8-29-2014 12:00 AM

Regulation of Metabotropic Glutamate Receptor 5 activity by Ca2+/Calmodulin-dependent Protein Kinase IIα

Fitore Raka, *The University of Western Ontario*

Supervisor: Dr. Stephen Ferguson, *The University of Western Ontario*

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

REGULATION OF METABOTROPIC GLUTAMATE RECEPTOR 5 ACTIVITY BY CA^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE II ALPHA

(Thesis format: Monograph)

By

Fitore, Raka

Graduate Program in Physiology and Pharmacology

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

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

ABSTRACT

The metabotropic glutamate receptor 5 (mGluR5) is a GPCR coupled to the heterotrimeric G protein $G\alpha_{q/11}$ and activates signaling pathways important for excitatory synaptic transmission. Emerging studies reveal that Amyloid β (Aβ) acts as an extracellular scaffold for mGluR5. We have identified Ca^{2+}/Calmodulin-dependent protein Kinase IIα (CaMKIIα) as an interacting protein of mGluR5. We hypothesize that CaMKIIα plays a role in mGluR5 signaling and Aβ produces differential effects on the regulation of mGluR5 by CaMKIIα. We find that overexpression of CaMKIIα significantly impairs mGluR5-mediated ERK1/2 phosphorylation but does not effect inositol phosphate formation or Ca^{2+} release. We find that Aβ increases the amount of co-immunoprecipitated CaMKIIα to mGluR5 and can activate mGluR5-mediated ERK1/2 phosphorylation via a PKC-dependent mechanism. mGluR5 and CaMKIIα are involved in learning and memory. Furthermore, Aβ and mGluR5 are implicated in Alzheimer's disease. Thus, investigating how these proteins work together could provide insight for developing treatments for Alzheimer's disease.

KEYWORDS: G protein-coupled receptors, Metabotropic Glutamate Receptors, $Ca^{2+/}$Calmodulin-dependent protein Kinase IIα, Receptor Signalling, Amyloid-β

ACKNOWLEDGEMENTS

I would like to acknowledge and sincerely thank my supervisor, Dr. Stephen Ferguson, for allowing me to pursue my research interests during my time in the lab. Your expertise, guidance and encouragement have enhanced the learning process of my Master's degree. I am thankful to Dr. Stephen Ferguson and my advisory committee member, Dr. Peter Chidiac for spending the time to read this thesis and providing useful suggestions.

I would like to express my gratitude to all of the members of the Ferguson Lab for your training, expertise, and making the lab an enjoyable place to work. A very special thanks goes out to Jessica Esseltine, whose training added considerably to my graduate experience.

Finally, a great deal of gratitude is due to my supporters outside of the lab. I especially thank Ahmed for his continuous support, encouragement and love.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Full Name
Aβ	Amyloid β
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
APP	Amyloid Precursor Protein
Bis-1	Bisindolymaleimide I
CAIN	Calcineurin inhbitor protein
CaM	Calmodulin
CaMKII	Calcium/calmodulin dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
DAG	Diacylglycerol
DHPG	(S)-3,5-Dihydroxyphenylglycine
D3	Dopamine 3
ECL	Enhanced chemiluminescence
ERK	Extracellular signal-regulated kinase
FMRP	Fragile X mental retardation protein
G Protein	Guanine nucleotide-binding proteins
GFP	Green Fluorescent Protein
GPCR	G protein coupled receptors
GRK	G protein-coupled receptor kinase
GTP	Guanosine-5'-trisphosphate
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
MEK	Mitogen-activated protein kinase kinase
mGluR	Metabotropic glutamate receptor
MPEP	2-Methyl-6-(phenylethynyl) pyridine
mRNA	Messenger RNA
NMDA	N-Methyl-D-aspartic acid
PBS	Phosphate buffered saline
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PP2A	Protein Phosphatase 2
PrP^C	Cellular prion protein
PSD	Post synaptic density
Pyk2	Proline-rich tyrosine kinase 2
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

CHAPTER 1

INTRODUCTION

1.1 G protein-coupled receptors

G protein-coupled receptors (GPCRs) make up the largest family of transmembrane receptors that function to transduce extracellular signals into intracellular responses (Lagerstrom and Schioth, 2008). GPCRs are composed of seven transmembrane domains and can be activated by a variety of stimuli including photons, odorants, hormones, amino acids, peptides and neurotransmitters (Hoon et al, 1999). Activation of GPCRs by agonist binding induces a conformational change in the receptor that allows GDP to be exchanged for GTP on the α subunit of the heterotrimeric GTP-binding protein (G protein), subsequently leading to the disassociation of the α subunit from the receptor and the G$\beta\gamma$ subunit. These subunits activate or inhibit a variety of signaling pathways in order to modulate cellular functions (Neer, 1995). Factors affecting GPCR regulation have been studied intensively since they regulate many physiological functions and their dysregulation contributes to the development of many disease states. Furthermore, it is estimated that over 40% of all pharmaceutical drugs target GPCRs or their downstream effectors making them a prime target for the development of novel therapeutic agents (Zhang and Xie, 2012).

GPCRs are classified into three main groups based on sequence homology. Class A includes the rhodopsin-like receptors, Class B includes the secretin/glucagon receptors, and Class C includes a unique member of GPCRs known as the metabotropic glutamate receptors (Lagerstrom and Schioth, 2008). The focus of this thesis will be on the metabotropic glutamate receptors.

1.2 Group I Metabotropic Glutamate Receptors

Glutamate is the major excitatory neurotransmitter in the central nervous system. It exerts its effects by activating two distinct types of receptors: the ionotropic glutamate receptors and the metabotropic glutamate receptors (Figure 1.1). Activation of the ionotropic glutamate receptors such as the N-methyl-D-aspartate (NMDA) and Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)/Kainate receptors mediates a fast and short lasting cellular response by opening cation channels. In order to produce a slow and long lasting cellular response, glutamate activates the metabotropic glutamate receptors (Conn and Pin, 1997; Nakanishi, 1992). There are eight metabotropic glutamate receptors divided into three subgroups based on the signaling pathways they activate, sequence homology and pharmacological characteristics. The Group I metabotropic glutamate receptors (mGluR1 and mGluR5) are excitatory and are coupled to $G\alpha_q$. The Group II (mGluR2 and mGluR3) and Group III metabotropic glutamate receptors (mGluRs4, mGluR6, mGluR7 and mGluR8) are similar in that they are involved in presynaptic inhibition by coupling to $G\alpha_i$. However, these receptors differ in their agonist selectivity (Nakanishi, 1994; Conn and Pin, 1997). The focus of this thesis will be on the Group I mGluRs.

1.2.1 Structure and functional domains

The general structure of Group I mGluRs consists of a long amino (N)-terminal extracellular domain tail, seven transmembrane domains separated by short intracellular and extracellular loops, and an intracellular carboxyl (C)-terminal domain that varies in length between splice variants (Pinn and Duvoisin, 1995). Interestingly, mGluRs share

very little sequence homology with prototypic GPCRs and thus have unique structural differences. Unlike prototypic GPCRs where ligand binding involves pocket formation by the seven transmembrane domains, glutamate binding to mGluRs involves the N-terminal domain (Chun et al., 2012). Group I mGluRs have a long N-terminal domain consisting of 600 amino acids. Structural studies have indicated that the glutamate binding site is located in this region and forms a 'clam shell' upon glutamate binding. This type of ligand binding is known as the venus fly trap model (Kunishima et al., 2000). Besides functioning as ligand binding domains, the N-termini of Group I mGluRs form disulphide bridges which allows for the formation of dimers (Beqollari and Kammermeier, 2010).

In contrast to prototypic GPCRs, where G protein coupling selectivity is mediated by the third intracellular loop, mGluRs have a highly conserved third intracellular loop, which does not determine G protein coupling selectivity. Instead, G protein coupling selectivity of mGluRs is mediated by the second intracellular loop where differences exist between different subtypes of mGluRs (Gomeza et al., 1996). In addition, the second intracellular loop is an important site for interaction with regulatory proteins such as G protein-coupled receptor kinase 2 (GRK2), proline-rich tyrosine kinase 2 (pyk2) and calcineurin inhibitor (CAIN) (Dhami et al., 2002, Nicodemo et al., 2010; Ferreira et al., 2009).

Diversity in the Group I mGluRs occurs as a result of alternative splicing mechanisms within the C-terminal tail. mGluR1 consists of four splice variants (mGluR1a, b, c and d) and mGluR5 consist of two splice variants (a and b). mGluR1a has a long C-terminal tail consisting of 313 amino acids. In contrast, the mGluR1b/c/d splice

variants have short C-terminus tails (20 residues for mGluR1b, 11 for mGluR1c and 26 for mGluR1d) (Conn and Pin, 1997). The differences in length contribute to the differences in affinity for agonists: mGluR1a has a higher affinity for agonist than the shorter mGluR1 splice variants (Flor et al., 1996). Moreover, splice variants display differential distribution in the central nervous system (Ferraguti et al., 1998). Both mGluR5a and mGluR5b splice variants have long C-terminal tails. The C-terminal tail of mGluR5a is 344 amino acids in length and mGluR5b contains an additional 32 amino acids (Joly et al, 1995) (Figure 1.1). Because of the large C-terminal tail of Group I mGluRs this region is important for interacting with many regulatory proteins. The C-terminus of Group I mGluRs encodes for binding motifs for PP1γ1, SIAH-1a, Homer and PDZ domain containing proteins (Enz, 2012). In addition, the C-terminal tail is important for G protein coupling (Francesconi and Duvoisin, 1998).

Pharmacologically, Group I mGluRs are selectively activated by the Group I mGluR agonist (*S*)-3,5-Dihydroxyphenylglycine (DHPG). The most potent agonist of the Group I mGluRs is quisqualic acid, although it can also activate the AMPA receptors. Currently there are no agonists that differentiate between mGluR1 and mGluR5, however the synthetic agonist CHPG is more selective for mGluR5 than mGluR1 (Doherty et al., 1997). There are also antagonists which are selective for the two receptor subtypes: 2-Methyl-6-(phenylethynyl) pyridine (MPEP) is non-competitive inhibitor of mGluR5 and LY367385 competitively inhibits mGluR1 (Gasparini et al., 1999; Clark et al., 1997).

1.2.2 Distribution

Group I mGluRs display differential distribution patterns throughout the central

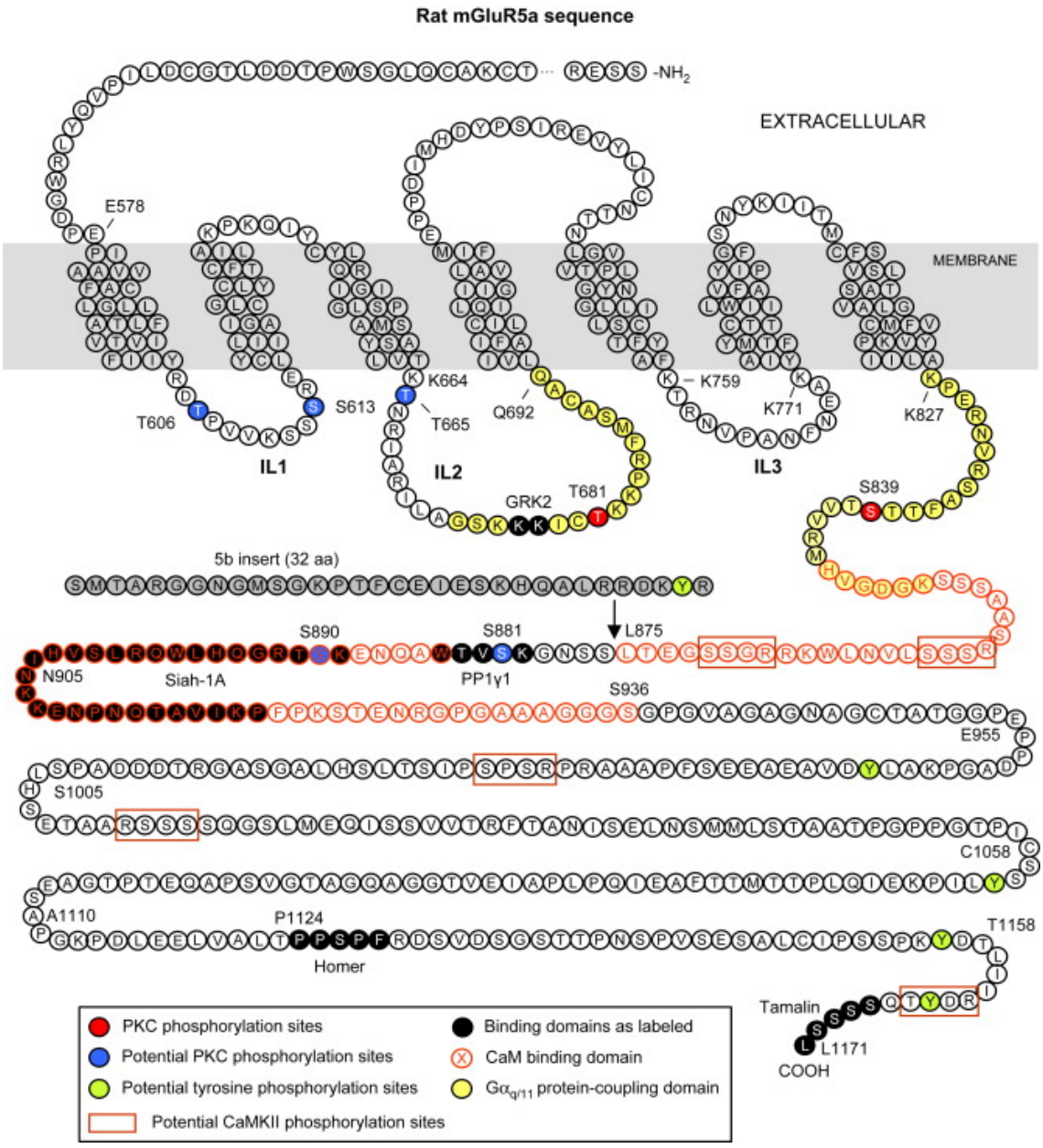

Figure 1.1 Schematic illustration of membrane topology of rat mGluR5. The mGluR5b splice variant contains an insert of 32 additional amino acids after L875. Several binding domains for interacting proteins are labeled as well as potential PKC and CaMKII phosphorylation sites. Residues involved in $G\alpha_{q/11}$ coupling are highlighted in yellow. (Taken from Mao et al., 2008)

nervous system which may contribute to the functional differences observed between mGluR1 and mGluR5 (Ferraguti and Shigemoto 2006). mGluR1 is highly expressed in the Purkinje cells of the cerebellum, CA3 pyramidal cells of the hippocampus, olfactory bulb, thalamus and substantia nigra. In contrast, mGluR5 is expressed in only 10% of the Golgi cells of the cerebellum, but is found to have a higher expression in the cortex, striatum, olfactory bulb and the CA1 and CA3 pyramidal cells of the hippocampus (Ferraguti and Shigemoto, 2006). The expression of Group I mGluRs in the hippocampus plays a role in long-term potentiation (LTP) and spatial learning (Neyman and Manahan-Vaughan, 2008; Balschun et al., 1999). Meanwhile, the predominant expression of mGluR1 in the cerebellum is reported to play a role in long- term depression (LTD) induction and motor learning (Aiba et al., 1994).

More complexity occurs as a result of differential distribution of mGluR1 splice variants. A prime example is the predominant expression of mGluR1a in the cerebral Purjinke cells and mGluR1b in the hippocampal pyramidal cells (Ferraguti et al., 1998). In contrast, only slight differences occur in the expression of the two mGluR5 isoforms although it has been shown that mGluR5a predominates in the young rat brain while mGluR5b is predominately found in the adult rat brain (Joly et al., 1995; Romano et al., 1996). In addition, mGluR5 expression is also observed in other cell-types of the brain such as astrocytes and microglia (Schools and Kimelberg, 1999; Biber et al., 1999). Besides the central nervous system, Group I mGluRs have been shown to be present in the rat spinal cord, heart cells, hepatocytes, osteoblasts and human melanocytes (Dhami and Ferguson, 2006).

At the subcellular level, the Group I mGluRs are predominately located on the

perisynaptic region of the post synaptic membrane where they can modulate ion channels to regulate neuronal excitability (Lujan et al., 1996). Interestingly, mGluR5 is also expressed on the intracellular membranes of the endoplasmic reticulum and nucleus, where it plays a role in regulating gene expression and activates signaling cascades distinict from cell surface counterparts (Kumar et al., 2012; Jong et al., 2009).

1.2.3 Group I metabotropic glutamate receptor signaling

As previously mentioned, Group I mGluRs are coupled to $G\alpha_{q/11}$. Upon agonist stimulation, Group I mGluRs change conformation allowing the exchange of GDP to GTP on the $G\alpha_{q/11}$ protein. The activation of the $G\alpha_{q/11}$ protein results in the activation of phospholipaseCβ (PLCβ) which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) on the plasma membrane producing the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). IP3 interacts with the IP3 receptor on the endoplasmic reticulum resulting in the release of Ca^{2+} from intracellular stores. The increase in both Ca^{2+} and DAG activates protein kinase C (PKC) which in turn can activate other downstream signaling pathways (Pinn and Duvoisin, 1997). Although Group I mGluRs are predominately coupled to $G\alpha_{q/11}$, they have also been shown to couple to other G proteins, such as $G\alpha_s$ and $G\alpha_{i/o}$ in a variety of cell lines (Franscesconi and Duvoisin 1998; Ribeiro et al., 2010a). Activation of $G\alpha_s$ stimulates adenylyl cyclase to produce cAMP, an important second messenger. Conversely, activation of $G\alpha_{i/o}$ negatively regulates adenylyl cyclase and inhibits cAMP formation.

Activation of PKC can lead to the activation of phospholipase D, phospholipase A_2, mitogen activated protein kinase (MAPK) as well as the modulation of a variety of

ion-channels (Dhami and Ferguson, 2006). mGluR1-mediated PKC activation has been shown to phosphorylate the NMDA receptor leading to increased NMDA receptor channel conductance, and therefore enhanced synaptic excitability (Skeberdis et al., 2001). In addition, PKC is believed to be involved in the two distinct types of Ca^{2+} responses. mGluR5-mediated Ca^{2+} signaling is unique from that of mGluR1 as mGluR5 activation results in high frequency Ca^{2+} oscillations, whereas activation of mGluR1 shows low frequency Ca^{2+} oscillations (Kawabata et al., 1998, Dale et al. 2001). The proposed mechanism involves the repetitive translocation of PKC between the cytoplasm and the plasma membrane in response to repetitive G protein coupling. The oscillation frequency is controlled by a single amino acid residue in the G protein coupling domain that differs between mGluR1 and mGluR5 (Dale et al., 2001). The activation of MAP kinases through Group I mGluRs involves a variety of mechanisms and will be discussed in detail below.

1.2.4 Activation of Mitogen Activated Kinases

Protein phosphorylation is a common post-transcriptional modification that functions to either activate or deactivate an enzyme and is widely seen in signaling cascades. The mitogen-activated protein (MAP) kinases are a family of serine/threonine kinases involved in the transduction of externally derived signals. This family includes the extracellular signal-regulated kinases ERK1 and ERK2, also known as p42/p44 MAP kinases. ERK1/2 can be activated by a variety of cell surface receptors and therefore acts as convergence point for integration of signals (Roskoski, 2012). Initially, ERK1/2 activation was studied in mitogenic signaling downstream of receptor tyrosine kinases. In

this signaling cascade, activation of Ras leads to the activation of the protein kinase c-raf which in turn activates the mitogen-activated protein kinase kinases (MEK1 and MEK2). MEK1/2 are dual function kinases that in turn carry out the phosphorylation and activation of ERK1/2 (Roskoski, 2012). Activated ERK1/2 have a variety of cellular and nuclear substrates that they act upon to regulate cell adhesion, migration, differentiation, proliferation, and transcription (Roskoski, 2012). Interestingly, this signaling pathway is upregulated in a variety of cancers (Pearson et al., 2001).

ERK1/2 phosphorylation can also be induced through activation of GPCRs including the Group I mGluRs through a number of mechanisms depending on cell type (Ferraguti et al., 1999; Choe and Wang, 2001; Karim et al., 2001). Group I mGluR-mediated ERK1/2 phosphorylation is observed in Chinese hamster ovary cell lines (Thandi et al., 2002). In this cell line mGluR5-mediated ERK1/2 activation is Ca^{2+}-independent, although other studies using striatal neurons have reported that Ca^{2+} release from intracellular stores via the activation of PLCβ contributes partially to ERK1/2 phosphorylation (Mao et al., 2005a). In striatal neurons mGluR5-mediated ERK1/2 activation is largely due to interaction with Homer proteins (Mao et al., 2005a). Homer1b/c proteins directly interact with mGluR5 to couple the receptor to ERK1/2 phosphorylation, since the disruption of the mGluR5-Homer1b/c interaction by TAT interference peptides inhibits ERK1/2 phosphorylation (Mao et al., 2005a). In rat striatal neurons mGluR5 mediated ERK1/2 phosphorylation appears to involve protein phosphatase 2A (PP2A) (Mao et al., 2005b). mGluR5 associates with PP2A and activation of mGluR5 inhibits this interaction and decreases PP2A activity. Consequently, the decrease in PP2A activity contributes to mGluR5-mediated ERK1/2

phosphorylation (Mao et al., 2005b). Our lab has found that pyk2 facilitates mGluR1-dependent ERK1/2 phosphorylation in mouse cortical neurons (Nicodemo et al., 2010)

In cortical astrocytes, mGluR5-mediated ERK2 activation is independent of PLCβ1 however and requires the transactivation of the EGF receptor, a receptor tyrosine kinase (Peavy et al., 2001). Interestingly, it appears that transactivation of receptor tyrosine kinases is a common mechanism by which GPCRs activate the ERK1/2 pathway as this mechanism is described for a variety of GPCRs such as the thrombin, angiotensin II, bradykinin, endothelin, and purinergic receptors (Daub et al., 1997; Soltoff, 1998; Adomeit et al., 1999; Della Rocca et al., 1999; Seo et al., 2000).

Another important pathway for ERK1/2 activation involves β-arrestins. β-arrestins are well known for their role in the desensitization and internalization of GPCRs (Ferguson 2001). However, they have also been reported to function as signal transducers by forming signaling complexes (Ferguson, 2001; Luttrell and Lefkowitz, 2002). Emery et al., (2010) found that sustained activation of ERK1/2 phosphorylation through mGluR1 is dependent on β-arrestin1. This signaling pathway is distinct from the G protein-mediated transient ERK1/2 phosphorylation and is shown to be neuroprotective (Emery et al., 2010). β-Arrestin-mediated ERK1/2 signaling has also been reported for other GPCRs including the angiotensin II type 1a receptor (Luttrell et al., 2001), D3 dopamine receptor (Beom et al., 2004), β2-adrenergic receptor (Luttrell et al., 1999; Shenoy et al., 2006) and the μ opioid receptor (Macey et al., 2006).

1.2.5 Regulation by interacting proteins

Protein interactions with GPCRs represent a large source for novel drug targets.

Group I mGluRs interact with a number of regulatory proteins that are important for receptor trafficking, protein scaffolding and coupling to distinct signaling cascades (Enz, 2012). Work done in our laboratory has characterized the interaction between GRK2, CAIN and Pyk2 with the second intracellular loop domain of Group I mGluRs (Dhami et al., 2002, Ferreira et al., 2009, Nicodemo et al., 2010). GRK2 interacts with the second intracellular loop, as well as the C-terminal tail of Group I mGluRs. This interaction functions to attenuate Group I mGluR-mediated signaling and contributes to phosphorylation-independent internalization of Group I mGluRs in striatal neurons (Dhami et al, 2002, 2004, 2005; Ribeiro et al., 2009). Similarly, CAIN interacts with the second intracellular loop and C-terminus tail and attenuates mGluR1-mediated signaling (Ferreira et al., 2009). As previously mentioned, the second intracellular loop is important for G protein coupling. It appears that the interaction between second intracellular loop interacting proteins disrupts coupling to $G\alpha_{q/11}$ thus attenuating the signaling response.

The long C-terminus tail of Group I mGluRs also harbours binding sites for many interacting proteins. The most studied are the Homer proteins, which are a family of multifaceted scaffolding proteins (Thomas 2002). Homer proteins bind to the proline rich region of the C-termius tail of Group I mGluRs and are proposed to physically link both mGluR1 and mGluR5 to the IP3 receptor thereby modulating Ca^{2+} release (Tu et al., 1998). In addition, the interaction between mGluR5 and Homer3 has been shown to prevent constitutive activity of the receptor (Ango et al., 2001). Other proteins interact with the C-terminal tail to modulate receptor cell surface expression and trafficking. Calmodulin (CaM) interacts with mGluR5 and is reported to play a role mGluR5 trafficking (Lee et al., 2008). Specifically, CaM overexpression increases cell surface

expression of mGluR5 while the knockdown of CaM decreases cell surface expression. CaM also competes with PKC for binding to the C-tail of mGluR5 and thus regulates PKC-mediated trafficking of mGluR5 (Lee et al., 2008).

1.2.6 Physiological roles of Group I mGluRs

Group I mGluRs have been extensively studied in experimental animals leading to the conclusion that they are involved in many aspects of brain function. In the brain, activation of Group I mGluRs plays a complex role that can be both neuroprotective and neurotoxic (Nicoletti et al., 1999; Caraci et al., 2012). Group I mGluRs have been implicated in the two forms of synaptic plasticity: LTP and LTD, indicating that are involved in the underlying mechanisms for learning and memory (Riedel and Reyman 1996, Ayala et al., 2009; Neyman and Vaughan, 2008). LTP involves a mechanism in which persistent synaptic activity leads to a pronounced increase in synaptic strength. In contrast, in LTD an absence of synaptic activity leads to a pronounced decrease in synaptic strength (Anwyl, 1999).

LTP is thought to occur in two phases: early phase LTP and late phase LTP. Early phase LTP is mediated by the activation of NMDA receptors which results in increased intracellular Ca^{2+} and subsequent activation of CaMKII (Lisman et al., 2002). CaMKII is responsible for strengthening of the synapse by increasing the number of AMPA receptors on the synaptic membrane (Lynch, 2004). Importantly, the insertion of AMPA receptors at the synapse occurs in a protein synthesis independent manner. This is achieved by the trafficking of existing nonsynaptic AMPA receptors to the synaptic membrane (Malinow, 2003). In contrast, late phase LTP depends on protein synthesis and

activation of mGluRs (Neyman and Vaughan, 2008). There is now a considerable amount of evidence that shows that certain forms of LTP are dependent on Group I mGluRs (Lu et al., 1997; Jia et a., 1998; Neyman and Vaughan, 2008). The perisynaptic localization of Group I mGluRs at excitatory synapses puts these receptors at an ideal location for regulating synaptic strength. One mechanism involves the mGluR5-mediated enhancement of NMDA receptor conductance (Attuci et al., 2001). In line with this, studies on mGluR5 knockout mice have revealed that NMDA-dependent LTP in the CA1 region of the hippocampus is reduced and that these mice exhibit impaired learning (Lu et al., 1997; Jia et a., 1998).

Besides their role in normal brain functions, Group I mGluRs have also been implicated in a variety of neurological disorders. Perhaps the most studied is the role of mGluR5 in the pathogenesis of Fragile-X syndrome. Fragile-X syndrome is the leading genetic cause of autism in which the gene encoding for Fragile-X mental retardation protein (FMRP) is silenced (Pieretti et al., 1991). FMRP is an RNA binding protein that selectively binds to 4% of mammalian mRNAs and represses their translation (Ashley et al., 1993). mGluR5 plays an important role in Fragile-X since both inhibition mGluR5 signaling and knockdown of mGluR5 improves Fragile-X phenotypes in FMRP knockout mice (Bear et al., 2004; Dölen and Bear, 2008). Additionally, mGluR-dependent LTD is exaggerated in the hippocampus of FMRP knockout mice which could impair synaptic plasticity and lead to memory deficits (Luscher and Huber, 2010)

Because of their involvement in LTP and LTD, it is no surprise that Group I mGluRs play a role in neurodegenerative diseases that affect learning and memory such as Huntington's and Alzheimer's diseases (Ribeiro et al., 2010). Huntington's disease is

an autosomal-dominant neurodegenerative disorder that affects muscle coordination, cognition and mental behavior (Ribeiro et al., 2011). Our lab has demonstrated that Group I mGluR signaling is altered in a knock-in mouse model of Huntington's disease (Ribeiro et al., 2010b; Ribeiro et al., 2014). Specifically, Group I mGluR mediated IP3 formation is decreased and Ca^{2+} release is increased compared to wild type control (Ribeiro et al., 2010b). Moreover, the mGluR5 antagonist MPEP is reported to prolong survival in a mouse model of Huntington's disease (Schiefer et al., 2004).

Several studies have implicated Group I mGluRs in Alzheimer's disease. Alzheimer's disease is a progressive neurodegenerative disease characterized by cognitive impairment, memory loss and behavioral changes (Tsai et al, 2005). Alzheimer's disease has a distinct pathology consisting of Amyloid β (Aβ) plaques and tangles of hyperphosphorylated tau (Holzman et al., 2011; Nelson et al., 2009; Hardy et al., 2002). Aβ can exert its effects by blocking LTP thereby reducing synaptic plasticity. Wang et al., (2004) found that the Aβ-mediated inhibition of LTP can be prevented by the mGluR5 antagonist MPEP. Recently it has been shown that genetic deletion of mGluR5 reverses spatial memory deficits in the APPswe/PS1E9 mouse model of Alzheimer's disease (Hamilton et al., 2014). Furthermore, genetic deletion of mGluR5 in this mouse model results in reduced Aβ oligomer formation compared to the APPswe/PS1E9 mice expressing mGluR5 (Hamilton et al., 2014). Taken together, these observations suggest that Group I mGluRs are important regulators of normal brain function and dysregulation of these receptors can be a factor in disease progression.

1.3 Amyloid β

Aβ is formed from the sequential cleavage of the amyloid precursor protein (APP)

by β- and γ-secretases (Vardy et al., 2005). APP is a transmembrane protein that can be processed by two alternate pathways. In the non-amyloidogenic pathway, α-secretase cleaves APP within the Aβ region thus preventing the formation of the Aβ peptide. In the amyloidogenic pathway, APP is initially cleaved by β-secretase resulting in the release of the N-terminal fragment, sAPPβ. The remaining C-terminal of APP is membrane bound and is cleaved by γ-secretase to produce Aβ peptide and amyloid intracellular domain (Figure 1.2) (Vardy et al., 2005; Chow et al., 2009). Once cleaved, Aβ peptide can form a variety of structures including monomers, oligomers and fibrils (Zhang et al., 2011). Cleavage by γ-secretase results in a number of Aβ peptide isoforms that vary in length between 39 and 43 amino acids. Aβ40 and Aβ42 are the most common Aβ species. However, Aβ42 peptides assemble more readily into oligomeric forms and are a major component of amyloid plaques (Selkoe 2006; Kellett and Hooper, 2009). Furthermore, Aβ42 oligomers correlate better with neurotoxicity than any other form of Aβ (Lacor et al., 2007; Butterfield 2002)

Aβ oligomers can exert their toxic affects by targeting synapses and contributing to synaptic dysfunction and synaptic loss (Lacor et al., 2007, Shankar et al., 2007). In addition, several studies have indicated that Aβ-mediated toxicity involves inhibition of LTP (Walsh et al., 2002). Aβ can suppress both early phase and late phase LTP which can explain the learning and memory deficits associated with Alzheimer's disease (Chen et al., 2000, Zhang et al., 2009; Li et al., 2009). The exact molecular mechanisms underlying Aβ-mediated toxicity are largely unknown. It is proposed that Aβ oligomers may exert their neurotoxic effects by interacting with several cell surface receptors

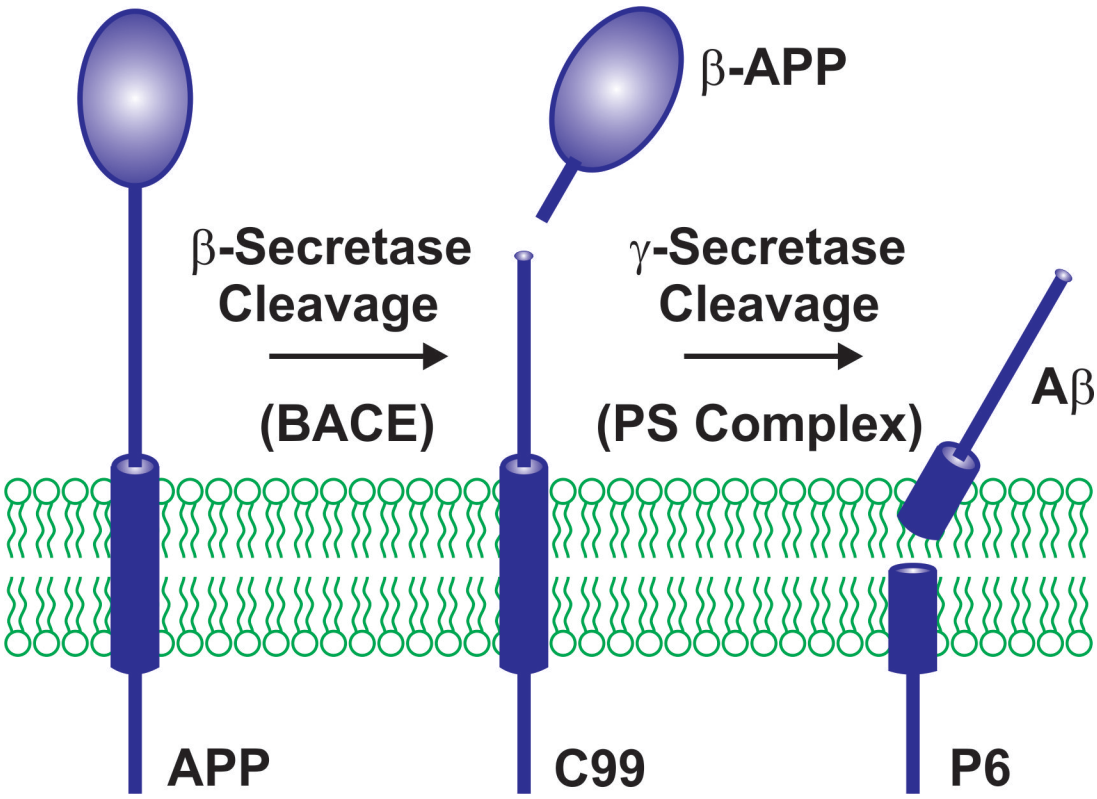

Figure 1.2. Schematic illustration of the β- and γ-site cleavage events required for Aβ formation. The amyloid precursor protein (APP) is a 100 kDa transmembrane protein that is initially cleaved at the beta site by the enzyme BACE. This releases the extracellular APP fragment β-APP leaving the C-terminal APP C99 fragment in the membrane. Cleavage at the beta site can be followed by a second cleavage within the membrane domain of APP by the γ-secretase complex to produce beta amyloid (Aβ). This "gamma-cleavage" determines the relative amount of the more toxic 42 amino acid form of Aβ produced.

(Dinamarca et al., 2012). Several studies have shown that Aβ can affect the function of NMDA receptors. Aβ binds to the NMDA receptor, contributes to NMDA receptor endocytosis and depresses NMDA receptor-evoked currents. (Snyder et al., 2005; Shankar et al., 2007). Aβ can also bind to the α7-nicotinic acetylcholine receptor (α7-nAChR) with high affinity (Wang et al., 2000). The same group discovered that the α7-nAChR can mediate Aβ peptide-induced tau protein phosphorylation (Wang et al., 2003). Aβ42 oligomers have been shown to bind with high affinity to the cellular prion protein (PrP^C) and reduce LTP in hippocampal slices of wildtype mice but not PrP^C-null mice, suggesting that PrP^C mediates Aβ42 oligomer toxicity (Lauren et al., 2009). PrP^C is a glycosylphosphatidylinositol-anchored membrane protein that is highly expressed within the central nervous system (Linden et al., 2007). Its pathogenic form, the abnormally folded scrapie prion protein PrP^{Sc}, is well known for its role in neurodegenerative disorders known as transmissible spongiform encephalopathies (Linden et al., 2007*)*. Normal physiological roles of PrP^C include neuroprotection to oxidative stress, cell adhesion and neurite growth (Linden et al., 2007).

Recent studies have shown that Aβ42 oligomers may produce their toxic effects through mGluR5 (Renner et al., 2010). Aβ42 oligomers induce clustering of mGluR5 resulting in limited lateral diffusion of the receptors and elevated Ca^{2+} levels that cause synaptic deterioration (Renner et al., 2010). Additionally, mGluR5 has been shown to be a co-receptor for the Aβ oligomer-PrP^C complex (Um et al., 2013). Aβ oligomers can increase intracellular Ca^{2+} in neurons in a manner that is dependent on both mGluR5 and PrP^C.

1.4 Ca^{2+}/ Calmodulin-dependent protein kinase II

Regulation of Ca^{2+} signaling is a vital component in the maintenance of cellular homeostasis. In the brain, Ca^{2+} signaling regulates many aspects of neuronal excitability by interacting with a variety of Ca^{2+} sensitive proteins (Zundorf and Reiser, 2011). Ca^{2+}/CaM-dependent protein kinase II (CaMKII) is a serine/threonine kinase that is sensitive to Ca^{2+} signaling since it is activated by Ca^{2+}/CaM binding when intracellular Ca^{2+} is increased. CaMKII comprises a family of 28 similar isoforms derived from four genes (α, β, γ, and δ). The expression of the γ and δ isorforms is ubiquitous whereas the α and β isoforms are restricted to neuronal tissue (Lisman et al., 2002). CaMKIIα and CaMKIIβ are highly abundant in the brain where they are estimated to make up 1-2% of the total protein (Erondu and Kennedy, 1985). Specifically, CaMKIIα/β is expressed in the cortex, striatum, cerebellum, hypothalamus, olfactory bulb and its highest expression is observed in the hippocampus (Erondu and Kennedy, 1985). At the subcellular level, CaMKIIα/β can be found in the cytoplasm as well as a protein dense region of the post-synaptic membrane known as the post synaptic density (PSD) (Hanson and Schulman, 1992). This thesis will focus on the CaMKIIα isoform.

1.4.1 Structure and activation of CaMKII

Each isoform of CaMKII consists of an autoinhibitory domain, a catalytic domain, a self-association domain and a variable segment. The self-association domain at the C-terminus allows for the assembly of a holoenzyme comprised of 12 subunits. The resulting structure resembles that of a hub and spoke in which the catalytic domains are arranged in a ring around a central scaffold formed by the self-association domains

(Lisman et al., 2002; Chao et al., 2011). Under basal conditions, the autoinhibitory domain binds to the catalytic domain, preventing any kinase activity. An increase in intracellular Ca^{2+} favours Ca^{2+}/CaM to bind to the autoinhibitory domain relieving the inhibition to the catalytic domain. This allows for the exposure of the Thr 286 site on the autoinhibitory domain (Thr 287 on CaMKIIβ), which can be phosphorylated by adjacent subunits of CaMKII in a process known as autophosphorylation. Once this site is phosphorylated the autoinhibitory domain can no longer inhibit the catalytic domain and the catalytic domain can remain active even after Ca^{2+}/CaM levels fall. This contributes to the persistent or autonomous activity of the kinase (Hanson and Schulman, 1992). Two molecules of Ca^{2+}/CaM are required to initiate the activation of the CaMKII holoenzyme. One binds to one subunit and activates it while the other Ca^{2+}/CaM molecule binds to an adjacent subunit in order to expose the Thr 286/287 site which gets phosphorylated by the activated subunit. In contrast, propagation of activating all 12 subunits requires binding of only one Ca^{2+}/CaM molecule to a subunit adjacent to the one that is already phosphorylated (Figure 1.3) (Lisman et al., 2002).

1.4.2 Role of CaMKII

CaMKII can phosphorylate a broad range of substrates and regulate a variety of cellular functions. In the heart, CaMKIIδ has been shown to be in involved in cardiomyocyte hypertrophy by regulating the expression of hypertrophic markers (Zhang and Brown, 2004). In the brain, CaMKII has been shown to be an important player in synaptic plasticity. The Threonine residue 286 on CaMKIIα in particular, has been shown to contribute to the induction of LTP (Lisman et al., 2002; Lee et al., 2009). It was found

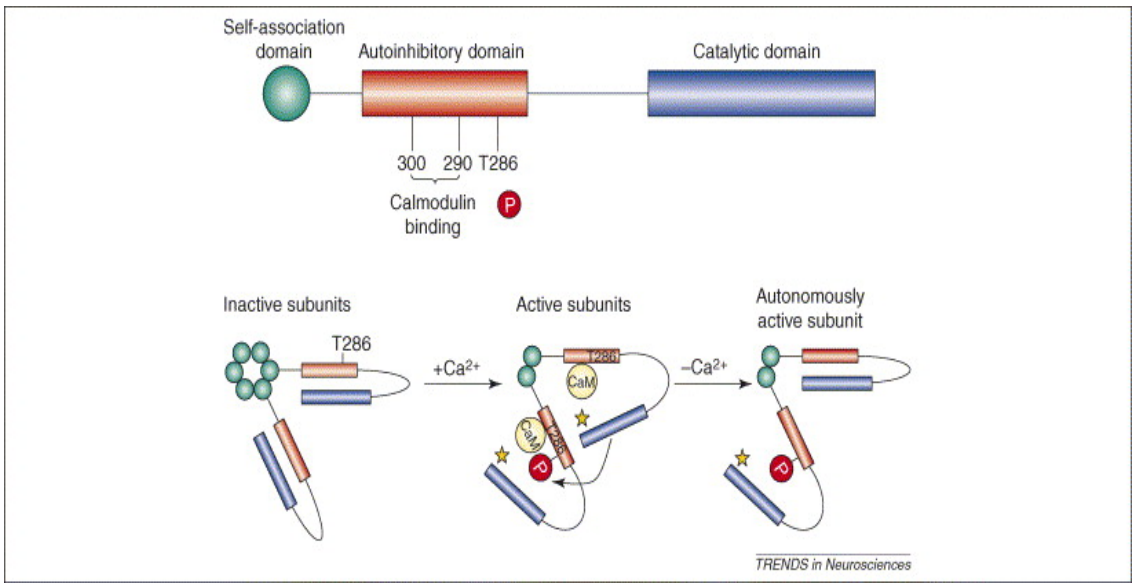

Figure 1.3 Representative diagram of CaMKII structure and activation. Each CaMKII subunit is composed of a self-association domain, a catalytic domain and an autoinhibitory domain. Twelve subunits of CaMKII join together at the self-association domain to form a holoenzyme. Under basal conditions, the autoinhibitory domain binds to the catalytic domain to inhibit its activity. Upon Ca^{2+}/CaM binding the catalytic domain becomes active and can phosphorylate an adjacent CaMKII subunit. Phosphorylation keeps the autoinhibitory domain from inhibiting the catalytic domain even after Ca^{2+}/ CaM fall resulting in autonomous activity of the enzyme. (Taken from Lisman et al., 2002)

that prevention of CaMKIIα activation by mutation of this residue results in impaired LTP induction and mice exhibiting this mutation show profound memory impairment (Giese et al., 1998; Lucchesi et al., 2011). Furthermore, mice exhibiting enhanced learning have increased activation of CaMKII (Lee and Silvia, 2009)

1.4.3 Regulation of ionotropic glutamate receptors.

CaMKII plays an important role in LTP by interacting with the ionotropic glutamate receptors. Once CaMKII is activated by increased intracellular Ca^{2+}, it can translocate from the cytoplasm to the PSD (Shen and Meyer, 1999). Here, CaMKII can bind to a variety of different proteins and receptors. The binding to the NMDA receptor has been key to elucidating the role of CaMKII in LTP induction. It is well established that stimulation of NMDA receptors in cultured neurons produces an increase in Ca^{2+} influx which activates CaMKII. Activated CaMKIIa can then translocate to the PSD where it binds to the NR2B subunit of the NMDA receptor (Strack et al., 1997; Leonard et al., 1999). The formation of the NMDA-CaMKII complex has been shown to play an important role in LTP since transgenic mice overexpressing a mutant form of NR2B which interferes with CaMKII binding have a 50% reduction in LTP and show learning impairments (Barria and Malinow, 2005; Zhou et al., 2007). Importantly, the binding of CaMKII to the NMDA receptor keeps the kinase in an active state (Bayer et al., 2001).

LTP induction and maintenance is also dependent on AMPA receptor activity. One mechanism involves the phosphorylation of AMPA receptors at a specific residue within the GluR1 subunit by CaMKII (Lee et al., 2000). The translocation of CaMKII to the PSD puts the kinase at an ideal location to phosphorylate the Ser 831 residue on the

GluR1 subunit of AMPA receptors leading to an increase in conductance of these channels and enhanced LTP (Barria et al., 1997; Poncer et al., 2002). Another mechanism involves the increase in AMPA receptors in the synapse. AMPA receptor trafficking to the synapse is positively regulated by the stargazin-PSD95 complex (Schnell et al., 2002; Bats et al., 2007). It is hypothesized that CaMKII potentiates this by phosphorylating stargazin which allows stargazin to bind to PSD95 thereby increasing the number of AMPA receptors at the cell surface (Lisman et al., 2012). These mechanisms work together to enhance LTP.

1.4.4 Regulation of G protein-coupled receptors

Emerging studies reveal that CAMKIIα can also regulate several GPCRs. CaMKIIα has been shown to regulate behavioral responses to cocaine by regulating the Dopamine 3 (D3) receptor, a GPCR coupled to $G\alpha_i$ (Liu et al., 2008). CaMKIIα binds to the long third intracellular loop of the D3 receptor and activation of CaMKIIα by $Ca^{2+/}$CaM, as well as Thr 286 autophosphorylation enhances the interaction to the D3 receptor. CaMKIIα phosphorylates the D3 receptor and contributes to receptor desensitization, thereby relieving the inhibition of cAMP accumulation induced by D3 receptor agonists (Liu et al., 2008). CaMKIIα-dependent desensitization has also been reported for the D1/D2 heterodimer (So et al., 2008) and the Histamine H1 receptor (Hishinuma and Ogura, 2000).

Another $G\alpha_i$ coupled GPCR, the M4 muscharinic acetylcholine receptor has been shown to be regulated by CaMKIIα (Guo et al., 2010). CaMKIIα binds to the second intracellular loop of the M4 receptor and potentiates its signaling. Interestingly, this interaction is enhanced by CaMKIIIα activation and autophosphorylation similar to that which occurs at the D3 receptor. Experiments using interference peptides designed to

block this interaction block the M4 receptor-mediated decrease in cAMP accumulation (Guo et al., 2010). Consequently, because motor activity in the brain is coordinated by M4 receptors, the CaMKIIα regulation of M4 receptor contributes to the integration of signals required for motor control (Guo et al., 2010).

CaMKIIα is well studied in context of the ionotropic glutamate receptors. However, very little is known about the effects of CaMKIIα on metabotropic glutamate receptors. Of all eight mGluRs, CaMKIIα has only been reported to regulate mGluR1 (Mundell et al., 2002; Jin et al., 2013a). CaMKIIα appears to play a role in mGluR1 internalization: inhibition of CaMKIIα blocks the glutamate-stimulated association of GRK2 with mGluR1a suggesting CaMKIIα plays a role in inhibiting mGluR1 internalization (Mundell et al., 2002). Recently, CaMKIIα has been shown to play a direct role in the regulation of mGluR1 by physically interacting with the C-terminus tail of mGluR1 (Jin et al., 2013a). Similar to that of the D3 dopamine and M4 muscarinic receptor, activation of CaMKIIα by Ca^{2+}/CaM binding as well as autophosphorylation potentiates CaMKIIα binding to mGluR1. In rat striatal neurons, stimulation with DHPG increases the amount of CaMKIIα co-immunoprecipitated with mGluR1a showing that this occurs in physiologically relevant tissue. Functionally, CaMKIIα phosphorylates mGluR1 at Thr 871 on the C-terminus tail and desensitizes mGluR1 mediated IP3 production in rat striatal neurons (Jin et al., 2013a). These studies suggest that CaMKIIα can be an important regulator of GPCR activity including mGluR1.

1.5 Preliminary data and rationale

In collaboration with Dr. Stephane Angers, we have identified CaMKIIα as a novel second intracellular loop (IL2) interacting protein of Group I mGluRs. A

membrane permeant Tat-IL2-FLAG peptide was used to screen for novel IL2 interacting proteins. Mouse cortical neurons were treated with the Tat-IL2-FLAG peptide and the Tat-IL2-FLAG peptide was subsequently immunoprecipitated with FLAG agarose beads. Immunoprecipitates were trypsinized and analyzed by Maldi-TOF Mass Spectroscopy. mGluR1-IL2 interacting proteins identified in the screen included CaMKII isoforms α, β, γ, δ.

Work done previously in our laboratory by Stephanie Kulhawy as part of her M.Sc. thesis has confirmed the interaction between CaMKIIα and Group I mGluRs in HEK 293 cells. When FLAG-mGluR1/5 was immunoprecipitated, co-immunoprecipitation of GFP-CaMKIIα occurred with both mGluR1a and mGluR5a and this interaction was independent of agonist stimulation with quisqualate. Additionally, CaMKIIα was shown to interact with mGluR5 in mouse hippocampal tissue indicating that this interaction takes place in physiologically relevant tissue. Furthermore, using a GST-pull down assay, it was established that the second intracellular loop of mGluR1 is sufficient for this interaction to occur.

When looking at the effects of CaMKIIα on mGluR1a signaling, it was found that CaMKIIα overexpression had no significant effect on mGluR1a-mediated ERK1/2 phosphorylation or IP3 formation. However the role of CaMKIIα on mGluR5-mediated signaling remains unknown. Additionally, it was found that CaMKIIα significantly enhances agonist-mediated mGluR5 internalization but not mGluR1 internalization suggesting that CaMKIIα has more of a pronounced effect on mGluR5. Therefore this thesis will focus on the role of CaMKIIα on mGluR5 activity.

1.6 Goals and Significance of Research

Interacting proteins play a vital part in GPCR regulation. As previously addressed, CaMKII has been shown to be an important regulator of GPCRs including mGluR1. Furthermore, it is well established that the second intracellular loop of Group I mGluRs is an important region for receptor regulation by interacting proteins. Preliminary data provide evidence for a role for CaMKIIα in mGluR5 internalization, however whether CaMKIIa plays a role in mGluR5 signaling is unknown. In this study we hypothesize that CaMKIIα plays a role in regulating mGluR5 mediated signaling and that Aβ oligomer interactions with mGluR5 may alter CaMKIIα/mGluR5 interactions. We hypothesized that Aβ oligomers will produce differential effects on the regulation of mGluR5 by CaMKII.

As previously mentioned, mGluR5 is involved in a variety of signaling pathways that can be both neuroprotective and neurotoxic. However, factors that differentiate between these outcomes are poorly understood. Furthermore, alterations in the regulation of mGluR5 may lead to the progression of several neurodegenerative diseases. Therefore, by elucidating the roles of its interacting proteins, such as CaMKIIα, on mGluR5 regulation, we may begin to better understand the mechanisms by which mGluR5 is regulated, as well as identify novel therapeutic targets for the treatment of neurodegenerative diseases related to mGluR5.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Human embryonic kidney (HEK293) cells were obtained from American Type Culture Collection (Manassas, VA, USA). Fetal Bovine Serum (FBS), minimum essential medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), 0.25% trypsin, and rabbit anti-GFP antibody were all purchased from Invitrogen Corporation (Burlington, ON, Canada). Goat anti-rabbit IgG (H+L)-HRP conjugate secondary antibody, ECL western blotting detection reagents, Dowex 1-X8 (formate form) resin 200-400 mesh and Bradford reagent for protein assays were all obtained from Bio-Rad (Mississauga, ON, Canada). Sheep anti-mouse IgG secondary antibody and Protein G Sepharose 4 Fast Flow were from GE Healthcare (Mississauga, ON, Canada). Phospho-p44/42 MAP kinase antibody and p44/42 MAP kinase antibody were purchased from Cell Signaling Technology (Mississauga, ON, Canada). Anti-FLAG antibody and rabbit anti-FLAG M2 Agarose were purchased from Sigma (St. Louis, MO, USA). Anti–mGluR5 rabbit IgG was purchased from Millipore (Billerica, MA, USA). Quisqualate and KN-93 were purchased from TOCRIS (Minneapolis, MN, USA). Myo-[3H] Inositol was purchased from Perkin Elmer (Waltham, MA, USA). Fura-2 AM and Zenon antibody labelling reagents were purchased from Molecular Probes (Billerica, MA, USA). $A\beta_{42}$ peptide was purchased from American Peptide (Vista, CA, USA). Bisindolymaleimide I (Bis-1) hydrochloride was purchased from Calbiochem (San Diego CA, USA). The GFP-CAMKIIα cDNA was a gift from Dr. Paul De Koninck (University Laval).

2.2 Cell culture and transfection

HEK 293 cells were maintained in MEM supplemented with 10% (v/v) fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. Cells were plated on 100 mm dishes and transfected using a modified Ca^{2+} phosphate method (Ferguson and Caron, 2004) with the amount of cDNA indicated in the *Figure Legends*. Briefly, cDNAs were diluted in 450 μL of sterile distilled water and 50 μL of 2.5M $CaCl_2$ was added to the mix. Next, 500 μL of 2X HEPES-balanced salt solution (0.28 M NaCl, 0.05M HEPES, 1.5 mM Na_2HPO_4, pH 7.10) was slowly dripped over the cDNA/$CaCl_2$ solution. The solution was mixed and immediately applied to cells grown to 75-85% confluency drop by drop. Cells were washed twice with PBS 16-18 hours after transfection and allowed to recover in MEM for 6 hours before being reseeded for experimentation. Experiments were performed the following day except otherwise indicated.

2.3 Co-immunoprecipitation

HEK 293 cells were transiently transfected with various cDNA constructs as described in the *Figure Legends*. One day after transfection, cells were starved for 1 hour in 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 $MgSO_4$, 2.5mM $CaCl_2$, pH 7.4) and subsequently stimulated as indicated in the *Figure Legends*. Cells were then lysed with ice-cold lysis buffer. (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100) and insoluble material was pelleted. FLAG-mGluR5 was immunoprecipitated from transfected HEK 293 cell lysates using FLAG M2-Agarose prepared as a 50:50 slurry with PBS. 200 μg of protein was

rotated with 50 μl of the slurry for 1 hour at 4°C. The beads were washed 3 times with PBS and eluted with 100 μl of SDS loading buffer containing β-mercaptoethanol. The eluted proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoprecipitated and co-immunoprecipitated proteins were detected by Western Blot.

2.4 GST pull down

GST-mGluR1a-IL2 and mutants were cloned into a pGEX4T1 vector and transformed into *E.coli* recombinant bacteria. *E. coli* bacteria were grown at 37°C with shaking until OD_{600} was 0.6-1.0. Cultures were then induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 3 hours at 23°C. Cells were pelleted and lysed in lysis buffer (500 mM NaCl, 0.5% NP-40, 50 mM Tris pH7.6, 5 mM EDTA, 5 mM EGTA) containing protease inhibitors (2 mM AEBSF, 50 mg/ml aprotinin, 20 mg/ml leupeptin) and sonicated (3 times for 10 seconds) at 4°C. Insoluble material was pelleted at 15000 g for 15 minutes at 4°C. 50 μl of Glutathione-Sepharose bead slurry was incubated overnight with 1 ml of solubilized protein to purify GST-fusion constructs. Glutathione-Sepharose beads were then washed 3 times in PBS and 500 μg of HEK 293 cell lystate overexpressing GFP-CAMKIIα was added to the GST-fusion peptide bound to matrix and rotated for 1 hour at 4°C. Glutathione-Sepharose beads were then washed 6 times in PBS and eluted with 3X SDS loading buffer containing β-mercaptoethanol. Samples were subjected to SDS-PAGE and membranes were immunoblotted with GFP to determine if GFP-CAMKIIα was pulled down with the GST-mGluR1a-IL2 peptides.

2.5 ERK1/2 phosphorylation

Twenty-four hours after transfection, cells were reseeded into 6-well dishes. The following day, cells were starved in DMEM overnight. On the day of experiment, cells were starved for an additional hour in HBSS. Cells were then stimulated at 37°C with either 50 μM Quisqualate or 100 nM Aβ42 oligomers as indicated in the *Figure Legends*. For experiments using the PKC inhibitor, 1 μM Bis-1 or DMSO (vehicle) was added 30 minutes prior to stimulating cells. Cells were subsequently lysed in lysis buffer (25 mM HEPES, 300 mM NaCl, 1.5 mM $MgCl_2$, 200 μM EDTA, 1% Triton-X) containing protease and phosphatase inhibitors (1 mM AEBSF, 25 mg/ml aprotinin, 10 mg/ml leupeptin, 10 mM NaF, 100 μM Na_3VO_4) rocking at 4°C. Lysates were collected and centrifuged at 15000 RPM for 15 minutes at 4°C. Protein concentration was determined using a Bradford protein assay. The lysates were mixed with SDS loading buffer containing β-mercaptoethanol prior to gel loading. ERK1/2 phosphorylation was determined by immunoblotting for phospho-ERK1/2 and Total-ERK1/2 and the ratio was normalized to basal levels.

2.6 Inositol phosphate formation

Forty-eight hours after transfection, inositol lipids were radiolabelled by incubating HEK293 cells overnight with 1 μCi/ml [^{3}H]myo-inositol in DMEM. Unincorporated [^{3}H]myo-inositol was removed by washing the cells 3X with warm HBSS. HEK 293 cells were then incubated for 1 hour in warm HBSS at 37°C and then for an additional 10 minutes in HBSS containing 10 mM LiCl. Subsequently, cells were treated with increasing concentrations of quisqualate (0-30 μM) for 30 minutes at 37°C.

The reaction was terminated by placing the cells on ice and adding 500 μl of 0.8 M perchloric acid and then neutralized with 400 μl of 0.72 M KOH and 0.6 M $KHCO_3$. Total [^{3}H]myo-inositol incorporated into the cells was determined by counting the radioactivity present in 50 μl of cell lysates. Total inositol phosphate was purified from the cell lysates by anion exchange chromatography using Dowex 1-X8 (formate form) 200-400 mesh anion exchange resin. [^{3}H]myo-inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation counter.

2.7 Calcium Imaging

HEK 293 cells were washed 2X in KRH buffer (125 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2.6 mM $MgSO_4$, 5 mM HEPES, pH 7.2). mGluR5 was labelled with rabbit anti-FLAG-conjugated Zenon Alexa Fluor 555 antibody for 20 minutes. Intracellular Ca^{2+} was then labeled with 5 μM fura-2 acetoxymethyl ester (AM) in KRH buffer for 30 minutes and washed off prior to loading onto PTI DeltaRam microscope. Cells expressing both FLAG-mGluR5 and GFP-CaMKII or GFP empty vector were selected to be imaged. Fluorescence intensity was examined by illuminating the cells with 340 and 380 nm and the intensity values at each excitation were recorded. After obtaining a one minute baseline, cells were stimulated with 30 μM Quisqualate for 5 minutes. Concentration of Ca^{2+} was obtained by using the formula $[Ca^{2+}] = Kd * [R-Rmin]/ [Rmax-R] * Fmax/Fmin$, where R is the Fura-2 340/380 ratio, Kd =.761 μM, Rmin=0.196, Rmax=6.907, and Fmax/Fmin=9.558, as determined by calibration experiments. Area under the curve was determined using GraphPad software.

2.8 Immunofluorescence confocal microscopy

Confocal microscopy was performed using a Zeiss LSM-510 laser scanning microscope equipped with a Zeiss 63X 1.4 numerical aperture oil immersion lens. Live cell imaging was performed on HEK293 cells in 35mm glass-bottomed plates. mGluR5 was labelled with rabbit anti-FLAG conjugated Zenon Alexa Fluor 647 antibody and PrP^C was labeled with mouse anti-PrP^C conjugated Zenon Alexa Fluor 555 IgG2B antibody. Visualization of labelled proteins with GFP-CaMKIIα was performed by triple excitation (488/543/647 nm), and emissions were collected using these filter sets: band pass from 505-530 (GFP), long pass at 560 (Alexa Fluor 555) and 660 (Alexa Fluor 647). For internalization experiments, FLAG-mGluR5 was labelled with rabbit anti-FLAG-conjugated Zenon Alexa Fluor 555 antibody. Visualization of antibody-labelled receptor with GFP-CaMKIIα was performed by dual excitation (488/543 nm) and emission band pass from 505-530 (GFP) and long pass at 560 (Alexa Fluor 555) filter sets. Receptor was stimulated with the addition of 30 μM Quisqualate (final concentration) for 20 minutes.

2.9 Immunoblotting

Nitrocellulose membranes were blocked for 1 hour with 10% milk in Tris-buffered Saline with Tween 20 (TBS-T) (150mM NaCl, 10 mM Tris HCl pH 7.0, and 0.05% Tween 20) and subsequently incubated overnight with primary antibody (1:1000) in TBS-T containing 3% milk. Membranes were washed 3X in TBS-T and then incubated with secondary horseradish peroxidase conjugated goat anti-rabbit IgG (1:5000) or sheep anti-mouse IgG (1:5000) in TBS-T containing 3% milk for 1 hour. Membranes were then rinsed 3X with TBS-T and incubated with enhanced chemiluminescence Western blotting

detection reagents and visualized using a Chemidoc Imaging System.

2.10 Aβ42 oligomer formation

Aβ42 peptides stored at -80°C were allowed to equilibrate to room temperature prior to dilution to 1 mM with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). HFIP was evaporated in a vacuum centrifuge in order to form Aβ42 peptide films and films were then stored at -80°C. Prior to use, Aβ42 peptide films were diluted in dimethylsulphoxide (DMSO) to 1 mM and sonicated for 10 minutes in a Branson sonicator. Aβ42 peptides were then subsequently diluted to 100 μM in ice-cold F-12 cell culture media (phenol free red), vortexed immediately for 30 seconds, and incubated at 4°C for 24 hours in order to form Aβ42 oligomers.

2.11 Statistical Analysis

The means ± S.E.M. are shown for values obtained for the number of independent experiments indicated in the *Figure Legends.* Immunoblots were quantified using Image Lab software. GraphPad Prism software was used to analyze data for statistical significance as well as to analyze and fit dose-response curves. Statistical significance was determined by either an unpaired two-tailed t-test or by one-way ANOVA followed by Tukey's or Dunnett's multiple comparison's test.

CHAPTER 3

RESULTS

3.1 Characterization of the interaction between CAMKIIα and mGluR5

CaMKIIα was previously shown to interact with mGluR1 (Jin et al., 2013a). We also previously identified CaMKIIα as an mGluR1/5 interacting protein following immunoprecipitation of the 2nd intracellular loop domain (IL2) of mGLuR1a/5a to identify mGluR1a/5a interacting proteins by proteomic analysis (Angers and Ferguson, unpublished observations). Consistent with this experiment, we found that GFP-CaMKIIα could be co-immunoprecipitated with the full-length FLAG-tagged mGluR5a in transfected HEK 293 cells in an agonist-independent manner (Fig. 3.1A).

Ca^{2+}/CaM has been demonstrated to bind to the regulatory domain of CaMKIIα to activate the kinase. This activation can regulate the affinity of CaMKIIα for its targets such as the NMDA receptor (Shen and Meyer, 1999) and previous studies showed that activation of CaMKIIα by Ca^{2+}/CaM increases its interaction to GPCRs such as the D3 receptor, the M4 muscarinic receptor and mGluR1a (Liu et al., 2008; Guo et al., 2010; Jin et al., 2013a). Therefore, we examined whether inhibition of CaMKIIα activity can decrease the association between CaMKIIα and FLAG-mGluR5a as determined by co-immunoprecipitation. KN-93 is a CaMKII inhibitor which prevents activation by binding to the Ca^{2+}/CaM binding site on the kinase (Sumi et al., 1991). Agonist stimulation of mGluR5a did not affect the amount of GFP-CaMKIIα co-immunoprecipitated with FLAG-mGluR5a and KN-93 treatment did not affect the amount of GFP-CaMKIIα co-immunoprecipitated with FLAG-mGluR5a indicating that this interaction was agonist-

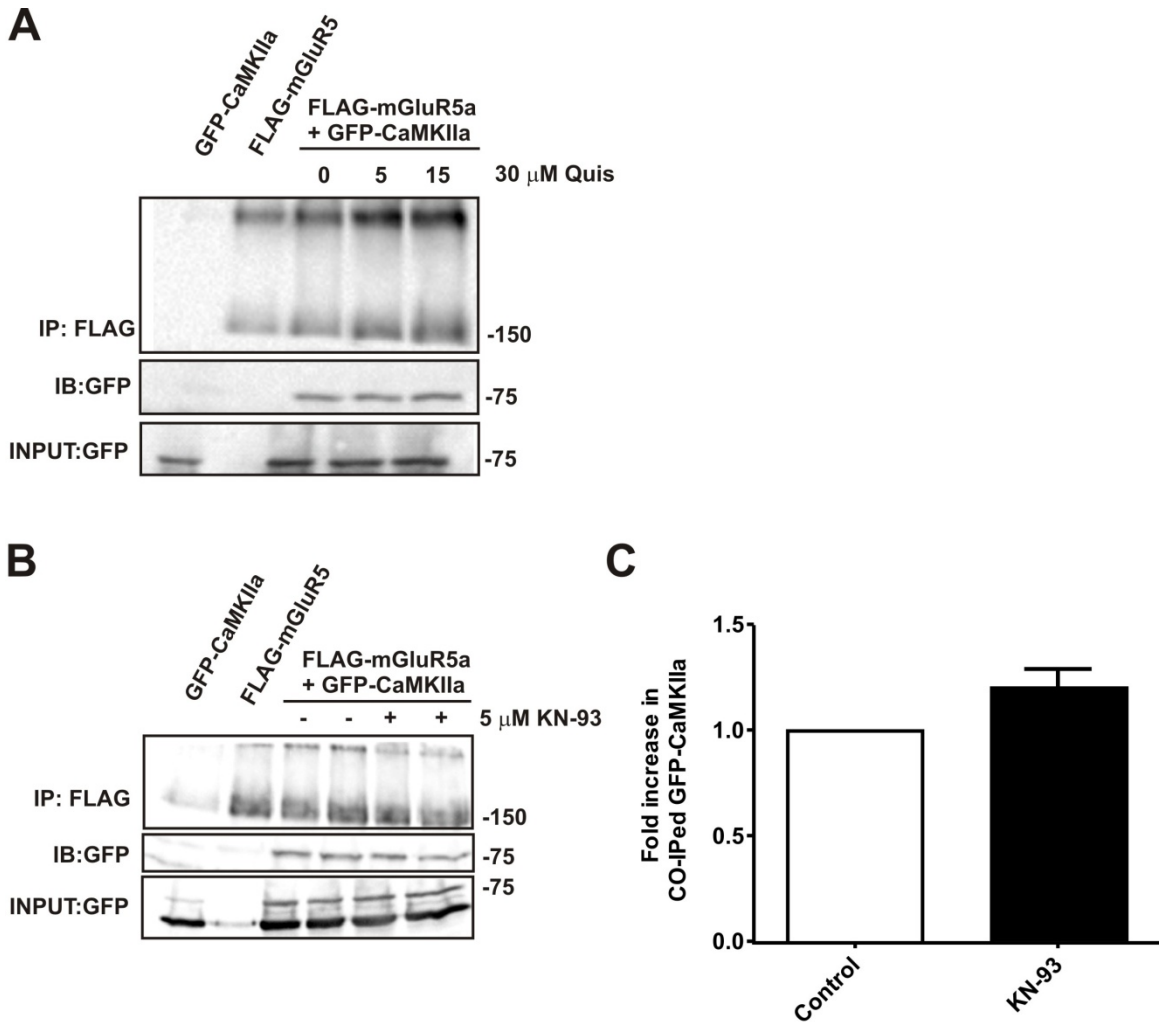

Figure 3.1. CaMKIIα co-immunoprecipitates with mGluR5 independent of agonist stimulation and CaMKIIα activity. (A) Representative immunoblot of GFP-CaMKIIα co-immunoprecipitated with FLAG-mGluR5a in HEK 293 cells transiently transfected with 2 μg of FLAG-mGluR5 and 0.5 μg of GFP-CaMKIIα as labelled. Cells were stimulated with 30 μM quisqualate for the indicated time points. **(B)** Representative immunoblot of GFP-CaMKIIα co-immunoprecipitated with FLAG-mGluR5a in HEK 293 cells transiently transfected with 2 μg of FLAG-mGluR5 and 0.5 μg of GFP-CaMKIIα as labelled. Cells were treated with 5 μM of the CaMKIIα inhibitor KN-93 for 1 hour. **(C)** Effect of KN-93 treatment was quantified by densitometry and had no significant effect on the amount of GFP-CaMKIIα co-immunoprecipitated with FLAG-mGluR5. Data are representative of the mean ± S.E.M of five independent experiments.

and CaMKIIa-activity independent (Fig. 3.1 B and C). GFP-CaMKIIα is localized in the cytosplasm when expressed alone in HEK293 cells and when co-expressed with FLAG-mGluR5 (Figure 3.2 A and B). Stimulation of mGluR5 with quisqualate does not alter the distribution of GFP-CaMKIIα (Fig. 3.2C).

We identified CaMKIIα as a novel mGluR1a/5a second intracellular loop (IL2) interacting protein, therefore we assessed whether CaMKIIα could be co-precipitated with a series of purified GST fusions protein corresponding to the IL2 of mGluR5a (Fig. 3.3A). We found that the CaMKIIα could be co-precipitated with the wild-type IL2 of mGluR5a (Fig. 3.3B). Alanine scanning mutagenesis of the mGluR5a IL2 did not result in mutations that completely prevented CaMKIIα association with the intracellular loop domain (Fig. 3.3B and C). However, we found that GFP-CaMKIIα displays reduced binding to GST-MUT2 compared to the wild type IL2 (Figure 3.3B and C). Interestingly, this region of mGluR1 was also important for binding to GRK2. More specifically, the binding site of GRK2 was localized to K691 and K692 (Dhami et al., 2005). We then tested the co-immunoprecipitation of GFP-CAMKIIα to the full length mGluR1b receptor and the K691A and K692A mutants within the MUT2 region. We found that these mutations were not sufficient to block binding to GFP-CAMKIIα (Figure 3.3D and E). Therefore, it appears that CaMKIIα is not localized to a specific region within the IL2 of mGluR1.

3.2 Effect of CAMKIIα on mGluR5-mediated signaling in HEK 293 cells

It has been well established that activation of Group I mGluRs turns on $G\alpha/_{q11}$ which results in the activation of PLC, IP3 formation and Ca^{2+} release from intracellular

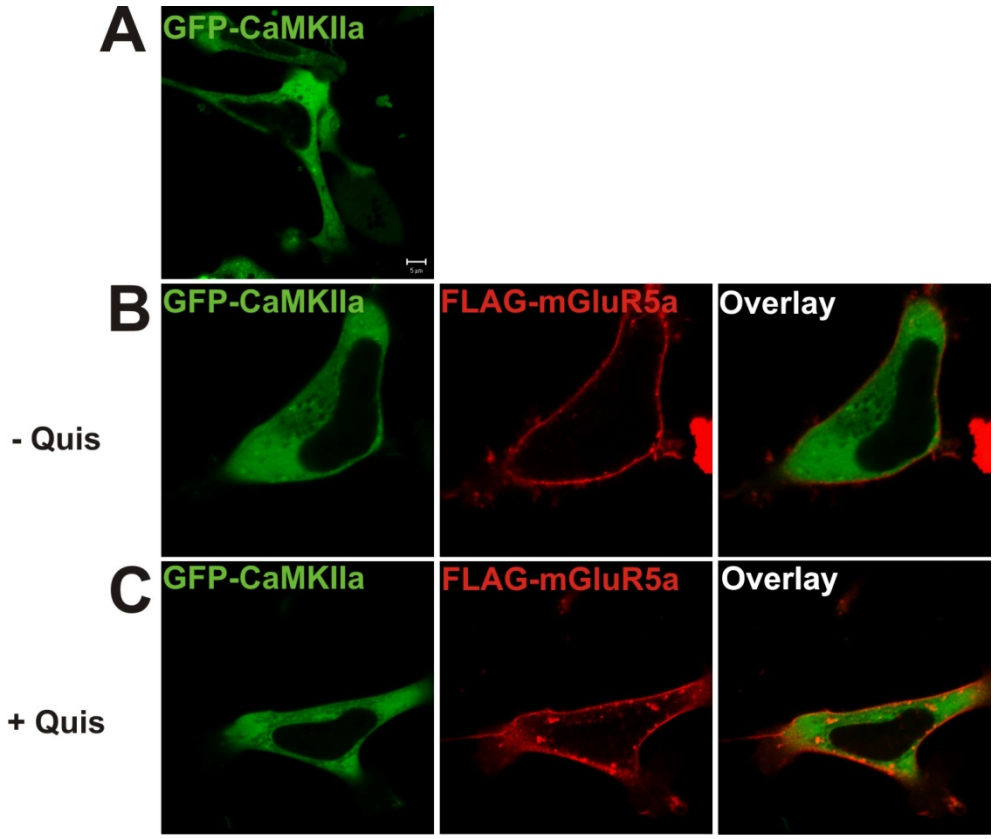

Figure 3.2. Localization of CaMKIIα and mGluR5a using confocal microscopy. (A) Representative confocal microscopy image showing the subcellular localization of GFP-CaMKIIα *(green)* when expressed alone HEK 293 cells. **(B)** Representative confocal image demonstrating the localization of FLAG-mGluR5 *(red)* labelled with Zenon Alexa Fluor 555-conjugated rabbit anti-FLAG antibody and GFP-CaMKIIα *(green)*. **(C)** Representative confocal image demonstrating the internalization of FLAG-mGluR5 *(red)* labelled with Zenon Alexa Fluor 555-conjugated rabbit anti-FLAG antibody and GFP-CAMKIIα *(green)* during 20 minutes of stimulation with quisqualate. HEK 293 cells were transfected with 2 μg of receptor and 0.5 μg of GFP-CaMKIIα plasmid cDNA. Data are representative of over 20 cells.

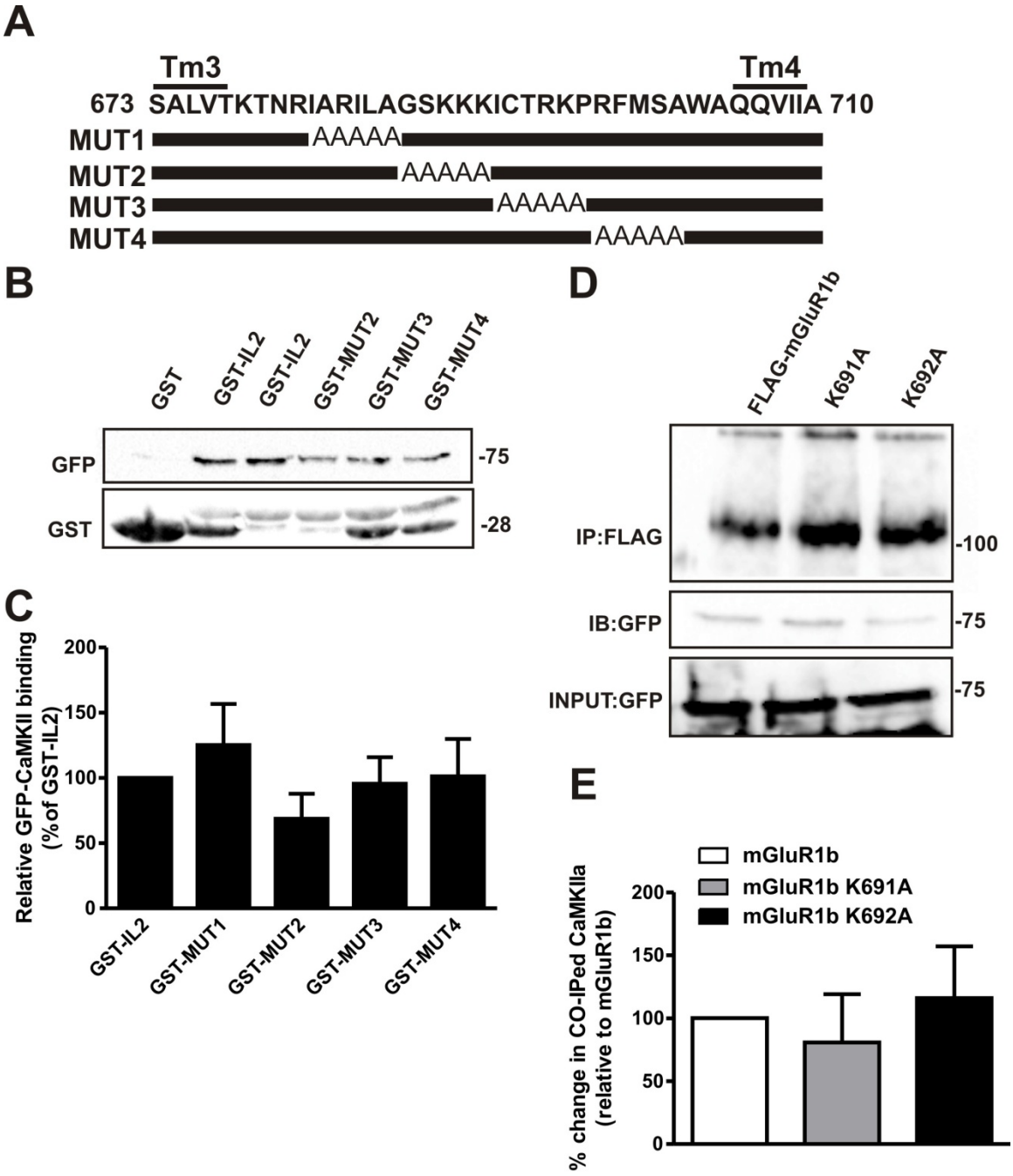
A
Tm3
Tm4
673 SALVTKTNRIARILAGSKKKICTRKPRFMSAWAQQVIIA 710
MUT1
MUT2
MUT3
MUT4
AAAAA
B
GST
GST-IL2
GST-IL2
GST-MUT2
GST-MUT3
GST-MUT4
GFP
GST
-75
-28
C
Relative GFP-CaMKII binding (% of GST-IL2)
200
150
100
50
0
GST-IL2
GST-MUT1
GST-MUT2
GST-MUT3
GST-MUT4
D
FLAG-mGluR1b
K691A
K692A
IP:FLAG
IB:GFP
INPUT:GFP
-100
-75
-75
E
mGluR1b
mGluR1b K691A
mGluR1b K692A
% change in CO-IPed CaMKIIa (relative to mGluR1b)
200
150
100
50
0

Figure 3.3. CaMKIIα interaction with GST-mGluR1-IL2 mutants.

(A) Schematic representation of the second intracellular loop (IL2) alanine scanning mutant GST peptides. **(B)** GST and GST-fusion proteins as labeled were purified from *E. coli* recombinant bacteria using Glutathione-Sepharose. Matrix bound GST-fusion peptides were incubated for 1 hour with lysate from HEK 293 cells overexpressing GFP-CaMKIIα and samples were analyzed by Western blotting. Shown is a representative immunoblot of GST-fusion peptides and associated GFP-CaMKIIα. **(C)** Quantification of the amount of recovered GFP-CaMKIIα was normalized to each GST-fusion protein and expressed as a percentage of GFP-CaMKIIα binding to GST-IL2. GFP-CaMKIIα showed reduced binding to GST-MUT2. Data shown are means ± S.E.M of four independent experiments. **(D)** Representative immunoblot of immunoprecipitated mGluR1b and mGluR1b mutants (K691A and K692A) with co-immunoprecipitated GFP-CaMKIIα**. (E)** Quantification of co-immunoprecipitated GFP-CaMKIIα analyzed using Image Lab software. Data shown are means ± S.E.M of three independent experiments.

stores (Dhami and Ferguson, 2006). CaMKIIα was previously found to desensitize mGluR1-mediated IP formation in striatal neurons (Jin et al., 2013a). Therefore, we examined whether GFP-CaMKIIα overexpression leads to an alteration of FLAG-mGluR5-mediated IP formation. Here we employed a mutant FLAG-mGluR5 cDNA construct that has a single amino acid mutation (A154V) in the glutamate binding region. This residue is analogous to Ala 168 in mGluR1 which when mutated to valine results in low basal IP formation, but can respond to agonist stimulation (Esseltine et al., 2012). Without GFP-CaMKIIα overexpression, we were able to produce a dose-response curve for quisqualate-induced IP formation with FLAG-mGluR5aA154V. However, we found that overexpression of GFP-CaMKIIα did not alter mGluR5a-mediated IP formation (Fig. 3.4A). Additionally, we tested whether overexpression of GFP-CaMKIIα had an effect on mGluR5-mediated Ca^{2+} release. It has been previously established that activation of mGluR5 induces Ca^{2+} oscillations (Kawabata et al., 1999; Dale et al., 2001). We observed Ca^{2+} oscillations in HEK 293 cells following activation of mGluR5a with quisqualate (Fig. 3.5A and B). We found that GFP-CaMKIIα overexpression did not affect the total amount of mGluR5-mediated Ca^{2+} release as quantified by area under the curve (Fig. 3.5C). GFP-CaMKIIα overexpression also did not affect the frequency of oscillations compared to control cells transfected with GFP despite the trend that there appears to be a higher frequency of oscillations with GFP- CaMKIIα overexpression (Fig. 3.5D).

In addition to IP3 formation and Ca^{2+} release, mGluR5 activation leads to the activation of ERK1/2. Therefore, we tested whether GFP-CaMKIIα overexpression might alter mGluR5-mediated ERK1/2 phosphorylation in HEK 293 cells. We found that

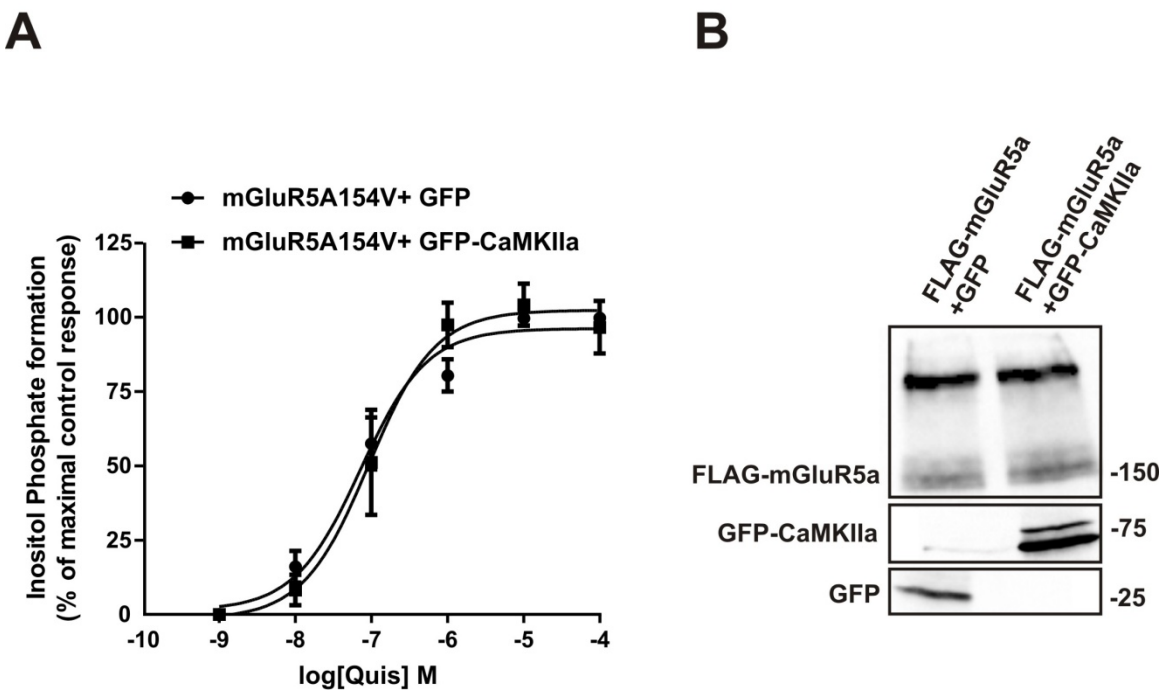

Figure 3.4. CaMKIIα does not have a significant effect on mGluR5-mediated inositol phosphate (IP) formation. (A) Dose response curves for mGluR5-mediated IP formation in response to treatment with increasing concentrations of quisqualate (0-30 μM) for 30 minutes in cells transfected with 2 μg of plasmid cDNA expressing FLAG-mGluR5A154V along with either 0.5 μg of plasmid cDNA expressing GFP-CAMKIIα or 0.5 μg of empty pEGFP vector. **(B)** Representative immunoblot showing equivalent FLAG-mGluR5 expression in GFP and GFP-CaMKIIα transfected cells. The data represent the mean ± S.E.M of five independent experiments.

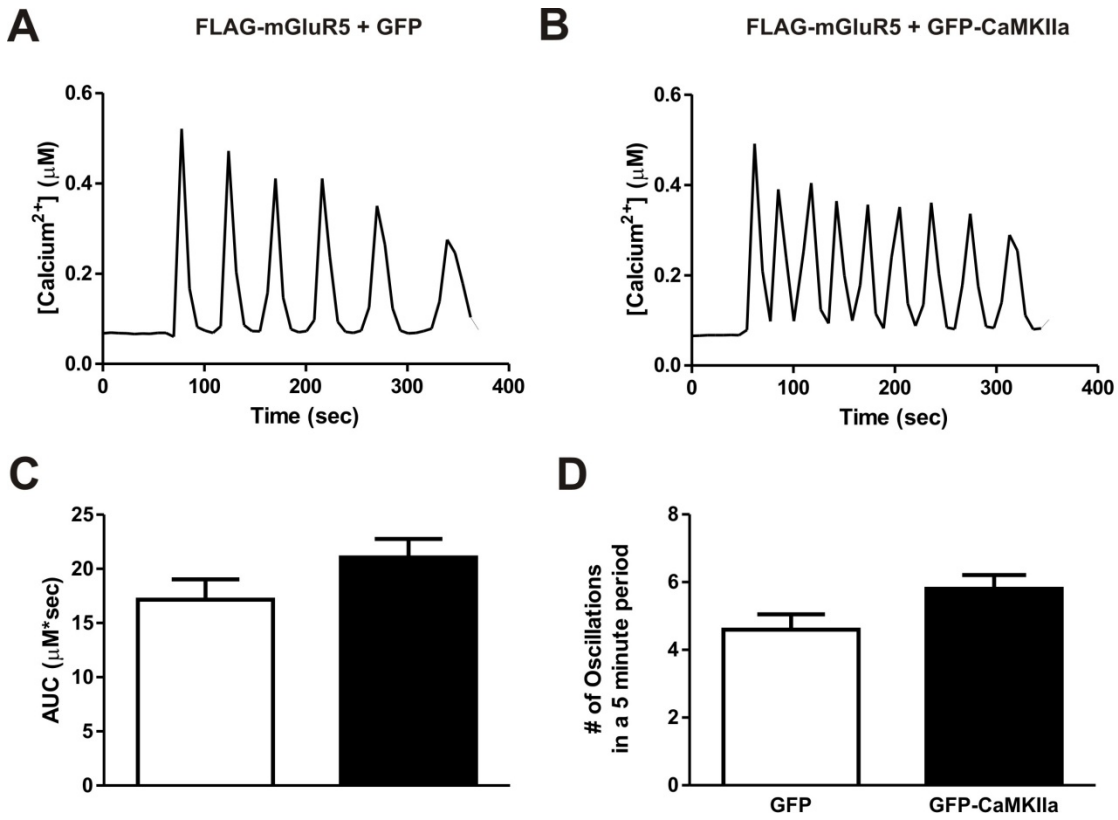

Figure 3.5. CaMKIIα has no affect on mGluR5-mediated Ca^{2+} release. Representative traces of the oscillatory Ca^{2+} response induced by 30 μM quisqualate in HEK 293 cells transiently transfected with 2 μg of pcDNA3.1 encoding FLAG-mGluR5a and 0.5 μg of cDNA encoding GFP **(A)** or GFP-CAMKIIα **(B)**. Cells were imaged to establish a baseline recording for 1 minute and then stimulated with 30 μM quisqualate for 5 minutes. **(C)** Quantification of the Ca^{2+} concentration represented as area under the curve (AUC). **(D)** Quantification of the frequency of oscillations as number of oscillations in a 5 minute period. Data is presented as mean ± S.E.M for 26-29 cells from four independent experiments.

overexpression of GFP-CaMKIIα resulted in a significant decrease in FLAG-mGluR5a mediated ERK1/2 phosphorylation and following 10 minutes of stimulation with 50 μM quisqualate this effect was statistically significant. (Fig. 3.6 A and B). Thus, CaMKIIα overexpression selectively attenuated mGluR5a-mediated ERK1/2 phosphorylation without affecting IP formation or Ca^{2+} release.

3.3 Effect of Aβ42 on mGluR5 activity

Several studies have shown that Aβ42 oligomers correlate better with cognitive impairment and synaptic loss than any other Aβ species (Selkoe, 2006). Current evidence suggests that the excitatory postsynaptic membrane represents a main target of Aβ toxicity (Lacor et al., 2007). Aβ oligomers can interact with a variety of cells surface receptors to modulate their function such as α7-nicotinergic acetylcholine receptor, NMDA and AMPA receptors, Eph2B and mGluR5 (Snyder et al., 2005; Hsieh et al., 2006, Cisse et al., 2011; Um et al., 2013). We tested the affect of Aβ42 oligomers on the interaction between CaMKIIα and mGluR5a. HEK 293 cells overexpressing FLAG-mGluR5a and GFP-CaMKIIα were treated with 200 nM Aβ42 oligomers for 0, 2, 5, 15, and 30 minutes and GFP-CaMKIIα binding to FLAG-mGluR5a was assessed by co-immunoprecipitation. We found that treatment with 200 nM Aβ42 oligomers increases the amount of GFP-CaMKIIα co-immunoprecipitated with mGluR5a over time and produced a significant increase in CaMKII co-immunoprecipitated with mGluR5a following 15 minutes of stimulation (Fig. 3.7A and B). Thus, Aβ42 oligomers can alter the association between mGluR5 and CaMKIIα. Aβ42 oligomers have been shown to induce mGluR5a-mediated Ca^{2+} release (Um et al., 2013). However whether Aβ42

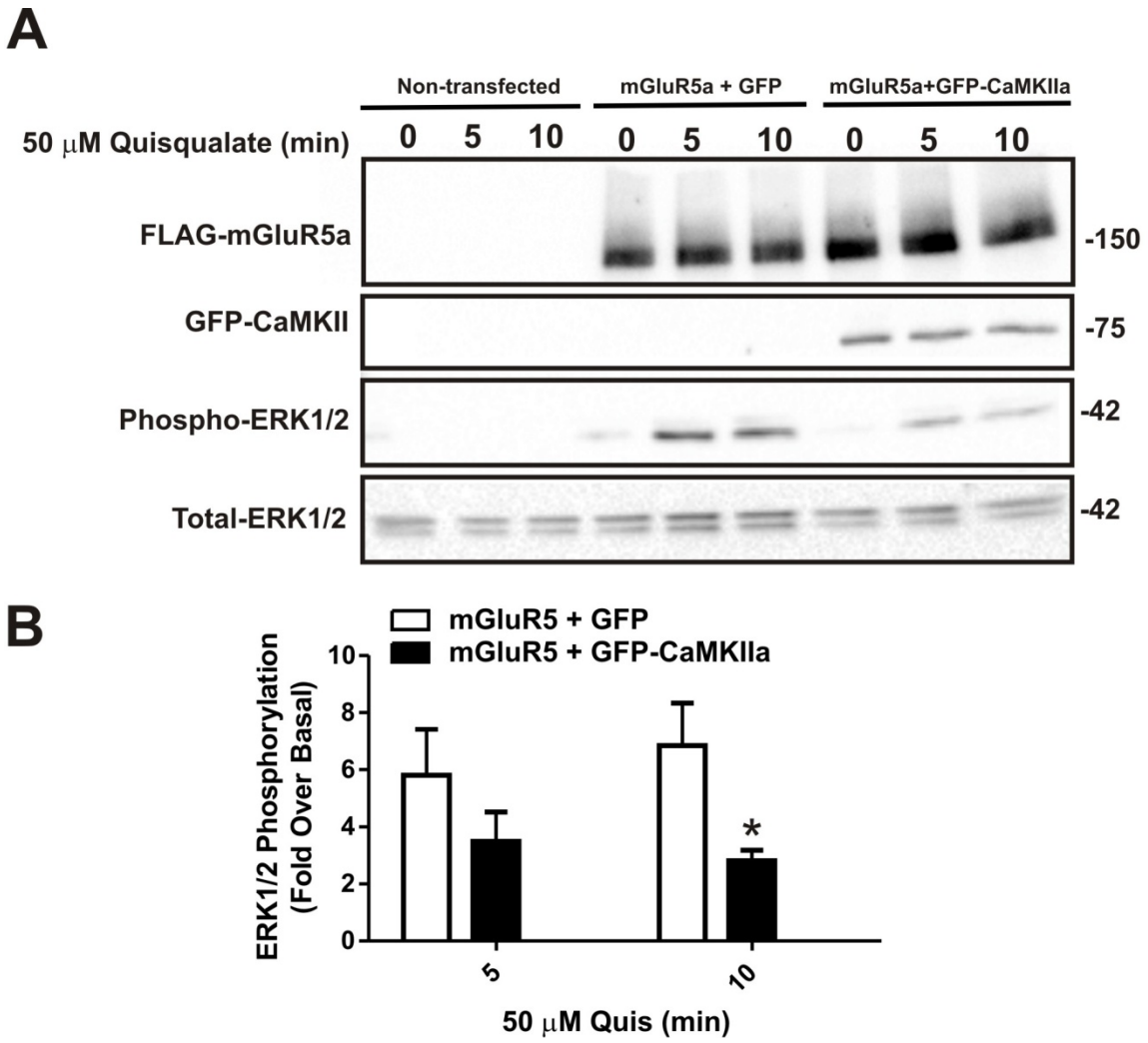

Figure 3.6. CAMKIIα overexpression significantly decreases mGluR5-mediated ERK1/2 phosphorylation. HEK 293 cells were transiently transfected with 2 μg of pcDNA3.1 encoding FLAG-mGluR5a and 0.5 μg cDNA expressing GFP-CaMKIIα or pEGFP vector as labeled. Cells were stimulated with 50 μM quisqualate for the indicated time points. **(A)** Representative immunoblot of FLAG-mGluR5a, GFP-CaMKIIα, Phospho-ERK1/2 and Total-ERK1/2. **(B)** Quantification of phosphorylated ERK1/2 compared to total ERK1/2 as fold-increase over basal. GFP-CAMKIIα significantly decreased ERK1/2 phosphorylation during 10 minutes of quisqualate stimulation as analyzed by Student's two-tailed t-test. *$p<0.05$. Data are mean ± S.E.M of five independent experiments.

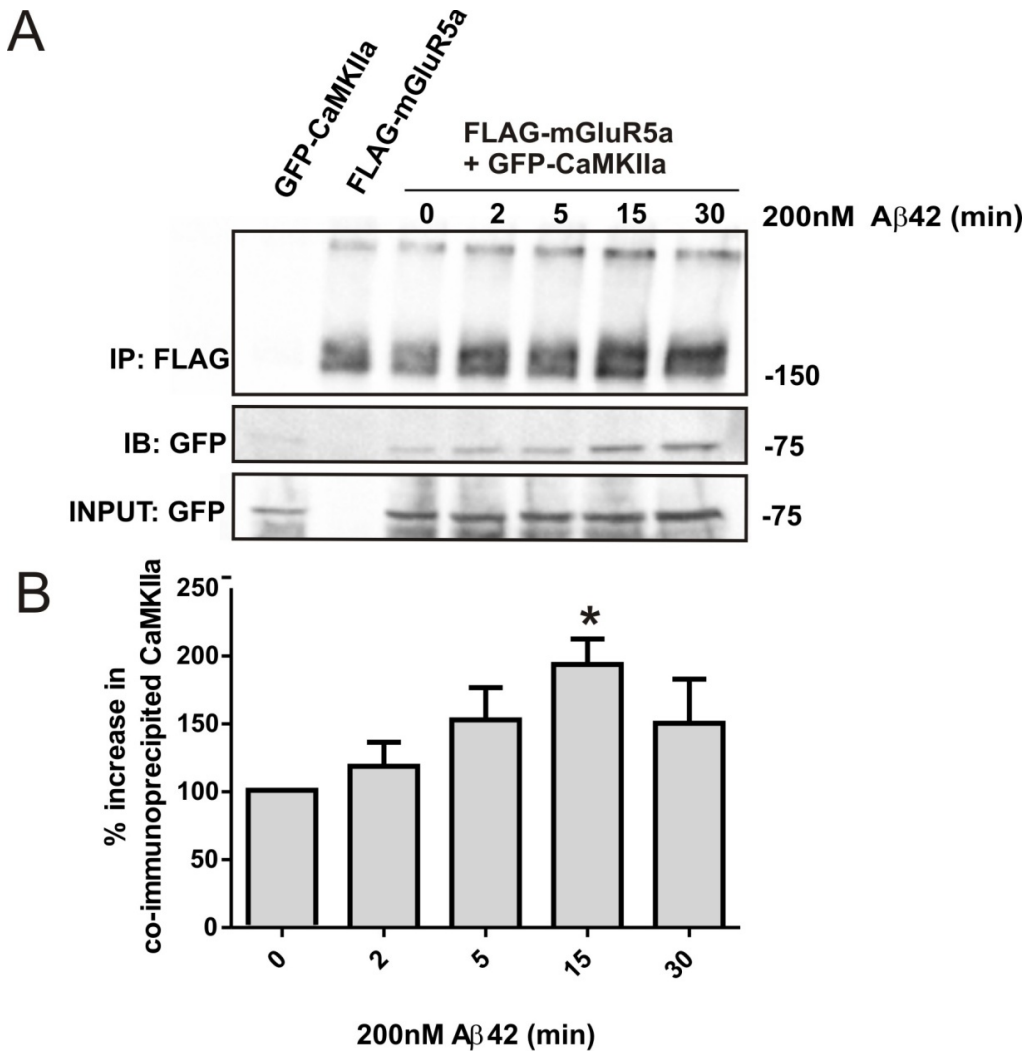

Figure 3.7. **Aβ42 oligomers increase the amount of co-immunoprecipitated CAMKIIα to mGluR5a.** HEK 293 cells were transiently transfected with 2 μg of pcDNA3.1 encoding FLAG-mGluR5a and 0.5 μg cDNA encoding GFP-CaMKIIα as labelled. Cells were treated with 200 nM Aβ42 oligomers for the indicated time points. **(A)** Shown is a representative immunoblot for immunoprecipitated FLAG-mGluR5a, co-immunoprecipitated GFP-CaMKIIα, and GFP-CaMKIIα inputs. **(B)** Quantification of co-immunoprecipitated GFP-CaMKIIα was done using Image Lab software. Treatment with Aβ42 oligomers for 15 minutes significantly increases the amount of co-immunoprecipitated GFP-CaMKIIα compared to untreated control. *p< 0.05. Data is presented as mean ± S.E.M fold increase over untreated control of five independent experiments.

oligomers can activate mGluR5a-mediated ERK1/2 phosphorylation was unknown. Therefore, we tested whether Aβ42 oligomers (100 nM) could activate ERK1/2 phosphorylation in HEK 293 cells overexpressing FLAG-mGluR5a. We found that treatment with Aβ42 oligomers for 5 minutes results in over 2-fold increase in ERK1/2 phosphorylation and over 4-fold increase with 10 minutes of stimulation in cells overexpressing FLAG-mGluR5a (Fig. 3.8A and B). Cells that were not transfected with the receptor did respond to Aβ42 oligomer treatment (Fig. 3.8A and B). In addition, it was found that overexpression of GFP-CaMKIIα did not impair the ERK1/2 phosphorylation induced by Aβ42 oligomers when compared to control cells expressing GFP (Fig.3.8A and B) suggesting that Aβ42 oligomers can differentially regulate mGluR5a-mediated ERK1/2 signaling.

ERK1/2 activation following Group I mGluR activation can occur through a variety of mechanisms as previously discussed in Chapter 1.2.4. Therefore, we tested whether Aβ42-mediated ERK1/2 activation occurred via a PKC-dependent mechanism. HEK293 cells were incubated in HBSS with DMSO (vehicle) or the PKC inhibitor bisindolymaleimide I (Bis-1) prior to stimulation with 30 μM quisqualate or 100 nM Aβ42 oligomers for 5 minutes and ERK1/2 phosphorylation was assessed. We found that PKC inhibition significantly reduced FLAG-mGluR5a mediated ERK1/2 phosphorylation induced by both quisqualate and Aβ42 oligomers (Figure 3.9A and B). Thus Aβ42-mediated ERK1/2 activation was PKC-dependent.

Aβ42 oligomers were shown to interact with PrP^C with high affinity and this complex could interact with mGluR5 to mediate signaling (Um et al., 2013). Using confocal microscopy we determined the effect of PrP^C and mGluR5a co-expression on

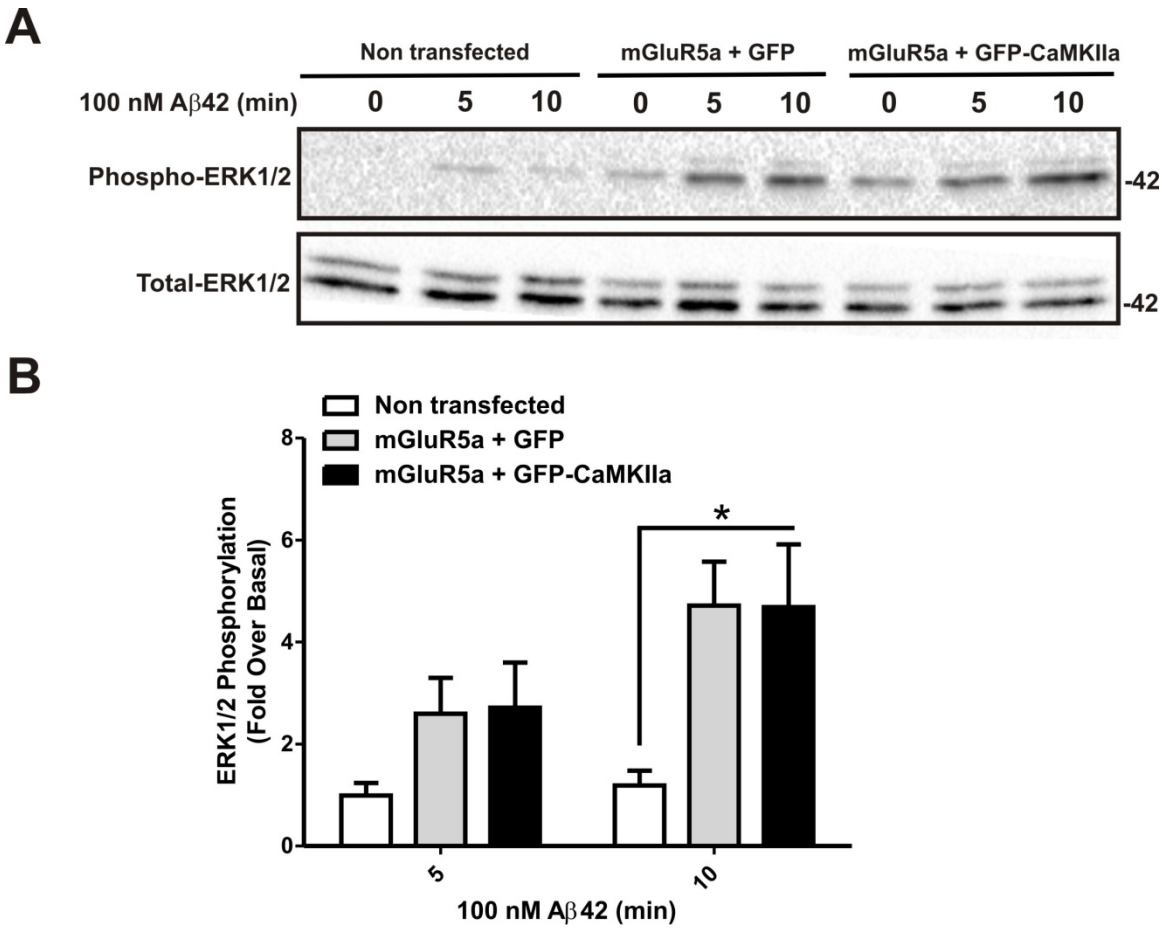

Figure 3.8. Aβ42 oligomers can activate mGluR5a-mediated ERK1/2 phosphorylation. HEK 293 cells were transiently transfected with 2 μg of pcDNA3.1 encoding FLAG-mGluR5 and 0.5 μg cDNA and pEGFP encoding CaMKIIα or pEGFP as labelled. Cells were stimulated with 100 nM Aβ42 oligomers for the indicated time points. **(A)** Representative immunoblot of FLAG-mGluR5, Phospho-ERK1/2 and Total-ERK1/2. **(B)** Quantification of phosphorylated ERK1/2 compared to total ERK1/2 as fold over basal. CaMKIIα has no significant effect on the mGluR5a-mediated ERK1/2 phosphorylation induced by 100 nM Aβ42 oligomers $p>0.05$. Data are mean ± S.E.M of four independent experiments.

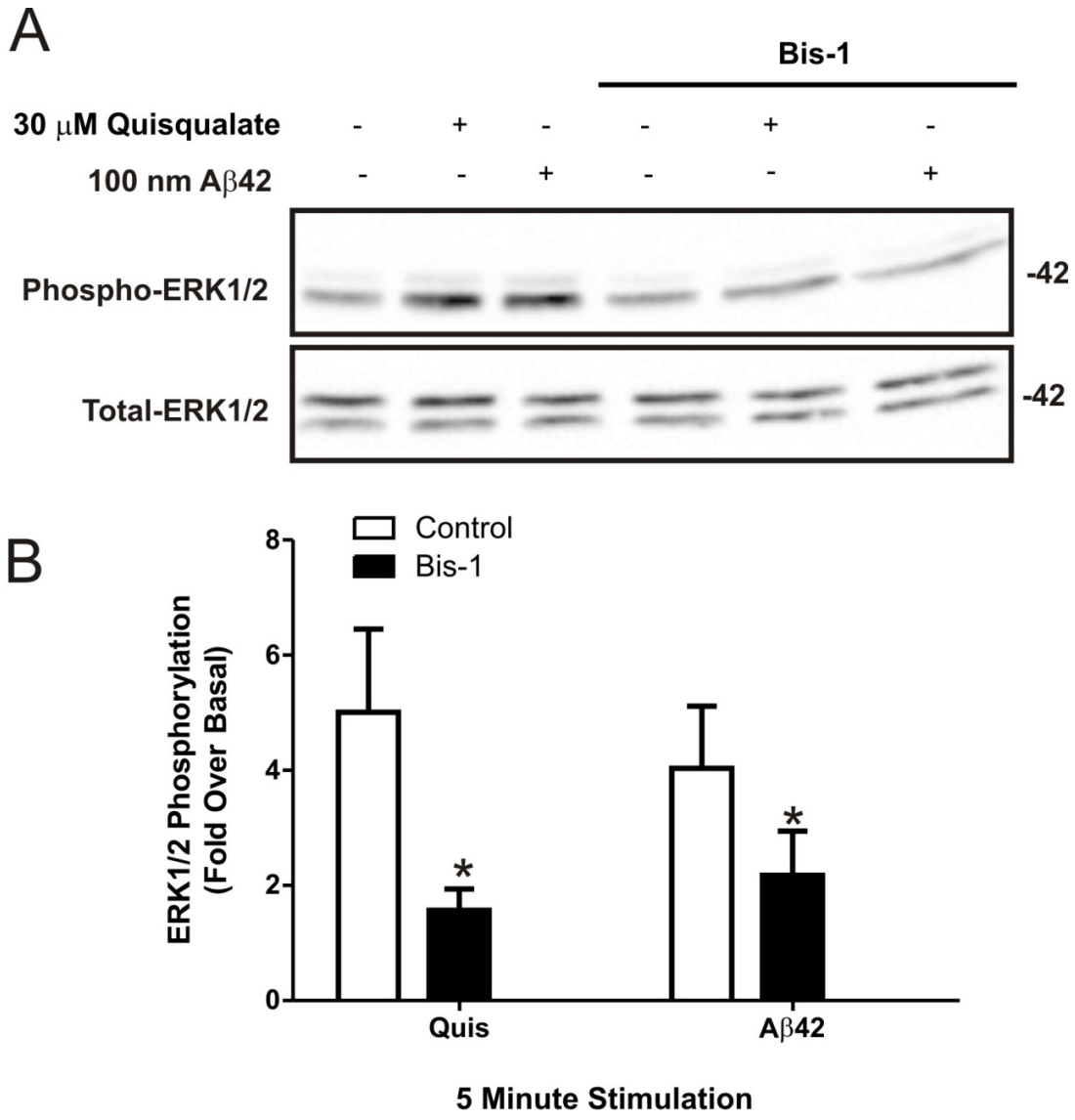

Figure 3.9. Aβ42 mediated ERK1/2 activation is PKC-dependent. HEK 293 cells were transiently transfected with 2 μg of pcDNA3.1 encoding FLAG-mGluR5 and stimulated with 30 μM quisqualate or 100 nM Aβ42 oligomers in the absence or presence of 1 μM bisindolymaleimide I (Bis-1). **(A)** Representative immunoblot of FLAG-mGluR5, phospho-ERK1/2 and total-ERK1/2. **(B)** Quantification of phosphorylated ERK1/2 compared to total ERK1/2 as fold over basal. PKC inhibition with Bis-1 significantly reduces mGluR5a-mediated ERK1/2 phosphorylation induced by quisqualate and Aβ42 oligomers *$p<0.05$. The data represent the mean ± S.E.M of seven independent experiments

CaMKIIα distribution. We found that cells co-expressing these cell surface proteins showed a higher percentage of redistribution of GFP-CAMKIIα into punctuate clusters (Fig.3.10A) compared to cells expressing PrP^C and mGluR5 individually. This effect did not occur in cells transfected with empty GFP vector. (Figure 3.10B). This suggests that CaMKIIα distribution may be affected by the mGluR5-PrP^C interactions.

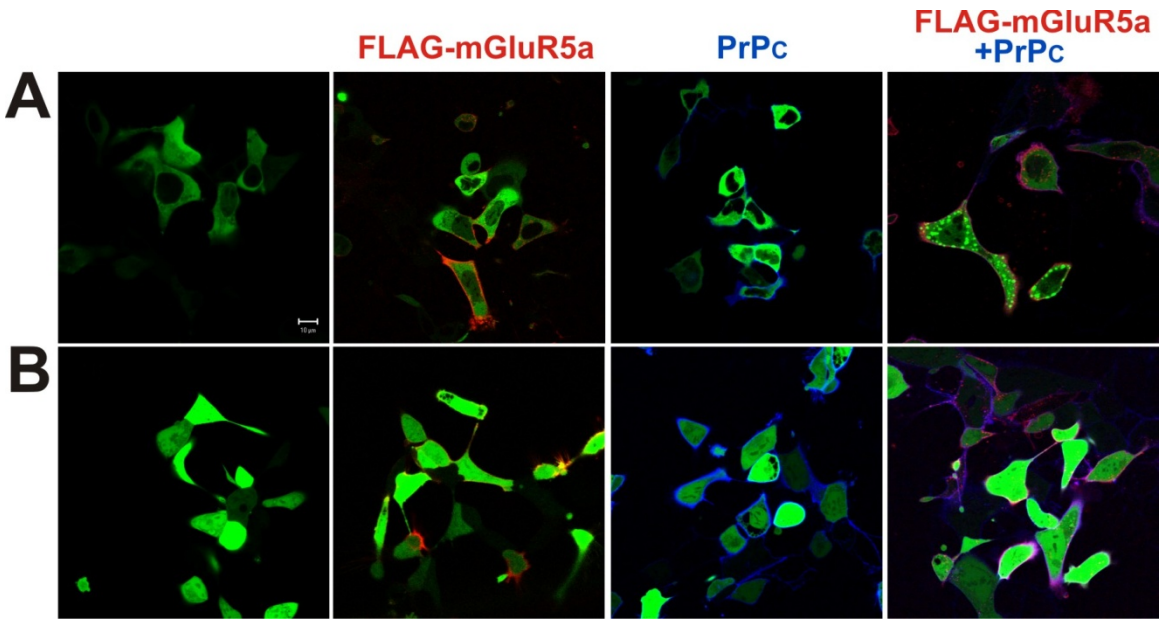

Figure 3.10. PrP^C overexpression results in redistribution of subcellular GFP-CaMKIIα. HEK 293 cells were transiently transfected with 2 μg of pcDNA3.1 encoding FLAG-mGluR5a, 2 μg of pcDNA3.1 encoding PrP^C and 0.5 μg of GFP-CaMKIIα or empty pEGFP vector (control). Representative confocal microscopy images showing cell surface FLAG-mGluR5a *(red)* labelled with Zenon Alexa Fluor 647-conjugated rabbit FLAG antibody, PrP^C *(blue)* labelled with Zenon Alexa Fluor 555-conjugated mouse PrP^C antibody and GFP-CaMKIIα **(A)** or empty pEGFP vector **(B).** Data are representative of over 70 cells for each transfection.

CHAPTER 4

DISCUSSION

The excitatory neurotransmitter glutamate mediates many physiological processes in the central nervous system through ionotropic and metabotropic glutamate receptors (Ozawa et al., 1998; Nakanishi, 1992). The Group I mGluRs play a key role in neuronal development, learning and synaptic transmission (Bordi and Ugolini, 1999; Nakanishi and Masu, 1994). Additionally, these receptors also have been implicated in a number of neurodegenerative diseases such as amyotrophic lateral sclerosis, Huntington's and Alzheimer's disease (Ribeiro et al., 2010a). Regulation of these receptors is a dynamic process involving many interacting proteins and scaffolding complexes (Enz, 2012). We have identified CaMKIIα as a novel IL2-interacting protein of Group I mGluRs. Our results reveal the following: 1) the interaction between CaMKIIα and mGluR5 is independent of CaMKIIα activity 2) CaMKIIα decreases mGluR5-mediated ERK1/2 phosphorylation, but not IP3 formation or Ca^{2+} release, 3) CaMKIIα binding to mGluR5 is increased in the presence of Aβ42 oligomers, and 4) Aβ42 oligomers can activate mGluR5-mediated ERK1/2 in a PKC dependent manner.

4.1 CAMKIIα interacts with mGluR5 independent of CAMKIIα activity.

Compared to prototypic GPCRs, the Group I mGluRs have small intracellular loops and a large C-tail which harbours many interacting proteins (Enz, 2012). Interestingly, C-tail interacting proteins such as GRK2, Pyk2 and CAIN can also interact with the second intracellular loop (Dhami et al., 2005; Nicodemo et al., 2010; Ferreira et al., 2009). This ability may result in differential outcomes in the regulation of these receptors. For example, the mGluR1a and mGluR1b splice variants reveal subtle

differences the attenuation of mGluR1 signaling by GRK2 (Dhami et al., 2005).

In a proteomic screen, we have identified CaMKIIα as a novel IL2 interacting protein of Group I mGluRs. The IL2 is also able to interact with the other CNS abundant CaMKII isoform, CaMKIIβ. The association between CaMKIIα and Group I mGluRs appears to be a direct interaction, since we have shown that the second intracellular loop peptide is sufficient for this interaction to occur. Additionally, CaMKIIα has been shown to interact with mGluR1 through the C-terminus tail demonstrating once more that interacting proteins of Group I mGluRs have multiple sites for interaction (Jin et al., 2013a). More specifically, this group found that CaMKIIα interacts with the proximal C-tail of mGluR1a and the interaction is localized between the residues K841 and N885 (Jin et al., 2013a). As a serine/threonine kinase, many binding partners of CaMKIIα are phosphorylation substrates. The region in mGluR1a that mediates CaMKIIα binding contains the CaMKIIα substrate recognition motif, RXXT (Jin et al., 2013a; White et al., 1998) and can be phosphorylated by CaMKIIα. mGluR5 also contains several CaMKII substrate recognition motifs. However whether they are phosphorylated by CaMKIIα is unknown. Therefore, future experiments are needed to determine if CaMKIIα phosphorylates mGluR5 and whether phosphorylation by CaMKIIα contributes to receptor regulation.

Another important characteristic of the interaction between mGluR5 and CaMKIIα is whether mGluR5 can bind to the activated form of CaMKIIα. Jin et al., (2013a) showed using in vitro assays that the presence of Ca^{2+} activates CaMKIIα and activated CaMKIIα has a higher affinity for mGluR1 as it displays greater binding to the receptor than the inactivated form. In addition, activated CaMKIIα has been shown to

increase the affinity for many targets including the D3 dopamine receptor and M4 muscarinic receptor (Liu et al., 2010; Guo et al., 2011). Moreover, the activation of CaMKIIα results in translocation from the cytoplasm to the post-synaptic density where it binds to the NMDA receptor and facilitates LTP (Strack et al., 1997). Thus, activation of CaMKIIα represents an important mechanism by which the kinase can regulate its association with binding partners. In contrast to these studies, we found that treatment with the CAMKIIα inhibitor KN-93 does not result in any difference in the amount of CaMKIIα co-immunoprecipitated with mGluR5a. This, along with the result that the interaction between CaMKIIα mGluR5 is independent of receptor activation, indicates that the association between CaMKIIα and mGluR5 is independent of CaMKIIα activity.

When two molecules of Ca^{2+}/CaM bind to two subunits of CaMKIIα, autophosphorylation of the enzyme can occur. CaMKIIα autophosphorylation results in persistent activity of CaMKIIα even after Ca^{2+}/CaM is no longer bound (Hanson and Schulman, 1992). The inhibitor KN-93 works by interacting with the Ca^{2+}/CaM binding site on CaMKIIα to prevent its activation by Ca^{2+}/CaM. However, this inhibitor does not prevent autophosphorylation of the kinase (Sumi et al., 1991). CaMKIIα autophosphorylation has been shown to lead to increased binding to the D3 dopamine receptor (Liu et al., 2010). It would be interesting to see whether autophosphorylated CaMKIIα interacts with mGluR5 and whether autophosphorylation of CaMKIIα contributes in the regulation of mGluR5.

4.2 Regulation of mGluR5 signaling by CaMKIIα

Interacting proteins of Group I mGluRs can be categorized according to the roles

they play such as scaffolding, cytoskeletal regulation and signaling. In the present study, we looked at the effects of CaMKIIα on mGluR5 signaling. It is well established that activation of Group I mGluRs activates the heterotrimeric $G\alpha_{q/11}$ protein which leads to formation of IP3 and Ca^{2+} release from intracellular stores. Jin and coworkers (2013a) established a role for CaMKIIα in regulating mGluR1-mediated IP formation in rat striatal slices. They found that blocking the interaction between mGluR5 and CaMKIIα by a Tat interference peptide results in a reduced desensitization response to DHPG. We previously showed that overexpression of CAMKIIα in HEK293 cells does not affect mGluR1a-mediated IP formation. However, this difference may be attributed to the differences between HEK293 cells and rat striatal neurons. In this study, we show that overexpression of CaMKIIα has no effect on mGluR5-mediated IP formation, but whether this occurs in neurons is unknown and represents an area for future study.

When looking at Ca^{2+} signaling, we observed no significant effect of CaMKIIa on mGluR5-mediated Ca^{2+} release despite the tendency for Ca^{2+} release and the frequency of Ca^{2+} oscillations to be increased in the presence of CaMKIIα. The number of Ca^{2+} oscillations is shown to be affected by the amount of cell surface receptor (Nash et al., 2002; Choi et al., 2011). A study in Chinese Hamster Ovary cells comparing the ability of receptor density and agonist concentration to affect mGluR5-mediated Ca^{2+} oscillations found that Ca^{2+} oscillation frequency is affected by receptor density and not agonist concentration (Nash et al., 2002). In support of this, mGluR5 induced Ca^{2+} oscillations are to be affected by CaM, an interacting protein that affects cell surface expression of mGluR5 (Choi et al., 2011). This group found that a mutant form of mGluR5 that disrupts CaM binding results in reduced cell surface expression, as well as reduced

frequency of Ca^{2+} oscillations. We have previously established that mGluR1 cell surface expression is unaffected by CaMKIIα. However, whether CaMKIIα affects mGluR5 cell surface expression is unknown. It is likely, that CaMKIIα has no effect on mGluR5 cell surface expression since both IP and Ca^{2+} seem to be unaffected CaMKIIα.

As previously mentioned, Group I mGluRs can also activate the ERK1/2 signaling cascade by a variety of mechanisms. CaMKII has been shown to affect DHPG-mediated ERK phosphorylation in striatal neurons (Choe and Wang, 2001). They found that treatment with the CaMKII inhibitor, KN-62, results in attenuation of DHPG-induced ERK phosphorylation in the rat striatum. DHPG can activate both mGluR1 and mGluR5, however this study did not determine the relative contribution of ERK phosphorylation caused by each receptor. We found that overexpression of CaMKIIα results in the attenuation of mGluR5-mediated ERK1/2 phosphorylation without affecting IP formation or Ca^{2+} release. This indicates that the regulation of mGluR5 by CaMKIIα may involve the participation of scaffolding proteins that selectively couple the receptor to distinct signaling pathways. This is similar to the effect produced by biased GPCRs ligands that activate select downstream signaling pathways (Luttrell and Kenakin, 2011).

4.3 Regulation of mGluR5 by Aβ oligomers

Soluble Aβ is a key player in the early pathogenesis of Alzheimer's disease. One of the proposed mechanisms of Aβ-mediated toxicity involves the disturbance of Ca^{2+} homeostasis (Khachaturian ZS, 1989; Green et al., 2009; Berridge MJ, 2010). Aβ has been shown to affect Ca^{2+} signaling in a manner dependent on mGluR5 (Renner et al., 2010; Grolla et al., 2013, Um et al., 2013). In hippocampal astrocytes, Aβ oligomer

treatment increases DHPG-mediated Ca^{2+} release (Grolla et al., 2013). Aβ oligomers can act as extracellular scaffolds of mGluR5 to reduce mobility and induce clustering of the receptor. This effect as well as mimicking clustering by artificial cross-linking leads to increased Ca^{2+} signaling in hippocampal neurons (Renner et al., 2010). Additionally, Aβ induced mGluR5-mediated Ca^{2+} signaling has been found by Um and coworkers (2013) to occur in a manner that is dependent on PrP^{C}.

In addition to inducing Ca^{2+} release, Aβ signaling can also lead to ERK1/2 activation. Chong et al., (2006) found that ERK1/2 can be activated by Aβ oligomers in a time and dose-dependent manner in rat hippocampal slices. Aβ oligomer-mediated ERK1/2 activation can also occur in SH-SY5Y cells via the α-7 nicotinic acetylcholine receptor (Young et al., 2009). We show that ERK1/2 activation can occur in HEK293 cells transfected with mGluR5 in response to stimulation with 100 nM Aβ42 oligomers. Furthermore, we found that treating cells with a PKC inhibitor prevented the ERK1/2 activation induced by Aβ42 oligomers suggesting that Aβ42 oligomer-induced ERK1/2 activation is PKC-dependent. The role of ERK1/2 activation in Alzheimer's disease is unclear. Some studies implicate ERK1/2 in the hyperphosphorylation of tau, a major hallmark of Alzheimer's disease, but this involvement is still controversial (Drewes et al., 1992; Ledesma et al., 1992; Lu et al., 1993; Pei et al., 2002). Additionally, we found that CaMKIIα overexpression does not have any appreciable effect on Aβ induced ERK1/2 activation as it did by conventional mGluR5 activation with quisqualate. This indicates that Aβ oligomers can differentially regulate mGluR5 signaling: while CaMKIIα appears to affect agonist-induced mGluR5 signaling, it does not appear to affect Aβ42 induced signaling (Summarized in Figure 4.1).

We also show that the presence of Aβ42 oligomers results in increased binding of CaMKIIα to mGluR5. As previously mentioned Aβ42 oligomers act as an extracellular scaffold for mGluR5 and thus can cause clustering of mGluR5 and reduce receptor mobility similar to that of artificially cross-linking the receptor (Renner et al., 2010). It is possible that the clustering of mGluR5 induced by Aβ42 oligomers can stabilize the interaction with CaMKIIα, thus leading to increased co-immunoprecipitated CAMKIIα to the receptor. To test this hypothesis, we could examine whether artificially cross-linking the receptor produces an increase in co-immunoprecipitated CaMKIIα with mGluR5. Interestingly, receptor cross-linking is a necessary step in the co-immunoprecipitation of some GPCR interacting proteins (Hall RA, 2004). Both Aβ42 oligomers and mGluR5 are involved in the pathogenesis of Alzheimer's disease (Selkoe, 2006; Hamilton et al., 2014). The regulation of mGluR5 by Aβ42 oligomers represents an area of future study in understanding how these two players contribute to the pathogenesis of Alzheimer's disease.

4.4 Role of CAMKIIα and mGluR5 in synaptic plasticity

LTP and LTD are well established forms of synaptic plasticity underlying memory and learning processes (Malenka and Bear, 2004). As previously mentioned, translocation of activated CaMKIIα to the PSD and binding to the NMDA receptor is important for LTP induction (Shen and Meyer, 1999). Additionally, NMDA dependent LTP in the CA1 region of the hippocampus is shown to involve mGluR5 and several studies indicate that mGluR5 is involved in late phase LTP, a form of synaptic plasticity

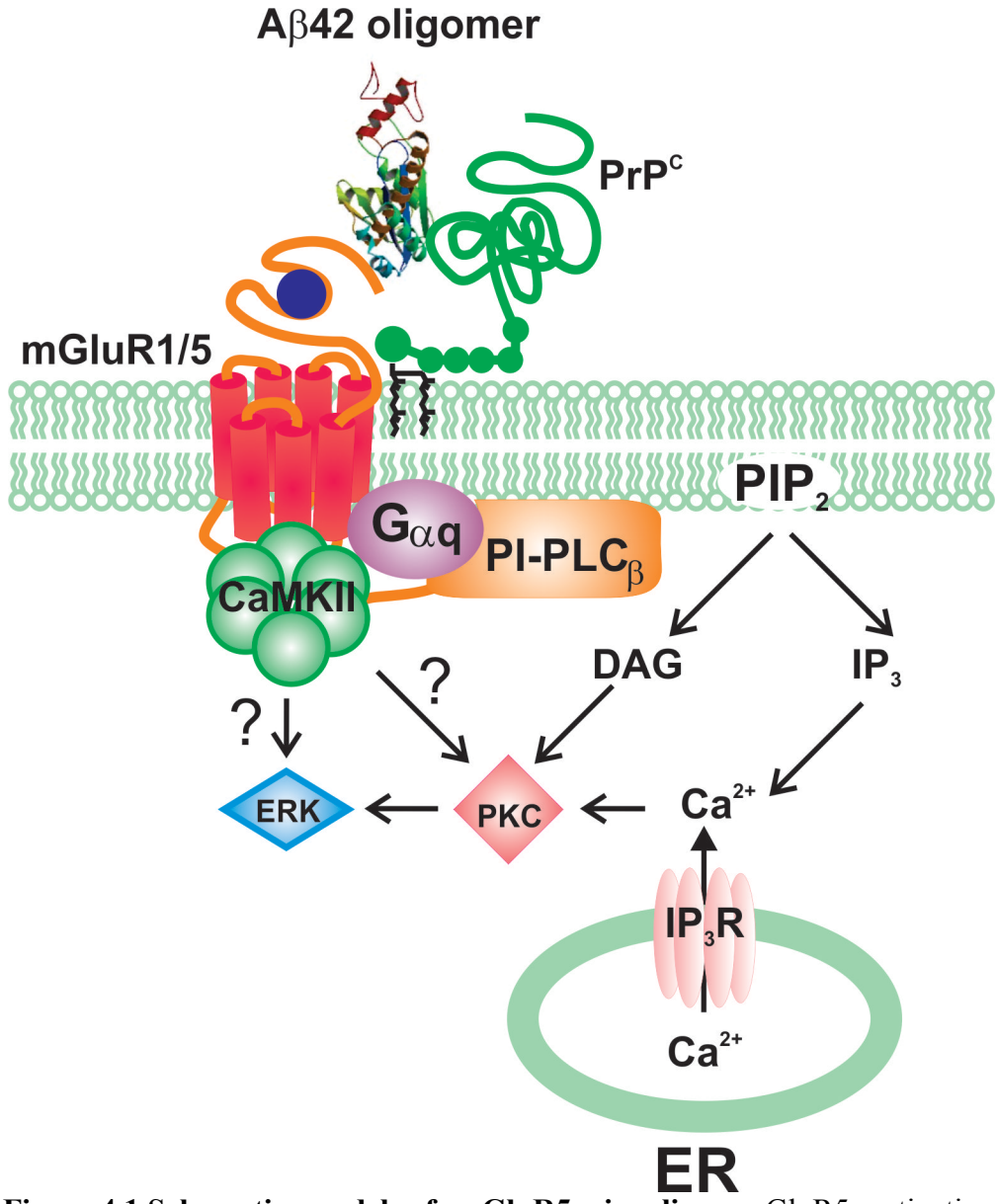

Figure 4.1 Schematic model of mGluR5 signaling. mGluR5 activation results in activation of PLC, IP3 formation, Ca^{2+} release from intracelluar stores and PKC activation followed by ERK1/2 phosphorylation. mGluR5 acts as an extracellular scaffold for the Aβ oligomer-PrP^C complex which allows for intracellular Ca^{2+} signaling. Ca^{2+} can activate PKC leading to ERK1/2 phosphorylation. We show that Aβ42 oligomers can activate mGluR5-dependent ERK1/2 phosphorylation in a PKC-dependent manner. CaMKIIα interacts with mGluR5 to attenuate agonist-stimulated ERK1/2 phosphorylation but not Aβ42–stimulated ERK1/2 phosphorylation however the exact mechanisms are unknown.

dependent on protein synthesis (Lu et al., 1997, Fransesconi et al., 2004). Both CaMKIIα and mGluR5 play an important role in LTP however how the interaction between CaMKIIα and mGluR5 plays a role in LTP is unknown and would be a key future area of study. Interestingly, mGluR5-dependent LTD may be attenuated by activation of CaMKII (Huang et al., 2013). This study showed that CaMKIIα activation resulted in the phosphorylation of Homer1b/c, thus impairing its interaction with mGluR5. Consequently, the disruption of this interaction attenuates mGluR5-mediated LTD (Huang et al., 2013). A direct role for CaMKIIα on mGluR5-dependent potentiation of NMDA receptors has been proposed by (Jin et al., 2013b). In this study, mGluR5-mediated Ca^{2+} release results in the dissociation of C-tail bound CaMKIIα and recruitment to the NMDA receptor where it can phosphorylate the NR2B subunit, thereby possibly effecting LTP (Jin et al., 2013b).

4.5 Summary

In summary, we have shown here that CaMKIIα can associate with mGluR5 independent of CaMKIIα activity. We demonstrated that CaMKIIα attenuates mGluR5-mediated ERK1/2 phosphorylation in the absence of attenuated mGluR5-mediated IP3 formation and Ca^{2+} release. Furthermore, we report that Aβ oligomers can increase binding of CaMKIIα to mGluR5 and can activate mGluR5-mediated ERK1/2 phosphorylation. Group I mGluRs are not only important for learning and memory but also for their contribution to neurodegenerative diseases. Therefore, understanding the mechanisms by which Group I mGluRs can be regulated can provide us with novel therapeutic targets to treat neurodegenerative diseases.

REFERENCE LIST

Abe, T., Sugihara, H., Nawa, H., Shigemoto, R., Mizuno, N., and Nakanishi, S. (1992). Molecular characterization of a novel metabotropic glutamate receptor mGluR5 coupled to inositol phosphate/Ca2+ signal transduction. The Journal of biological chemistry *267*, 13361-13368.

Adomeit A, Graness A, Gross S, Seedorf K, Wetzker R, Liebmann C. (1999) Bradykinin B2 receptor-mediated mitogen-activated protein kinase activation in COS-7 cells requires dual signaling via both protein kinase C pathway and epidermal growth factor receptor transactivation. *Molecular and Cellular Biology 19*, 5289–5297.

Aiba, A., Kano, M., Chen, C., Stanton, ME., Fox, GD., Herrup, K., Zwingman, TA., and Tonegawa, S. (1994). Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. Cell *79*, 377–388.

Ango, F., Prezeau, L., Muller, T., Tu, JC., Xiao, B., Worley, PF., Pin, JP, Bockaert, J., and Fagni, L. (2001). Agonist-independent activation of metabotropic glutamate receptors by the intracellular protein Homer. Nature *411*, 962–965.

Anwyl, R. (2009). Metabotropic glutamate receptor-dependent long-term potentiation. Neuropharmacology *56*.

Ashley, CT., Wilkinson, KD., Reines, D., and Warren, ST. (1993). FMR1 protein: conserved RNP family domains and selective RNA binding. Science *262*, 563-566.

Attucci, S., Carla, V., Mannaioni, G., and Moroni, F. (2001). Activation of type 5 metabo- tropic glutamate receptors enhances NMDA responses in mice cortical wedges. British Journal of Pharmacology *132*, 799–806.

Ayala, JE., Chen, Y., Banko, JL., Sheffler, DJ., Williams, R., Telk, AN., Watson, NL., Xiang, Z., Zhang, Y., Jones, PJ., Lindsley, CW., Olive, MF., and Conn PJ. (2009). mGluR5 positive allosteric modulators facilitate both hippocampal LTP and LTD and enhance spatial learning. Neuropsychopharmacology *34*, 2057–2071.

Balschun, D., Manahan-Vaughan, D., Wagner, T., Behnisch, T., Reymann, K. G., and Wetzel, W. (1999). A specific role for group I mGluRs in hippocampal LTP and hippocampus-dependent spatial learning. Learn. Mem. *6*, 138–152.

Barria, A., and Malinow, R. (2005). NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. Neuron *48*, 289-301.

Bats, C., Groc, L., and Choquet, D. (2007). The interaction between Stargazin and PSD-95 regulates AMPA receptor surface trafficking. Neuron *53*, 719-734.

Bayer, K., De Koninck, P., and Schulman, H. (2002). Alternative splicing modulates the frequency-dependent response of CaMKII to Ca(2+) oscillations. The EMBO journal *21*, 3590-3597.

Bear, MF., Huber, KM., and Warren, ST. (2004) The mGluR theory of fragile X mental retardation. Trends Neuroscience *27*, 370-377.

Beom, S., Cheong, D., Torres, G., Caron, M.G., and Kim, K.M. (2004). Comparative studies of molecular mechanisms of dopamine D2 and D3 receptors for the activation of extracellular signal-regulated kinase. J. Biol. Chem. *279*, 28304 –28314.

Beqollari, D., and Kammermeier, P. (2010). Venus fly trap domain of mGluR1 functions as a dominant negative against group I mGluR signaling. Journal of Neurophysiology *104*, 439-448.

Berridge, M. J. (2010). Calcium hypothesis of Alzheimer's disease. Pflugers Arch. *459*, 441–449

Biber, K., Laurie, DJ., Berthele, A., Sommer, B., Tolle, TR., Gebicke-Harter, PJ., van, CD., and Boddeke, HW. (1999). Expression and signaling of group I metabotropic glutamate receptors in astrocytes and microglia. Journal of Neurochemistry *72,*1671-1680.

Bordi, F., and Ugolini, A. (1999). Group I metabotropic glutamate receptors: implications for brain diseases. Prog. Neurobiol. *59*, 55-79.

Butterfield, DA. (2002). Amyloid beta-peptide (1-42)-induced oxidative stress and neurotoxicity: implications for neurodegeneration in Alzheimer's disease brain. A review. Free Radic Res. *36*, 1307-1313.

Caraci, F., Battaglia, G., Sortino, MA., Spampinato, S., Molinaro, G., Copani, A., Nicoletti, F., and Bruno, V. (2012). Metabotropic glutamate receptors in neurodegeneration/neuroprotection: Still a hot topic? Neurochem. Int *61*, 559–565.

Chao, L., Stratton, M., Lee, I.-H., Rosenberg, O., Levitz, J., Mandell, D., Kortemme, T., Groves, J., Schulman, H., and Kuriyan, J. (2011). A mechanism for tunable autoinhibition in the structure of a human Ca2+/calmodulin- dependent kinase II holoenzyme. Cell *146*, 732-745.

Chen, QS., Kagan, BL., Hirakura, Y., and Xie, CW. (2000) Impairment of hippocampal long-term potentiation by Alzheimer amyloid beta-peptides. Journal of Neuroscience Research *60*, 65-72.

Choe, ES., and Wang, JQ. (2001) . Group I metabotropic glutamate receptors control

phosphorylation of CREB, Elk-1 and ERK via a CaMKII-dependent pathway in rat striatum. Neurosci Lett *313*,129 –132.

Choi, KY., Chung, S., and Roche KW (2011). Differential binding of calmodulin to group I metabotropic glutamate receptors regulates receptor trafficking and signaling. Jornal of Neuroscience *31*, 5921–5930.

Chong, YH., Shin, YJ., Lee, EO., Kayed, R., Glabe, CG., and Tenner, AJ. (2006). ERK1/2 activation mediates Abeta oligomer-induced neurotoxicity via caspase-3 activation and tau cleavage in rat organotypic hippocampal slice cultures. Journal of Biolgical Chemistry *281*, 20315–20325.

Choquet, D. (2010). CaMKII triggers the diffusional trapping of surface AMPARs through phosphorylation of stargazin. Neuron *67*, 239-252.

Chow, VW., Mattson, M.P., Wong, P.C., and Gleichmann, M. (2009) An Overview of APP Processing Enzymes and Products. Neuromolecular Medicine.

Chun, L., Zhang, W.-h., and Liu, J.-f. (2012). Structure and ligand recognition of class C GPCRs. Acta pharmacologica Sinica *33*, 312-323.

Cissé, M., Halabisky, B., Harris, J., Devidze, N., Dubal, DB., Sun, B., Orr, A., Lotz, G., Kim, DH., Hamto, P., Ho, K., Yu, GQ., Mucke, L. (2011). Reversing EphB2 depletion rescues cognitive functions in Alzheimer model. Nature *469*, 47–52

Clark, BP., Baker, SR., Goldsworthy, J., Harris, JR., and Kingston, AE. (1997) (+)-2-Methyl-4-carboxyphenylglycine (LY367385) selectively antagonises metabotropic glutamate mGluR1 receptors. Bioorg. Med. Chem. Lett. *7*, 2777-2780.

Conn, P., and Pin, J. (1997). Pharmacology and functions of metabotropic glutamate receptors. Annual review of pharmacology and toxicology *37*, 205-237.

Dale, L., Babwah, A., Bhattacharya, M., Kelvin, D., and Ferguson, S. (2001). Spatial temporal patterning of metabotropic glutamate receptor-mediated inositol 1,4,5-triphosphate, calcium, and protein kinase C oscillations: protein kinase C-dependent receptor phosphorylation is not required. The Journal of biological chemistry *276*, 35900-35908.

Daub, H., Wallasch, C., Lankenau, A., Herrlich, A., and Ullrich, A. (1997) Signal characteristics of G-protein-transactivated EGF receptor. EMBO Journal *16*, 7032–7044.

Della Rocca, GJ., Maudsley,S., Daaka, Y., Lefkowitz RJ., LuttrellLM. (1999). Pleiotropic coupling of G-protein-coupled receptors to the mitogen-activated protein kinase cascade. Role of focal adhesions and receptor tyrosine kinases. Journal of Biological Chemistry *274*, 13978–13984.

Dhami GK, and Ferguson SS (2006) Regulation of metabotropic glutamate re- ceptor signaling, desensitization and endocytosis. Pharmacol Ther *111*, 260 –271.

Dhami, G., Anborgh, P., Dale, L., Sterne-Marr, R., and Ferguson, S. (2002). Phosphorylation-independent regulation of metabotropic glutamate receptor signaling by G protein-coupled receptor kinase 2. The Journal of biological chemistry *277*, 25266-25272.

Dhami, G., Babwah, A., Sterne-Marr, R., and Ferguson, SS. (2005). Phosphorylationindependent regulation of metabotropic glutamate receptor 1 signaling requires g proteincoupled receptor kinase 2 binding to the second intracellular loop. The Journal of biological chemistry *280*, 24420-24427.

Dhami, G., Dale, L., Anborgh, P., O'Connor-Halligan, K., Sterne-Marr, R., and Ferguson, S. (2004). G Protein-coupled receptor kinase 2 regulator of G protein signaling homology domain binds to both metabotropic glutamate receptor 1a and Galphaq to attenuate signaling. The Journal of biological chemistry *279*, 16614-16620.

Dinamarca, MC., Ríos, JA., and Inestrosa, NC. (2012). Postsynaptic receptors for amyloid-β oligomers as mediators of neuronal damage in Alzheimer's disease. Frontiers in Physiology *3*, 464.

Doherty, AJ., Palmer, MJ., Henley, JM., Collingridge, GL., and Jane, DE. (1997) (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) activates mGlu5, but not mGlu1, receptors expressed in CHO cells and potentiates NMDA responses in the hippocampus. Neuropharmacology *36,* 265-267.

Dolen, G., Bear, MF. (2008) Role for metabotropic glutamate receptor 5 (mGluR5) in the pathogenesis of fragile X syndrome. Journal of Physiology *586,* 1503-1508.

Emery, A., Pshenichkin, S., Takoudjou, G., Grajkowska, E., Wolfe, B., and Wroblewski, J. (2010). The protective signaling of metabotropic glutamate receptor 1 Is mediated by sustained, beta-arrestin-1-dependent ERK phosphorylation. The Journal of biological chemistry *285*, 26041-26048.

Enz, R. (2007) The trick of the tail: protein-protein interactions of metabo- tropic glutamate receptors. Bioessays *29*, 60–73

Enz, R. (2012) Metabotropic glutamate receptors and interacting proteins: evolving drug targets. Curr Drug Targets *13*:145–156

Enz, R. (2012). Structure of metabotropic glutamate receptor C-terminal domains in contact with interacting proteins. Frontiers in molecular neuroscience *5*, 52.

Erondu, N., and Kennedy, M. (1985). Regional distribution of type II Ca2+/calmodulindependent protein kinase in rat brain. The Journal of neuroscience : the official journal of the Society for Neuroscience *5*, 3270-3277.

Esseltine, JL., Willard, MD., Wulur, IH., Lajiness, ME., Barber, TD., and Ferguson, SS. (2013). Somatic Mutations in GRM1 in Cancer Alter Metabotropic Glutamate Receptor 1 Intracellular Localization and Signaling. Mol Pharmacol *83*, 770–780

Ferguson, S., and Caron, M. (2004). Green fluorescent protein-tagged beta-arrestin translocation as a measure of G protein-coupled receptor activation. Methods in molecular biology (Clifton, NJ) *237*, 121-126.

Ferguson, SS. (2001). Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacol Rev *53*, 1–24.

Ferraguti, F., and Shigemoto, R. (2006). Metabotropic glutamate receptors. Cell and tissue research *326*, 483-504.

Ferraguti, F., Baldani-Guerra, B., Corsi, M., Nakanishi, S., and Corti, C. (1999). Activation of the extracellular signal-regulated kinase 2 by metabotropic glutamate receptors. The European journal of neuroscience *11*, 2073-2082.

Ferraguti, F., Conquet, F., Corti, C., Grandes, P, Kuhn, R., and Knopfel, T. (1998). Immunohistochemical localization of the metabotropic glutamate receptor mGluR1' in the adult rodent forebrain. Evidence for a differential distribution of mGluR1 splice variants. J Comp Neurol *400*, 391– 407.

Ferreira, L., Dale, L., Ribeiro, F., Babwah, A., Pampillo, M., and Ferguson, S. (2009).
Flor, PJ., Gomeza, J., Tones, MA., Kuhn, R., Pin, JP. And Knopfel, T. (1996). Journal of Neurochemistry *67*, 58-63.

Flor, P. J., Gomeza, J., Tones, M. A., Kuhn, R., Pin, J. P. & Knopfel, T. (1996). The C-terminal domain of the mGluR1 metabotropic glutamate receptor affects sensitivity to agonists. Journal of Neurochemistry *67*, 58–63.

Francesconi, A., and Duvoisin, R. (1998). Role of the second and third intracellular loops of metabotropic glutamate receptors in mediating dual signal transduction activation. The Journal of Biological Chemistry *273*, 5615-5624.

Francesconi, W., Cammalleri, M., Sanna, PP. (2004). The metabotropic glutamate receptor 5 is necessary for late- phase long-term potentiation in the hippocampal CA1 region. Brain Research. *1022*, 12–18.

Gasparini, F., Lingenhohl, K., Stoehr, N., Flor, PJ., Heinrich, M., Vranesic, I., Biollaz, M., Allgeier, H., Heckendorn, R., Urwyler, S., Varney, M. A., Johnson, EC., Hess, SD., Rao, SP., Sacaan, AI., Santori, EM., Velicelebi, G., and Kuhn, R. (1999). 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu5 receptor antagonist. Neuropharmacology *38*, 1493-1503.

Giese, K., Fedorov, N., Filipkowski, R., and Silva, A. (1998). Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. Science *279*, 870-873.

Gomeza, J., Joly, C., Kuhn, R., Knöpfel, T., Bockaert, J., and Pin, J. (1996). The second intracellular loop of metabotropic glutamate receptor 1 cooperates with the other intracellular domains to control coupling to G-proteins. The Journal of biological chemistry *271*, 2199-2205.

Green, K. N. (2009). Calcium in the initiation, progression and as an effector of Alzheimer's disease pathology. J. Cell. Mol. Med. *13* 2787–2799.

Grolla, AA., Sim, JA., Lim, D., Rodriguez, JJ., Genazzani, AA., and Verkhratsky, A. (2013). Amyloid-beta and Alzheimer's disease type pathology differentially affects the calcium signalling toolkit in astrocytes from different brain regions. Cell Death Dis. *9*, 623

Groves, J., Schulman, H., and Kuriyan, J. (2011). A mechanism for tunable autoinhibition in the structure of a human Ca2+/calmodulin- dependent kinase II holoenzyme. Cell *146*, 732-745.

Gunther, EC., Strittmatter, SM. (2010). Beta-amyloid oligomers and cellular prion protein in Alzheimer's disease. J Mol Med (Berl) *88*, 331-338.

Guo, ML., Fibuch, EE., Liu, XY., Choe, ES., Buch, S., Mao, LM., and Wang, JQ. (2010). CaMKIIalpha interacts with M4 muscarinic receptors to control receptor and psychomotor function. The EMBO Journal *29*, 2070-2081.

Hall, RA. (2004). Co-immunoprecipitation as a strategy to evaluate receptor-receptor or recetptor-protein interactions. G Protein-Coupled Receptor–Protein Interactions.165-178

Hamilton, A., Esseltine, JL., DeVries RA., Cregan SP., and Ferguson, SS. (2014). Metabotropic glutamate receptor 5 knockout reduces cognitive impairment and pathogenesis in a mouse model of Alzheimer's disease. Molecular Brain *7*, 40.

Hanson, PI., and Schulman, H.(1992). Neuronal Ca2+/calmodulin-dependent protein kinases. Annual Reviews Biochemistry *61*, 559-661.

Hardy J, Selkoe DJ. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science *297*, 353–356.

Hishinuma, S., and Ogura, K. (2000). Ca2+/Calmodulin-Mediated Regulation of the Desensitizing Process in Gq Protein-Coupled Histamine H1 Receptor-Mediated Ca2+ Responses in Human U373 MG Astrocytoma Cells. Journal of Neuro Chemistry *75*, 772-781.

Holtzman, DM., Morris, JC., and Goate, AM. (2011). Alzheimer's disease: the challenge of the second century. Science Translational Medicine *3*, 77.

Hsieh, H., Boehm, J., Sato, C., Iwatsubo, T., Tomita, T., Sisodia, S., and Malinow, R. (2006). AMPAR removal underlies Abeta-induced synaptic depression and dendritic spine loss. Neuron *52*, 831–843

Hudmon, A., and Schulman, H. (2002). Neuronal CA2+/calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function. Annual review of biochemistry *71*, 473-510.

Jia, Z., Lu, YM., Henderson, J., Taverna, F., Romano, C., Abramow-Newerly, W., Wojtowicz, M., and Roder J.(1998). Selective Abolition of the NMDA Component of Long-Term Potentiation in Mice Lacking mGluR5. Learning and Memory *5*, 331-343.

Jin, DZ., Guo, ML., Xue, B., Fibuch, EE., Choe, ES., Mao, LM., and Wang, JQ. (2013a). Phosphorylation and feedback regulation of metabotropic glutamate receptor 1 by calcium/calmodulin-dependent protein kinase II. Journal of Neuroscience *33*, 3402-3412.

Jin, DZ., Guo, ML., Xue, B., Mao, LM., and Wang, JQ. (2013b). Differential regulation of CaMKIIα interactions with mGluR5 and NMDA receptors by Ca2+ in neurons. Journal of Neurochemistry *127*, 620-631.

Joly, C., Gomeza, J., Brabet, I., Curry, K., Bockaert, J., and Pin, J. (1995). Molecular, functional, and pharmacological characterization of the metabotropic glutamate receptor type 5 splice variants: comparison with mGluR1. The Journal of neuroscience : the official journal of the Society for Neuroscience *15*, 3970-3981.

Jong, YJ., Kumar, V., and O'Malley, KL. (2009). Intracellular metabotropic glutamate receptor 5 (mGluR5) activates signaling cascades distinct from cell surface counterparts. Journal of Biological Chemistry *284*, 35827–35838.

Karim, F., Wang, C., and Gereau, R. (2001). Metabotropic glutamate receptor subtypes 1 and 5 are activators of extracellular signal-regulated kinase signaling required for inflammatory pain in mice. The Journal of neuroscience: the official journal of the Society for Neuroscience *21*, 3771-3779.

Kawabata, S., Kohara, A., Tsutsumi, R., Itahana, H., Hayashibe, S., Yamaguchi, T., and Okada, M. (1998). Diversity of calcium signaling by metabotropic glutamate receptors. The Journal of biological chemistry *273*, 17381-17385.

Kellett, KAB., and Hooper, NM. (2009). Prion protein and Alzhemier Disease. Prion *3*, 190-194.

Khachaturian ZS.(1989). Calcium, membranes, aging, and Alzheimer's disease. introduction and overview. Ann N Y Acad Sci. *9*, 1–4.

Kumar V., Fahey PG., Jong YJ., Ramanan N., and O'Malley K. L. (2012). Activation of intracellular metabotropic glutamate receptor 5 in striatal neurons leads to up-regulation of genes associated with sustained synaptic transmission including Arc/Arg3.1 protein. Journal of Biological Chemistry *287*, 5412–5425

Kunishima, N., Shimada, Y., Tsuji, Y., Sato, T., Yamamoto, M., Kumasaka, T., Nakanishi, S., Jingami, H., and Morikawa, K. (2000). Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. Nature *407*, 971-977.

Lagerström, M., and Schiöth, H. (2008). Structural diversity of G protein-coupled receptors and significance for drug discovery. Nature Review Drug Discovery *4*, 339-357.

Lauren, J., Gimbel, DA., Nygaard, HB., Gilbert, JW., and Strittmatter, SM. (2009). Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. Nature *457*, 1128-1132.

Lee, H., Barbarosie, M., Kameyama, K., Bear, M., and Huganir, R. (2000). Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. Nature *405*, 955-959.

Lee, H., Zhu, X., O, M., and Webber, K. (2004). The role of metabotropic glutamate receptors in Alzheimer's disease. *Acta. Neurobiol. 64*, 89-98.

Lee, JH., Lee, J., Choi, KY., Hepp, R., Lee, J. Y., Lim, MK., Chatani-Hinze, M., Roche, PA., Kim, DG., Ahn, YS., Kim, CH., and Roche, KW. (2008). Calmodulin dynamically regulates the trafficking of the metabotropic glutamate receptor mGluR5. PNAS *105*, 12575–12580.

Lee, S.-J.R., Escobedo-Lozoya, Y., Szatmari, E., and Yasuda, R. (2009). Activation of CaMKII in single dendritic spines during long-term potentiation. Nature *458*, 299-304.

Lee, YS., and Silva, A. (2009) The molecular and cellular biology of enhanced cognition. Nature Review Neuroscience *10*, 126–140.

Leonard, AS., Lim, IA., Hemsworth, DE., Horne, MC., Hell, JW. (1999). Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-D-aspartate receptor. PNAS *96*, 3239–3244.

Li, S., Hong, S., Shepardson, N.E., Walsh, DM., Shankar, G.M., and Selkoe, D. (2009). Soluble oligomers of amyloid Beta protein facilitate hippocampal long-term depression by disrupting neuronal glutamate uptake. Neuron *62,* 788-801.

Linden, R., Martins, VR., Prado, MA., Cammarota, M., Izquierdo, I., Brentani, RR. (2008). Physiology of the prion protein. Physiology Review *88*, 673-728.

Lisman, J., Schulman, H., and Cline, H. (2002). The molecular basis of CaMKII functionin synaptic and behavioural memory. Nature reviews Neuroscience *3*, 175-190.

Lisman, J., Yasuda, R., and Raghavachari, S. (2012). Mechanisms of CaMKII action in long-term potentiation. Nature reviews Neuroscience *13*, 169-182.

Liu XY, Mao LM, Zhang GC, Papasian CJ, Fibuch EE, Lan HX, Zhou HF, Xu M, Wang JQ (2009). Activity-dependent modulation of limbic dopamine D3 receptors by CaMKII. Neuron *61*, 425– 438.

Lu, YM., Jia, Z., Janus, C., Henderson, J., Gerlai, R., Wojtowicz, M., and Roder J.(1997) Mice lacking metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term potentiation (LTP), but normal CA3-LTP. Journal of Neuroscience *17*, 5196–5205.

Lucchesi, W., Mizuno, K., and Giese, K. (2011). Novel insights into CaMKII function and regulation during memory formation. Brain research bulletin *85*, 2-8.

Lujan R., N., Z., Roberts, J. D., Shigemoto, R., Somogyi, P. (1996). Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. European journal of neuroscience 8, 1488-1500.
Luttrell LM, Kenakin TP. Refining efficacy: allosterism and bias in G protein-coupled receptor signaling. Methods Mol Biol (2011). *756*, 3–3510.

Luttrell, LM., and Lefkowitz, RJ. (2002). The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci 115*, 455–465.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ. (1999). Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. Science *283*, 655–6110.

Luttrell, LM., Roudabush, FL., Choy, EW., Miller, WE., Field, ME., Pierce, KL., Lefkowitz, RJ. (2001). Activation and targeting of extracellular signal-regulated kinases by β-arrestin scaffolds. PNAS *98*, 2449-2454.

Lynch, M. (2004). Long-term potentiation and memory. Physiological Reviews *84*, 87-136.

López-Bendito, G., Shigemoto, R., Fairén, A., and Luján, R. (2002). Differential distribution of group I metabotropic glutamate receptors during rat cortical development. Cerebral cortex *12*, 625-638.

Lüscher, C., and Huber, K. (2010) Group 1 mGluR-dependent long-term depression (mGluR-LTD): mechanism and implications for circuitry and disease. Neuron *65*, 445-459.

Macey, TA., Lowe, JD., and Chavkin, C. (2006). Mu opioid receptor activation of ERK1/2 is GRK3 and arrestin dependent in striatal neurons. Journal of Biological Chemistry *281*, 34515-34524.

Malinow, R. (2003). AMPA receptor trafficking and long-term potentiation. Philos Trans R Soc Lond B Biol Sci *358*, 707–714.

Malenka, R., and Bear, M. (2004). LTP and LTD: an embarrassment of riches. Neuron *44*, 5-21.

Malinow R. (2003). AMPA receptor trafficking and long-term potentiation. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *358*, 707–714.

Mao, L., Yang, L.,Tang, Q., Samdani, S., Zhang, G., and Wang, JQ. (2005a). The scaffold protein Homer1b/c links metabotropic glutamate receptor 5 to extracellular signal-regulated protein kinase cascades in neurons. Journal of Neuroscience *25*, 2741-2752.

Mao, L., Yang, L., Arora, A., Choe, ES., Zhang, G., Liu, Z., Fibuch, EE., and Wang, JQ. (2005b). Role of protein phosphatase 2A in mGluR5-regulated MEK/ERK phosphorylation in neurons. Journal of Biological Chemistry *280*, 12602-12610.

Mao, L., Liu, X., Zhang, G., Chu X., Fibuch, E., Wang, L., Liu, Z., and Wang JQ. (2008). Phosphorylation of group I metabotropic glutamate receptors (mGluR1/5) in vitro and in vivo. Neuropharmacology *55*, 403-408.

Mockett, B., Guévremont, D., Wutte, M., Hulme, S., Williams, J., and Abraham, W. (2011). Calcium/calmodulin-dependent protein kinase II mediates group I metabotropic glutamate receptor-dependent protein synthesis and long-term depression in rat hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 7380-7391.

Mundell, S., Matharu, A.-L., Pula, G., Holman, D., Roberts, P., and Kelly, E. (2002). Metabotropic glutamate receptor 1 internalization induced by muscarinic acetylcholine receptor activation: differential dependency of internalization of splice variants on nonvisual arrestins. Molecular pharmacology *61*, 1114-1123.

Nakanishi, S. (1992). Molecular diversity of glutamate receptors and implications for brain function. Science *258*, 597-603.

Nakanishi, S., and Masu, M. (1994). Molecular diversity and functions of glutamate receptors. Annu. Rev. Biophys. Biomol. Struct. *23*, 319-348.

Nash MS, Schell MJ, Atkinson PJ, Johnston NR, Nahorski SR, Challiss RA. (2002). Determinants of metabotropic glutamate receptor-5-mediated Ca2+ and inositol 1,4,5-trisphosphate oscillation frequency: receptor density versus agonist concentration. J Biol Chem *277*, 35947–35960

Neer, EJ. (1995). Heterotrimeric G proteins: Organizers of Transmembrane Signals. Cell *80*, 249-257.

Nelson, P.T., Braak, H., and Markesbery, W.R. (2009) Neuropathology and cognitive impairment in Alzheimer disease: a complex but coherent relationship. *J. Neuropathol. Exp. Neurol. 68***,** 1–14.

Neyman, S., and Manahan-Vaughan, D. (2008). Metabotropic glutamate receptor 1 (mGluR1) and 5 (mGluR5) regulate late phases of LTP and LTD in the hippocampal CA1 region in vitro. European Journal of Neuroscience *6,* 1345-1352.

Nicodemo, A., Pampillo, M., Ferreira, L., Dale, L., Cregan, T., Ribeiro, F., and Ferguson, S. (2010). Pyk2 uncouples metabotropic glutamate receptor G protein signaling but facilitates ERK1/2 activation. Molecular Brain *3*, 4.

Nicoletti, F., Bruno,V., Catania, MV., Battaglia, G., Copani, A., Barbagallo, G., Cena, V., Sanchez-Prieto, J., Spano, PF., and Pizzi, M. (1999). Group-I Metabotropic Glutamate Receptors: Hypotheses to Explain Their Dual Role in Neurotoxicity and Neuroprotection. Neuropharmacology *38*, 1477–1484.

Niswender, C.M., and Conn, P.J. (2010). Metabotropic glutamate receptors: phsyiology, pharmacology, and disease. Annu. Rev. Pharmacol. Toxicol. *50*, 295-322.

Opazo, P., Labrecque, S., Tigaret, C., Frouin, A., Wiseman, P., De Koninck, P., and Choquet, D. (2010). CaMKII triggers the diffusional trapping of surface AMPARs through phosphorylation of stargazin. Neuron *67*, 239-252.

Ozawa S, Kamiya H, Tsuzuki K. (1998). Glutamate receptors in the mammalian central nervous system. *Progress in Neurobiology*. *54*, 581–618.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, BE., Karandikar, M., Berman, K. and Cobb, MH. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocrine Review *22*, 153–183.

Peavy, RD., Chang, MS., Sanders-Bush, E., and Conn, PJ. (2001). Metabotropic glutamate receptor 5-induced phosphorylation of extracellular signal-regulated kinase in astrocytes depends on transactivation of the epidermal growth factor receptor. Journal of Neuroscience *21,* 9619-9628.

Pieretti, M., Zhang, FP., Fu, YH., Warren, ST., Oostra BA., Caskey, CT., and Nelson DL. (1991). Absence of expression of the FMR-1 gene in Fragile X syndrome. Cell *66*, 817–822.

Pin, J.P., and Duvoisin R. (1995). The metabotropic glutamate receptors: structure and function. Neuropharmacology *34*, 1-26.

Poncer, JC., Esteban, JA., and Malinow, R. (2002). Multiple mechanisms for the potentiation of AMPA receptor-mediated transmission by alpha- Ca21/Calmodulin-dependent protein kinase II. Journal of Neuroscience *22*, 4406– 4411.

Renner M, Lacor PN, Velasco PT, Xu J, Contractor A, Klein WL, Triller A. (2010). Deleterious effects of amyloid beta oligomers acting as an extracellular scaffold for mGluR5. Neuron *66*, 739-754.

Ribeiro FM, Ferreira LT, Paquet M, Cregan T, Ding Q, Gros R, Ferguson SSG. (2009). Phosphorylation-independent regulation of metabotropic glutamate receptor 5 desensitization and internalization by G protein-coupled receptor kinase 2 in neurons. J Biol Chem *7*, 23444–23453.

Ribeiro, FM., Paquet, M., Cregan, SP., and Ferguson, SSG. (2010a). Group 1 metabotropic glutamate receptor signaling and its implication in neurological disease. CNS Neurol Discord Drug Targets *9*, 574–595.

Ribeiro, F., Paquet, M., Ferreira, L., Cregan, T., Swan, P., Cregan, S., and Ferguson, S. (2010b). Metabotropic glutamate receptor-mediated cell signaling pathways are altered in a mouse model of Huntington's disease. The Journal of neuroscience : the official journal of the Society for Neuroscience *30*, 316-324.

Ribeiro, F., Pires, R., and Ferguson, S. (2011). Huntington's disease and Group I metabotropic glutamate receptors. Molecular neurobiology *43*, 1-11.

Riedel G, Reymann KG. (1996). Metabotropic glutamate receptors in hippocampal long-term potentiation and learning and memory. Acta Physiol. Scand *157*, 1–19

Romano, C., Sesma, M., McDonald, C., O'Malley, K., Van den Pol, A., and Olney, J. (1996). Distribution of metabotropic glutamate receptor mGluR5 immunoreactivity in rat brain. The Journal of comparative neurology *355*, 455-469.

Roskoski R., Jr. ERK1/2 MAP kinases: structure, function, and regulation. (2012) Pharmacology Research *66*, 105–143.

Schiefer, J., Sprunken, A., Puls, C., Luesse, H.G., Milkereit, A., Milkereit, E., Johann, V., and Kosinski, CM. (2004). The metabotropic glutamate receptor 5 antagonist MPEP and the mGluR2 agonist LY379268 modify disease progression in a transgenic mouse model

of Huntington's disease. Brain Research 1019, 246-254.

Schnell. E., Sizemore, M., Karimzadegan, S., Chen, L., Bredt, DA., and Nicoll, RA. Direct interactions between PSD-95 and stargazin control synaptic AMPA receptor number. (2002) PNAS *99*, 13902-13907.

Schools, GP., and Kimelberg, HK. (1999). mGluR3 and mGluR5 are the predominant metabotropic glutamate receptor mRNAs expressed in hippocampal astrocytes acutely isolated from young rats. Journal of Neuroscience Research *58,* 533-543.

Selkoe, DJ. (2006). Toward a comprehensive theory for Alzheimer's disease. Hypothesis: Alzheimer's disease is caused by the cerebral accumulation and cytotoxicity of amyloid β-protein. Ann NY Acad Sci *924*, 17-25.

Seo, B., Choy EW., Maudsley, S., Miller, WE., Wilson, BA., and L uttrell, LM. (2000) Pasteurella multocida toxin stimulates mitogen-activated protein kinase via Gq/11-dependent transactivation of the epidermal growth factor receptor. Journal of Biological Chemistry *275*, 2239–2245.

Shankar, GM., Bloodgood, BL., Townsend, M., Walsh, DM., Selkoe, DJ., and Sabatini BL. (2007). Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. Journal of Neuroscience *27*, 2866-2875.

Shen, K., and Meyer, T. (1999). Dynamic Control of CaMKII Translocation and Localization in Hippocampal Neurons by NMDA Receptor Stimulation. Science *284*, 162-167.

Shenoy, SK., Drake, MT., Nelson, CD., Houtz, DA., Xiao, K, Madabushi S., Reiter, E., Premont, RT., Lichtarge O, and Lefokowitz RJ. (2006) beta- arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. Journal of Biological Chemistry *281*, 1261–1273.

Shigemoto, R., Kinoshita, A., Wada, E., Nomura, S., Ohishi, H., Takada, M., Flor, P., Neki, A., Abe, T., Nakanishi, S.*, et al.* (1997). Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience *17*, 7503-7522.

Shrivastava AN, Kowalewski JM, Renner M, Bousset L, Koulakoff A, Melki R, Giaume C, Triller A. (2013) β-amyloid and ATP-induced diffusional trapping of astrocyte and neuronal metabotropic glutamate type-5 receptors. Glia. *61*, 1673-1686

Skeberdis, VA., Lan, J., Opitz, T., Zheng, X., Bennett, MV., and Zukin, RS. (2001). mGluR1-mediated potentiation of NMDA receptors involves a rise in intracellular calcium and activation of protein kinase C. Neuropharmacology *40,* 856-865.

Snyder, EM., Nong, Y., Almeida, CG., Paul, S., Moran, T., Young-Choi, E., Nairn, AC., Salter, MW., Lombroso, PJ., Gouras, GK., and Greengard, P. (2005). Regulation of NMDA receptor trafficking by amyloid-β. Nature Neuroscience *8*, 1051-1058.

So, CH., Verma, V., O'Dowd, BF., and George SR. (2007). Desensitization of the Dopamine D1 and D2 Receptor Hetero-Oligomer Mediated Calcium Signal by Agonist Occupancy of Either Receptor. Molecular Pharmacology *72*, 450-462.

Soltoff, SP. (1998) Related adhesion focal tyrosine kinase and the epider- mal growth factor receptor mediate the stimulation of mitogen- activated protein kinase by the G-protein-coupled P2Y2 receptor. Journal of Biological Chemistry *273*, 23110-23117.

Strack, S., Choi, S., Lovinger, D., and Colbran, R. (1997). Translocation of autophosphorylated calcium/calmodulin-dependent protein kinase II to the postsynaptic density. The Journal of Biological Chemistry *272*, 13467-13470.

Sumi M., Kiuchi K., Ishikawa T., Ishii A., Hagiwara M. (1991). The newly synthesized selective Ca2+/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. Biochem. Biophys. Res. Commun. *181*, 968–975

Thandi, S., Blank, JL., and Challiss, RA. (2002) Group-I metabotropic glutamate receptors, mGlu1a and mGlu5a, couple to extracellular signal-regulated kinase (ERK) activation via distinct, but overlapping, signalling pathways. Journal of Neurochemistry. *83,* 1139–1153.

Thomas U. (2002). Modulation of synaptic signalling complexes by Homer proteins. J. Neurochem. *81*, 407–41310.

Tu, J., Xiao, B., Yuan, J., Lanahan, A., Leoffert, K., Li, M., Linden, D., and Worley, P. (1998). Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. Neuron *21*, 717-726.

Tsai, V. W., Scott, H. L., Lewis, R. J., and Dodd, P. R. (2005). The role of group I metabotropic glutamate receptors in neuronal excitotoxic- ity in Alzheimer's disease. Neurotox. Res. *7*, 125–141

Um JW, Kaufman AC, Kostylev M, Heiss JK, Stagi M, Takahashi H, Kerrisk ME, Vortmeyer A, Wisniewski T, Koleske AJ, Gunther EC, Nygaard HB, Strittmatter SM. (2013). Metabotropic Glutamate Receptor 5 Is a Coreceptor for Alzheimer Ab Oligomer Bound to Cellular Prion Protein. Neuron. *79*, 887-902.

Vardy ERLC, Catto AJ, Hooper NM. (2005). Proteolytic mechanisms in amyloid-beta metabolism: therapeutic implications for Alzheimer's disease. Trends Mol Med *11*, 464-472.

Walsh, DM., Klyubin., I, Fadeeva, JV., Cullen, WK., Anwyl, R., Wolf, MS., Rowan, MJ., Selkoe, DJ. (2002). Naturally secreted oligomers of amyloid bold beta protein potently inhibit hippocampal long-term potentiation in vivo. Nature *416*, 535–539.

Wang, HY., Lee, DH., Davis, CB., and Shank, RP. (2000). Amyloid peptide Aβ(1-42) binds selectively and with picomolar affinity to α7 nicotinic acetylcholine receptors. Journal of Neurochemistry *75*, 1155–1161.

Wang, HY., Li, W., Benedetti, NJ., and Lee, DHS. (2003). α7-nicotinic acetylcholine receptors mediate β-amyloid peptide-induced tau protein phosphorylation. Journal of Biological Chemistry *278*, 31547–31553.

Wang, Q., Walsh, DM., Rowan, MJ., Selkoe, DJ., and Anwyl, R. (2004). Block of long-term potentiation by naturally secreted and synthetic amyloid beta-peptide in hippocampal slices is mediated via activation of the kinases c-Jun Nterminal kinase, cyclin-dependent kinase 5, and p38 mitogen-activated protein kinase as well as metabotropic glutamate receptor type 5. Journal of Neuroscience *24,* 3370-3378.

White, RR., Kwon, YG., Taing, M., Lawrence DS., Edelman, AM. (1998). Definition of optimal substrate recognition motifs of Ca2+-calmodulin-dependent protein kinases IV and II reveals shared and distinctive features. J Biol Chem. *273,* 3166–3179.

Willard, SS., Koochekpour S. (2013). Glutamate, Glutamate Receptors, and Downstream Signaling Pathways. Int J Biol Sci. *9*, 948-959.

Young K. F., Pasternak S. H., Rylett R. J. (2009). Oligomeric aggregates of amyloid beta peptide 1–42 activate ERK/MAPK in SH-SY5Y cells via the alpha7 nicotinic receptor. *Neurochem. Int. 55*, 796–801

Zhang, J.F., Qi, JS., and Qiao, JT. (2009). Protein kinase C mediates amyloid betaprotein fragment 31-35-induced suppression of hippocampal late-phase longterm potentiation in vivo. Neurobiol. Learn. Mem *91,* 226-234.

Zhang, R., and Xie, X. (2012). Tools for GPCR drug discovery. Acta Pharmacology Sin *33*, 372-384.

Zhang, T., and Brown, JH. (2004). Role of Ca2+/calmodulin-dependent protein kinase II in cardiac hypertrophy and heart failure. Cardiovascular Research *63*, 476-486.

Zhao, D., Watson, JB., Xie, CW. (2004). Amyloid beta prevents activation of calcium/calmodulin-dependent protein kinase II and AMPA receptor phosphorylation during hippocampal long-term potentiation. J Neurophysiol. *92*, 2853-2858.

Zhou, Y., Takahashi, E., Li, W., Halt, A., Wiltgen, B., Ehninger, D., Li, G., Hell, JW., Kennedy, MB., and Silva AJ. (2007). Interactions between the NR2B receptor and CaMKII modulate synaptic plasticity and spatial learning. Journal of Neuroscience *27*, 13843-13853.

Zundorf, G., and Reiser, G. (2011). Calcium dysregulation and homeostasis of neural calcium in the molecular mechanisms of neurodegenerative diseases provide multiple targets for neuroprotection. Antioxidants and Redox Signaling *14*, 1275-1288.

NAME: Fitore Raka

POST-SECODNARY EDUCATION

Master of Science — 2012-2014
University of Western Ontario
Physiology and Pharmacology

Honours Bachelor of Medical Science — 2008-2012
University of Western Ontario
Honors Specialization in Physiology

SCHOLARSHIPS

Ontario Graduate Scholarship ($15, 000)	2013
CIHR Strategic Training Program in Vascular Research ($12,000/year)	2012-2014
Western Graduate Research Scholarship ($8,000/year)	2012-2014
Western Admission Scholarship of Distinction ($1, 500)	2008

RELATED EXPERIENCE

Teaching Assistant — 2012-2014
University of Western Ontario
Department of Physiology and Pharmacology

Student Researcher — 2012
Robarts Research Institute – Dr. Stephen Ferguson

POSTER PRESENTATIONS

Role of CaMKIIa in the regulation of Metabotropic Glutamate Receptor 5. Presented at London Health Research Day, London ON., 2014.

Regulation of Metabotropic Glutmate Receotir 5 activity by CaMKIIa. Presented at Great Lakes GPCR Retreat, Cleveland OH., 2013