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SPINOPHILIN REGULATION OF GROUP I METABOTROPIC GLUTAMATE RECEPTOR ACTIVITY

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SPINOPHILIN REGULATION OF GROUP I METABOTROPIC GLUTAMATE RECEPTOR ACTIVITY

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ABSTRACT

Group I metabotropic glutamate receptors (mGluR1 and mGluR5) are activated by glutamate, the major excitatory neurotransmitter in the brain. mGluR1/5 stimulation causes the release of calcium from intracellular stores thereby activating calcium-regulated downstream effector enzymes that produce long-lasting changes in synaptic activity that are implicated in memory, learning, and neurodegenerative diseases. Therefore, understanding the precise molecular mechanisms underlying mGluR1/5 desensitization is important in understanding the physiological response caused by these receptors. Recently, we have discovered a novel Group I mGluR-interacting protein, spinophilin. Spinophilin is a multifunctional protein that regulates the endocytosis of some GPCRs through its interaction with the receptors third intracellular loop domain. We find that spinophilin over-expression decreases agonist-stimulated internalization of mGluR1a, does not affect mGluR1a-dependent activation of phospholipase C leading to inositol phosphate formation, but results in attenuated ERK1/2 phosphorylation. Thus, this study provides evidence that spinophilin regulates the signalling and internalization of Group I mGluRs.

Keywords: metabotropic glutamate receptor, G protein-coupled receptor, spinophilin, internalization, extracellular signal-regulated kinase, signalling

DEDICATION

I would like to thank my family and friends for all of their love and support.

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
AD	Alzheimer's disease
AIDA	(RS)-1-aminoindan-1,5-dicarboxylic acid
ALS	Amyotrophic lateral sclerosis
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AR	Adrenergic receptor
Ca^{2+}	Calcium
CaMKII	Ca^{2+}/calmodulin-dependent protein kinases II
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CNS	Central Nervous System
CPCCOEt	7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester
DAG	Diacylglycerol
DHPG	3,5-dihydroxyphenylglycine glutamate
ECL	Enhanced chemiluminescence
ERK	Extracellular signal-regulated kinase
F-actin	Actin Filament
GAP	GTPase-activating protein
GEFs	Guanine nucleotide exchange factors
GFP	Green fluorescence protein
GPCR	G protein-coupled receptor
G-protein	Guanine nucleotide binding protein
GRK	G protein-coupled receptor kinase
Group I mGluRs	Group I Metabotropic Glutamate Receptors
HBSS	HEPES-balanced salt solution
HEK 293 Cells	Human Embryonic Kidney Cells
IP	Inositol Phosphate
IP_3	inositol 1,4,5-triphosphate
LTD	Long-term synaptic depression
LTP	Long-term synaptic potentiation
LY 367385	(S)-(+)-alpha-amino-4-carboxy-2-methylbezeneacetic acid
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium
mGluR	Metabotropic glutamate receptor
MPEP	2-methyl-6-(phenylethynyl)-pyridine
NMDA	N-methyl-D-aspartate receptor

PBS	Phosphate Buffered Saline
PDZ	PSD-95/Discs large/ZO-1 homology
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKD	Protein kinase D
PLD2	Phospholipase D2
PP1	Protein phosphatase 1
PSD	Post synaptic density
Pyk2	Proline-rich tyrosine kinase 2
RalGDS	Ral guanine nucleotide exchange factor
RFP	Red fluorescence protein
RGS	Regulators of G-protein signalling
RH	RGS homology
RPM	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Src	Proto-oncogene tyrosine-protein kinase

CHAPTER 1
INTRODUCTION

1.1 G-Protein Coupled Receptors

Currently, 50% of pharmacotherapeutics are targeted towards G-protein coupled receptors (GPCRs) (Gesty-Palmer and Luttrell, 2011). GPCRs reside on the plasma membrane, where they play a role in a cell's ability to sense its local environment via interaction with different types of stimuli including photons, odorants, tastants, amino acids, saccharides, fatty acids, complex polypeptides and neurotransmitters (Vilardaga, 2010). GPCRs are seven transmembrane domain proteins that have an amino terminus that is exposed to the extracellular environment and a carboxyl terminus that is exposed to the intracellular environment. Extracellular signalling molecules are recognized by the extracellular domain which binds to these molecules inducing a change in receptor conformation that allows the initiation of an intracellular signalling cascade. The signalling cascade occurs as a consequence of the interaction of heterotrimeric guanine nucleotide binding proteins (G-proteins) with the GPCR carboxyl terminus and intracellular loops. GPCRs have been classified into three families; family A (e.g. rhodopsin like receptors), family B (e.g. secretin/glucagon receptors) and family C (e.g. metabotropic glutamate receptors). Metabotropic glutamate receptors (mGluRs) are unique from other GPCRs due to variation of sequence and structural homology (Dhami and Ferguson, 2006; Jacoby et al., 2006; Niswender and Conn, 2010).

1.2 Metabotropic Glutamate Receptors

Glutamate is the major excitatory neurotransmitter in the brain, it is essential for many physiological processes, including integrative brain function and neuronal cell development (Bordi and Ugolini, 1999; Nakanishi, 1994). It has been found to interact with both ionotropic and metabotropic glutamate receptors (mGluRs). When ionotropic receptors including: N-methyl-D-aspartate receptors (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and kainite type cation channels, become stimulated by glutamate they produce a fast and short cellular response by increasing the amount of sodium and calcium ions in the cell (Nakanishi, 1994). When mGluRs become stimulated by glutamate they also cause an influx of calcium in the cell, and activate downstream effector enzymes that produce long-lasting changes in cellular activity (Figure 1.1) (Bordi and Ugolini, 1999; Ferraguti et al., 1999; Nakanishi, 1994; Niswender and Conn, 2010; Wang, J., et al., 2004; Wang, J., et al., 2007). There are eight types of mGluRs, which are divided into three subgroups based on sequence homology and G protein activation. Group I contains two subtypes mGluR1 and mGluR5, Group II contains mGluR2 and mGluR3 and Group III contains mGluR4, mGluR6, mGluR7, and mGluR8. Upon agonist stimulation, Group I mGluRs couple to the heterotrimeric $G\alpha_{q/11}$ protein causing the activation of phospholipase Cβ, while Groups II and III negatively regulate adenylyl cyclase via $G\alpha_{i/o}$ (Bordi and Ugolini, 1999; Conn and Pin, 1997). Group I metabotropic receptors are of primary focus in this thesis, and will be discussed further in the introduction.

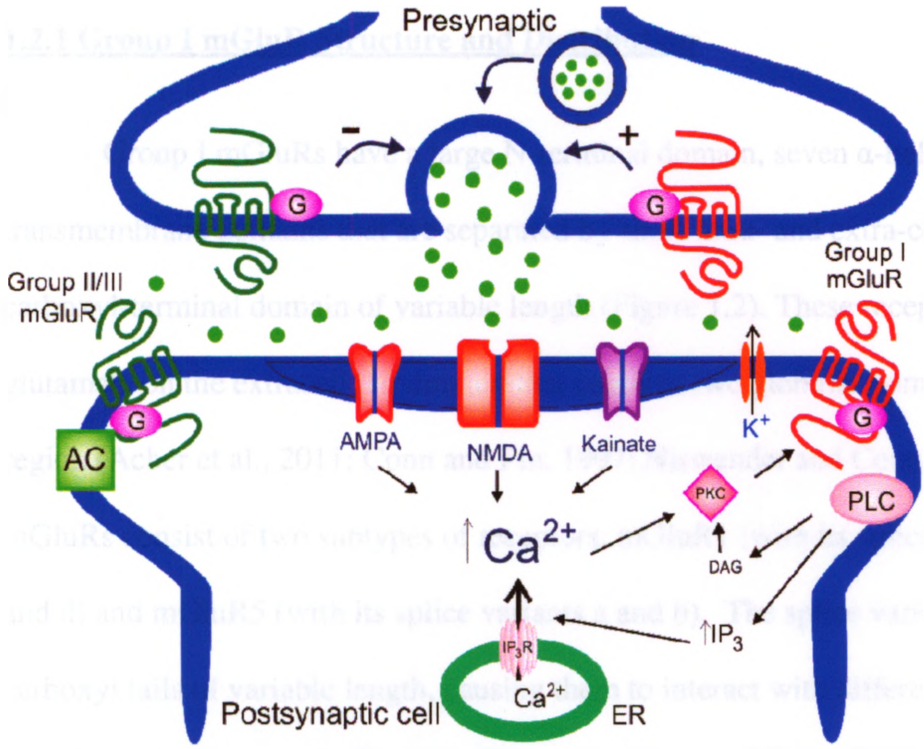

Figure 1.1: Glutamate Signalling in the Synapse. Glutamate interacts with both ionotropic receptors and mGluRs. When ionotropic receptors including: NMDA receptors, AMPA receptors, and kainite type cation channels, become stimulated by glutamate they produce a fast and short cellular response by increasing the amount of calcium in the cell. When mGluRs become stimulated by glutamate, the $G\alpha_{q/11}$ protein activates PLC, resulting in the formation of IP_3 and DAG. The release of IP_3 activates the IP_3 receptor on the ER, causing the release of Ca^{2+} from intracellular stores. The increase in Ca^{2+} and DAG causes the activation of PKC, allowing PKC to phosphorylate its respective substrates (Bordi and Ugolini, 1999; Conn and Pin, 1997; Nakanishi, 1994). Abbreviations: AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Ca^{2+}, calcium; DAG, diacylglycerol; ER, endoplasmic reticulum; IP_3, inositol 1,4,5-triphosphate; mGluRs, metabotropic glutamate receptors; NMDA, N-methyl-D-aspartate receptors; PKC, protein kinase C; PLC, phospholipase C.

1.2.1 Group I mGluR Structure and Distribution

Group I mGluRs have a large N-terminal domain, seven α-helical transmembrane domains that are separated by short intra- and extra-cellular loops and a carboxyl-terminal domain of variable length (Figure 1.2). These receptors interact with glutamate on the extracellular domain that contains two globular domains with a hinge region (Acher et al., 2011; Conn and Pin, 1997; Niswender and Conn, 2010). Group I mGluRs consist of two subtypes of receptors, mGluR1 (with its splice variants a, b, c and d) and mGluR5 (with its splice variants a and b). The splice variants express carboxyl tails of variable length, causing them to interact with different protein complexes and ultimately function differently (Conn and Pin, 1997; Niswender and Conn, 2010). For example, mGluR1a and mGluR5a have long carboxyl tails that are shown to interact with protein phosphatase 1γ, Siah-1A, Homer and PDZ proteins, whereas mGluR1b lacks the amino acid motifs required for these protein interactions (Croci et al., 2003; Kammermeier and Ikeda, 2001; Kammermeier, 2008; Kitano et al., 2002; Niswender and Conn, 2010; Ronesi and Huber, 2008). Additionally, Group I mGluRs couple to G proteins primarily via their second intracellular loop domains as well as their carboxyl tails (Conn and Pin, 1997; Niswender and Conn, 2010).

In the central nervous system (CNS), Group I mGluRs are located in the hippocampus, cortex, thalamus and cerebellum (Byrnes et al., 2009). mGluR1 is highly expressed in the CA3 region and the dentate gyrus of the hippocampus and Purkinje

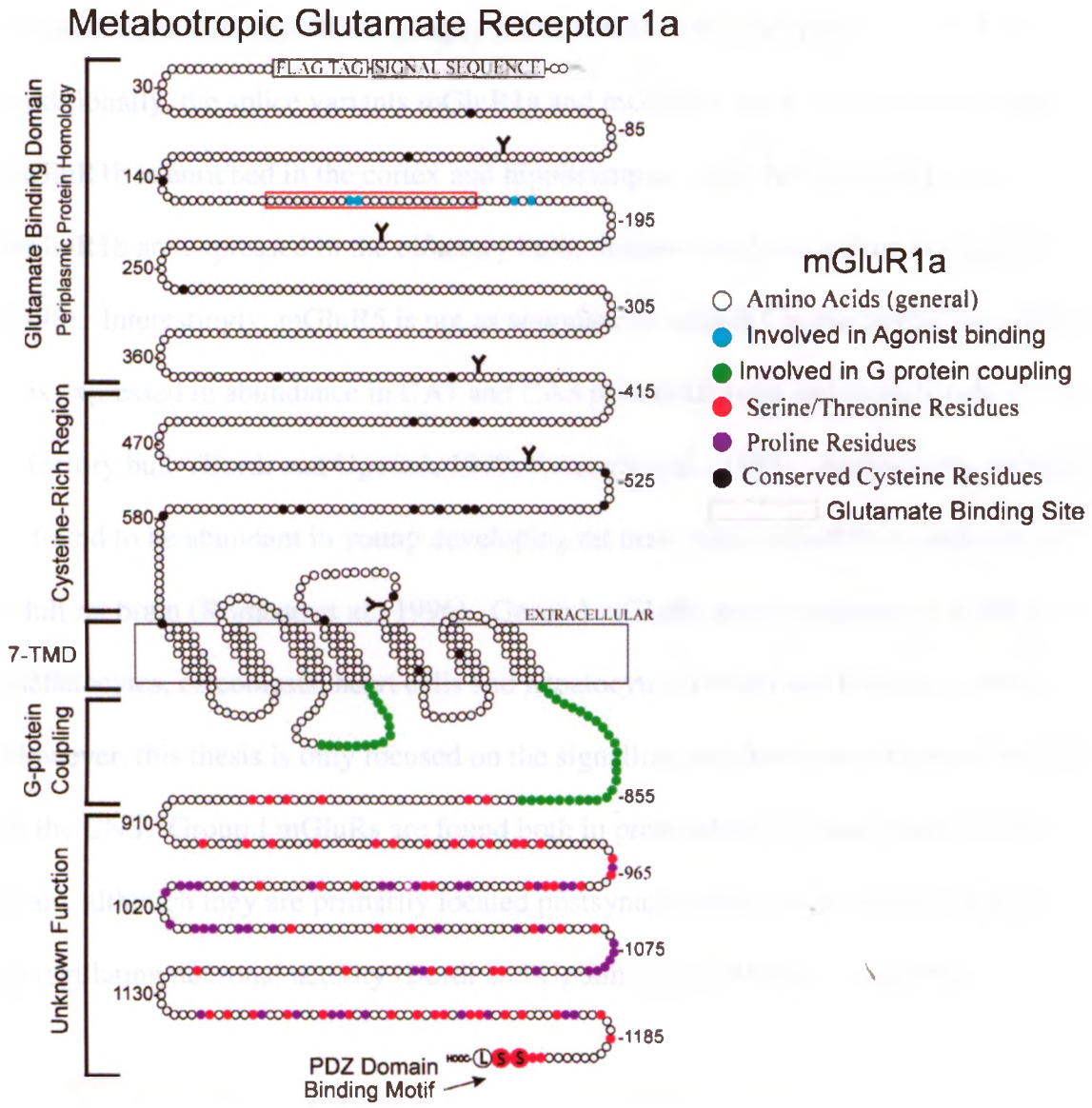

Figure 1.2: Schematic of the Secondary Amino Acid Sequence of mGluR1a.

Illustrated is the glutamate binding domain, transmembrane topology, and the regions involved in agonist binding and G protein coupling (Dhami and Ferguson, 2006). The amino acid residues of the PDZ binding motif are also illustrated. Abbreviations: PDZ, PSD-95/Discs large/ZO-1 homology; TMD, transmembrane domain.

cells in the cerebellum, but it is also expressed in lower quantities in the olfactory bulb, amygdala, thalamus and basal ganglia (Martin et al., 1992; Shigemoto et al., 1992). Additionally, the splice variants mGluR1a and mGluR1b are distributed differently, mGluR1b is enriched in the cortex and hippocampus, while both mGluR1a and mGluR1b are expressed in the olfactory bulb, thalamus and cerebellum (Ferraguti et al., 1998). Interestingly, mGluR5 is not as abundant as mGluR1 in the cerebellum, although it is expressed in abundance in CA1 and CA3 pyramidal cells and granule cells in the olfactory bulb (Bordi and Ugolini, 1999; Romano et al., 1995). Additionally, mGluR5a is found to be abundant in young developing rat brain, and mGluR5b is predominant in adult rat brain (Romano et al., 1996). Group I mGluRs are also expressed in the skin, melanocytes, osteoblasts, heart cells and hepatocytes (Dhami and Ferguson, 2006). However, this thesis is only focused on the signalling and function of Group I mGluRs in the CNS. Group I mGluRs are found both in presynatpic and postsynaptic sites in the brain, although they are primarily located postsynaptically, where they play a large role in regulating neuronal activity (Bordi and Ugolini, 1999; Martin et al., 1992).

1.2.2 Signalling by Group 1 Metabotropic Glutamate Receptors

The primary signalling cascade mediated by Group I mGluRs is through coupling to the heterotrimeric $G\alpha_{q/11}$ proteins, which ultimately causes an increase of intracellular calcium and stimulation of second messenger dependent proteins that produce long lasting changes inside the cell (Dhami and Ferguson, 2006; Kawabata et al., 1998; Wang, J., et al., 2007). After mGluR1/5 agonist stimulation, the receptor changes its conformation allowing the exchange of GDP to GTP on the heterotrimeric

$G\alpha_{q/11}$ protein, this causes the disassociation of the $G\alpha$·GTP and $G\beta\gamma$ proteins, allowing them to interact with and activate their respective effector enzymes (Ferguson, 2001; Nakanishi, 1994). The $G\alpha_{q/11}$ proteins activate phospholipase Cβ1, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) from the plasma membrane resulting in the formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). The release of IP_3 activates the IP_3 receptor on the endoplasmic reticulum, causing the release of calcium (Ca^{+2}) from intracellular stores. The increase in Ca^{+2} and DAG causes the activation of protein kinase C (PKC), allowing PKC to phosphorylate its respective substrates. The activation of mGluR1/5 causes an oscillatory production of DAG, IP_3, and Ca^{+2}, which leads to oscillations in PKC activation (Dale et al., 2001; Dale et al., 2002; Kawabata et al., 1998). Interestingly, the frequency of oscillations differs between mGluR1 and mGluR5, which translate to different PKC oscillations (Dale et al., 2001; Kawabata et al., 1998). Several agonists have been characterized for Group I mGluRs, the most potent being quisqualate, followed by 3,5-dihydroxyphenylglycine glutamate (DHPG) which both primarily activate $G\alpha_{q/11}$ proteins, but have also been shown to couple mGluR1/5 with $G\alpha_s$ and $G\alpha_{i/o}$ proteins, which leads to increased cAMP formation and the release of arachidonic acid (Aramori and Nakanishi, 1992; Bordi and Ugolini, 1999; Conn and Pin, 1997; Dale et al., 2002). Coupling to the heterotrimeric $G\alpha_{q/11}$ proteins also leads to the activation of effector proteins, such as protein kinase C (PKC), protein kinase D (PKD), proline-rich tyrosine kinase 2 (Pyk2), proto-oncogene tyrosine-protein kinase (Src), Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII) and extracellular signal-regulated kinases (ERK). mGluR1/5 signalling also leads to the potentiation of voltage-dependent calcium channels and the inhibition of potassium

conduction (Dale et al., 2001; Dhami and Ferguson, 2006; Krueger et al., 2010; Lu et al., 1999; Nicodemo et al., 2010; Wang, J., et al., 2004).

1.2.3 Activation of Mitogen-Activated Protein Kinases

Agonist stimulation of Group I mGluRs also causes the stimulation of p44 and p42 mitogen-activated protein kinases (MAPK). Two proteins from the MAPK family have been found to be phosphorylated by mGluR1/5 stimulation, extracellular signal-regulated kinase 1 (ERK1) and 2 (ERK2). The activation of these proteins allows them to transduce the activity of extracellular and intracellular signals into long lasting changes in the CNS by regulating cellular activities and gene transcription (Wang, J., et al., 2007). Studies have found that DHPG stimulation increases ERK phosphorylation in the spinal cord, hippocampus and glia (Gallagher et al., 2004; Karim et al., 2001; Peavy and Conn, 1998). Additionally, mGluR5 signalling causes ERK2 phosphorylation in astrocytes (Peavy et al., 2001). The activation of MAPK can occur in many ways, some studies have found that it is primarily through a calcium independent route, our group has found that Pyk2 is required for ERK1/2 phosphorylation in cortical neurons, while others have found that PKC and Homer proteins also contribute to ERK1/2 phosphorylation (Ferraguti et al., 1999; Mao et al., 2005; Nicodemo et al., 2010; Wang, J., et al., 2007). Additionally, other studies have found that internalization of mGluR1 by β-arrestin causes a direct activation of MAPK, while others have shown that ERK phosphorylation can occur by the Gβγ subunits and Src tyrosine kinases (Emery et al., 2010; Iacovelli et al., 2003; Luttrell et al., 1997; Wang, J., et al., 2007). Consequently, it is clear that Group I mGluRs activate MAPKs via multiple molecular pathways, thereby

connecting extracellular and intracellular signals to genomic responses that are required for long lasting changes in neuronal plasticity (Ferraguti et al., 1999).

1.2.4 Role of Group I Metabotropic Glutamate Receptors

When metabotropic glutamate receptors become stimulated by an agonist, such as glutamate they produce signals that cause long-lasting changes in the cell (Dhami and Ferguson, 2006; Gallagher et al., 2004; Kawabata et al., 1998). As a consequence, Group I mGluRs are involved in the two classical forms of synaptic plasticity, long-term depression (LTD) and long-term potentiation (LTP) (van Dam et al., 2004). LTD induced by glutamate signalling involves a quick cellular response by NMDA receptors while long-term maintenance is mediated by mGluR1/5 (Bashir et al., 1993; Clement et al., 2009; van Dam et al., 2004). Studies have found that mice lacking mGluR1 experience a severe motor deficit due to loss of LTD induction in the cerebellum. Additionally, these mice exhibited severe learning impairments, with an impaired mossy fibre LTP (Conquet et al., 1994). Similarly, exposure to an mGluR1 antagonist (S)-(+)-alpha-amino-4-carboxy-2-methylbezeneacetic acid (LY 367385) impairs corticostriatal LTD (Gubellini et al., 2001). Blockade of either mGluR1 or mGluR5 has also prevented LTP induction in hippocampal slices (Le Duigou and Kullmann, 2011).

Due to the effect Group I mGluRs have on synaptic plasticity, they have been implicated in changes in synaptic activity that play a role in memory, learning, and neurodegenerative diseases including; Huntington's disease, amyotrophic lateral sclerosis (ALS), and Alzheimer's disease (Bordi and Ugolini, 1999; Byrnes et al., 2009; Ribeiro et al., 2011; Riedel, 1996). Additionally, mGluR1/5 receptors are implicated in

excitotoxicity caused by glutamate over-stimulation, which leads to neuronal cell death and ischemia (Calabresi et al., 1999). However, the exact role Group I mGluRs play in neuroprotection or neurodegeneration remain unclear (Pshenichkin et al., 2008). mGluR5 has been shown to protect against excitotoxcity leading to cell death in cerebellular neurons by decreasing NMDA-induced activation of nitric oxide synthase (Llansola and Felipo, 2010). However, other studies have found that mGluR1/5 enhanced NMDA receptor mediated neuronal toxicity in cortical cells (Bruno et al., 1995). Due to the effects Group I mGluRs have on synaptic plasticity, potential therapeutics for neurological diseases are targeted towards these receptors. For example, mGluR1 antagonists (RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA), 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt), and LY367385 have been shown to reduce neuronal cell death in cortical cultures (Faden et al., 2001). Similarly, mGluR5 antagonists 2-methyl-6-(phenylethynyl)-pyridine (MPEP) and SIB-1893 have also been shown to reduce neuronal cell death in response to NMDA exposure (O'Leary et al., 2000). However, the precise mechanisms that contribute to the neuroprotective or neurotoxic effects of Group I Metabotropic Glutamate Receptors still need to be defined.

1.2.5 The Regulation of G-protein Signalling

The signalling of Group I mGluRs is regulated at many levels under physiological and pathological conditions, for example G-protein signalling is controlled by Regulators of G-protein Signalling (RGS) proteins. RGS proteins terminate G-protein signalling by acting as GTPase-activating proteins (GAPs) that accelerate Gα-

GTP hydrolysis. The RGS proteins are divided into four groups based on sequence homology, R4, R7, R12 and RZ. The R4 group contains RGS 1-5, 8, 13, 16, 18, and 21, these proteins are able to function as GAP proteins and interact with other regulatory proteins, including the third intracellular loop of GPCRs (Magalhaes et al., 2011). RGS2 and RGS4 interact with $G\alpha_{q/11}$ proteins and therefore regulate mGluR1/5 signalling. Studies have found RGS4 to block $G\alpha_{q/11}$ activation of phospholipase Cβ and mGluR5a inhibition of potassium currents in hippocampal neurons (Hepler et al., 1997; Saugstad et al., 1998). Similarly, RGS2 has been shown interact with $G\alpha_{q/11}$-GTP and decrease mGluR1a inhibition of potassium currents (Kammermeier and Ikeda, 1999). The regulation of Group I mGluR signalling, trafficking and distribution is extremely complex and involves many proteins, it is important to understand these mechanisms in order to determine the role mGluR1/5 play in the CNS.

1.3 GPCR Desensitization and Endocytosis

GPCR signalling begins a feedback mechanism causing receptor desensitization and endocytosis which protects against both acute and chronic receptor stimulation (Ferguson, 2007). The classical model of desensitization involves uncoupling of the GPCR and the G-proteins by receptor phosphorylation, this is mediated by second-messenger protein kinases or G protein-coupled receptor kinases (GRKs). Second messenger protein kinases, protein kinase C (PKC) or protein kinase A (PKA), can phosphorylate both receptors exposed to agonist and non-agonist activated receptors, which prevents subsequent activation. However, GRKs only phosphorylate the serine and threonine resides in the third intracellular loop and carboxyl-terminal domains of

agonist activated receptors (Ferguson, 2007). Upon GRK interaction, the receptor is then stabilized in a conformation state that promotes the interaction of arrestins. Arrestins preferentially bind to agonist activated and GRK-phosphorylated GPCRs. Upon arrestin interaction, coupling to the G-protein is diminished and endocytosis begins by targeting clathrin-coated vesicles to the receptor (Dale et al., 2001; Dale et al., 2002; Ferguson et al., 1996; Ferguson, 2001; Ferguson, 2007). Upon endocytosis by clathrin-coated vesicles, receptors can undergo resensitization by undergoing dephosphorylation in endosomes and are subsequently recycled back to the cell surface. Receptors may also be targeted for degradation in lysosomes which causes a down regulation of receptor signalling (Figure 1.3) (Ferguson, 2001). However, metabotropic glutamate receptors are unique from other GPCRs, in view of the fact that they undergo both phosphorylation-dependent and -independent desensitization (Dhami and Ferguson, 2006).

1.3.1 Desensitization of Group I Metabotropic Glutamate Receptors

Group I mGluRs undergo various feedback mechanisms that protect against over stimulation and cell death. Internalization can occur by multiple mechanisms depending on the initiation of internalization and the splice variant (Dhami et al., 2002). Additionally, signal attenuation can be mediated by a variety of second messenger-dependent proteins including: PKC, calmodulin kinase II, GRKs, and optineurin (Anborgh et al., 2005; Catania et al., 1991; Dale et al., 2000; Mundell et al., 2003). mGluR1/5 desensitization is attenuated when PKC is inhibited, indicating an important

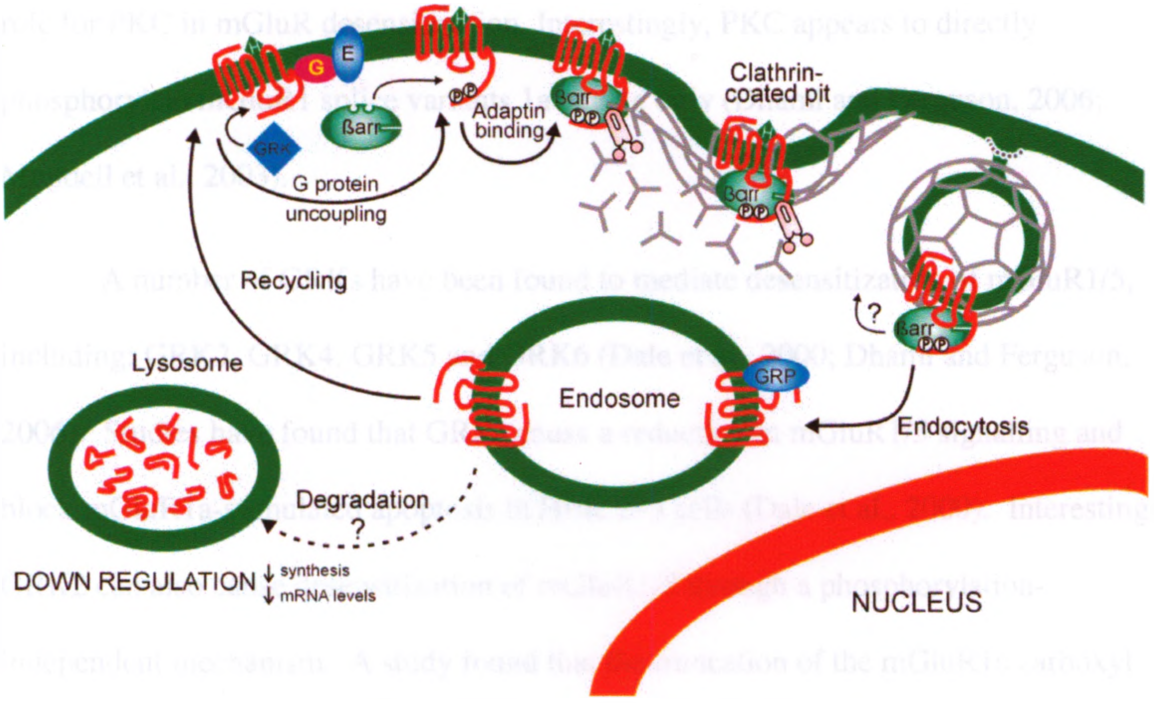

Figure 1.3: Schematic of GPCR Desensitization and Resensitization. Upon GRK phosphorylation, the receptor is then stabilized in a conformation state that promotes the interaction of β-arrestin. Endocytosis begins by targeting clathrin-coated vesicles to the receptor, where receptors can undergo resensitization by undergoing dephosphorylation in endosomes and are subsequently recycled back to the cell surface. Receptors may also be targeted for degradation in lysosomes which causes a down regulation of receptor signalling (Dhami and Ferguson, 2006). Abbreviations: βarr, β-arrestin; E, effector enzyme; G, heterotrimeric G protein; GRK, G protein-coupled receptor kinase; GRP, G protein-coupled receptor phosphatase; H, hormone; P, phosphate group.

role for PKC in mGluR desensitization. Interestingly, PKC appears to directly phosphorylate mGluR1 splice variants 1a and 1c only (Dhami and Ferguson, 2006; Mundell et al., 2003).

A number of GRKs have been found to mediate desensitization of mGluR1/5, including: GRK2, GRK4, GRK5 and GRK6 (Dale et al., 2000; Dhami and Ferguson, 2006). Studies have found that GRKs cause a reduction in mGluR1/5 signalling and block mGluR1a-stimulated apoptosis in HEK 293 cells (Dale et al., 2000). Interestingly, GRK2 can also cause desensitization of mGluR1/5 through a phosphorylation-independent mechanism. A study found that the truncation of the mGluR1a carboxyl tail prevents receptor phosphorylation but not desensitization by GRK2 (Dhami et al., 2002). Similarly, the splice variant mGluR1b, which contains a shorter C-tail than mGluR1a, is not phosphorylated by GRK2 but still undergoes GRK2 mediated desensitization (Dhami et al., 2002). Phosphorylation-independent desensitization occurs by GRK2 interaction on the second intracellular loop and C-tail of mGluR1a, but only requires an interaction with the second intracellular loop of mGluR1b (Dhami et al., 2005).

GRK2 has an amino-terminal RGS homology (RH) domain, a central catalytic domain and a carboxyl-terminal Gβγ binding pleckstrin homology domain, allowing it to interact with the receptor, $G\alpha_{q/11}$, and Gβγ simultaneously, and thereby preventing subsequent signalling cascades (Figure 1.4) (Ferguson, 2007). Furthermore, expression of only the N-terminal domain of GRK2 has been shown to attenuate both constitutive and agonist-dependent mGluR1a and mGluR1b signalling (Dhami et al., 2002). The GRK2 RH domain inhibits the signalling cascades induced by the $G\alpha_{q/11}$ protein by

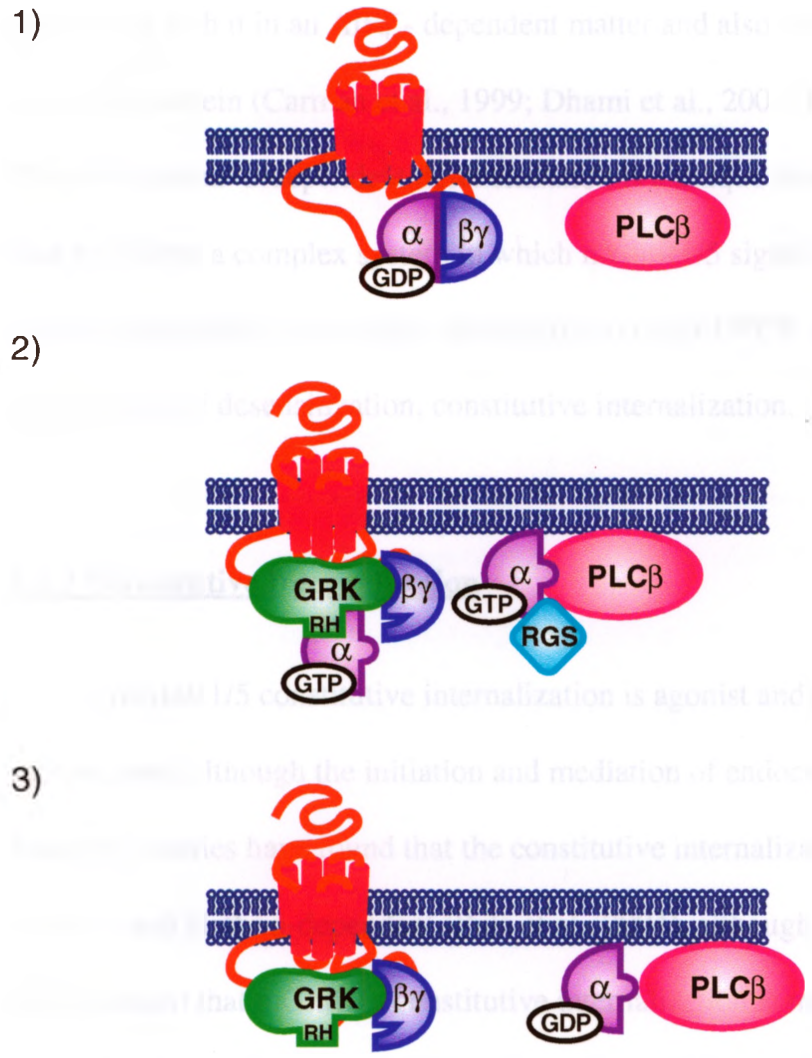

Figure 1.4: A Schematic Representation of GRK2 Mediated Desensitization of Group I Metabotropic Glutamate Receptors. (1) The inactive receptor with the Gα-GDP protein bound. (2) The active receptor bound with the Gα-GTP protein, which causes the G-protein to disassociate allowing GRK2 to interact with the receptor, the Gα-GTP protein through the GRK2-RH domain, and the Gβγ subunit. (3) GRK interaction maintains the receptor in an inactivated state, preventing the Gα-GDP protein to interact with the receptor. Abbreviations: GRK, G protein-coupled receptor kinases; PLCβ, phospholipase Cβ; RGS, Regulators of G-protein signalling (Ferguson, 2007).

interacting with it in an AlF_4^-- dependent matter and also functions as a weak GTPase-activating protein (Carman et al., 1999; Dhami et al., 2002; Ferguson, 2007). Phosphorylation–independent desensitization is a unique feature of Group I mGluRs, thus providing a complex system in which mGluR1/5 signalling is regulated. In addition to phosphorylation-independent desensitization, this GPCR family undergoes another unique form of desensitization, constitutive internalization.

1.3.2 Constitutive Internalization

mGluR1/5 constitutive internalization is agonist and phosphorylation independent, although the initiation and mediation of endocytosis remains unclear. Recently, studies have found that the constitutive internalization of mGluR1a is β-arrestin- and clathrin-dependent (Pula et al., 2004), although previous studies have demonstrated that mGluR1a constitutive internalization is mediated by clathrin-coated vesicles but is β-arrestin and dynamin independent (Figure 1.5) (Dale et al., 2001). Similarly, mGluR5a agonist-independent internalization also remains unknown, studies have found that mGluR5a and mGluR1a both co-localize with clathrin during agonist-independent internalization (Bhattacharya et al., 2004; Dale et al., 2001), yet it has been suggested that mGluR5 constitutive internalization is not mediated by clathrin-coated pits (Fourgeaud et al., 2003). Work from our laboratory has shown that mGluR1/5 constitutive internalization is β-arrestin-independent and is mediated by the association with Ral (small GTP binding protein) and phospholipase D2 (PLD2). More specifically, mGluR1/5 has been shown to scaffold a protein complex containing Ral, Ral guanine nucleotide exchange factor (RalGDS) and PLD2, and this complex appears to be a

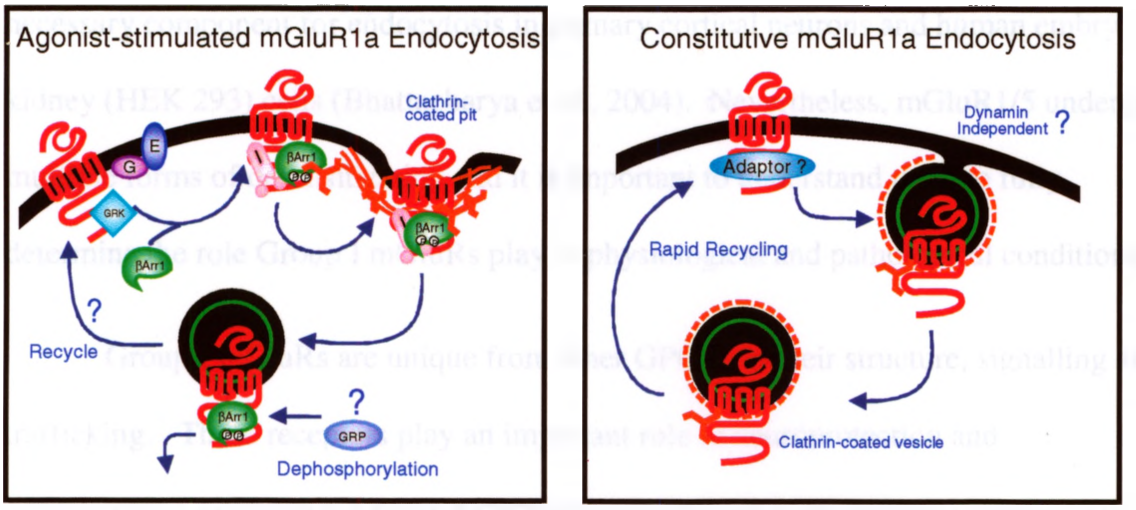

Figure 1.5: Schematic Representation of Agonist-stimulated and Constitutive Endocytosis of mGluR1a. 1) Group I mGluRs undergo the classical model of desensitization that involves receptor phosphorylation, and subsequent β-arrestin interaction. Upon β-arrestin interaction, endocytosis begins by clathrin-coated vesicles targeted to the receptor. 2) Group I mGluR constitutive endocytosis is agonist and phosphorylation independent, although the initiation and mediation of endocytosis remains unclear. Studies have found that the constitutive internalization of mGluR1a is mediated by clathrin-coated vesicles but is β-arrestin and dynamin independent. Abbreviations: βarr, β-arrestin; E, effector enzyme; G, heterotrimeric G protein; GRK, G protein-coupled receptor kinase; GRP, G protein-coupled receptor phosphatase.

necessary component for endocytosis in primary cortical neurons and human embryonic kidney (HEK 293) cells (Bhattacharya et al., 2004). Nevertheless, mGluR1/5 undergoes multiple forms of desensitization and it is important to understand them to fully determine the role Group I mGluRs play in physiological and pathological conditions.

Group I mGluRs are unique from other GPCRs in their structure, signalling and trafficking. These receptors play an important role in neuroprotection and neurotoxicity, although the precise mechanism is still yet to be defined (Pshenichkin et al., 2008). Additionally, interacting protein partners have been shown to alter the signalling and may alter the physiological effects induced by Group I mGluRs (Croci et al., 2003; Kammermeier and Ikeda, 2001; Kammermeier, 2008; Kitano et al., 2002; Ronesi and Huber, 2008). A novel protein, spinophilin has recently been identified to interact with Group I mGluRs and this novel protein may regulate the signalling and trafficking of these receptors.

1.4 Spinophilin

Spinophilin is a scaffolding protein with multiple domains that is used to bring protein partners into close proximity to respective substrates. Spinophilin was first discovered in the heads dendritic spines and found to interact with protein phosphatase 1(PP1) α and γ, thus it was called spinophilin (Allen et al., 1997). At the same time, another group discovered the same protein to interact with actin filament (F-actin) with similar organization to neurabin, thus it was named neurabin-II, which is now also called spinophilin (Satoh et al., 1998). Spinophilin is closely related to and interacts with its homologue neurabin, having 51% identical amino acids and 74% functional similarities

(Sarrouilhe et al., 2006). In addition to an actin-binding domain and a protein phosphatase 1 binding and regulatory domain, spinophilin also has a PSD-95/Discs large/ZO-1 homology (PDZ) domain, a receptor-interacting domain, three putative Src homology 3 domains, a potential leucine/isoleucine zipper motif and three coiled-coil domains (Figure 1.6) (Sarrouilhe et al., 2006).

Spinophilin has been referred to as a scaffolding protein due to its many binding domains, which allow it to mediate protein-protein interactions used for regulating neuronal cell signalling. Recently, spinophilin has been found to interact with two subfamilies of GPCRs; the dopamine receptors and the α-adrenergic receptors (Brady et al., 2003; Sarrouilhe et al., 2006; Smith et al., 1999). Furthermore, spinophilin has been found to scaffold other protein partners into close proximity to these receptors such as RGS proteins 1, 2, 4 and 16 (Sarrouilhe et al., 2006; Wang et al., 2005). It is clear that spinophilin has potential to alter cell signalling by mediating protein-protein interactions, which demonstrates spinophilin's important role in synaptic plasticity (Table 1.1).

1.4.1 Spinophilin and Dendritic Spines

Dendritic spines are protrusions from the dendritic shaft that receive the majority of excitatory signals in the central nervous system. Changes in number, size and shape are associated with learning, development and changes in synaptic behaviour and activity (Feng et al., 2000). Spinophilin plays an important role in organization and intracellular transport in spine remodelling and morphology by stabilizing and bundling F-actin (Hsieh-Wilson et al., 2003; Satoh et al., 1998). Studies have found that

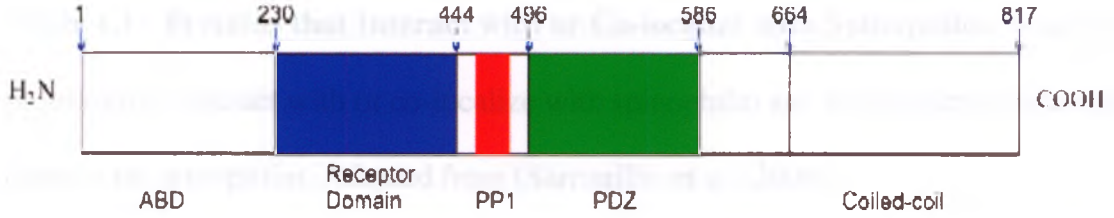

Figure 1.6: A Schematic Representation of the Binding Domains of Full Length Spinophilin. Spinophilin is scaffolding protein that is able to mediate a variety of protein-protein interactions. It is able to interact with F-actin through the actin binding domain, GPCRs through the receptor interacting domain, and PDZ containing proteins through the PDZ domain. Additionally, spinophilin interacts with and regulates protein phosphatase 1 (PP1) through the PP1 binding site. Abbreviations: ABD, actin binding domain; PP1BS, protein phosphatase 1 binding site; PDZ, PSD-95/Discs large/ZO-1 homology domain (Wang et al., 2005).

Table 1.1: Proteins that Interact with or Co-localize with Spinophilin. Summary of proteins that interact with or co-localize with spinophilin and the respective interacting domain on spinophilin. Adapted from (Sarrouilhe et al., 2006).

Partner protein	Spinophilin motif
F-actin	ABD 1[b]
Neurabin	Coiled-coil domain
Spinophilin	Coiled-coil domain
Doublecortin	Coiled-coil domain
DCAMKL1	Coiled-coil domain
PP1α, PP1β, PP1γ1	R-K-I-H-F motif
$p70^{S6k}$	PDZ domain
Tiam 1	Amino acids 444–817
Ras-GRF1	Amino acids 444–817
Lfc	Coiled-coil domain
RGS2	Amino acids 480–525
RGS1, RGS4, RGS16	Unknown
GAIP	Unknown
D2 dopamine receptor	Receptor binding domain
α_{1B}-, α_{2A}-, α_{2B}-, α_{2C}-Adrenergic receptors	Receptor binding domain
RYR1, RYR2	LIZ motif
TRPC5 and 6	Unknown
ARF	Amino acids 605–726
TGN 38	Coiled-coil domain
AMPA-type glutamate receptor	Unknown
E-cadherin	Unknown

Abbreviations: ABD, actin binding domain; DCAMKL1, doublecortin and calcium/calmodulin-dependent protein kinase-like 1; GRF, guanine nucleotide exchange factor; Lfc, Lbc [lymphoid blast crisis]'s first cousin; LIZ, leucine/isoleucine zipper; PDZ, postsynaptic density-95/discs large/zona occludens-1 motif; PP1, protein phosphatase 1; RGS, regulator of G-protein signalling; RYR, ryanodine receptor; TGN38, *trans*-Golgi network protein 38; TRP, the transient receptor potential.

spinophilin deficient mice display an increase in spine density during development *in vivo* and *in vitro*. Furthermore, spinophilin knockout mice display more protrusions from the dendritic shafts but the same number of nerve terminals as wild type mice (Feng et al., 2000). This indicates that spinophilin may regulate spine retraction or decrease initial outgrowth of spines from the dendrite. Nevertheless, it is clear that spinophilin plays an important role in the central nervous system by regulating spine morphology and development.

1.4.2 Distribution of Spinophilin

Spinophilin is a ubiquitously expressed protein found in the spleen, lung, kidney, and testis, but it is primarily expressed in the CNS. Spinophilin is primarily found in the hippocampus, specifically the stratum oriens, stratum lacunosum- moleculare and hilus (Allen et al., 1997; Sarrouilhe et al., 2006). Interestingly, the phosphorylation state of spinophilin greatly affects its distribution in the CNS. Spinophilin has phosphorylation sites for many kinases, including mitogen-activated protein kinase ERK2, CaMKII, PKC and PKA. The consensus phosphorylation sites for these proteins are within spinophilin's actin binding domain, causing spinophilin to disassociate from actin upon phosphorylation (Baucum et al., 2010; Feng et al., 2000; Hsieh-Wilson et al., 2003). In rat spinophilin there are seven phosphorylation sites in the actin-binding domain; Ser15 (ERK-site), Ser17 (cyclin-dependent kinase 5 site), Ser94 [cAMP-dependent protein kinase (PKA-site)], Ser100 (CaMKII-site), Ser116 (CaMKII-site), Ser177 (PKA-site) and Ser205 (ERK-site) (Uematsu et al., 2005). Upon phosphorylation in the actin binding domain, spinophilin disassociates from actin and

relocates so that it is no longer distributed in the dendritic spines (Grossman et al., 2002). Upon CaMKII phosphorylation on Ser-100, spinophilin becomes enriched at the synaptic plasma membrane but not in the post synaptic density (PSD) (Grossman et al., 2004). Similarly, upon PKA phosphorylation on Ser-177, spinophilin relocates from the PSD and becomes enriched in the cytosol (Hsieh-Wilson et al., 2003). Furthermore, studies have found that spinophilin regulates GPCRs in a dephosphorylated state. For example, PKA phosphorylation decreases the interaction between spinophilin and both the α_2-adrenergic receptor and F-actin (Xu et al., 2008). Interestingly, PKA phosphorylation does not disrupt the interaction between spinophilin and protein phosphatase 1 (PP1), which may provide a mechanism in which the spinophilin-PP1 complex relocates to regulate other protein partners (Hsieh-Wilson et al., 2003; Uematsu et al., 2005).

1.4.3 Spinophilin and Protein Phosphatase 1

Protein phosphatase 1 is a serine/threonine phosphatase that is also located in the dendritic spines, where it is involved in the regulation of hippocampal LTD and LTP (Hsieh-Wilson et al., 1999; Morishita et al., 2001). Spinophilin has been shown to anchor PP1 and bring it to close proximity to interacting proteins, causing a wide array of secondary interactions (Ragusa et al., 2011). Three isoforms of PP1, PP1α, PP1β and PP1γ1, interact with spinophilin on amino acid residues 417 to 494 (Allen et al., 1997; Sarrouilhe et al., 2006). Spinophilin preferentially binds to the PP1γ subunit, which upon interaction suppresses the phosphorylase phosphatase activity and thereby inactivates PP1. Interestingly, PP1 also dephosphorylates spinophilin which regulates

the function and distribution of spinophilin (Sarrouilhe et al., 2006; Uematsu et al., 2005). Studies have found that spinophilin targets PP1 to respective substrates and allows PP1 to dephosphorylate those proteins, indicating that spinophilin and PP1 interact transiently to allow the reactivation of PP1 (Hsieh-Wilson et al., 1999). For example, spinophilin forms a complex with PP1, allowing it to interact and dephosphorylate proteins and receptors including: actin, ionotropic glutamate receptors, and dopamine receptors (Hsieh-Wilson et al., 1999; Ragusa et al., 2010).

1.4.4 Spinophilin and Glutamate Receptors

Spinophilin has been shown to regulate synaptic transmission by targeting PP1 to ionotropic glutamate receptors including: N-methyl-D-aspartate receptor (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Spinophilin mediates the dephosphorylation of the AMPA and NMDA receptors by anchoring PP1 to the postsynaptic densities (PSD) where the receptors are found, this keeps the receptors in a low activity state (Feng et al., 2000; Yan et al., 1999). Studies have found that the regulation of PP1 on NMDA receptors is diminished in spinophilin knockouts (Feng et al., 2000), indicating that spinophilin is responsible for the enrichment of PP1 in the PSD and contributes to the regulation of ionotropic glutamate receptors.

1.4.5 Spinophilin and the α_2-Adrenergic Receptors

The α_2-adrenergic receptors (α_2AR) are a family of GPCRs that are coupled to the heterotrimeric $G\alpha_{i/o}$ proteins, which upon agonist stimulation cause the inhibition of adenylyl cyclase and voltage gated calcium currents. Physiologically, α_2AR stimulation

results in lower blood pressure and a sedative, hypnotic effect (Lu et al., 2010). Unlike Group I mGluRs, the α_2AR only internalizes by receptor phosphorylation and subsequent β-arrestin interaction with the third intracellular loop. Therefore, interactions that occur on the third intracellular loop are important for cell surface retention or internalization (Wang and Limbird, 2002). Recently, studies have found spinophilin to interact with all three subtypes of the α_2-adrenergic receptors (α_{2A}AR, α_{2B}AR, α_{2C}AR) on the third intracellular loop (Brady et al., 2003; Sarrouilhe et al., 2006). Upon α_2AR agonist stimulation, the $G\alpha_{i/o}$ proteins disassociate and the Gβγ proteins become activated, spinophilin is then recruited to the plasma membrane where it recognizes the α_2AR- Gβγ complex and interacts with the receptor (Brady et al., 2005; Wang, Q., et al., 2004). Spinophilin competes against GRK2 for the third intracellular loop, preventing receptor phosphorylation and subsequent β-arrestin interactions. Therefore, spinophilin causes the α_2AR to remain on the plasma membrane, which decreases receptor recycling, and the rate of receptor resensitization (Figure 1.7). In addition to preventing internalization, spinophilin also decreases the acceleration of the MAPK pathway initiated by β-arrestin-induced endocytosis (Wang, Q., et al., 2004).

Interestingly, Lu *et al.* (2010) found that spinophilin knockout mice display an increased hypotensive, sedative, and hypnotic effect in response to α_2AR stimulation. These results suggest that deletion of spinophilin may cause an enhanced α_2AR- G protein coupling that causes the physiological effects observed. Furthermore, Lu *et al.* (2010) found that spinophilin and arrestin dynamically regulate α_2AR phosphorylation,

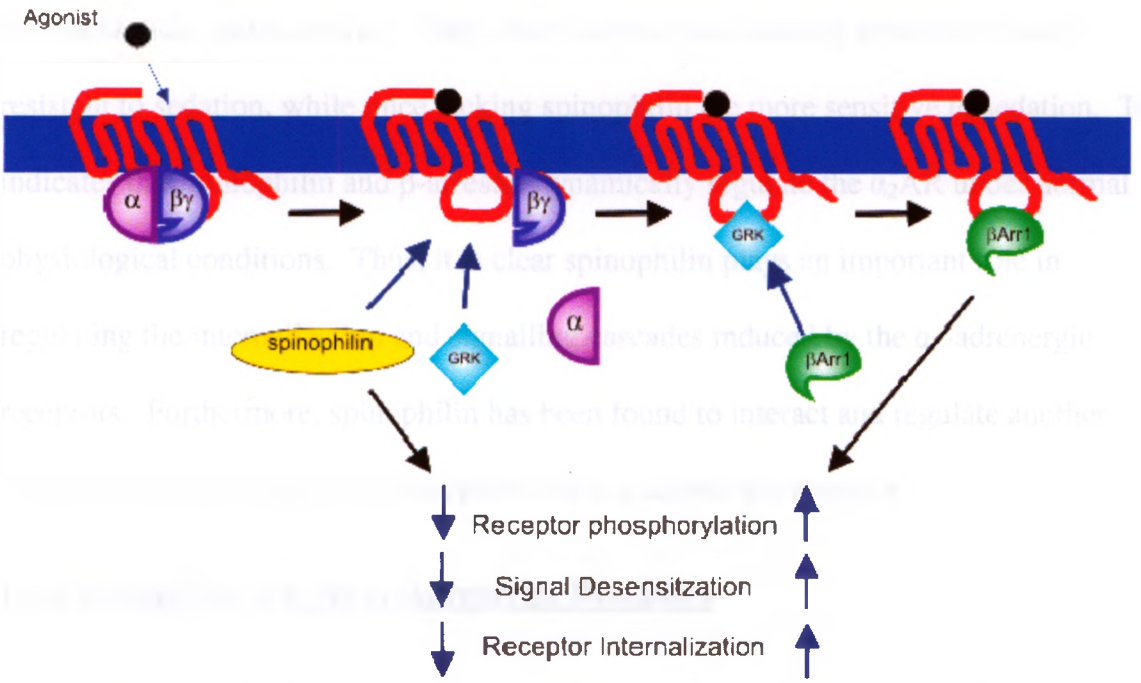

Figure 1.7: Proposed Model of α-adrenergic Receptor Interactions with G-proteins, Spinophilin, GRK and Arrestin. Spinophilin competes against GRK for the third intracellular loop of the agonist activated α-adrenergic receptor. If spinophilin interacts with the receptor first, it prevents receptor phosphorylation by GRK and subsequent β-arrestin interaction, thereby decreasing receptor internalization and signal desensitization (Wang, Q., et al., 2004). Abbreviations: βarr, β-arrestin; GRK, G protein-coupled receptor kinase.

internalization, and signalling. They observed that mice lacking arrestin are more resistant to sedation, while mice lacking spinophilin are more sensitive to sedation. This indicates that spinophilin and β-arrestin dynamically regulate the α_2AR under normal physiological conditions. Thus, it is clear spinophilin plays an important role in regulating the internalization and signalling cascades induced by the α_2-adrenergic receptors. Furthermore, spinophilin has been found to interact and regulate another subfamily of the α-adrenergic receptors, the α_{1B}-adrenergic receptor.

1.4.6 Spinophilin and the α_1-Adrenergic Receptors

The α_{1B}-adrenergic receptor (α_{1B}AR) is coupled to the heterotrimeric $G\alpha_{q/11}$ protein, which upon agonist stimulation activates phospholipase C resulting in the formation of IP_3 and DAG, thereby causing the subsequent release of calcium from intracellular stores. Recently, Wang *et al.* (2005) found that spinophilin interacts with the third intracellular loop of the α_{1B}AR, and regulates $G\alpha_{q/11}$ signalling by recruiting RSG2 to the plasma membrane. As mentioned earlier, the $G\alpha_{q/11}$ protein is regulated by RGS2 which is a $G\alpha_{q/11}$ specific GTPase (Chen et al., 1997; Kammermeier and Ikeda, 1999). Spinophilin interacts with the α_{1B}AR via the receptor interacting domain, tethers the α_{1B}AR to the cell surface via its coiled-coil domains and regulates the $G\alpha_q$ signalling by interacting to the N-terminus of RGS2. Studies have found that the co-expression of spinophilin with the α_{1B}AR increases the maximum calcium current induced by receptor stimulation but decreased the rate of activation and spontaneously inactivated signalling with continuous stimulation. Additionally, spinophilin was found to be less effective in regulating calcium currents in RGS2 deficient cells and RGS2 was less effective in

inhibiting calcium currents in spinophilin knockout mice (Wang et al., 2005; Wang, X., et al., 2007). This indicates that spinophilin plays an important role in regulating the signalling and trafficking of the α_{1B}-adrenergic receptor, and provides a new mechanism in which RGS2 regulates GPCRs.

1.4.7 Spinophilin and the Dopamine Receptors

There are five types of dopamine receptors and they are classified in two groups based on the structure, function and pharmacology. D1-like receptors (D1 and D5) are coupled to heterotrimeric $G\alpha_s$ proteins and have a short third cytoplasmic loop and long a carboxyl-terminal tails. While, D2-like receptors (D2, D3 and D4) couple to heterotrimeric $G_{i/o}$ proteins and have a large third cytoplasmic loop and short carboxyl-terminal tails. Spinophilin has been found to interact to the third intracellular loop of the D2 receptor in a yeast two hybrid assay and a GST pull down (Smith et al., 1999; Wang, X., et al., 2007). However, very little research has been done to determine the relevance of this interaction. One study found that chronic antipsychotic treatment targeted to D2 receptors significantly increased expression of spinophilin (Kabbani and Levenson, 2006), while another study found that D2 stimulation decreased the basal level of phosphorylated-Ser94 spinophilin (Uematsu et al., 2005). Similarly, agonist stimulation of D1 receptors causes signalling cascades that alter spinophilin function. Studies found that agonist stimulation of D1 receptors increases PKA phosphorylation of Ser-94 spinophilin (Uematsu et al., 2005). Moreover, spinophilin knockout mice display a diminished ability to phosphorylate AMPA receptors in response to D1 receptor

activation (Allen et al., 2006). Thus, it is clear that spinophilin plays an important role in dopamine signalling observed in physiological and pathological conditions.

1.4.8 Role of Spinophilin

Spinophilin is required for LTD, the regulation of receptors and channels and the formation of dendritic spines (Sarrouilhe et al., 2006). Spinophilin knockout mice have a small reduction in body weight, a reduced hippocampus and exhibit reduced LTD and neuronal apoptosis (Feng et al., 2000). As a consequence, spinophilin has been linked to many neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease. In disease states such as AD, there is a deficit in higher order cognitive processes, with a primary dysfunction in spines causing synaptic loss. A dysfunction in spines can be attributed to changes in spine morphology, receptor distribution, and changes in synaptic signalling; furthermore, these mechanisms have been shown to be regulated by spinophilin (Feng et al., 2000; Hsieh-Wilson et al., 2003; Sarrouilhe et al., 2006; Satoh et al., 1998). Similarly, Parkinson's disease results in dopamine depletion, which causes morphological alterations in striatal medium spiny neurons and impairment of synaptic plasticity. Furthermore, studies have found that chronic striatal dopamine depletion decreases PP1γ activity by increasing interaction with spinophilin (Brown et al., 2008).

1.5 Goals and Significance of Research

As previously addressed, many studies have demonstrated that spinophilin has the ability to interact with and regulate receptors found in the central nervous system (Sarrouilhe et al., 2006). Studies have found that spinophilin interaction alters the

signalling and trafficking of α-adrenergic receptors (Brady et al., 2005; Lu et al., 2010; Wang et al., 2005). Similarly, spinophilin has been found to regulate ionotropic glutamate receptors by mediating dephosphorylation through protein phosphatase 1 recruitment (Feng et al., 2000; Yan et al., 1999). Recently, our lab found spinophilin using a tandem affinity purification proteomic screen to interact with the carboxyl tail of mGluR1a and mGluR5a. Furthermore, studies have revealed that the regulation of Group I mGluR signalling is a dynamic process that involves a variety of proteins that scaffold to the carboxyl-tail of mGluR1/5 receptors (Kammermeier and Ikeda, 2001; Kammermeier, 2008; Kitano et al., 2002; Ronesi and Huber, 2008). Interestingly, there has yet to be an experiment that investigates whether spinophilin interacts with Group I mGluRs and if this interaction regulates mGluR1/5 signalling. Therefore, my thesis will test the hypothesis that spinophilin associates with Group I mGluRs and this interaction regulates the signalling and trafficking of these receptors.

Group I mGluR signalling has been implicated in a number of neurodegenerative diseases including; Huntington's disease, ALS and Alzheimer's disease (Bordi and Ugolini, 1999; Byrnes et al., 2009; Ribeiro et al., 2011; Wang, J., et al., 2004). However, the exact role mGluRs play in neuroprotection or neurodegeneration remain unclear (Pshenichkin et al., 2008). Thus, a clear understanding of how Group I mGluR interacting proteins regulate receptor signalling is required for the development of novel therapeutics targeted towards neurodegenerative diseases associated with mGluR1/5 signalling.

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

Human embryonic kidney (HEK293) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Tissue culture reagents, Alexa Fluor 555, Alexa Fluor 633 antibody labelling reagents, and GFP antibody were purchased from Invitrogen (Burlington, ON, Canada). Quisqualate was purchased from Tocris Cookson Inc. (Ellisville, MO, USA). EZ-Link Sulfo-NHS-SS-Biotin and immobilized NeutrAvidin beads were from Pierce Biotechnology. The rabbit anti-mGluR1 and –mGluR5 antibodies are from Milipore. [*myo*-^{3}H]Inositol was acquired from PerkinElmer Life (Waltham, MA). Dowex 1-X8 (formate form) resin 200 to 400 mesh, DC protein assay kit, horseradish peroxidase-conjugated anti-rabbit, and anti-goat IgG secondary antibodies were from Bio-Rad Laboratories (Mississauga, ON, Canada). Goat anti-spinophilin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The protein G-Sepharose beads and chemiluminescence Western blotting detection reagents were purchased from GE Healthcare (Oakville, ON, Canada). The spinophilin cDNA clone was a kind gift from Dr. Wang (University of Alabama). Rabbit polyclonal phospho-p42/44 MAP kinase (Thr202/Tyr402) and p42/44 MAP kinase were obtained from Cell Signaling Technology (Pickering, ON, Canada). The rabbit anti-FLAG antibody and all other biochemical reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Cell Culture

HEK 293 cells were maintained in Eagle's minimal essential medium supplemented with 10% (v/v) heat inactivated fetal bovine serum (Invitrogen, Burlington, ON, Canada) and 50 µg/ml gentamicin. Cells seeded in 100-mm dishes were transfected using a modified calcium phosphate method with cDNA as indicated in the *Figure Legends*. After transfection (18 h), cells were washed with phosphate buffered saline (PBS), pooled and reseeded on appropriate dishes.

Primary neuronal cultures were prepared from the hippocampus of embryonic day 18 CD1 mouse embryos. Cells were plated on 15 mm glass coverslips coated with poly-L-ornithine (Invitrogen) in Neurobasal media with B-27 (Gibco) and N2 (Gibco) supplements, 2 mM glutamax, 50 µg/ml penicillin, and 50 mg/ml streptomycin for 5 h at 37°C and 5% CO_2 in a humidified incubator to permit cell attachment. Neurobasal medium was subsequently replaced with culture medium that was replenished every 3 days, cells were cultured for up to 21 days *in vitro*. Culture medium consisted of Neurobasal medium supplemented with B27, 0.5 U/mL penicillin, 0.05 mg/mL streptomycin, 10 µm MK-801, 25 mm KCl and 5 pg/mL glial-derived neurotrophic factor. All animal procedures were approved by the University of Western Ontario Animal Care Committee.

2.3 Cell Transfection

HEK 293 cells were transfected using a modified calcium phosphate method in 100 mm dishes. 2 µg of cDNA indicated in *Figure Legends* was added to 450 µL sterile distilled water, followed by the addition of 50 µL 2.5 *M* $CaCl_2$. Then 500 µL of 2X

HEPES-balanced salt solution (16.4 g NaCl, 0.38 M final; 11.9 g HEPES, 0.05 M final; 0.21 g Na_2HPO_4, 1.5 mM final) was added drop-wise to the mixture. The final mixture was added drop-wise across the surface of the 100-mm cell culture dish. The cells were incubated with the transfection overnight at 37°C in a humidified atmosphere of 95% air, 5% CO_2.

Hippocampal neurons were transfected using a modified calcium phosphate method in 15 mm glass coverslips. 4 μg of cDNA indicated in *Figure Legends* was added to 23 μL sterile distilled water, followed by the addition of 2.25 μL 2 *M* $CaCl_2$. Then 22.3 μL of 2X HEPES-balanced salt solution was added drop-wise to the mixture. The reaction was allowed to precipitate for 20 minutes in the dark. The final mixture was added drop-wise across the surface of the cell culture dishes containing only neurobasal medium. The cells were incubated with the transfection for 20-60 minutes at 37°C in a humidified atmosphere of 95% air, 5% CO_2. After incubation the transfection was washed off by changing the medium and experiments were performed the next day.

2.4 Co-immunoprecipitation

HEK 293 cells were transiently transfected with various cDNA constructs as described in the *Figure Legends*. After transfection, the cells were incubated for 5, 10, 15 min in HEPES-buffered saline solution (HBSS) at 37°C with or without 30 μM of quisqualate as indicated in the *Figure Legends*. The cells were then placed on ice, washed twice with ice-cold phosphate-buffered saline (PBS), and lysed with ice-cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10 μg/ml leupeptin, and 5 μg/ml aprotinin). The

lysates were placed on a rocking platform for 15 min at 4°C and centrifuged at 15,000 RPM for 15 min at 4°C to pellet insoluble material. Cleared supernatant containing 250-1000 μg of protein were incubated with 25 μl of Protein G Sepharose beads (GE Healthcare) containing either anti-mGluR1 or –mGluR5 antibodies (Milipore) for 1 h rotating at 4°C to immunoprecipitate the receptor. After incubation, the beads were washed three times with PBS, and proteins were solubilised in a 3× SDS sample buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted to identify co-immunoprecipitated GFP-tagged spinophilin using a primary polyclonal rabbit anti-GFP (1:1000 dilution; Invitrogen/Molecular Probes) followed by a horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:10,000; Bio-Rad). Receptor and spinophilin protein expression was determined by immunoblotting 10 μg of protein from each cell lysate used for immunoprecipitation. Proteins were detected using chemiluminescence with the enhanced chemiluminescence kit from GE Healthcare.

2.5 Immunofluorescence Confocal Microscopy

Confocal microscopy was performed using a Zeiss LSM-510 laser scanning microscope equipped with a Zeiss 63× 1.4 numerical aperture oil immersion lens (Carl Zeiss Inc., Thornwood. NJ). Live-cell imaging was performed on HEK 293 cells in untreated 35-mm glass-bottomed plates. Cells were kept at 37°C using a heated microscope stage. mGluR1a/5a was labelled with rabbit anti-FLAG (Sigma-Aldrich)-conjugated Zenon Alexa Fluor 555 antibody (Invitrogen). Receptor was stimulated with the addition of 30 μM of quisqualate (final concentration). Visualization of antibody-

labelled receptor with GFP-spinophilin was performed by dual excitation (488/543 nm) and emission band pass from 505 to 530 (GFP) and long pass at 560 (Alexa Fluor 555) filter sets.

Live imaging was performed on primary hippocampal cultures in 15-mm glass coverslips. Cells were kept at 37°C using a heated microscope stage. mGluR5 was labelled with rabbit anti-FLAG (Sigma-Aldrich)-conjugated Zenon Alexa Fluor 555 antibody (Invitrogen). Visualization of antibody-labelled receptor with GFP-spinophilin was performed by dual excitation (488/543 nm) and emission band pass from 505 to 530 (GFP) and long pass at 560 (Alexa Fluor 555) filter sets.

2.6 Internalization Assay

HEK 293 cells transfected with 2 μg of FLAG-mGluR1a/5a and either 2 μg of GFP or GFP-spinophilin were incubated with HBSS for one hour at 37°C. The cells were then washed with and incubated for 20 min on ice in HBSS, then incubated with 0.5 mg/ml sulfo-NHS-SS-biotin (Pierce Biotechnology) in HBSS for 1 h on ice to biotinylate cell surface proteins. Biotinylation was quenched by incubating cells with 100 mM glycine in HBSS for 30 min on ice. Cells were washed again with HBSS, then incubated for 5 and 15 min in HEPES-buffered saline solution (HBSS) at 37°C with 30 μM of quisqualate (Tocris), to cause internalization. Cells were then returned to ice to stop mGluR1a/5a internalization. To measure biotin bound to mGluR1a/5a that has internalized, residual biotin bound to membrane proteins that remained at the cell surface was removed by incubating cells with 100 mM sodium 2-mercaptoethanesulfonate (MesNa) in HBSS for 45 min. Cells were then washed three

times with HBSS, then lysed with ice-cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10 μg/ml leupeptin, and 5 μg/ml aprotinin). Lysates were centrifuged at 15 000 RPM for 15 min at 4 °C, then equal amounts of protein from each sample were incubated with Neutravidin beads (Pierce Biotechnology) for 1.5 h at 4 °C. After incubation, the beads were washed three times with PBS, and proteins were solubilized in a 3× SDS sample buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted for mGluR1a/5a. Cells subjected to biotinylation, but not incubated at 37 °C to allow receptor internalization, underwent treatment with MesNa as a control to assess biotin stripping efficiency. Cells that were biotinylated, but not stimulated and not stripped with MesNa were a positive control to show total plasma membrane receptor.

2.7 Inositol phosphate formation

HEK293 cells were transiently transfected with 2 μg of FLAG-mGluR1a and with 2 μg of either GFP or GFP-spinophilin. Forty-eight hours after transfection, inositol lipids were radiolabeled by incubating HEK293 cells overnight with 1 μCi/ml [^{3}H]myo-inositol (PerkinElmer Life) in Dulbecco's modified Eagle's medium (Invitrogen). Unincorporated [^{3}H]myo-inositol was removed by washing the HEK293 cells with HBSS. HEK293 cells were then preincubated for 1 hr in HBSS at 37°C and then preincubated in 500 μl of HBSS containing 10 mM LiCl for an additional 10 min at 37°C. HEK293 cells were subsequently treated in either the absence or the presence of increasing concentrations (0-100 μM) of quisqualate (Tocris) for 30 min at 37°C. The

reaction was stopped on ice by adding 500 µl of 0.8 M perchloric acid and then neutralized with 400 µl of 0.72 M KOH and 0.6 M KHCO3. The total [^{3}H]myo-inositol incorporated into the cells was determined by counting the radioactivity present in 50 µl of the cell lysate. Total inositol phosphate was purified from the cell extracts by anion exchange chromatography using Dowex 1-X8 (formate form) 200-400 mesh anion exchange resin (Bio-Rad). [^{3}H]Inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system (LS 6500; Beckman Coulter, Fullerton, CA).

2.8 ERK1/2 immunoblots

HEK293 cells were transiently transfected with 2 µg of FLAG-mGluR1a with 2 µg of either GFP or GFP-spinophilin. The cells were then stimulated for 0, 2, 5, 10 and 15 minutes with 30 µM of quisqualate. Following stimulation, the cells were placed on ice and washed with cold PBS. The cells were lysed using with ice-cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100) containing protease and phosphatase inhibitors (1 mM AEBSF, 10 µg/ml leupeptin, and 5 µg/ml aprotinin, 1 M NaF, 100 µM Na_3VO_4). The cells were incubated with the lysis buffer for 15 minutes at 4°C and centrifuged at 15000 RPM for 15 minutes to pellet insoluble material. The protein content of 50 µg samples of cell lysate was determined using a protein assay kit (Bio-Rad). The lysates were then solubilized in a 3× SDS sample buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted for phospho-p42/44 MAP kinase (Thr202/Tyr402) antibody (diluted 1:1000), p42/44 MAP kinase antibody (1:1000) (Cell Signalling),

rabbit anti-Flag antibody (1:1000) (Sigma) and goat anti-spinophilin (1:200) (Santa Cruz).

2.9 Immunoblotting

Membranes were blocked with 10% milk in wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.0, and 0.05% Tween 20) for 1 h and then incubated overnight with antibodies stated above in wash buffer containing 3% milk. Membranes were rinsed three times with wash buffer and then incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000), and rabbit anti-goat IgG (1:2500) (Bio-Rad) in wash buffer containing 3% skim milk for 1 h. Membranes were rinsed three times with wash buffer and incubated with enhanced chemiluminescence Western blotting detection reagents (GE Healthcare).

2.10 Statistical Analysis

The means ± S.E.M. are shown for values obtained for the number of independent experiments indicated in the *Figure Legends*. Quantification of immunoblots of biotinylated receptors was performed using Scion Imaging using immunoblots of similar exposures, and data expressed as mean pixel intensity. GraphPad Prism software (Graph Pad, San Diego, CA) was used to analyze data for statistical significance, as well as to analyze and fit dose-response and time course data. The statistical significance was determined by either an unpaired two tailed t-test, or by one-way ANOVA analysis of variance followed by Dunnett's multiple comparison test.

CHAPTER 3
RESULTS

3.1 Identification of Spinophilin as a Group I Metabotropic Glutamate Receptor-interacting Protein

We used the carboxyl tails (C-tails) of mGluR1a and mGluR5a to identify Group I mGuR-interacting proteins in a tandem affinity purification proteomic screen and identified spinophilin as a novel interactor. In addition to spinophilin, we identified PP1γ, Homer1b, Homer2, Homer 3 and GIPC as mGluR1a/5a-interacting proteins. The mGluR1a and mGluR5a -C tails were previously shown to bind phosphatase 1γ (PP1γ), Siah-1A, Homer and tamalin, a PDZ domain containing protein (Croci et al., 2003; Kammermeier and Ikeda, 2001; Kammermeier, 2008; Kitano et al., 2002; Ronesi and Huber, 2008). The mGluR1a/5a C-tails encode both PP1γ and PDZ binding motifs and spinophilin encodes a PDZ domain and is the regulatory subunit for protein phosphatase 1γ (Hsieh-Wilson et al., 1999; Sarrouilhe et al., 2006). Therefore, we first tested whether spinophilin could be co-immunoprecipitated with either mGluR1a or mGluR5a. We found that GFP epitope-tagged spinophilin was co-immunoprecipitated from HEK 293 cells transiently transfected with either FLAG-tagged mGluR1a or mGluR5 and GFP-spinophilin (Figure 3.1). This confirmed that spinophilin was an mGluR1a- and mGluR5a-interacting protein.

Previous studies found that spinophilin was recruited to the α_2-adrenergic receptor in response to agonist stimulation (Brady et al., 2003; Brady et al., 2005). Therefore we examined whether agonist treatment increased the association between

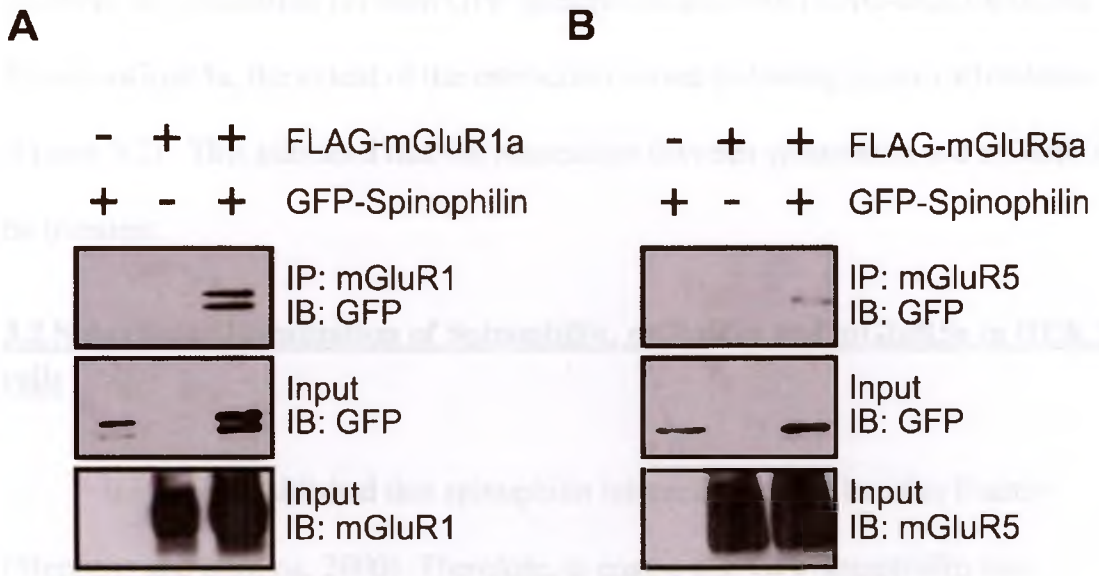

Figure 3.1: Co-immunoprecipitation of Spinophilin with Group I mGluRs. Western blots demonstrating the co-immunoprecipitation of GFP-spinophilin with FLAG-mGluR1a and FLAG-mGluR5a in HEK 293 cells. Western blots also demonstrate the expression of receptors and spinophilin from cell lysates with anti-mGluR1, anti-mGluR5 and anti-GFP antibodies. HEK 293 cells were transfected with 2 μg of GFP-spinophilin along with either 2 μg of FLAG-mGluR1a and FLAG-mGluR5a cDNA. The data is representative of 3 independent experiments.

spinophilin and mGluR1/5 by co-immunoprecipitation. Although we consistently observed an association between GFP-spinophilin and both FLAG-mGLuR1a and FLAG-mGluR5a, the extent of the interaction varied following agonist stimulation (Figure 3.2). This indicated that the association between spinophilin and mGluR1/5 may be transient.

3.2 Subcellular Localization of Spinophilin, mGluR1a and mGluR5a in HEK 293 cells

It is well established that spinophilin interacts with and bundles F-actin (Stephens and Banting, 2000). Therefore, to ensure that GFP-spinophilin was appropriately localized in HEK 293 cells, we transfected GFP-spinophilin in HEK 293 cells and stained for actin (phalloidin). These cells were then imaged by confocal microscopy. We found that spinophilin and actin were clearly co-localized in HEK 293 cells (Figure 3.3). Previous studies demonstrated that spinophilin co-localized with the α_2-adrenergic receptor on the cell surface of polarized Madin-Darby canine kidney cells (Brady et al., 2003). Therefore, we examined the subcellular localization of GFP-spinophilin in cells co-expressing FLAG-mGluR1a or FLAG-mGluR5a. We found that the co-expression of both FLAG-mGluR1a (Figure 3.4A) and FLAG-mGluR5a (Figure 3.4B) increased the localization of GFP-spinophilin at the cell surface.

3.3 Co-localization of Spinophilin and mGluR5 in Hippocampal Neurons

To further determine the subcellular localization of spinophilin, hippocampal neurons were transfected to over-express GFP-spinophilin and red fluorescence protein (RFP) as a non-specific protein to fill the processes in neurons allowing a clear

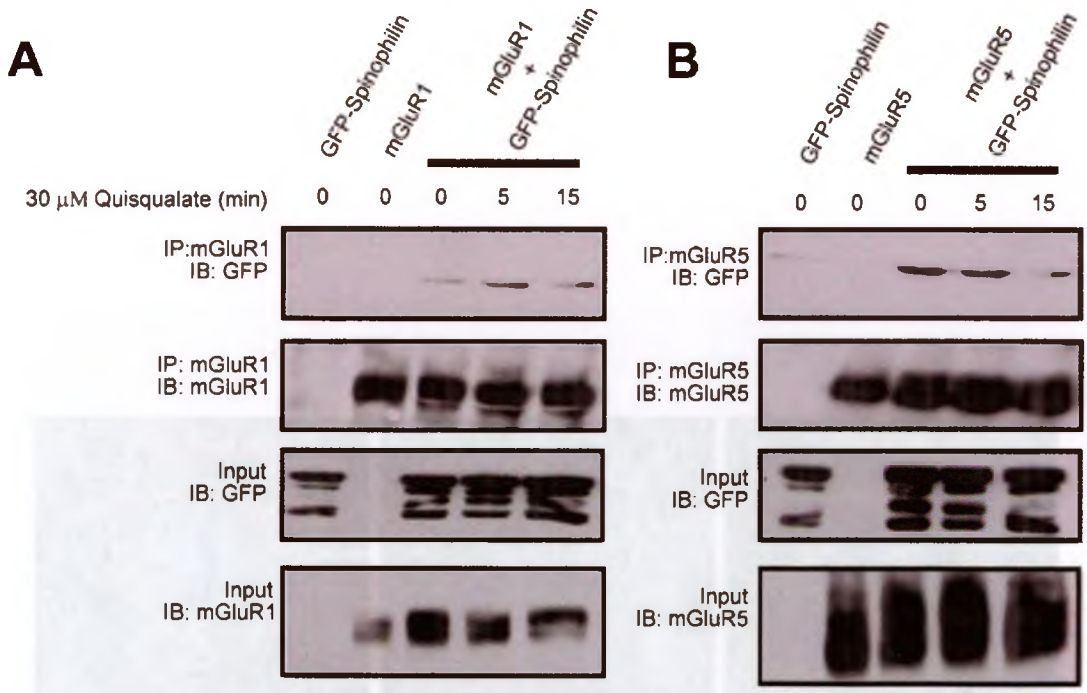

Figure 3.2: Co-immunoprecipitation of Spinophilin with Group I mGluRs During Agonist Stimulation. **(A)** Western blots demonstrating the immunoprecipitation of the receptors FLAG-mGluR1a and the co-immunoprecipitation of GFP-spinophilin in HEK 293 cells with 30 μM of quisqualate stimulation for 0, 5, 15 minutes. Western blots also demonstrate the expression of receptors and spinophilin from cell lysates with anti-mGluR1 and anti-GFP antibodies. **(B)** Western blots demonstrating the immunoprecipitation of the receptors FLAG-mGluR5a and the co-immunoprecipitation of GFP-spinophilin in HEK 293 cells with 30 μM of quisqualate stimulation for 0, 5, 15 minutes. Western blots also demonstrate the expression of receptors and spinophilin from cell lysates with anti-mGluR5 and anti-GFP antibodies. HEK 293 cells were transfected with 2 μg of GFP-spinophilin along with either 2 μg of FLAG-mGluR1a and FLAG-mGluR5a cDNA. The data is representative of 3 independent experiments.

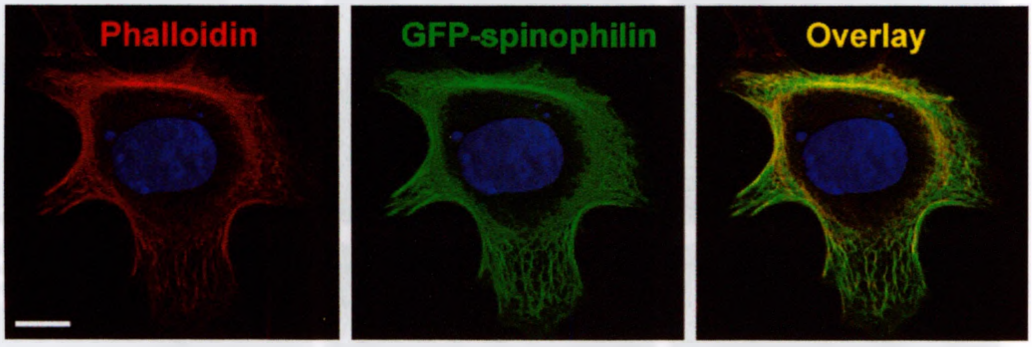

Figure 3.3: Expression of Spinophilin in HEK 293 Cells. Confocal images of GFP-spinophilin (green), phalloidin (red), Hoechst (blue), and co-localization (yellow). HEK 293 cells were transfected with 2 μg of GFP-spinophilin and labelled with phalloidin and Hoechst and visualized using confocal microscopy. The data is representative of 3 experiments. Scale bars, 10 μm.

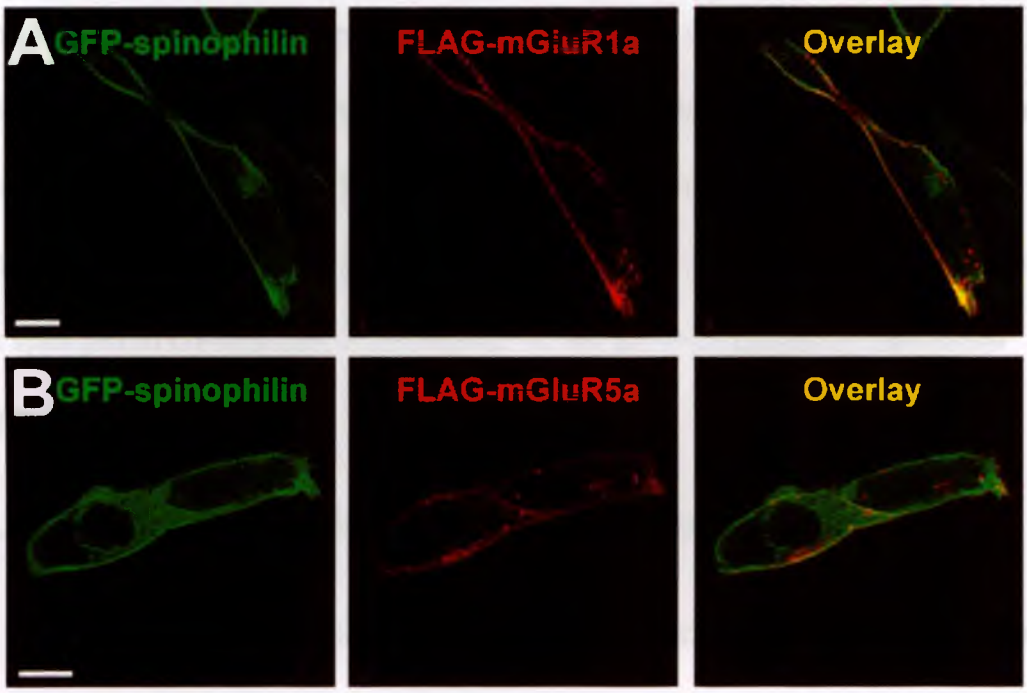

Figure 3.4: Expression of Spinophilin and Group I mGluRs in HEK 293 Cells. (A) Confocal images of live HEK 293 cells transfected with GFP-spinophilin (green) and FLAG-mGluR1a (red). Co-localization is indicated in the overlay images (yellow). **(B)** Confocal images of live HEK 293 cells transfected with expressing GFP-spinophilin (green) and FLAG-mGluR5a (red). Co-localization is indicated in the overlay images (yellow). Cells are transfected with 2 μg of cDNA encoding GFP-spinophilin and 2 μg of cDNA encoding either FLAG-mGluR1a or FLAG-mGluR5a and are stained with Alexa Fluor-conjugated 555 rabbit polyclonal antibody at 4°C. The data is representative of 5 experiments. Scale bars, 10 μm.

indication of the spines. GFP-spinophilin was found to be primarily localized within synaptic spines in hippocampal neuronal dendrites (Figure 3.5A). To determine whether GFP-spinophilin was co-localized with Group I mGluRs at synaptic densities, hippocampal neurons were transfected to express both FLAG-mGluR5a and GFP-spinophilin. Live neurons expressing FLAG-mGluR5a were labelled with Alexa Fluor 555-conjugated FLAG antibody. Consistent with the observation that spinophilin co-localizes with mGluR1/5 in HEK 293 cells, co-localization of the mGluR5 and spinophilin was found along the dendritic processes (Figure 3.5B). The data indicates that spinophilin co-localizes with mGluR5 in the dendritic spines of transfected hippocampal neurons.

3.4 Agonist-stimulated mGluR5a and Spinophilin Internalization

Spinophilin was previously shown to be recruited to the plasma membrane after α_2AR agonist stimulation, where it then recognizes the activated α_2AR- G$\beta\gamma$ complex and tethers the receptor to the plasma membrane (Brady et al., 2003; Brady et al., 2005). Additionally, spinophilin could be co-immunoprecipitated with both mGluR1a and mGluR5a and was co-localized with both receptors. Therefore, we tested whether spinophilin over-expression altered agonist-mediated internalization of mGluR1a and mGluR5a using a cell surface biotinylation assay. To determine the extent of mGluR1a/5a internalization, cell-surface proteins were biotinylated on ice, warmed to 37 °C, and treated with 30 μM quisqualate for 0, 5, 15 min. The internalization of mGluR1a/5a in response to agonist was determined as the proportion of total biotinylated receptor that was resistant to MESNA stripping following agonist treatment.

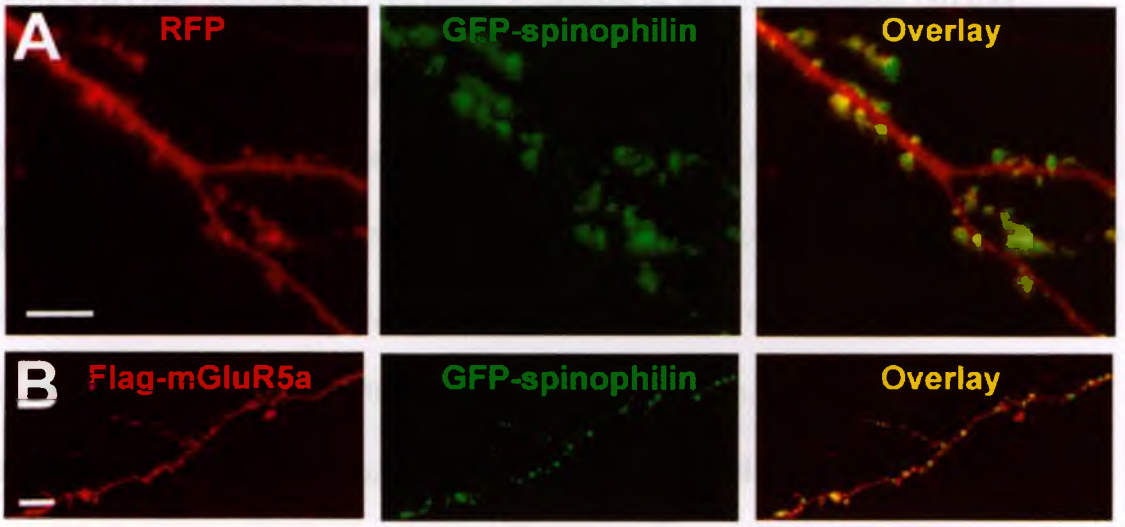

Figure 3.5: Spinophilin is Expressed Primarily along the Spines in Primary Mouse Hippocampal Neurons. (A) Confocal images of live mouse hippocampal neurons transfected with GFP- spinophilin (green) and red fluorescence protein (red). **(B)** Confocal images of live mouse hippocampal neurons transfected with GFP-spinophilin (green) and FLAG-mGluR5a labelled with Alexafluor 555 conjugated FLAG antibody (red). Co-localization is shown in the overlay image (yellow). Neurons transfected with 4 µg of cDNA encoding GFP-spinophilin, and either 2 µg of cDNA encoding red fluorescence protein or 4 µg of FLAG-mGluR5a cDNA were imaged by confocal microscopy. The data is representative of 3 experiments. Scale bars, 10 µm.

We found that, the internalization of FLAG-mGluR1a was significantly reduced in the presence of GFP- spinophilin following 5 minutes of agonist treatment (30 μM quisqualate), but was not significantly different from GFP transfected control at 15 minutes (Figure 3.6). In contrast, the internalization of FLAG-mGluR5a was not significantly reduced in the presence of GFP-spinophilin following 5 minutes of agonist treatment (30 μM quisqualate), but exhibited a trend towards reduced internalization following 15 minutes agonist treatment (Figure 3.7). Thus, similar to what was previously observed for the α_2AR, spinophilin antagonized FLAG-mGluR1a internalization.

3.5 Effect of Spinophilin on mGluR1a-mediated Signalling in HEK 293 cells

It is well established that Group I mGluRs activate the heterotrimeric $G\alpha_{q/11}$ protein which leads to the activation of phospholipase C, causing the formation of inositol 1, 4, 5 trisphosphate (IP_3) and diacylglycerol (DAG), which subsequently causes the release of calcium from intracellular stores (Kawabata et al., 1998). Spinophilin has been found to interact with the α_{1B} - adrenergic receptor (α_{1B}AR), which is also coupled to the heterotrimeric $G\alpha_{q/11}$ protein, and functions to enhance α_{1B}AR-stimulated calcium currents by attenuating α_{1B}AR endocytosis (Wang et al., 2005). We have shown that spinophilin decreases mGluR1a internalization following 5 minutes of agonist stimulation, therefore, we examined whether the GFP-spinophilin over-expression leads to an alteration of FLAG-mGluR1a-mediated inositol phosphate (IP) formation. In the absence of GFP-spinophilin, increasing amounts of quisqualate treatment on HEK 293 cells over-expressed with FLAG-mGluR1 resulted in a dose-dependent increase

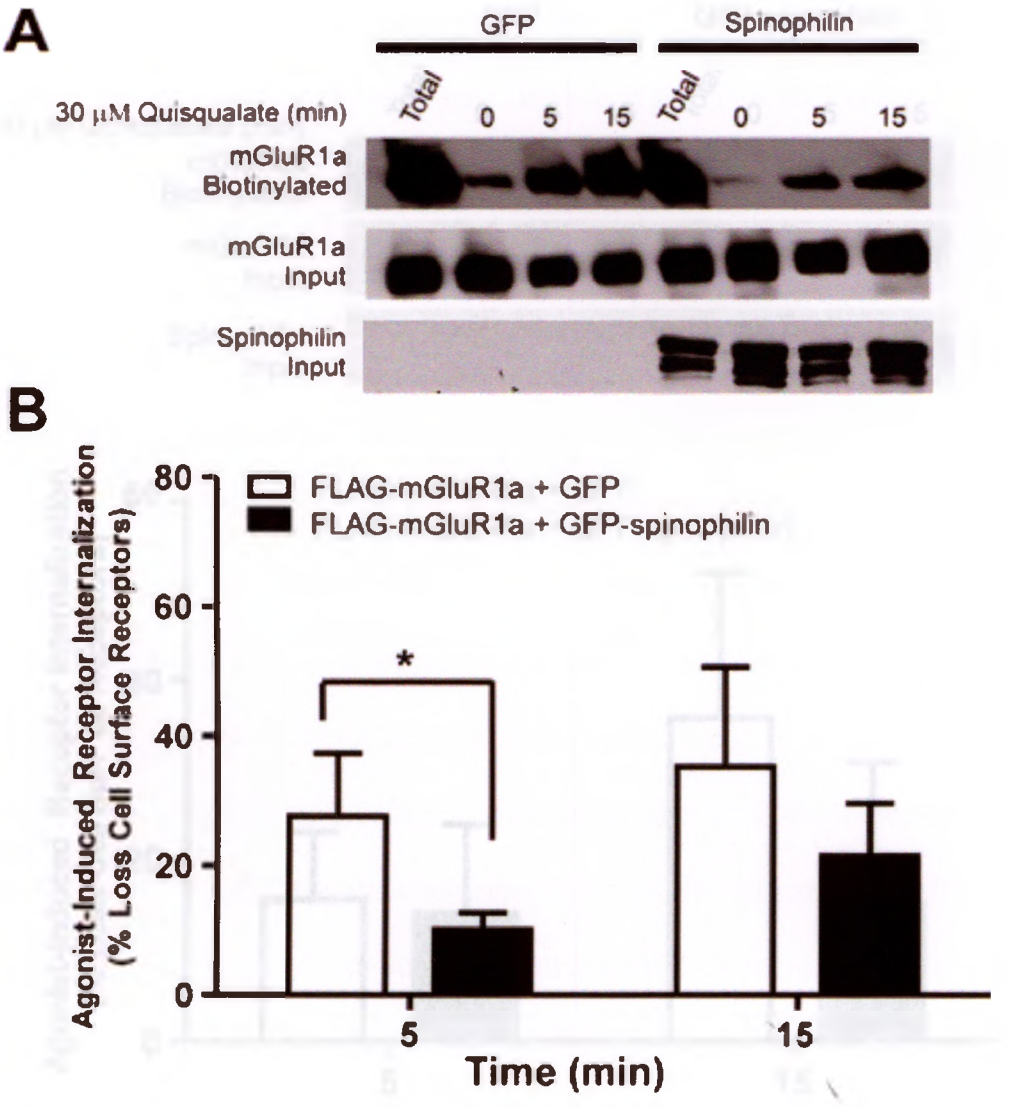

Figure 3.6: Internalization of mGluR1a with Spinophilin. **(A)** Shown is a representative immunoblot demonstrating the agonist-stimulated internalization of FLAG-mGluR1a in the presence of either GFP or GFP-spinophilin following 5 and 15 minutes of agonist treatment with 30 μM quisqualate. **(B)** Densitometry analysis of the immunoblot showing the extent of loss of cell surface protein expression. The data represents the mean ± the SEM for 6 independent experiments. *$P<0.05$, two-tailed unpaired t-test.

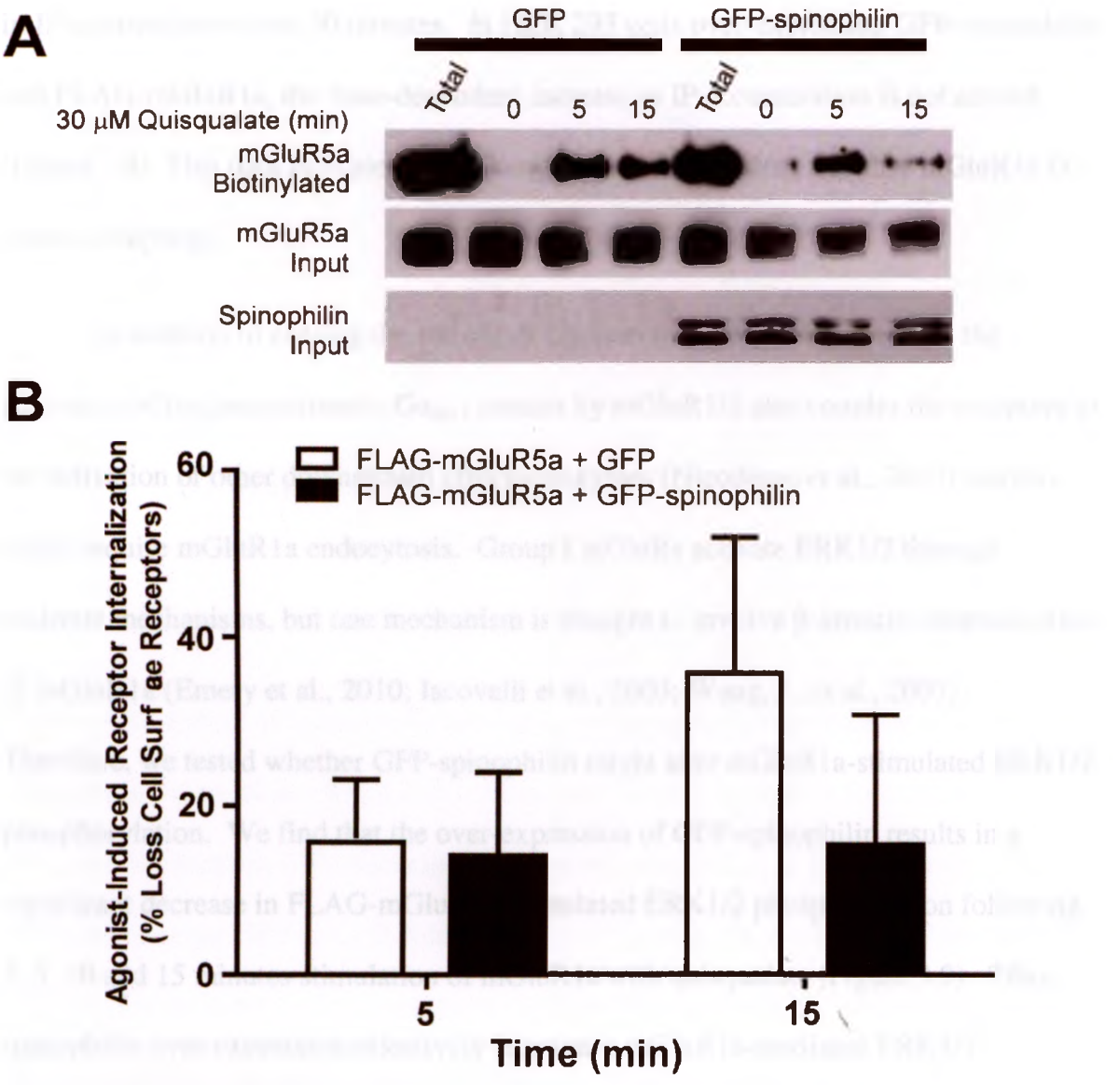

Figure 3.7: Internalization of mGluR5a with Spinophilin. **(A)** Shown is a representative immunoblot demonstrating the agonist-stimulated internalization of FLAG-mGluR5a in the presence of either GFP or GFP-spinophilin following 5 and 15 minutes of agonist treatment with 30 μM quisqualate. **(B)** Densitometry analysis of the immunoblot showing the extent of loss of cell surface FLAG-mGluR5a protein expression. The data represents the mean ± the SEM for 5 independent experiments.

in IP accumulation over 30 minutes. In HEK 293 cells over-expressing GFP-spinophilin and FLAG-mGluR1a, the dose-dependent increase in IP accumulation is not altered (Figure 3.8). This data indicates that spinophilin interaction does not alter mGluR1a G-protein coupling.

In addition to causing the release of calcium from intracellular stores, the activation of the heterotrimeric $G\alpha_{q/11}$ protein by mGluR1/5 also couples the receptors to the activation of other downstream effector enzymes (Nicodemo et al., 2010) and this might require mGluR1a endocytosis. Group I mGluRs activate ERK1/2 through multiple mechanisms, but one mechanism is thought to involve β-arrestin internalization of mGluR1a (Emery et al., 2010; Iacovelli et al., 2003; Wang, J., et al., 2007). Therefore, we tested whether GFP-spinophilin might alter mGluR1a-stimulated ERK1/2 phosphorylation. We find that the over-expression of GFP-spinophilin results in a significant decrease in FLAG-mGluR1a-stimulated ERK1/2 phosphorylation following 2, 5, 10 and 15 minutes stimulation of mGluR1a with quisqualate (Figure 3.9). Thus, spinophilin over-expression selectively attenuates mGluR1a-mediated ERK1/2 phosphorylation without affecting receptor G-protein coupling.

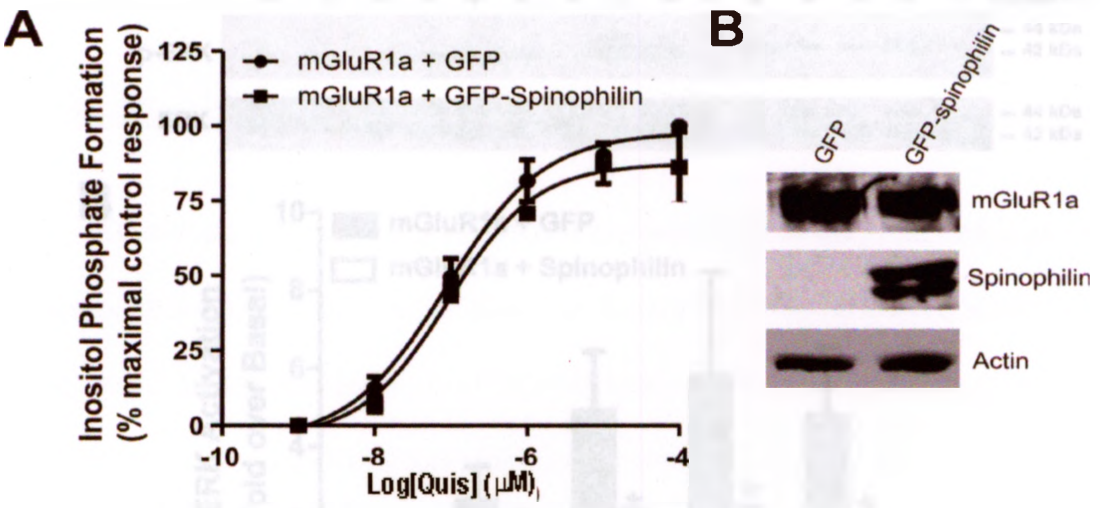

Figure 3.8: Effect of Spinophilin on Agonist-stimulated mGluR1a Inositol Phosphate Formation. **(A)** mGluR1-stimulated inositol phosphate formation in response to treatment with increasing concentrations of quisqualate for 30 min in the presence of either GFP or GFP-spinophilin. The log EC_{50} for mGluR1 is -7.058 and the log EC_{50} for mGluR1 and spinophilin is -7.033. **(B)** Immunoblot showing equivalent FLAG-mGluR1a expression in GFP and GFP-spinophilin transfected HEK 293 cells. The data represents the mean ± the SEM for 6 independent experiments.

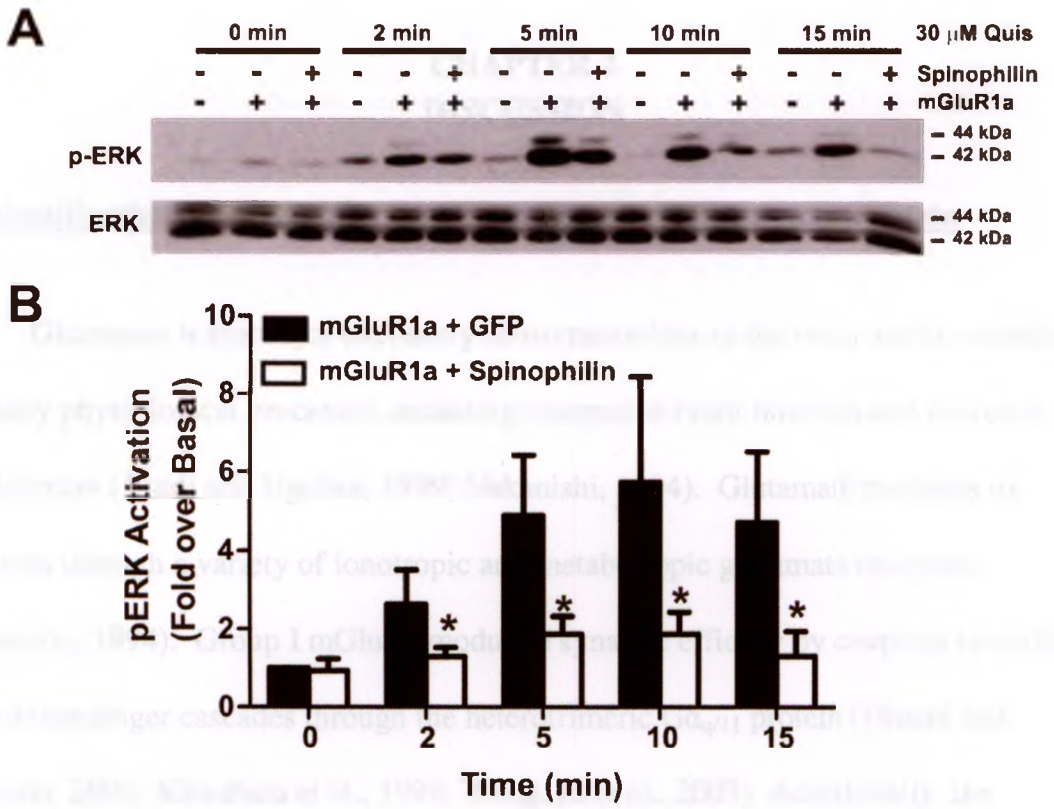

Figure 3.9: Effect of GFP-spinophilin Over-expression on mGluR1a-stimulated ERK1/2 Phosphorylation. **(A)** Representative immunoblot for FLAG-mGluR1a stimulated ERK1/2 phosphorylation in response to 30 μM quisqualate stimulation for 0, 2, 5, 10 and 15 min in the presence of either GFP or GFP-spinophilin. Shown are immunoblots for phosphorylated (p-ERK) and total ERK. **(B)** Densitometry analysis of the immunoblots showing the mean ± SD of the change in ERK1/2 phosphorylation for 6 independent experiments. *$P<0.05$, ANOVA followed by paired t-test.

CHAPTER 4
DISCUSSION

4.1 Identification of Spinophilin as a Group I mGluR Regulatory Protein

Glutamate is the major excitatory neurotransmitter in the brain and is essential for many physiological processes, including integrative brain function and neuronal cell development (Bordi and Ugolini, 1999; Nakanishi, 1994). Glutamate mediates its functions through a variety of ionotropic and metabotropic glutamate receptors (Nakanishi, 1994). Group I mGluRs modulate synaptic efficacy by coupling to multiple second messenger cascades through the heterotrimeric $G\alpha_{q/11}$ protein (Dhami and Ferguson, 2006; Kawabata et al., 1998; Wang, J., et al., 2007). Additionally, the activation of these receptors has been implicated in a number of neurodegenerative diseases including: Huntington's disease, ALS and Alzheimer's disease (Bordi and Ugolini, 1999; Byrnes et al., 2009; Ribeiro et al., 2011; Wang, J., et al., 2004). However, the exact role that mGluRs play in both neuroprotection and neurodegeneration remain unclear (Pshenichkin et al., 2008).

Protein-protein interactions have been shown to play an important role in regulating Group I mGluR signal transduction in the central nervous system and it is now established that mGluR1a and mGuR5a act as molecular scaffolds for the recruitment of multiple regulatory signalling complexes. For example, mGluR1a and mGluR5a have been shown to interact with phosphatase 1γ, Siah-1A, Homer and PDZ proteins. Furthermore, these interactions play an important role in the regulation of Group I mGluR signalling cascades (Croci et al., 2003; Kammermeier and Ikeda, 2001;

Kammermeier, 2008; Kitano et al., 2002; Ronesi and Huber, 2008). We have found a new Group I-interacting protein, spinophilin that binds to both the mGluR1a and mGluR5a C-tails. Spinophilin has been shown to regulate ionotropic glutamate receptors through protein phosphatase 1 (PP1γ), as well as interact with a number of GPCRs through a receptor interacting domain (Sarrouilhe et al., 2006; Smith et al., 1999; Uematsu et al., 2005; Wang et al., 2005; Yan et al., 1999). Furthermore, spinophilin encodes a PDZ domain, which may mediate the interaction with mGluR1a/5a (Sarrouilhe et al., 2006). Similarly, Group I mGluRs have a PP1γ domain and a PDZ domain at the end of their C-tails (Croci et al., 2003; Kitano et al., 2002). Therefore, the purpose of this thesis was to determine if spinophilin interacts with mGluR1a and mGluR5a and to examine whether this interaction is of importance for regulating both mGluR1a and mGluR5a trafficking and signalling.

A clear understanding of how Group I mGluR interacting proteins regulate receptor signalling is required for the development of effective therapeutics to treat neurodegenerative diseases associated with mGluR1a/5a signalling. In attempt to determine the potential role spinophilin has in the regulation of Group I mGluR signalling and internalization, we have made several key observations: 1) spinophilin associates with mGluR1a and mGluR5a and this association may be dynamically regulated by agonist activation, 2) spinophilin antagonizes agonist-stimulated mGluR1a internalization , 3) spinophilin does not alter inositol phosphate formation induced by mGluR1a stimulation, and 4) spinophilin prevents ERK1/2 phosphorylation in response to mGluR1a activation.

4.2 Interaction between Spinophilin and Group I mGluRs

Group I mGluRs function as molecular scaffolds for the recruitment of intracellular and extracellular signalling complexes. mGluR1 splice variants (a-d) and mGluR5 splice variants (a and b) are of variable length and therefore function differently and interact with distinct protein complexes. mGluR1a and mGluR5a have extremely long C-tails that are known to interact with several proteins including: protein phosphatase 1γ, Siah-1A, Homer and PDZ proteins. In contrast, mGluR1 and mGluR5 splice variants lack the extended C-tails and thus do not have the capacity to mediate the essential protein interactions required for Group I mGluR-dependent neuronal plasticity (Croci et al., 2003; Kammermeier and Ikeda, 2001; Kammermeier, 2008; Kitano et al., 2002; Ronesi and Huber, 2008). For example, protein phosphatase 1 (PP1γ) has been shown to regulate mGluR1/5-mediated ERK1/2 phosphorylation (Voulalas et al., 2005). Additionally, PP1γ has been shown to interact with AMPA receptors, and regulates their low activity state by allowing their dephosphorylation (Yan et al., 1999). The recruitment of PP1γ to ionotropic glutamate receptors is also mediated by spinophilin, which recruits PP1γ to these receptors and also regulates PP1γ catalytic activity (Hsieh-Wilson et al., 1999; Ragusa et al., 2011; Yan et al., 1999). Thus, spinophilin has the capacity to regulate post synaptic receptors by recruiting enzymes to the receptor complex.

Work done in our laboratory has identified spinophilin, using a tandem affinity purification proteomic screen, as a protein that interacts with the carboxyl tails of mGluR1a and mGluR5a. To confirm these findings, our initial experiments tested

whether spinophilin associated with both mGluR1a and mGluR5a. We observed that GFP epitope-tagged spinophilin co-immunoprecipitated with both FLAG-tagged mGluR1a and -mGluR5a. There are two potential mechanisms by which spinophilin may interact with mGluR1a and mGluR5a. Spinophilin either forms a protein complex with PP1γ that is scaffolded to the Group I mGluR PP1γ binding motif or the spinophilin PDZ domain interacts directly with the mGluR1a and mGluR5a PDZ C-tail binding motifs. Further analysis of the mechanisms underlying the interaction between spinophilin and Group I mGluRs, will require the mutation of both the PP1γ and PDZ motifs in the mGluR1a and mGluR5a C-tails and the investigation of spinophilin interactions with mGluR1b which lacks these motifs. Similarly, co-immunoprecipitation experiments with spinophilin, PP1γ and mGluR1/5 need to be conducted. This will help determine whether PP1γ mediates the association of spinophilin with mGluR1a and mGluR5a.

The regulation by spinophilin is determined on the basis of its phosphorylation state (Baucum et al., 2010; Feng et al., 2000; Grossman et al., 2002; Grossman et al., 2004; Hsieh-Wilson et al., 2003; Uematsu et al., 2005). For example, following PKA phosphorylation of Ser-177, spinophilin relocates from the post synaptic density (PSD) and becomes enriched in the cytosol (Hsieh-Wilson et al., 2003). Phosphorylation at this site also decreases the ability of spinophilin to regulate the trafficking of the α_{2A}-adrenergic receptor (Xu et al., 2008). Therefore, future experiments are required to allow the assessment of whether spinophilin phosphorylation is required for spinophilin binding and regulation of Group I mGluRs.

4. 3 Role of Spinophilin in the Agonist Induced Internalization of mGluR1

Spinophilin interacts with and regulates the internalization and signalling of the α_2AR (Brady et al., 2003; Brady et al., 2005; Wang and Limbird, 2002; Wang, Q., et al., 2004). More specifically, spinophilin retains the α_2AR at the plasma membrane by competing with GRK2 for binding to the third intracellular loop and prevents subsequent β-arrestin-dependent and clathrin-mediated endocytosis of the receptor (Brady et al., 2003; Wang and Limbird, 2002; Wang, Q., et al., 2004). Wang Q., *et al.* (2004) found that spinophilin and β-arrestin dynamically regulate α_2AR phosphorylation, internalization and signalling both *in vitro* and *in vivo*. Therefore, we tested whether over-expression of spinophilin prevents the agonist-induced internalization of both mGluR1a and mGluR5a. We show that spinophilin antagonizes mGluR1a endocytosis without increasing G protein-mediated signalling. This suggests the possibility that spinophilin might not compete for GRK2 binding to Group I mGluRs. However, this possibility remains to be determined.

Group I mGluRs undergo internalization by a number of distinct mechanisms depending on how internalization is initiated and the splice variant studied. Both mGluR1a and mGluR5a are suggested to undergo internalization via both a classical model of internalization (a GRK-phosphorylation- and β-arrestin-dependent pathway), as well as agonist-stimulated and constitutive pathways that are GRK2-phosphorylation- and β-arrestin-independent (Dhami and Ferguson, 2006). However, spinophilin does not attenuate mGluR1a G protein signalling, it is also possible that by competing with GRK2 to bind Group I mGluRs, it prevents the phosphorylation-independent

mGluR1a/5a internalization (Ribeiro et al., 2009). Future experimentation will be essential for understanding the relative roles of GRK2 and spinophilin in the regulation of the internalization of Group I mGluRs.

Spinophilin tethers the α-adrenergic receptor to the cell surface via its coiled-coil interactions and by preventing GRK2 interaction (Wang et al., 2005). As a consequence, spinophilin has been described as a scaffolding protein that provides a mechanism for receptors to interact with protein partners and the cytoskeleton (Brady et al., 2003; Sarrouilhe et al., 2006). Consistent with this observation, we find that spinophilin over-expression antagonizes the internalization of mGluR1a. To date, a variety of protein interactions with the mGluR1a/5a C-tails have been reported to regulate the trafficking and signalling of these receptors (Croci et al., 2003; Kammermeier and Ikeda, 2001; Kammermeier, 2008; Kitano et al., 2002; Ronesi and Huber, 2008). For example, Homer proteins interact with the C-tail of Group I mGluRs (Kammermeier, 2008; Ronesi and Huber, 2008). Similar to spinophilin, Homer proteins function as scaffolds promoting the formation of mGluR1/5 regulated protein complexes at the post synaptic density (Magalhaes et al., 2011). Whether spinophilin contributes in a similar way to the scaffolding of synaptic protein complexes with mGluR1a/mGluR5a at post synaptic densities remains to be determined.

4.4. Role of Spinophilin in mGluR1a-mediated Inositol Phosphate Formation

Group I mGluRs activate the heterotrimeric $G\alpha_{q/11}$ protein which leads to the: 1) activation of phospholipase Cβ, 2) formation of IP_3 and DAG, 3) release of calcium from intracellular stores and 4) activation of effector enzymes such as protein kinase C

and ERK1/2 (Dhami and Ferguson, 2006; Kawabata et al., 1998; Wang, J., et al., 2007). We found that spinophilin over-expression attenuated mGluR1a-mediated ERK1/2 phosphorylation without affecting inositol phosphate formation in response mGluR1a stimulation. This indicates that the interaction between spinophilin and mGluR1a results in the antagonism of the activation of ERK1/2 signalling without affecting $G\alpha_{q/11}$-mediated inositol phosphate formation. This suggests that the formation of intracellular scaffolding protein complexes with mGluR1a may bias intracellular signalling pathways activated by mGluR1a. This is akin to the observed effect of biased GPCR ligands that influence distinct GPCR conformations to activate selective set of intracellular signalling pathways (Gesty-Palmer and Luttrell, 2011; Niswender and Conn, 2010).

In addition to retaining the α_{1B}AR to the plasma membrane by preventing β-arrestin-mediated α_{1B}AR endocytosis, spinophilin also recruits RGS2 to the signalling complex to prevent the prolonged activation of signalling cascades induced by the $G\alpha_{q/11}$ protein (Wang et al., 2005). Additionally, Wang, X., *et al.* (2005) found that spinophilin is less effective in regulating calcium currents in RGS2 deficient cells and RGS2 is less effective in inhibiting calcium current in spinophilin deficient cells. To determine whether spinophilin regulates mGluR1a signalling by recruiting RGS2, future experiments examining the over-expression and knockdown of both RGS2 and spinophilin on mGluR1a signalling is required.

4.5 Role of Spinophilin in mGluR1-Mediated ERK1/2 Activation

It is well established that MAPK activation by Group I mGluRs involves different protein partners and a selection of distinct signalling pathways (Emery et al.,

2010; Iacovelli et al., 2003; Wang, J., et al., 2007). Voulalas *et al.* (2005) have reported that PP1γ regulates MAPK activation induced by mGluR1/5 stimulation. Specifically, they have shown that the blockade of PP1γ by phosphatase inhibitors increases mGluR1/5-mediated ERK1/2 phosphorylation. Consequently, the potential association of spinophilin in a complex with PP1 and Group I mGluRs may contribute to the direct inactivation of ERK1/2 phosphorylation. To confirm this hypothesis, mGluR1a-stimulated ERK1/2 phosphorylation needs to be assessed following the over-expression and knockdown of both PP1γ and spinophilin proteins.

4.6 Summary

In summary, we have shown here that spinophilin associates with mGluR1a and mGluR5a, potentially via its association with PP1γ or mGluR1a/5a the PDZ binding motifs. Moreover, we demonstrate that spinophilin antagonizes both mGluR1a endocytosis and ERK1/2 phosphorylation. This occurs in the absence of attenuated mGluR1a-stimulated inositol phosphate formation. These observations indicate that the interaction of proteins with the intracellular face of Group I mGluRs can influence the agonist-stimulated coupling of receptors to distinct intracellular signalling pathways. This opens the window to specifically target protein interfaces required for selective activation of unique signalling pathways that may be inhibited to treat neurodegenerative diseases.

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