characterization revealed a tri-basic residue cluster at residues Lys^{866}-Arg^{867}-Arg^{868} necessary for CaMKII binding to this portion of the receptor. Triple alanine mutation of these residues or addition of CaM to the binding reaction resulted in a loss of CaMKII binding in vitro and a reduction in CaMKII binding to full-length $mGlu_5$.

Mouse forebrain lysates contain CaMKIIα-mGlu$_5$ complexes

In order to confirm that mGlu$_5$ specifically associates with CaMKII in the brain, we incubated forebrain lysates from WT or CaMKIIα-KO mice with a CaMKIIα-specific monoclonal antibody or a control IgG. The resulting immune complexes were isolated and then immunoblotted for mGlu$_5$ and CaMKIIα. Note that the mGlu$_5$ antibody used for these studies recognizes an epitope that is shared by the two known mGlu$_5$ splice variants, mGlu$_{5a}$ and mGlu$_{5b}$, which are differentially expressed during development (Minakami et al., 1995; Romano et al., 1996). Input samples from WT and CaMKIIα-KO tissue prepared in parallel contain similar levels of the monomeric and dimeric forms of mGlu$_5$ (Figure 3.2), although the ratio of monomeric and dimeric species varied between independent experiments (not shown). CaMKIIα complexes isolated from WT mouse forebrain contained both monomeric and dimeric forms of mGlu$_5$, with the ratio of these forms reflecting variability in the ratio detected in the inputs. However, very little mGlu$_5$ could be detected in IgG control complexes isolated from WT tissue, or in CaMKIIα complexes isolated from CaMKIIα-KO tissue (Figure 3.2). Thus, mGlu$_5$ is a component of the CaMKIIα complexes present in mouse brain lysates.

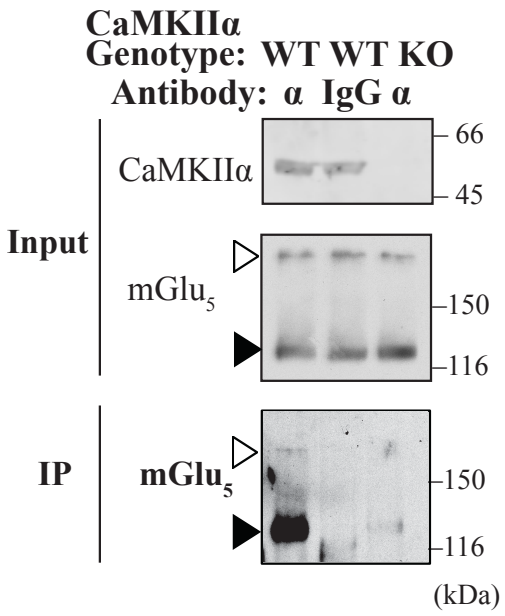

Figure 3.2. *Co-immunoprecipitation of $mGlu_5$ with mouse forebrain CaMKIIα*. Solubilized fractions from WT or CaMKIIα-KO mouse forebrain were immunoprecipitated using CaMKIIα-specific (α) or control (IgG) antibodies, as indicated. Inputs and immune complexes were analyzed by immunoblotting: $mGlu_5$ was detected only in immune complexes isolated from WT tissue using the CaMKIIα antibody. Open and closed arrowheads indicate dimeric and monomeric species of $mGlu_5$. Representative of 3 similar experiments.

CaMKIIα directly binds to the $mGlu_5$ C-terminal domain

Like most prior studies, we chose to use the $mGlu_{5a}$ splice variant for our binding studies. It was previously reported that residues 827-964 of the $mGlu_{5a}$-CTD bind to inactive CaMKIIα, but that CaMKII autophosphorylation disrupted the interaction (Jin et al., 2013b). In order to confirm this finding, we generated a GST-tagged $mGlu_{5a}$-CTD construct containing residues 827-964 (GST-$mGlu_{5a}$-CTD) for use in glutathione agarose co-sedimentation experiments (Figure 3.3). Initial studies detected weak binding of inactive CaMKIIα to GST-$mGlu_{5a}$-CTD that was not consistently

above background binding to a GST negative control (data not shown). Therefore, we systematically tested interactions of GST-mGlu$_{5a}$-CTD with CaMKIIα in various activation states.

Purified CaMKIIα was autophosphorylated in the presence of Ca^{2+}/CaM *in vitro* at either 4°C or at 30°C. Similar total levels of Thr286 autophosphorylation were detected by immunoblotting following incubation at either 4°C or at 30°C (Figure 3.3A), but the 30°C autophosphorylation reduced the electrophoretic mobility of CaMKIIα. These observations are consistent with prior studies showing that incubation at 4°C results in selective Thr286 autophosphorylation (McNeill and Colbran, 1995), whereas incubation at 30°C allows for extensive autophosphorylation at several other sites (Baucum et al., 2015).

We then performed glutathione-agarose cosedimentation experiments to test the interaction of GST-mGlu$_5$-CTD with CaMKIIα in these different activation states (after terminating the autophosphorylation reactions by chelating metal ions with excess EGTA and EDTA). The selective Thr286-autophosphorylation (4°C) protocol resulted in a robust enhancement of CaMKIIα binding to GST-mGlu$_5$-CTD relative to the non-phosphorylated kinase, but this interaction was substantially reduced following more extensive *in vitro* phosphorylation at 30°C (Figure 3.3B). The short exposure times used for development of these immunoblots failed to detect weak binding of inactive CaMKIIα to GST-mGlu$_5$-CTD. In combination, these data show that while activation and Thr286 autophosphorylation of CaMKIIα strongly enhances binding to the mGlu$_{5a}$-CTD, the interaction can be weakened by autophosphorylation at additional non-Thr286 sites. Although we largely focused on the interaction between mGlu$_{5a}$ and CaMKIIα, I also generated data showing that the mGlu$_{5b}$-CTD (residues 827-996), and full-length receptor are capable of binding to CaMKIIα in vitro and in HEK293 cells, respectively.

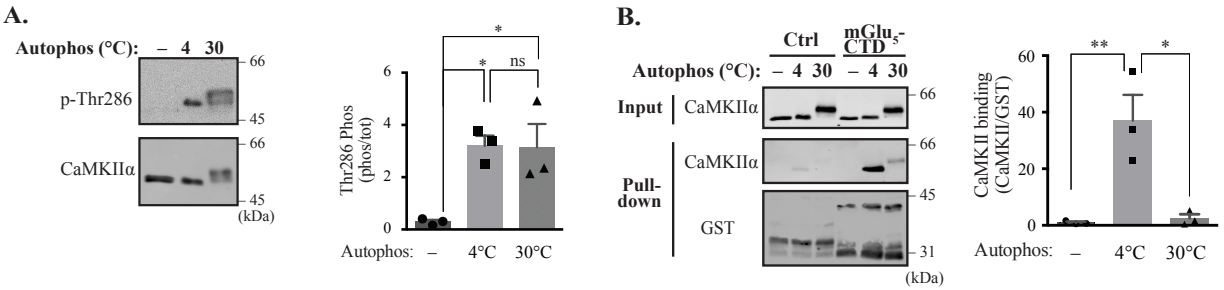

Figure 3.3. *CaMKII autophosphorylation at Thr286 enhances binding to the mGlu$_5$ C-terminal domain*. **A.** *Autophosphorylation of purified CaMKIIα.* Purified CaMKIIα was incubated with *Mg*$(C_2H_3O_2)_2$, $CaCl_2$, CaM, and ATP for either 90 s at 4°C or 10 min at 30°C and samples were immunoblotted for total or phospho-Thr286 CaMKII. While quantitative analysis (right) indicated that there was a similarly robust Thr286 autophosphorylation using these two conditions, the 10 min/30°C incubation resulted in a substantial reduction in electrophoretic mobility due to phosphorylation at additional unidentified sites. Data are plotted as the mean ± SEM (n = 3) and analyzed using a one-way ANOVA (p = 0.0167, F = 8.746, R square = 0.7446) with Sidak's *post-hoc* test for multiplicity adjusted *p* values: Control vs. 4°C, p = 0.031. Control vs. 30°C, p = 0.035. 4°C vs. 30°C, p = 1.00 **B.** *The GST-mGlu$_{5a}$-CTD binds CaMKIIα following selective autophosphorylation at Thr286*. GST-mGlu$_{5a}$-CTD was incubated with purified CaMKIIα that had been preincubated as in panel A, and complexes were isolated using glutathione agarose. Immunoblot analyses revealed that CaMKIIα binding to the CTD was strongly enhanced by selective Thr286 autophosphorylation at 4°C, but that the autophosphorylation of additional sites on CaMKII at 30°C substantially reduced binding. Data are plotted as the mean ± SEM (n = 3) and were analyzed using a one-way ANOVA (p = 0.005, F = 2.477, R square = 0.829) with Sidak's *post-hoc* test for multiplicity adjusted *p* values: Control vs. 4°C, p = 0.009. 4°C vs. 30°C, p = 0.011. Control vs. 30°C, p = 1.00. Data are plotted as the mean ± SEM

Binding of activated CaMKIIα to the GST-mGlu$_{5a}$-CTD is disrupted by Ca^{2+}/CaM

Ca^{2+}/CaM binds to residues 889-917 within the CTD of mGlu$_{5a}$ with important functional consequences (Choi et al., 2011; Lee et al., 2008; Minakami et al., 1997). Moreover, it was previously reported that excess Ca^{2+}/CaM disrupts the binding of inactive CaMKIIα to the mGlu$_{5a}$-CTD (Jin et al., 2013b). Therefore, we tested whether excess Ca^{2+}/CaM also disrupts the binding of activated CaMKIIα to GST-mGlu$_{5a}$-CTD. Thr286-autophosphorylated CaMKIIα (4°C protocol) robustly binds to GST-mGlu$_{5a}$-CTD, as noted above, but this interaction was essentially eliminated by inclusion of excess Ca^{2+}/CaM in the binding assay (Figure 3.4). Thus, binding of activated

CaMKIIα to the $mGlu_{5a}$-CTD is also blocked by Ca^{2+}/CaM, suggesting that multiple Ca^{2+} sensitive proteins are involved in the regulation of $mGlu_5$ signaling.

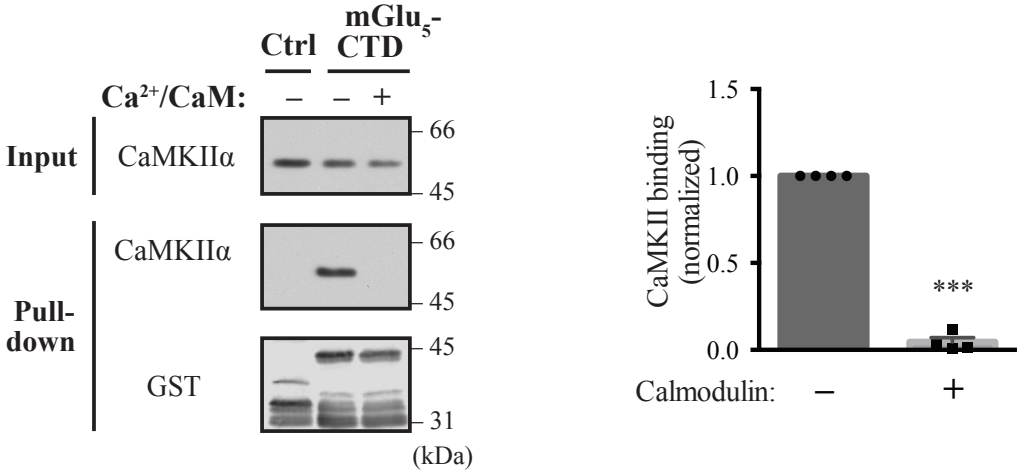

Figure 3.4 *Binding of activated CaMKIIα to GST-$mGlu_{5a}$-CTD is disrupted by Ca^{2+}/CaM*. Purified CaMKIIα was autophosphorylated for 90 s at 4°C and then incubated with GST-$mGlu_{5a}$-CTD in the absence or presence of excess Ca^{2+}/CaM. Complexes were isolated using glutathione-agarose and then immunoblotted as indicated. Data are plotted as the mean ± SEM; excess Ca^{2+}/CaM significantly reduced CaMKIIα binding ($p < 0.0001$ relative to theoretical value of 1.00 by one-sample t-test; $n = 4$).

Identification of a CaMKIIα-binding determinant in the $mGlu_5$-CTD

As an initial approach to identify key CaMKIIα binding determinants in the $mGlu_{5a}$-CTD, we compared residues 827-964 of $mGlu_5$ with CaMKIIα-binding domains that have been previously identified in other proteins. Our lab recently showed that activated CaMKIIα binds to the N-terminal domains of $Ca_V1.2$ and $Ca_V1.3$ L-type voltage gated Ca^{2+} channels, and that this interaction is disrupted by mutation of three basic residues (Arg^{83}-Lys-Arg^{85}) to alanine (Wang et

al., 2017). Similar tri-basic residue motifs are also present within CaMKIIα binding domains that have been previously identified in the intracellular loops of the D_2 and D_3 dopamine receptor (Liu et al., 2009; Zhang et al., 2014), and the $mGlu_1$-CTD (Jin et al., 2013a). Notably, the CaMKIIα-binding fragment of the $mGlu_{5a}$-CTD also contains a tribasic residue motif (residues Lys^{866}-Arg^{867}-Arg^{868}) (Figure 3.5A). We found that substituting alanines for Lys^{866}-Arg^{867}-Arg^{868} in the $mGlu_{5a}$-CTD essentially abolished the binding of activated CaMKIIα to GST-$mGlu_5$-CTD *in vitro* (Figure 3.5b). These data identify a key determinant for CaMKII-binding to the CTD of $mGlu_{5a}$.

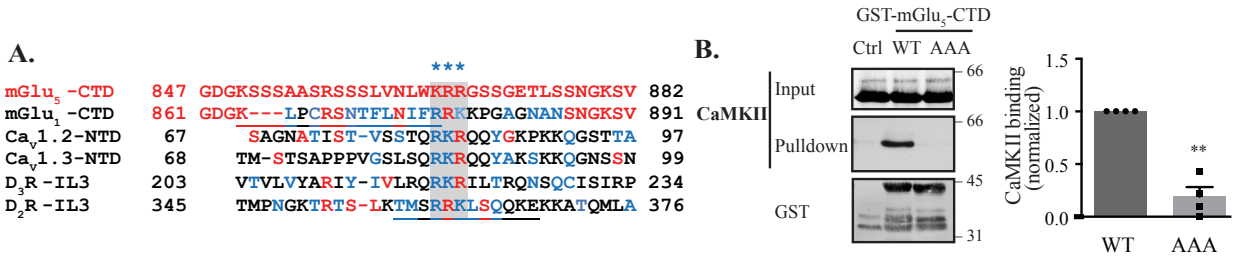

Figure 3.5 *Identification of CaMKII-binding determinants in the $mGlu_{5a}$-CTD*. A. Alignment of part of the $mGlu_{5a}$-CTD with amino acid sequences surrounding known CaMKII-binding domains. Tribasic residue motifs (highlighted) were identified within CaMKII-binding domains from other proteins, as well as within the CaMKII binding fragment in the CTD of $mGlu_{5a}$. Mutation of $R^{83}K^{84}R^{85}$ to AAA in the $Ca_V1.3$ N-terminal domain (NTD) disrupts the binding of CaMKII (Wang et al., 2017). The red and blue fonts indicate residues in each domain that are identical and homologous, respectively, with residues in the $mGlu_{5a}$ sequence. Underlined residues in the $mGlu_1$-CTD and the D_2 dopamine receptor (IL3: third intracellular loop) demark the sequences of synthetic peptides that were shown to compete for CaMKII binding (Jin et al., 2013a; Zhang et al., 2014). B. *Mutation of the tribasic residue motif in the $mGlu_{5a}$-CTD disrupts CaMKII binding*. Thr286 autophosphorylated CaMKIIα (90 s/4°C protocol) was incubated with GST-$mGlu_{5a}$-CTD (WT or with a $K^{866}R^{867}R^{868}$ to AAA mutation) and complexes were analyzed as in Fig. 1. The $K^{866}R^{867}R^{868}$/AAA mutation essentially abolishes CaMKII binding Data are plotted as the mean ± SEM ($p = 0.003$ by a one sample t-test; $n = 4$).

CaMKIIα activation increases CaMKIIα-mGlu$_{5a}$ association in heterologous cells

In order to better understand the interaction of CaMKIIα with full-length mGlu$_{5a}$ we conducted co-immunoprecipitation experiments from lysates of transfected HEK293A cells. We first tested the hypothesis that CaMKIIα activation would increase the association with full-length mGlu$_{5a}$, as with *in vitro* binding of CaMKIIα to GST-mGlu$_{5a}$-CTD. We expressed mApple-tagged WT CaMKIIα in the absence or presence of mGlu$_{5a}$ with an N-terminal HA-epitope tag in HEK293A cells. Prior to HA immunoprecipitation, the cell lysates were split into two aliquots and pre-incubated with either excess EGTA and EDTA or with Ca^{2+}/CaM, Mg^{2+}, ATP, and phosphatase inhibitors to stimulate CaMKIIα autophosphorylation. CaMKII activation in the lysates resulted in a robust increase in autophosphorylation at Thr286, without the large shift in electrophoretic mobility that was observed following autophosphorylation of purified CaMKIIα at 30°C (Figure 3.6). HA-immununoprecipitation from the two preincubated lysates yielded similar amounts of the monomeric and dimeric species of HA-mGlu$_{5a}$, but CaMKIIα activation resulted in a statistically significant ~3-fold increase in the amount of co-immunoprecipitated CaMKIIα (Figure 3.6). These data show that full-length mGlu$_{5a}$ preferentially interacts with activated WT CaMKIIα.

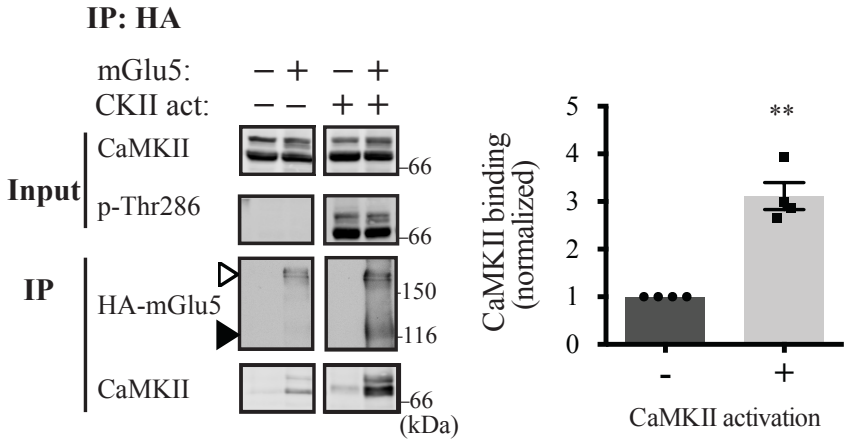

Figure 3.6 *CaMKII activation enhances interaction with full length mGlu$_5$.* Solubilized fractions of HEK293A cells expressing HA-tagged mGlu$_{5a}$ and/or mApple-tagged WT CaMKIIα (as indicated above lanes) were pre-incubated with Ca^{2+}/CaM, $MgAc_2$, and ATP in the presence or absence of excess EDTA (–/+ activation, respectively) and then immunoprecipitated using antibodies to the HA epitope. Lysates and immune complexes were analyzed by immunoblotting, as indicated. CaMKIIα activation results in robust Thr-286 autophosphorylation, which increases CaMKIIα association with HA-mGlu$_{5a}$ Data are plotted as the mean ± SEM ($p = 0.043$; one-sample t-test; $n = 4$). Open and closed arrowheads indicate dimeric and monomeric species of mGlu$_{5a}$.

Association of activated CaMKIIα with full-length mGlu$_{5a}$ requires Lys^{866}-Arg^{867}-Arg^{868}

We next investigated whether the association of activated CaMKIIα with full-length mGlu$_{5a}$ involves the CTD. In order to avoid complications that might arise from preincubating cell lystates to activate WT-CaMKIIα, we used an mApple-tagged constitutively active T286D/T305A/T306A triple mutant of CaMKIIα (mApple-CA-CaMKIIα); the phospho-mimetic T286D mutation results in constitutive CaMKIIα activity and the phospho-null T305A/T306A mutations prevent CaMKIIα phosphorylation at these sites, which interferes with binding of Ca^{2+}/CaM and α-actinin (Jalan-Sakrikar et al., 2012). The mApple-CA-CaMKIIα was expressed alone, or co-expressed with either HA-mGlu$_{5a}$ or HA-mGlu$_{5a}$-AAA (with Lys^{866}-Arg^{867}-Arg^{868} mutated to alanines). HA-

immunoprecipitation from cell lysates confirmed a robust association of mApple-CA-CaMKIIα with WT $mGlu_{5a}$ that was partially (~50%) reduced by the triple alanine mutation in the CTD (Figure 3.7). These data demonstrate that the Lys^{866}-Arg^{867}-Arg^{868} residues in the $mGlu_{5a}$-CTD play an important role in the association of activated CaMKIIα with the full-length $mGlu_5$ receptor.

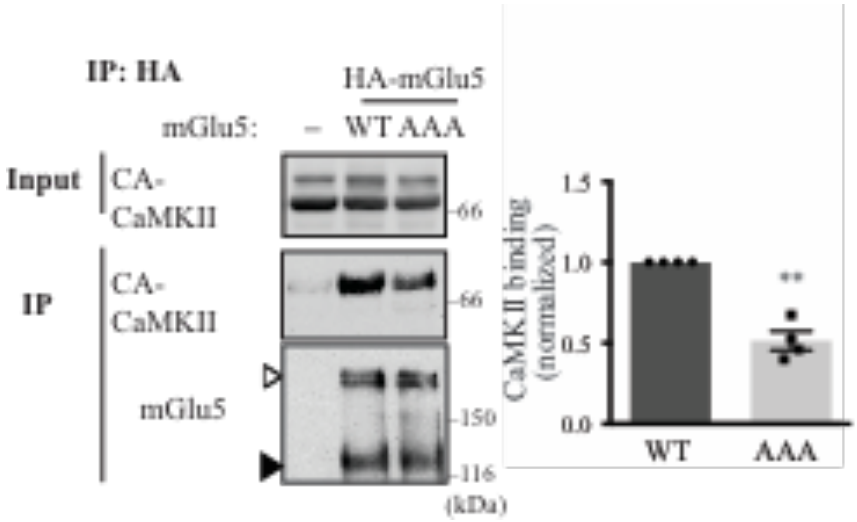

Figure 3.7 *CaMKII association with full-length $mGlu_{5a}$ is disrupted by mutation of the CTD tribasic residue motif*. Solubilized fractions of HEK293A cells expressing HA-$mGlu_5$ (WT or with the $K^{866}R^{867}R^{868}$/AAA mutation) and mApple-tagged CA-CaMKIIα were immunoprecipitated using antibodies to the HA epitope. Lysates and the immune complexes were analyzed by immunoblotting, as indicated. The $K^{866}R^{867}R^{868}$/AAA mutation reduced the association of CA-CaMKIIα with HA-$mGlu_5$ Data are plotted as the mean ± SEM ($p = 0.028$; one sample t-test; $n = 4$).

Discussion

In a previous report, the membrane proximal region of the $mGlu_{5a}$-CTD was shown to bind *inactive* CaMKII (Jin et al., 2013b). Here we extend these findings by further characterizing the physical and functional relationship between these key regulators of synaptic transmission. We confirmed

that CaMKIIα and $mGlu_5$ specifically interact in mouse brain. However, our data show that $mGlu_{5a}$-CTD residues 827-964 bind more strongly to CaMKIIα in an active, Thr286-autophosphorylated conformation, but that this interaction is disrupted by excess Ca^{2+}/CaM or by robust CaMKII autophosphorylation at additional undefined sites.

There is a growing appreciation that specific physiological actions of CaMKII are modulated in part through dynamically-regulated interactions with CaMKAPs. Several CaMKAPs preferentially interact with activated conformations of CaMKII; these CaMKAPs can be sub-classified based on differences between the amino acid sequences of their CaMKII-binding domains. CaMKII-binding domains in the NMDAR GluN2B subunits and calcium channel β1 and β2 subunits resemble the CaMKII regulatory domain (Grueter et al., 2008; Jiao et al., 2008; Strack et al., 2000). In contrast, the amino acid sequence of a CaMKII-binding domain in densin has similarity with a naturally occurring CaMKII inhibitor protein (CaMKIIN) (Jiao 2011). Here, we show here that the binding domain for activated CaMKII in the $mGlu_{5a}$-CTD does not resemble these CaMKAPs. Rather, this novel interaction requires three basic residues (Lys^{866}-Arg^{867}-Arg^{868}), similar to the recently identified interaction of activated CaMKII with the N-terminal domains of L-type voltage-gated Ca^{2+} channels (Wang et al., 2017). Interestingly, triple basic residue motifs can also be identified in CaMKII-binding domains of other GPCRs, including intracellular loops of the $G\alpha_i$-coupled D_2 and D_3 dopamine receptors (Zhang et al., 2014; Liu et al., 2009), and the CTD of the $mGlu_1$ receptor (Jin et al., 2013a; Jin et al., 2013b), which also couples to $G\alpha_{q/11}$ (Figure 3). Thus, it will be interesting to investigate the role of these triple basic residue motifs in CaMKII binding to additional GPCRs.

One unusual aspect of CaMKII binding to the $mGlu_{5a}$-CTD is that, while the *in vitro* interaction requires CaMKIIα activation and Thr286 autophosphorylation, additional autophosphorylation at non-Thr286 sites following incubation at 30°C reduces the binding. Our recent proteomics analyses of purified CaMKIIα autophosphorylated *in vitro* using a similar 30°C protocol detected 17 autophosphorylation sites, in addition to Thr-286 (Baucum et al., 2015). Presumably the autophosphorylation at one or more of these non-Thr286 sites interferes with *in vitro* CaMKIIα binding to $mGlu_{5a}$. While this is a potentially interesting finding, parallel proteomics analyses of CaMKII isolated from mouse brain failed to detect phosphorylation at many of these *in vitro* sites (Baucum et al., 2015). However, it is possible that this observation explains why Jin and colleagues found that autophosphorylated CaMKII did not bind to $mGlu_{5a}$ *in vitro* (Jin et al., 2013b) because their autophosphorylation reactions were incubated at 30°C.

Our data show that CaMKIIα activation enhances the association with full-length $mGlu_{5a}$, and that this interaction involves the Lys^{866}-Arg^{867}-Arg^{868} motif in the CTD. However, triple alanine substitution of CTD residues 866-868 reduced the interaction by only ~50%, suggesting that CaMKII may interact with additional regions in $mGlu_{5a}$ or bind to the receptor through an indirect interaction. Indeed, a CaMKII interaction with the second intracellular loop of $mGlu_5$ has been previously reported (Raka et al., 2015). In order to determine the effect of CaMKII binding to the $mGlu_5$-CTD we used the tools generated in this chapter to study the role of CaMKII on $mGlu_5$ signaling.

Chapter IV

Consequences of CaMKII-mGlu$_5$ binding on mGlu$_5$ receptor localization and signaling

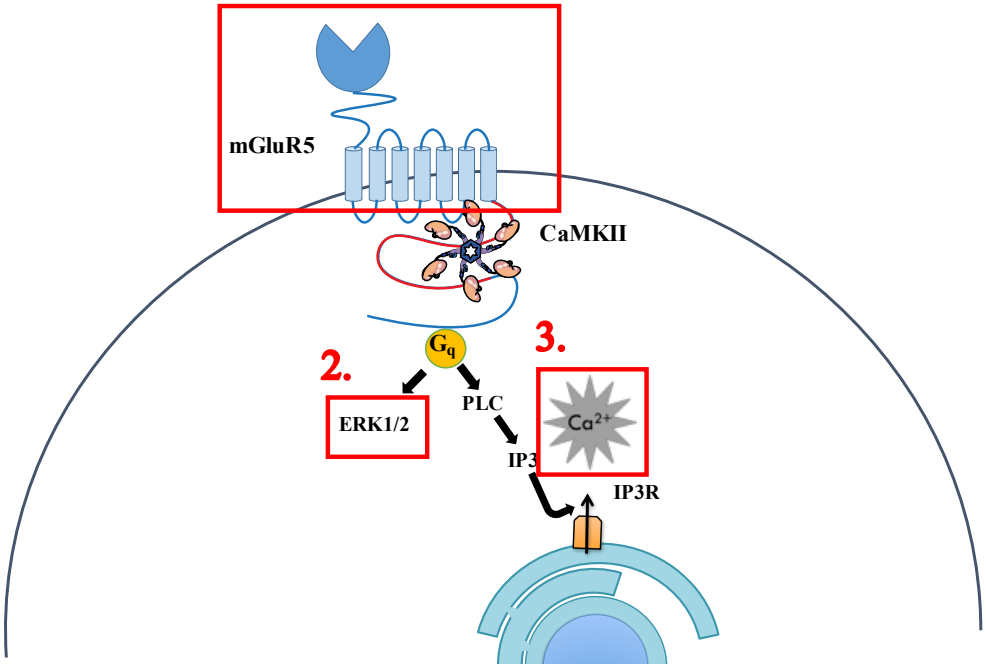

Figure 4.1 *Overview of work presented in Chapter IV.* Schematic representation of mGlu$_5$-dependent downstream signaling that include activation of PLC and Ca^{2+} release from intracellular stores and activation of a Src/ERK-cascade. In this chapter I examine the effect of CaMKII on three readouts of mGlu$_5$ function including (1) surface expression, (2) ERK1/2 activation, and (3) Ca^{2+} release from intracellular stores in a heterologous cell system.

Introduction

Work to characterize the CaMKII binding domain in the mGlu$_5$-CTD made it possible to determine the effect of CaMKII binding on receptor localization and signaling in a heterologous cell system.

$mGlu_5$ activation has many downstream effects that can be controlled in a number of different ways including surface expression, protein binding, and phosphorylation. Here, I examined the role of CaMKII in three readouts of $mGlu_5$ activation including the CaMKII effect on surface expression, ERK1/2 activation, and Ca^{2+} release from intracellular stores (Figure 4.1). CaMKII had significant effects on all three of these readouts including an increase in basal $mGlu_5$ surface expression disrupted by mutation of the CaMKII binding site on the $mGlu_5$-CTD, an increase in ERK1/2 activation, and a reduction in the initial peak Ca^{2+} amplitude but prolonged $mGlu_5$-mediated Ca^{2+} signals. These results were published in the same manuscript as the results shown in Chapter III (Marks et al., 2018). All figures in this chapter with the exception of Figure 4.1 and Figure 4.3 were published in this manuscript

CaMKIIα increases basal $mGlu_{5a}$ surface expression

Since the CTD is known to modulate $mGlu_5$ cell surface expression and consequently $mGlu_5$ signaling, we investigated the effect of CaMKIIα on the cell-surface expression of full-length $mGlu_{5a}$. Intact HEK293A cells expressing $mGlu_5$ with or without mApple-CA-CaMKIIα were incubated with Sulfo-NHS-SS-Biotin to biotinylate all surface-expressed proteins. Streptavidin-conjugated magnetic beads were then used to isolate cell-surface proteins from cell lysates. Immunoblototting of total cell lysates and isolated cell-surface proteins revealed that the co-expression of mApple-CA-CaMKIIα increased the proportion of $mGlu_{5a}$ expressed on the cell-surface by 3.0±0.7-fold (SEM) under basal conditions ($p = 0.036$; one-sample t-test vs. hypothetical value of 1) (Figure 4.2). In order to determine whether CaMKIIα interaction with the $mGlu_{5a}$-CTD is important for this effect we examined the cell surface expression of $mGlu_{5a}$-AAA, in which Lys^{866}-Arg^{867}-Arg^{868} in the CTD were replaced with alanines. In the absence of co-

expressed CaMKII, the surface expression of $mGlu_{5a}$-AAA was not significantly different from those of WT $mGlu_{5a}$ (1.6±0.6-fold (SEM); n = 5; p = 0.35; one-sample t-test vs. hypothetical value of 1). Moreover, the co-expression of mApple-CA-CaMKIIα had no effect on cell-surface expression of $mGlu_{5a}$-AAA. These data demonstrate that interaction with the $mGlu_{5a}$-CTD is necessary for CaMKIIα-mediated increases in $mGlu_{5a}$ cell-surface expression.

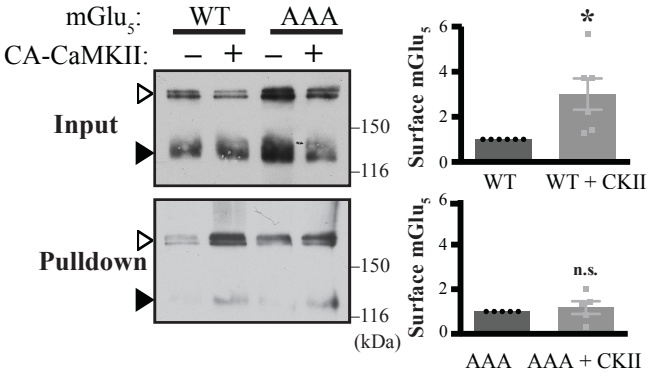

Figure 4.2 *CaMKII enhances the cell-surface expression of $mGlu_{5a}$ via interaction with the CTD*. Cell-surface biotinylation analyses of HEK293A cells expressing $mGlu_{5a}$ (WT or with $K^{866}R^{867}R^{868}$/AAA mutation in the CTD) with either mApple or mApple-tagged CA-CaMKIIα. The co-expression of CA-CaMKIIα increased steady-state surface expression levels of WT $mGlu_{5a}$ (p = 0.036; one sample t-test; n = 6), but not of the $K^{866}R^{867}R^{868}$/AAA mutant. Data are plotted as the mean ± SEM (p = 0.569; one sample t-test; n = 5).

CA-CaMKIIα increases glutamate stimulated ERK activation

To investigate the effect of CaMKIIα on $mGlu_{5a}$ signaling through the activation of the ERK-cascade we measured glutamate-induced ERK activation (phosphorylation) in populations of 293A-$5a^{LOW}$ cells that stably express $mGlu_{5a}$ and were transiently transfected to co-express

mApple or mApple-tagged CaMKIIα (WT, kinase dead (K42R), or CA (T286D/305/306AA). Cells were glutamate starved then treated with 100 μM glutamate for 5 minutes. The reaction was stopped and the cell lysates were probed for CaMKII, and total and phosphorylated levels of ERK to look for ERK activation. The glutamate response was unaffected by co-expression of m-Apple, mApple-WT-CaMKIIα, mApple-KD-CaMKIIα, but the co-expression of mApple-CA-CaMKIIα increased glutamate-stimulated ERK activation (Figure 4.3). I attempted to replicate these studies in transiently-transfected cells, which would allow us to determine the role of the CaMKII-CTD interaction. However, despite extensive efforts to optimize my procedure, my analyses were frustrated by considerable variability in basal levels of ERK activation and glutamate-induced ERK activation levels, likely reflecting variability in the transfection and expression of the WT and mutated receptor.

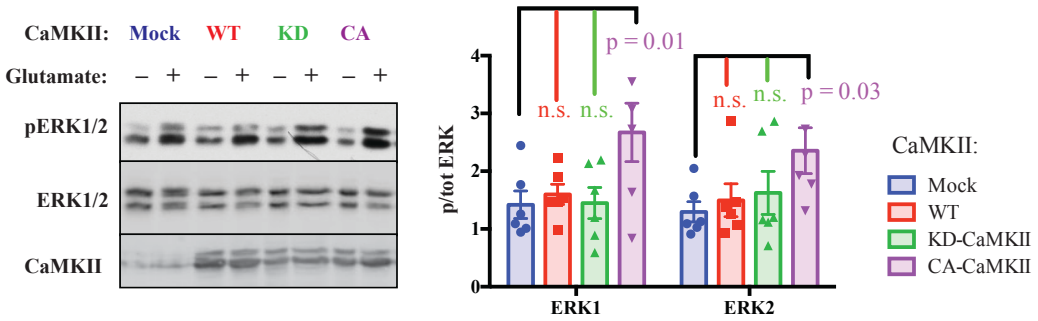

Figure 4.3 *CA-CaMKII enhances mGlu5 ERK1/2 activation.* Stable cells expressing low amounts of $mGlu_5$ (293A-5a^{LOW} cells) were transfected with an empty vector (Mock), WT, kinase dead (KD-CaMKII with catalytic mutation K42R) or CA-CaMKII (T286D/AA) and treated with 100 µM glutamate for 5 min. Cell lysates were immunoblotted for total and p-ERK1/2. Only the presence of CA-CaMKII had a statistically effect to enhance glutamate-stimulated ERK1/2 activation by $mGlu_5$ Two-way repeated measures ANOVA with Sidak's multiple comparisons test n = 6 Source of variation ERK1/2 p = 0.7393, transfection p = 0.0002, ERK1: Mock vs WT p = 0.95, mock vs. K42R p = 0.99, mock vs. T286D/AA p = 0.01, ERK2: Mock vs WT p = 0.9309, Mock vs K42R p = 0.7591, mock vs T286D/AA p = 0.03.

CaMKIIα reduces $mGlu_{5a}$-stimulated peak Ca^{2+} mobilization

To investigate the effect of CaMKIIα on $mGlu_{5a}$ signaling, we measured glutamate-induced Ca^{2+} mobilization in populations of 293A-5a^{LOW} cells that stably express $mGlu_{5a}$ and were transiently transfected to co-express mApple or mApple-tagged CaMKIIα (either WT or CA). A similar fraction of the total cells expressed detectable levels of mApple-tagged WT- or CA-CaMKIIα in each transfection (typically ~60%). After loading glutamate-starved cells with fluo-4-AM, a fluorescent Ca^{2+} indicator, we measured fluorescence responses of total cell populations to increasing glutamate concentrations (0.01-100 µM) (Figure 4.4A). An overlay of raw traces from cells expressing mApple, mApple-WT-CaMKIIα, or mApple-CA-CaMKIIα in a representative experiment is shown in Figure 4.4B. Peak Ca^{2+} responses (increased fluorescence) at each glutamate concentration were expressed as a ratio to the maximum response to a saturating concentration of glutamate (100 µM) in mApple-expressing control cells for each individual

experiment, and then data were averaged across 5 independent experiments. Glutamate increased the peak fluorescence in a concentration-dependent manner, with an apparent EC_{50} of 0.38±0.03 μM in control cells, similar to previous analyses (Hammond et al., 2010; Schoepp et al., 1999). The glutamate response was unaffected by co-expression of mApple-WT-CaMKIIα, but the co-expression of mApple-CA-CaMKIIα reduced peak Ca^{2+} responses at the highest concentrations of glutamate by approximately 20%, without affecting the apparent EC_{50} (Figure 4.4C). As an alternative measure of Ca^{2+} responses, we determined the area under the curve of the initial Ca^{2+} peak at the highest glutamate concentration. There was no difference in area under the curve between cells expressing mApple or mApple-CaMKIIα-WT, but the co-expression of mApple-CA-CaMKIIα significantly reduced the area under the curve (Control, 109.2±2.7; WT, 101.3±7.4; CA, 82.6±4.1. One-way ANOVA, p = 0.011, F=6.280. Sidak's *post hoc* test for multiplicity adjusted *p* values: WT vs. control, p = 0.51, CA vs. control, p = 0.0073) (data not shown). Since mApple-CA-CaMKIIα is expressed in only a fraction of the cell population in each well, the measured reductions in maximal Ca^{2+} responses presumably under-estimate the actual impact of expressing CA-CaMKIIα in each cell. However, these data cannot differentiate whether this effect reflects decreased Ca^{2+} mobilization within each cell or a decrease in the fraction of responsive cells. Nevertheless, the data indicate that the co-expression of CA-CaMKIIα but not WT-CaMKIIα can reduce $mGlu_{5a}$-stimulated peak Ca^{2+} mobilization.

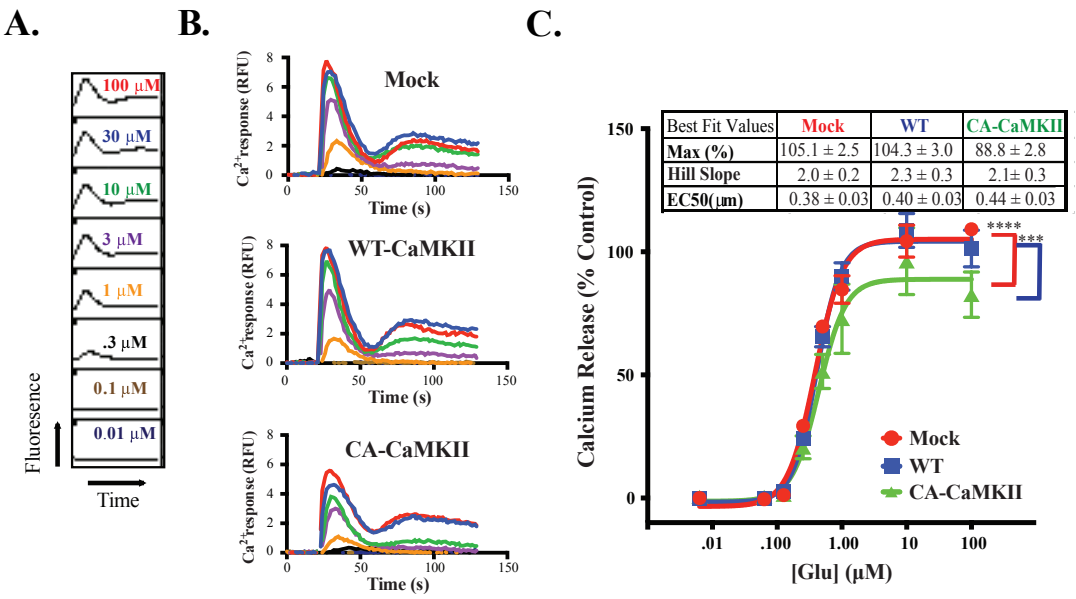

Figure 4.4 *CaMKIIα regulates $mGlu_{5a}$-stimulated Ca^{2+} mobilization in 293A-$5a^{LOW}$ cells*. Time courses of intracellular Ca^{2+} responses to glutamate were measured by changes in Fluo-4 fluorescence in stable 293A-$5a^{LOW}$ cells in 96 well plates. A. *Time courses of Ca^{2+} responses*. Example of calcium responses to increasing glutamate concentrations collected in a row of 8-wells. B. Overlay of individual Ca^{2+} responses to increasing concentrations of glutamate (labeled by colors in Panel A) from 293A-$5a^{LOW}$ cells transiently transfected to express mApple control, mApple-CaMKIIα-WT or mApple-CA-CaMKIIα from a representative experiment. C. *Concentration response curves*. Initial peak Ca^{2+} responses (ΔF/F0) at each concentration were normalized to the maximal glutamate-stimulated response in control (mApple-transfected) cells within each experiment. Normalized Ca^{2+} responses are plotted as the mean ± SEM (n = 5 experiments) as a function of glutamate concentration. The expression of CaMKIIα-WT had no impact on the Ca^{2+} response curve but the expression of CA-CaMKIIα reduced peak Ca^{2+} responses (multiple comparisons two-way ANOVA: Sources of Variation: CaMKII $p < 0.0001$, Interaction $p = 0.029$. Tukey's *post hoc* test for multiplicity adjusted *p* values: mApple vs. WT, $p = 0.926$, mApple vs. CA-CaMKIIα, $p < 0.0001$. WT vs. CA-CaMKIIα, $p = 0.0002$). The inset table shows the maximum response (Max), EC_{50} (µM), and Hill coefficient (± SEM) obtained by fitting the data in GraphPad Prism.

CaMKIIα prolongs $mGlu_{5a}$-mediated Ca^{2+} signaling

To address caveats associated with studies investigating the effects of mApple-CA-CaMKIIα on Ca^{2+} mobilization in 293A-$5a^{LOW}$ cells, we also examined Ca^{2+} mobilization in single HEK293A cells transfected to express full-length $mGlu_{5a}$ with either mApple alone (control) or mApple-CA-CaMKIIα. After loading all cells with Fura-2-AM, a ratiometric Ca^{2+} indicator, single cells were selected for analysis based on the presence of mApple as a marker of transfection. Application of 100 μM glutamate to cells co-expressing soluble mApple or mApple-CA-CaMKIIα with WT $mGlu_{5a}$ produced an initial peak of Fura-2 fluorescence followed by highly variable changes of fluorescence over the next 10 min (Figure 4.5A).

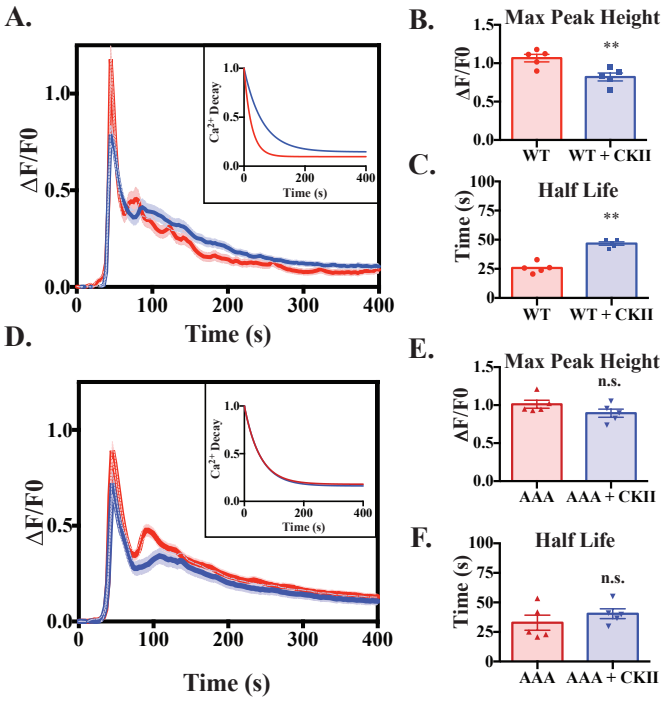

Figure 4.5. *CaMKIIα binding to the CTD is required for modulation of mGlu$_{5a}$-stimulated Ca^{2+} mobilization*. HEK293A cells were transiently transfected to express mGlu$_{5a}$ (WT or K^{866}RR^{868}/AAA) with either mApple or mApple-CA-CaMKIIα for single-cell Fura-2 Ca^{2+} imaging (see Methods). **A, D.** *Representative data from a single experiment*. Averaged normalized changes in fluorescence from 58-114 cells (ΔF/F0: mean ± SEM) expressing mGlu$_{5a}$-WT (**A**) or mGlu$_5$-K^{866}R^{867}R^{868}/AAA (**D**) in the presence (blue lines) or absence (red lines) of mApple-CA-CaMKIIα. The inset graphs show line-fits for time courses of the decline of Ca^{2+} signals from the peak ΔF/F0 under each condition. **B, C, E, F.** *Summary data*. The bar graphs depict mean ± SEM values for peak Ca^{2+} signals (ΔF/F0) (**B, E**) and half-lives for the decline in Ca^{2+} signals (**C, F**) with super-imposed data points from each experiment (n = 5). Expression of constitutively-active mApple-CA-CaMKII decreases the peak Ca^{2+} signal but increases the half-life of the Ca^{2+} signal with mGlu$_{5a}$-WT (B, p = 0.009. C, p = 0.001), but has no significant effect on the mGlu$_{5a}$-K^{866}R^{867}R^{868}/AAA mutant that disrupts CaMKII binding to the CTD (E, p = 0.155. F, p = 0.415). Paired Student's t-tests were used for statistical comparisons in each panel.

In a majority of cells in each group (53-68%) Ca^{2+} signals waned over time, sometimes with a secondary shoulder, but subpopulations of the cells displayed clear Ca^{2+} oscillations that either

returned to baseline between oscillations (10-21%) or were super-imposed on a more sustained Ca^{2+} elevation (18-25%) (Figure 4.6A-B).

However, the percentage of WT $mGlu_{5a}$ cells exhibiting Ca^{2+} oscillations was unaffected by the co-expression of mApple-CA-CaMKIIα (Figure 4.6C). Since it is unclear whether oscillating and non-oscillating cells have different physiological effects, we developed an approach to analyze the responses of all cells (both oscillating and non-oscillating) across 5 independent experiments, revealing that the initial peak fluorescence was significantly reduced ($p = 0.009$) in cells expressing mApple-CA-CaMKIIα vs. cells expressing mApple alone (Figure 4.5A), consistent with data from stably transfected cell populations (Figure 4.4). Moreover, the Ca^{2+} signal was relatively prolonged in cells expressing mApple-CA-CaMKIIα vs. cells expressing mApple alone, as reflected by a statistically-significant increase in the half-life of the fluorescence signal (Figure 4.5C). We also analyzed responses in subsets of the cells within each population that exhibited at least three baseline Ca^{2+} oscillations (Figure 4.7).

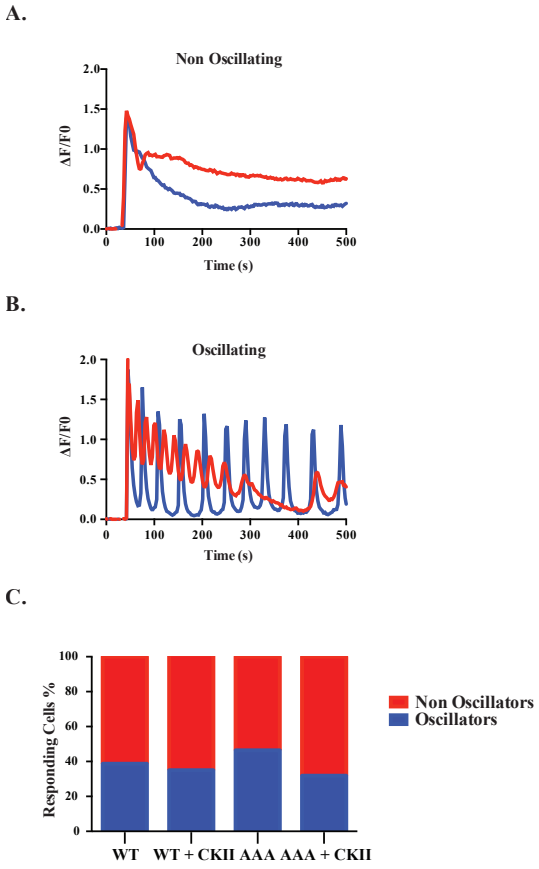

Figure 4.6 *Variability of glutamate-induced mGlu$_5$ Ca^{2+} responses in transfected HEK293A cells.* Data reported is from data summarized in Fig. 7. Responses of individual cells could be divided into two main categories: **A**. *Non-oscillators*: An initial peak of Ca^{2+} that is sustained or returns to baseline over time (blue), in some cases with second shoulder (red), with no clear oscillations. **B**. *Oscillating cells:* The initial Ca^{2+} peak decays in an oscillatory pattern (up to ~25 oscillations in 10 min) that may (blue) or may not (red) return to baseline between successive oscillations. **C**. *Distribution of responding cells between non-oscillating or oscillating categories for each transfection condition*. The ratio of non-oscillating to oscillating HEK293A cells was not affected by co-expression of mApple-CA-CaMKIIα with WT mGlu$_{5a}$ ($p = 0.28$ by Fisher's exact test) or by mutation of Lys^{866}-Arg^{867}-Arg^{868} in the CTD to alanines in mGlu$_{5a}$-AAA ($p = 0.62$).

There was no statistically significant difference in the total number of $mGlu_{5a}$-mediated Ca^{2+} oscillations between transfection conditions (Figure 4.7B). However, co-expression of mApple-CA-CaMKIIα reduced the relative rate of decay of peak Ca^{2+} signals in successive oscillations (Figure 4.7C). Co-expression of mApple-CA-CaMKIIα also increased the frequency of Ca^{2+} oscillations, as reflected by a reduction of the inter-event intervals (Figure 4.7D). In combination, these data indicate that CaMKIIα can reduce the amplitude of initial $mGlu_{5a}$-dependent Ca^{2+} mobilization while extending the relative duration of Ca^{2+} signals and increasing the frequency of oscillations when they are present.

To test the hypothesis that CaMKIIα binding to the $mGlu_{5a}$-CTD is necessary for the modulation of Ca^{2+} mobilization, we examined the effect of co-expressing mApple-CA-CaMKIIα with $mGlu_{5a}$-AAA, in which Lys^{866}-Arg^{867}-Arg^{868} in the CTD were replaced with Ala (Figure 7D). This CTD mutation had little effect on glutamate-stimulated Ca^{2+} mobilization in cells expressing mApple. Moreover, the co-expression of mApple-CA-CaMKIIα with $mGlu_{5a}$-AAA had no statistically significant effect on either the initial peak (Figure 4.5E) or the duration (Figure 4.5F) of the glutamate-stimulated Ca^{2+} signal relative to control cells expressing mApple alone. Furthermore, the CTD mutation had no statistically significant effect on the responses of cells displaying baseline Ca^{2+} oscillations (Figure 4.6D), but abrogated the CaMKII-dependent modulation, as reflected by a lack of effect on the peak height decay of successive Ca^{2+} oscillations (Figure 4.7C) and the Ca^{2+} oscillation frequency (Figure 4.7D). These data indicate that binding to the $mGlu_{5a}$-CTD is important for both the increase of initial peak Ca^{2+} signals and for the prolonged Ca^{2+} signaling induced by co-expression of CA-CaMKIIα.

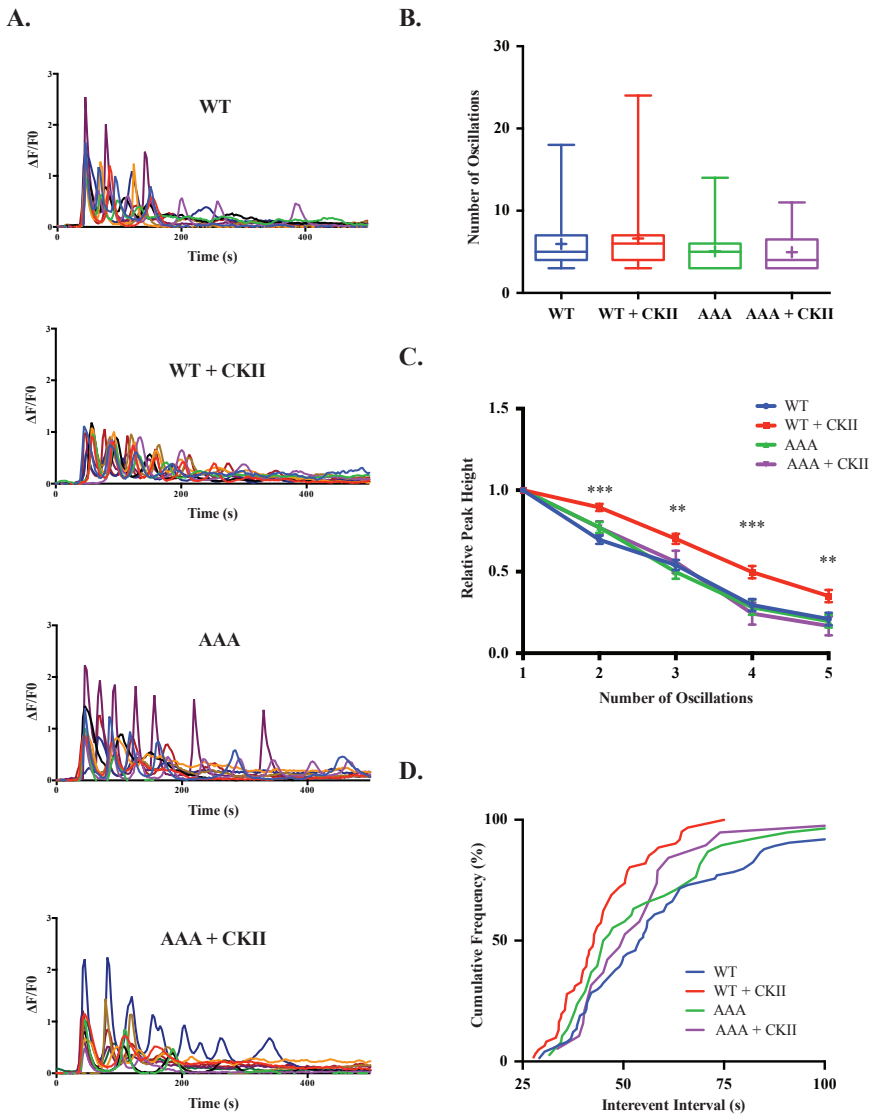

Figure 4.7. *Ca^{2+} responses in baseline-oscillating HEK293A cells.* Expressing $mGlu_5$-WT or $mGlu_5$-AAA with either mApple or mApple-CA-CaMKII **A**. Overlays of ten representative Ca^{2+} responses for each transfection condition from cells selected for ≥3 baseline oscillations. **B**. There were no statistically significant differences in the total number of oscillations recorded over 10 min between transfection conditions (total numbers of baseline-oscillating cells: $mGlu_5$-WT, 78; $mGlu_5$-WT+CKII, 62; $mGlu_5$-AAA, 38; $mGlu_5$-AAA+CKII, 21. One-way ANOVA: $p = 0.08$). The error bars depict the range between the minimum (3) and maximum number of oscillations, boxes indicate the 25-75th percentile, lines within each box indicate the median, and the "+" sign within each box indicates the mean. **C**. Peak responses for the first 5 oscillations were normalized to the first Ca^{2+} peak and plotted as the mean ± SEM for each transfection condition. Co-expression

of mApple-CA-CaMKII significantly slows the rate of decay of successive peak Ca^{2+} responses in cells expressing $mGlu_{5a}$-WT, but has no effect in cells expressing $mGlu_{5a}$-AAA (2-way repeated measures ANOVA . CaMKII effect, $p<0.0001$, Sidak's test for multiplicity adjusted *p* values: $mGlu_{5a}$ vs $mGlu_{5a}$-WT+CaMKII $p = 0.0003$, $mGlu_{5a}$ vs. $mGlu_{5a}$-AAA $p>0.999$, $mGlu_{5a}$-AAA vs $mGlu_{5a}$-AAA + CaMKII $p>0.999$). **D**. Cumulative probability curves for mean inter-event intervals in baseline-oscillating cells. Co-expression of mApple-CA-CaMKII significantly decreases inter-event intervals between Ca^{2+}-oscillations in cells expressing $mGlu_{5a}$-WT but not $mGlu_{5a}$-AAA (Kruskal Wallis test $p = 0.006$, Dunn's test for multiplicity adjusted *p* values: WT-$mGlu_{5a}$ vs WT-$mGlu_{5a}$ + CaMKII $p = 0.003$, WT-$mGlu_{5a}$ vs $mGlu_{5a}$-AAA $p>0.999$, $mGlu_{5a}$-AAA vs $mGlu_{5a}$-AAA + CKII $p>0.999$).

Discussion

The data in this chapter indicate that CaMKII binding to the CTD exerts complex effects on $mGlu_{5a}$ surface expression and downstream Ca^{2+} mobilization. Our analyses in heterologous cells indicate that CaMKIIα interaction with the CTD is critical for several novel functional effects of CaMKII on $mGlu_{5a}$ signaling. First, we show here that CaMKIIα can increase cell surface expression of $mGlu_{5a}$. Second, we found that CaMKIIα has complex effects on $mGlu_{5a}$-dependent Ca^{2+} mobilization. As noted previously, $mGlu_5$ activation can induce temporally-diverse intracellular Ca^{2+} responses in heterologous cells and in neurons (Flint et al., 1999; Jong and O'Malley, 2017; Kim et al., 2005; Mao and Wang, 2003; Uematsu et al., 2015). The co-expression of CaMKII had little effect on the proportion of cells exhibiting different oscillatory or non-oscillatory response patterns. However, we found that the co-expression of CA-CaMKIIα reduces the amplitude of the initial peak Ca^{2+} signals (Figure 4.4C, Figure 4.5B), but prolongs the duration of the Ca^{2+} signals (Figure 4.5C, Figure 4.7C) in both the total responding cell population or only in cells that exhibit baseline Ca^{2+} oscillations. The co-expression of CA-CaMKIIα also increases the frequency of baseline Ca^{2+} oscillations (Figure 4.7D). All of these effects are prevented by the triple alanine substitution for Lys^{866}-Arg^{867}-Arg^{868} in the CTD (Figure 4.5 D-F, Figure 4.7C,D).

Presumably, the effect of CaMKIIα to increase basal cell-surface expression contributes to the prolongation of Ca^{2+} signaling, but the mechanisms underlying the reduced initial peak Ca^{2+} signal, observed in both stable 293A-5a^{LOW} cell populations and in single transiently-transfected cells, remains unclear. Taken together, our data show that binding of CaMKIIα can play an important role in modulating cellular responses to $mGlu_{5a}$ activation. Further examination into the contribution of these mechanisms in synaptic plasticity and neuronal Ca^{2+} signaling are warranted in future studies.

Interestingly, cell surface expression of $mGlu_{5a}$ is also modulated by direct binding of Ca^{2+}/CaM to the CTD and prolongs $mGlu_5$-mediated Ca^{2+} signaling (Lee et al., 2008), similar to the effects of CaMKIIα binding to the CTD reported herein. The Ca^{2+}/CaM binding domain involved in mediating these effects is located 30-40 residues C-terminal to the tribasic residue motif that is critical for CaMKII binding. Nevertheless, we found that Ca^{2+}/CaM competes for binding of activated CaMKII to the $mGlu_{5a}$-CTD *in vitro* (Figure 4.3C). Taken together, our data suggest an intriguing model in which the binding of CaM might confer a relatively transient Ca^{2+}-dependent modulation of $mGlu_{5a}$ surface expression and signaling, but that increased CaMKIIα autophosphorylation at Thr286 would result in sustained binding to the CTD and longer-term modulation of $mGlu_{5a}$ surface expression and Ca^{2+} mobilization. Since Thr286 autophosphorylation of CaMKII is sensitive to changes in the source, duration, or frequency of Ca^{2+} signals originating from multiple channels (Pasek et al., 2015), such as those occurring during synaptic plasticity, as well as to the regulated activities of protein phosphatases, this may provide a mechanism for cross-talk with other signaling pathways.

As noted above, CaMKII has also been shown to interact with a membrane proximal region in the CTDs of $mGlu_1$ (Jin et al., 2013a), and the CaMKII-binding domain in $mGlu_1$ contains a tri-basic residue motif, similar to the motif we have here identified as being critical for CaMKII binding to the $mGlu_{5a}$-CTD. However, CaMKII was shown to desensitize $mGlu_1$ signaling whereas we found that CaMKII prolongs $mGlu_5$ signaling. This apparently differential modulation of $mGlu_1$ and $mGlu_5$ by CaMKII may contribute to their distinct neuronal roles (Mannaioni et al., 2001; Valenti et al., 2002; Volk et al., 2006). Interestingly, the effects of CaMKII on $mGlu_1$ signaling are mediated in part by phosphorylation at Thr871, which lies near the CaMKII-binding domain. Therefore, it will be interesting to investigate whether phosphorylation is required for the effects of CaMKII on $mGlu_5$ signaling, as well as the physical interaction demonstrated here.

The effects of CaMKIIα on $mGlu_{5a}$ must also interface with the known modulation of $mGlu_5$ signaling by other mechanisms. Prior studies have shown that several protein kinases modulate $mGlu_5$ via the CTD. PKC phosphorylates Ser901 in the $mGlu_{5a}$-CTD to inhibit Ca^{2+}/CaM binding and antagonize the aforementioned modulation by Ca^{2+}/CaM (Lee et al., 2008). In addition, PKA phosphorylates Ser870 in $mGlu_{5a}$, prolonging Ca^{2+} mobilization, similar to the effects of CaMKII reported here, and enhancing ERK activation (Uematsu et al., 2015). However, it was previously reported that CaMKII reduces $mGlu_5$-stimulated ERK1/2 activation and increases agonist-induced $mGlu_5$ internalization (Raka et al., 2015). It is possible that the enhanced agonist-induced internalization in part results from the increased basal surface expression reported here (Figure 4.6). Although the mechanistic relationships between these different modes of $mGlu_5$ regulation remain to be more clearly established, the convergence of Ca^{2+}/CaM, CaMKII, PKA and PKC actions within an ~60 amino acid region in the long CTD (345 amino acids) suggests that the

actions of $mGlu_5$ are tightly controlled across different time frames, presumably fine-tuning neuronal responses such as different forms of synaptic plasticity.

CHAPTER V

CaMKII phosphorylation of the $mGlu_5$-CTD

Introduction

As reviewed in Chapter I, regulation of $mGlu_5$ signaling by phosphorylation has been well studied and phosphorylation of the $mGlu_5$-CTD has been defined by a number of kinases including PKA and PKC. These phosphorylation sites have broad physiological consequences for $mGlu_5$ signaling, including modulation of Ca^{2+} oscillations, surface expression, and ERK activation. It was previously reported that residues 827-964 of the $mGlu_{5a}$-CTD bind to CaMKII, and, as reported in Chapter III, we identified a mutation that could disrupt this binding. To date, no reports have presented evidence on the ability of CaMKII to phosphorylate $mGlu_5$. Therefore, we began to research if CaMKII was capable of phosphorylating the CTD of $mGlu_5$ using biochemical and proteomic approaches. Here, I report evidence that CaMKII phosphorylates both $mGlu_{5a}$ and $mGlu_{5b}$ isoforms of $mGlu_5$ *in vitro*. In addition to abolishing CaMKII binding to the CTD of $mGlu_5$, the triple-alanine mutation at residues 866-868 also disrupts CaMKII phosphorylation of the $mGlu_5$-CTD. Similar to binding, phosphorylation of $mGlu_5$ by CaMKII can be blocked by the presence of Ca^{2+}/CaM in both isoforms, although with different potencies. Using a proteomic approach, I identified putative sites of phosphorylation and began to confirm these sites *in vitro*.

These studies are important because they establish that CaMKII is capable of phosphorylating $mGlu_5$. Few studies have examined both $mGlu_{5a}$ and $mGlu_{5b}$ and there is little evidence supporting differences between these two receptor isoforms. We have identified a $mGlu_{5b}$-specific

phosphorylation site and shown differences in the potency of CaM required to inhibit CaMKII phosphorylation. These findings may lead to a better understanding of $mGlu_{5a}$ and $mGlu_{5b}$ regulation across development.

CaMKII phosphorylates purified $mGlu_{5a}$ and $mGlu_{5b}$ *in vitro*

To determine if CaMKII is capable of phosphorylating the $mGlu_5$-CTD, we used GST-fusion constructs of both $mGlu_{5a}$ and $mGlu_{5b}$ in the presence of pre-autophosphorylated CaMKII and radio-labeled [γ-^{32}P] ATP. I was able to demonstrate that both isoforms of $mGlu_5$ can be phosphorylated by CaMKII *in vitro* (Figure 5.1B). I also wanted to determine if the binding site identified in Chapter III was necessary for CaMKII to phosphorylate the $mGlu_{5a}$-CTD. We found that substituting alanines for Lys^{866}-Arg^{867}-Arg^{868} in the $mGlu_{5a}$-CTD essentially abolished the *in vitro* phosphorylation of GST-$mGlu_5$-CTD by CaMKII (Figure 4.1C).

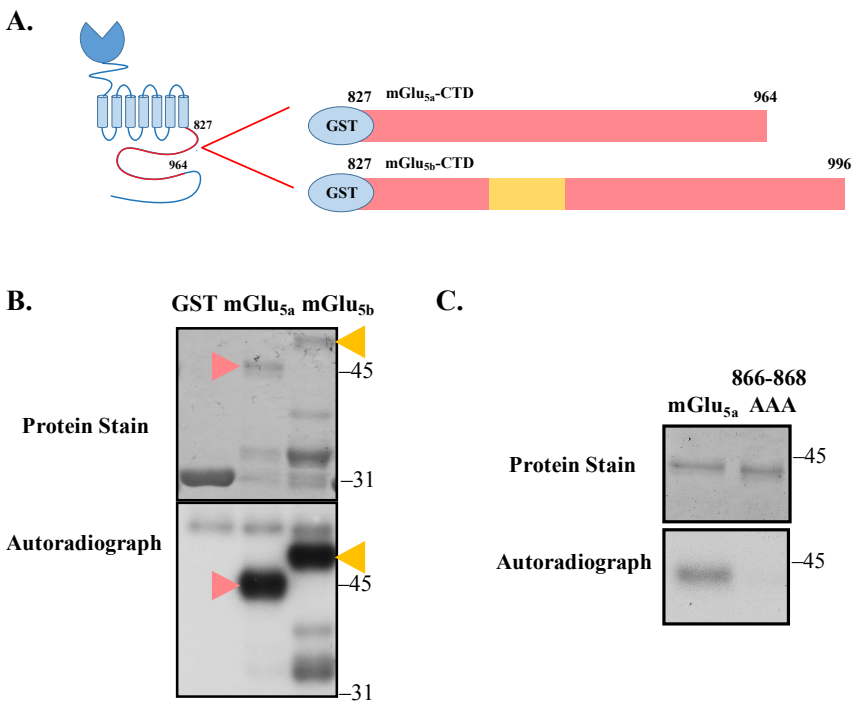

Figure 5.1 *CaMKII phosphorylates the CTD of mGlu$_{5a}$ and mGlu$_{5b}$* A. Schematic representation of GST-fusion proteins of the mGlu$_{5a}$ and mGlu$_{5b}$ CTD. These constructs are identical except for a 32 amino acid insert that is specific to mGlu$_{5b}$ denoted in yellow. B. *In vitro* phosphorylation of the GST-fusion constructs of the mGlu$_{5a}$-CTD and mGlu$_{5b}$-CTD with pre-autophosphorylated CaMKII show that CaMKII can phosphorylate both mGlu$_{5a}$ and mGlu$_{5b}$. Full length GST-fusion constructs are denoted with red and yellow arrows, respectively. C. A representative protein stain and autoradiograph from an *in vitro* CaMKII phosphorylation assay where triple alanine mutation of the tribasic residues Lys^{866}-Arg^{867}-Arg^{868} previously shown to disrupt CaMKII binding to the mGlu$_5$-CTD also disrupts CaMKII phosphorylation of the mGlu$_5$-CTD. Representative of three independent experiments.

CaM inhibits CaMKII phosphorylation of mGlu$_{5a}$ and mGlu$_{5b}$

Because the presence of Ca^{2+}/CaM is capable of breaking the binding interaction between mGlu$_{5a}$ and CaMKII, we also decided to test if this was true for CaMKII phosphorylation of the mGlu$_5$-CTD. Using the GST fusion constructs for mGlu$_{5a}$ and mGlu$_{5b}$, we performed an *in vitro* phosphorylation assay with increasing concentrations of CaM (Figure 5.2). The presence of CaM

was capable of inhibiting phosphorylation of both $mGlu_{5a}$ and $mGlu_{5b}$, but the CaM concentration dependence of this effect was different between $mGlu_{5a}$ and $mGlu_{5b}$. Inhibition of phosphorylation on $mGlu_{5a}$ was much more sensitive to CaM than $mGlu_{5b}$. This presents an interesting difference between $mGlu_{5a}$ and $mGlu_{5b}$ that was previously unknown. This may be due to the additional 32-amino acid insert specific to $mGlu_{5b}$.

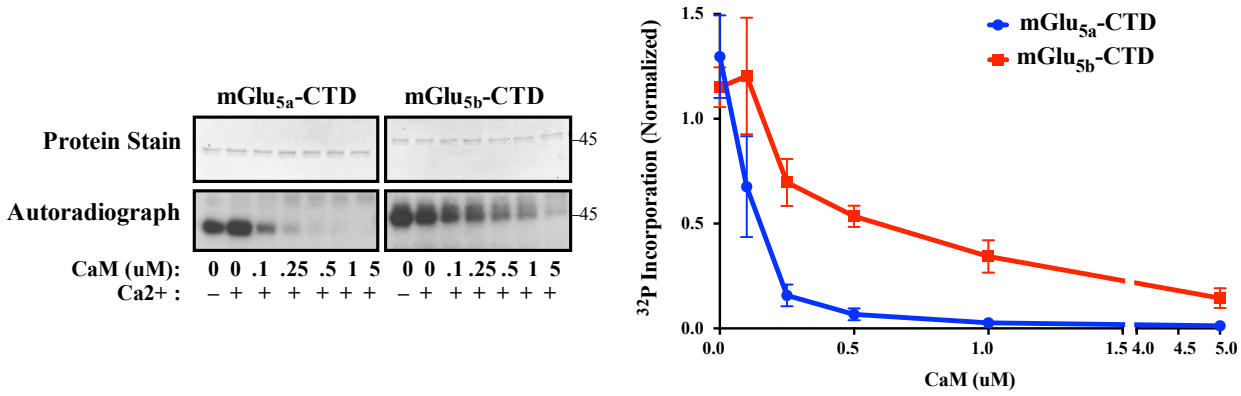

Figure 5.2 *CaM inhibits CaMKII phosphorylation of the $mGlu_{5a/b}$-CTD with different potencies.* Phosphorylation of GST-$mGlu_{5a}$-CTD and GST-$mGlu_{5b}$-CTD *in vitro* by CaMKII alone, in the presence of Ca^{2+}, or with increasing concentrations of CaM. The phosphorylation of both $mGlu_5$-CTD constructs were largely reduced by the presence of CaM. The left panel shows the protein stain and autoradiograph from a single experiment. The ^{32}P incorporation was measured by autoradiograph signals normalized to the phosphorylation of the GST-fusion construct in the $-Ca^{2+}$/CaM condition. Normalized phosphorylation responses are plotted as the mean ± SEM in the right panel (n = 3 experiments). CaM had a significant effect on the phosphorylation of both $mGlu_{5a}$ and $mGlu_{5b}$-CTDs, The CaM-dependent inhibition of $mGlu_5$-CTD constructs were also significantly different. (multiple comparisons two-way ANOVA: Sources of Variation: CaM $p<0.0001$, GST-$mGlu_{5a}$-CTD vs. GST-$mGlu_{5b}$-CTD, $p = 0.0226$, Interaction $p = 0.0973$.)

Identifying the region of CaMKII phosphorylation on $mGlu_{5a}$

To determine which portion of the $mGlu_{5a}$-CTD was being phosphorylated, I generated overlapping truncations of the GST-tagged $mGlu_{5a}$-CTD constructs designated C1-C4 containing residues 827-873, 853-899, 879-925, and 905-964, respectively (Figure 5.3A). We saw that *in vitro* CaMKII phosphorylated C1, C2, and C3 constructs, suggesting CaMKII can phosphorylate multiple residues on the $mGlu_5$-CTD, at least *in vitro* because there are regions within C1 and C3 that do not overlap. C1 and C2 were the most strongly phosphorylated of the constructs. C4, the most C-terminal truncation did not contain detectable phosphorylation. From this study we deduced that phosphorylation occurs at residues between 827-905.

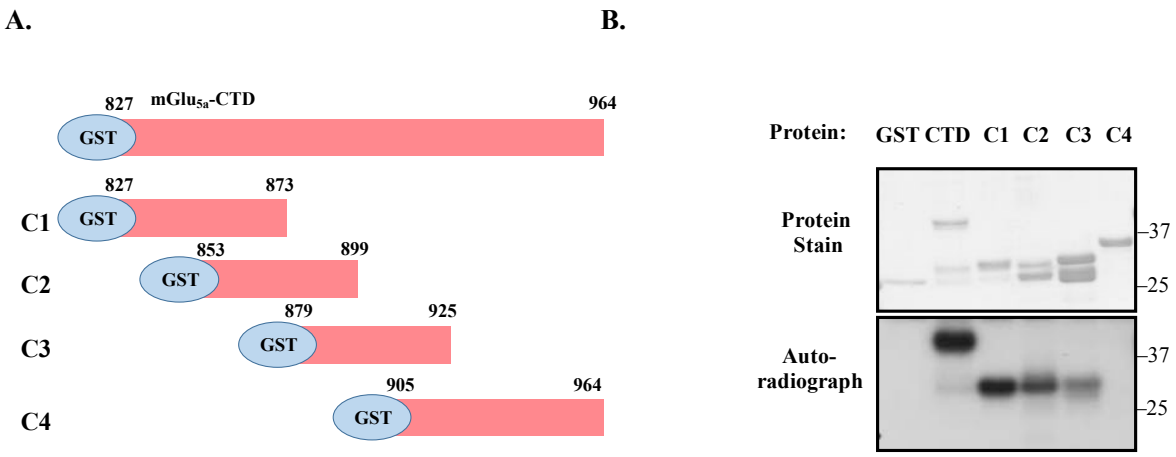

Figure 5.3 *CaMKII phosphorylation of $mGlu_5$-CTD truncations.* A. Schematic showing the four overlapping truncations of the GST-tagged $mGlu_{5a}$-CTD constructs designated C1-C4 containing the residues denoted. B. A representative protein stain and autoradiograph from an *in vitro* CaMKII phosphorylation assay of GST, GST-$mGlu_5$-827-964, and C1-C4 using preautophosphorylated CaMKII. CaMKII can phosphorylate C1, C2, and C3. Respresentative of three independent experiments.

Proteomic analysis of CaMKII phosphorylation sites on the mGlu$_5$-CTD

We submitted mGlu$_{5a}$ and mGlu$_{5b}$ fragments phosphorylated using nonradioactive ATP for proteomics analysis to identify detectable phosphorylation sites. Tryptic digestion was performed prior to mass spectral analysis. In Figure 5.5A the expected tryptic digestion of the mGlu$_{5a}$-CTD is diagrammed. We identified several sites of phosphorylation by CaMKII denoted in red in Figure 5.5A.

A.

mGlu5a

827 880

KPER NVR SAFTTSTVVR MHVGDGK SSSAASR SSSLVNLWKR RGSSGETLSSNGK

881 926

SVTWAQNEK STR GQHLWQR LSVHINK K ENPNQTAVIK PFPK STENR

B.

Amino Acids	Sequence	Phosphorylated Spectral Counts
834-843	SAFTTSTVVR	5.6%
858-867	SSSLVNLWKR	5.5%
868-880	RGSSGETLSSNGK	62.1%
881-889	SVTWAQNEK	4.2%

Figure 5.4 *Identification of putative phosphorylation sites by mass spectroscopy* **A.** The sequence of the mGlu$_{5a}$-CTD region of interest with tryptic digestion sites denoted with arrows. All residues that were detected as phosphorylated are highlighted in red in both panels **B.** Using a crude quantitative assessment made from the relative number of times a peptide is identified in its phosphorylated vs non-phosphorylated state we detected three peptides identified about 5% of the time as phosphorylated, but the peptide containing residues 868-880 was detected more than 60% in a phosphorylated state. For this reason, we targeted residues on this peptide for phospho-null mutations and in vitro phosphorylation assays.

In agreement with the *in vitro* radiolabeled phosphorylation assay, no phosphorylation was detected after amino acid 905. We identified four peptides that contained phosphorylation sites after incubation with CaMKII (Figure 5.5b). Using a crude quantitative assessment made from the relative number of times a peptide is identified in its phosphorylated vs non-phosphorylated state, we determined that three of these peptides were identified about 5% of the time as phosphorylated, but more than 60% of the peptide containing amino acids 868-880 was identified in a phosphorylated state. This suggests that the major CaMKII site of phosphorylation lies within this region of the $mGlu_{5a}$-CTD.

Verifying CaMKII phosphorylation sites on the $mGlu_5$-CTD

We examined the proteomics data and found the most commonly identified phosphorylation site on the 868-880 peptide was Ser871. We were able to confirm Ser871 phosphorylation in the mass spectral data by identifying characteristic fragmentation patterns of the phosphorylated peptide (Figure 5.6B). In order to test whether this was a major site of CaMKII phosphorylation we generated a serine to alanine phosphoinhibitory mutation of Ser870 and Ser871. We decided to proceed initially with a double alanine mutation because CaMKII can phosphorylate a neighboring site in single point mutation experiments. We followed our mass spectral analysis with an in vitro phosphorylation of the $mGlu_5$-CTD-Ser-870/871-Ala construct. We were able to demonstrate that mutation of these sites reduced CaMKII phosphorylation of the $mGlu_5$-CTD by about 65% in three independent experiments (Figure 5.6 C,D). This suggests that Ser870/Ser871 (one or both residues) are site(s) of phosphorylation by CaMKII *in vitro*. As is visible in figure 5.6C, one caveat to this study is that the Ser-870/871-Ala construct was largely degraded in these experiments, and much more degraded than the WT samples. Because of this, this study needs to be repeated in

proteins that have been re-purified to produce more intact constructs with comparable degradation. However, because of the agreement of the proteomic and biochemical data, Ser871 is a great target for future studies of CaMKII regulation of $mGlu_5$ by CaMKII.

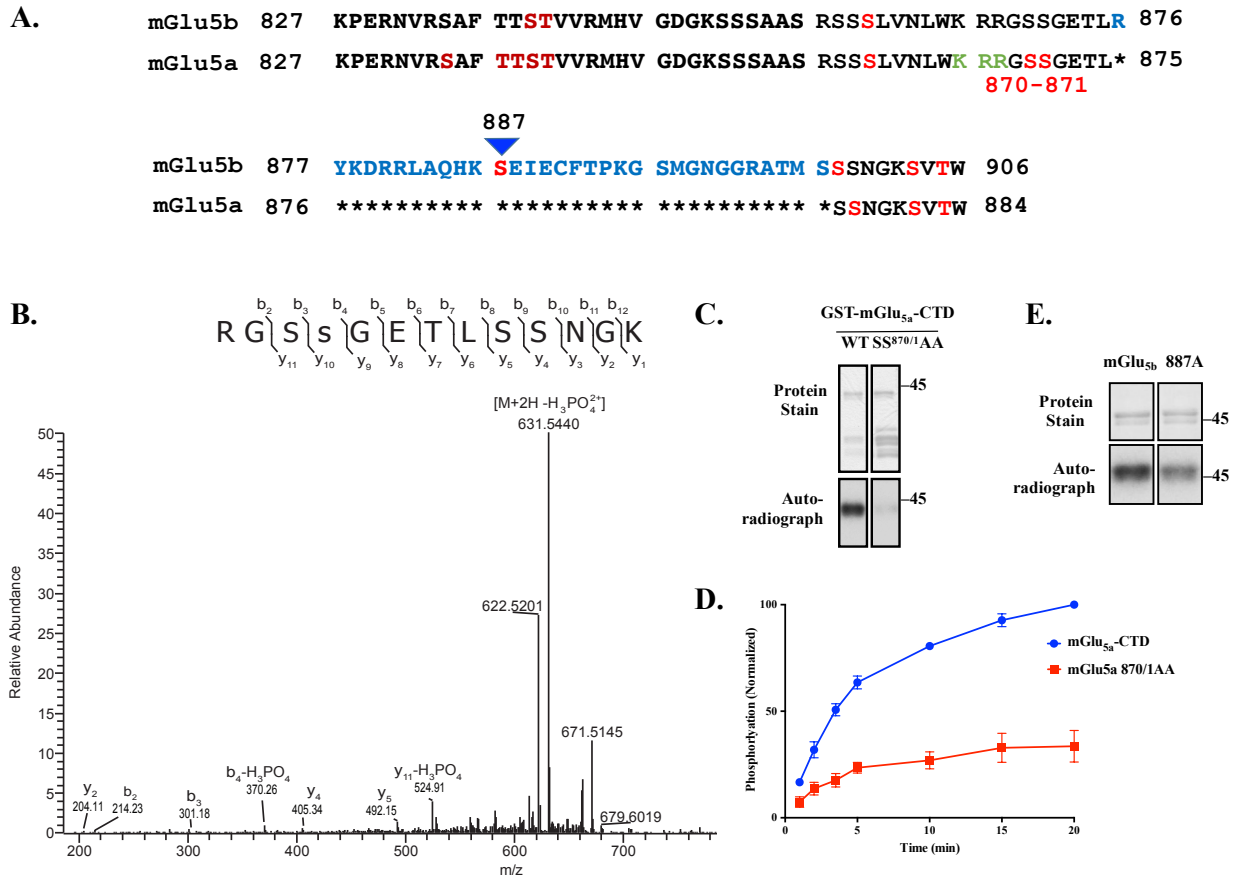

Figure 5.5 *Verification of mass spectroscopy identified phosphorylation sites on the $mGlu_5$-CTD* A. Alignment of the $mGlu_{5a}$ and $mGlu_{5b}$ peptide sequences. spanning the phosphorylated residues detected in mass spectroscopy experiments. As noted in Figure 5.5 we detected the following sites in $mGlu_{5a}$: S834 T837 T838 S839 T840 S860 S870 S871 S876 S877 S881 T883 The $mGlu_{5b}$ specific sequence is highlighted in blue. In $mGlu_{5b}$ we did not detect all of the same sites as we identified in $mGlu_{5a}$ including S834, T837, S870, S871, S877. We also identified a site within the $mGlu_{5b}$ specific sequence as a potential CaMKII phosphorylation site S887 denoted with a blue arrow. The tribasic residue sequence shown in Chapter III to disrupt CaMKII binding to $mGlu_{5a}$ and disrupt CaMKII phosphorylation of $mGlu_5$ in Chapter IV is denoted in green. *B*. A spectrum from the LC-MS/MS showing the phosphorylation of Ser871 of the $mGlu_{5a}$-CTD Purified GST-

tagged $mGlu_{5a}$-CTD was incubated with 10 nM purified mouse CaMKII 30^{o}C for 20 minutes with nonradioactive ATP. Samples were then resolved by SDS-PAGE and the gel was stained by colloidal blue. Bands corresponding to the full-length proteins were excised and submitted for mass spectrometry analysis. Data in panel B were annotated by Christian Marks and verified by Kristie Rose, Ph.D. C. Protein stain and autoradiograph showing simultaneous mutation of the identified phosphorylation sites Ser870/1 to alanine largely reduces CaMKII phosphorylation. A representative protein stain and autoradiograph is shown from three independent experiments. D. Time course data showing the normalized ^{32}P incorporation into GST-$mGlu_{5a}$-CTD or GST-$mGlu_{5a}$-CTD with a Ser870/1 to alanine mutation. The time course of ^{32}P incorporation into $mGlu_{5a}$ vs. $mGlu_{5a}^{870/1AA}$ were significantly different (Two-way ANOVA: Sources of Variation: $mGlu_{5a}$ vs $mGlu_{5a}$ 870/1AA $p = 0.0008$, Time $p<0.0001$, Interaction $p<0.0001$) E. Mutation of the $mGlu_{5b}$ specific phosphorylation site S887 to a phosphoinhibitory alanine reduced $mGlu_{5b}$ phosphorylation by an average of 40% in two independent experiments.

During our proteomic analyses we also investigated the phosphorylation of the $mGlu_{5b}$ construct by CaMKII. Interestingly, not all of the phosphorylation sites identified in the analysis of $mGlu_{5a}$ were identified in $mGlu_{5b}$ (Figure 5.6A.). These include residue Ser871, the most commonly detected phosphorylation site on $mGlu_{5a}$-CTD construct. In addition to this, we identified phosphorylation of a serine residue present in a CTD sequence unique to $mGlu_{5b}$. This residue, Ser887, caught our attention because there are very few differences that have been shown between the $mGlu_{5a/b}$ isoforms. In order to test if Ser887 was a site of CaMKII phosphorylation *in vitro* we generated a $mGlu_{5b}$-CTD-Ser887/Ala phosphoinhibitory mutation. When we tested this construct for phosphorylation we saw that in two experiments that $mGlu_{5b}$-CTD-Ser887/Ala reduced $mGlu_{5b}$ phosphorylation by ~40% in two independent experiments (Figure 5.6E).

While mass spectroscopy is a great tool for identifying phosphorylation sites, the absence of the detection of phosphorylation is not enough to remove the possibility that other sites not detected are being phosphorylated. Sites can be missed because of poor peptide detection or fragmentation during analysis.

Discussion

Phosphorylation of Group I mGlu receptors has been studied for a number of different kinases including PKC and PKA. PKC phosphorylation at Ser839 has been reported as the site responsible for $mGlu_5$ Ca^{2+} oscillations and also plays a role in receptor desensitization (Kawabata et al., 1996; Nakahara et al., 1997). The role of PKC phosphorylation of $mGlu_5$ is an example of a phosphorylation site dynamically regulated by the presence of CaM on the $mGlu_5$-CTD (Lee et al., 2008; Minakami et al., 1997). The binding site for CaM also overlaps with other regulators of $mGlu_5$ surface expression including CaMKII and the E3 ligase Siah-1a (Jin et al., 2013b; Ko et al., 2012). The importance of CaM on $mGlu_5$ has been demonstrated including the finding that CaM activity is required for $mGlu_5$-mediated ERK1/2 activation, Arc expression, and LTD in the hippocampus (Sethna et al., 2016). It seems that tight regulation of this portion of the $mGlu_5$-CTD is important because of the multiple proteins that have been shown to bind in this region. It seems that CaM and other Ca^{2+} sensitive proteins like CaMKII can regulate the binding of other partners to $mGlu_5$ to accurately control phosphorylation of the receptor.

In this chapter, I demonstrate that CaMKII is capable of phosphorylating both $mGlu_{5a}$ and $mGlu_{5b}$, at least *in vitro*. The finding that triple alanine mutation of Lys^{866}-Arg^{867}-Arg^{868} disrupts phosphorylation of CaMKII leaves unanswered questions about work done in the previous chapters of this thesis, and opens the door to determine the roles of both CaMKII binding and phosphorylation. It will be important to establish if the effects that we saw from disrupting CaMKII binding are a result of CaMKII phosphorylation of the $mGlu_5$-CTD. Future studies should be able to create a phosphoinhibitory $mGlu_5$ construct that is still capable of binding to CaMKII to tease apart the roles of CaMKII binding and phosphorylation on receptor function.

Our proteomics study identified a number of potential phosphorylation sites on the $mGlu_5$-CTD and we identified Ser871 as a likely candidate for phosphorylation on $mGlu_{5a}$. A recent study identified PKA phosphorylation of $mGlu_5$ and identified at the neighboring residue Ser870 (Uematsu et al., 2015). The phosphoinhibitory alanine mutation at Ser870 also significantly reduced the number of $mGlu_5$-transfected HEK cells that responded with Ca^{2+} oscillations and reduced total number of Ca^{2+} oscillations per cell. This is distinct from what we see with CaMKII, but interesting considering that we suspect that the neighboring Ser871 may be a major site of CaMKII phosphorylation. There are some issues with the experiment showing the effect of the phosphoinhibitory mutation of Ser870-871 presented in this dissertation because of extensive GST-fusion protein degradation. This experiment needs to be repeated to determine if $mGlu_5$ degradation effects phosphorylation of the $mGlu_5$ construct. If we are able to verify this finding, phosphorylation of this site poses the possibility of an exciting project to look at the linkage between $mGlu_5$ phosphorylation by CaMKII and PKA. The literature suggests that while CaMKII seems to promote $mGlu_5$ Ca^{2+} signaling, that PKA seems to have the opposite effect. Phosphorylation by these kinases at these neighboring sites may cause major differences in $mGlu_5$ activity during neuronal activation.

We also identified a CaMKII phosphorylation site that is specific to $mGlu_{5b}$. This is exciting because there is little known about the differences between two $mGlu_5$ isoforms. The developmental regulation of $mGlu_5$ isoform expression suggests that there are important to receptor function.

The finding that CaM is capable of blocking both phosphorylation and binding of $mGlu_5$ is also of interest because it confirms that CaM can compete for CaMKII binding to the $mGlu_5$-CTD. If the

major site of phosphorylation on $mGlu_{5a}$ is Ser871 and CaMKII phosphorylation is blocked by the presence of CaM, it is likely that PKA phosphorylation at Ser870 can also be affected by the presence of CaM. This could suggest that a small section of the $mGlu_5$-CTD presents a system within where CaM, CaMKII, PKA and PKC can all compete for binding, but the CaM effect on CaMKII phosphorylation is much less potent on $mGlu_{5b}$. This leads me to hypothesize that $mGlu_{5b}$ is less sensitive to the competing effects of CaM and CaMKII. We were also unable to detect phosphorylation of Ser871 in our mass spectroscopy analysis of $mGlu_{5b}$. The presence of the 32-amino acid insert in the CTD lies close to the residues necessary for CaMKII binding and Ser871. This insert may change the way that CaMKII binds to the receptor or disrupt phosphorylation of Ser871. I would hypothesize that the $mGlu_{5b}$-specific residues reduce the effect of CaM binding on $mGlu_5$ regulation and allow for CaMKII-mediated modulation of the receptor even in the presence of CaM. This might make mGlu5b more accessible to modulation by CaMKII. Additionally, it is possible if CaM can block phosphorylation of both PKC and PKA, that $mGlu_{5b}$ receptor regulation by CaMKII would be preferred during intracellular Ca^{2+} influx. Further understanding of this difference may shed light on the developmental changes that we see in synaptic plasticity.

Chapter VI

Regulation of Neuronal Ca^{2+} Signaling

Introduction

Long-term changes in synaptic function require changes in synaptic proteins. Some proteins play direct roles in synaptic transmission while others serve as structural scaffolds within the synapse. Functional changes require shifts in the availability, distribution, and post-translational modifications of the PSD proteins. Accordingly, functions related to the protein composition of the PSD underlies synaptic physiology and malfunction can cause neuronal diseases.

In my studies I decided to focus on the role of some of these synaptic regulators in controlling intracellular changes in neuronal Ca^{2+}. First, I have examined the role of CaMKII in intracellular increases in Ca^{2+} through activation of different sources. Here we chose to focus on Ca^{2+} increases dependent on known CaMKII binding partners including $mGlu_{1/5}$ and LTCCs.

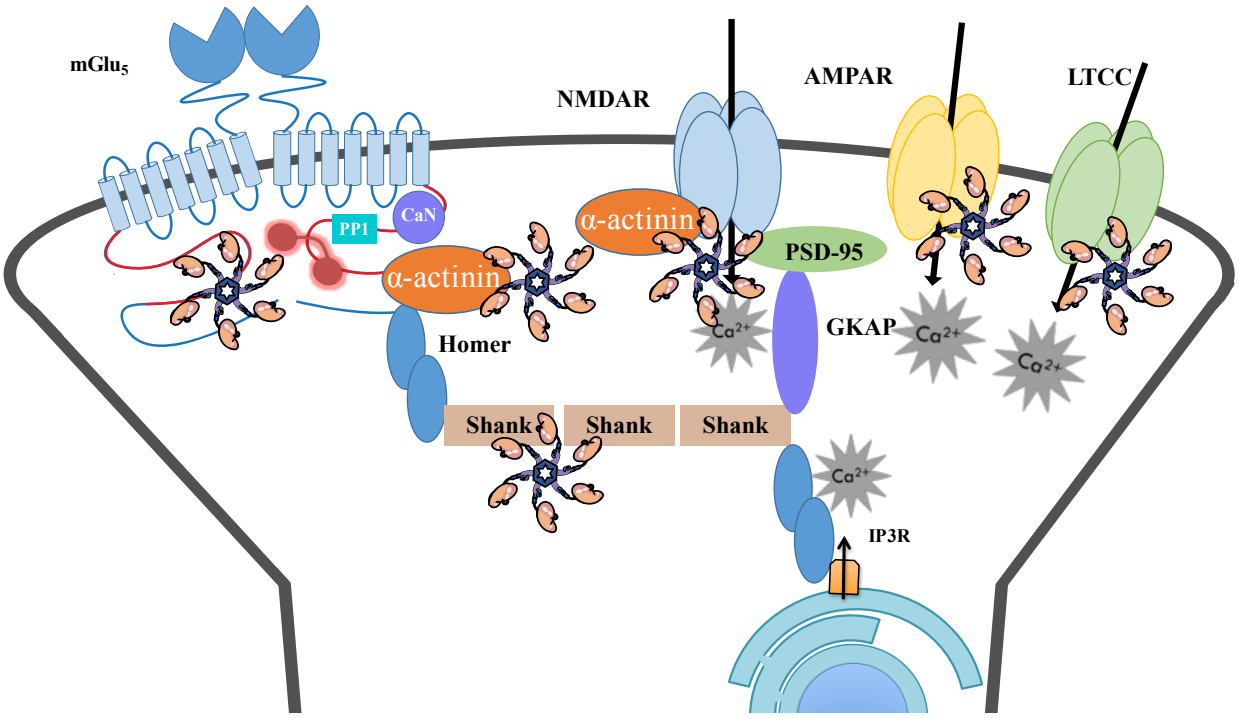

Figure 6.1. *CaMKII-associated macro-molecular protein complex composition.* The macro-molecular complexes formed with CaMKII are tightly regulated and important to proper synaptic signaling. Levels of Ca^{2+} influx through the signaling of ion channels within this complex are important for proper control of CaMKII activity and other Ca^{2+} regulated $mGlu_5$ binding partners like CaM and CaN. Multiple proteins in this complex can be regulated by CaMKII binding and phosphorylation including LTCCs, SHANK, α–actinin, NMDARs, and AMPARs. These proteins also indirectly connect $mGlu_5$ to modulators of intracellular Ca^{2+} like the NMDAR and IP3R.

CaMKII has many binding partners in the PSD, including some structural scaffolding proteins (Figure 6.1). Our lab has unpublished evidence that CaMKII is capable of binding and phosphorylating SHANK-3, an important PSD scaffolding protein. There is evidence that SHANK-3 is important in the regulation of dendritic spine morphology and synaptic plasticity (Durand et al., 2012) and has been linked to autistic phenotypes. Group I mGlu receptors directly bind the scaffolding protein Homer to form indirect binding interactions with Shank and the IP_3 and ryanodine receptors to control intracellular Ca^{2+} release from the endoplasmic reticulum (Feng

et al., 2002; Tu et al., 1999; Tu et al., 1998). The LTCC $Ca_V1.3$ also has a SHANK binding domain that is important for Ca^{2+}-dependent long-range signaling to the nucleus (Zhang et al., 2005). CaMKII, Shank-3, Homer, $mGlu_5$ and the LTCC $Ca_V1.3$ form a complex. We hypothesized that disruption of this complex would have effects on Ca^{2+} signals generated through both LTCCs or $mGlu_5$. To study this, we measured Ca^{2+} signals in cells expressing endogenous SHANK-3 or shRNA knockdown of Shank-3. These studies show that CaMKII can differentially regulate neuronal Ca^{2+} signals in hippocampal neurons depending on the source of neuronal excitation. Additionally, we have begun to gather evidence to show that SHANK-3 is important for proper Ca^{2+} influx.

Characterizing $mGlu_{1/5}$ mediated Ca^{2+} responses in hippocampal neurons

In order to study the role of $mGlu_5$ in generating Ca^{2+} responses in our hippocampal culture system, I began to characterize neuronal Ca^{2+} responses in the presence of the Group I mGlu receptor agonist DHPG by measuring somatic changes in Fura-2 fluorescence. The neurons were imaged in 5 mM K^+ Tyrode's solution with DHPG alone, or with different receptor and channel inhibitors. In the presence of DHPG alone, most neurons exhibited a single Ca^{2+} spike that was reduced to levels below the baseline in about 150 seconds (Figure 6.2A). Only two of twenty-six imaged neurons responded with a sustained response (Figure 6.2A, orange trace). To look more specifically at Group I mGlu receptor-mediated responses, we incubated neurons in 5 mM K^+ Tyrode's solution APV and CNQX to block the activation of NMDA- and AMPA-type glutamate receptors, and with tetrodotoxin (TTX) to inhibit voltage-dependent sodium channels before stimulation with DHPG. In this condition, application of DHPG should activate both $mGlu_1$ and $mGlu_5$ receptors. Inhibition of NMDARs, AMPARs, and voltage-dependent sodium channels

resulted in a prolonged Ca^{2+} signal with a larger area under the curve than with DHPG alone (Figure 6.2B). To show that this response was specific to Group I mGlu receptors, we proceeded to simultaneously inhibit $mGlu_1$ and $mGlu_5$. In the presence of the $mGlu_5$ specific antagonist MPEP and $mGlu_1$ NAM VU0469650 (VU50), DHPG cause no increase in intracellular Ca^{2+} as expected (Figure 6.2C).

When we inhibited $mGlu_5$ with MPEP to exhibit a $mGlu_1$-specific response, we saw no increases in intracellular Ca^{2+} throughout the 10-minute experiment (Figure 6.2D). Inhibition of $mGlu_1$ alone (to exhibit $mGlu_5$-mediated responses) produced two types of neuronal responses. About half of the cells exhibited larger single-peaked Ca^{2+} responses (14/30 cells) that returned to baseline (Figure 6.2E) and the half of the cells responded with smaller sustained Ca^{2+} responses (Figure 6.2F) (16/30 cells). This data suggests that DHPG-mediated Ca^{2+} responses in our primary hippocampal neuron system require the activation of $mGlu_5$ to produce intracellular Ca^{2+} release. After this initial characterization we performed all DHPG neuronal Ca^{2+} imaging experiments in the presence of APV, CNQX, TTX and VU50 to elicit $mGlu_5$-specific responses. All of the traces in Figure 6.2 came from one day's experiments. I am reporting them in this dissertation because I think they pose valuable information in the future of this project, but these studies do need to be repeated in order to more formally report these results.

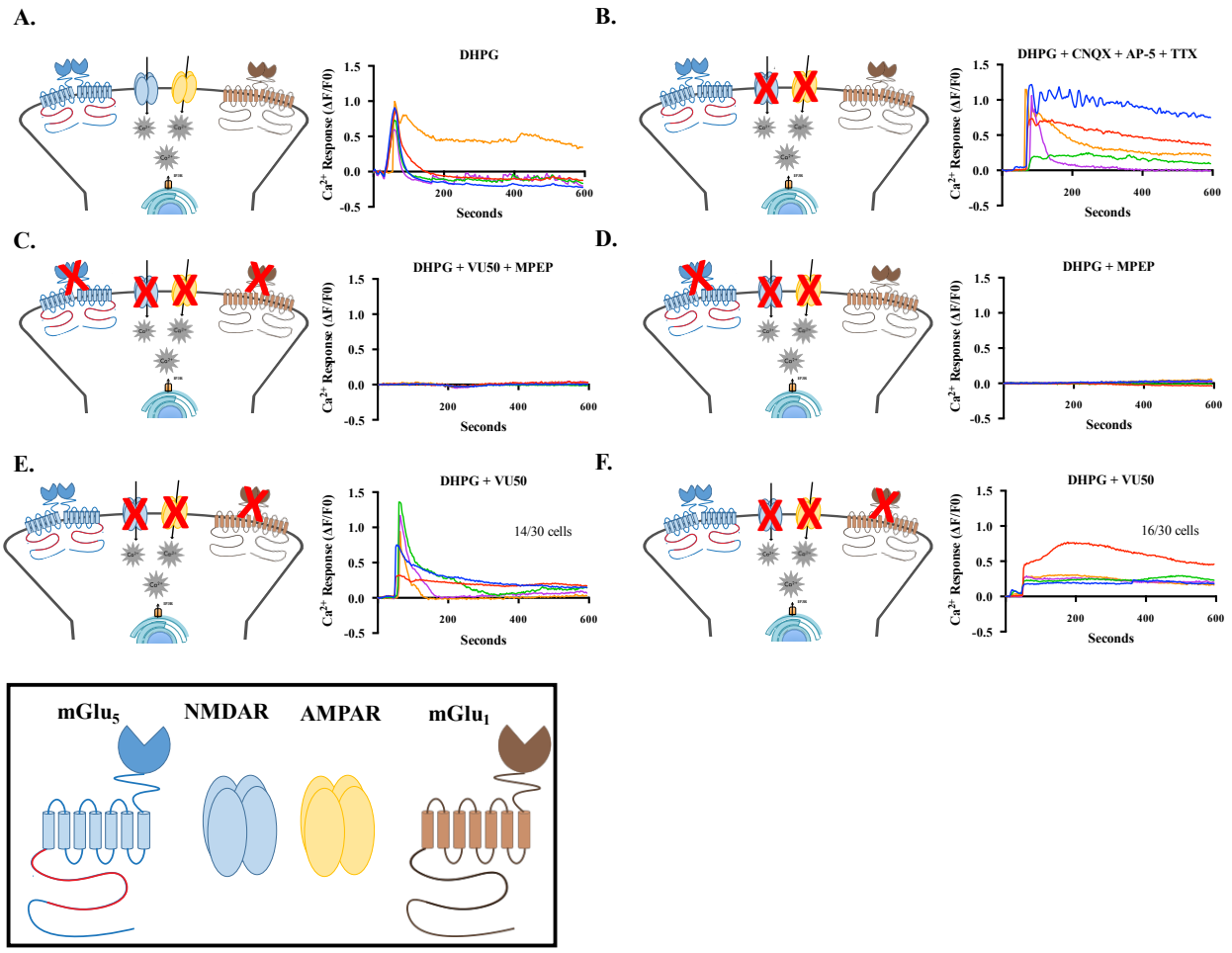

Figure 6.2 *Characterizing Group I mGlu receptor Ca^{2+} responses in primary hippocampal cultures.* Ca^{2+} imaging experiments in primary hippocampal neuronal cultures (DIV 14). Neurons were incubated with Tyrode's solution for 30 seconds and switched to Tyrode's solution containing 100 uM DHPG for 10 minutes and the Ca^{2+} responses were measured in the presence of **A.** DHPG alone resulting in a single-spiked increase in intracellular Ca^{2+}. 2/26 neurons responded with a sustained response (i.e., orange trace). All other conditions **(B-F)** were pre-incuabted in Tyrode's solution with the inhibitors CNQX, AP-5, and TTX in the presence or absence of the indicated mGlu receptor specific inhibition and stimulation with DHPG. B. Blockade of AMPA, NMDARs and voltage-dependent sodium channels shifted Ca^{2+} influx from a single peaked increase to a more sustained Ca^{2+} signal. **C.** The presence of VU50 and MPEP to block both $mGlu_1$ and $mGlu_5$ completely abolished DHPG-mediated intracellular Ca^{2+} increases **D.** MPEP alone had a similar effect and eliminated DHPG-mediated Ca^{2+} responses **E,F.** VU50 produced two types of Ca^{2+} responses. **E.** 14/30 cells exhibited larger single-peaked Ca^{2+} responses **F.** The other half of cells responded with smaller sustained Ca^{2+} responses (16/30). Example traces from a single experiment are plotted.

CaMKII-KD reduces $mGlu_5$-mediated Ca^{2+} responses in hippocampal neurons

To understand the role of CaMKII in neuronal $mGlu_5$-mediated Ca^{2+} responses, we used a CaMKII knock-down and rescue strategy. In our earlier HEK-293 experiments we saw that the presence of CaMKII reduced peak $mGlu_5$ Ca^{2+} amplitudes and prolonged $mGlu_5$ signals (Chapter IV); therefore, we hypothesized that CaMKII-KD would enhance $mGlu_5$ Ca^{2+} amplitudes and lead to faster termination of Ca^{2+} signals in CaMKII-KD neurons.

Neurons were transfected with control shRNA or shRNAs to knockdown CaMKIIα and CaMKIIβ expression. In cells transfected with control-ShRNA, DHPG produced a fast, sustained increase in intracellular Ca^{2+}. This response was specific to $mGlu_5$, because addition of MPEP at the end of the experiment quickly reduced intracellular Ca^{2+} levels back to baseline. Knockdown of CaMKII and CaMKII expression significantly reduced $mGlu_5$-dependent somatic peak Ca^{2+} amplitudes in response to DHPG. Moreover, the re-expression of shRNA-resistant constitutively active CaMKIIα (CA-CaMKIIα^R) rescued the reduction in peak amplitude seen with CaMKIIα/β knockdown.

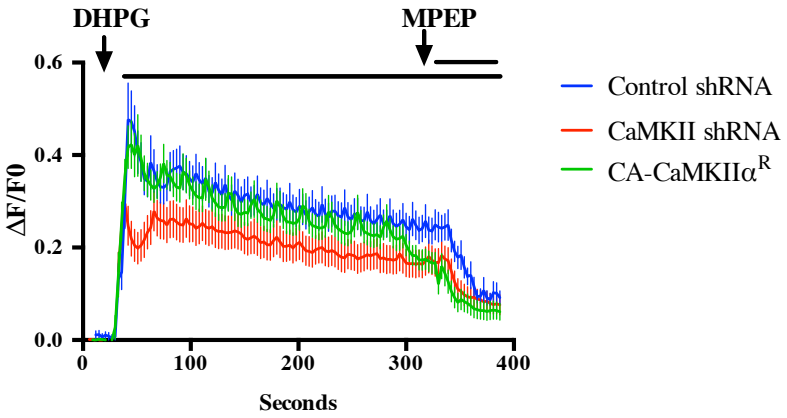

Figure 6.3 *CaMKII-KD reduces neuronal mGlu$_5$-mediated Ca^{2+} responses.* Neurons were transfected with control shRNA, CaMKII shRNA, or CaMKII shRNA and CA-CaMKII rescue. Neurons were preincubated in Tyrode's solution containing CNQX, APV, and VU-50 before imaging. Neurons were imaged for 30 seconds in Tyrode's solution with inhibitors to elicit mGlu$_5$ specific responses and switched to Tyrode's solution containing DHPG and the Ca^{2+} responses were measured. To show the mGlu$_5$ specificity of this response, MPEP was added to the plate at the end of the experiment. Average traces from 3 independent experiments are plotted as the change in fluorescence over baseline fluorescence.

This is not the result that we would have expected based on our HEK-293 data. Neurons are a much more complicated system than HEK293 cells, and mGlu$_5$ signaling is supported by proper formation of the complex macro-molecular structures of the PSD (Figure 6.1). We suspect that CaMKII-KD may have effects on other proteins present in synaptic signaling. To determine the specificity of this result we followed by testing the effect of CaMKII-KD in LTCC-mediated responses.

The role of CaMKII in somatic LTCC-mediated Ca^{2+} influx

In collaboration with Xiaohan Wang, a former graduate student in the Colbran lab, I began to study the role of CaMKII in LTCC dependent Ca^{2+} signaling. This work was published in the Jounal of Biological Chemistry (Wang et al., 2017). To study the effect of CaMKII on LTCC somatic Ca^{2+}

responses, we used a stimulation paradigm to induce LTCC-dependent increases of Ca^{2+} concentrations. Neurons were pre-incubated in 5 mM K^+ Tyrode's solution containing APV and CNQX to block the activation of NMDA- and AMPA-type glutamate receptors, and with TTX to inhibit voltage-dependent sodium channels before stimulation. Neuronal depolarization was induced by replacing the solution with 40 mM K^+ Tyrode's solution in the presence of APV, CNQX and TTX. The addition of 40 mM K^+ induced a significant increase in intracellular (somatic) Ca^{2+}, which is largely blocked by 10 μM nimodipine, a highly selective LTCC antagonist (Figure 6.3). Transfection of shRNAs to knockdown CaMKIIα and CaMKIIβ expression had had no significant effect on LTCC-dependent somatic Ca^{2+} responses to stimulation with 40 mM K^+ Tyrode's solution. The re-expression of shRNA-resistant wild-type CaMKIIα (WT-CaMKIIα^R) also had no effect (Figure 6.3). LTCC channel activation can induce new mRNA transcription, a phenomenon called excitation-transcription (E-T) coupling often measured by the phosphorylation of the transcription factor CREB. Although we saw no effect on somatic Ca^{2+} levels in response to 40 mM K+, we did see significant deficits in LTCC-mediated activation of CREB (Wang et al., 2017). This suggests that, even though total Ca^{2+} levels were not changed under these conditions, smaller changes in the LTCC nanodomain are important for the proper control of Ca^{2+} signaling to mediate the proper downstream effects.

While CaMKII-KD had no effect on LTCC-specific influxes, we see a reduction in $mGlu_5$-dependent signaling in the absence of CaMKII. These finding show that there are unique effects of CaMKII on Ca^{2+} sources when measured by changes in somatic Ca^{2+} influx.

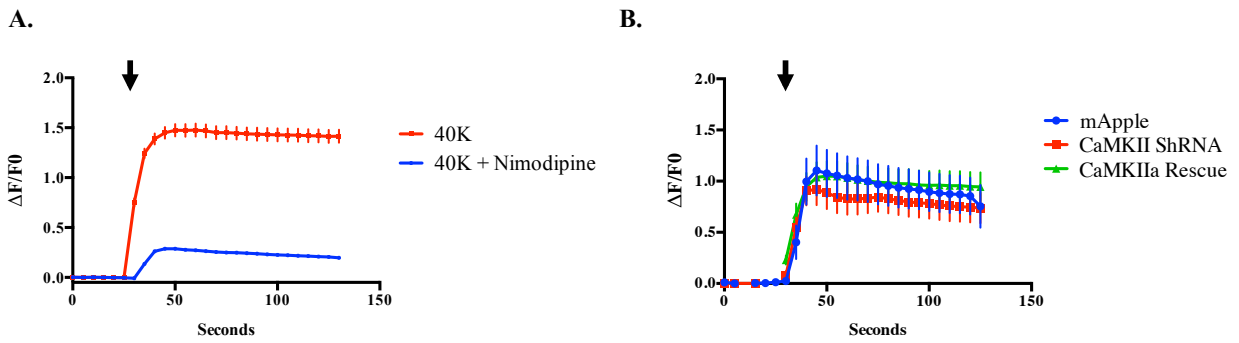

Figure 6.4 *CaMKII-KD does not affect somatic LTCC-mediated Ca^{2+} influx* **A.** Ca^{2+} imaging showing that nimodipine largely prevents the high K^{+}-induced increase of somatic Ca^{2+}. Neurons were incubated with Tyrode's solution containing 5 mM KCl (5K) for 30 seconds and switched to Tyrode's solution containing 40 mM KCl for 2 minutes in the absence (40K control, n = 193) or presence (40K+NIM, n = 215) of 10 mM nimodipine. The black arrow indicates the buffer switch. A total of 5 dishes from two independent cultures were analyzed per group. B. CaMKIIa/b knockdown or re-expression/rescue has no effect on high K^{+}-induced increases of somatic Ca^{2+}. Cultured hippocampal neurons were transfected with mApple only (n = 17), mApple with CaMKIIα/β shRNA (n = 23), or mApple/CaMKIIα/β shRNA with shRNA-resistant $CaMKII\alpha^{R}$-WT (n = 26) There was no significant difference between conditions by Two-way ANOVA. Sources of variation are as follows Interaction p = 0.40 Transfection p = 0.582 time p < 0.0001. All data were plotted as mean ± S.E.M. Figures were adapted from Wang et. al, 2017

Shank-3 Knockdown Reduces LTCC- and $mGlu_5$-mediated Ca^{2+} Responses

We thought it might be possible that the reductions in somatic $mGlu_5$ Ca^{2+} responses and changes in LTCC E-T coupling (Wang et al., 2017) that we saw in response to CaMKII-KD might involve mechanisms other than the direct regulation of $mGlu_5$ and LTCCs. Because CaMKII, $mGlu_5$, LTCC are part of a bigger PSD molecular complex, we decided to study another protein that was identified by our lab as a major component of CaMKII complexes by mass-spectroscopy (Baucum et al., 2015). Shank-3, an important synaptic scaffolding protein, can bind to both LTCC and indirectly to $mGlu_5$ to regulate PSD composition. Our lab has also confirmed a direct interaction

between CaMKII and Shank-3 (Tyler Perfitt, unpublished results). Therefore, we decided to investigate the role of Shank-3 in both $mGlu_5$- and LTCC- mediated Ca^{2+} responses.

For these experiments, we measured somatic changes in Fura-2 fluorescence in hippocampal neurons with control shRNA or shRNA-KD of Shank-3 in response to LTCC-mediated depolarization and $mGlu_5$-mediated responses to DHPG. Unlike knockdown of CaMKII, knockdown of Shank-3 caused significant reduction in both LTCC- and $mGlu_5$-mediated somatic Ca^{2+} responses (Figure 6.3). When cells were transfected with Shank-3 shRNA, 40 mM K^+ treatment resulted in a significant reduction in Ca^{2+} influx (Figure 6.3 A,B). This was also the case for neurons treated with DHPG (Figure 6.3 C,D). These results emphasize the result that the PSD macromolecular complexes are important for proper control of intracellular Ca^{2+} influx.

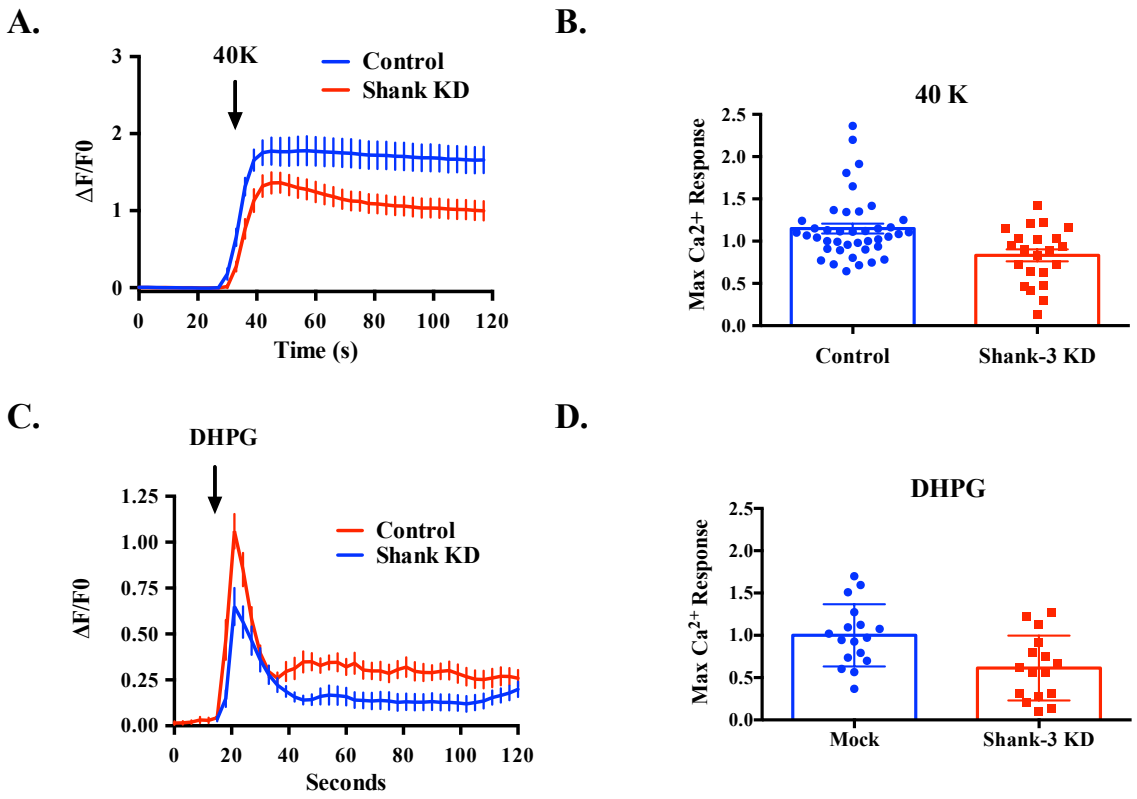

Figure 6.5 Shank-3 KD affects both LTCC- and $mGlu_5$-mediated Ca^{2+} responses Somatic levels of high potassium concentrations (A., B.) or DHPG-induced (C.,D.) transfected with a control shRNA or with shRNA to knock-down Shank-3 protein levels. A. Depolarization induced Ca^{2+} entry was measured by Fura-2 fluorescence in hippocampal neurons from three independent cultures (Control n = 41, Shank3-KD n = 22) pre-incubated with 5K Tyrode's solution containing APV, CNQX, and TTX. The black arrow indicated the buffer switch to 40K Tyrodes. Shank-KD caused a significant reduction in the 40K Ca^{2+} response by Two-way ANOVA Source of Variation Interaction $p < 0.0001$ Time $p < 0.0001$ Shank-KD $p = 0194$ B. Peak Ca^{2+} signals for each cell were normalized to the day of the experiment and plotted. Shank-3 KD caused a significant reduction in LTCC Ca^{2+} responses. In an unpaired t test $p = 0.001$. C. To measure the effect of Shank-3 expression on $mGlu_5$ responses and Ca^{2+} signals DHPG-induced Ca^{2+} entry was measured by Fura-2 fluorescence in hippocampal in one experimental day (Control n = 17, Shank3-KD n = 16) pre-incubated with 5K Tyrode's solution containing APV, CNQX, TTX, and VU-50. The black arrow indicates the buffer switch. Shank-3 KD also reduced $mGlu_5$-mediated Ca^{2+} influx (Two-way ANOVA Interaction $p < 0.0001$ Time $p < 0.0001$ Shank-3-KD, $p = 0.010$). D. Peak Ca^{2+} signals for each cell were normalized to the control and plotted. Shank-3 KD reduced peak DHPG-induced Ca^{2+} levels by an unpaired T-test ($p = 0.0059$). All data were plotted as mean ± S.E.M.

Discussion

Ca^{2+}influx into neurons through channels or by activation of receptors plays an important role in proper synaptic function. Understanding the molecular mechanisms that underlie the specificity of signaling downstream of these channels and receptors is critical to understanding their biological roles.

Here, we show the effect of CaMKII on regulating Ca^{2+} release from different sources in neurons. In Chapters III-V I discuss the role of CaMKII on $mGlu_5$ *in vitro* and in a heterologous cell system, but it is important to understand the role of CaMKII in a neuronal context. The macro-molecular complexes formed between CaMKII, $mGlu_5$, Homer, SHANK-3, and other Ca^{2+} regulating proteins within the synapse increases the complexity of the system and introduces a number of unknown variables within these experiments (Figure 6.1).

In order to study the role of CaMKII in these processes, I first focused on Group I mGlu receptors. I began to characterize Group I mGlu receptor responses in the presence of a number of inhibitors. I was also able to execute an experimental paradigm to study $mGlu_5$ specific responses. In data collected from heterologous cells, we reported that CaMKII was capable of reducing $mGlu_5$-mediated intracellular Ca^{2+} signals while prolonging the relative Ca^{2+} signal. From these data we hypothesized that knockdown of CaMKII in neurons would produce larger $mGlu_5$-mediated signals than control cells. Instead, we saw significant deficits in the Ca^{2+} response to $mGlu_5$-specific activation in neurons when CaMKII was knocked down. Reducing CaMKII in this neuronal system is likely to have many effects because of the large number of CaMKII interacting proteins. The importance of CaMKII in synaptic regulation has been demonstrated and therefore I propose using KD and re-expression of WT $mGlu_5$ or $mGlu_5$ 866-868AAA (Chapter III-IV) to

reduce CaMKII binding to $mGlu_5$. In this way we could use an experimental design that would preserve the interaction of CaMKII with other binding partners and eliminate a number of unknown variables introduced by long-term knockdown of CaMKII. We did not examine $mGlu_5$-mediated activation of transcription in the absence of CaMKII, but future studies should examine if CaMKII knockdown can also impair $mGlu_5$-mediated transcriptional activation. I would hypothesize that CREB activation would be impaired by CaMKII-KD similar to what we saw in LTCC experiments.

To study the effect of CaMKII on LTCC responses we began by measuring LTCC-specific responses by stimulating neurons with 40 mM K^+ in the presence of AMPAR and NMDAR antagonists. Knock-down of CaMKII had no effect on LTCC-mediated Ca^{2+} responses measured in the soma of neurons, but there was an impairment of long-range signaling to the nucleus as measured by the activation of E-T coupling (Wang et al., 2017). LTCC mediated E-T coupling seems to be independent of increases in nuclear Ca^{2+} concentrations, but seems to depend on Ca^{2+} signals within the LTCC nanodomain. Examining Ca^{2+} within neuronal spines may reveal differences when CaMKII is knocked down, but these changes were undetectable using our methods. Using more targeted Ca^{2+} imaging should help us to determine if there are smaller changes in LTCC nano-domain Ca^{2+} that can explain the E-T coupling deficits that we see with the CaMKII knockdown. I propose imaging dendritic spines for these experiments with GCaMP-X-tagged receptors described recently (Yang et al., 2018). These findings emphasize that local LTCC signaling is important to mediate the proper downstream effects of LTCC activation even when global changes are not detectable.

Lastly, we studied the role of SHANK-3 in LTCC-mediated depolarization and $mGlu_5$-mediated Ca^{2+} responses. The presence of Shank-binding motifs in $Ca_V1.3$ sequence is both necessary and sufficient for synaptic clustering of $Ca_V1.3$ L-type Ca^{2+} channels and important to LTCC E-T coupling (Zhang et al., 2005). Knockdown of Shank3 has also been shown to reduce $mGlu_5$ surface expression, CREB phosphorylation, and synaptic plasticity (Verpelli et al., 2011). This evidence led us to hypothesize that disruption of this synaptic scaffolding protein by shRNA-KD of Shank-3 would cause reductions in LTCC and $mGlu_5$ Ca^{2+} signals. Unlike knockdown of CaMKII, knockdown of Shank-3 caused significant reduction in both LTCC- and $mGlu_5$-mediated somatic Ca^{2+} responses. These data suggest that the experimental procedures we describe to measure Ca^{2+} responses in our primary hippocampal neurons are a good model to study LTCC- and $mGlu_5$-specific Ca^{2+} responses. We saw specific changes with CaMKII knockdown where global Ca^{2+} was not changed after activation of LTCCs, but was impaired in $mGlu_5$-signaling. Additionally, Shank-3 is an important component of proper LTCC- and $mGlu_5$-mediated Ca^{2+} release. The complex macro-molecular structures of the PSD are important to proper signaling within the cell. Future studies may be able to determine more carefully dissect the role of CaMKII on $mGlu_5$-specific signaling in neurons by using the $mGlu_5$ construct that we identified that is capable of reducing CaMKII binding and phosphorylation of $mGlu_5$.

Chapter VII

The effect of $mGlu_5$ on CaMKII signaling

Introduction

Most of the work in this dissertation has surrounded focused on understanding the role of CaMKII in modulating $mGlu_5$. In this chapter I investigate questions surrounding the second aim of this project to understand the role of $mGlu_5$ on regulating CaMKII. CaMKII activation is necessary for its interaction with many proteins. Because CaMKII is a critical regulator of numerous synaptic processes, $mGlu_5$-dependent modulation of CaMKII *in vivo* might have important implications for synaptic regulation. Because $mGlu_5$ activation leads to intracellular Ca^{2+} release, I hypothesized that $mGlu_5$ activation could work as a feedback regulator of CaMKII activation in neurons. $mGlu_5$-induced Ca^{2+} responses are modulated by a number of different $mGlu_5$ binding proteins including the Ca^{2+} sensitive CaM and CaMKII, but the effect of $mGlu_5$ on CaMKII activity and function is not well understood.

This work began the initial steps necessary to determine how activation of these two proteins are linked in normal physiology. First, we looked for basal differences in CaMKII activation in WT or $mGlu_5$-KO mice.

To determine the CaMKII response to $mGlu_5$ activation, we generated data showing the effect of DHPG treatment on CaMKII activation in acutely isolated brain slices. Lastly, we used *in vitro*

tools to understand the role of the $mGlu_5$-CaMKII interaction on CaMKII activation.

Changes in CaMKII activation in $mGlu_5$-KO mice

CaMKII and $mGlu_5$ have both been shown to regulate synaptic function in the hippocampus and the striatum. Distinct physiological stimuli are required for bidirectional synaptic plasticity in these brain regions, but differences in the underlying signaling mechanisms are poorly understood. To begin to understand how $mGlu_5$ might be regulating CaMKII activity in the brain, we compared CaMKII phosphorylation in micro-dissected striatum and hippocampus from WT mice and mice lacking $mGlu_5$ ($mGlu_5$-KO). We used a fractionation protocol to separate subcellular fractions containing cytosolic (S1), extrasynaptic (S2), and PSD (S3) proteins to probe brain lysate for levels of total and activated CaMKII.

We probed these samples for total CaMKII and pThr-286 to measure the level of CaMKII autophosphorylation as a measurement of CaMKII activation. In WT mice, the levels of basal CaMKII autophosphorylation are relatively low, but the absence of $mGlu_5$ in the $mGlu_5$-KO mice resulted in significant increases in CaMKII autophosphorylation at Thr286 in all of the synaptic fractions in both brain regions (Figure 7.1)

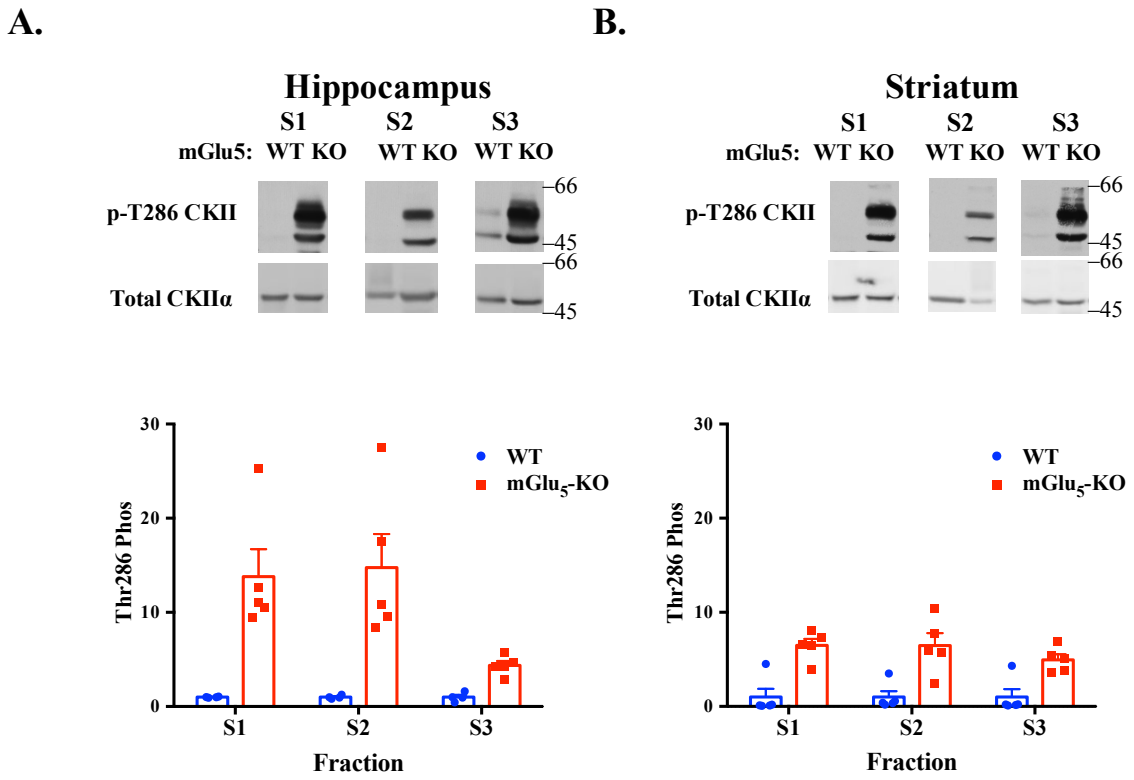

Figure 7.1 *Subcellular fractionation from WT or mGlu$_5$ knockout mice forebrains.* The hippocampus and striatum were microdissected from WT, and mGlu$_5$-KO mice and fractionated to isolate S1 (cytosolic), S2 (membrane-associated), and S3 (PSD-associated) proteins. All three fractions were then immunoblotted with a CaMKII and phospho-Thr286 antibody. The WT average Thr286/Total CaMKII was taken and used to normalize all values of Thr286/total CaMKII signals. A. Immunoblot showing p-Thr286 and total CaMKII levels in S1, S2, an S3 of WT (n = 4) and KO mice (n = 5) hippocampi showing that CaMKII autophosphorylation at Thr286 is increased in all three fractions. B. Quantification of the immunoblots where mGlu$_5$-KO significantly increased the autophosphorylation of CamKII by Two-way ANOVA (Interaction p = 0.0065, Fraction p = 0.0065, Genotype p = 0.0052) B. Representative data of striatal extracts where CaMKII autophosphorylation in also increased in the striatum of mGlu$_5$ KO mice. By Two-way Anova Interaction p = 0.2789, Fraction p = 0.2789, Genotype p = 0.0014. Each individual data point is a different mouse analyzed on the same immunoblot.

The effect of $mGlu_5$ activation on CaMKII autophosphorylation in brain slices

Results from the literature show both increases and decreases in CaMKII activation after treatment with DHPG depending on the time points examined and the brain region used for analysis (Jin et al., 2013a; Mockett et al., 2011). To compare the response of CaMKII to Group I mGlu receptor activation in the hippocampus and the striatum, we generated data showing the effect of DHPG treatment on CaMKII Thr286 phosphorylation in acutely isolated brain slices. Brain slices containing the hippocampus or the striatum were incubated with 100 μM DHPG for 20 min and a punch was gathered for analysis. Quantification of immunoblotts for total and pT286 CaMKII showed that, after $mGlu_{1/5}$ activation, there was a trend for a decrease in CaMKII phosphorylation at Thr-286 in the hippocampus and a statistically significant reduction in Thr-286 activation in the striatum. These data are in agreement with Figure 7.1 where $mGlu_5$-KO mice had increased levels of basal CaMKII autophosphorylation. Taken together, these data suggest that $mGlu_5$ can reduce the levels of CaMKII autophosphorylation in both the hippocampus and the striatum.

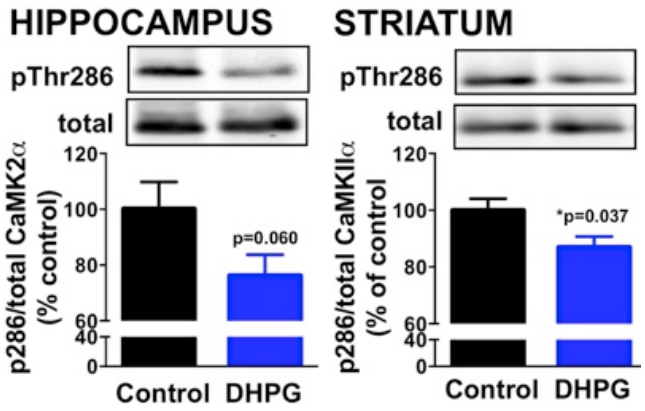

Figure 7.2. *DHPG reduces CaMKII activation in acute slice pharmacology experiments.* A. Acutely isolated brain slices containing the hippocampus or the striatum were incubated with 100 μM DHPG for 20 min. Punches were homogenized and immunoblotted for total and pT286 CaMKII. Work in this figure was generated by Dr. Johanna Gandy.

The effect of mGlu$_5$-CTD on CaMKII activation *in vitro*

The effect seen in Figure 7.1 and 7.2 led us to question if CaMKII binding to mGlu$_5$ could have a direct role in regulating CaMKII activation. To investigate this, we used *in vitro* tools to understand the role of the mGlu$_5$-CaMKII interaction on CaMKII activation. We first pre-incubated purified CaMKII with GST-mGlu$_{5a}$-CTD or GST alone and performed an CaMKII autophosphorylation assay to determine if the presence of mGlu$_5$ was capable of altering CaMKII autophosphorylation. Interestingly, we found that the presence of mGlu$_{5a}$-CTD increased CaMKII autophosphorylation compared to GST (Figure 7.3A). To understand this mechanistically, we continued our studies using an assay to measure phosphorylation of a CaMKII synthetic substrate, syntide, in the presence of GST-mGlu$_{5a}$-CTD and increasing concentrations of CaM. The GST-mGlu$_{5a}$-CTD dramatically enhanced cooperativity of CaMKII activation by CaM as measured by an increase in the Hill constant (Fig. 7.3B). These findings suggest that mGlu$_5$ can enhance the switch-like

activation of CaMKII by suppressing activation until a threshold concentration of Ca^{2+}/CaM is exceeded.

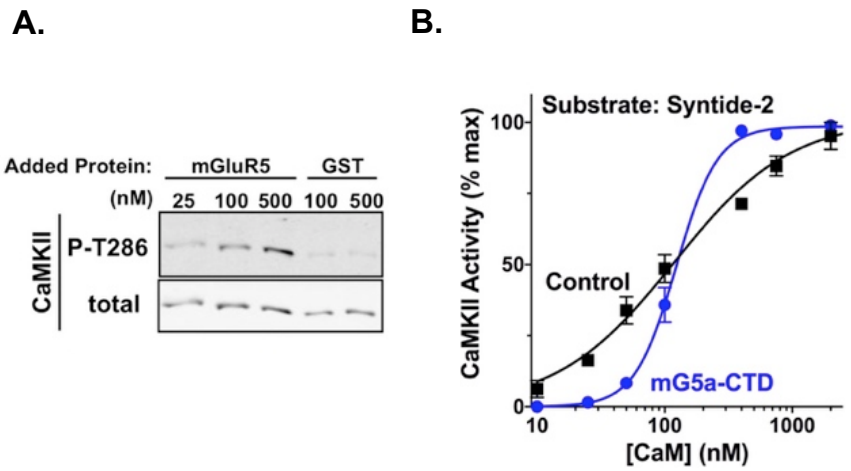

Figure 7.3 *The $mGlu_5$-CTD enhances CaMKII autophosphorylation and increases the apparent cooperativity for CaMKII activation by CaM.* A. CaMKII was preincubated with the indicated concentrations of either GST-mG5-CTD or GST. Reactions were initiated by addition of Ca^{2+}, CaM, and ATP for 1 minute on ice. These conditions are designed to limit the rate of Thr286 phosphorylation so differences can be detected. Samples were immunoblotted for total and pT286 CaMKII. B. CaMKII was preincubated with GST-$mGlu_5$-CTD or GST on ice. Reactions were started by addition of indicated concentrations of CaM, along with 2 mM Ca^{2+}, 0.4 mM [γ-^{32}P]ATP, and 0.2 mM syntide-2. After 10 min at 30C, ^{32}P incorporation into syntide-2 was measured. GST-mG5-CTD increases the Hill constant from 1.1 to 3.1, indicating an increase in apparent cooperativity for activation by CaM.

Discussion

The results presented in this chapter support the idea that $mGlu_5$ can suppress CaMKII activation at low levels of intracellular Ca^{2+}. I report here an enhancement of CaMKII activation in $mGlu_5$ KO mice, a reduction in CaMKII autophosphorylation after $mGlu_5$ activation in acute brain slices, and the ability of $mGlu_5$ to enhance CaMKII cooperativity. Thus, $mGlu_5$ may suppress CaMKII

activity under basal conditions (low Ca^{2+}/CaM), and enhance switch-like activation in response to modest increased in Ca^{2+}. Such a mechanism could enhance the switch-like responsiveness of the pool of CaMKII bound to $mGlu_5$ in cells.

The effect of $mGlu_5$ activation on CaMKII autophosphorylation at Thr286 has not been clear based on previous findings from the literature. It was been reported that there is a quick reduction CaMKII autophosphorylation in hippocampal synaptoneurosomes treated with DHPG that is followed by a biphasic increase in CaMKII (Mockett et al., 2011) and another lab showed increases in CaMKII activation after DHPG application in striatal brain slices (Jin et al., 2013a). Our studies show in both the hippocampus and the striatum that $mGlu_5$ activation reduces CaMKII autophosphorylation at Thr286. Currently, we are unable to explain the difference in these findings, but they may be due to differences in experimental design. It is also possible that the developmental switch between $mGlu_{5a}$ and $mGlu_{5b}$ differentially regulates the role of $mGlu_5$ activity on CaMKII across different age ranges. It will be interesting to examine differences in basal autophosphorylation of CaMKII at Thr286 in the brains of $mGlu_5$ KO mice relative to WT mice and the role of $mGlu_5$ activation in affecting CaMKII autophosphorylation more systematically across development. We also saw that the $mGlu_5$-CTD was capable of directly affecting CaMKII activation and cooperativity for CaM. I would like to repeat these experiments with the GST-$mGlu_5$-866-868AAA mutant to confirm that CaMKII binding to $mGlu_5$ is important for this result. It will also be useful to determine whether mutation of a Ser901 PKC phosphorylation site to Asp, which blocks Ca^{2+}/CaM-binding to the CTD, prevents enhanced CaMKII activation. Low levels of $mGlu_5$ activation may play a role in inhibiting CaMKII activation during baseline levels of neuronal activity. Lack of $mGlu_5$ is also likely to disrupt the proper composition of the molecular PSD complexes that are tightly regulated in dendritic spines.

Examining the effect of pharmacological inhibition of $mGlu_5$ on CaMKII activation over time may be helpful in addressing some of the issues posed by long-term knockout of $mGlu_5$. Additionally, our slice pharmacology studies and those done by other laboratories have examined the effect of DHPG which activates both $mGlu_1$ and $mGlu_5$. A clearer picture of $mGlu_5$ activation on CaMKII activity can be deduced by using specific protocols to activate $mGlu_5$ such as in our neuronal Ca^{2+} imaging experiments presented in Chapter VI.

Chapter VIII

Discussion and Future Directions

Summary

The work presented in this document provides new insights into the complex regulation of CaMKII and its interacting proteins. The multiprotein complexes present in the dendrites of neurons are highly regulated and specific. This work has begun to unravel the importance of some of these interactions. In this dissertation I focused on the CaMKII and $mGlu_5$ interaction and signaling relationship.

When I began these studies, it had been reported that the membrane proximal region of the $mGlu_{5a}$-CTD bound to *inactive* CaMKII (Jin et al., 2013b). I focused on the novelty of this type of interaction because there are few proteins known to bind to CaMKII in their inactive conformation. I hoped to further characterize the physical and functional relationship between these key regulators of synaptic transmission because of the evidence of their functional interaction in physiology (discussed on pg. 51). We confirmed that CaMKIIα and $mGlu_5$ specifically interact in mouse brain. However, the binding interaction between $mGlu_{5a}$ and CaMKII was weak in my initial *in vitro* studies using inactive kinase. I was able to show that the $mGlu_{5a}$-CTD residues 827-964 bind more strongly to CaMKIIα in an active, Thr286-autophopshorylated conformation, but that this interaction is disrupted by excess Ca^{2+}/CaM or by robust CaMKII autophosphorylation at additional undefined sites. I showed that the interaction requires three basic residues (Lys^{866}-Arg^{867}-Arg^{868}) on the $mGlu_5$-CTD.

I tested the hypothesis that CaMKII modulates signaling via an interaction with $mGlu_5$ through binding or phosphorylation of the receptor by measuring changes in $mGlu_5$ surface expression, ERK1/2 activation, and Ca^{2+} signaling. Active CaMKII increases basal $mGlu_5$ surface expression and ERK activation in heterologous cells.

Careful control of intracellular Ca^{2+} is important to processes of development, synaptic remodeling, transcriptional regulations, and control of synaptic strength (Brini et al., 2014). The diverse effects of Ca^{2+} in neurons can be specific to the cell-type and source of calcium. Understanding the precise control of this signaling mechanism has been a major motivation for pursuing this work. To study the CaMKII control of $mGlu_5$ Ca^{2+} signaling in a simple system I looked at changes in $mGlu_5$-mediated Ca^{2+} signals in a heterologous cell system and showed that CaMKII decreases the initial amplitude of $mGlu_5$ Ca^{2+} release, but prolongs the relative Ca^{2+} signal. CaMKII also increases $mGlu_5$ oscillation frequency in cells responding with baseline oscillations. All of these effects are prevented by the triple alanine substitution for Lys^{866}-Arg^{867}-Arg^{868} in the CTD.

To understand the regulation of neuronal Ca^{2+} levels we used cultured hippocampal neurons. Based on our HEK cell studies we expected that knockdown of CaMKII would result in enhanced $mGlu_5$ signaling in neurons, but we saw reduced $mGlu_5$-specific Ca^{2+} signals. To determine if this effect was specific to $mGlu_5$, we also tested the effect of CaMKII knockdown on LTCC influx. Knockdown of CaMKII did not affect global LTCC Ca^{2+} signals, but did result in impairments in LTCC E-T coupling. In order to determine a mechanism of how the macro-molecular complex associated with $mGlu_5$, CaMKII, and LTCC might be playing a role in these results, we looked at the effect of shRNA knockdown of an important scaffolding protein, Shank-3. Unlike CaMKII,

knockdown of Shank-3 caused impairments in both LTCC- and $mGlu_5$-mediated Ca^{2+} signals. We also have unpublished evidence that Shank-3 knockdown can impair LTCC E-T coupling (unpublished, Tyler Perfitt).

It had never been demonstrated that CaMKII was capable of phosphorylating $mGlu_5$. I performed *in vitro* phosphorylation assays to show that CaMKII can phosphorylate both isoforms of $mGlu_5$. Similar to binding, Ca^{2+}/CaM can compete with CaMKII to inhibit CaMKII phosphorylation of the $mGlu_5$-CTD, but this effect on $mGlu_{5a}$ was much more potent than on $mGlu_{5b}$. I was also able to identify putative sites of phosphorylation of both $mGlu_{5a}$ and $mGlu_{5b}$.

In the last chapter I show work surrounding the second aim of this project to understand the role of $mGlu_5$ on regulating CaMKII. I hypothesized that $mGlu_5$ activation could work as a feedback regulator of CaMKII activation in neurons. To determine the CaMKII response to $mGlu_5$ activation we showed that $mGlu_5$ activation in acutely isolated brain slices led to decreases in CaMKII activation. Additionally, $mGlu_5$ KO mice show largely elevated levels of basal CaMKII autophosphorylation at Thr286, and lastly we show that the $mGlu_5$-CaMKII interaction directly affects CaMKII cooperativity for CaM.

CaMKII binding studies

Contrary to a previous publication (Jin et al., 2013b), we were able to show for the first time that CaMKII activation results in stronger binding to $mGlu_5$. This is true for $mGlu_{5a}$, but we have not undertaken these same studies in $mGlu_{5b}$. An obvious future direction is to systematically test the $mGlu_{5b}$ interaction with CaMKII under different activation states. This is of particular interest because the $mGlu_{5b}$ specific insert (starting at residue 876) lies very close to the tri-basic residues

necessary for CaMKII binding (Lys^{866}-Arg^{867}-Arg^{868}) and the putative phosphorylation site identified on $mGlu_{5a}$ (Ser871) in Chapter V. For this reason, the interaction between $mGlu_{5a}$ and $mGlu_{5b}$ may be different. It will be interesting to test if triple Ala mutation of Lys^{866}-Arg^{867}-Arg^{868} in $mGlu_{5b}$ disrupts CaMKII binding.

The tribasic residues important for the interaction between $mGlu_5$ and CaMKII are similar to a tribasic residue stretch necessary for other CaMKII interactions on the $Ca_V1.3$ NTD (Wang et al., 2017), and Shank-3 (Tyler Perfitt, unpublished). Other GPCRs that bind CaMKII also include tribasic residues within their CaMKII binding regions, including $mGlu_1$ and the D_2 dopamine receptor (Jin et al., 2013a; Zhang et al., 2014) (Figure 3.5). We believe that these tribasic residue motifs may constitute a new type of CaMKII binding domain. These tri-basic residue stretches are not sufficient to bind CaMKII on their own and the flanking residues are probably an important aspect in CaMKII binding to these regions.

It was reported that $mGlu_5$ could only interact with inactive kinase and that CaMKII autophosphorylation inhibited this reaction (Jin et al., 2013b). Using different autophosphorylation protocols for CaMKII (Chapter III), I was able to show that active CaMKII can bind more strongly to the $mGlu_5$-CTD depending on the autophosphorylation conditions (Figure 3.2). Extensive autophosphorylation at unidentified sites on CaMKII reduced the CaMKII interaction with $mGlu_5$ (Figure 3.2 & Jin et al, 2013b), but not with $mGlu_1$ (Jin et al., 2013a). Because of these reported differences, I would hypothesize that the $mGlu_1$ and $mGlu_5$ interactions are distinct and that CaMKII regulates these two receptors at different sites of interaction (Figure 8.1). In addition to binding to $mGlu_1$, it was also published that CaMKII was capable of binding to the $mGlu_5$ IL-2. I was unable to confirm this result (data not shown). Future studies should determine other sites of

CaMKII binding on the $mGlu_5$ full-length receptor because triple alanine mutation of Lys^{866}-Arg^{867}-Arg^{868} was unable to completely disrupt the CaMKII interaction with full-length receptor.

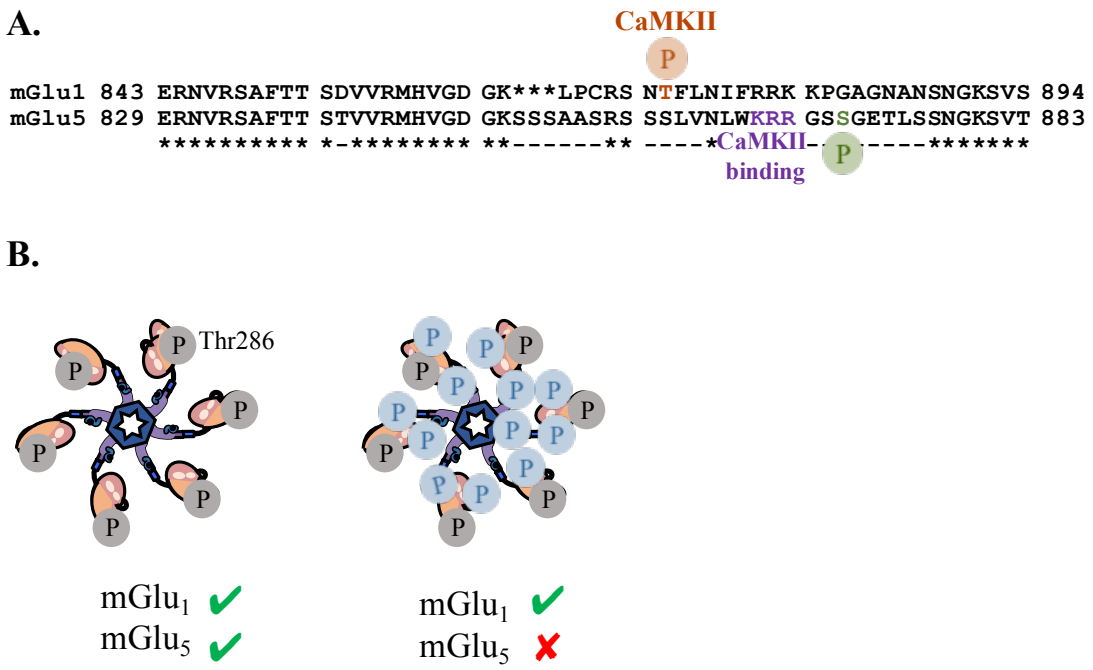

Figure 8.1 *$mGlu_1$ and $mGlu_5$ interactions with CaMKII.* A. Alignment of the CTD region of $mGlu_1$ and $mGlu_5$. The CaMKII phosphorylation site on $mGlu_1$ (Jin et al., 2013a) is marked as well as the CaMKII phosphorylation site on $mGlu_5$ detected in our studies (Chapter V). B. Schematic showing that both $mGlu_1$ and $mGlu_5$ are capable of binding to CaMKII in their Thr286 autophosphorylated state, but CaMKII autophosphorylation at unidentified sites that exceed Thr286 phosphorylation has no effect on CaMKII binding to $mGlu_1$, but disrupts the interaction with $mGlu_5$ (reported by Jin et al., 2013a, b). The differences in sequence homology, suspected phosphorylation sites, and ability to bind to CaMKII in different autophosphorylation conditions leads me to hypothesize that these interactions occur in distinct binding regions.

It is not clear what CaMKII autophosphorylation sites affect the interaction between $mGlu_5$ and CaMKII. It is known that CaMKII does have multiple autophosphorylation sites, but the most well

studied have been Thr286, 305, and 306. We demonstrate in our studies that Thr286 phosphorylation does not differ between a 90 second or 10 minute autophosphorylation protocol, but there are large increases at unidentified sites in this extensive autophosphorylation protocol (Figure 3.3). This is an important area of study because imbalances in CaMKII autophosphorylation have been linked to altered synaptic plasticity and neuronal dysfunction in disease models (Table 8.1). For example, increased phosphorylation at both Thr286 and Thr305/306, and defects in hippocampal LTP and learning were observed in a mouse model of Angelman's mental retardation syndrome (AS) (Weeber et al., 2003). This is similar to what we see in $mGlu_5$-KO mice where CaMKII autophosphorylation at Thr286 is largely increased in the hippocampus and striatum.

A recent study analyzed CaMKII autophosphorylation sites using a proteomics approach and identified multiple novel phosphorylation sites including Ser78, Thr261, Ser275, Ser315, Thr320/Thr321, Ser331, and Thr378 (Baucum et al., 2015). These newly identified sites of autophosphorylation are great targets to understand how CaMKII self-regulation may systematically control protein specific interactions. There is evidence that CaMKII autophosphorylation sites other than Thr286, 305, and 306 play a role in synaptic function. A group recently showed that phosphorylation of CaMKII at Ser331 inhibited CaMKII activity and is reversibly regulated in cocaine-associated memory reconsolidation and extinction (Rich et al., 2016). Another autophosphorylation site, Thr253, did not alter CaMKII activity, but did alter CaMKII targeting and the CaMKII interactome (Skelding et al., 2010). Testing phosphomimetic mutations of CaMKII for their interactions with $mGlu_5$ and other CaMKAPs may help us to understand how CaMKII autophosphorylation can regulate CaMKII targeting in neurons. I also report here that the $mGlu_5$-CTD can enhance CaMKII cooperativity (Chapter VII). Enhanced

CaMKII cooperativity may lead CaMKII to be more susceptible to excess phosphorylation in the presence of $mGlu_5$. Activation of CaMKII by its interaction with $mGlu_5$ could also direct CaMKII to other targets such as GluN2B. This hypothesis is supported by work showing that $mGlu_5$ activation leads to a reduction in the CaMKII association with $mGlu_5$ and increased association with GluN2B (Jin et al., 2013b). If this is the case, the composition of the indirect interface between $mGlu_5$ and the NMDAR is likely very important to proper function.

CaMKII-$mGlu_5$ interaction on $mGlu_5$ receptor localization and signaling

The regulation of $mGlu_5$-CTD by interacting proteins can regulate cell surface localization and intracellular receptor signaling as reviewed in Chapter I. The $mGlu_5$-CTD contains binding sites for many proteins that can occupy overlapping binding domains and compete for binding to induce regulatory control of $mGlu_5$ signaling (Figure 1.8). In our initial heterologous cell studies we examined the role of overexpressing CaMKII on $mGlu_5$ signaling.

We began our studies in HEK293 cells because they represent a much simpler system than neurons. In this system, I demonstrated that CA-CaMKIIα can increase cell surface expression of $mGlu_{5a}$ and effect $mGlu_{5a}$-dependent Ca^{2+} mobilization. Co-expression of CA-CaMKIIα reduced the amplitude of $mGlu_{5a}$ mediated initial Ca^{2+} peaks but prolonged the duration of the $mGlu_5$-generated Ca^{2+} signal. These effects were prevented by the triple alanine substitution for Lys^{866}-Arg^{867}-Arg^{868} in the CTD. These findings introduce questions about how CaMKII is capable of modulating $mGlu_5$ Ca^{2+} signals.

$mGlu_5$–mediated Ca^{2+} induction activates a number of downstream signaling pathways, and involves a number of Ca^{2+}-sensing feedback regulators including CaM, CaMKII, CaN, and PLC.

Understanding what is known about the generation of $mGlu_5$-mediated signals and incorporating the effects that CaMKII has on $mGlu_5$ Ca^{2+} oscillations allows us to propose a model for what is happening in HEK293 cells in the presence of $mGlu_5$ and CaMKII.

The activation of Group I mGlu receptors leads to increases in membrane-bound diacylglycerol IP_3 concentrations, resulting in the release of Ca^{2+} from intracellular stores, and the activation of PKC. $mGlu_5$ activation has most often been reported to give rise to repetitive base-line separated Ca^{2+} oscillations, although we see a number of different types of responses in both HEK293 cells and in neurons as reported in Chapters IV and VI.

A number of different hormones and neurotransmitters activate signaling pathways that communicate through increases in intracellular Ca^{2+}. The dynamics of Ca^{2+} oscillations have been studied in order to understand how cells are capable of decoding the universal Ca^{2+} signal. This leads to the complicated question of how different stimuli working through the same messenger systems can generate specificity. Some mechanisms of control include spatial and temporal patterns coded into the signal. For example, NMDAR LTP and LTD are both triggered by post-synaptic elevations in Ca^{2+}. Additionally, CaMKII can also play a role in both LTP and LTD (Pi et al., 2010). This is evidence that Ca^{2+} channels and Ca^{2+} dependent proteins can induce opposing processes by interpreting specific Ca^{2+} signals. Simulations have shown how different frequency and amplitudes of neuron stimulation affect activation of different Ca^{2+} sensors. While Ca^{2+} activates both CaN and CaMKII at all frequencies, higher frequencies shift the relative activation from CaN to CaMKII (Li et al., 2012). CaMKII has also been described as a frequency detector, as differences in Ca^{2+} spike frequency can cause differences in the extent of CaMKII activity (De Koninck and Schulman, 1998). Changes in Ca_{2+} influx patterns and different interpretation of the

signal can then alter downstream functional outputs such as gene expression (Dolmetsch et al., 1998). The ability of CaMKII to alter the amplitude and relative duration of $mGlu_5$ Ca^{2+} signals in cells has the potential to alter the cellular interpretation of $mGlu_5$ activation.

$mGlu_5$-mediated Ca^{2+} oscillations are accompanied by IP_3 oscillations in single cells. Many studies have focused on a PKC-dependent "dynamic desensitization" model whereby PKC can phosphorylate the receptor initiating cycles of activation/deactivation as first suggested by early studies of $mGlu_5$ (Kawabata et al., 1996). The simplest model was proposed as follows: 1. agonist activation of $mGlu_5$ increases IP_3 and diacylglycerol production, 2. Ca^{2+} is released from intracellular stores by IP_3 3. increases in diacylglycerol and Ca^{2+} activate PKC to inhibit IP_3 production by phosphorylation of $mGlu_5$ 4. Ca^{2+} levels consequently fall and PKC activity is no longer sustained, and 5) dephosphorylation by protein phosphatases resets the system to allow IP_3 production to cycle again (Kawabata et al., 1996)

Later, the hypothesis surrounding $mGlu_5$ oscillations was expanded to include input resulting from Ca^{2+} induced Ca^{2+} release (CICR) in addition to dynamic uncoupling. Receptor stimulation has been shown to initiate Ca^{2+} oscillations in both PKC-dependent and-independent mechanisms (Dale et al., 2001; Kawabata et al., 1996). This led to the IP_3-induced Ca^{2+} signaling "slide rule" (Nash et al., 2002). This model proposes a continuum of Ca^{2+} responses relative to increases in IP_3 concentration where low concentrations sensitize IP3Rs and lead to CICR. Here, it is thought that the frequency of Ca^{2+} oscillations is dependent on agonist concentration. Both IP_3 and Ca^{2+} regulate IP_3 receptors, with Ca^{2+} exerting a biphasic effect that facilitates Ca^{2+} release at low concentrations but is inhibitory as its levels rise (Adkins and Taylor, 1999). Levels of stimulation too great to drive oscillatory behavior lead to a peak-plateau response (i.e. Figure 4.6A, red trace)

These hypotheses do not take into consideration the multiple proteins that affect $mGlu_5$ surface expression and signaling in neurons. However, looking at these simple models and other examples from the literature, we can begin to hypothesize the role that CaMKII might be playing in our heterologous cell studies.

When we examined Ca^{2+} response curves to glutamate, CA-CaMKIIα did not affect the apparent EC_{50} value, but did increase $mGlu_5$ surface expression and ERK activation. Other studies have reported similar effects to these in the presence of a phosphoinhibitory mutation of a PKC site, Ser901, or in the presence of $mGlu_5$ binding partners Norbin and α-actinin-1 (Cabello et al., 2007; Lee et al., 2008; Wang et al., 2009). In the case of the mutation of Ser901, the authors propose that increases in $mGlu_5$ surface expression and prolonged half-life of $mGlu_5$ Ca^{2+} signals were a result of enhanced CaM binding to the $mGlu_5$-CTD to stabilize surface expression.

CaMKII affects the initial Ca^{2+} signal amplitude of $mGlu_5$ signals. This could be explained by two possible scenarios: 1. CaMKII could cause a reduction in the receptor "on" signal, or 2. CaMKII could promote a faster "off" signal. In the first scenario, CaMKII binding or phosphorylation could reduce $mGlu_5$ coupling to G_q to reduce initial IP_3 and DAG production. Alternatively, CaMKII could promote a faster "off signal" to induce smaller productions in IP_3 by enhancing PKC phosphorylation of site responsible for stopping $mGlu_5$ oscillations (Ser839) to promote the off signal. CaMKII could also play a similar role to that predicted of CaM, causing enhanced receptor surface stability and spatial restrictions that block PKC phosphorylation at Ser901. In this way CaM and CaMKII could play complementary roles as discussed in Chapter III.

One property of $mGlu_5$ oscillations resulting from dynamic uncoupling is that, when a threshold concentration of orthosteric agonist is reached, oscillation frequency is unchanged by increasing

agonist concentration, but can be modulated by receptor surface expression (Nash et al., 2002). CaMKII is capable of increasing $mGlu_5$ surface expression; therefore, I predicted that in cells expressing oscillatory patterns that we would see an increase in Ca^{2+} oscillation frequency. When we analyzed cells responding with at least 3 baseline oscillations in response to glutamate, we saw that CA-CaMKII caused a significant increase in the frequency of oscillations (average time between peaks: $mGlu_5$ 65 seconds, $mGlu_5$ + CA-CaMKII 46 seconds, Figure 4.7D). However, I would hypothesize that CaMKII is capable of affecting $mGlu_5$-mediated Ca^{2+} signals in a more complex way than simple increased surface expression. To my knowledge, there are no studies that show an effect of increased $mGlu_5$ surface expression causing a reduction in $mGlu_5$ Ca^{2+} amplitudes.

I would hypothesize that the reduction in the intracellular Ca^{2+} amplitudes correlate with reductions in IP_3 production, but this measurement alone would not reveal a difference between the two hypotheses that I have offered (reduced "on"/enhanced "off" signals). Activation of $mGlu_5$ results in the synchronized repetitive cytosol to plasma membrane translocation of PKC in a heterologous cell system (Dale et al., 2001) and in astrocytes (Codazzi et al., 2001). This translocation could present a spatial restriction for PKC phosphorylation of $mGlu_5$, allowing for other Ca^{2+} sensors like CaM or CaMKII to regulate the receptor. It would be interesting to determine if CA-CaMKII changed the frequency or extent of PKC translocation in response to $mGlu_5$ activation. It would also be interesting to look at the translocation of CaMKII during $mGlu_5$ activation in both HEK293 cells and neurons. Previous experiments have used co-expression of fluorescently tagged CaMKII and a genetically encoded Ca^{2+} indicator, GCaMP2, to simultaneously measure Ca^{2+} dynamics and spatial and temporal accumulation of CaMKII (Lemieux et al., 2012). Combining the live imaging of CaMKII and PKC might reveal if these two

proteins translocate in similar or distinct patterns to the cell membrane in cells expressing $mGlu_5$. To isolate the effect of CaMKII on this system, one could examine how pharmacological inhibition of PKC or PKC phospho-null $mGlu_5$ mutations affect the $mGlu_5$ signal in the presence of CA-CaMKII.

We have shown that CaMKII is capable of both binding and phosphorylation of the $mGlu_5$-CTD and both are blocked by triple alanine mutation of Lys^{866}-Arg^{867}-Arg^{868}; therefore, our studies cannot conclude whether the CaMKII-specific effects that we see in HEK293 cells are induced because of CaMKII binding or through phosphorylation at the $mGlu_5$-CTD. Future directions to tease apart these systems might identify the CaMKII sites of phosphorylation on the $mGlu_5$-CTD to determine if these effects were due to binding or phosphorylation of the receptor.

It was recently reported that PKA can phosphorylate Ser870 on $mGlu_5$ and that this phosphorylation is required for $mGlu_5$ activation of ERK signaling and intracellular Ca^{2+} oscillations (Uematsu et al., 2015). This site is directly adjacent to Ser871, a site of CaMKII phosphorylation on $mGlu_5$ identified in our proteomics analysis. It was reported that a phosphomimetic mutation of Ser870 allowed for normal ERK activation, but phosphoinhibitory mutation of this site inhibited $mGlu_5$ ERK signaling and Ca^{2+} responses. The coupling of $mGlu_5$ with $G_{q/11}$-protein is an important step in activation of downstream ERK signaling; therefore, it is possible that PKA and CaMKII are able to modulate the $G_{q/11}$-protein-coupling region in the $mGlu_5$-CTD. Phosphorylation of these two sites by PKA and CaMKII might affect receptor signaling during different cellular stimulation conditions. A helpful assay to better understand the interaction of the kinases capable of phosphorylating $mGlu_5$ might be to perform sequential phosphorylation with PKA, PKC, and CaMKII in a simple *in vitro* system. For instance, an initial

phosphorylation of the $mGlu_5$-CTD with PKA could reduce a sequential phosphorylation by CaMKII or vice-versa. Understanding preferential phosphorylation *in vitro* could help us to make more educated hypotheses about the regulation of CTD binding and phosphorylation in cellular systems.

Strict control of $mGlu_5$ activity is important for proper synaptic function; therefore, it is important to study these processes in a neuronal context. The simple model of $mGlu_5$ Ca^{2+} signal regulation by the two methods of CICR and dynamic uncoupling to PKC becomes much more complicated as we examine the $mGlu_5$-CTD interacting partners in neurons. There are a number of proteins that can compete with CaM binding to the $mGlu_5$-CTD including CaMKII and α-actinin (Cabello et al., 2007; Jin et al., 2013b; Marks et al., 2018). Interestingly, α-actinin, a scaffolding protein that links actin filaments, was shown to bind $mGlu_{5b}$ in the CTD region near the Ser901 (Ser 933 in $mGlu_{5b}$) PKC phosphorylation site. The authors showed that α-actinin increased $mGlu_5$ surface expression and ERK activation (Cabello et al., 2007). Previous work from our lab showed α-actinin is also a CaMKAP that can cause Ca^{2+}-independent activation of CaMKII (Jalan-Sakrikar et al., 2012). Binding of α-actinin can mimic CaM binding to CaMKII to activate binding to a subset of substrates in vitro and in intact cells. In addition, α-actinin can also compete with CaM for binding to the NMDAR to promote CaMKII binding (Merrill et al., 2007). Based on this work, I propose experiments to determine if α-actinin is capable of increasing the phosphorylation of $mGlu_5$ by CaMKII. The α-actinin, CaMKII, $mGlu_5$ complex could allow for CaMKII regulation of $mGlu_5$ even when intracellular levels of Ca^{2+}/CaM are not capable of fully activating CaMKII.

The binding site for CaM also overlaps with other regulators of $mGlu_5$ surface expression including Norbin, and the E3 ligase Siah-1a (Ishikawa et al., 1999; Ko et al., 2012; Wang et al.,

2009). These proteins can bind within a very small region of $mGlu_5$ to modulate receptor function. It seems as though CaM, Norbin, α-actinin, and CaMKII can all promote increased receptor surface expression. Although α-actinin was not tested for effects on mediating $mGlu_5$-dependent Ca^{2+} release, all of these other $mGlu_5$ interaction proteins increased Ca^{2+} signal half-lives, similar to our findings with CaMKII. Alternatively, PKC phosphorylation at Ser901 inhibited CaM binding and enhanced Siah-1A binding to increase receptor internalization and lysosomal degradation.

I would to acknowledge that although I was able to identify effects of CA-CaMKII on $mGlu_5$ signaling in heterologous cells, these effects were not immediately evident using WT-CaMKII. In surface expression, ERK activation, and Ca^{2+} signaling studies we only identified the effect of CaMKII on $mGlu_5$ when we used CA-CaMKII. This is not surprising because we showed that active CaMKII binds to $mGlu_5$ much more strongly than inactive kinase, and basal levels of CaMKII activation in HEK293 cells is very low. Neurons have detectable Thr286 autophosphorylation levels that may be able to regulate $mGlu_5$ even under baseline activity.

Based on our work in HEK293 cells, we hypothesized that CaMKII knockdown in neurons would lead to enhanced amplitude of Ca^{2+} influx. When we examined Ca^{2+} responses in neurons we saw that CaMKII knockdown reduced global $mGlu_5$-mediated Ca^{2+} signals, but not LTCC influx. The macro-molecular complexes formed between CaMKII, $mGlu_5$, Homer, Shank-3, and other Ca^{2+} regulating proteins within the synapse increase the complexity of the system and introduce a number of unknown variables within these experiments. Considering the complexity of signaling in this system, is not surprising that our results in neuronal Ca^{2+} imaging studies differ from what we would expect from heterologous cell studies.

Our neuronal Ca^{2+} imaging studies confirm that we can distinguish source-specific changes in neuronal Ca^{2+}. Although knockdown of CaMKII had no effect on LTCC-mediated Ca^{2+} responses measured in the soma of neurons, there was an impairment of long-range signaling to the nucleus as measured by the activation of E-T coupling (Wang et al., 2017). These findings highlight that *local* LTCC signaling is important to mediate the proper downstream effects of LTCC activation even when global changes are not detectable. Future experiments should examine if CaMKII knockdown can also impair $mGlu_5$-mediated transcriptional activation, as was seen in LTCC experiments. Tyler Perfitt, a graduate student in our lab, has begun to collect these data and has confirmed that activation of $mGlu_5$ leads to CREB activation in neurons (data not shown).

A physical interaction as well as a functional interaction between $mGlu_5$ and LTCCs was demonstrated in a study where $mGlu_5$ and LTCCs were co-immunoprecipitated from brain lysates and $mGlu_5$ facilitated depolarization-evoked calcium currents (Kato et al., 2012). Another link between these two proteins is that they are both in complex with the scaffolding protein Shank. Shank is important for $Ca_V1.3$ LTCC E-T coupling (Zhang et al., 2005) and $mGlu_5$ surface expression, CREB phosphorylation, and synaptic plasticity (Verpelli et al., 2011).

We were able to show an effect on both LTCC- and $mGlu_5$-mediated Ca^{2+} responses with the knockdown of the scaffolding protein Shank-3. These data show that our methods to measure Ca^{2+} responses in our primary hippocampal neurons are a good model to study LTCC- and $mGlu_5$-specific Ca^{2+} responses. There may be some changes that we are unable to detect with our current methods. It will be interesting to use more targeted Ca^{2+} imaging to determine the effect of CaMKII on local LTCC signaling, as discussed in Chapter VI.

In addition to the kinase activity of CaMKII, it is also thought to function as an organizer of synaptic proteins (Hell, 2014; Incontro et al., 2018). The molecular composition and proximity of CaMKII to substrates within the synapse cause highly selective regulation by CaMKII (Tsui et al., 2005; Tsui and Malenka, 2006). Spatial barriers of substrate phosphorylation are often overcome by translocation, or by anchoring to the substrate or a nearby protein. Due to the high degree of $mGlu_5$ regulation by CaMKAPs such as CaM, α-actinin, Shank, and Homer, long-term knockdown of CaMKII may have unexpected indirect effects on receptor signaling (Cabello et al., 2007; Guo et al., 2015; Minakami et al., 1997; Mizutani et al., 2008).

The importance of CaMKII in synaptic regulation has been demonstrated and knock-down of CaMKII is not the most specific manipulation that we can make to answer questions about the effect of CaMKII on $mGlu_5$ in neurons. Pharmacological inhibition of CaMKII offers an acute perturbation of the system that may be more telling, but I propose that knockdown of $mGlu_5$ and re-expression of the $mGlu_5$ binding mutant with Ala mutation at Lys^{866}-Arg^{867}-Arg^{868} may preserve the interaction of CaMKII with other synaptic binding partners and eliminate the unknown effects of long-term CaMKII knockdown. Although this manipulation would preserve CaMKII binding interactions, it will be important to determine if this mutant disrupts $mGlu_5$ association with other regulating proteins.

This work on $mGlu_5$, CaMKII, and other neuronal regulators has added to the evidence supporting the importance of proper synaptic signaling complexes. These studies also enhance our understanding of the cellular responses associated with $mGlu_5$ activation. The interactions of multiple proteins regulating CaMKII-dependent processes that will necessitate the development of new tools to explore the role of individual protein contribution to proper neuronal signaling and

function. While there is good evidence that modulation of $mGlu_5$ could be helpful to many neuronal disorders, some drugs have lacked efficacy in clinical trials (Scharf et al., 2015). Understanding the full pharmacological impact of $mGlu_5$ ligands may help in the development of more efficacious medical interventions with fewer adverse effects or greater efficacy.

Differences between $mGlu_5$ isoforms

Another exciting aspect of this project was the identification of previously unknown differences between $mGlu_{5a}$ and $mGlu_{5b}$. There is a clear developmental regulation of $mGlu_5$ isoforms. $mGlu_{5a}$ is most highly expressed in early postnatal development, and $mGlu_{5b}$, which contains a 32 amino acid CTD insert is the dominant variant in adulthood (Romano et al., 1996). Little is known about functional differences between these two receptors. One study demonstrated that $mGlu_{5a}$ hinders the acquisition of mature neuronal traits and $mGlu_{5b}$ promotes the elaboration and extension of neurites (Mion et al., 2001). Studies have shown comparable pharmacological profiles of these splice variants. Differential regulation of $mGlu_5$ variants by CaMKII could provide a better understanding of developmental changes in synaptic plasticity mechanisms. Studies have shown that LFS in rats P9-12 produces an NMDAR and $mGlu_5$ LTD that switches to $mGlu_5$ receptor dependent LTP in older animals (Lante et al., 2006). DHPG treatment in rats P8-15 showed a presynaptically induced form of LTD independent of protein synthesis that changed in older rats (P21-35) to a form of LTD dependent on AMPAR endocytosis and protein synthesis (Nosyreva and Huber, 2005). The study showing the role of CaMKII in DHPG mediated LTD were performed in young adult mice (P42-70) (Mockett et al., 2011). Changes in Ca^{2+} responses also occur throughout postnatal development in the central nucleus of the inferior colliculus where larger Ca^{2+} responses were seen at P6 and lower peak plateau responses were see after P13 (Martinez-Galan

et al., 2012). These changes correlate with the switch of the $mGlu_{5a/b}$ isoforms and regulation of CaMKII could explain some aspects of these changes.

We identified different sites of phosphorylation by CaMKII within both $mGlu_5$ isoforms, but also established that $mGlu_{5a}$ phosphorylation by CaMKII was much more potently inhibited by the presence of CaM than $mGlu_{5b}$. I would hypothesize that the $mGlu_{5b}$ specific sequence in the $mGlu_5$-CTD results in a separation of the CaM and CaMKII binding sites on the $mGlu_5$-CTD, resulting in differential regulation of $mGlu_{5b}$ (Figure 8.2). $mGlu_{5a}$ binding to CaM, α-actinin, and CaMKII may have similar effects in $mGlu_{5a}$, but I would predict that the separation of these binding sites can cause differences in the CaMKII regulation of $mGlu_{5b}$.

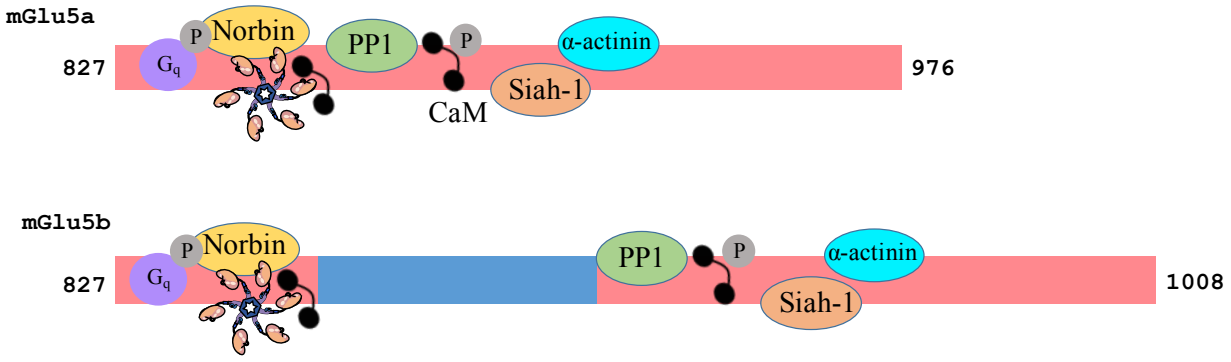

Figure 8.2 *$mGlu_{5a/b}$ specific residues may affect regulation by mGlu5 binding partners.* Schematic demonstrating how the $mGlu_{5b}$-specific residues are capable of separating protein binding sites. $mGlu_5$ has two CaM binding sites separated by the $mGlu_{5b}$ specific insert. The C-terminal CaM binding site is agreed upon, but there is some debate about if the N-terminal binding site exists. The first paper characterizing these two sites did show that the C-terminal CaM site has a higher binding affinity (Minakami et al., 1997). This explains why the CaMKII phosphorylation is more potently inhibited in $mGlu_{5a}$ than in $mGlu_{5b}$.

To study this, I propose first looking at the interaction between CaMKII and $mGlu_5$ throughout

development. The use of younger mice will bias the interaction of CaMKII with $mGlu_{5a}$ while older mice will reveal more information about interactions of CaMKII with $mGlu_{5b}$. In our studies we have routinely used mice of P30-60. Using tissue homogenates fractionated into cytosolic, extra-synaptic membrane proteins, and synaptic proteins (as in Figure 7.1), future studies could include a co-immunoprecipitation using a $mGlu_5$ or CaMKII antibody and subsequent immunoblot analysis for CaMKII and $mGlu_5$. I would predict that although $mGlu_5$ expression is reduced in older mice, that there will be a higher ratio of CaMKII associated with $mGlu_5$ in older mice expressing higher levels of $mGlu_{5b}$.

I also propose using slice pharmacology in acutely isolated brain slices from WT and $mGlu_5$-null mice to compare effects of $mGlu_5$ activation on Thr286 autophosphorylation, CaMKII association with $mGlu_5$, NMDARs and phosphorylation of multiple physiologically relevant downstream substrates such as AMPARs across multiple age ranges. These tissues can also be used to look for ERK and mTOR activation by immunoblotting for phosphorylation of ERK1/2 at Thr202/Tyr204, mTOR at Ser2448, and phosphorylation of the transcription factor CREB at Ser133 which has been shown to transmit $mGlu_5$ signaling to target DNA transcription (Mao et al., 2008a). $mGlu_5$-KO slices can be used as a control to confirm the specificity of our pharmacological manipulations.

Although studies have shown that CaMKII inhibition reduces $mGlu_5$-LTD, these studies used CaMKII inhibitors KN62 and KN93 (Mockett et al., 2011). These inhibitors are peptides based on the autoinhibitory region of CaMKII, but these drugs cannot discriminate between CaMKII and CaMKIV, have off-target effects on voltage-gated K^+ and Ca^{2+} channels, and do not inhibit autonomous activity of the kinase (Pellicena and Schulman, 2014). I propose performing DHPG-induced LTD in the presence of a cell-permeable inhibitor tatCN-21, a CaMKII inhibitor that has

no known off-target effects to confirm the result that CaMKII does play a role in DHPG-induced LTD. I would predict that blockade of CaMKII in DHPG-induced LTD will have bigger effects in mice >P15 compared to mice P9-12. These studies will reveal the role of CaMKII in regulating $mGlu_{5a}$ vs $mGlu_{5b}$ signaling. It will also be important to perform single-cell Ca^{2+} imaging with a construct of $mGlu_{5b}$. We could perform these studies in parallel with cells transfected with $mGlu_{5a}$ to determine differences in $mGlu_5$ isoform regulation by CaMKII.

We have begun to look at Ca^{2+} signals in cultured hippocampal neurons, but it is also possible to perform these types of experiments in brain slices. If we used acutely isolated brain slices we could then use genetic manipulations already available in our lab such as $mGlu_5$ KO mice, CaMKII KO mice, and Thr286A-KI mice, in which the mutation of CaMKIIα Thr286 to Ala abrogates Thr286 autophosphorylation, preventing autonomous kinase activity and reducing synaptic targeting of CaMKIIα. We could also use genetically encoded GCaMP to target and measure changes in specific cell populations. Oscillations in intracellular Ca^{2+}concentrations mediated through $mGlu_5$ play a critical role in neuronal development in the neocortex (Flint et al., 1999). We could examine how acute inhibition of CaMKII or use of Thr286Ala-KI alters $mGlu_5$ responses across different age ranges.

$mGlu_5$ control of CaMKII activation

In this dissertation I provide results that support the idea that $mGlu_5$ can suppress CaMKII activation at low levels of intracellular Ca^{2+}. These findings include an enhancement of CaMKII activation in $mGlu_5$ KO mice, a reduction in CaMKII autophosphorylation after $mGlu_5$ activation in acute brain slices, and the ability of $mGlu_5$ to enhance CaMKII cooperativity. Thus, $mGlu_5$ may suppress CaMKII activity under basal conditions (low Ca^{2+}/CaM), and enhance switch-like

activation in response to intracellular Ca^{2+} increases. Such a mechanism could enhance the switch-like responsiveness of the pool of CaMKII bound to $mGlu_5$ in cells.

CaM is important in the induction of synaptic plasticity because of its ability to translate Ca^{2+} signals and regulate different binding proteins including a number of enzymes and receptors (Xia and Storm, 2005). Evidence has suggested that smaller increases in Ca^{2+} work through CaM to activate CaN and lead to LTD, and higher Ca^{2+} concentrations lead to LTP through CaMKII activation and increases in dendritic protein synthesis (Li et al., 2012). However, other studies have shown activated CaMKII can promote both LTP and LTD (Pi et al., 2010), suggesting that the control of these processes are much more complicated than the simple activation of singular protein kinases and phosphatases.

Although there are high concentrations of CaM within the cell, the immense number of CaM binding proteins renders the total CaM concentration significantly less than that its partners and creates competition for CaM (Sanabria et al., 2008). Both the frequency and amplitude of Ca^{2+} signals can control how CaM binds to its many partners. Some proteins, such as neurogranin, are capable of binding CaM in its Ca^{2+} free state (apoCaM) and these proteins are thought to localize CaM to the synapse for proper control and targeting of CaM once Ca^{2+} has entered the cell (Romano et al., 2017; Xia and Storm, 2005). Increases in intracellular Ca^{2+} cause release of CaM from neurogranin. The role of neurogranin in CaMKII regulation has been recently demonstrated because neurogranin KO mice show reduced levels of CaMKII autophosphorylation and activity. This is predicted to be because of reduced CaM docking in the synapse (Pak et al., 2000; Zhong and Gerges, 2010). Additionally, neurogranin overexpression increases synaptic concentrations of

CaM and lowers the threshold of Ca^{2+} signaling and causes increases in synaptic strength (Zhong et al., 2009).

Activation of mGlu receptors and PKC activation leads to phosphorylation of neurogranin, releasing apoCaM for targeting to neuronal substrates (Ramakers et al., 1997). This unique interaction may explain a way that neurogranin can specifically integrate the $mGlu_5$ signal to CaM sensitive processes including activation of the CaM-dependent CaN and CaMKII.

NMDAR activation can enhance $mGlu_5$ receptor-mediated IP_3 production through $mGlu_5$ dephosphorylation by CaN (Alagarsamy et al., 1999). CaN has also been shown to bind to the $mGlu_5$-CTD (Alagarsamy et al., 2005). The ability of CaM and CaN to bind $mGlu_5$ may play a role in precise control of CaN regulation of this process.

We showed that $mGlu_5$ activation in acutely isolated brain slices resulted in a reduction in CaMKII autophosphorylation at Thr286 in both the hippocampus and the striatum. Other reports showing the effect of $mGlu_5$ activation on CaMKII autophosphorylation have not been consistent. One study saw a quick reduction CaMKII autophosphorylation in hippocampal synaptoneurosomes treated with DHPG, followed by a biphasic increase in CaMKII (Mockett et al., 2011) while another showed increases in CaMKII activation after DHPG application in striatal brain slices (Jin et al., 2013a). Our studies show in the hippocampus and the striatum that $mGlu_5$ activation reduces CaMKII autophosphorylation at Thr286. Currently, we are unable to explain the difference in these findings, but they may be due to differences in experimental design. It is also possible these mechanisms are different across development as discussed in the previous section. We saw dramatic increases in CaMKII autophosphorylation in our $mGlu_5$-KO mice at an age range encompassing P30-60, but it will be interesting to see how autophosphorylation of CaMKII at

Thr286 in the brains of $mGlu_5$ KO mice relative to WT mice more systematically across development.

When reviewing the literature, I was able to identify other models of neuronal disorders that caused large increases in CaMKII autophosphorylation including Fragile X Syndrome (Guo et al., 2015) (Table 8.1). Another example of CaMKII hyperautophosphorylation was examined in ATRX knockout mice. Mutations in the gene encoding ATRX cause mental retardation disorders, including α-thalassemia X-linked mental retardation syndrome. These mice show increased CaMKII activity associated with decreased expression of PP1 (Shioda et al., 2011). This may also give us insight into the role of $mGlu_5$ in controlling basal CaMKII autophosphorylation.

Model	CaMKII Autophos.	Reference
Alzheimer's Disease	Decreased	Min et al., 2013
Angelman's syndrome	Increased	Weeber et al., 2003
ATRX KO	Increased	Shioda et al., 2011
Autism Model E183V CaMKII	Decreased	Stephenson et al., 2017
Fragile X Syndrome	Increased	Guo et al., 2015
mGlu5 KO mice	Increased	Figure X.X
Neurogranin KO Mice	Decreased	Pak et al., 2000
Parkinson's Disease	Increased	Picconi et al., 2004

Table 8.1 *Aberrant CaMKII autophosphorylation in models of disease.* Examples of rodent models of disease and genetic protein deletions that cause deleterious effects with reported changes in CaMKII autophosphorylation. Models of neuronal disorder are associated with both increased and decreased CaMKII activation.

Interactions have been demonstrated between $mGlu_5$ and PP1 at $mGlu_5$ residues 880-884 (KSVTW) and a physical association with CaN, mentioned earlier in this chapter (Alagarsamy et al., 2005; Croci et al., 2003). Unlike PP1, there is no evidence for dephosphorylation of CaMKII by CaN, but CaN may indirectly modulate CaMKII by enzymes that activate PP1 such as DARP-32 (Colbran, 2004). PP1 dephosphorylation of CaMKII increases CaMKII cooperativity for Ca^{2+}/CaM providing ultrasensitive responsiveness to increasing concentrations (Bradshaw et al., 2003). PP1 has been hypothesized to form a molecular switch controlling synaptic plasticity (Lisman and Zhabotinsky, 2001; Zhabotinsky, 2000). It was proposed the translocation of Thr286-autophosphorylated CaMKII to the PSD causes saturation of local PP1 and allows for sustained Thr286 autophosphorylation. Inhibition of PP1 before stimulation with DHPG enhances $mGlu_5$-mediated ERK2 activation, and this may explain why we see increases in ERK activation in the presence of CA-CaMKII (Voulalas et al., 2005). CaMKII recruitment of Thr286-autophosphorylated CaMKII to $mGlu_5$ may also increase $mGlu_5$ ERK signaling by causing a saturation of local $mGlu_5$-docked PP1.

This emphasizes the importance of the role of the $mGlu_5$-CTD as a docking station of proteins that are capable of modulating its activity including CaMKII and PP1. I would hypothesize that CaMKII docked near $mGlu_5$, perhaps bound to α-actinin is kept in its inactive conformation through dephosphorylation by PP1 docked on $mGlu_5$. This model would support the idea that at low intracellular Ca^{2+} concentrations, $mGlu_5$ is capable of inhibiting CaMKII autophosphorylation.

To test this hypothesis, I propose using acutely isolated brain slices to look at the autophosphorylation status of CaMKII after DHPG treatment in the absence or presence of PP1

inhibitors in WT and $mGlu_5$ KO mice. Where we previously saw a decrease in Thr286 autophosphorylation I would suspect to see increases in kinase activation in the presence of PP1 inhibition. Future directions could also use alanine mutagenesis of the PP1 binding site on $mGlu_5$ residues 880-884 (KSVTW) to determine the role of PP1 docking on regulation of CaMKII activity. Interestingly, the $mGlu_{5b}$ specific sequence separates the CaMKII binding site from the PP1 binding site. If PP1 does cause dephosphorylation of CaMKII near $mGlu_5$, this effect may be bigger in the context of $mGlu_{5a}$ than in $mGlu_{5b}$ due to a spatial restriction imposed by the $mGlu_{5b}$ specific sequences.

Other proteins have been shown to cause increased cooperativity or Ca^{2+}-independent activation of CaMKII. For instance, CaMKII binding to $Ca_V2.1$ channels induces Ca^{2+}-independent activity of the kinase, which induces CaMKII autophosphorylation and phosphorylation of the neuronal substrate synapsin-1 (Magupalli et al., 2013). Because multiple specific dynamic interactions with individual PSD proteins regulate CaMKII activity-dependent targeting and its role in synaptic structure, tools that block or enhance CaMKII binding to individual proteins are now necessary to explore the individual roles of these interactions in disease and for the development of therapeutics.

Previously, I discussed the effect of CaMKII in $mGlu_5$-mediated Ca^{2+} amplitude and duration. This effect likely translates to differential activation of Ca^{2+}-sensitive proteins. This promotes the specificity of Ca^{2+} signals where small elevations in cytosolic Ca^{2+} can activate high affinity sensors, but reduce the activation of a low-affinity Ca^{2+} sensors (or sensors with a greater activation threshold). In the $mGlu_5$ complex it seems that multiple Ca^{2+} sensors could be present including CaM, CaMKII, and CaN. A complex harboring a number of Ca^{2+}-binding molecules within close

proximity presents a situation where sensors with range of Ca^{2+} sensitivities are docked and ready to respond based on the amplitude of cellular Ca^{2+} responses.

Final summary statement

The work presented in this dissertation has highlighted the importance of the CaMKII interaction with the $mGlu_5$-CTD in heterologous cells, cultured neurons, and mouse brains.

By defining a CaMKII binding domain on $mGlu_5$ and identifying possible phosphorylation sites on the $mGlu_5$-CTD, I have generated new tools to continue studies surrounding this interaction.

We have begun to study the importance of this interaction in cultured neurons to understand the interaction of $mGlu_5$ and CaMKII in a synaptic context. These studies will allow us to understand the mechanisms important to proper regulation of synaptic activity. This work has laid the groundwork for further exploration of the role of CaMKII in regulating synaptic macro-molecular complexes that integrate Ca^{2+} signals into functional changes in synaptic strength.

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