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The Role of Ca2+/Calmodulin Dependent Protein Kinase II Alpha in Group 1 Metabotropic Glutamate Receptor Endocytosis and Signalling

Stephanie C. Kulhawy
The University of Western Ontario

Supervisor
Dr. Stephen Ferguson
The University of Western Ontario

Graduate Program in Physiology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

THE ROLE OF Ca^{2+}/CALMODULIN DEPENDENT PROTEIN KINASE II ALPHA IN GROUP 1 METABOTROPIC GLUTAMATE RECEPTOR ENDOCYTOSIS AND SIGNALLING

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Stephanie Chanel Kulhawy

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THE UNIVERSITY OF WESTERN ONTARIO
School of Graduate and Postdoctoral Studies

CERTIFICATE OF EXAMINATION

Supervisor

_____
Dr. Stephen Ferguson

Supervisory Committee

_____
Dr. Sean Cregan

_____
Dr. John MacDonald

_____
Dr. Nica Borradaile

Examiners

_____
Dr. John DiGuglielmo

_____
Dr. Rob Gros

_____
Dr. Greg Kelly

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ABSTRACT

Group 1 metabotropic glutamate receptors (mGluR1 and mGluR5) are G-protein coupled receptors (GPCRs) activated by glutamate. mGluR1/5 couples to $G\alpha_{q/11}$ and releases Ca^{2+} from the endoplasmic reticulum. Ca^{2+}/calmodulin-dependent protein kinase II alpha (CaMKIIα) can be activated by $G\alpha_{q/11}$-mediated Ca^{2+} release through binding of Ca^{2+}/calmodulin. Results from a proteomic screen identified CaMKII as a novel mGluR-interacting protein. Therefore, we hypothesized that CaMKIIα associates with group 1 mGluRs and this association alters mGluR1/5 signalling and internalization. Firstly, we demonstrated the novel association between CaMKIIα and mGluR1/5 by co-immunoprecipitation of transiently transfected proteins in HEK293 cells and of endogenous proteins in mouse hippocampal tissue. Next, we showed that the second intracellular loop of the mGluR1a receptor is sufficient for this association. Furthermore, CaMKIIα significantly enhances agonist-induced internalization of group 1 mGluRs. Yet, it does not appear that CaMKIIα plays a significant role in receptor signalling by either ERK1/2 phosphorylation or inositol phosphosphate formation. Both CaMKIIα and mGluR1/5 play an important role in memory, learning and synaptic transmission. Understanding how these two players work together could provide a mechanism for reducing excitotoxicity through desensitization of mGluR1/5 by CaMKIIα.

KEYWORDS: G protein-coupled receptor, metabotropic glutamate receptor, Ca^{2+}/calmodulin dependent protein kinase II alpha, endocytosis, extracellular signal-regulated kinase, inositol phosphate formation

CO-AUTHORSHIP

All experiments were performed by myself, expect for some of the inositol phosphate formation experiments, which were completed by Dr. Christie Godin. Experiments in Figure 3.6C and two experiments in Figure 3.6A were completed by Dr. Godin. As well, all cell surface expression as determined by flow cytometry was done with the assistance of Dr. Godin. All experiments were performed in the laboratory of Dr. Stephen Ferguson at the Robarts Research Institute, Western University.

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
AMPA	Alpha-amino-3-hydroxy-5-methyl-4- isoxazole-propionic acid
CaMKII	Calcium/calmodulin dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
DAG	Diacylglycerol
DHPG	(S)-3,5-Dihydroxyphenylglycine
ECL	Enhanced chemiluminescence
ERK	Extracellular signal-regulated kinase
FMRP	Fragile X mental retardation protein
G Protein	Guanine nucleotide-binding proteins
GPCR	G protein coupled receptors
GRK	G protein-coupled receptor kinase
HBS	HEPES-buffered saline
HBSS	HEPES-balanced salt solution
HEK	Human embryonic kidney
IL	Intracellular loop
IP3	Inositol 1,4,5-trisphosphate
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptor
MS	Multiple sclerosis
NMDA	N-Methyl-D-aspartic acid
PBS	Phosphate buffered saline
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	cAMP dependent protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
PSD	Post synaptic density
Pyk2	Proline-rich tyrosine kinase 2
RalGDS	Ral guanine nucleotide dissociation stimulator
RGS	Regulators of G protein signalling
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Short hairpin ribonucleic acid

CHAPTER 1

INTRODUCTION

1.1 G protein-coupled receptors

G protein-coupled receptors (GPCRs) are seven transmembrane (7TM) embedded receptors that respond to a wide variety of stimuli: odor, taste, light, hormones and neurotransmitters. These extracellular signals are relayed by coupling of GPCRs to heterotrimeric guanine nucleotide-binding proteins (G proteins). Activated G proteins then mediate downstream effector pathways through second messengers. GPCRs are the target of over 50% of all prescription drugs and yet only approximately 4% of GPCRs are currently targeted (Tyndall Jd and Sandilya R, 2005). Therefore, GPCRs are a key area of research for expanding the understanding and treatment of diseases. There are six subfamilies within the GPCR superfamily: Class A of rhodopsin-like receptors; Class B includes secretin receptors; Class C, also called glutamate family, includes metabotoropic glutamate receptors (mGluR), $GABA_B$ and Ca^{2+} sensing receptors; Class D of pheromone receptors; Class E of cAMP receptors and Class F of frizzled and smoothened receptors (Kolakowski; Lagerström and Schiöth, 2008). Class C receptors and specifically mGluRs will be the focus of this thesis. This class of receptors bears little sequence homology and is structurally distinct from prototypic GPCRs (Chun et al., 2012).

1.2 Metabotropic glutamate receptors

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS). It functions in learning and memory as well as neurodegenerative disorders (Fonnum, 1984; Lau and Tymianski, 2010; Nakanishi, 1992). Glutamate signals are received at the post-synaptic membrane by two types of receptors: ionotropic and metabotropic. N-methyl-D-aspartate receptor (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), and kainate receptors are all ionotropic receptors, which respond to agonist stimulation by opening cation channels. mGluRs are GPCRs that mediate changes in the post-synaptic cell through second messenger signalling pathways (Figure1.1) (Conn and Pin, 1997; Nakanishi, 1992).

There are eight mGluRs, which are grouped into three subclassifications by sequence homology and G protein coupling (Conn and Pin, 1997; Ferraguti and Shigemoto, 2006). Group 1 mGluRs (mGluRs 1 and 5) activate phospholipase Cβ through coupling to $G\alpha_{q/11}$, while groups 2 and 3 mGluRs (mGluRs 2 and 3; mGluRs 4,6,7, and 8 respectively) negatively regulate adenylyl cyclase though coupling to $G\alpha_{i/o}$. It is group 1 mGluRs, which will be the focus of this project. mGluR5 was discovered and characterized after mGluR1 (Abe et al., 1992). At this time, it was grouped together with mGluR1 because of its similarities in sequences and ligand specificity. These receptors are endogenously activated by glutamate or synthetic analog quisqualate and are specifically activated by (S)-3,5-dihydroxyphenylglycine (DHPG). Agonist activation of group 1 mGluRs signals through $G\alpha_{q/11}$ to mediate intracellular Ca^{2+} release (Mizuno and Itoh, 2009).

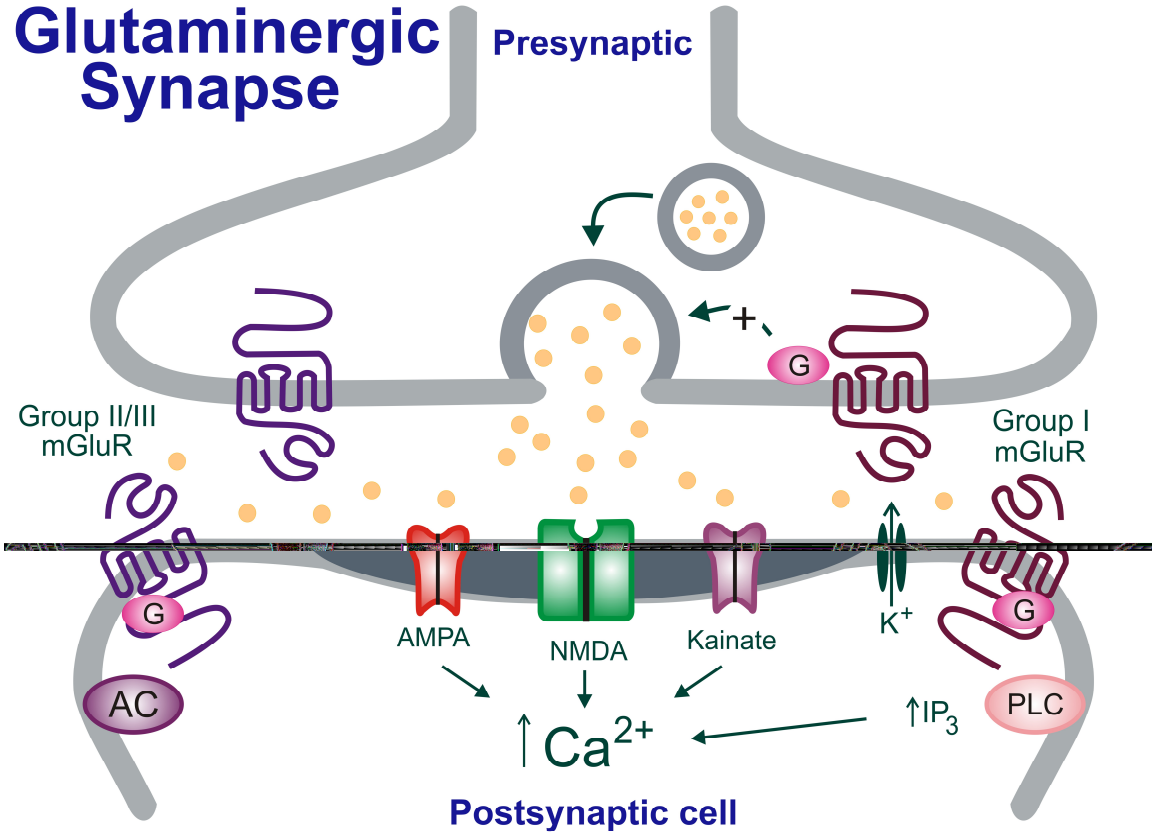

Figure 1.1. Glutamate receptor-mediated calcium release at the synapse. Glutamate interacts with both ionotropic glutamate receptors (iGluRs), NMDAR, AMPAR and Kainite-R, as well as group 1 metabotropic glutamate receptors (mGluRs), mGluR1 and mGluR5, to mediate increased intracellular Ca^{2+} upon glutamate stimulation. iGluRs respond to agonist stimulation through the opening of cation channels. Extracellular Ca^{2+} then flows according to its gradient into the cell through these channels. mGluR1/5 couples to $G\alpha_{q/11}$, which stimulates phospholipase Cβ to hydrolyze phophatydylinositol bisphosphate into second messengers: diacylglyceral (DAG) and inositol-1,4,5-trisphosphate (IP3). IP3 releases Ca^{2+} from endoplasmic reticulum by activating the IP3 receptor. (Dhami and Ferguson, 2006)

1.2.1 Structure and functional domains

There are four known splice variants of mGluR1 (a, b, c, and d) and two known of mGluR5 (a and b). mGluR1a has the longest carboxyl-terminal tail (318 amino acids), while mGluR1b, 1c, and 1d have shorter tails (20, 11 and 26 amino acids in length) (Conn and Pin, 1997; Pin et al., 1992; Tanabe et al., 1992). Therefore, mGluR1a is more efficient in coupling with $G\alpha_{q/11}$ as compared to the shorter carboxyl-terminal tail variants (Pin et al., 1992; Prézeau et al., 1996). mGluR5 variants also vary in C-terminal tail length 5a with 32 amino acids and 5b with 50 amino acids (Joly et al., 1995). My project focuses on mGluR1a and mGluR5a.

Unlike prototypic GPCR agonists, glutamate, is not bound in a pocket formed by the 7TMs, but instead by the extracellular N-terminal domain, which makes a 'clam-shell' like shape, also known as the venus fly trap model (Kunishima et al., 2000; Niswender and Conn, 2010). This large extracellular domain is also required for receptor dimerization (Beqollari and Kammermeier, 2010). More specifically, the receptors form a homodimer through covalent linkage at the receptor's Cys 140 residue. Group 1 mGluRs, especially mGluR1a, have a large intracellular C-terminal tail domain important for receptor scaffolding as it contains the Homer binding domain and the PDZ binding domain (reviewed in (Enz, 2012; Magalhaes et al., 2012; Ritter and Hall, 2009).

For prototypic GPCRs, the third intracellular loop (IL-3) is important for G protein selectivity (Blüml et al., 1994). However, the IL-3 region of mGluRs is highly conserved and, therefore, not likely responsible for G protein selectivity. Instead, the IL-2 has been implicated as a key player in G protein selectivity because it is highly variable among subtypes of mGluRs, thus allowing for different G protein selectivity among subtypes

(Gomeza et al., 1996; Niswender and Conn, 2010). Furthermore, experimental evidence has confirmed that the IL-2 of the mGluR1/5 is involved in G protein coupling (along with the IL-3 and C-terminal tail) (Francesconi and Duvoisin, 1998; Hermans and Challiss, 2001). Francesconi and Duvoisin (1998) isolated specific domains in the IL-2 that were important for $G\alpha_q$ selectivity (Thr 695, Lys 697 and Ser 702) and $G\alpha_s$ selectivity (Pro 698, Cys 694-Thr 695). Our lab has also shown that the IL-2 interacts with CAIN (Ferreira et al., 2009), Pyk2 (Nicodemo et al., 2010) and is also the primary binding site of GRK2 (Dhami et al., 2004).

1.2.2 Cellular and subcellular distribution

Group 1 mGluRs, mGluR1 and mGluR5, have distinct expression patterns in the CNS, which yields an anatomical basis for their divergent functions (see Ferraguti and Shigemoto (2006) for a review). mGluR5 is highly expressed throughout the hippocampus, especially in the CA1 and CA3 pyramidal cells and the granular cells of the dentate gyrus (Shigemoto et al., 1997). Some isoforms of mGluR1 are expressed in the CA3 pyramidal cells and the granular cells of the dentate gyrus. However, mGluR1a is expressed mostly in the CA1 interneurons of the hippocampus (Shigemoto et al., 1997) and is essential for long-term potentiation initiation in these interneurons (Lapointe et al., 2004; Perez et al., 2001). mGluR1a is highly expressed in the Purkinje cells of the cerebellar cortex, where mGluR1a is required for long-term depression (LTD) and motor coordination (Ichise et al., 2000). Comparatively, mGluR5 is not expressed in the Purkinje cells and is expressed only in a small portion of the Golgi cells in the cerebellar cortex (Négyessy et al., 1997). Expression of mGluR5 is much higher than mGluR1 in both the cortex (Romano et al., 1995) and the striatum (Ribeiro et al., 2010). Research from our lab suggested that

mGluR5 desensitization in the striatum plays a neuroprotective role in the early, asymptomatic phase of Huntington's disease and a neurotoxic role in later stages of the disease (Ribeiro et al., 2010; Ribeiro et al., 2011). In general, mGluRs are expressed in the neuronal cells of the CNS, however some expression of mGluR5 has been found in astrocytes (Balázs et al., 1997).

At the synapse, group 1 mGluRs are located predominately post-synaptic just outside of the post-synaptic density (López-Bendito et al., 2002; Lujan R., 1996; Shigemoto et al., 1997). This makes mGluR1/5 particularly attractive for the study of post-synaptic modification and synaptic plasticity. Overall, group 1 mGluRs are usually localized to somatodendritic regions of neurons. However, this expression pattern is altered in multiple sclerosis (MS) (Geurts et al., 2003). Geurts et al. revealed heightened mGluR1a expression in neuronal axons both in lesions and in normal appearing white matter of MS brains, suggesting a possible role for mGluRs in MS pathology. Localization of mGluRs in the CNS is important for understanding their role in normal neuronal functioning as well as the aberrant localization that can contribute to disease pathology.

1.2.3 G protein coupling and effector signalling

Group 1 mGluRs are coupled predominately to $G\alpha_{q/11}$, which will be the focus of this thesis ($G\alpha_{q/11}$ signalling is reviewed in Mizuno and Itoh (2009)). mGluR1a can also couple to other G proteins ($G\alpha_s$ and $G\alpha_{i/o}$) and stimulate adenylate cyclase, which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (Francesconi and Duvoisin, 1998); conversely, mGluR5a does not stimulate the cAMP pathway (Abe et al., 1992). Agonist binding to mGluR1/5 stabilizes the receptor conformation that promotes the exchange of GDP to GTP on the Gα subunit of the

heterotrimeric G protein. This allows Gα-GTP and Gβγ subunits to dissociate and activate effector enzymes. $G\alpha_{q/11}$ activates phospholipase Cβ (PLC-β) to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) producing two second messengers: diacylglyceral (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG activates protein kinase C (PKC) and IP3 releases Ca^{2+} from the IP3-regulated intracellular stores such as the endoplasmic reticulum. Group 1 mGluR coupling to heterotrimeric $G\alpha_{q/11}$ activates effector proteins such as protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), extracellular signal-regulated kinase (ERK), and proline-rich tyrosine kinase 2 (Pyk2) (Choe and Wang, 2001; Mockett et al., 2011; Nicodemo et al., 2010).

Receptor subtypes express different signal patterning, which can lead to divergent downstream signalling of mGluR-1 and -5. Oscillatory mGluR1/5 coupling to $G\alpha_{q/11}$ and activation of PLC causes oscillations in IP formation, Ca^{2+}, and PKC activation (Dale et al., 2001a; Kawabata et al., 1998). Both Ca^{2+} and PKC oscillations are distinct between mGluR1 and mGluR5. mGluR1a-mediated Ca^{2+} oscillations are lower in frequency when compared to mGluR5a oscillations (Kawabata et al., 1998). Oscillations of Ca^{2+} are important for activation of downstream effector proteins such as CaMKII (Bayer et al., 2002; Chao et al., 2011; Koninck, 1998) and PKC. PKC oscillations are distinct between receptor subtypes mGluR1a and -5a. A single residue in the G protein-coupling domain regulates this receptor subtype specific pattern of PKC oscillation (Dale et al., 2001a).

1.2.4 Activation of mitogen-activated protein kinases

Activation of group 1 mGluRs stimulates the mitogen-activated protein kinase (MAPK) pathway specifically the phosphorylation of extracellular regulated kinase 1 and 2 (ERK1/2) (Choe and Wang, 2001; Ferraguti et al., 1999; Karim et al., 2001). Once

activated, EKR1/2 translocates to the nucleus where it facilitates gene expression through regulation of specific transcription factors, thereby effecting long-term changes in the CNS at the cellular level. ERK1/2 activation can promote cell protection or cell death pathways depending on the level and duration of stimulation (reviewed in (Agell et al., 2002; Mebratu and Tesfaigzi, 2009). There is still much to discover about mGluR-mediated ERK1/2 phosphorylation. To date there are a number of molecular pathways that have been shown to contribute to mGluR-mediated ERK1/2 phosphorylation (reviewed in Wang et al. (2007)). mGluR-mediated activation can occur via by both G protein-dependent and -independent mechanisms. In terms of G protein-dependent mechanism, studies have found that mGluR1a (Ferraguti et al., 1999) and mGluR5 (Chen et al., 2012) activate ERK in a PKC dependent manner. Conversely, Mao et al. (2005) found that IP3 and Ca^{2+} and not DAG and PKC were important for some forms of ERK activation by mGluR5. In addition to this G protein-dependent mechanism, Mao et al. goes on to describe a second mechanism that accounts for a greater amount of ERK1/2 activation. This mechanism is G protein-independent and requires Homer1b/c (Mao et al., 2005). In addition, mGluRs appear to signal through receptor tyrosine kinases as well as non-receptor tyrosine kinases (reviewed in Wang et al. (2007)). Our lab found that Pyk2, a non-receptor tyrosine kinase, associates with mGluR1a's second intracellular loop and facilitates ERK1/2 phosphorylation (Nicodemo et al., 2010). Pyk2 activates ERK1/2 in a PKC-, calmodulin- and Src-dependent manner. Emery et al. (2010) described a G protein- and PLC-independent mechanism of ERK phosphorylation by mGluR1a. This requires β-arrestin-1 and Dynamin, which suggests there may be a role for the internalization of mGluR1 by β-arrestin in ERK activation. This mechanism produces sustained ERK phosphorylation,

compared to a transient form accounted for by G protein-dependent mechanisms. Furthermore, this group elucidated a ligand bias whereby glutamate, not quisqualate nor DHPG, activates the G protein-independent ERK activation pathway (Emery et al., 2012; Emery et al., 2010). Emery suggests that it is this sustained ERK activation and not the transient G protein-dependent ERK activation that has a neuroprotective quality. Moreover, ERK1/2 activation is required for mGluR-mediated LTD. ERK1/2 regulates the initiation of protein translation in many cell types and is thus thought to play a central role in expression of mGluR-mediated LTD through protein translation (Gallagher et al., 2004; Volk et al., 2006).

1.3 GPCR desensitization and endocytosis

Receptor desensitization is a protective mechanism from over stimulation and potential neuronal death by excitotoxicity. Neurodegenerative diseases such as Amyotrophic Lateral Sclerosis, Alzheimer's Disease, Multiple Sclerosis and Huntinton's Disease are all mediated in part by excitotoxicity (D'Antoni et al., 2011; Geurts et al., 2003; Ribeiro et al., 2010). That desensitization is a naturally occurring neuroprotective mechanism makes it an exciting field of study for development of disease treatment. Desensitization is a feedback mechanism whereby excessive acute or chronic over stimulation leads to a reduced receptor response over time. The mechanism of GPCR desensitization can occur within seconds of agonist stimulation beginning with phosphorylation of the receptor, within minutes the receptor can internalize and within hours it can be down regulated. The extent of receptor desensitization depends on the level and duration of agonist stimulation as well as fine-tuning by receptor interacting proteins (reviewed in Ferguson, 2001; Kelly et al., 2008).

The prototypic model of GPCR desensitization (Figure 1.2) begins with receptor phosphorylation, which promotes uncoupling of the G protein from the receptor. Serine and threonine residues within the intracellular loops and C-terminal tail are phosphorylated. This receptor phosphorylation can be accomplished by second messenger-dependent protein kinases, protein kinase C (PKC) and cAMP-dependent protein kinase (PKA), or G protein-coupled receptor kinases (GRKs) (Benovic et al., 1985; Benovic et al., 1986). Homologous (agonist-dependent) desensitization refers to reduced response from the stimulated receptor, whereas heterologous (agonist-independent) desensitization occurs when the stimulation of one receptor leads to the reduced response in another receptor. Second messenger-dependent protein kinases phosphorylate receptors regardless of receptor conformation and can contribute to both agonist-dependent and -independent desensitization (Clark et al., 1988; Kelly et al., 2008). Conversely, GRKs selectively phosphorylate receptors in the agonist-activated conformation, and are implicated in homologous desensitization (Benovic et al., 1986). β-arrestins preferentially bind to GRK-phosphorylated receptors more so than unphosphorylated or second messenger phosphorylated (Ferguson et al., 1996; Lohse et al., 1992). Binding of β-arrestins furthers desensitization in two main ways: it sterically uncouples the receptor from the G protein and promotes internalization by recruiting clathrin and β2-adaptin, which facilitates endocytosis by clathrin-coated pits (Zhang et al., 1996). Internalized receptors then undergo resensitization or down regulation. Resensitized receptors are dephosphorylated and recycled back to the cell surface. Down regulation involves the reduction of the cell's receptor inventory through proteolytic degradation of existing receptors and reduced gene expression (Ferguson, 2001; Kelly et al., 2008; Ritter and Hall, 2009).

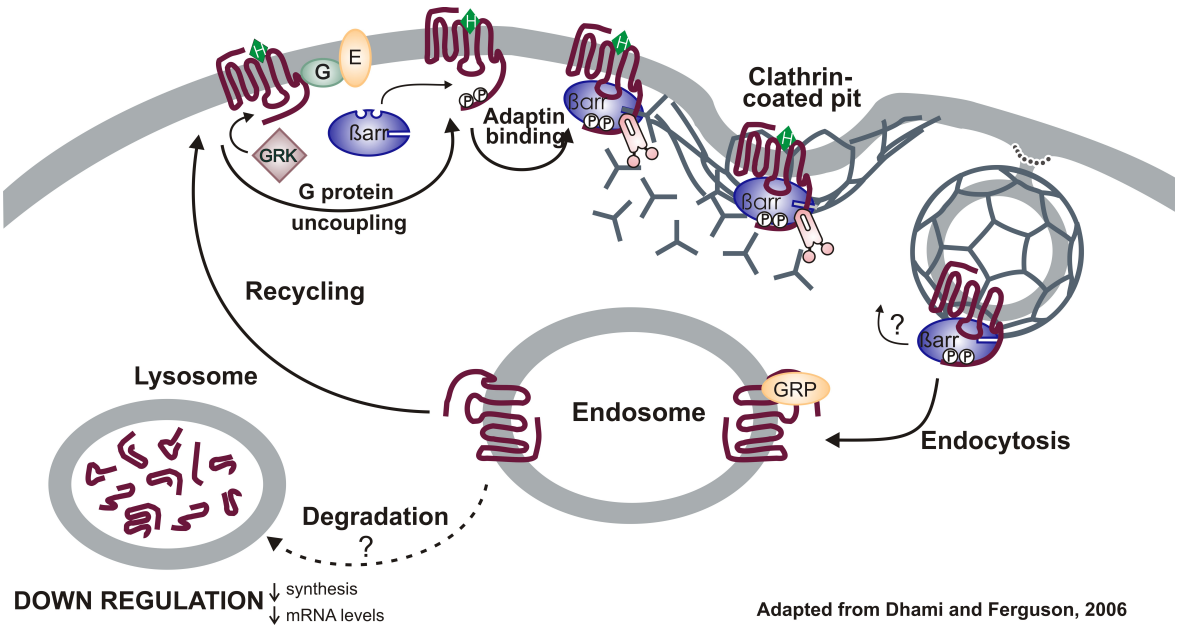

Figure 1.2. Prototypic model of GPCR desensitization. Receptor desensitization begins when the receptor is phosphorylated by second messenger dependent protein kinases, like protein kinase C (PKC), or G protein-coupled Receptor Kinases (GRKs). This is followed by β-arrestin binding, which promotes G protein uncoupling. β-arrestin recruits clathrin and β2-adaptin, facilitating endocytosis by clathrin coated pits. (Ferguson, 2001; Dhami and Ferguson, 2006; Ritter and Hall, 2009)

1.3.1 Group 1 mGluR desensitization and endocytosis

mGluRs are structurally and functionally distinct from other GPCRs. Likewise, the mechanism of mGluR desensitization and endocytosis is also distinct. mGluRs are able to undergo both phosphorylation-dependent and -independent desensitization (reviewed in Dhami and Ferguson (2006)). PKC contributes to desensitization of group 1 mGluRs by direct phosphorylation of the receptor (Gereau and Heinemann, 1998; Herrero et al., 1994; Mundell et al., 2002). For mGluR5, two residues in the C-terminal tail, Ser 881 and Ser 890, seem to be especially important for PKC-mediated desensitization (Gereau and Heinemann, 1998). For mGluR1, PKC-mediated desensitization occurs by phosphorylation of Thr 695 in the second intracellular loop, which disrupts the $G\alpha_{q/11}$ pathway while having no effect on the $G\alpha_{i/o}$ (Francesconi and Duvoisin, 2000). In addition to PKC, Optineuron (Anborgh et al., 2005), PKA (Mundell et al., 2004) and CaMKIIα (Mundell et al., 2002) have been shown to contribute to mGluR desensitization and internalization. Furthermore, many GRK isoforms have been shown to mediate desensitization of mGluR1 (Dale et al., 2000; Sallese et al., 2000). Sallese et al. 2000 found that GRK4 contributed to mGluR1a desensitization and internalization in HEK293 cells and cultured Purkinje cells. Dale et al. (2000) found that in HEK293 cells GRK2 and 5 contributed to mGluR1a desensitization and internalization and that this process can protect against cell death.

GRK2-mediated mGluR1 desensitization departs from the prototypic model of receptor desensitization as it can be mediated via a β-arrestin and phosphorylation independent mechanism. β-arrestin is not required for mGluR1a desensitization; however, it is required for mGluR1a agonist-dependent internalization (Dale et al., 2001b). Phosphorylation-independent desensitization of mGluR1 was demonstrated in our lab by

multiple experimental methods. Expression of a catalytically inactivate mutant of GRK2-K220R still attenuated mGluR1 signalling (Dale et al., 2000). Furthermore, Dhami et al. (2002) used an mGluR1 mutant with a truncated C-terminal tail, which prevented GRK2 phosphorylation but not desensitization. These findings suggest that it is the regulators of G protein signaling (RGS) homology (RH) domain of GRK2 and not the catalytic domain that mediates mGluR-$G\alpha_{q/11}$ uncoupling. GRK2 attenuates receptor signalling by binding the receptor, $G\alpha_{q/11}$ and $G\beta\gamma$ simultaneously (Ferguson, 2007; Tesmer et al., 2005). GRK2 interacts with mGluR1 at the second intracellular loop and the C-terminal tail. Mutation of either amino acid residues Lys 691 and Lys 692 within the second intracellular loop disrupts interaction as well as desensitization of the receptor by GRK2 (Dhami et al., 2005). However, GRK2 regulation of mGluR5a appears to be phosphorylation dependent and that a residue in the C-terminal tail T840 seems to be important for the interaction (Sorensen and Conn, 2003). Phosphorylation-independent desensitization, although not prototypic, is possible for other GPCRs such as follicle-stimulating hormone receptor, $5HT_{1b}$ receptor and the parathyroid hormone receptor (reviewed in Ferguson, 2007).

1.3.2 Constitutive internalization of mGluRs

Group 1 mGluRs constitutively internalize in an agonist, phosphorylation, and GRK2-independent manner (reviewed in Dhami et al., (2006)). There is some conflict in the literature about whether β-arrestin is required for this constitutive internalization. Dale et al. (2001a, b) reported β-arrestin- and dynamin-independent constitutive internalization, whereas Pula et al. (2004) reported a β-arrestin-dependent mechanism. However, both groups agree that constitutive internalization of mGluR1a occurs through clathrin-coated vesicles. The role of clathrin in mGluR5 constitutive internalization is unclear. Fourgeaud

et al. (2003) reported that mGluR5a internalizes in a clathrin-independent mechanism. However, Dale et al. (2001a,b) and Bhattacharya et al. (2004) found significant colocalization of mGluR1a and 5a with clathrin in endocytic vesicles. Together these findings suggest that both clathrin-dependent and -independent mechanism contribute to mGluR1/5 internalization. Bhattacharya et al. (2004) found that Ral (small GTP-binding protein), Ral guanine nucleotide dissociation stimulator (RalGDS) and PLD2 play a role in mGluR1/5 constitutive internalization. RalGDS is common to both agonist-dependent and -independent internalization (Bhattacharya et al., 2002; Bhattacharya et al., 2004). Ral/PLD2 act as an adaptor for constitutive internalization, whereas β-arrestin acts as an adaptor for agonist-stimulated internalization. Recruitment of β-arrestin by Ral-GDS in agonist-independent endocytosis may explain the finding of Pula et al. (2004) of β-arrestin-dependent constitutive internalization. Moreover, group 1 mGluRs can undergo multiple mechanisms of endocytosis, each contributing to the complex role that mGluRs play receptor signalling.

1.4 Regulation of mGluRs by interacting proteins

In addition to proteins that contribute to receptor endocytosis, receptor interaction with regulatory molecules further tunes the complex process of receptor signalling (reviewed in Magalhaes et al. (2012); Ritter and Hall (2009)). RGS proteins increase the GTP hydrolysis rate on the $G\alpha_{i/o}$ and $G\alpha_{q/11}$ subunits of the heterotrimeric G protein complex. This diminishes the signalling capacity of these G protein-regulated signalling pathways (reviewed in Hollinger and Hepler (2002)). More specifically, RGS2 and RGS4 have been implicated in regulation of group 1 mGluRs. RGS2 alters mGluR1a-mediated inhibition of Ca^{2+} currents and M-type potassium currents (Kammermeier and Ikeda,

1999). RGS4 inhibits receptor-mediated ion currents by blocking mGluR1a and mGluR5a-mediated activation of PLCB by $G\alpha_{q/11}$ (Saugstad et al., 1998).

G protein-independent interacting proteins

Scaffolding proteins play an indirect role in GPCR signalling. They facilitate protein-signalling pathways by tethering interacting proteins in close proximity to one another thereby increasing the chances of interaction. Many GPCRs are considered to play a role as scaffolding proteins forming agonist-independent signal transduction complexes, termed 'signalsomes'. mGluR interacting proteins include Homer, calmodulin, PDZ proteins (Tamalin, NHERF-1, NHERF-2, and CAL) (Brakeman et al., 1997; Kitano et al., 2002; Paquet et al., 2006; Ting et al., 2012).

Perhaps the best characterized scaffolding complex for mGluRs is that of Homer, IP3R and Shank. The Homer protein family is encoded by 3 genes (Homer1, Homer2 and Homer3), which yield many Homer isoforms (reviewed in Shiraishi-Yamaguchi and Furuichi (2007)). All Homer isoforms share an N-terminal EVHI domain, which recognizes and binds the mGluR as well as the IP3R. Homer1a is the short-protein form; Homer1b/c, Homer2a/b, Homer3a/b are long-protein forms. Long proteins refer to those who have a C-terminal coiled-coil domain, which is required for multimerization of Homers into tetramers. Only long Homer proteins possess the ability to link proteins, such as mGluR to IP3R. Homer1b proteins bind to mGluR1/5 and IP3R, structurally linking the two proteins together by Homer multimerization (Tu et al., 1998). Homer 1a protein lacks the coiled-coil domain and therefore acts as a dominant negative protein to uncouple mGluR1/5 from the IP3R (Kammermeier, 2008; Kammermeier et al., 2000; Tu et al., 1998). Homer 1a is an activity-induced isoform that is rapidly up regulated following

seizure (Brakeman et al., 1997; Ting et al., 2012). Shank proteins coordinate with Homer proteins to facilitate mGluR1/5 signalling. Shanks crosslink Homer and PSD-95 to cluster mGluR5 at the PSD (Tu et al., 1999). Shank1b and Homer1b facilitate mGluR1/5-mediated Ca^{2+} signalling (Sala et al., 2005) and induce spine maturation and enlargement as well as translocation of IP3R to the PSD (Sala et al., 2001). Homer1a reverses these spine maturation effects (Sala et al., 2003) and also down regulates synaptic AMPARs (Hu et al., 2010). The Homer-Shank complex provides an example whereby scaffolding proteins coordinate signalling in a G protein-independent manner.

1.5 Physiological role of group 1 mGluRs

Group 1 mGluRs are mediators of synaptic plasticity as they contribute to both long-term potentiation (LTP) and LTD by regulation of protein synthesis (Neyman and Manahan-Vaughan, 2008; Pfeiffer and Huber, 2006). mGluRs play an important role in the synaptic plasticity that leads to learning and memory, yet dysregulation of these processes can be manifested as memory related diseases or cognitive impairment. Likewise, these receptors are implicated in neurodegeneration characteristic of Alzheimer's Disease (Lee et al., 2004), excitotoxicity in Huntington's Disease (Ribeiro et al., 2010), and excessive LTD characteristic of Fragile X mental retardation syndrome (Bear et al., 2004).

Long-term potentiation

Lomo (1966) first proposed LTP as a mechanism behind learning, acquisition of new information, and memory, retention of this information. LTP is defined as an activity-dependent long lasting increase in synaptic efficacy (reviewed in Lynch, 2004; Malenka and Bear, 2004). Simply put, LTP is a mechanism of strengthening an active synaptic connection, thus potentiating future signal transmission at this synapse. There are two

phases of LTP: Early LTP (E-LTP), which lasts for hours, and long lasting (L-LTP), which can last for weeks. The basic mechanism of E-LTP is that the activation of NMDAR increases intracellular Ca^{2+}, leads to CaMKII phosphorylation and the insertion of AMPAR receptors thereby strengthening the synapse. L-LTP mediates longer-term changes to synaptic activity through local protein translation and gene regulation. Ionotropic glutamate receptors (NMDAR and AMPAR) tend to be the focus of much LTP research. However, mGluRs play an important role in LTP as well and more specifically in the protein translation required of L-LTP (reviewed in Anwyl (2009)).

mGluR-mediated LTP is generally believed to be ionotropic receptor-dependent. Recent findings have unearthed ionotropic receptor-independent mGluR-LTP in interneurons of the hippocampus (Le Duigou and Kullmann, 2011). Here, postsynaptic mGluR1a activation is necessary for interneuron LTP induction (Lapointe et al., 2004; Perez et al., 2001). Potentiation of interneurons increases inhibition on pyramidal neurons, thus providing an adaptive mechanism for regulation of the CA1 pyramidal neurons (Lapointe et al., 2004). When group 1 mGluRs are activated together with ionotropic receptors, a higher level of LTP is achieved as compared to ionotropic receptors alone. Group 1 mGluR's contribution to LTP is only triggered with prolonged high frequency stimulation (Wu et al., 2008). This finding together with the perisynaptic localization of group 1 mGluRs suggests that these receptors are activated by excessive glutamate release causing spillover to innervate these receptors located outside of the PSD. mGluRs are of particular importance for late phase and persistence of LTP, whereby mGluRs trigger local, transcription-independent protein synthesis (Raymond et al., 2000). Furthermore, Job and

Eberwine (2001) demonstrated that DHPG stimulation could trigger protein synthesis in dendrites that were isolated from the soma.

Long-term depression

LTD is defined as an activity-dependent long lasting decrease in synaptic efficacy (reviewed in Bellone et al., 2008; Collingridge et al., 2010; Ito, 1989; Malenka and Bear, 2004). This can include reduced post-synaptic sensitivity to glutamate, reduced synaptic conductance by internalization of AMPA receptors and reduced individual channel conductance. There are multiple mechanisms for LTD induction. The two main mechanisms are NMDA-mediated and mGluR-mediated LTD. These two forms of LTD can coexist in the same neurons and have specifically been observed together in CA1 pyramidal neurons (Oliet et al., 1997). Unlike NMDAR-mediated LTD, mGluR-mediated LTD is not easily reversible and may be a precursor to synapse elimination (Bear et al., 2004; Oliet et al., 1997). Group 1 mGluR-mediated LTD decreases synaptic efficacy through redistribution and internalization of AMPAR (Snyder et al., 2001). mGluR-LTD mediates these effects through alterations in local protein translation of pre-existing mRNA in the dendrites (Huber et al., 2000). A proposed mechanism for translation initiation requires activation of PI3K, Akt, and mTor (Hou and Klann, 2004). Gallagher et al. 2004 suggests that ERK may play a role the mGluR1/5-mediated alterations in protein synthesis. Stimulation of either mGluR1 or mGluR5 is sufficient to induce LTD, including reduced synaptic strength and ERK activation. However, mGluR1 and not mGluR5 is required for LTD expression and its associated decrease in AMPA receptor expression (Volk et al., 2006).

Aberrant mGluR-mediated LTD has been implicated in the pathology of Fragile X syndrome. Experimentally, mGluR5 knockdown in Fragile X mouse model can reduce Fragile X phenotypes (Dölen et al., 2007). Additionally, there is currently a phase III clinical trial underway for the use of an mGluR5 antagonist as treatment for Fragile X (for results from phase II trials see Jacquemont et al. (2011). In Fragile X syndrome, Fragile X mental retardation protein (FMRP), which usually represses mRNA translation of specific proteins, is lost leading to exaggerated LTD in CNS development, specifically in the hippocampus. Fragile X syndrome is characterized by developmental delay and cognitive impairment and is a known cause of autism spectrum disorders.

NMDAR-mediated LTD is unaffected in Fragile X mice; however, mGluR-mediated LTD is exaggerated. Under normal conditions, mGluR stimulation initiates a feedback loop that stimulates FMRP translation, which inhibits further mGluR-mediated protein translation (reviewed Dölen and Bear (2008)). Without FMRP, mGluR signalling is unchecked, resulting in increased translation of pre-existing mRNA leading to the exaggerated mGluR-LTD found in Fragile X syndrome. This is supported by Nakamoto et al. (2007), who described an excessive mGluR5-mediated internalization of AMPAR in Fragile X.

1.6 Ca^{2+}/calmodulin-dependent protein kinase II

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a Ca^{2+}-activated enzyme that has been extensively studied in context with learning and memory since its discovery (Schulman and Greengard, 1978a, b). Although initially extracted from membranes of nerve terminals it has since been deemed ubiquitously expressed. It is, however, enriched in the brain and specifically at the synapses. CaMKII is a serine/threonine kinase whose

substrates contribute to a wide variety of cellular processes including metabolism, gene expression, neurotransmitter synthesis and release, cytoskeletal organization, intracellular Ca^{2+} homeostasis, membrane current as well as synaptic plasticity by way of long-term potentiation and long-term depression (Hudmon and Schulman, 2002).

CaMKII is a well-established player in some processes that contribute to learning and memory. For example, CaMKII is required and sufficient for LTP induction and spatial learning tasks. CaMKIIα mutant mice were impaired in LTP formation as well as spatial learning tasks such as Morris Water Maze (Silva et al., 1992a; Silva et al., 1992b). Furthermore, CaMKII is emerging as a player in LTD. It has recently been implicated in the protein translation required of mGluR-LTD (Mockett et al., 2011). All together CaMKII plays a significant role in plasticity of the glutaminergic synapse. It is well established to regulate ionotropic glutamate signalling and is just starting to be studied in the context of metabotropic glutamate receptor signalling. Furthermore, CaMKII is suspected to be a so-called memory molecule. That is, it has been suggested as a molecular mechanism behind neuronal memory because it possesses a unique ability to autophosphorylate. Autophosphorylated CaMKII, or autonomous CaMKII, remains persistently activated after a transient Ca^{2+} signal. Likewise, CaMKII autophosphorylation is essential for LTP initiation as NMDA-LTP was abolished in mice expressing mutant CaMKIIα lacking the ability to be autophosphorylated (Giese et al., 1998).

1.6.1 Distribution and expression

There are 4 genes that express CaMKII (α, β, γ, and δ), which yields 28 similar isoforms. All isoforms of CaMKII are expressed in the brain; however, α and β are the predominate isoforms in the brain, while β and δ are predominate in the cerebellum. It is

the α isoform that will be the focus of this thesis. CaMKIIα constitutes 2% of total protein in the hippocampus, 1.3% of total cortical protein and 0.7% of total striatal protein (Coultrap and Bayer, 2012; Erondu and Kennedy, 1985; Hudmon and Schulman, 2002; Lisman et al., 2002). Within the brain, CaMKII is highly expressed at the synapse and specifically at the Post Synaptic Density (PSD). CaMKII was initially discovered as a membrane protein (Schulman and Greengard, 1978a, b), but is now known to be present in the cytosol as well. Upon activation, CaMKII translocates from the cytosol to the PSD where it can coordinate processes linked to synaptic plasticity (Hudmon et al., 2005; Strack et al., 1997). The translocation and activation of CaMKII is specific to the synapse that is activated (Lee et al., 2009; Zhang et al., 2008).

1.6.2 Structure and functional domains

Each CaMKII subunit is composed of three domains: 1) the N-terminal kinase domain; 2) the regulatory domain, which contains the autoinhibitory domain, Ca^{2+}/calmodulin binding domain and important phosphorylation residues Thr 286, Thr 305 and Thr 306; and 3) the C-terminal self-association domain (reviewed in (Coultrap and Bayer, 2012; Hudmon and Schulman, 2002; Lisman et al., 2002)). Twelve individual subunits associate to form a dodecomeric holoenzyme. The holoenzyme structure has most simply been compared to the spokes in a wheel. The self-association domains gather at the wheel's hub and the N-terminal catalytic domains of each subunit radiate outwards like spokes. Recently, the crystal structure of CaMKII was published, this confirms and builds upon the currently understanding of holoenzyme organization (Chao et al., 2011) (Figure 1.3b). Inactivated CaMKII is in equilibrium between two conformations: compact and extended. In the compact state the kinase domain folds back on the self-association domain

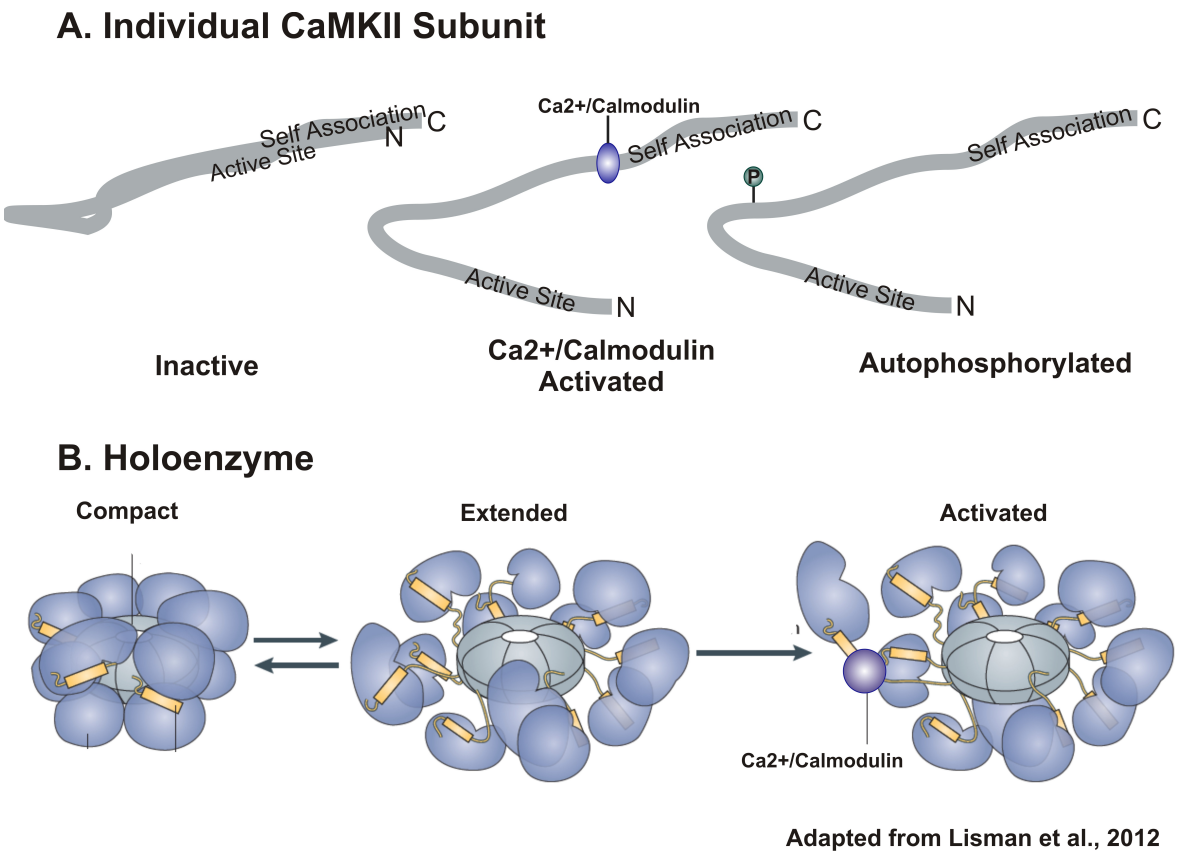

Figure 1.3. CaMKII regulation. A, Above depicts a single subunit of CaMKII. A CaMKII subunit is inactivated as the autoinhibitory domain binds and inactivates the active site of the catalytic domain. $Ca^{2+/}$Calmodulin binds and activates CaMKII. Its binding region overlaps the psuedosubstrate region and thus releases the active site allowing substrate phosphorylation. Additionally, binding of $Ca^{2+/}$Calmodulin exposes the autophosphorylation domain located in the autoinhibitory domain (Thr 286 in the alpha isoform and Thr 287 in other isoforms). Neighboring subunits can phosphorylate CaMKII. Once autophosphorylated this residue results in a persistently activated CaMKII molecule (reviewed in Hudmon and Schulman, 2002). B, 12 CaMKII subunits join together to form a dodecomeric holoenzyme. This enzyme cycles between its extended and compact conformation. Only in its extended conformation is it accessable to CaM binding and subsequent autophosphorylation (Lisman et al., 2012).

yielding the regulatory (CaM-binding) domain inaccessible. In the extended conformation, the kinase domain is stretched out, no longer blocking the regulatory domain, thus allowing Ca^{2+}/calmodulin to bind. Within the dodecomeric holoenzyme each subunit individually moves between compact and extended, but a subunit in the extended conformation that is bound to Ca^{2+}/calmodulin influences the neighboring subunits to also move into the extended conformation allowing Ca^{2+}/calmodulin to bind. Only when two subunits are bound to Ca^{2+}/calmodulin can autophosphorylation occur.

Within each subunit, the autoinhibitory domain acts as a psuedosubstrate by binding and inactivating the active site of the catalytic domain (reviewed in Hudmon and Schulman (2002)). Upon activation, Ca^{2+}/calmodulin binds overlapping the psuedosubstrate region and thus releases the active site allowing substrate phosphorylation. Additionally, binding of Ca^{2+}/calmodulin exposes the autophosphorylation domain, Thr 286 in CaMKIIα isoform and Thr 287 in other CaMKII isoforms, located in the autoinhibitory domain, which can be phosphorylated by neighboring subunits (Bradshaw et al., 2002). Once this residue is phosphorylated, CaMKII is persistently activated, termed autonomous (Figure 1.3a). After Thr 286 phosphorylation, residues Thr 305, Thr 306 residues within the calmodulin-binding domain can be phosphorylated. Phosphorylation at either site prevents reactivation of CaMKII by blocking the calmodulin-binding domain. This phosphorylation opposes the effects of Thr 286 phosphorylation by reducing the autonomous activity (Pi et al., 2010). Phosphorylation at Thr 305 and 306 are important to determine if CaMKII contributes to LTD or LTP (see below).

1.6.3 Regulation of CaMKII activity

CaMKIIα responds to increases in intracellular Ca^{2+} as it is activated by the Ca^{2+}/calmodulin complex. Activating increases in intracellular Ca^{2+} can be achieved at the glutamatergic synapse by activation of ionotropic receptors, which allows the influx of extracellular Ca^{2+}. Additionally, CaMKIIα can be activated through G_q-coupled GPCR-mediated Ca^{2+} release (Ng et al., 2010). Once activated CaMKII is free to phosphorylate downstream substrate targets, some of which will further affect CaMKII activation by regulating Ca^{2+} concentrations (IP3R, Ca^{2+}/ATPase, AMPAR and NMDAR) (Hudmon and Schulman, 2002).

Activated CaMKIIα undergoes autophosphorylation, which can both increase and decrease CaMKII activity. Autophosphorylation at the Thr 286 residue allows CaMKII to retain some level of activation after the Ca^{2+} spike (Colbran and Brown, 2004). Thr 286 phosphorylated CaMKII has been implicated in long-term potentiation and neuronal plasticity as it triggers translocation to the PSD where it phosphorylates PSD proteins such as AMPA receptors (Strack et al., 1997). Autophosphorylation at Thr 305 and Thr 306 reduces autonomous activity of CaMKII. It also inhibits binding to PSD and weakens synapses it could provide a mechanism for LTD regulation by CaMKII (Pi et al., 2010). Within the PSD, CaMKII binds directly to the NMDA receptor subunit NR2B. The NR2B subunit binds in the catalytic domain of CaMKII and potentiates CaMKII signalling in two main ways (Bayer et al., 2001). First, it keeps the enzymatic active site exposed and able to bind to substrates for phosphorylation. Secondly, it keeps the Thr 286 residue exposed so that it can be autophosphorylated and thus maintained in its Ca^{2+}/calmodulin-independent

activated state. The CaMKII-NMDA complex is important for LTP induction and learning as reviewed in Lisman et al. (2012).

CaMKII is also regulated by endogenous proteins, which negatively regulate its activity. CaM-KIIN is a naturally expressed CaMKII inhibitor found in the brain. It binds to the catalytic site of activated or autophosphorylated CaMKII with a high degree of specificity. It binds in the same location as NR2B, but unlike NR2B it blocks the active site and prevents substrate phosphorylation (Chang et al., 1998; Coultrap and Bayer, 2011; Lucchesi et al., 2011). Another protein, α-actinin, also contributes to the regulation of CaMKII. It mimics calmodulin as it binds to the regulator domain. CaMKII bound to α-actinin has limited activity and is Ca^{2+}-independent; however, the α-actinin binding represses some of the functions of CaMKII and also inhibits activation by Ca^{2+}/calmodulin (Jalan-Sakrikar et al., 2012). For experimental purposes, specific pharmacological inhibitors have been developed to inhibit CaMKII activity. Both KN-62 and KN-93 inhibit Ca^{2+}-dependent activity of CaMKII by blocking calmondulin binding (Sumi et al., 1991) (Tokumitsu et al., 1990). Autocamtide-2-related inhibitory peptide (AIP) is another CaMKII inhibitor that is 500 times more potent than KN-93 and, unlike KN-93 or KN-62, it also inhibits automomously active CaMKII in addition to Ca^{2+}-dependent CaMKII activity (Ishida et al., 1995).

1.6.4 CaMKIIα regulation of glutamate receptors

CaMKIIα plays a significant role in the regulation of the glutamatergic synapse. Most studied is the relationship between CaMKIIα and the ionotropic glutamate receptors. However, emerging studies have implicated CaMKII in the regulation of metabotropic receptors as well. It is well established that CaMKII is required for LTP initiation and

plays a significant role in its expression. Activated CaMKII translocates from the cytoplasm to activated synapses (Zhang et al., 2008). Specifically, CaMKIIα has been shown to translocate in response to NMDAR activation (Hudmon et al., 2005). This translocation is partially driven by diffusion and by binding to PSD proteins such as the NR2B subunit of NMDAR (Strack et al., 2000). The direct binding of CaMKII to the NR2B subunit of the NMDA receptor holds CaMKII in its constitutively active form (Bayer et al., 2001). This CaMKII-NMDA complex is important for LTP induction as transgenic mice expressing mutant NR2B subunits, block the CaMKII interaction, and have impaired LTP (Barria and Malinow, 2005). CaMKII then facilitates synaptic strengthening by increasing individual AMPA receptor channel conductance by phosphorylating Ser 831 on the AMPAR GluR1 subunit (Derkach et al., 1999; Lee et al., 2000). Trafficking of AMPAR to the synapse is positively regulated by the AMPAR-associating protein complex Stargazin-PSD95 (Bats et al., 2007). Phosphorylation of Stargazin triggers AMPAR capture at the synapse (Tomita et al., 2005). CaMKII is shown to phosphorylate Stargazin in synapses of cultured neurons and may be responsible for triggering the AMPAR capture characteristic of LTP (Opazo et al., 2010; Tsui and Malenka, 2006).

Little is known about the role of CaMKII in regulation of mGluR activity. However, there is mounting evidence that suggests CaMKII may play a significant role in metabotropic glutamate receptor regulation in addition to ionotropic glutamate receptor regulation. Mundell et al. 2002 elucidated a novel role for CaMKIIα in activation of mGluR1 internalization. They found that CaMKII inhibition significantly attenuated carbachol-induced heterologous mGluR1a internalization. In addition, they found that

CaMKII inhibition significantly attenuated glutamate-induced homologous internalization of mGluR1c. Very recently group 1 mGluR activation by DHPG was shown to cause a transient increase in phosphorylation of CaMKIIα (Mockett et al., 2011). Mockett et al. also found that CaMKII inhibitors (KN-62, KN-93 and AIP) attenuate DHPG-mediated LTD and protein synthesis. This suggests a role for CaMKII in group 1 mGluR-dependent LTD by regulating protein translation.

1.7 Hypothesis

CaMKIIα associates with group 1 mGluRs and this association alters mGluR1/5 signalling and internalization.

1.8 Specific questions

This present thesis builds on preliminary results from a proteomic screen that elucidated a potential association between group 1 mGluRs and CaMKIIα. We set out to explore these specific questions to better understand the relationship between group 1 mGluRs and CaMKIIα: 1) Confirm the association between group 1 mGluRs and CaMKII; 2) Examine the effect of CaMKII on group 1 mGluR trafficking; 3) Examine the effect of CaMKII on mGluR1a signalling.

1.9 Relevance

As discussed previously, CaMKIIα can be phosphorylated by mGluR1/5 stimulation and plays a role in mGluR-LTD, likely through mGluR-mediated protein translation (Mockett et al., 2011). It is also known that CaMKIIα can alter heterologous mGluR internalization. However, it is not yet known whether or not mGluR1/5 can associate with CaMKIIα and if CaMKIIα plays a significant role in receptor signalling and

homologous internalization. Both CaMKIIα and group 1 mGluRs play an important role in the maintenance of memory, learning and synaptic transmission. Understanding how these two players work together is an important bridge to further our current understanding of post-synaptic modification. This study could also have important implications for neurodegenerative diseases providing a potential mechanism for reducing excitotoxicity through desensitization of mGluR1/5 by CaMKII.

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

Adult CD-1 mice were from Charles River (Wilmington, MA). Human Embryonic Kidney (HEK293) Cells were from American Type Culture Collection (Manassas, VA). Cell culture reagents were from Invitrogen (Burlington, ON): Minimal Essential Media (MEM), Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) and 0.25% Trypsin-EDTA. OmniPur Bovine Serum Albumin (BSA) was from VWR (Mississauga, ON). L-Quisqualic Acid and KN-93 were from Tocris Bioscience (Minneapolis, MN). Biotinylation reagents EZ-Link Sulfo-NHS-SS-Biotin and NeutrAvidin Agarose Resin as well as Pierce ECL Western Blotting Substrate and SuperSignal West Dura Chemiluminescent Substrate were purchased from Thermo Scientific (Rockford, IL). Myo-[^{3}H]Inositol and Phosphorus-32 Radionuclide were from Perkin Elmer (Waltham, MA). Protein G Sepharose beads were from GE Healthcare (Oakville, ON). ANTI-FLAG M2 Affinity Gel and Dowex 1X8 formate 200-400 mesh resin were from Sigma-Aldrich (St. Louis, MO). DC Protein Assay Kit was from BioRad Laboratories (Mississauga, ON). Kodak X-Omat Blue Film was from Fisher Scientific (Ottawa, ON).

Primary Antibodies: CaMKII (G-1) [Santa Cruz Biotechnology (Santa Cruz, CA): sc-5306]; CaMKII (pan), Phospho-CaMKII (Thr286), CaMKIIα, Phospho-p44/42 MAPK, p44/42 MAPK [Cell Signaling Technology (Danvers, MA): 3362S, 3361S, 3357S, 9101S, 9102S]; mGluR1 and mGluR5 [Millipore (Billerica, MA): 07-617, AB5675]; GFP

(Invitrogen: A-6455). Secondary Antibodies: Mouse (GE Healthcare) and Rabbit (BioRad).

GFP-CaMKIIα construct was from Dr. Paul De Koninck (Laval University, Quebec, Canada). CaMKIIα shRNA was from Dr. Kenichi Okamoto (Mount Sinai, Toronto, Canada).

2.2 Cell culturing and transfections

HEK293 cells were used in our investigation because they express a number of proteins required for mGluR receptor signaling and endocytosis. Specifically they express GRKs, Arrestins and PKC, which are proteins involved in endocytosis (Atwood, B., 2011). HEK293 cells were cultured in MEM with 8% FBS. Cells were plated on 100 mm dishes and transfected using a modified Ca^{2+} phosphate method (Ferguson and Caron, 2004) with cDNA amounts indicated in *Figure Legends*. For transfection, cDNA was diluted to 450 μL in sterile distilled water, 50 μL 2.5 M $CaCl_2$ added, 500 μL 2X HEPES-Buffered Saline (0.38 M NaCl, 0.05 M HEPES, 1.5 mM Na_2HPO_4, pH 7.05) added drop-wise and mixed gently before transfection mixture was added to cells. Cells were washed 16-20 hours post transfection and then allowed to recover in new media before experimentation. For co-immunoprecipitation, cells recovered for 24 hours. For all other experiments, cells recovered for 6-8 hours and then were reseeded into 6-well dishes and allowed to recover for 18 hours.

2.3 Immunoblotting

Acrylamide gels (10%) were run using a Hoefer gel system, and then transferred to 0.45 μM nitrocellulose membrane using a semidry transfer apparatus. Membranes were blocked for 1 hour in Tris-Buffered Saline and Tween-20 (TBS-T) with 10% milk, and

then incubated with primary antibody as described, later in the methods, in TBS-T with 5% milk overnight. Blots were washed with TBS-T, incubated with secondary antibody (BioRad, Rabbit 1:10,000 and GE Healthcare, Mouse 1:2500) in TBS-T with 5% milk for 1 hour, washed again, treated with ECL Western Blotting Substrate and exposed on film.

2.4 HEK293 cell co-immunoprecipitation

HEK293 cells were transiently transfected with cDNA of FLAG tagged receptor (mGluR1a or mGluR5a) and either pEGFP (control) or GFP-CaMKIIα (3 μg of receptor and 0.5 μg of GFP constructs). Cells were stimulated for 0, 2 and 10 minutes with 50 μM quisqualate in HEPES-Balanced Salt Solution (HBSS: 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM Hepes, 11 mM Glucose, 116 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, pH 7.4) at 37°C. Cells were washed on ice with cold Phosphate-Buffered Saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2) and then lysed on a rocking platform for 15 minutes at 4˚C for using 0.1% Triton-X 100 lysis buffer (0.025 M Hepes, 300 mM NaCl, 1.5 mM MgCl, 0.2 mM EDTA, 0.1% Triton-X) with added protease inhibitors: 1 mM AEBSF, 10 μg/ml leupeptin, and 5 μg/ml aprotinin. Lysate was collected and centrifuged at 15,000 RPM for 15 minutes at 4˚C. 250 μg of each lysate was incubated with FLAG-agarose beads (50 μl bead slurry) for 1 - 2 hours. Beads were washed three times with cold PBS. Samples were eluted using 3x SDS sample buffer with 2-mercaptoethanol and separated by SDS-PAGE. Membranes were blotted for GFP (1:1000) to test co-immunoprecipitation of GFP-CaMKIIα with FLAG-mGluR1/5, and then immunoblotted for mGluR1 (Millipore, 1:1000) and mGluR5 (Millipore, 1:1000).

2.5 Hippocampus co-immunoprecipitation

The hippocampus was removed from CD-1 adult mice and placed in 0.5% Triton-X 100 lysis buffer with protease inhibitors. It was then homogenized using a Polytron and allowed to solubilize for 2 hours while rotating at 4˚C. Lysate was then centrifuged at 15,000 RPM for 15 minutes at 4˚C and 1 mg of protein was incubated with protein G-Sepharose with mGluR5 antibody (Millipore, 1.5 μg) or Rab11 antibody (control) to immunoprecipitate mGluR5. Samples were eluted using 3x SDS sample buffer with 2-mercaptoethanol and separated by SDS-PAGE. Membranes were immunoblotted for immunoprecipitated mGluR5 (Millipore, 1:1000) and co-immunoprecipitated CaMKII (Santa Cruz, 1:250).

2.6 GST-mGluR1a fusion protein purification and pull-down assay

GST-Fusion protein purification and pull-down assay was conducted similarly to Dhami et al. (2005). GST-mGluR1a-IL-2 and GST control peptide was generated in *E. coli* recombinant bacteria grown at 37°C until OD_{600} was 0.6-1.0. Cultures were then induced with 1mM isopropyl 1-thio-β-D-galactopyranoside at 15°C for 2 hours. Cells were pelleted and resuspended in PBS with (1mM AEBSF, 25 μg/ml Aprotinin, 10 μg/ml Leupeptin, 10 μg/ml Pepstatin A). Sonication (3 times for 30 seconds) at 4°C was then used to lyse the cells. 1% Triton X-100 was added to the lysate and incubated with rocking at 4°C for 30 minutes. Insoluble materials were pelleted at 12,000 g for 10 minutes at 4°C and the supernatant containing proteins was aliquoted and stored at -80°C. 50 μl of Glutathione-Sepharose bead slurry was incubated overnight with 1 ml of solubilized protein to purify GST-Fusion constructs. Beads were then washed and resuspended in 100 μl of lysis buffer. HEK293 cells transiently transfected with GFP-CaMKIIα (0.5 μg cDNA) was lysed and

centrifuged. For the pull-down assay, GST-Fusion peptide bound to matrix was incubated with 500 μg of GFP-CaMKIIα HEK293 cell lysate at 4°C for 2-4 hours. Samples were then washed extensively and eluted using 3x SDS sample buffer containing 2-mercaptoethanol. Analysis was done by SDS-PAGE and immunoblotted for CaMKII (Santa Cruz, 1:250) to determine if GFP-CaMKIIα was pulled down with the GST-mGluR1a peptides.

2.7 Biotinylation internalization assay

Biotinylation internalization assay was conducted similarly to Ferreira et al. (2009). HEK293 cells were transiently transfected with receptor (FLAG-mGluR1a and FLAG-mGluR5a) and either pEGFP (control) or GFP-CaMKIIα (3 μg of receptor and 0.5 μg of GFP constructs). Cells were serum starved for 1 hour in HBSS at 37°C on the morning of the experiment. Cells were washed and incubated on ice for 20 minutes in HBSS. Plasma membrane proteins were biotinylated at 4°C with EZ-Link Sulfo-NHS-SS-Biotin in HBSS and then incubated at 4°C in 100mM glycine in HBSS for 30 minutes to quench biotinylation. Cells were then stimulated with 50 μM quisqualate for 0, 5 and 15 minutes, which allowed the receptor to internalize. Remaining cell surface biotin was stripped using 100 mM sodium 2-mercaptoethanesulfonate (MesNa) in TE Buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.6) with 0.2% BSA. A control without stimulation or stripping was kept on ice and used to assess amount of total cell surface receptor. Cells were lysed, biotinylated protein pulled down with NeutrAvidin agarose resin (50 μl bead slurry), eluted with 3x SDS sample buffer containing 2-mercaptoethanol, separated by SDS-PAGE and immunoblotted for receptors, mGluR1 and mGluR5 (Millipore, 1:1000). Internalization of the receptor at various time points with and without the presence of over expressed

CaMKIIα was compared to control. Results expressed as percent cell surface internalization.

Biotinylation internalization assay was modified slightly for mGluR1a experiments. Following serum starving, cells were pretreated for 1 hour with or without 1.0 μM KN-93 in HBSS. For stimulation, cells were stimulated with 50 μM quisqualate for 0 and 15 minutes.

2.8 mGluR1/5 ERK1/2 activation

ERK activation assay was conducted similarly to Esseltine et al. (2011). HEK293 cells were transiently transfected with receptor (FLAG-mGluR1a and FLAG-mGluR5a) and either pEGFP (control) or GFP-CaMKIIα (3 μg of receptor and 0.5 μg of GFP constructs). Cells were first serum starved overnight in DMEM supplemented with 0.1% BSA and then serum starved for an additional hour in HBSS on the morning of the experiment. Cells were stimulated with quisqualate (50 μM) in HBSS at 37°C for 0 or 5 minutes. Samples were washed with cold HBSS and lysed using 1% Triton-X 100 lysis buffer with protease inhibitors plus phosphatase inhibitors (1 M NaF and 100 μM Na_3VO_4). Next, 30-50 μg of each sample were separated by SDS-PAGE. ERK1/2 phosphorylation was determined by immunoblotting for phosphorylated ERK and compared to total ERK (Cell Signaling, 1:000).

2.9 Inositol phosphate formation

Inositol phosphate formation assay was conducted similarly to Dale et al. (2001b). HEK293 cells were transiently transfected with FLAG-mGluR1a and either pEGFP (control) or GFP-CaMKIIα (3 μg of receptor and 0.5 μg of GFP constructs). Cell were incubated overnight with 1 μCi/ml myo-[^{3}H]inositol in DMEM to radiolabel inositol lipids.

Cells were washed, and then preincubated in HBSS at 37°C for 1 hour. Cells were then incubated for an additional 10 minutes at 37°C with 500 µl of HBSS with 10mM LiCl. Next, cells were incubated with or without quisqualate at increasing concentrations (0-100 µM) for 30 minutes. After stimulation, the reaction was stopped by placing samples on ice and adding 500 µl 0.8 M perchloric acid, which was neutralized with 400 µl of 7.2 M KOH and 0.6 M $KHCO_3$. The radioactivity of a 50 µl sample of cell lysate was counted by liquid scintillation using a Beckman LS 6500 scintillation system to determine the total myo-[^{3}H]inositol incorporated into the cells. Anion exchange chromatography, using Dowex 1X8 formate 200-400 mesh resin, was used to extract total inositol phosphate from cells. [^{3}H]Inositol Phosphate formation was then determined by liquid scintillation using a Beckman LS 6500 scintillation system.

2.10 Statistical analysis

Immunoblots were quantified using Scion Imaging software. Densitometry values were normalized for protein expression. GraphPad Prism software was used for statistics and graph generation. Statistical analysis was performed using a One-way Analysis of Variance followed by a Tukey's post hoc test to determine which means were significantly different ($p < 0.05$) from one another.

CHAPTER 3

RESULTS

3.1 CaMKIIα identified as a novel group 1 mGluR associating protein

The intracellular loop (IL) 2 domain of mGluR1a is known to contain a critical residue for G protein selectivity (Hermans and Challiss, 2001). Our lab has previously studied CAIN (Ferreira et al., 2009), Pyk2 (Nicodemo et al., 2010) and GRK2 (Dhami et al., 2004) interactions with this domain. Therefore, we wanted to continue our study of IL-2 binding proteins by screening for novel associating proteins to suggest new potential functions of this important domain. In collaboration with Dr. Stephane Angers, we utilized a membrane permeant Tat-IL2-FLAG peptide to treat 100 x 10^8 mouse cortical neurons for 1 hour following which the neurons were solublized, the Tat-IL2-FLAG peptide was immunoprecipated with FLAG sepharose beads, immunoprecipitates trypsinized and analyzed by Maldi-TOF Mass Spectroscopy. mGluR1 IL2 interacting proteins identified in the screen included CaMKII isoforms α, β, γ, and δ as well as other kinases (including MAPK) and components of endocytotic machinery (including clathrin heavy chain and dynactin1/2).

Our initial experiments were focused on validating the potential interaction between CaMKIIα and group 1 mGluRs (mGluR1a and mGluR5a). First, we assessed whether GFP-CaMKIIα could be co-immunoprecipitated from HEK 293 cells that were co-transfected to overexpress either FLAG-mGluR1a or FLAG-mGluR5. We found that GFP-CaMKIIα could be co-immunoprecipitated with FLAG-mGluR1a in the absence of agonist stimulation (Fig. 3.1A). Moreover, the treatment of the cells with 50 μM

quisqualate for either 2 or 10 min did not increase the association of GFP-CaMKIIα with the receptor (Fig. 3.1A). Similarly, GFP-CaMKIIα could be co-immunoprecipitated with FLAG-mGluR5 in the absence of agonist and agonist treatment did not increase GFP-CaMKIIα interactions with FLAG-mGluR5 (Fig. 3.1B). To assess whether endogenous CaMKII interacts with endogenous mGluR5, we immunoprecipitated CaMKII with a CaMKII-specific antibody from mouse hippocampal tissue lysates and blotted for mGluR5. We found that mGluR5 was effectively co-immunoprecipitated with CaMKII from hippocampal tissue (Fig. 3.2). To confirm that the interaction between CaMKIIα and mGluR1a/5 was mediated by IL2 we used purified GST-Fusion protein containing the IL-2 portion of mGluR1a and incubated this with HEK293 cell lysate expressing GFP-CaMKIIα. We found that GST-IL2, but not GST alone effectively precipitated CaMKIIα from HEK 293 cell lysates (Fig. 3.3). As a positive control we assessed whether GRK2 could also be precipitated with GST-IL2 and found that similar to that reported by Dhami et al., (2005) GST-IL2 also precipitates GRK2 from HEK 293 cell lysates (Fig. 3.3). Taken together these experiments confirm a novel interaction between CaMKIIα and group I mGluRs, which is mediated by the receptor IL2.

3.2 Role of CaMKIIα in group 1 mGluR internalization

CaMKII regulates heterologous internalization of mGluR1a in response to activation of the m1 muscarinic acetylcholine receptor (Mundell et al., 2002). Specifically, Mundell demonstrated that CaMKII inhibition prevented heterologous-stimulated mGluR1a internalization, but they were unable to demonstrate a significant regulatory role for CaMKIIα in homologous mGluR1a internalization. In continuation of this work, we investigated the role of CaMKIIα in homologous internalization of group 1 mGluRs.

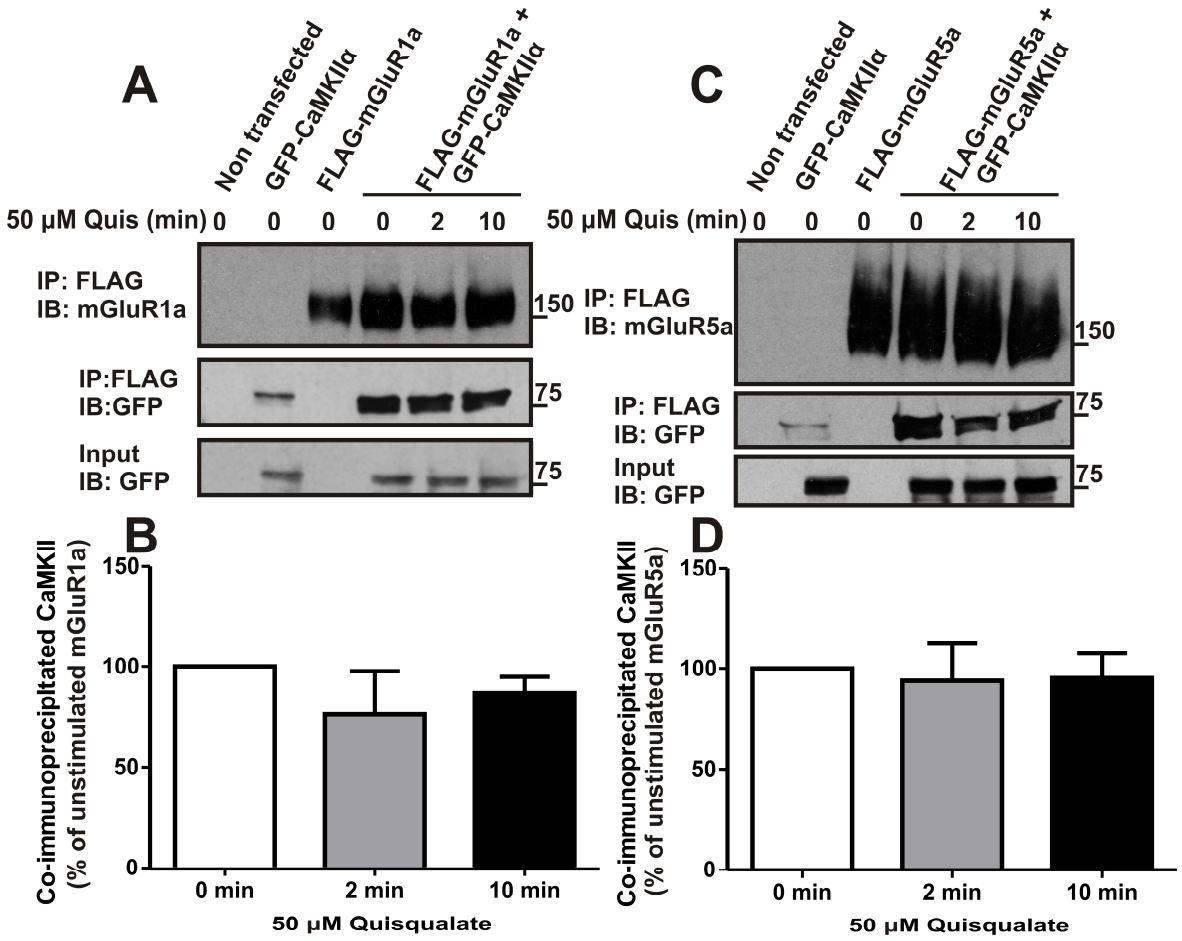

Figure 3.1. Co-immunoprecipitation of GFP-CaMKIIα with FLAG-mGluR1a and FLAG-mGluR5a. HEK293 cells were transiently transfected as labeled with 0.5 μg of cDNA mGFP encoding CaMKIIα or pEGFP and 3 μg of pcDNA3.1 encoding (A) FLAG-mGluR1a or (C) FLAG-mGluR5a. Cells were stimulated with 50 μM quisqualate then lysed. Lysates were incubated with mouse FLAG-agarose to immunoprecipitate FLAG-tagged receptor. Shown are representative immunoblots for immunoprecipitated receptor with rabbit anti-mGluR1 or rabbit anti-mGluR5 and for GFP to assess if GFP-CaMKIIα was co-immunoprecipitated with the receptor. B, D) Immunoblots for co-immunoprecipitated CaMKII were analyzed by densitometry. Agonist treatments were compared to unstimulated. $p > 0.05$. Data are averaged means ± S.E.M. of six independent experiments.

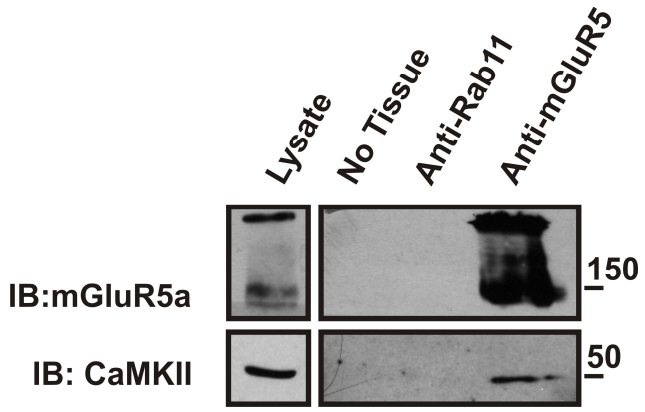

Figure 3.2. Co-immunoprecipitation of endogenous CaMKII with mGluR5a in mouse hippocampal tissue. 1 mg of adult CD-1 mouse hippocampal tissue lysate was incubated with Protein G-Sepharose and rabbit anti-mGluR5. Samples were analyzed by western blot. Shown are representative immunoblotted for immunoprecipitated receptor with rabbit anti-mGluR5 and co-immunoprecipitated CaMKII with mouse anti-CaMKII. Representative immunoblots of four independent experiments.

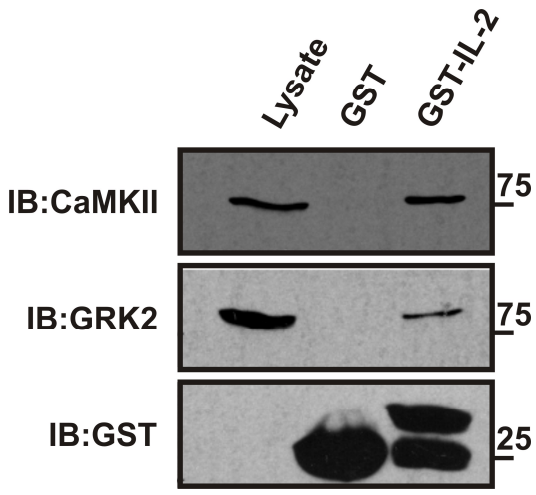

Figure 3.3. Purified GST-fusion mGluR1a IL-2 peptide precipitates GFP-CaMKIIα. Protein purification and lysate preparation: GST-fusion peptides were generated in *E. coli* recombinant bacteria, induced with isopropyl 1-thio-β-D-galactopyranoside, sonicated, centrifuged and purified using Glutathione-Sepharose. HEK293 cells were transiently transfected with 0.5μg of cDNA mGFP encoding GFP-CaMKIIα. Cells were lysed and centrifuged. Affinity pull-down assay: GST-Fusion peptides GST-mGluR1a IL-2 and GST control were each incubated with 500 μg of GFP-CaMKIIα lysate. GRK2 is known to bind to the IL-2 and is used here as a positive control for IL-2 binding. Samples were analyzed by western blot. Shown are representative immunoblotted with mouse anti-CaMKII and rabbit anti-GRK2 to determine if GFP-CaMKIIα and GRK2 was pulled down with the purified GST-mGluR1a peptide. Representative immunoblots of three independent experiments.

3.2.1 CaMKIIα enhances agonist-stimulated mGluR5a internalization

Our initial studies examined whether quisqualate-mediated internalization of mGluR5a was altered following GFP-CaMKIIα overexpression using a biotinylation internalization assay. Cell surface proteins were biotinylated on ice, and then warmed to 37℃ and stimulated with 50 μM quisqualate for 0, 5 and 15 minutes of stimulation in the presence or absence of ectopically expressed GFP-CaMKIIα. Internalized mGluR5 following stimulation was compared to total cell surface mGluR5 expression. CaMKIIα co-expression significantly increased mGluR5a internalization at 15 minutes of agonist stimulation (Fig. 3.4). mGluR5a co-expressed with GFP-CaMKIIα exhibited a five-fold increase of agonist-induced loss of cell surface receptor as compared to mGluR5a co-expressed with GFP-control.

3.2.2 CaMKIIα enhances agonist-stimulated mGluR1a internalization

As an extension of our work examining mGluR5a internalization, we tested whether the CaMKII inhibitor KN-93 would inhibit mGluR1a internalization following 15 min of agonist activation. A biotinylation internalization assay was conducted as described above except that cells were pretreated with either 1 μM KN-93 or HBSS as a control. Figure 3.5 shows mGluR1a internalization was increased two fold in the presence of overexpressed CaMKIIα and this increased internalization was attenuated following KN-93 treatment, although the attenuation did not reach a statistically significant value. Overall, the data supports the conclusion that CaMKIIα overexpression enhances group 1 mGluR internalization and suggests that this may be dependent upon CaMKII catalytic activity.

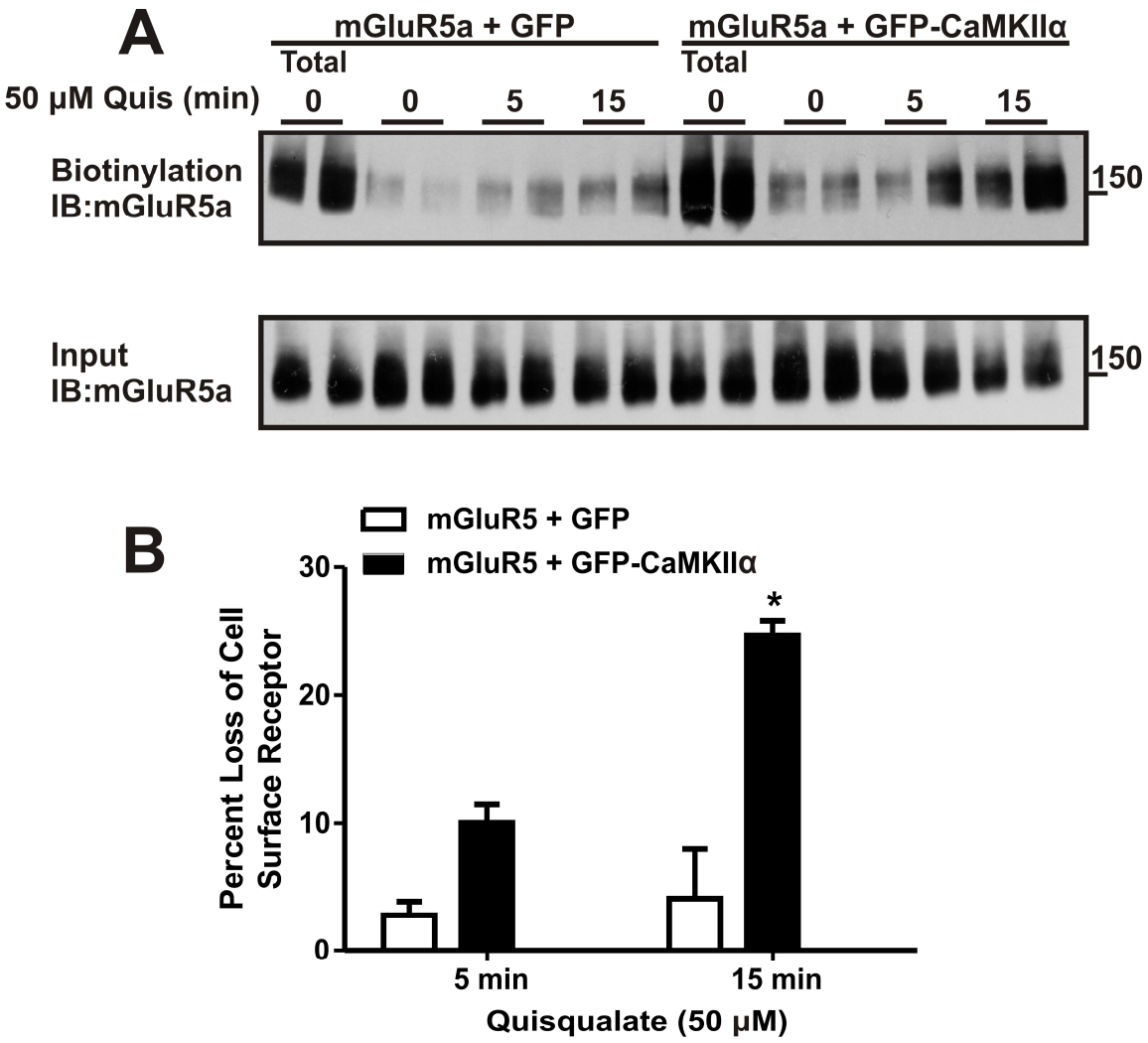

Figure 3.4. CaMKIIα significantly enhances of mGluR5a internalization at 15 minutes of quisqualate stimulation. A, shown are representative immunoblots for rabbit anti-mGluR5. HEK293 cells were transiently transfected with 0.5 μg of cDNA mGFP encoding CaMKIIα or pEGFP and 3 μg of pcDNA3.1 encoding FLAG-mGluR5a. Cell surface proteins were biotinylated. Cells were stimulated (50 μM quisqualate) and then biotin was stripped from the cell surface with MesNa (100 mM). Lysates were incubated with NeutrAvidin-agarose to pull out biotin and associated proteins. B, Immunoblots were analyzed by densitometry. Internalized mGluR5 was compared to total cell surface receptor. *, $p < 0.05$. Data are averaged means ± S.E.M. of four independent experiments completed in duplicate.

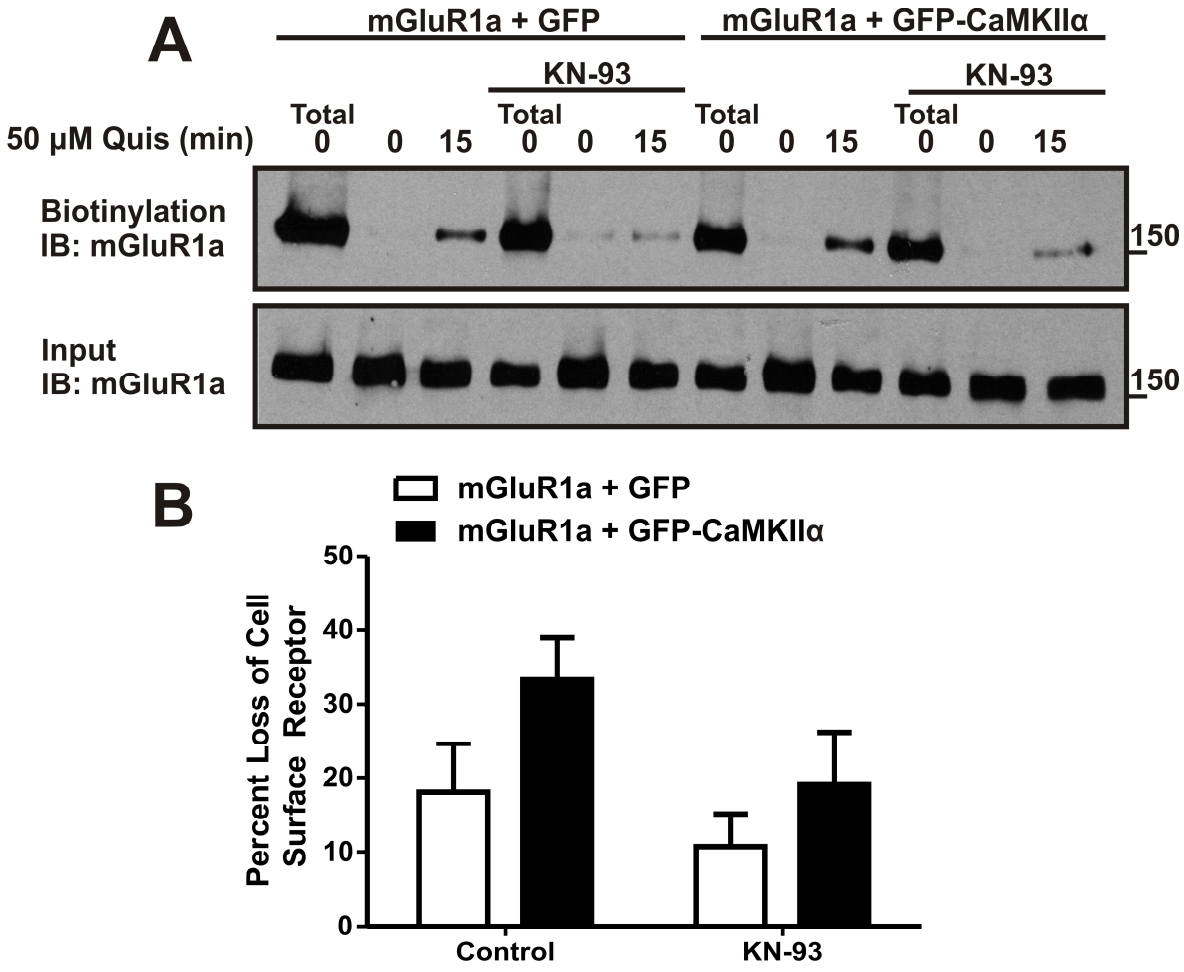

Figure 3.5. CaMKIIα does not significantly enhance agonist-stimulated mGluR1a internalization. A, shown are representative immunoblots for rabbit anti-mGluR1a. HEK293 cells were transiently transfected with 0.5 μg of cDNA mGFP encoding CaMKIIα or pEGFP and 3 μg of pcDNA3.1 encoding FLAG-mGluR1a. Cells were pretreated with or without 1 μM KN-93 for 1 hour. Cell surface proteins were biotinylated. Cells were stimulated with 50 μM quisqualate for 15 minutes and then biotin was stripped from the cell surface with MesNa (100 mM). Lysates were incubated with NeutrAvidin-agarose to pull out biotin and associated proteins. B, Immunoblots were analyzed by densitometry. Internalized mGluR1 was compared to total cell surface receptor. $p > 0.05$. Data are mean ± S.E.M. of seven independent experiments.

3.3 Effect of CaMKIIα on mGluR1a signalling

Group I mGluRs are coupled via $G\alpha_{q/11}$ to the activation of phospholipase C and the generation of diacylglycerol and inositol phosphate (IP). CaMKIIα is a kinase that could contribute to the phosphorylation and desensitization of group I mGluR G protein coupling. We focused our investigation into mGluR signalling on mGluR1a. Signalling for this receptor is better established in this lab and therefore allowed us to compare these results with previous lab data. Therefore, we assessed the role of CaMKIIα in FLAG-mGluR1a-stimulated IP formation in HEK 293 cells. We found that the overexpression of GFP-CaMKII resulted in a statistically insignificant trend of increase in FLAG-mGluR1a-stimulated IP formation in response to increasing concentrations of quisqualate (Fig. 3.6A), without affecting basal IP formation in FLAG-mGluR1a expressing cells (Fig. 3.6B). The treatment of cells with shRNA to knockdown endogenous CaMKIIα expression (Fig. 3.6C) did not result in an alteration in the dose-response curve for quisqualate-stimulated IP formation in HEK 293 cells transfected to express FLAG-mGluR1a (Fig. 3.6D), and did not affect basal IP formation (Fig. 3.6E). Furthermore, CaMKIIα shRNA treatment did not significantly alter FLAG-mGluR1a expression (Fig. 3.6F). Taken together, these results indicate that CaMKIIα does not contribute to the regulation of mGluR1a G protein coupling.

To assess whether CaMKIIα interactions with mGluR1a might alter signalling via alternative cell signalling pathways, we investigated whether CaMKIIα overexpression might alter FLAG-mGluR1a-stimulated ERK1/2 phosphorylation. We found that the overexpression of GFP-CaMKIIα did not alter FLAG-mGluR1a-mediated ERK1/2

phosphorylation in response to the treatment of cells with 50 μM quisqualate for 5 min (Fig. 3.7). Furthermore, treatment of cells with 1 μM KN-93 had no effect on the extent of FLAG-mGluR1a-stimulated ERK1/2 phosphorylation in either the absence or presence of overexpressed GFP-CaMKIIα (Fig. 3.7). These results suggest that, similar to G protein coupling, CaMKIIα does not contribute to the regulation of mGluR1a-mediated ERK1/2 phosphorylation.

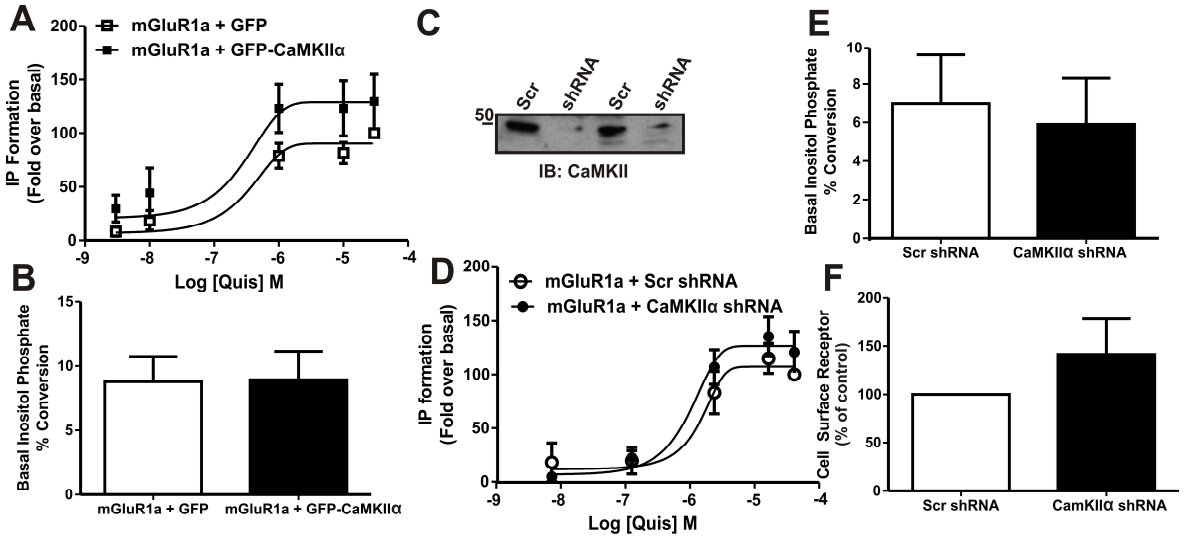

Figure 3.6. CaMKIIα does not have a significant effect on mGluR1a-mediated inositol phosphate (IP) formation. A, shown is FLAG-mGluR1a-induced IP formation stimulated with increasing concentrations of quisqualate (0–30 μM) for 30 minutes in the presence of either GFP (control) or GFP-CaMKIIα. B, shown is basal IP formation without quisqualate stimulation. HEK293 cells were transiently transfected with 0.5 μg of cDNA mGFP encoding CaMKIIα or pEGFP and 3 μg of pcDNA3.1 encoding FLAG-mGluR1a. $p > 0.05$. Values expressed at mean ± S.E.M. for four-seven independent experiments. C, representative immunoblots for mouse anti-CaMKII confirms shRNA knockdown of CaMKII. HEK293 cells were transiently transfected with 3 μg of pcDNA3.1 encoding FLAG-mGluR1a and with 1 μg of either scramble control or CaMKIIα shRNA 72 hours before experiment. D, shown is FLAG-mGluR1a-induced IP formation stimulated with increasing concentrations of quisqualate (0–30 μM) for 30 minutes with or without CaMKIIα knock down by shRNA. E, shown is basal IP formation without quisqualate stimulation. F, receptor cell surface expression was analyzed by flow cytometry to confirm that shRNA treatment did not alter receptor cell surface expression. $p > 0.05$. Values expressed at mean ± S.E.M. for four independent experiments.

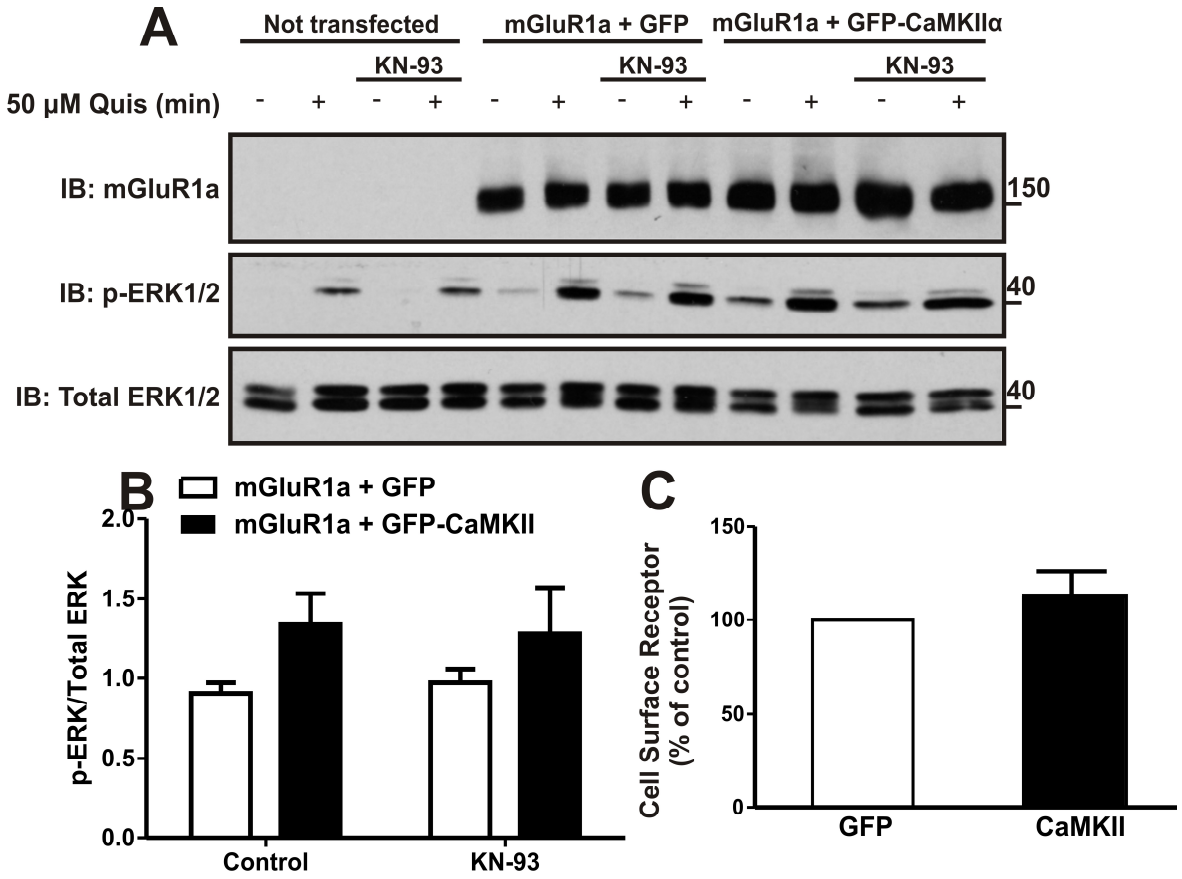

Figure 3.7. CaMKIIα does not have a significant effect on mGluR1a-mediated ERK1/2 phosphorylation. A, shown are representative immunoblots for rabbit anti-ph-ERK1/2 and rabbit anti-total-ERK1/2. HEK293 cells were transiently transfected with 0.5 μg of cDNA mGFP encoding CaMKIIα or pEGFP and 3 μg of pcDNA3.1 encoding FLAG-mGluR1a. After transfection, cells were serum starved in DMEM overnight and for an additional hour on the morning of the experiment in HBSS. Cells were stimulated with 50 μM quisqualate for 5 minutes. B, immunoblots were analyzed by densitometry. Phospho-ERK1/2 compared to total ERK1/2. $p > 0.05$. C, receptor cell surface expression was analyzed by flow cytometry to confirm that GFP-CaMKIIα expression did not alter receptor cell surface expression. Data are mean ± S.E.M. of five independent experiments.

CHAPTER 4

DISCUSSION

The complex and sometimes controversial relationship between CaMKIIα and mGluRs is important for the better understanding of mGluR-mediated LTD and related plasticity. We first identified CaMKIIα as a potential mGluR interacting protein through a proteomic screen for novel mGluR associating proteins. We hypothesized that CaMKIIα associates with group 1 mGluRs and this association would alter mGluR1/5 signalling and internalization. Our studies have revealed several key findings: 1) CaMKIIα associates with both mGluR-1a and -5a in an agonist independent manner, 2) CaMKIIα enhances agonist-stimulated internalization of group 1 mGluRs, 3) CaMKIIα does not play a significant role in group 1 mGluR signalling through either IP3 or ERK1/2 phosphorylation.

4.1 Association between CaMKIIα and group 1 mGluRs

This present thesis demonstrates the novel association of CaMKIIα with group 1 mGluRs. First, we confirmed this association in HEK293 cells (Figure 3.1). We next replicated this experiment in adult mouse hippocampal tissue (Figure 3.2). This second experiment showed that the interaction could take place with endogenously expressed proteins in physiologically relevant tissue. Finally, we confirmed that the second intracellular loop of mGluR1a is sufficient for the interaction - that it does not require the full-length receptor (Figure 3.3). This last finding provides new functional significance for the IL-2 domain. Beyond the bounds of this current thesis we would like to further characterize this novel interaction. We would localize the specific binding domain within

the IL-2 required for CaMKIIα association by using four alanine scanning GST-Fusion IL-2 peptides similar to that performed in Dhami et al. (2005).

The data suggests that the association between CaMKIIα and mGluRs is agonist independent, which means that CaMKIIα binds to both the agonist bound and unbound receptor conformation. Similarly, this brings one to question if the receptor binds preferentially to activated CaMKIIα. Furthermore, does this interaction have significant implications for the regulation of CaMKIIα? We know that the interaction of CaMKIIα with NR2B enhances the autonomously active state of CaMKIIα (Bayer et al., 2001) and that this CaMKII-NR2B complex is essential for LTP (Barria and Malinow, 2005; Lisman et al. 2012). Could it be possible that CaMKIIα's interaction with metabotropic glutamate receptors could also hold CaMKIIα in a persistently activated state? Mockett et al. (2011) discussed that CaMKIIα phosphorylation is dynamically regulated by mGluRs. That mGluRs associate with CaMKIIα could suggest a direct regulation of CaMKIIα activity and phosphorylation beyond activation by mGluR-mediated Ca^{2+} release.

4.2 Role of CaMKII in group 1 mGluR internalization

CaMKIIα is known to associate with and contribute to desensitization of ionotropic glutamate receptors, NMDAR and AMPAR (Colbran and Brown, 2004) and $G\alpha_{q/11}$ coupled GPCRs, D1/D2-R (So et al., 2007) and D3-R (Liu et al., 2009). Together, these findings allow us to reasonably suspect that the association of CaMKIIα with mGluRs could have similar implications for the mGluR family of receptors. The next goal of this current thesis was to investigate the role of CaMKIIα in mGluR desensitization.

Our results show that CaMKIIα plays a significant role in agonist-induced mGluR internalization (Figures 3.4 and 3.5). These results are similar to those found by (Guetg et

al., 2010) that CaMKIIα significantly enhances agonist-induced internalization of another class C GPCR, the $GABA_B$ receptor. In our results, a more exaggerated enhancement of receptor internalization was observed for mGluR5a as compared to mGuR1a. This result for mGluR1a is congruent with previously published findings. Mundell et al. (2002) did not find a significant difference in glutamate-induced internalization of mGluR1a with treatment of KN-93, a CaMKII inhibitor; however, he did find significance in the internalization of the mGluR1c variant. Perhaps this receptor variant dependent difference could be explained by their structural differences - specifically the length of their C-terminal tail. Mundell et al. (2002) observed that mGluR-1a internalized more slowly than shorter C-tail splice variants (mGluR-1b and -1c). He did, however, find significant heterologous internalization for all tested mGluR1 splice variants (mGluR-1a, -1b, and -1c), which he suggested may have to do with a more drastic increase in intracellular Ca^{2+} to activate CaMKIIα. Together, these findings suggest that CaMKIIα plays a significant role in group 1 mGluR internalization; however, this role is more pronounced for shorter tail variants (mGluR-1c and mGluR-5a). It would be interesting to study the effect of CaMKIIα on mGluR-1b and -1c variants using a co-expression model compared to Mundell's KN-93 inhibition. We predict that the agonist-induced internalization of mGluR1c would be enhanced by CaMKIIα similarly to our results for mGluR5a.

4.3 Role of CaMKIIα in group 1 mGluR signalling

We moved on to explore the effects of CaMKIIα on mGluR signalling because of the potential role for CaMKIIα in mGluR signalling attenuation through desensitization. We also wanted to see if overexpression of CaMKIIα could decrease inositol phosphate formation because CaMKIIα is known to promote mGluR-IP3R uncoupling by

phosphorylation of Homer3. CaMKIIα phosphorylates Homer3, which reduced Homer3's affinity for its substrates including mGluR1a, phosphorylation of Homer3 changes mGluR initiated Ca^{2+} signalling pattern by uncoupling of mGluR1a from the IP3R (Mizutani et al., 2008). However, we did not find a significant difference between IP3 formation with co-expression of GFP-CaMKIIα compared to GFP control (Figure 3.7).

Furthermore, we found no significant change in ERK1/2 phosphorylation with co-expression of CaMKIIα or with pretreatment by KN-93 (Figure 3.8). Our findings in this study are contradictory to earlier findings, which state that CaMKIIα inhibitor, KN-62, decreased DHPG-mediated ERK phosphorylation (Choe and Wang, 2001). It should be pointed out that we used a different inhibitor KN-93 compared to KN-62. They also used immunoreactive imaging in rat striatal neurons and we used quantitative western blotting in HEK293 cell lysates. However, it still remains unclear if CaMKIIα plays a role in mGluR-mediated ERK signalling and if this is the route to protein synthesis required for expression of mGluR-LTD. Based on our findings it does not appear that CaMKIIα plays a significant role in mGluR signalling through either ERK or IP3. Therefore, it would be interesting to determine whether AKT or mTOR signalling pathways play a role in mGluR1/5-mediated protein transcription and LTD.

4.4 A potential physiological role of CaMKII in mGluR1a LTD

The relationship between CaMKIIα and mGluR1a-mediated signalling has been hinted at for sometime; however, conflicting results have delayed its full knowledge coming to fruition. Choe et al. (2001) published that CaMKIIα mediates DHPG-stimulated phosphorylation of Elk-1, ERK and CREB. Yet, Schnabel et al. (1999) published that treatment of CaMKII inhibitor, KN-62, facilitates mGluR-LTD. These two studies are

contradictory because phosphorylation of Elk-1, ERK and CREB are required for receptor mGluR-LTD but KN-62 blocks CaMKII's ability to phosphorylate its substrates. This topic was further clouded by the larger discussion of the role of Ca^{2+}, an indirect activator of CaMKII, in mGluR-LTD. Schnabel et al. (1999) went on to rule out Ca^{2+} as a mediator of LTD. This was supported by Fitzjohn et al. (2001) and again by Kasten et al. (2012). Another study showed evidence of Ca^{2+}-dependent mGluR-LTD (Holbro et al., 2009). Connelly et al. (2011) suggested two forms of mGluR-LTD that are distinctly regulated: agonist and synaptic. Agonist mGLuR-LTD was regulated by Ca^{2+} whereas synaptic mGluR-LTD was not. This could help to explain why sometimes mGluR-LTD is dependent on Ca^{2+} and other times it is not. Moreover, mGluR-mediated Ca^{2+} release is required for some forms of LTD and also has been shown to activate CaMKIIα. CaMKIIα is known to be activated by other $G\alpha_{q/11}$ coupled GPCRs via IP3-mediated Ca^{2+} release: D1/D2-R (Ng et al., 2010). Furthermore, it has recently been found to be activated by group 1 mGluRs (Mockett et al., 2011) and again through mGluR5 (Moriguchi et al., 2009).

CaMKIIα plays a role in mediating group 1 mGluR internalization and desensitization (Mundell et al., 2002). This was again confirmed in our results. In addition, there is now mounting evidence that implicates CaMKIIα in mGluR1a-mediated protein translation and eventual expression of synaptic specific LTD (Mockett et al., 2011; Park et al., 2008). Mockett suggested a role for CaMKIIα in mGluR-mediated protein synthesis required by LTD through regulating phosphorylation of initiation factor (elF4E). Park suggested it works through and elongation factor (eEF2). Other studies have suggested that CaMKIIα plays a role in mGluR-mediated ERK activation in LTD (Choe and Wang,

2001). Our results showed no CaMKII-mediated change in ERK1/2 phosphorylation. It remains unclear if CaMKII plays a role in mGluR-mediated LTD through regulation of protein synthesis. Further studies are required to better understand the relationship between these two important regulators of synaptic plasticity.

4.5 Summary

This thesis elucidates the novel association between CaMKIIα and group 1 mGluRs. More specifically CaMKIIα interacts within the second intracellular loop of mGluRs this opens up potential regulatory and functional significance for this domain among class C GPCRs. From our results it seems that CaMKIIα plays an important role in receptor internalization. This effect appears to be enhanced in shorter tail variants such as mGluR5a as compared to mGluR1a.

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CURRICULUM VITAE

Name: **Stephanie Chanel Kulhawy**

Post-Secondary Education and Degrees:

2010-Present	Master of Science Physiology UNIVERSITY OF WESTERN ONTARIO
2006-2010	Honours Bachelor of Medical Science Specialization Medical Sciences UNIVERSITY OF WESTERN ONTARIO

Scholarships and Awards:

2010-2012	CIHR - Strategic Training Program in Vascular Research ($12 000/year)
2010-2011	HSFR Program Grant in Vascular Bio - Grad Student Funding ($3330)
2010-2012	Schulich Graduate Scholarship, UWO ($8 000/year)
2009	John D. Shultz Scholarship, HSFO ($4000)

Related Experience:

2009-2010	Student Researcher Robarts Research Institute - Dr. Stephen Ferguson
2010-2012	Teaching Assistant Physiology and Pharmacology, Western University - Tom Stavraky
2010-Present	Outreach Committee; New Student Representative (2010-2011) Phys & Pharm Graduate Student Council

Conferences:

Great Lakes GPCR Retreat: 2010, 2011*, 2012*
Phys & Pharm Research Day and Stevenson Memorial Lecture: 2010*, 2011*, 2012*
** denotes poster presentation*

Poster Presentation Award:

Kulhawy, S.C., Godin, C., Ferguson, S.S.G. The role of Calcium/Calmodulin dependent protein kinase II alpha in group 1 metabotropic glutamate receptor endocytosis and signaling (2012) Phys & Pharm Research Day and Stevenson Memorial Lecture. The University of Western Ontario, London ON.