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## Modulation of G Protein-Coupled Receptor Intracellular Trafficking and Signal Transduction

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Graduate Program in Physiology  
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy  
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**MODULATION OF G PROTEIN-COUPLED RECEPTOR INTRACELLULAR  
TRAFFICKING  
AND SIGNAL TRANSDUCTION**

**(Spine Title: GPCR Intracellular Trafficking and Signal Transduction)  
(Thesis format: integrated article)**

**by**

**Jessica Lynn Esseltine**

**Graduate Program**

**in**

**Physiology**

**Submitted in partial fulfillment of the requirements for the degree of Doctor of  
Philosophy**

**School of Graduate and Postdoctoral Studies**

**The University of Western Ontario**

**London, Ontario, Canada**

**April, 2012**

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## **Abstract**

The seven transmembrane-spanning G Protein-coupled Receptor (GPCR) super family is the largest family of cell-surface receptors, comprising greater than 650 members. GPCRs represent the primary targets of most therapeutic drugs. Desensitization, endocytosis and recycling are major mechanisms of receptor regulation and intracellular trafficking of GPCRs is linked to the Rab family of small G proteins. In the present study, we examined whether multiple Rab GTPase regulate receptor trafficking through endosomal cellular compartments as a consequence of their direct association with GPCRs. We find that Rab4, Rab7 and Rab11 all bind to the last 10 amino acid residues of the angiotensin II Type 1 (AT<sub>1</sub>R) carboxyl-terminal tail. We show that the Rab GTPases compete with one another for receptor binding and that Rab4 effectively displaces Rab11 from the receptor. In contrast, Rab11 overexpression does not prevent Rab4 binding to the AT<sub>1</sub>R. Overexpression of wild-type Rab4, but not Rab11, facilitates AT<sub>1</sub>R dephosphorylation, and a constitutively active Rab4-Q67L mutant reduces AT<sub>1</sub>R desensitization and promotes AT<sub>1</sub>R resensitization. We also find that Rab8, a RabGTPase involved in the regulation of secretory/recycling vesicles, modulation of the actin cytoskeleton and cell polarity, interacts with the carboxyl-terminal tail of metabotropic glutamate receptor 1 (mGluR1a) and attenuates mGluR1a signalling and endocytosis in a protein kinase C-dependent manner. Finally, we have examined several previously uncharacterised but naturally occurring mutations in mGluR1a that have been associated with cancer that may alter mGluR1a signalling. We find that mutations found within the ligand binding domain of mGluR1a result in both decreased cell surface expression and basal inositol 1,4,5, trisphosphate formation and bias mGluR1a signalling via the ERK1/2 pathway. Additional mGluR1a mutations localized to the mGluR1a glutamate

binding site, intracellular regulatory domains and Homer binding site also result in changes in mGluR1a subcellular localization, signalling and cell morphology. Taken together, these results indicate that GPCR signalling is significantly modulated by the association of intracellular regulatory proteins that can be influenced by receptor structure.

## **Key Words**

G Protein-Coupled Receptors

Angiotensin II Type 1 Receptor

Metabotropic Glutamate Receptor

Heterotrimeric G Protein

Desensitization

Receptor Signalling

Phosphorylation

Endocytosis

Resensitization

Plasma Membrane Recycling

Intracellular Membrane Compartments

Receptor Trafficking

Rab GTPase

Small G Proteins

Receptor Carboxyl-Terminal Tail Domains

Human Embryonic Kidney 293 Cells

Hippocampal Neurons

## **Co-Authorship**

Data presented in Chapter 2 are published elsewhere. Data presented in Chapters 3 and 4 have been submitted for publication. Ms. Lianne Dale and Dr. Ana Cristina Magalhaes assisted me in creating the Rab4 mutant cDNA constructs used in chapter 2. Mutant AT<sub>1</sub>R constructs used in chapter 2 were created by me. All mGluR1 mutant cDNA constructs used in chapter 4 were created by the Oncology group at Eli Lilly and Company and provided to me through collaboration with Dr. Melinda Willard and Dr. Thomas Barber. All other constructs were previously generated by the Ferguson Lab.

I performed all experiments in Chapter 2 with the assistance of Ms. Lianne Dale in both the experimental design and troubleshooting. In Chapter 3 Dr. Fabiola M. Ribeiro contributed Figure 3.4A and 3.5A. Drs. Thomas Barber and Melinda D. Willard of Eli Lilly and Company contributed Figure 4.7 and 4.8B in Chapter 4.

Dedication

For William, of course!

## **Acknowledgements**

Steve, thank you for your support, financially and otherwise during my degree. Thank you for the four years I spent as a teaching assistant for phys 3140 and it meant a great deal to me to give your last lecture this year. Thank you for allowing me to travel during my degree, especially for Australia. You provided an excellent atmosphere to foster independent thinking and research, and I owe much of my scientific competence to that atmosphere. I appreciate you recommending me for your collaboration with Eli Lilly and Company—I have gained valuable connections and resources because of that.

Lianne, how can I convey the impact you have had these past five years! Thank you for answering my gazillion questions, for patiently walking me through troubleshooting, endless scientific talks, analyzing and interpreting data, teaching me how to think critically and objectively. Mostly, thank you for being my friend, for laughing with me, and listening. Thank you for sticking up for me and for your unconditional support and advice, especially through the rough times.

Ana, thank you for your advice and guidance. Thank you for keeping me safe in Brazil and wow-thank you for taking me on a family vacation with you! I also appreciate your unconditional love and support as well. You are a brilliant and dedicated scientist and I know you will excel at whatever you do.

Steve and Jane, thank you for writing endless letters of reference for me. Because of the great things you said in them, I was very successful in securing scholarships. And Jane, thank you also for allowing me to give two of your lectures - I really enjoy teaching and found them to be a treat.

Thank you to all the other members of my lab, for teaching me new things every day.



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## LIST OF ABBREVIATIONS

<b>Abbreviation</b>	<b>Full Name</b>
<b>AngII</b>	Angiotensin II
<b>AP-2</b>	Adaptor protein-2 complex
<b>ATP</b>	Adenosine triphosphate
<b>AT<sub>1</sub>R</b>	Angiotensin II Type 1 receptor
<b>Barr</b>	Beta arrestin
<b>β<sub>2</sub>AR</b>	Beta 2 adrenergic receptor
<b>BSA</b>	Bovine serum albumin
<b>cAMP</b>	Cyclic adenosine monophosphate
<b>cDNA</b>	Complementary deoxyribonucleic acid
<b>C-tail</b>	Carboxyl-terminal tail
<b>DMEM</b>	Dulbecco's minimal Eagle's medium
<b>DNA</b>	Deoxyribonucleic acid
<b>EDTA</b>	Ethylene diamine tetra-acetic acid
<b>EEA 1</b>	Earl endosomal antigen 1
<b>EGFP</b>	Enhanced green fluorescence protein
<b>ERK1/2</b>	Extracellular signal-regulated kinase 1/2
<b>FBS</b>	Fetal bovine serum
<b>G protein</b>	Guanine nucleotide-binding protein
<b>GABA</b>	γ-aminobutyric acid receptor
<b>GAP</b>	GTPase activating protein
<b>GDF</b>	GDP-dissociation factor
<b>GDI</b>	GDP-dissociation inhibitor
<b>GDP</b>	Guanosine diphosphate
<b>GPCR</b>	G protein-coupled receptor
<b>GRK</b>	G protein-coupled receptor kinase
<b>GRP</b>	G protein-coupled receptor phosphatase
<b>GST</b>	Glutathione <i>S</i> -transferase
<b>GTP</b>	Guanosine triphosphate
<b>GTPase</b>	Guanine nucleotide binding protein
<b>HBSS</b>	HEPES-buffered saline solution

<b>HEK 293</b>	Human embryonic kidney 293
<b>IP3</b>	Inositol (3,4,5)-trisphosphate
<b>kDa</b>	Kilodalton
<b>LSM</b>	Laser-scanning microscope
<b>MAPK</b>	Mitogen-activated protein kinase
<b>MEK</b>	Mitogen-activated protein kinase
<b>MEM</b>	Eagle's minimal essential medium with Earl's salts
<b>mGluR</b>	Metabotropic glutamate receptor
<b>mRNA</b>	Messenger ribonucleic acid
<b>optineurin</b>	Optineurin
<b>PBS</b>	Phosphate buffered saline
<b>PI3K</b>	Phosphatidyl inositol-3-OH kinase
<b>PKA</b>	Protein kinase-A
<b>PKC</b>	Protein kinase-C
<b>PLC</b>	Phospholipase C
<b>PLD</b>	Phospholipase D
<b>RGS</b>	Regulator of G protein signalling
<b>RIPA</b>	Radio-immune precipitation assay
<b>RNA</b>	Ribonucleic acid
<b>Rpm</b>	Rotations per minute
<b>RTK</b>	Receptor tyrosine kinase
<b>SDS</b>	Sodium dodecyl sulfate
<b>TBS</b>	Tris-buffered saline
<b>TGN</b>	<i>Trans</i> -Golgi network
<b>VSMC</b>	Vascular smooth muscle cells
<b>Y2H</b>	Yeast two-hybrid
<b>YFP</b>	Yellow fluorescence protein

**CHAPTER 1**  
**INTRODUCTION**

## **1.1. THE G PROTEIN-COUPLED RECEPTOR SUPERFAMILY**

The seven transmembrane-spanning G Protein-Coupled Receptor (GPCR) superfamily is the largest family of cell-surface receptors that is comprised of more than 650 receptor proteins. Based on structure-function and crystallographic structure of rhodopsin and, more recently the  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR), GPCRs are characterised by extracellular amino-terminal regions, followed by seven transmembrane-spanning domains separated by three extracellular and three intracellular loop domains and finally an intracellular carboxyl-terminal tail (Palczewski et al., 2000; Cherezov et al., 2007; Rasmussen et al., 2011; Tebben and Schnur, 2011). GPCRs are categorized into six classes based on sequence homology, ligand activation and G protein coupling: the largest class A or rhodopsin-like receptors; class B, secretin-like, many of which are regulated by peptide hormones from the glucagon hormone family and class C, the metabotropic glutamate receptors characterized by the large venus fly trap like amino terminus ligand binding pocket; Class D the  $G_{\alpha_i}$ -associated pheromone receptors; Class E the *D. discoideum* - specific cAMP receptors; and finally the Frizzled/Smoothed family, activated by Wnt ligands (Bockaert and Pin, 1999; Foord et al., 2005; Fredriksson et al., 2003; Kolakowski, 1994; Sharman et al., 2011; Bockaert and Pin, 1999).

## **1.2. RECEPTOR SIGNALLING**

### **1.2.1. G Protein-Dependent Signal Transduction Paradigm**

GPCRs couple to and activate cognate heterotrimeric guanine-nucleotide binding (G) proteins, which in turn transduce the GPCR signal through coupling to downstream effector molecules (Neer, 1995). Heterotrimeric G proteins are comprised of  $\alpha$ ,  $\beta$  and  $\gamma$

subunits, which associate with the plasma membrane in a variety of ways, including lipid modification and association with membrane-bound proteins (Casey, 1994). Also called molecular switch proteins, the  $\alpha$  subunit of the G protein cycles between the inactive guanosine 5'-diphosphate (GDP) bound state and becomes activated when GDP is exchanged for guanosine 5'-triphosphate (GTP) (Neer, 1995). In addition to the G protein's endogenous GTP hydrolysis activity, several proteins assist the G protein through this cycle including: 1) guanine nucleotide exchange proteins (GEFs), which facilitate the exchange of GDP for GTP, and 2) GTPase activating proteins (GAPs), which enhance GTP hydrolysis and regulators of G protein signalling (RGS) (Siderovski, 2005). The classical GPCR signalling paradigm begins with ligand activation of the GPCR, which induces a conformational change in the receptor, such that it can associate with its cognate heterotrimeric G protein and act as a GEF by exchanging GDP for GTP on the  $\alpha$  subunit, thereby activating it. Once activated, the heterotrimeric G protein dissociates into the functional  $\alpha$  and the  $\beta\gamma$  subunits to target downstream effector molecules.

There are four classes of  $\alpha$  subunits (Neer, 1995; Gilman, 1987).  $G_{\alpha_s}$  activates membrane-bound adenylyl cyclase (AC) stimulating the formation of the second messenger molecule cyclic adenosine 5' monophosphate (cAMP) and the activation of cAMP-dependent protein kinase-A (PKA). Conversely,  $G_{\alpha_i}$  inhibits AC, decreasing cAMP generation and decreasing PKA activity.  $G_{\alpha_{q/11}}$  proteins activate phospholipase C (PLC), which in turn hydrolyzes the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PtdIns (4,5) $P_2$ , or  $PIP_2$ ), into inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> then activates endoplasmic reticulum localized IP-gated

calcium channels, releasing intracellular calcium stores, which triggers protein kinase C (PKC) translocation to the plasma membrane, where it is co-activated by DAG. Finally,  $G\alpha_{12/13}$  activates Rho and other small G proteins leading to the rearrangement of the actin cytoskeleton (Suzuki et al., 2009). The activation of these second messenger-dependent kinases triggers a phosphorylation cascade of membrane associated, intracellular and nuclear signalling scaffolding proteins and transcription factors leading to immediate and long term functional changes in the cell.

### **1.2.2. G Protein-Independent and Non-Classical Signal Transduction**

In addition to these classical heterotrimeric G protein signalling pathways,  $G\alpha_{q/11}$ -coupled receptor-mediated activation of PKC also triggers the phosphorylation of Raf-1, thus activating mitogen activated kinases (MAPK) and extracellular signal-related kinases (ERK) (Luttrell, 2002). ERK activation by GPCRs is also mediated by adaptor molecules such as  $\beta$ -arrestin, transactivation of receptor tyrosine kinases and ion channels, downstream activation of intracellular tyrosine and serine/threonine kinases such as Src and Pyk2 and  $G\beta\gamma$ -mediated mechanisms (Luttrell, 2002; van Biesen et al., 1995). For example, Pyk2 uncouples metabotropic glutamate receptor G protein signalling, but facilitates ERK1/2 activation (Nicodemo et al., 2010). Meanwhile, endothelin-1-induced ERK activation and is predominantly regulated by EGFR transactivation (Kodama et al., 2003). Activated MAP kinases then regulate the phosphorylation and activation of transcription factors related to cell growth.

### **1.2.3. Agonists and Functional Agonism**

Ligands are characterized via their affinity for cognate receptors, and their efficacy of signalling and functional responses. Therefore, ligands can be classified as full agonists (eliciting maximal possible receptor response), partial agonists (sub-maximal response), neutral antagonists (occupies ligand binding region of receptor, yet elicits no signalling response, positive or negative), or inverse agonists (reduced ligand-independent, basal receptor activity) (Kenakin, 2002). Traditionally, ligand functionality was considered to be constant at a given receptor, eliciting the same response regardless of receptor location, and differences in receptor response were considered to be due to differentially expressed signal transduction machinery. For example, several GPCRs, including the vasopressin and angiotensin II receptors have been shown to couple to multiple G proteins generating divergent signal transduction pathways (Gudermann et al., 1996). Additionally, although  $\beta$ -arrestin was traditionally thought only to participate in receptor desensitization and endocytosis, it is now clear that this adaptor molecule can facilitate signal transduction through multiple pathways including MAPK, Src and PI3K (Rajagopal et al., 2010).

However, emerging evidence suggests that ligands can induce unique, ligand-specific receptor conformations resulting in differential activation of signal transduction pathways. Additionally, ligands with the same intrinsic activity can display different relative potency to divergent signalling pathways activated by the same receptor. This phenomenon, sometimes termed functional selectivity or biased agonism has been reviewed by Kenakin and Miller, 2010, Rajagopal et al., 2010 and Kenakin, 2011. Ligand-induced functional selectivity has been well-characterized for dopamine and

serotonin receptors. Activation of the 5-HT<sub>2C</sub> receptor by agonists such as quipazine preferentially activates PLC while other agonists including LSD activate PLA<sub>2</sub> (Berg et al., 1998).

One way biased agonists may function is by stabilizing a receptor conformation favourable for association with different downstream molecules. For example, the  $\beta$ AR inverse agonist carvedilol triggers  $G_{\alpha s}$ -independent,  $\beta$ -arrestin-dependent EGFR activation leading to downstream ERK phosphorylation while  $\beta_2$ AR activation by cyclopentylbutanephine biases the receptor toward  $\beta$ -arrestin-dependent MAPK activation relative to isoproterenol (Azzi et al., 2003; Noma et al., 2007; Drake et al., 2008).

Multiple amino acid residues in various GPCRs have been shown to play a role in functional selectivity. For example,  $\mu$ -opioid receptor stimulation by different agonists results in site-specific patterns of serine (ser) and threonine (thr) phosphorylation in the second intracellular loop of the receptor, resulting in differential receptor endocytosis patterns and targeting the receptor toward either PKC $\epsilon$  or ERK signal transduction pathways (Doll et al., 2011; Zheng et al., 2011). Mutation of histidine 393 in the sixth transmembrane domain of the dopamine D<sub>2L</sub> receptor abolishes the functional selectivity exhibited by the wild type receptor whereas ligand modification at the site of histidine 393 interaction biases the signalling towards ERK1/2 phosphorylation (Tschammer et al., 2011).

#### **1.2.4. Receptor Desensitization**

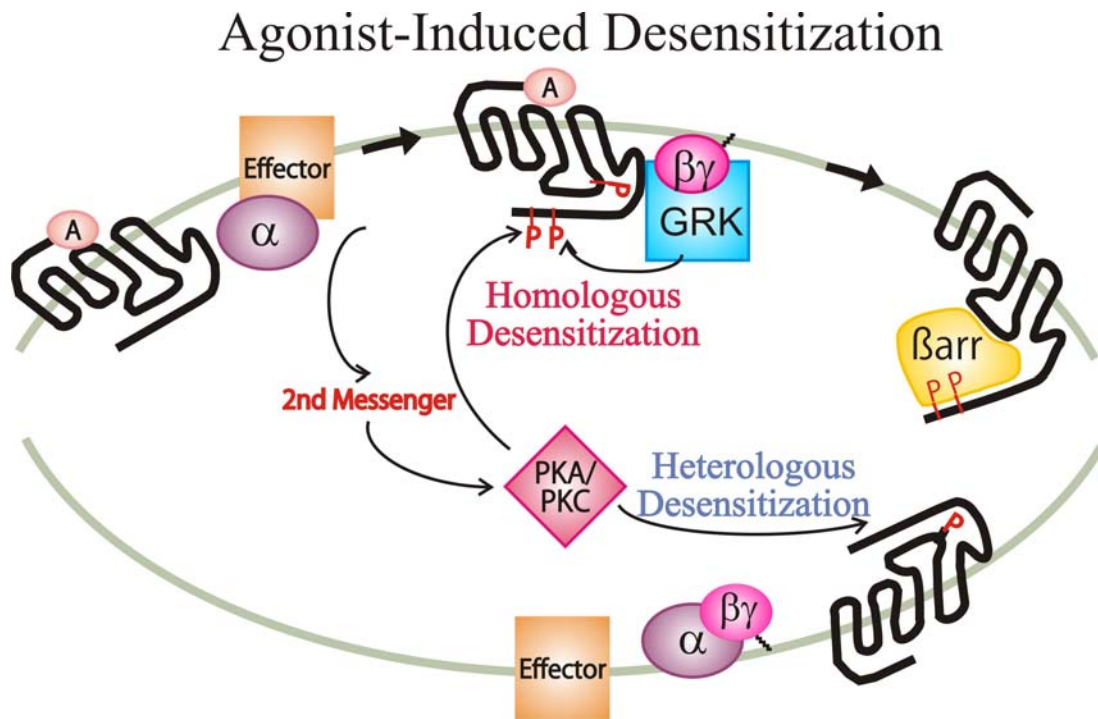
Ligand activation of GPCRs also results in a cascade of events ultimately leading to decreased receptor signalling. This process, termed desensitization, is crucial to



prevent aberrant or chronic receptor over-stimulation. There are multiple mechanisms of receptor desensitization ranging from immediate G protein uncoupling to degradation of the receptors and transcriptional down-regulation (Ferguson, 2001).

#### **1.2.4.1. Receptor Phosphorylation**

Phosphorylation is the most rapid desensitization response to receptor activation, occurring within seconds to minutes after receptor activation. GPCR phosphorylation occurs mainly at Ser and Thr residues within the carboxyl-terminal tail and third intracellular loop (Ferguson and Caron, 1998; Kohout and Lefkowitz, 2003; Krupnick and Benovic, 1998; Pierce et al., 2002). The addition of large, negatively charged phosphates to the intracellular loops and C-tail of receptors desensitizes receptor signalling by interfering with G protein-coupling, as well as facilitating recruitment of adaptor proteins for internalization. Receptor phosphorylation can be carried out both homologously and heterologously by second messenger-dependent protein kinases (PKA and PKC) or homologously by G protein-coupled receptor kinases (GRKs) (Figure 1.1.). Phosphorylation by either type of kinase can lead to decreased receptor signalling. However, GRK phosphorylation results in desensitization via recruitment of the adaptor molecules called  $\beta$ -arrestins (Benovic et al., 1987; Lohse et al., 1990b; Pippig et al., 1993).  $\beta$ -arrestins associate exclusively with ligand-activated and GRK-phosphorylated GPCRs and functions in two ways to desensitize the receptor: first,  $\beta$ -arrestin physically uncouples the receptor from the G protein and secondly they recruit the endocytic machinery required to facilitate the endocytosis of the receptor.



**Figure 1.1. Schematic diagram illustrating the agonist-induced desensitization of GPCRs via phosphorylation.** Agonist binding triggers second messenger generation, leading to activation of second messenger-dependent kinases (PKA and PKC). Receptor activation also allows for the recruitment of G protein-coupled receptor kinases (GRK), which associate with and phosphorylate exclusively agonist-activated receptors (homologous desensitization). In addition to homologously phosphorylating agonist-activated receptors, second messenger-dependent protein kinases can also phosphorylate non-activated receptors (heterologous desensitization).

#### **1.2.2.4. Second-Messenger-Dependent Protein Kinase**

GPCR-stimulated second messenger generation of molecules such as cAMP and  $ca^{2+}$  leads to the activation of second messenger dependent kinases, which in turn phosphorylate downstream target proteins. Second messenger dependent kinases PKC and PKA function to transfer the high energy  $\gamma$  phosphate from adenosine 5' triphosphate (ATP) to target serine and threonine residues contained within specific consensus sites of proteins (Taylor et al., 1988). Protein phosphorylation cascades generate short and long term changes in cell signalling and function, however, these kinases can also feedback phosphorylate GPCRs within their intracellular loops and carboxyl terminal tails (Smith, 1998; Eason et al., 1995; Bouvier et al., 1988). However, so long as the necessary consensus sequence is present on the target GPCR, second messenger dependent kinases do not discriminate upon receptor activation state or, indeed receptor type. This process is termed heterologous desensitization (Hausdorff et al., 1989; Lohse et al., 1990a).

#### **1.2.4.2. G Protein-Coupled Receptor Kinases**

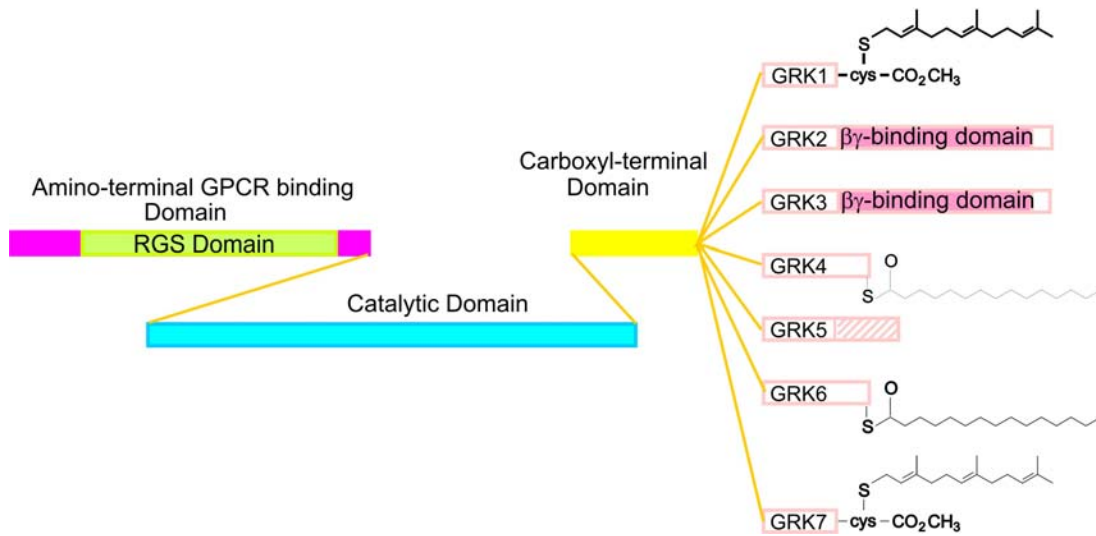
The GRK family of protein kinases contain a central catalytic domain flanked by a carboxyl terminal membrane targeting domain and an amino terminal RGS-like domain hypothesised to be responsible for substrate recognition (Pitcher et al., 1998; Stoffel et al., 1997). The seven GRK family members are sub-classified into three groups based on sequence homology and functional similarity (Figure 1.2). The groups are: 1) visual GRKs, GRK1 (rhodopsin kinase) and GRK7, (Shichi and Somers, 1978; Weiss et al., 1998), 2) GRK2 ( $\beta$  adrenergic receptor kinase 1,  $\beta$ ARK1) and GRK3 ( $\beta$  adrenergic receptor kinase 2,  $\beta$ ARK2) (Benovic et al., 1986; Benovic et al., 1991) and 3) GRK4, GRK5 and GRK6 (Benovic and Gomez, 1993; Kunapuli and Benovic, 1993; Premont et

al., 1994; Salles et al., 1994). The plasma membrane targeting of the different GRK groups is mediated by mechanisms involving their carboxyl-terminal domains. GRK1 and GRK7 are farnesylated at CAAX motifs in their carboxyl termini while GRK2 and GRK3 contain a carboxyl-terminal  $\beta\gamma$ -subunit binding pleckstrin homology domain (Pitcher et al., 1998). The GRK5 carboxyl-terminal domain contains a stretch of 46 basic amino acids that allow plasma membrane phospholipid interactions and finally, GRK4 and GRK6 are palmitoylated at cysteine residues (Premont et al., 1996; Shaw, 1996; Stoffel et al., 1994; Stoffel et al., 1998).

#### **1.2.4.3. Arrestin**

GRK phosphorylation is usually not sufficient for GPCR desensitization and requires the association of arrestin molecules to agonist-activated, GRK-phosphorylated GPCRs. There are four arrestin family members categorized into two groups; 1) visual and cone arrestin are expressed exclusively in the retina while 2)  $\beta$ -arrestin-1 (arrestin 2) and  $\beta$ -arrestin-2 (arrestin 3) are ubiquitously expressed, though they show enhanced localization in neuronal tissues (Ferguson, 2001; Krupnick and Benovic, 1998; Lefkowitz, 1993). Arrestins associate preferentially with agonist-activated, GRK-phosphorylated GPCRs, as opposed to unphosphorylated or second messenger-dependent kinase phosphorylated receptors (Lohse et al., 1990b; Lohse et al., 1992).  $\beta$ -arrestin is an important player in clathrin-mediated GPCR internalization. Once  $\beta$ -arrestin associates with agonist-activated and GRK-phosphorylated GPCRs, it recruits AP2 and clathrin, initiating receptor endocytosis (Ferguson et al., 1996; Goodman et al., 1996; Laporte et al 1999). The role of  $\beta$ -arrestin in GPCR desensitization and endocytosis is discussed in detail in section 1.3.2.1.

# GRK Homology



**Figure 1.2. Schematic representation of the GRK family structure.** The amino-terminus contains the conserved regulator of G protein signalling (RGS) homology domain (RH) while the divergent carboxyl-terminal domains participate in GRK plasma membrane targeting. GRK1 and 7 are farnesylated within the CAAX motifs, GRK4 and 6 are palmitoylated and GRK5 contains a 46 basic amino acid stretch that allows association with membrane phospholipids. Finally, GRK2 and 3 contain G $\beta\gamma$  binding motifs similar to pleckstrin homology domains. Figure adapted from Ferguson, 2001.

#### **1.2.4.4. Phosphorylation-Independent Desensitization**

Phosphorylation of intracellular residues of agonist activated receptors is the most widely studied and best understood form of receptor desensitization. However, it is now appreciated that phosphorylation-independent mechanisms of receptor desensitization also exist. Phosphorylation-independent desensitization has been shown for the endothelin A and B receptors, as well as the follicle-stimulating hormone receptor, histamine H<sub>2</sub> receptor, m<sub>3</sub> muscarinic acetylcholine and metabotropic glutamate receptor 1a (mGluR1a) (Dhami et al., 2002; Ferguson, 2007; Fernandez et al., 2011; Reiter et al., 2001; Shibasaki et al., 1999; Willets et al., 2004).

GRK catalytically inactive mutants are sufficient for desensitization of 5HT<sub>1B</sub> and parathyroid hormone receptors. While GRK2 does phosphorylate agonist activated mGluR1a, physical association between GRK2, Gαq/11 and mGluR1a is sufficient for this receptor's desensitization in a phosphorylation independent manner, partially through the GRK2 RGS homology (RH) domain (Dhami et al., 2002; Flannery and Spurney, 2001; Lembo et al., 1999). Other proteins have been implicated in phosphorylation-independent GPCR desensitization. For example, the huntingtin-binding and Rab8 effector molecule optineurin has been shown to associate with group I mGluRs to physically uncouple the receptor from G protein (Anborgh et al., 2005).

#### **1.2.4.5. GPCR Down-Regulation**

In addition to the immediate phosphorylation by GRKs and second messenger-dependent kinases and clathrin-mediated internalization of activated receptors, other down-regulation mechanisms are employed to decrease the total cellular complement of receptors in response to prolonged or repeated agonist stimulation. Receptor down-

regulation is achieved via proteosomal and lysosomal GPCR degradation, modulation of receptor gene transcription, RNA stability and translation (Tsao and von Zastrow, 2000; Bouvier et al., 1989).

#### **1.2.4.6. GPCR Proteolytic Degradation**

Two specific compartments contribute to the proteolytic degradation of GPCRs, the proteasome and lysosome (Hislop et al., 2011; Clague and Urbe, 2010). Proper cell signalling requires selective trafficking of membrane proteins to lysosomes for degradation. A major mechanism of lysosomal targeting involves protein ubiquitination on lysine residues and sorting via the endosomal sorting complex required for transport (ESCRT) machinery (Katzmann et al., 2001). Proteosomal degradation of cytosolic proteins is signalled by polyubiquitination whereas multiple monoubiquitination or polyubiquitination signals membrane proteins for lysosomal sorting and degradation. Ubiquitinated proteins are not recycled back to the plasma membrane, but instead are sorted into intraluminal vesicles in endosomes, forming multivesicular bodies. When these MVBs fuse with lysosomes, lipases degrade the ILV membrane as well as the transmembrane proteins (Marchese et al., 2008; Katzmann et al., 2002). Mu-opioid (MOR) and  $\beta_2$ AR both undergo agonist-dependent ubiquitination and receptor degradation and  $\beta_2$ AR mutation of Lys residues internalizes normally, but does not downregulate in response to prolonged agonist exposure (Hislop et al., 2011; Xiao and Shenoy, 2011). However, several GPCRs including delta-opioid (DOR) have been shown to target to lysosomes independent of ubiquitination (Tanowitz and Von Zastrow, 2002).

#### **1.2.4.7. Transcriptional Downregulation**

Total cell receptor loss involves not only degradation of existing receptor proteins, but also alterations in receptor transcription and translation. Interestingly, GPCR activation of second messenger dependent signalling may function as positive or negative feedback regulators of GPCR gene and protein expression. For example, cAMP activation induces PKA-mediated  $\beta_2$ AR phosphorylation and also decreases the level of  $\beta_2$ AR mRNA, ultimately decreasing receptor population while serotonin treatment of C6 glioma cells leads to a PKC-dependent decrease in 5HT2A receptor mRNA levels (Anji et al., 2001; Bouvier et al., 1989). Other GPCRs, including AT<sub>1</sub>R, dopamine D1 and thyroid-stimulating hormone receptors also show agonist-induced decreases in receptor mRNA (Collins et al., 1992).

### **1.3. GPCR TRAFFICKING**

Intracellular trafficking is a crucial mechanism to ensure proper targeting and function of receptor signalling and deregulated trafficking has been shown to play a role in multiple disorders and pathologies. This section will highlight the major steps in receptor intracellular trafficking as it relates to function and signalling.

#### **1.3.1. ER/Golgi to Plasma membrane**

As transmembrane proteins, GPCRs are synthesized and processed in the endoplasmic reticulum (ER). Folding and post-translational modifications such as glycosylation and ubiquitination, oligomerization take place in this compartment as well as association with accessory proteins (Braakman and Bulleid, 2011). Accumulating evidence suggests that receptor dimerization is necessary for correct plasma membrane targeting for some GPCRs and there are reports of dimerization mutations in hormone-

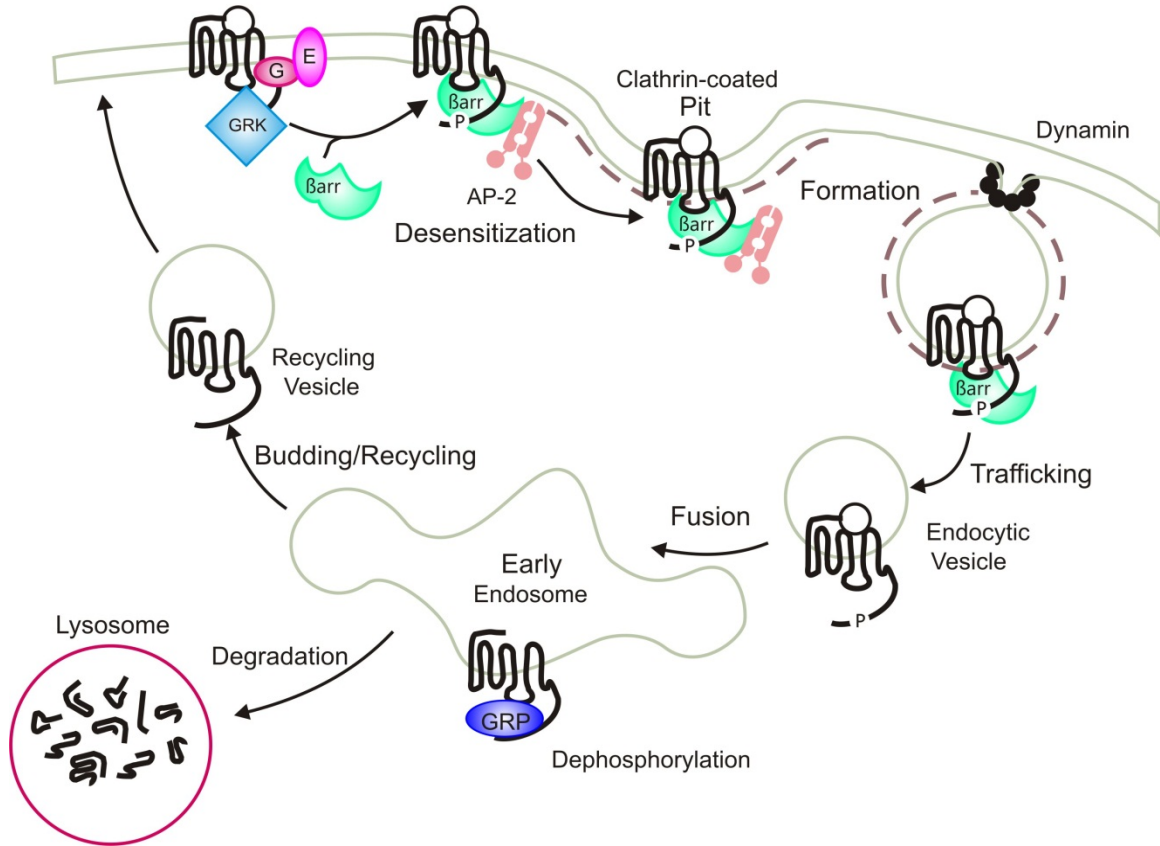


activated GPCRs that result in ER retention. One example of this is heterodimerization of GABA<sub>B</sub> receptor subunits, which masks an ER retention sequence in the C-terminus of GABA<sub>B</sub> and allows the dimer to reach the plasma membrane (Margeta-Mitrovic et al., 2000). It is becoming clear that proper receptor targeting and function is dependent on dimer formation. For example, the GABA<sub>B</sub> receptor requires both GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits in order to reach the plasma membrane as well as activate G proteins (Duthey et al., 2002; Pin et al., 2004). Dimerization is also essential for receptor-G protein coupling. GABA<sub>B1</sub> requires co-expression with GABA<sub>B2</sub> in order to couple functionally to the G-protein signalling cascade (Galvez et al., 2001; Margeta-Mitrovic et al., 2000). This is not just due to plasma membrane localization as a mutant form of GABA<sub>B1</sub> that lacks its ER retention signal and can reach the cell surface on its own still requires GABA<sub>B2</sub> for functional activity (Margeta-Mitrovic et al., 2000). Additionally, heterodimerization between the  $\alpha_{1D}$ - and  $\alpha_{1B}$ -adrenoceptors, was shown to be necessary for the proper cell-surface expression of the  $\alpha_{1D}$ AR subtype (Hague et al., 2006).

### **1.3.2. Receptor Internalization**

The internalization and intracellular trafficking of GPCRs is a highly regulated process that, in addition to contributing to GPCR desensitization, is also required for receptor dephosphorylation and resensitization (Ferguson, 2001). GPCRs may internalize in either a constitutive manner or in response to agonist activation: once the receptor is desensitized at the cell surface, it must then be internalized into the cell where it is sequestered or targeted for resensitization or degradation (Figure 1.3) (Ferguson, 2001). There are multiple means of receptor internalization employed by the cell including lipid rafts, caveolin, pinocytosis and the most common, clathrin-mediated endocytosis.

# GPCR Internalization and Trafficking



**Figure 1.3. Clathrin-mediated endocytosis and intracellular trafficking of agonist-activated receptors.** Ligand-activated receptors are phosphorylated on intracellular residues by G protein-coupled receptor kinases (GRKs) and bound by the adaptor protein, β-arrestin (βarr). β-arrestin then recruits the clathrin adaptor protein, AP2 as well as clathrin, which assembles to form clathrin-coated pits. The GTPase dynamin severs the pit from the membrane, forming a clathrin coated vesicle. Once internalized, clathrin uncoats from the vesicle, during which arrestin may or may not also dissociate from the receptor. The vesicle is then trafficked to early endosomes, where the acidic pH facilitates agonist dissociation from the receptor and phosphates are removed by G protein-coupled receptor phosphatases (GRP). Internalized receptors may be sequestered in early endosomes, trafficked to the lysosome and degraded or recycled back to the plasma membrane as a fully functional receptor.

### **1.3.2.1. Clathrin-mediated Endocytosis**

Clathrin is a triskelion membrane coat protein that when assembled, forms a polyhedron lattice, which surrounds membrane and encourages invagination and clathrin-coated pit formation (Hinrichsen et al., 2006; Kirchhausen and Harrison, 1981). Clathrin coated vesicles transport molecules from the plasma membrane, endosomes and trans-Golgi network. Several adaptor molecules, including adaptor protein-2 (AP-2), epsin and  $\beta$ -arrestin recruit clathrin and other accessory proteins to the site of invagination and facilitate clathrin assembly (Wolfe and Trejo, 2007).  $\beta$ -arrestin is an important player in clathrin-mediated GPCR internalization. Once  $\beta$ -arrestin associates with agonist-activated and GRK-phosphorylated GPCRs, it recruits AP2 and clathrin, initiating receptor endocytosis (Ferguson et al., 1996; Goodman et al., 1996; Laporte et al 1999). Once the clathrin coated pit is formed, the GTPase dynamin severs the pit from the membrane, thus forming a clathrin-coated vesicle (Ungewickell and Hinrichsen, 2007; Praefcke and McMahon, 2004).

### **1.3.3. Early Endosomal Sorting and Trafficking**

Once the coated vesicle is internalized, clathrin disassembles and uncoats the vesicle (McMahon and Boucrot, 2011). Internalized receptor is trafficked to the early endosome where it can be sorted for retention, recycling or degradation. A key determinant of receptor fate occurs in the process of uncoating, when  $\beta$ -arrestin may or may not also dissociate from the internalized receptor (Oakley et al., 2000). Class A receptors, such as the  $\beta_2$  adrenergic receptor and  $\alpha_{1b}$  adrenergic receptor contain few phosphorylation sites and therefore form a transient association with  $\beta$ -arrestin 1 and 2. However, class B receptors, including AT<sub>1</sub> and V2 vasopressin receptors contain multiple

phosphorylation sites and form high affinity, prolonged association with arrestins (Anborgh et al., 2000; Oakley et al., 2000; Zhang et al., 1996). The presence of clusters of phosphorylated Ser and Thr residues in some GPCRs may stabilize the interaction with arrestins (Oakley et al., 2000).  $\beta$ -arrestin dissociation is assumed to allow access for protein phosphatases to cleave phosphates from the receptor, which in turn allows the receptor to recycle and resensitize at the cell surface, but there is no direct experimental evidence to support this assumption (Lefkowitz, 1998). However, if  $\beta$ -arrestins cannot dissociate, phosphatases are unlikely to access the phosphorylated receptor and dephosphorylate and resensitize the receptor (Ferguson and Caron, 1998; Ferguson et al., 1998; Ferguson, 2001; Ferguson, 2007).

#### **1.3.3.1. Recycling Endosomes**

Recycling and resensitization of internalized receptors to the plasma membrane represents a much more efficient way to re-initialize signal transduction than novel receptor synthesis. GPCR sorting into the recycling pathway can either occur via a default trafficking mechanism or through a regulated process. Studies of nutrient receptors supported the idea that receptor recycling occurs via a default “bulk” membrane flow from endosomes to the plasma membrane (Gruenberg, 2001; Gruenberg and Stenmark, 2004; Maxfield and McGraw, 2004; Mayor et al., 1993). However, this model does not allow for the dependency of GPCR recycling on specific protein interactions (Anborgh et al., 2000; Cao et al., 1999; Dale et al., 2004; Seachrist et al., 2000). Many GPCRs, including  $\beta_2$ AR,  $\mu$ -opioid and endothelin receptors contain specific plasma membrane sorting sequences, necessary for endocytic delivery back to the cell surface (Cao et al., 1999; Paasche et al., 2005; Tanowitz and von Zastrow, 2003). Many GPCR

recycling sequences, including those found on the  $\beta_1$ AR and  $\beta_2$ AR encode motifs recognized by a variety of postsynaptic density 95/disc large/zonula occludens-1 (PDZ)-containing proteins. Other protein trafficking molecules, such as Rab small G proteins (discussed in detail below) also differentially regulate receptor recycling.

#### **1.3.3.2. Lysosomes**

Lysosomes are acidic intracellular membrane-bound organelles distinguished from endosomes by their lack of mannose-6-phosphate receptor (Luzio et al., 2007). Lysosomes contain lysosomal-associated proteins, lysosomal integral membrane proteins I and II, lysosomal acid phosphatase and acid hydrolases, which function optimally at acidic pH. As mentioned above, a major mechanism for endosome-lysosome trafficking involves the ESCRT machinery targeting ubiquitinated GPCRs to the lysosome. For example, lysines in the third intracellular loop (Lys-263 and Lys-270) and in the carboxyl tail (Lys-348, Lys-372, and Lys-375) of the  $\beta_2$ AR are involved in ubiquitination and lysosomal degradation (Xiao and Shenoy, 2011). There are, however, multiple other mechanisms of lysosomal targeting, including GPCR-associated sorting proteins (GASP) and sorting nexin-1 (Gaborik and Hunyady, 2004; Tanowitz and von Zastrow, 2002). Additionally, lysosomal targeting may involve Rab5 and/or Rab7 as discussed in detail in later sections (Seachrist and Ferguson, 2003).

#### **1.3.4. Role of the Receptor Carboxyl-Terminal Tail in Trafficking**

While it is well known that the C-tails of GPCRs mediate receptor desensitization through phosphorylation of serine and threonine residues and the association with  $\beta$ -arrestin, there is also much evidence to support the importance of GPCR C-tails in the coordination of receptor intracellular trafficking. Chimeric receptor constructs

demonstrate specific residues that determine the fate of different receptors. For example, chimeric receptors in which the C-tails of  $\beta_2$ AR and AT<sub>1</sub>R were exchanged demonstrated that the C-tail of  $\beta_2$ AR does not form a stable association with  $\beta$ -arrestin, which may account for this receptor's rapid and efficient recycling (Anborgh et al., 2000). Meanwhile, although wild type AT<sub>1</sub>R is neither dephosphorylated nor recycled, a chimeric AT<sub>1</sub>R containing the  $\beta_2$ AR C-tail does dissociate from  $\beta$ -arrestin and promotes partial receptor recovery.

Many proteins involved in protein scaffolding and/or transport associate with GPCR C-tails. Molecules such as PDZ-containing proteins associate with GPCRs via protein modular domains commonly found in the carboxyl-terminal tail to mediate protein targeting (Cao et al., 1999; Magalhaes et al., 2011; Romero et al., 2011; Xia et al., 2003). The Homer family of proteins contain Enabled/VASP Homology-1 (EVH-1) domains that associate with a proline-rich region in the C-tails of mGluR1/5 and  $\alpha_{1D}$ AR (Roche et al., 1999). Homer proteins regulate the targeting of mGluRs to different subcellular compartments, mediate their insertion into the plasma membrane and facilitate receptor activation (Ango et al., 2002; Ciruela et al., 1999; Coutinho et al., 2001; Roche et al., 1999; Tadokoro et al., 1999).

Other protein trafficking molecules have also been shown to associate with the C-tail of GPCRs to mediate receptor localization. For example, Rab5a has been shown to interact with the AT<sub>1A</sub>R carboxyl-terminal tail and retain the receptor in Rab5a-positive early endosomes (Seachrist et al., 2002; Dale et al., 2004). Rab11 binding to the thromboxane A<sub>2</sub> receptor is mediated by residues 335-345 that are localized within the central region of the thromboxane A<sub>2</sub> receptor carboxyl-terminal tail and Rab11 binding

to the  $\beta_2$ AR involves a bipartite binding motif, with arginine 333 and lysine 348 representing the essential amino acid residues mediating Rab11 binding to the receptor (Hamelin et al., 2005; Parent et al., 2009).

#### **1.4. EFFECTS OF INTRACELLULAR TRAFFICKING ON GPCR SIGNALLING**

Agonist-activation of signal transduction cascades through GPCRs comprises both G protein-dependent and -independent signalling resulting in parallel signalling cascades and complex signalling networks. Because cells express hundreds of different receptors, a mechanism to organize signal cascades must be put in place. One major mechanism of spatiotemporal signal organization includes protein intracellular trafficking (Jean-Alphonse and Hanyaloglu, 2011). In addition to rapid and effective signal desensitization, the endocytosis and intracellular trafficking of GPCRs can spatially and temporally determine G protein-dependent and independent signalling pathways. Receptors such as the  $\beta_2$ AR, which recycle quickly and efficiently also resensitize to persistent or repeated agonist activation whereas receptors such as  $AT_1R$ , which are retained in early endosomes remain desensitized much longer (Anborgh et al., 2000; Oakley et al., 2000). However, chronic GPCR stimulation may cause altered GPCR trafficking away from the recycling to the degradative pathway leading to receptor downregulation (Hislop et al., 2011; Xiao and Shenoy, 2011). Conversely, GPCR recycling has been shown to actually change the signalling of the receptor. For example,  $\beta_2$ AR endocytosis and recycling switches the receptor's traditional coupling with  $G\alpha_s$  to  $G\alpha_i$  (Wang et al., 2007).

Membrane trafficking of GPCRs provides novel compartments for G protein signalling. GPCR signalling via heterotrimeric G proteins is traditionally associated with the plasma membrane. However, recent studies indicate that GPCRs may also associate with and signal through their cognate G proteins at intracellular sites. Membrane permeable agonists also suggest that GPCRs such as the V2 vasopressin receptor and estrogen receptor GPR30 signal from the ER/Golgi (Revankar et al., 2005; Robben et al., 2009). Meanwhile, the  $\beta_2$ AR has been shown to be pre-associated with its heterotrimeric G proteins as well as adenylyl cyclase II in the ER (Dupre et al., 2006; Dupre et al., 2007).

Few GPCRs have been shown to co-localize with their G proteins at the endosomes and display persistent, internalization-dependent G protein signalling. In *S. cerevisiae*, the  $G\alpha$  protein translocates to the endosome where it stimulates PI3K, while in mammalian cells, lysophosphatidic acid treatment causes  $G\beta\gamma$  to associate with Rab11a, PI3K and PKT at the endosome (Garcia-Regalado et al., 2008; Slessareva and Dohlman, 2006; Slessareva et al., 2006). FRET-based measurement of the cAMP sensitive EPAC molecule showed that parathyroid hormone receptor (PTHr) and thyroid-stimulating hormone receptor (TSHR) internalization is required for sustained  $G\alpha_s$  signalling from the endosomal compartment (Calebiro et al., 2010a; Calebiro et al., 2010b; Calebiro et al., 2009; Jalnik and Moolenaar, 2010).

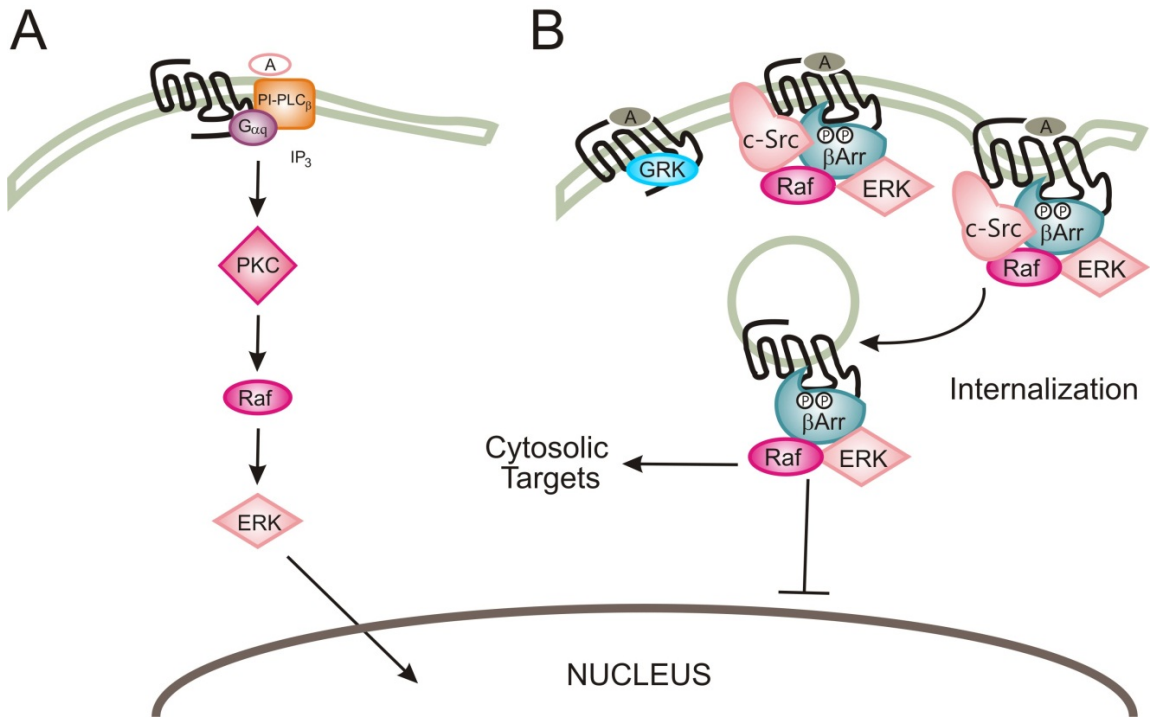
It is also well established that GPCRs can mediate non-G protein signalling pathways from endosomes, mainly via GPCR/ $\beta$ -arrestin complexes, that scaffolds association with MAPK molecules and tyrosine kinases (DeWire et al., 2007). This was first reported in studies inhibiting receptor internalization when overexpression of either



dominant negative  $\beta$ -arrestin or dynamin prohibited  $\beta_2$ AR-induced ERK activation (Daaka et al., 1998; Lin et al., 1998; Luttrell et al., 1997). It was later shown that  $\beta$ -arrestin couples  $\beta_2$ AR to Src in order to mediate ERK1/2 phosphorylation (Luttrell et al., 1999). Similarly, phosphorylation of protease-activated receptor-2 (PAR-2) is not required for  $\beta$ -arrestin recruitment and internalization, but a phosphorylation-deficient PAR-2 mutant is unable to facilitate  $\beta$ -arrestin-dependent MAPK activation (DeFea et al., 2000). Meanwhile, PAR-2 can increase PI3K activity through a  $G\alpha_q$ /calcium-dependent pathway involving PYK2 and Src, while inhibiting PI3K activity through a  $\beta$ -arrestin-dependent mechanism (Wang and DeFea, 2006). It has been proposed that  $\beta$ -arrestin facilitates ERK activation by recruiting and scaffolding members of the MAPK pathways to internalizing vesicles or endosomes (DeWire et al., 2007; Luttrell et al., 2001; DeFea et al., 2000). Moreover, anchoring activated ERK to endosomes may prevent ERK translocation to the nucleus, thus encouraging cytoplasmic ERK signalling (Figure 1.4). This suggests an important spatial role, as well as temporal control, for GPCR membrane trafficking.

Less well understood are the direct and indirect roles of GPCR signal transduction on regulation of trafficking machinery, though it has been proposed that receptors participate in modulation of their own intracellular trafficking (Seachrist et al., 2002; Yudowski et al., 2009).  $AT_{1A}R$  activation causes GTP binding of Rab5a, while PKA activation by  $\beta_2$ AR regulates Rab4, but not Rab11 recycling pathways and  $\beta_2$ AR also modulates the Rab8 geranyl-geranylation, altering the ability of Rab8 to associate

## Receptor-mediated ERK Signalling



**Figure 1.4. GPCR-mediated activation of ERK1/2 signalling.** **A)** Agonist-activation of receptors leads to downstream activation of protein kinase-C (PKC), which can activate Raf, initiating the MAP kinase cascade. Phosphorylated and activated ERK then translocates to the nucleus where it regulates gene transcription through phosphorylation of transcription factors. **B)**  $\beta$ -Arrestin associates with agonist-activated, GRK-phosphorylated receptors and acts as a scaffold, recruiting tyrosine kinases such as Src, as well as components of the MAP kinase cascade.  $\beta$ -arrestin-mediated scaffolding retains activated ERK in the cytosol where it phosphorylates target proteins.

with membranes, and thus its activity. Additionally, p38 MAPK activation stimulates the formation of Rab5-guanine dissociation inhibitor (GDI) complexes, thereby increasing endocytosis (Lachance et al., 2011).

## **1.5. ANGIOTENSIN II TYPE 1 RECEPTOR**

The Angiotensin II receptor family includes angiotensin II type 1 (AT<sub>1</sub>R), the focus of this thesis, and type 2 (AT<sub>2</sub>R) receptors. The AT<sub>1</sub>R mediates the cardiovascular effects of the AngII peptide hormone including vasoconstriction, angiogenesis, atherosclerosis, glomerulosclerosis and cardiac cell growth and hypertrophy. AT<sub>2</sub> receptors, on the other hand are expressed in the fetus, injured tissue, adult brain and affect vascular tone and growth oppositely to AT<sub>1</sub> receptors (de Gasparo et al., 2000).

### **1.5.1. AT<sub>1</sub>R Signalling**

The AT<sub>1</sub>R is coupled through G $\alpha_{q/11}$  to the activation of phospholipase C $\beta$  resulting in the formation of diacylglycerol and inositol 1,4,5 trisphosphate leading to the release of intracellular calcium stores and the activation of PKC (de Gasparo et al., 2000). AT<sub>1</sub>R also couples to G $\alpha_{o/i}$  in some tissues leading to inactivation of adenylyl cyclase and decreased production of cyclic AMP and can also couple to the pertussis insensitive G $\alpha_{12/13}$ , which mediate AngII-induced L-channel activation (de Gasparo et al., 2000). AT<sub>1</sub>R can also mediate G protein-independent signalling mechanisms, such as mitogen activated protein kinases (MAPK), JAK/STAT kinases, tyrosine kinase activation (Pyk2, Src) and transactivation of growth factor receptors. Multiple amino acid residues involved in G protein-coupling and phosphorylation have been isolated (Figure 1.5).



### **1.5.2. AT<sub>1</sub>R Desensitization, Endocytosis and Intracellular Trafficking**

Agonist-activation of the AT<sub>1</sub>R also results in the attenuation of receptor signalling as the consequence of receptor phosphorylation by G protein-coupled receptor kinases (GRKs) and PKC. Agonist activation and GRK-mediated phosphorylation of the AT<sub>1</sub>R facilitates the recruitment of the cytosolic adaptor protein,  $\beta$ -arrestin, which functions to sterically uncouple the AT<sub>1</sub>R from the heterotrimeric G protein and targets the AT<sub>1</sub>R for clathrin-mediated endocytosis (Freedman et al., 1995; Ferguson et al., 1995; Ferguson et al., 1996; Opperman et al., 1996; Krupnick and Benovic, 1998; Ferguson, 2001; Ferguson, 2007). Once internalized, GPCRs may be either sequestered in early endosomes, dephosphorylated and recycled back to the plasma membrane or targeted to lysosomes for degradation (Ferguson, 2001; Gáborik and Hunyady, 2004; Seachrist and Ferguson, 2003). In the case of the AT<sub>1</sub>R, the receptor is internalized as a complex with  $\beta$ -arrestin and is retained in the early endosomal compartment and is not readily dephosphorylated and recycled (Anborgh et al., 2000).

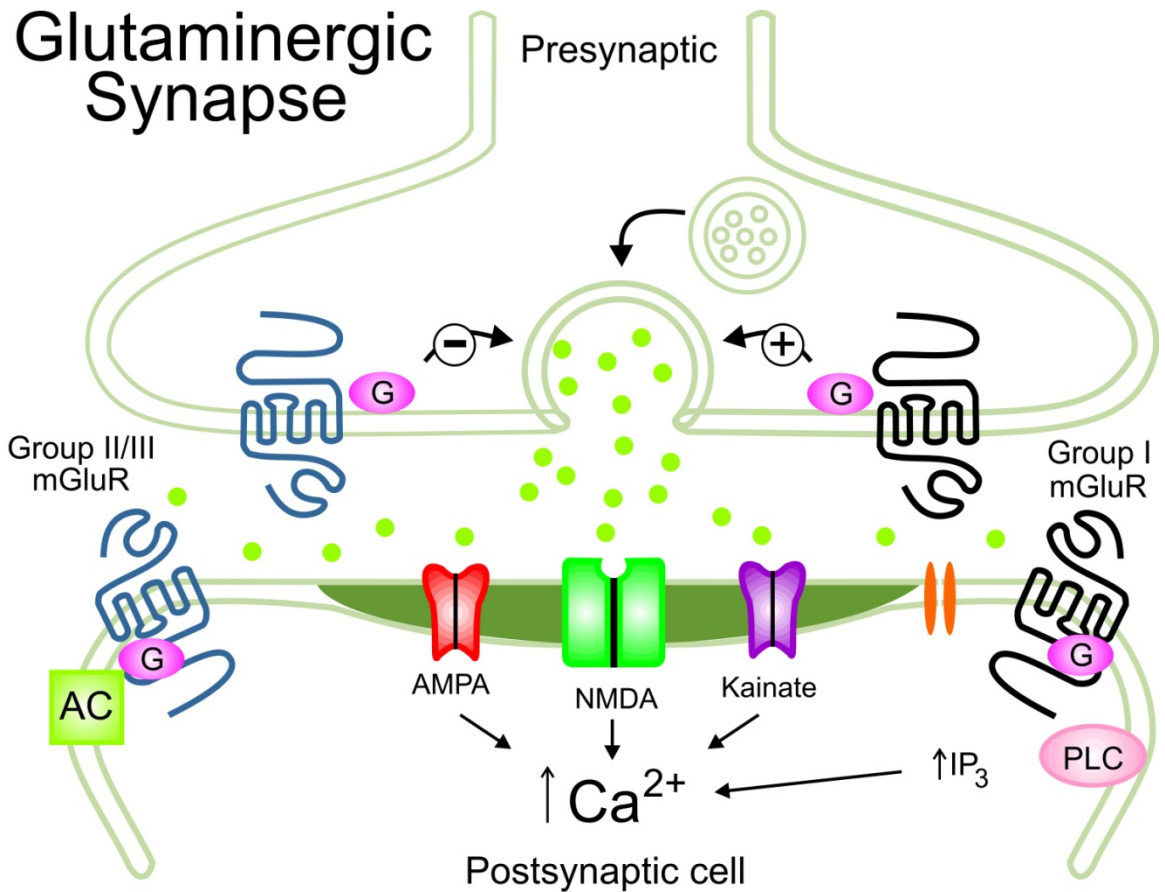
## **1.6. THE METABOTROPIC GLUTAMATE RECEPTOR FAMILY**

Glutamate is a major excitatory neurotransmitter whose actions are mediated through two types of receptors: ionotropic and metabotropic glutamate receptors (Figure 1.6). Ionotropic glutamate receptors are ligand-gated cation channels subdivided into N-methyl-*D*-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and Kainite receptors based in agonist preference. Metabotropic glutamate receptors (mGluR) are G protein-coupled receptors, which play an important role in processes of synaptic plasticity, such as learning and memory, neuronal development, and neurodegeneration. mGluRs are categorized into three classes based on sequence

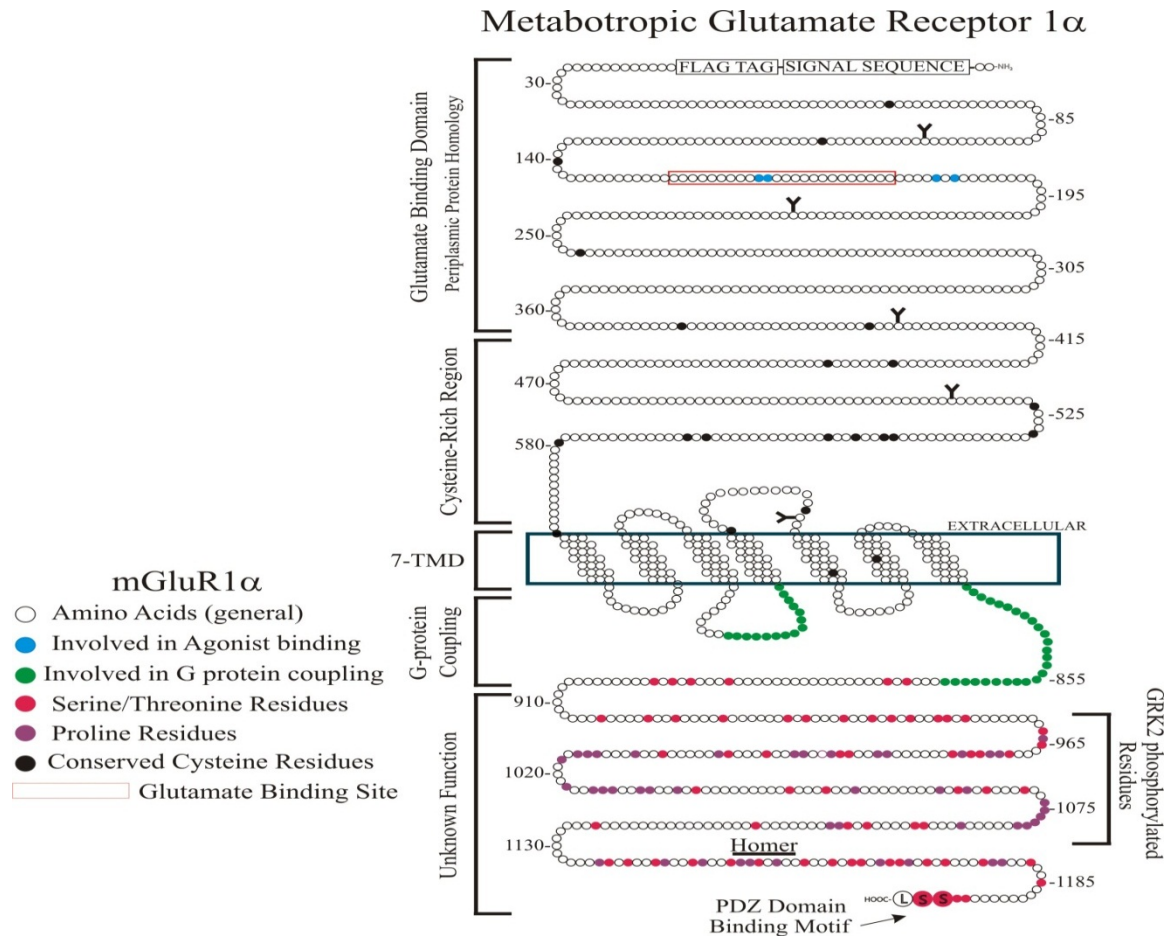
homology and G protein coupling (Niswinder and Conn, 2010). Group I mGluRs, including mGluR1a, the focus of this thesis, and mGluR5 are primarily located perisynaptically on the postsynaptic cell (Niswinder and Conn, 2010). The prototypic mGluR1 has five splice variants (mGluR1a-e), which differ primarily in the length of the carboxyl terminus (Conn and Pin, 1997). There are no alterations in G protein-coupling between these variants as this is mediated by residues within the second and third intracellular loops as well as the membrane proximal region of the c-tail (Figure 1.7). Group II and III receptors are localized to both presynaptic and postsynaptic terminals and are negatively coupled to adenylyl cyclase and inhibit action of L-, N- and P/Q-type VDCC, thus activating hyperpolarizing potassium currents. Activation of these receptors leads to presynaptic inhibition of the release of neurotransmitters, including glutamate (Conn and Pin, 1997).

### **1.6.1. Group I mGluR Signalling**

Group I mGluRs are coupled through G $\alpha_q$  to activate PLC production of IP<sub>3</sub> and DAG generation leading to intracellular calcium release and PKC activation. These receptors can also functionally activate a number of G protein-independent signal transduction pathways including tyrosine kinases, mitogen activated kinases, ion channels and other phospholipases. The tyrosine kinase Pyk2 uncouples mGluR G-protein signalling, but facilitates extracellular signal-regulated kinases (ERK) 1/2 activation (Dhami and Ferguson, 2006; Kim et al., 2008; Niswinder and Conn, 2010; Ribeiro et al., 2010). ERK phosphorylates transcription factors such as cAMP response element-binding protein (CREB) and Elk-1, facilitating immediate early gene expression (Nicodemo et al., 2010). Via G proteins, mGluRs activate K<sup>+</sup>, Ca<sup>2+</sup> and nonselective



**Figure 1.6. Schematic depiction of the glutamatergic synapse.** Depolarization of the presynaptic cell results in the membrane translocation of secretory vesicles, which release glutamate into the synaptic cleft. Presynaptic Group I mGluRs (mGluR1/5) enhance glutamate secretion while presynaptic Group II and III mGluRs inhibit secretion. Glutamate diffuses through the synaptic cleft to activate numerous postsynaptic glutamate receptors, ultimately resulting in increased calcium release in the postsynaptic cell. Ionotropic glutamate receptor channels, including AMPA, NMDA and Kainate-type channels are located in the postsynaptic density while metabotropic glutamate receptors are predominately found perisynaptically.



**Figure 1.7. Snake model depicting amino acid residues of the metabotropic glutamate receptor 1 (mGluR1).** The mGluRs possess a large bi-lobed extracellular amino-terminal, which folds to form the characteristic “venus fly trap” domain involved in agonist binding. This agonist-binding domain is linked via a Cysteine-rich region to the heptahelical transmembrane domains, linked by the intracellular loop domains collectively responsible for G protein activation. Ligand binding to a receptor dimer stabilises a closed conformation of the lobes, triggering intracellular signal transduction by stabilising the two receptors in an active conformation. The carboxyl-terminal tail contains numerous serine and threonine residues, which may be phosphorylated by second messenger-dependent kinases as well as G protein-coupled receptor kinases. The C-tail is also involved in the regulation of receptor function through interaction with intracellular proteins including the Homer family and calmodulin. Figure adapted from Dhama and Ferguson, 2006.



cation channels have been shown to mediate long-term potentiation, as well as long-term depression in neurons, involving mGluR1/5 activation or inhibition (respectively) of AMPAR- or NMDAR-mediated transmission (Mao et al., 2008). mGluR activation of phospholipase A2 (PLA2) can occur via PKC or ERK (Rozenjurt, 2007; Stella et al., 1994). PLA2 hydrolyses glycerophospholipids generating arachidonic acid, which can then be converted into pro-inflammatory eicosanoids. Phospholipase D (PLD) is activated in both PKC-dependent and independent manners PLD activation by mGluRs in astrocytes is dependent on PKC and small G proteins of the ARF family (Anwyl, 1999).

### **1.6.2. mGluR1 Desensitization, Endocytosis and Intracellular Trafficking**

Desensitization and endocytosis of mGluRs abandons the GPCR paradigm as mGluR1 undergoes constitutive, as well as agonist-induced endocytosis and mGluR1 desensitization includes phosphorylation-dependent and -independent mechanisms (Ferguson, 2007; Dhimi and Ferguson, 2006; Bhattacharya et al., 2004; Dhimi et al., 2005, Dhimi et al., 2004; Dhimi et al., 2002). Phosphorylation-independent mGluR desensitization involves the GRK2 RGS homology domain associating with both the receptor second intracellular loop and the alpha subunit of the heterotrimeric G protein (Bhattacharya et al., 2004; Dale et al., 2000; Dhimi and Ferguson, 2006; Ferguson, 2001b; Ferguson, 2007). Phosphorylation-dependent mGluR desensitization occurs via receptor phosphorylation by second messenger-dependent kinases (PKC and CamKII) (Dale et al., 2000; Dhimi et al., 2002; Dhimi et al., 2004). To date, many proteins have been implicated in the desensitization of the mGluRs including GRK2, second messenger dependent kinases, scaffolding and trafficking molecules, such as, the huntingtin-interacting protein optineurin (Anborgh et al., 2005; Mundell et al., 2004; Mundell et al.,

2003; Schoepp and Johnson, 1988). The endocytosis of mGluR1 is equally complex, with differing partners contributing to constitutive versus agonist-induced internalization and even different endocytic machinery depending on the agonist (Dhami and Ferguson, 2006). PKC activation is important for glutamate-induced mGluR1a internalization while mGluR1 constitutive internalization can be mediated by scaffolding RalA along with phospholipase D2 (PLD2) at the receptor (Bhattacharya et al., 2004a).

### **1.7. SMALL G PROTEINS**

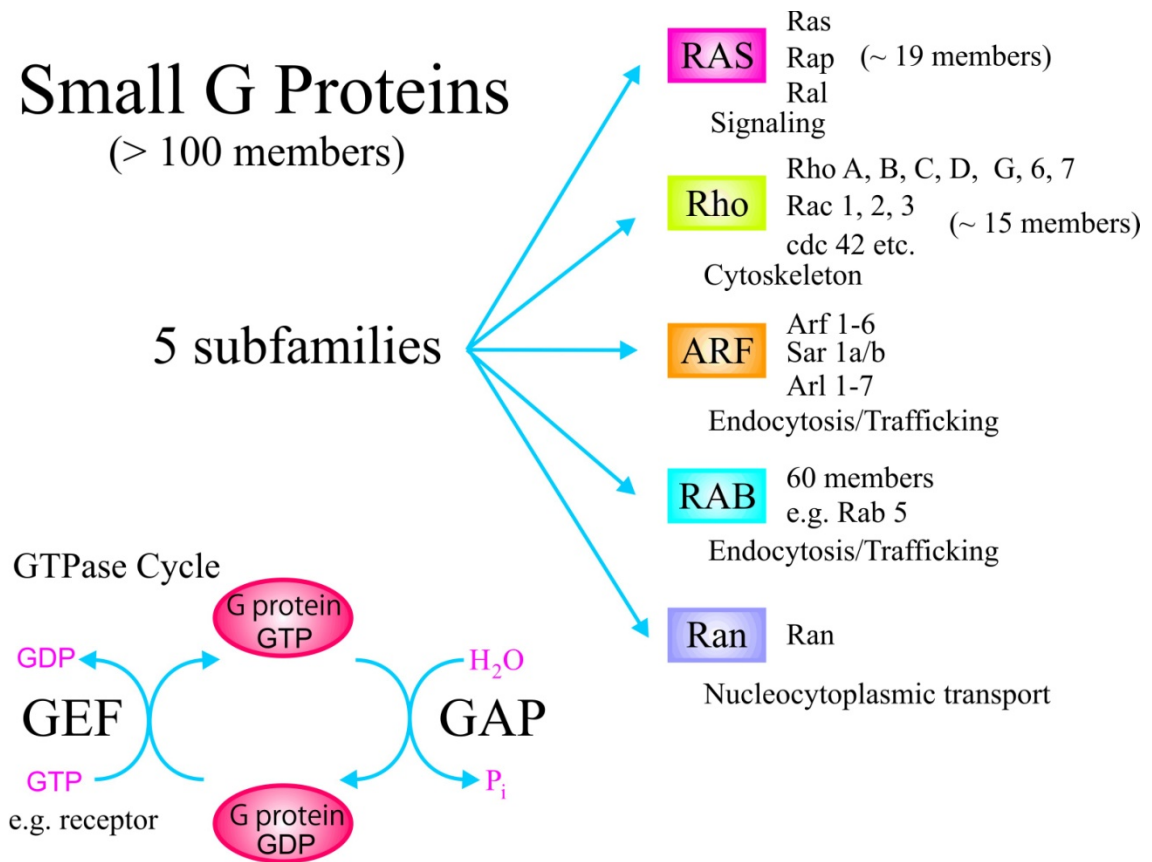
The small, monomeric GTP-binding superfamily contains more than 100 members with molecular masses ranging from 20 to 30 kDa, classified into five subfamilies based on structural similarity: Ras, Rho, Ran Arf and Rab family GTPases (Figure 1.8) (Bhattacharya et al., 2004b; Mundell et al., 2003; Exton, 1998). Small G proteins play essential roles in signal transduction, growth regulation, cell motility and intracellular trafficking among other cellular processes. Ras family members (Ras, Rap and Ral) typically regulate cell signalling events that lead to alterations in gene transcription while the Rho family also regulates the actin cytoskeleton (Takai et al., 2001; Wettschureck and Offermanns, 2005; Sah et al., 2000). Ran members regulate microtubule organization and protein transport between the nucleus and cytoplasm (Bos, 1998). The Rab and Arf families control the formation, fusion, and movement of vesicular traffic between different membrane compartments (Bos, 1998).

As guanine nucleotide binding proteins, members of this superfamily cycle between the guanosine triphosphate (GTP) bound “active” state and the guanosine diphosphate (GDP) bound “inactive state” and possess intrinsic GTP hydrolysis activity (Lundquist, 2006; Zerial and McBride, 2001). In addition to their inherent GTPase

# Small G Proteins

(> 100 members)

5 subfamilies



**Figure 1.8. The Ras superfamily of small G proteins.** G proteins (also called GTPases) cycle between the guanosine triphosphate (GTP)-bound active form and the guanosine diphosphate (GDP)-bound inactive forms. In addition to the inherent hydrolyzing activity of G proteins, GTP hydrolysis is assisted by GTPase accelerating proteins (GAP) while the exchange of GDP for GTP is facilitated by guanine exchange factors (GEF). A large class of G protein GEFs include GPCRs themselves. The superfamily of Ras small G proteins are categorized into five subfamilies, including Ras, Raf, Rho, Arf, Rab and Ran. While the Ras subfamily participates mainly in signal transduction events, Arf, Rab and Ran family members facilitate intracellular membrane transport and Rho members associate with the cytoskeleton.

activity, many members of this family associate with accessory proteins including GTPase activating (GAP) proteins, which accelerate GTP hydrolysis, as well as guanine exchange factors (GEF), which facilitate the exchange of GDP for GTP (Bos, 1998; Takai et al., 2001).

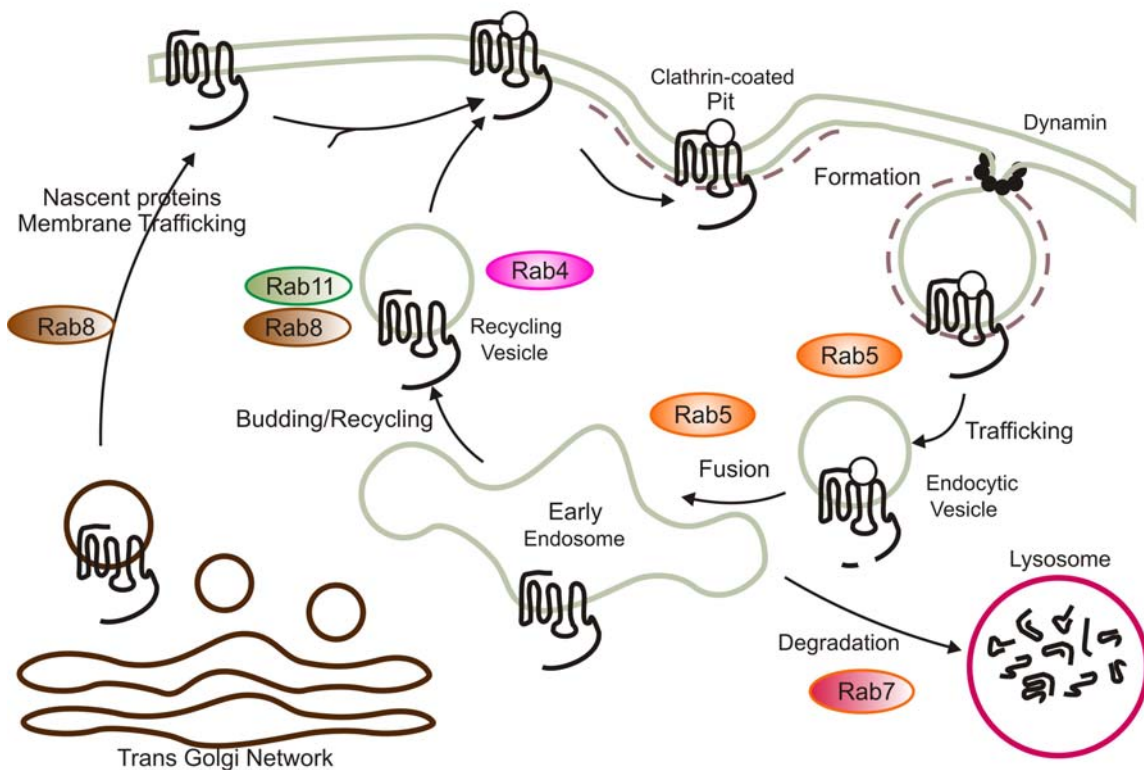
### **1.7.1. Overview of the Rab Family of Small GTPases**

Ras-like in Brain (Rab) are the largest group of small Ras-like G proteins comprising 11 members in yeast and over 60 in mammals (Ross, 2008; Rossman et al., 2005). Rab family members regulate all aspects of intracellular membrane trafficking from vesicular targeting, docking and fusion events. They participate in the transport of nascent proteins from the trans-Golgi network (TGN), the exocytosis and endocytosis of proteins, endocytic sorting and lysosomal degradation of membrane bound proteins (Seachrist and Ferguson, 2003; Zerial and McBride, 2001; Stenmark, 2009). Rabs reversibly associate with membranes via hydrophobic geranylgeranyl groups attached to two C-terminal Cysteine residues. Rab escort proteins (REPs) capture newly synthesized Rabs and present them to geranylgeranyl transferase before targeting them to the appropriate membrane. GDP dissociation inhibitors (GDIs) recognize Rab-GDP and prevent GDP release, while simultaneously chaperoning geranylgeranylated Rabs in the cytosol and mediating their delivery to membranes or recycling them back to the cytosol. GDI displacement factors (GDFs) recognize specific Rab-GDI complexes and promote GDI release, thereby facilitating the association of Rabs with relevant membrane domains (Hutagalung and Novick, 2011; Pfeffer and Aivazian, 2004).

### **1.7.1.1. Intracellular Localization of Rab Proteins**

A unique and useful characteristic of Rabs is their tendency to occupy distinct and predictable membrane microdomains (Figure 1.9) (Hutagalung and Novick, 2011; Stenmark, 2009; Zerial and McBride, 2001). Rab1 and Rab2 are localized to the endoplasmic reticulum and pre-Golgi area and mediate ER-Golgi trafficking. Rab 6, 33 and 40 are found at the Golgi and mediate intra-Golgi trafficking while Rab8 mediates constitutive biosynthetic trafficking from the trans-Golgi network to the cell surface and has also been implicated in Rab11 mediated recycling (Wang and Wu, 2012; Knodler et al., 2010). Rab32 controls mitochondrial fission while Rab13 assembles epithelial cell tight junctions. Autophagosome formation is regulated by Rabs 33 and 24 (Chua et al., 2011; Marzescot and Zarhaoui, 2005; Alto et al., 2002). Rab3, 26, 27 and 37 regulate exocytic events and Rab27 mediates the translocation of melanosomes to the cell periphery (Fukuda, 2008; Strom et al., 2002). Rab5 is localized to the plasma membrane, early endosomes and phagosomes, where it participates in clathrin-mediated endocytosis of plasma membrane proteins (Seachrist and Ferguson, 2003). Rab21 is implicated in integrin endocytosis (Pellinen et al., 2006). Rab4 is localized to early endosomes and recycling endosomes and is responsible for “fast” recycling of plasma membrane proteins while Rab11, which is located in the perinuclear region and Rab35 mediates “slow” recycling of plasma membrane proteins (Chua et al., 2010; Seachrist and Ferguson, 2003). Rab7 and Rab9 are located in late endosomes and lysosomes and traffic proteins for degradation (Zerial and McBride, 2001). This thesis focuses on the management of GPCRs by Rabs involved in the endocytic pathway including Rab4, Rab5, Rab7, Rab8 and Rab11 and will henceforth focus on these Rab family members (Figure 1.9).

## GPCR Trafficking by Rab GTPases



**Figure 1.9. Rab Family members coordinate all aspects of endocytic trafficking.** Rab5 participates in clathrin-mediated endocytosis and early endosomal trafficking of endocytic vesicles while Rab7 mediates the late endosomal and lysosomal trafficking of proteins for degradation. Rab4 mediates the “fast” recycling route, directly from early endosomes to plasma membrane while Rab11 mediates the “slow” perinuclear recycling route. Rab8 participates in the plasma membrane targeting of nascent proteins and may coordinate with Rab11 for recycling.

### **1.7.1.2. Structure of Rab Proteins**

Like other members of the Ras superfamily of small G proteins, Rabs contain the GTPase fold and COOH-terminal to the fold is the hyper-variable region as well as the CAAX boxes containing two cysteines, which are posttranslationally modified with the addition of a geranylgeranyl group, allowing the Rab to associate with membranes (Ng and Tang, 2008; Stenmark, 2009; Zerial and McBride, 2001). The switch I and II regions of Rabs are the area of nucleotide binding, and both switch regions make contact with the  $\gamma$  phosphate of GTP. Effector molecules likely associate with the heterogeneous switch domains along with the  $\alpha 3/\beta 5$  loop (a loop that connects  $\alpha$  helix 3 with  $\beta$  sheet 5) that lies adjacent to the switch II domain. Rab GTPases are differentiated from other members of the Ras superfamily by a Rab-specific amino acid sequence F1-F5. Rab GTPases are evolutionarily well conserved with 55-75% identity between orthologs from yeast and mammals (Hutagalung and Novick, 2011; Pfeffer and Aivazian, 2004).

### **1.7.1.3. Rab Interacting-Proteins**

Rabs mediate targeting, docking and fusion of their cognate vesicles via association with effector molecules and Rab interacting proteins (Zerial and McBride, 2001). Membrane tethering complexes often contain GEFs for Rabs that serve to recruit them. For example, the Rab5 effector Rabaptin 5 is complexed with the Rab5 GEF Rabex5, which amplifies Rab5 activation in microdomains of endosomal membranes while Rab5 association with effectors Rabenosyn 5 and Early Endosomal Antigen 1 (EEA1) recruit members of the SNARE complex to coordinate tethering, docking and fusion (Hutagalung and Novick, 2011).

#### **1.7.1.4. Mechanisms of Rab Action**

GTP bound Rabs can activate sorting adaptors to sort a receptor into budding vesicles and via recruitment of PI kinases and phosphatases can alter PI composition and trigger uncoating. Rabs can also mediate vesicle transport along actin filaments or microtubules by recruiting motor adaptors or binding directly to motors. For example, the plus-end-directed, actin-based motor protein myosin Vb facilitates protein trafficking in Rab11a-specific recycling vesicles (Hutagalung and Novick, 2011; Pfeffer and Aivazian, 2004; Stenmark, 2009; Zerial and McBride, 2001). Rabs mediate vesicle tethering by recruiting tethering factors that interact with SNARES or their regulators. Finally, following membrane fusion, the Rab hydrolyzes GTP, thus associating with GDP and therefore targeted by a GDI back to the donor membrane and then to a GDF.

Rab activity can be manipulated by specific functional mutations. These mutations are design based on well-characterized amino acid substitutions in the p21ras GTPase. Dominant negative and constitutively active Rab mutations are very useful tools, which are frequently utilized to study the effect of Rab function in regulation of membrane trafficking.

Dominant negative Rab mutations are created by substituting an amino acid in the N-terminal (equivalent to p21ras S17N) or C-terminal (equivalent to p21ras N116I) of the Rab nucleotide binding domain (Millman et al., 2008; Volpicelli et al., 2002). This forces the Rab to be constitutively associated with a guanine nucleotide exchange factor, thus preventing Rab activation and rendering the Rab defective in guanine nucleotide binding.

Constitutively active Rab mutations are created by substituting an amino acid in the GTPase region, equivalent to the p21ras Q61L mutant, which exhibits reduced



GTPase activity (Stenmark et al., 1994). Thus, the Rab does not hydrolyse GTP to GDP and its activity is effectively non-regulated.

### **1.7.2. Rabs and Receptor Intracellular Trafficking**

An explosion of Rab-mediated GPCR trafficking studies in recent years has expanded our understanding of the different members of this family of small G proteins affects the intracellular targeting of GPCRs.

#### **1.7.2.1. Rab4**

Rab4 is involved in the transport of protein from early endosomes to the plasma membrane via a direct, “fast” recycling route directly from early endosomes (Table 1.1.). Rab4 is involved in the recycling of the AT<sub>1</sub>R, somatostatin receptor 3, corticotropin releasing factor 1 $\alpha$  receptor and  $\beta$ 2 adrenergic receptor among others (Esseltine et al., 2011; Yudowski et al., 2009; Holmes et al., 2006; Odley et al., 2004; Seachrist et al., 2000). Although the AT<sub>1</sub>R is not known to be efficiently recycled, fluorescence resonance energy transfer (FRET) microscopy shows that during the early recycling stage, internalized AT<sub>1</sub>Rs are associated with Rab4 in the cytoplasm whereas during the mid-recycling stage, AT<sub>1</sub>Rs are associated with both Rab4 and Rab11 in the perinuclear compartments (Esseltine et al., 2011; Hunyady et al., 2002; Seachrist et al., 2000). Internalized CRF1 $\alpha$  receptor transits from Rab5-positive early endosomes to Rab4-positive recycling endosomes and CRF1 $\alpha$  receptor resensitization is blocked by the overexpression of wild-type Rab5 and Rab4 GTPases (Holmes et al., 2006). Meanwhile, the apelin-13 internalized receptor is rapidly recycled to the cell surface through a Rab4-dependent mechanism, while dominant negative Rab4 causes the receptor to be trafficked

**Table 1.1. Rab4-mediated GPCR trafficking and signalling**

<b>Receptor</b>	<b>Proposed Function in Receptor Trafficking and Signalling</b>	<b>References</b>
Somatostatin 3	Rapid SSTR3 trafficking	Tower-Gilchrist et al., 2011
Angiotensin II type 1	Facilitates AT <sub>1</sub> R dephosphorylation, thereby decreasing desensitization and increasing resensitization	Hunyady et al., 2002; Esseltine et al., 2011
Corticotropin releasing factor 1 alpha	Rab4 overexpression blocked CRF1 $\alpha$ R resensitization	Holmes et al., 2006
Oxytocin receptor		Conti et al., 2009
Mu-opioid receptor	Recycling of phosphorylated MOR	Wang et al., 2008
Apelin	Rab4 rapidly recycled apelin-13 receptor while Rab4DN caused receptor trafficking to lysosomes	Lee et al., 2010
Thyrotropin releasing factor	Localized with both phosphorylated and dephosphorylated TRH	Jones and Hinkle, 2009
Glucagon	Facilitates actin- and arrestin-dependent GR recycling	Krilov et al., 2008
CXC chemokine 2	CA RhoB reroutes CXCR2 from Rab11 to Rab4 recycling	Neel et al., 2007
Prostaglandin D2	Specifically recycles DP2 receptor, not CRHT2	Gallant et al., 2007
Neurokinin	Rab4 disruption attenuated NK1R resensitization	Roosterman et al., 2004
Beta 2 adrenergic	$\beta_2$ AR activation results in increased Rab4-positive vesicle fusion with PM	Yudowski et al., 2009;
	Transgenic Rab4DN decreased catecholamines response and caused abnormal accumulation of $\beta_2$ AR in the sarcoplasm	Seachrist et al., 2000;
Beta 1 adrenergic	Transgenic overexpression of Rab4 in the mouse myocardium increased $\beta$ AR in the plasma membrane and cAMP production leading to cardiac hypertrophy	Odley et al., 2004 Filipeanu et al., 2006

to lysosomes (Lee et al., 2010). Somatostatin receptor 3 traffics through Rab4-, and Rab11-containing endosomes and expression of the inactive Rab4/S22N, and Rab11/S25N inhibits receptor trafficking (Tower-Gilchrist et al., 2011). Similarly, Rab4 or Rab11 dominant-negative mutants and small interfering RNA both significantly impair the recycling of the wild-type  $\mu$ -opioid receptor (Wang et al., 2008).

#### **1.7.2.2. Rab5**

Rab5 is one of the more intensely studied members of the large and diverse family of Rab GTPases (Table 1.2.). Enriched at the plasma membrane and early endosomes, Rab5 coordinates the endocytosis of proteins via clathrin-coated vesicles and transport to early endosomes where proteins may be sorted for retention, recycling or degradation (Seachrist and Ferguson, 2003). Rab5a has been shown to interact with the AT<sub>1A</sub>R carboxyl-terminal tail and retain the receptor in Rab5a-positive early endosomes (Dale et al., 2004; Seachrist et al., 2002). Co-expression of cannabinoid receptor 2, muscarinic acetylcholine receptor M4 and human NPY receptors with dominant negative Rab5 results in a significant reduction in receptor internalization (Grimsey et al., 2011; Lecat et al., 2011; Volpicelli et al., 2001). Conversely, although dominant-negative Rab5-S34N did inhibit receptor internalization, Rab5 and TRH receptor do not colocalize at the plasma membrane immediately after TRH addition, but overlap extensively by 15 min (Jones and Hinkle, 2009). Cysteinyl leukotriene receptor is internalized through a clathrin- arrestin- and Rab5-dependent pathway and internalized adenosine A (2A) receptors also co-localize with clathrin and Rab5 (Parhamifar et al., 2009; Mundell et al., 2000). Both phosphorylated and nonphosphorylated  $\mu$ -opioid receptor internalize via Rab5-dependent pathway after agonist stimulation (Wang et al., 2008).

**Table 1.2. Rab5-mediated GPCR trafficking and signalling**

<b>Receptor</b>	<b>Proposed Function in Receptor Trafficking and Signalling</b>	<b>References</b>
Cannabinoid 2	Rab5DN attenuated CB2 internalization	Grimsey et al., 2011
angiotensin II type 1	Rab5a associates with last 10 amino acids and Rab5aDN or truncated AT <sub>1A</sub> R prevents AT <sub>1A</sub> R trafficking into large, hollow cored vesicular structures. AT <sub>1A</sub> R activation facilitates Rab5a GTP binding.	Seachrist et al., 2001; Dale et al., 2004; Esseltine et al., 2011
corticotropin releasing factor	Rab5 overexpression blocked CRF1 $\alpha$ R resensitization	Holmes et al., 2006
Melanocortin		Roy et al., 2011
Cysteinyl leukotriene 1	Clathrin, arrestin-3, and Rab5 mediates internalization of CysLT(1)R	Parhamifar et al., 2010
Dopamine 2	Rab5aCA facilitates while Rab5aDN attenuates $\beta$ -arrestin-mediated D2R Endocytosis and ERK1/2 activation	Iwata et al., 2005
Neurokinin	Rab5aDN caused retention of the NK1R in early endosomes	Roosterman et al., 2004
CXC chemokine 2	Rab5DN mutant decreases CXCR2 endosomal sequestration	Fan et al., 2003
Endothelin A		Bremnes et al., 2000; Paasche et al., 2001
Endothelin B		Bremnes et al., 2000;
Oxytocin		Conti et al., 2009
mu-opioid	Rab5 facilitates phosphorylated and nonphosphorylated MOR internalization	Wang et al., 2008
C5a anaphylatoxin chemotactic		Cui et al., 2009
Thyrotropin releasing	Rab5 and phosphorylated TRHR colocalized at 15 min and dephosphorylated receptor colocalized with Rab4 but not with Rab5. Rab5DN inhibited receptor internalization.	Jones and Hinkle, 2009
Platelet activating factor	PAFR activation triggered signal-regulating kinase-1/MAPK kinase-3/p38 MAPK assembly with Rab5a and Rab GDI, thus activating Rab5a	McLaughlin et al., 2008
Somatostatin 1		Roosterman et al., 2007
TPbeta	Amino acids 335-344 of the TPbeta C-tail essential for the directing the	Hamelin et al., 2005

<p>Adenosine (2B) Neurotensin 1 Beta 2 adrenergic</p>	<p>receptor from the Rab5-positive vesicles to the perinuclear recycling endosome Involved in <math>\beta</math> arrestin-1 mediated endocytosis of A(2B)AR</p>	<p>Mundell et al., 2000 Vandenbulcke et al., 2000 Seachrist et al., 2000</p>
<p>Ghrelin Human prostacyclin</p>	<p>Rab5DN impaired <math>\beta_2</math>AR internalization and <math>\beta_2</math>AR-positive vesicles remained closely associated with plasma membrane. Rab5CA internalized <math>\beta_2</math>AR to enlarged endosomes</p> <p>Rab5a associates with C-tail of hIP in agonist-dependent manner and hIP stimulation triggered Rab5a translocation from cytosolic to membrane fraction.</p>	<p>Holliday et al., 2007 O’Keeffe et al., 2008</p>
<p>Rhodopsin</p>	<p>Dynamin- and Rab5-dependent endocytosis necessary to prevent early onset of rhabdomere degeneration in <i>Drosophila</i>.</p>	<p>Pinal and Pichaud, 2011</p>
<p>M4 muscarinic</p>	<p>Rab5DN inhibits m4 endocytosis while Rab5CA enhances m4 intracellular distribution and produces enlarged vacuoles</p>	<p>Volpicelli et al., 2001</p>
<p>Neuropeptide Y Metabotropic glutamate 1</p>	<p>Facilitates clathrin-mediated endocytosis of NPY receptor Rab5b siRNA prevents DHPG-mediated LTD in neurons. Group I mGluRs are linked to Rab5b synthesis</p>	<p>Lecat et al., 2011 Baskys et al., 2005</p>

### **1.7.2.3. Rab7**

Rab7 is enriched at late endosomes and lysosomes where it mediates the trafficking of targeted proteins to the lysosome for degradation (Table 1.3.). CXCR2 type 1 PDZ binding motif truncation increases ligand-mediated receptor degradation and Rab7 dominant negative overexpression prevents CXCR2 from trafficking to lysosomes (Baugher and Richmond, 2008; Fan et al., 2003). Rab7 also targets the apelin-13 receptor to lysosomes and Rab7 overexpression can change the fate of AT<sub>1A</sub>R from Rab5-mediated sequestration to Rab7-mediated degradation (Lee et al., 2010; Dale et al., 2004).

### **1.7.2.4. Rab8**

Rab8 is localized to Golgi, vesicles and membrane ruffles and is involved in trafficking of basolateral proteins in polarized epithelial cells, as well as neurite outgrowth (Table 1.4.) (Ng and Tang, 2008). Rab8 has been shown to regulate ionotropic glutamate AMPA receptor synaptic delivery and recycling in rat hippocampal spines and is documented in the polarized transport of rhodopsin in photoreceptor cells (Brown et al., 2007; Gerges et al., 2004; Moritz et al., 2001; Deretic, 1997). Rab8 has recently been shown to directly associate with different regions of the  $\alpha_{2B}$ - and  $\beta_2$ -adrenergic receptors and a GDP-bound dominant negative Rab8 mutant blocks cell surface expression and ERK1/2 activation of  $\alpha_{2B}$ AR but not  $\beta_2$ AR (Dong et al., 2010). Rab8 has also been implicated in non-clathrin mediated endocytosis and is associated with macropinosomes generated at ruffling membrane domains (Hattula et al., 2006).

**Table 1.3. Rab7-mediated GPCR trafficking and signalling**

<b>Receptor</b>	<b>Proposed Function in Receptor Trafficking and Signalling</b>	<b>References</b>
Apelin Angiotensin II type 1	Rab7 targeted the receptor to lysosomes Rab7 associates with last 10 amino acids of AT <sub>1</sub> R and wild-type Rab7 and Rab7CA increased AT <sub>1</sub> R lysosomal targeting and degradation	Lee et al., 2010 Seachrist et al., 2001; Dale et al., 2004; Esseltine et al., 2011
GABA(A) Prostaglandin EP4	EP(4), $\gamma$ -secretase and Rab7 co-localised after agonist stimulation in cells and also in the brain of wild-type mice but not of EP(4) receptor null mice.	Davis et al., 2010 Hoshino et al., 2009
C5a anaphylatoxin CXC chemokine 2	CXCR2 type 1 PDZ binding motif truncation increases ligand-mediated receptor degradation	Cui et al., 2009 Fan et al., 2003; Baugher and Richmond 2008
Beta 2 adrenergic Neurotensin 1	PI3K inhibitors reroute $\beta_2$ AR from recycling to degradative pathways	Awwad et al., 2007 Vandenbulcke et al., 2000

**Table 1.4. Rab8-mediated GPCR trafficking and signalling**

<b>Receptor</b>	<b>Proposed Function in Receptor Trafficking and Signalling</b>	<b>References</b>
Alpha 2B adrenergic Beta 2 adrenergic	DN Rab8 reduced $\alpha_{2B}$ AR plasma membrane expression $\beta_2$ AR modulates the Rab geranyl-geranylation DN Rab8 reduced $\beta_2$ AR plasma membrane expression	Dong et al., 2010 Lachance et al., 2011; Dong et al., 2010
CXC chemokine 4 CCR5	CXCR4-CCR5 homodimer transport from endoplasmic reticulum to plasma membrane when CD4 co-receptor is present	Charette et al., 2011
Rhodopsin	Rab8DN caused rapid retinal degeneration in <i>Xenopus</i>	Moritz et al., 2001

### 1.7.2.5. Rab11

Rab11 is another extensively studied protein transport molecule (Table 1.5.). Rab11 is localized to early endosomes and the perinuclear regions and is crucial for recycling and resensitization of many GPCRs and other receptors (Seachrist and Ferguson, 2003). Like Rab4, Rab11 mediates protein trafficking from endosomes back to the plasma membrane. However, unlike the direct Rab4 route from early endosomes to plasma membrane, Rab11 coordinates a “slow” recycling route through the perinuclear region and Rab11 and has often been shown to coordinate with or be in competition with Rab4 for recycling (Esseltine et al., 2011; Hunyady et al., 2002). For example, Rab11 mediates the recycling of nonphosphorylated MOR, while Rab4 mediates phosphorylated  $\mu$ -opioid receptor recycling (Wang et al., 2008). Dominant negative Rab11 causes decreased  $\beta_2$ AR membrane expression and mediates recycling of constitutively internalized TP $\beta$  receptor (Parent et al., 2009; Hamelin et al., 2005; Theriault et al., 2004). Rab11 also participates in M4 muscarinic receptor recycling via myosin Vb, a Rab11a effector (Volpicelli et al., 2002). In addition to recycling, Rab11 has been shown to mediate other antrograde trafficking pathways and along with Rab6, Rab3 and Rab8, Rab11 is also linked to post-Golgi trafficking of rhodopsin and coordinates CXCR4-CCR5 homodimer transport from endoplasmic reticulum to the plasma membrane (Sato et al., 2005; Deretic, 1997).



**Table 1.5. Rab11-mediated GPCR trafficking and signalling**

<b>Receptor</b>	<b>Proposed Function in Receptor Trafficking and Signalling</b>	<b>References</b>
Somatostatin 3 Cannabinoid 2 Angiotensin II type 1	Regulates slow receptor trafficking Rab11DN impaired receptor return to plasma membrane Rab11 associates with AT1R C-tail to mediate recycling	Tower-Gilchrist et al., 2011 Grimsey et al., 2011 Dale et al., 2004; Esseltine et al., 2011
Beta 2 adrenergic	$\beta_2$ AR modulates the Rab geranyl-geranylation endoplasmic reticulum to plasma membrane PI3K inhibitors reroute $\beta_2$ AR from recycling to degradative pathways DN Rab11 causes decreased $\beta_2$ AR membrane expression Actin-dependent recycling of $\beta_2$ AR	Lachance et al., 2011  Parent et al., 2009 Millman et al., 2008 Roy et al., 2011
Melanocortin 2 Human prostacyclin	Rab11a increased recycling of hIP, while Rab11DN impaired recycling. Interaction between hIP and Rab11a via Val299-Gln320 sequence within the hIP C-tail domain	Wikstrom et al., 2008;
CXC chemokine 4	G $\alpha$ 13 and Rho mediate actin-dependent trafficking of CXCR4 into the Rab11 compartment	Reid et al., 2010 Kumar et al., 2011
CCR5 Vasopressin CXC chemokine 2	CXCR4-CCR5 homodimer transport from ER to PM  RhoB CA mutant reroutes CXCR2 from Rab11 to Rab4 recycling	Charette et al., 2011 Innamorati et al., 2001 Fan et al., 2003; Neel et al., 2007
LPAR	LPA promotes Rab11a interaction with G $\beta\gamma$ , activating PI3K and AKT Recycling of nonphosphorylated MOR	Garcia-Regaldo et al., 2008
Mu-opioid		Liang et al., 2008; Wang et al., 2008
Delta-opioid	DN Rab11 abolished the ability of Src blockers to prevent DOR desensitization	Archer-Lahlou et al., 2009
C5a anaphylatoxin Glucagon	Facilitates actin- and arrestin-dependent GR recycling	Cui et al., 2009 Krilov et al., 2008

Calcium sensing	DN Rab11a decreased CaR-mediated PTHrP secretion but not CaR-dependent ERK1/2	Reyes-Ibarra et al., 2007
Somatostatin 1 TPbeta	Rab11 interacts directly with the first intracellular loop and the C-tail of TPbeta and mediates recycling of constitutively internalized TPbeta receptor	Roosterman et al., 2007 Hamelin et al., 2005; Theriault et al., 2004
Neurokinin 1 M4 muscarinic Chemoattractant homologous receptor expressed on TH2 cells	Rab11aDN inhibited NK1R recycling Regulates M4 recycling via myosin Vb, a Rab11a effector Specifically recycles CRTH2, not DP2 receptor	Roosterman et al., 2004 Volpicelli et al., 2002 Gallant et al., 2007
Neurotensin 1 GhrelinR Rhodopsin	Rhodopsin colocalizes with Rab11 and disruption of Rab11 activity inhibits rhabdomere morphogenesis and rhodopsin-positive vesicles accumulate in the cytosol.	Vandenbulcke et al., 2000 Holliday et al., 2007 Satoh et al., 2005
Endothelin A Endothelin B		Paasche et al., 2001 Paasche et al., 2001

### **1.7.3. Rabs and Receptor Signalling**

#### **1.7.3.1. Rab4**

Previously, it has been shown that the dephosphorylation and resensitization of the  $\beta_2$ AR occurs as the receptor transits between the Rab5-positive early endosome and the Rab4-positive rapid recycling endosome (Seachrist et al., 2000). Transgenic overexpression of Rab4 in the mouse myocardium significantly increased the number of  $\beta$ AR in the plasma membrane and augmented cAMP production at the basal level and in response to isoproterenol stimulation (Odley et al., 2004). Expression of dominant negative Rab4 impaired  $\beta_2$ AR responsiveness to endogenous and exogenous catecholamine and Rab4 inhibition prevented resensitization after isoproterenol-induced *in vivo* adrenergic desensitization (Filipeanu et al., 2006). Moreover, it has been reported that phosphorylated  $\mu$ -opioid receptor is preferentially recycled through Rab4-positive endosomes (Wang et al., 2008). CRF1 $\alpha$  receptor resensitization was blocked by the overexpression of wild-type Rab5 and Rab4 GTPases and dephosphorylated receptor colocalized with Rab4 but not with Rab5 (Holmes et al., 2006).

#### **1.7.3.2. Rab5**

As stated above, dephosphorylation and resensitization of the  $\beta_2$ AR occurs as the receptor transits between the Rab5-positive early endosome and the Rab4-positive rapid recycling endosome and CRF1 $\alpha$  receptor resensitization was blocked by the overexpression of wild-type Rab5 (Holmes et al., 2006; Seachrist et al., 2000). Rab5 has also been shown to be involved in other aspects of GPCR signalling. Interestingly, Purvanov et al., 2010 demonstrate an interaction of *Drosophila* Rab5 and the G protein

Go, *in vitro* and *in vivo*. Purified Rab5 and Go proteins associate with each other and Go contributes to Rab5 activation and endosome fusion. Serotonin, by cooperating with mGluRs, regulates synaptic plasticity through a mechanism dependent on p38 MAPK/Rab5-mediated enhancement of AMPA receptor internalization in a clathrin/dynamin-dependent manner (Zhong et al., 2008). Further studies established a functional link between phosphatidic acid-derived DAG and the activation of p38 mitogen-activated protein kinase and the subsequent phosphorylation of the Rab5 effector EEA1, which has been demonstrated to be required for the induction of MOR endocytosis (McLaughlin et al., 2008). Additionally, Rab5b plays an important role in Group I mGluR-mediated neuroprotection and synaptic plasticity. While DHPG treatment of neurons typically leads to long term depression (LTD) in Rab5b siRNA treated neurons, DHPG no longer causes LTD. Additionally, group I mGluRs are linked to Rab5b synthesis (Baskys et al., 2007; Arnett et al., 2004).

### **1.7.3.3. Rab8**

Rab8 has been shown to coordinate with Rab11 to modulate plasma membrane targeting of receptors. Like Rab11, Rab8 associates with the actin motor protein myosin Vb to regulate trafficking (Roland et al., 2007).  $\beta_2$ AR also modulates the geranyl-geranylation of Rab8, thus facilitating the Rab association with membranes and Rab8 activity (Lachance et al., 2011). Dominant negative or depletion of Rab8 by siRNA significantly attenuates ERK1/2 activation by  $\alpha_{2B}$ AR but not  $\beta_2$ AR and inhibits plasma membrane delivery of  $\alpha_{2B}$ AR from the TGN (Parent et al., 2009). Our lab has previously demonstrated that the Rab8 effector molecule, optineurin associates with and desensitizes mGluR1 IP3 signalling (Anborgh et al., 2005). Rab8 also coordinates with Rab11 to

mediate the synaptic delivery of AMPARs during long-term potentiation and constitutive receptor cycling (Brown et al., 2007; Gerges et al., 2004). After Rab11 targets AMPAR-containing endosomes from the dendritic shaft into spines, Rab8 directs receptor insertion into the synaptic membrane (Brown et al., 2007).

#### **1.7.3.4. Rab11**

In addition to recycling and resensitizing receptors to the plasma membrane, Rab11 has been shown in recent years to participate in many other aspects of receptor signalling. For example, DN Rab11 causes decreased  $\beta_2$ AR membrane expression through decreased receptor recycling, but additionally,  $\beta_2$ AR itself also modulates the geranyl-geranylation of Rab11, thus determining the membrane association state of the Rab and its activity (Lachance et al., 2011). Rab11 also coordinates with other signalling and regulatory molecules including heterotrimeric G proteins and other small G proteins. G $\alpha$ 13 and Rho mediate actin-dependent trafficking of CXCR4 into the Rab11 compartment while a RhoB constitutively active mutant reroutes CXCR2 from Rab11 to Rab4 recycling pathways (Kumar et al., 2011; Reid et al., 2010; Neel et al., 2007; Fan et al., 2003). Additionally, LPA promotes Rab11a interaction with G $\beta\gamma$ , causing PI3K recruitment and AKT phosphorylation (Garcia-Regaldo et al., 2008).

### **1.8. HYPOTHESIS AND SPECIFIC OBJECTIVES**

In recent years, our lab and others have provided increasingly convincing evidence that Rab small G proteins comprise a major component of intracellular trafficking machinery and it is now apparent that GPCR trafficking actively affects signal transduction. Rabs directly associate with, and actively direct GPCR signalling through intracellular localization of receptors and signalling molecules. Our research presented

here further supports the **hypothesis that intracellular trafficking of GPCRs can actively contribute to and alter receptor signal transduction.**

The aim of this thesis is to elucidate the role that receptor trafficking plays in signal transduction by addressing the following three questions:

- 1. Do multiple Rab proteins associate with AT<sub>1</sub>R to alter receptor desensitization or resensitization?**
- 2. Does Rab8 associate with mGluR1 to modulate its intracellular trafficking and signalling?**
- 3. What alterations do previously uncharacterized mutations in mGluR1 exhibit in mGluR1 intracellular localization and signal transduction?**

The data presented in chapters 2, 3 and 4 of this thesis summarize my research aimed at answering important questions regarding the regulation of two prototypic GPCRs.

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## **CHAPTER 2.**

### **RAB GTPASES BIND AT A COMMON SITE WITHIN THE ANGIOTENSIN II TYPE I RECEPTOR CARBOXYL-TERMINAL TAIL: EVIDENCE THAT RAB4 REGULATES RECEPTOR PHOSPHORYLATION, DESENSITIZATION AND RESENSITIZATION<sup>1</sup>**

<sup>1</sup> A version of this chapter has been published: Esseltine JL, Dale LB and Ferguson SS (2011) Rab GTPases bind at a common site within the angiotensin II type I receptor carboxyl-terminal tail: Evidence that Rab4 regulates receptor phosphorylation, desensitization and resensitization. *Mol Pharm* 79:175-84.



## 1.1. INTRODUCTION

The angiotensin II type 1 receptor (AT<sub>1</sub>R) is a member of the G protein-coupled receptor (GPCR) superfamily, the largest family of integral membrane receptors and represents an important pharmacological target for drug therapy in hypertension (Hoffman and Lefkowitz, 1996). The AT<sub>1</sub>R is coupled through G $\alpha_{q/11}$  to the activation of phospholipase C $\beta$  resulting in the formation of diacylglycerol and inositol 1,4,5 trisphosphate leading to the release of intracellular calcium stores and the activation of PKC. Agonist activation of the AT<sub>1</sub>R also results in the attenuation of receptor signalling as the consequence of receptor phosphorylation by GRKs and PKC. Agonist activation and GRK-mediated phosphorylation of the AT<sub>1</sub>R facilitates the recruitment of the cytosolic adaptor protein,  $\beta$ -arrestin, which functions to sterically uncouple the AT<sub>1</sub>R from the heterotrimeric G protein and targets the AT<sub>1</sub>R for clathrin-mediated endocytosis (Benovic et al., 1987; Freedman et al., 1995; Ferguson et al., 1995; Ferguson et al., 1996; Opperman et al., 1996; Krupnick and Benovic, 1998; Ferguson, 2001; Ferguson, 2007). Once internalized, GPCRs may be either sequestered in early endosomes, dephosphorylated and recycled back to the plasma membrane or targeted to lysosomes for degradation (Ferguson, 2001; Gáborik and Hunyady, 2004; Seachrist and Ferguson, 2003). In the case of the AT<sub>1</sub>R, the receptor is internalized as a complex with  $\beta$ -arrestin and is retained in the early endosomal compartment and is not readily dephosphorylated (Anborgh et al., 2000).

The Rab subfamily of small Ras-like GTPases regulate the intracellular trafficking of proteins between intracellular compartments through their ability to regulate vesicular targeting, docking and fusion (Seachrist and Ferguson, 2003; Gáborik

and Hunyady, 2004). Rab protein function is in turn tightly regulated at the level of protein expression, localization, membrane association, and activation. Different Rab isoforms regulate different aspects of intracellular trafficking such as internalization (Rab5), recycling (Rab4 and Rab11) and degradation (Rab7) and different GPCRs are known to preferentially traffic through certain Rab pathways (Seachrist et al., 2000; Hunyady et al., 2002; Seachrist et al., 2002; Dale et al., 2004; Hamelin et al., 2005; Holmes et al., 2006; Wang et al., 2008; Li et al., 2008; Parent et al., 2009). For example, Rab5a has been shown to interact with the AT<sub>1</sub>R carboxyl-terminal tail and retain the receptor in Rab5a-positive early endosomes. Nevertheless, overexpression of either Rab7 or constitutively active Rab11 can redistribute AT<sub>1</sub>R into either Rab7-positive late endosomes or Rab11-positive recycling endosomes, respectively (Seachrist et al., 2000; Dale et al., 2004). Additionally, although AT<sub>1</sub>R is not readily dephosphorylated and efficiently recycled, there is evidence to suggest that the receptor can be recycled via both slow (Rab11-mediated) and rapid (Rab4-mediated) pathways (Hunyady et al., 2002; Li et al., 2008). Rab binding to a GPCR is not unique to the AT<sub>1</sub>R, as Rab11 has been shown to bind to the  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR), thromboxane A<sub>2</sub> receptor and prostacyclin receptor (Seachrist et al., 2002; Hamelin et al., 2005; Parent et al., 2009; Reid et al., 2010). Emerging evidence suggests that Rab interactions with these GPCRs are also critical for regulating both the trafficking and activity of these receptors. For example, previous studies with the  $\beta_2$ AR have shown that the transit of the receptor from the Rab5-positive early endosome to the Rab4-positive recycling endosome is required for the dephosphorylation of the receptor (Seachrist et al., 2000).

In the present study, we have investigated whether other Rab GTPases (Rab4, Rab7 and Rab11) can interact with AT<sub>1</sub>R carboxyl-terminal tail and compete with Rab5 for binding. We report here that Rab4, Rab5, Rab7 and Rab11 each compete for an overlapping site in the last 10 amino acid residues of the AT<sub>1</sub>R carboxyl-terminal tail and that proline residue 354 and Cysteine residue 355 represent important amino acid residues involved in Rab protein binding. Moreover, we find that overexpression of either wild-type or constitutively active Rab4, but not Rab11, promotes AT<sub>1</sub>R dephosphorylation. The overexpression of a constitutively active Rab4 mutant also results in reduced AT<sub>1</sub>R desensitization and promotes AT<sub>1</sub>R resensitization. Taken together, our data indicate that multiple Rab GTPases are able to associate with their cargo and that the activity of the AT<sub>1</sub>R may be regulated by the interaction of different Rab GTPases at the carboxyl-terminal Rab binding site.

## **2.2. EXPERIMENTAL PROCEDURES**

### **2.2.1. Materials**

*myo*-(<sup>3</sup>H)Inositol and (<sup>32</sup>P)orthophosphate were acquired from PerkinElmer Life Sciences (Waltham, MA). Dowex 1-X8 (formate form) resin 200–400 mesh was purchased from BioRad (Mississauga, ON). Rabbit anti-GST, -Rab4 (sc-26562), -Rab5a (sc-312) and -Rab11 (sc-309) antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA) and goat anti-GST as well as ECL Western blotting detection reagents were purchased from GE Healthcare (Oakville, Ontario, Canada). Horseradish peroxidase-conjugated anti-rabbit and anti-goat IgG secondary antibody was from BioRad (Mississauga, ON). QuikChange<sup>TM</sup> site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Rabbit anti-FLAG antibody, M2 anti-FLAG agarose and all other biochemical reagents were

purchased from Sigma-Aldrich (St. Louis, MO).

### **2.2.2. DNA Construction**

An AT<sub>1</sub>R mutants lacking the distal 10 amino acids (AT<sub>1</sub>R-C1) was generated using the QuikChange<sup>TM</sup> Site-directed mutagenesis kit (Stratagene) to introduce a stop codon after residue 319 in the AT<sub>1</sub>R carboxyl-terminal tail. Subsequently, primers were designed for mutagenesis such that amino acid residues within the last 10 amino acid residues of the AT<sub>1</sub>R tail were mutated in pairs to alanine residues using the QuikChange Site-directed mutagenesis kit.

### **2.2.3. Cell Culture**

Human embryonic kidney (HEK) 293 cells were maintained in Eagle's minimal essential medium supplemented with 10% (v/v) heat inactivated fetal bovine serum (Invitrogen, Burlington, ON) and 50 µg/ml gentamicin. Cells seeded in 100 mm dishes were transfected using a modified calcium phosphate method as described previously (Ferguson and Caron, 2004). Following transfection (18 h), the cells were incubated with fresh medium and allowed to recover for 24 hrs for co-immunoprecipitation studies. Otherwise, they were allowed to recover for 6-8 hrs and re-seeded into 24-well dishes and then grown an additional 18 hrs prior to experimentation.

### **2.2.4. Co-Immunoprecipitation**

HEK 293 cells were transiently transfected with FLAG-tagged AT<sub>1</sub>R and either GST-tagged Rab4, Rab4-Q67L, Rab4-S22N, Rab5, Rab7, Rab7-Q67L, Rab7-N125I, Rab11, Rab11-Q70L or Rab11-S25N. Following transfection, the cells were incubated for 20 minutes in HEPES balanced salt solution (HBSS) at 37°C with or without 100 nM AngII.

The cells were then placed on ice, washed two times with ice-cold phosphate-buffered saline (PBS) and lysed with cold-lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 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 15000 x g for 15 min at 4°C to pellet insoluble material. Cleared supernatant containing 250 µg protein were incubated with 25 µL of FLAG M2-affinity beads (Sigma) for 1h rotating at 4°C to immunoprecipitate FLAG-AT<sub>1</sub>R. Following incubation, the beads were washed twice with lysis buffer and twice with PBS, and proteins were solubilized in a 3X SDS sample buffer containing 2-mercaptoethanol (BME). Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane and immuno-blotted to identify co-immunoprecipitated GST-tagged Rab proteins using a primary polyclonal rabbit or goat anti-GST antibody (1:1000 dilution, Santa Cruz, GE Healthcare) followed by a horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:10000, BioRad) or secondary anti-goat (1:2500, BioRad). Receptor and Rab protein expression was determined by immunoblotting 10 µg of protein from each cell lysate used for immunoprecipitation. Proteins were detected using chemiluminescence with the ECL kit from GE Healthcare.

### **2.2.5. Whole cell phosphorylation**

AT<sub>1</sub>R phosphorylation was measured as described previously (Anborgh et al., 2000). HEK 293 cells were transiently transfected with FLAG-AT<sub>1</sub>R along with either pEGFP (control), GFP-tagged Rab4, Rab4Q67L, Rab4S22N, Rab5, Rab5-S34N, Rab5-Q79L, Rab11, Rab11Q70L or Rab11S25N. Seventy-two hours post transfection cells were rinsed twice and incubated at 37°C for one hour in phosphate-free HBSS (5 mM

NaHCO<sub>3</sub>, 20 mM HEPES, 11 mM glucose, 116 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, pH 7.4). Cells were then incubated at 37°C for one hour in 100 µCi/mL [<sup>32</sup>P]orthophosphate, and treated for 10 min with and without 100 nM AngII, rinsed and allowed to recover at 37°C for 0, 20 or 40 min in phosphate-free HBSS. Cells were placed on ice and lysates were collected in the presence of protease inhibitors (0.1 mM PMSF, 10 µg/ml leupeptin, and 5 µg/ml aprotinin) and phosphatase-inhibitors (10mM NaF and 10mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>) and incubated with M2 anti-FLAG affinity agarose for 2-3 hours to immunoprecipitate receptor protein. Beads were washed and bound proteins were solubilized in SDS-PAGE sample buffer. Equal amounts of receptor protein, as determined by protein measurement and flow cytometry were separated by SDS-PAGE and receptor phosphorylation was determined via autoradiography at -80°C.

#### **2.2.6. Measurement of inositol phosphate formation**

Desensitization of AT<sub>1</sub>R signalling of inositol phosphate was measured as described previously (Olivares-Reyes et al., 2001) with some modifications. HEK 293 cells were transiently transfected with the cDNAs as described. Forty-eight hours post-transfection cells were incubated overnight in inositol-free DMEM with 100 µCi/mL *myo*-(<sup>3</sup>H)Inositol. Cells were washed twice and incubated for one hour in warm HBSS then preincubated for 3 min at 37°C in either HBSS (lacking LiCl) alone or with 100 nM AngII (desensitizing stimulus). After a brief acid wash (50 mM glycine, 150 mM NaCl, pH 3.0), cells were washed twice and were then incubated with either 10 mM LiCl alone or 10 mM LiCl with 100 nM AngII for 10 min. The resensitization of AT<sub>1</sub>R-mediated IP<sub>3</sub> formation was assessed in the same fashion except that desensitized cells were allowed to recover for 30 min prior to the second incubation with either 10 mM LiCl

alone or 10 mM LiCl with 100 nM AngII for 10 min. Cells were placed on ice and the reaction was stopped with 500  $\mu$ L of perchloric acid and was neutralized with 400  $\mu$ l of 0.72 M KOH, 0.6 M KHCO<sub>3</sub>. Total cellular (<sup>3</sup>H)inositol incorporation was determined in 50  $\mu$ l of cell lysate. Total inositol phosphate was purified by anion exchange chromatography using Dowex 1-X8 (formate form) 200-400 mesh anion exchange resin and (<sup>3</sup>H)inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system.

### **2.2.7. Statistical Analysis**

Densitometric data were normalized first for protein expression and the maximum value was set to 100, with all other values displayed as percentage thereof. One-way analysis of variance test (ANOVA) was performed to determine significance, followed by a post-hoc Tukey multiple comparison test or Bonferroni's multiple comparisons test to determine which means were significantly different ( $p < 0.05$ ) from one another.

## **2.3. RESULTS**

### **2.3.1. Rab4, Rab5, Rab7 and Rab11 all interact with the AT<sub>1</sub>R**

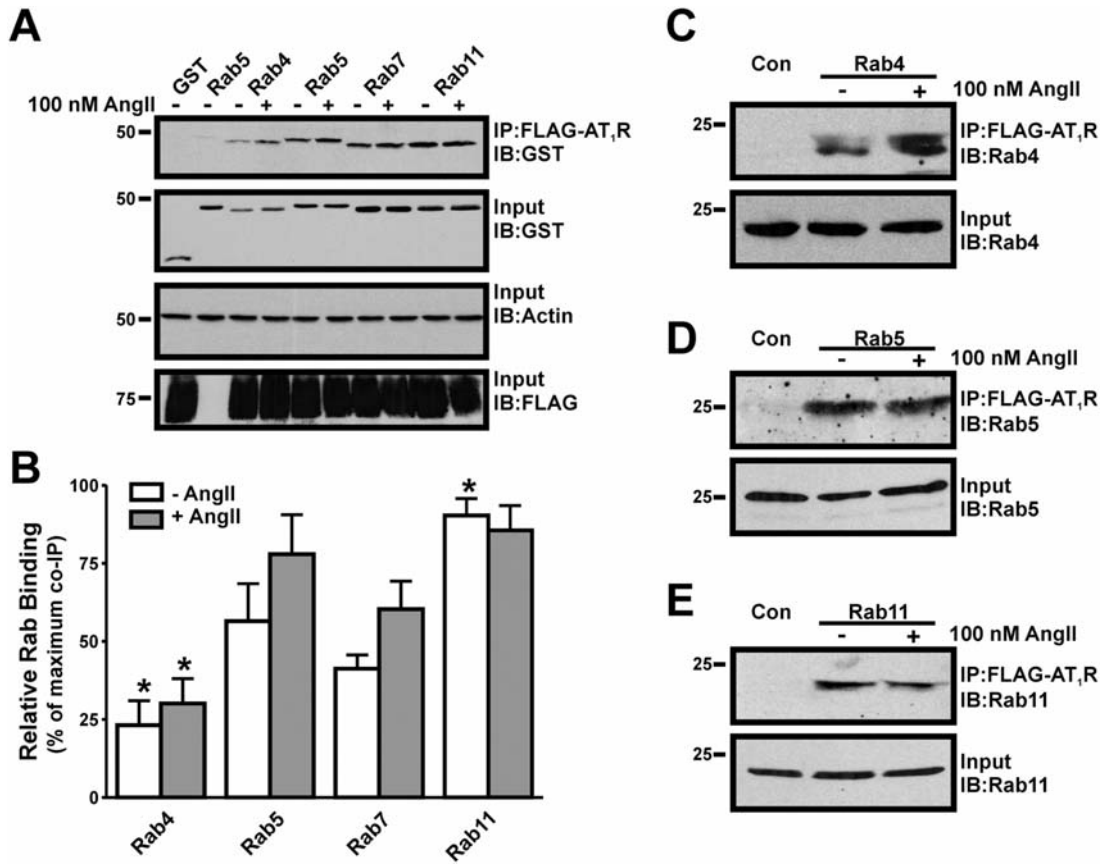
Previous research showed direct association between Rab5a and AT<sub>1</sub>R, as well as co-localization of the AT<sub>1</sub>R in Rab7- and Rab11-positive endosomes following Rab GTPase overexpression (Seachrist et al., 2002; Dale et al., 2004). Thus, we investigated whether Rab binding to the human AT<sub>1</sub>R C-tail was either exclusive to Rab5 or was also observed for Rab4, Rab7 and Rab11. HEK293 cells were transiently transfected with FLAG-AT<sub>1</sub>R and either GST-tagged Rab4, Rab5, Rab7 or Rab11. We find that similar to what we observed previously for Rab5a, each of the GST-Rab4, GST-Rab7 and GST-

Rab11 proteins could be co-immunoprecipitated with the FLAG-AT<sub>1</sub>R from HEK 293 cells (Figure 2.1A and 1B). We found that in the absence of agonist treatment significantly more GST-Rab11 and significantly less Rab4 protein could be co-immunoprecipitated with the FLAG-AT<sub>1</sub>R, when compared to GST-Rab5 (Figure 2.1A and 1B). Treatment of cells with 100 nM AngII to activate the FLAG-AT<sub>1</sub>R resulted in a small and statistically insignificant increase in GST-Rab5 and GST-Rab7 binding to the receptor, but had no effect on the association of either Rab4 or Rab11 (Figure 2.1A and 1B). We also examined whether endogenous Rab4, Rab5 and Rab11 could be co-immunoprecipitated with the FLAG-AT<sub>1</sub>R from HEK 293 cells. We found that Rab4 could be co-immunoprecipitated and that agonist stimulation increased Rab4 co-immunoprecipitation with the FLAG-AT<sub>1</sub>R by  $1.6 \pm 0.3$  fold ( $P < 0.05$ ) (Figure 2.1C). However, agonist treatment had no effect upon the co-immunoprecipitation of either Rab5 or Rab11 with the receptor (Figure 2.1D and 1E).

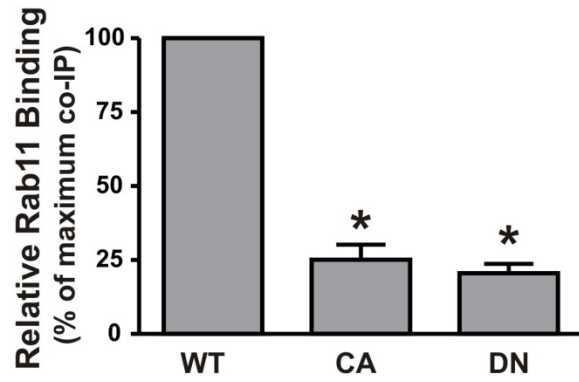
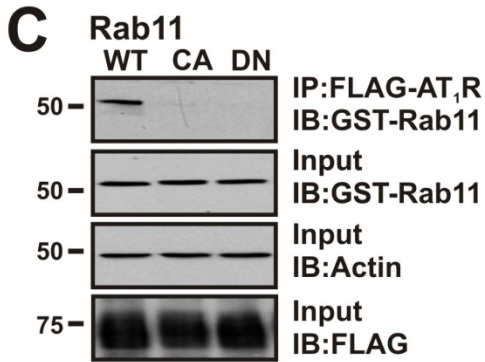
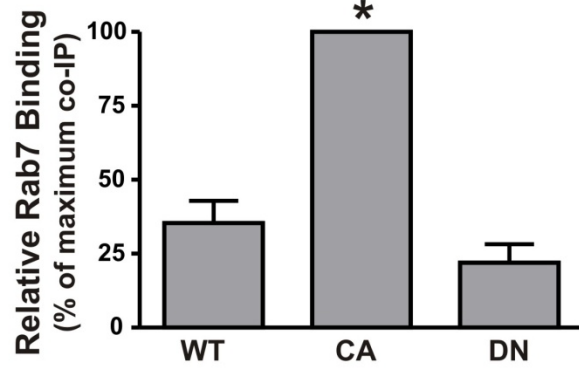
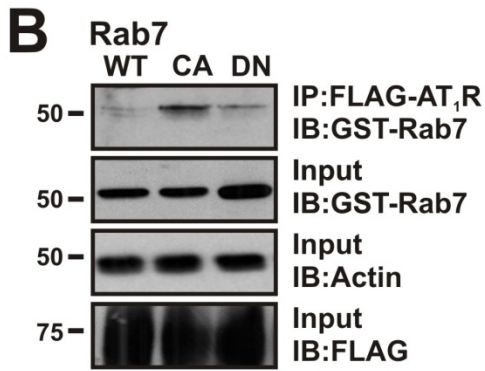
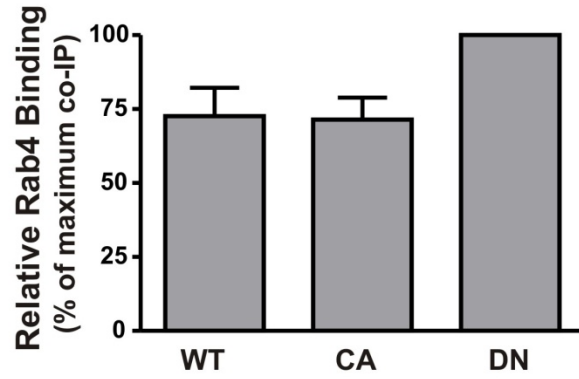
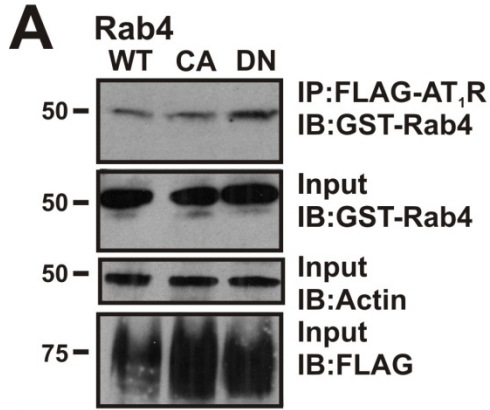
The rat AT<sub>1A</sub>R was previously shown to preferentially bind to the GDP-bound form of Rab5 (Rab5-S34N) and the GDP-bound form of Rab11 interacted specifically with the thromboxane A<sub>2</sub> receptor (Seachrist et al., 2002; Hamelin et al., 2005). We found that wild-type Rab4, dominant-negative Rab4-S22N, and constitutively active Rab4-Q67L did not exhibit a preference for binding to the FLAG-AT<sub>1</sub>R (Figure 2.2A). In contrast, constitutively active Rab7-Q67L mutant exhibited preferential binding to the FLAG-AT<sub>1</sub>R (Figure 2.2B). Unlike what was previously observed for the thromboxane



**Figure 2.1. Rab4, Rab4, Rab7 and Rab11 each co-immunoprecipitate with AT<sub>1</sub>R.** (A) Representative immunoblot showing the co-immunoprecipitation of GST-Rab4, GST-Rab5, GST-Rab7 and GST-Rab11 with the FLAG-AT<sub>1</sub>R from HEK 293 cells in the absence (-) and presence (+) of 100 nM AngII treatment for 20 min. (B) Densitometric analysis of GST-Rab4, GST-Rab5, GST-Rab7 and GST-Rab11 co-immunoprecipitated with the FLAG-AT<sub>1</sub>R from HEK 293 cells in the absence (-) and presence (+) of 100 nM AngII treatment for 20 min. Data represents the mean  $\pm$  SD of 5 independent experiments. Data were normalized for both individual Rab protein expression levels and normalized to maximum Rab protein binding to the AT<sub>1</sub>R in each experiment. \* $p < 0.05$  compared to Rab5 co-immunoprecipitated with the AT<sub>1</sub>R and correspondingly treated. (C) Immunoblot demonstrating the co-immunoprecipitation of endogenous Rab4 protein with the FLAG-AT<sub>1</sub>R from HEK 293 cells in the absence (-) and presence (+) of 100 nM AngII treatment for 20 min. Rab4 co-immunoprecipitated with GFP antibody (Con) is used as a control. Data represents the mean  $\pm$  SD of 4 independent experiments. (D) Immunoblot demonstrating the co-immunoprecipitation of endogenous Rab5 protein with the FLAG-AT<sub>1</sub>R from HEK 293 cells in the absence (-) and presence (+) of 100 nM AngII treatment for 20 min. Rab5 co-immunoprecipitated with GFP antibody (Con) is used as a control. Data represents the mean  $\pm$  SD of 4 independent experiments. (E) Immunoblot demonstrating the co-immunoprecipitation of endogenous Rab11 protein with the FLAG-AT<sub>1</sub>R from HEK 293 cells in the absence (-) and presence (+) of 100 nM AngII treatment for 20 min. Rab11 co-immunoprecipitated with GFP antibody (Con) is used as a control. Data represents the mean  $\pm$  SD of 4 independent experiments.



**Figure 2.2. Co-immunoprecipitation of wild-type, dominant negative and constitutively active Rab4, Rab7 and Rab11 GTPases with the AT<sub>1</sub>R.** (A) Representative immunoblot and densitometric analysis showing the co-immunoprecipitation of GST-Rab4 (WT), constitutively active GST-Rab4-Q67L (CA) and dominant-negative GST-Rab4-S22N (DN) with FLAG-AT<sub>1</sub>R from HEK 293 cells. (B) Representative immunoblot and densitometric analysis showing the co-immunoprecipitation of GST-Rab7 (WT), constitutively active GST-Rab7-Q67L (CA) and dominant-negative GST-Rab7-N125I (DN) with FLAG-AT<sub>1</sub>R from HEK 293 cells. \*p< 0.05 compared to wild-type Rab7 co-immunoprecipitated with the AT<sub>1</sub>R. (C) Representative immunoblot and densitometric analysis showing the co-immunoprecipitation of GST-Rab11 (WT), constitutively active GST-Rab11-Q70L (CA) and dominant-negative GST-Rab7-S25N (DN) with FLAG-AT<sub>1</sub>R from HEK 293 cells. \*p< 0.05 compared to wild-type Rab11 co-immunoprecipitated with the AT<sub>1</sub>R. Data represents the mean ± SD of 3-5 independent experiments. All data were normalized for individual Rab protein expression levels in each experiment.

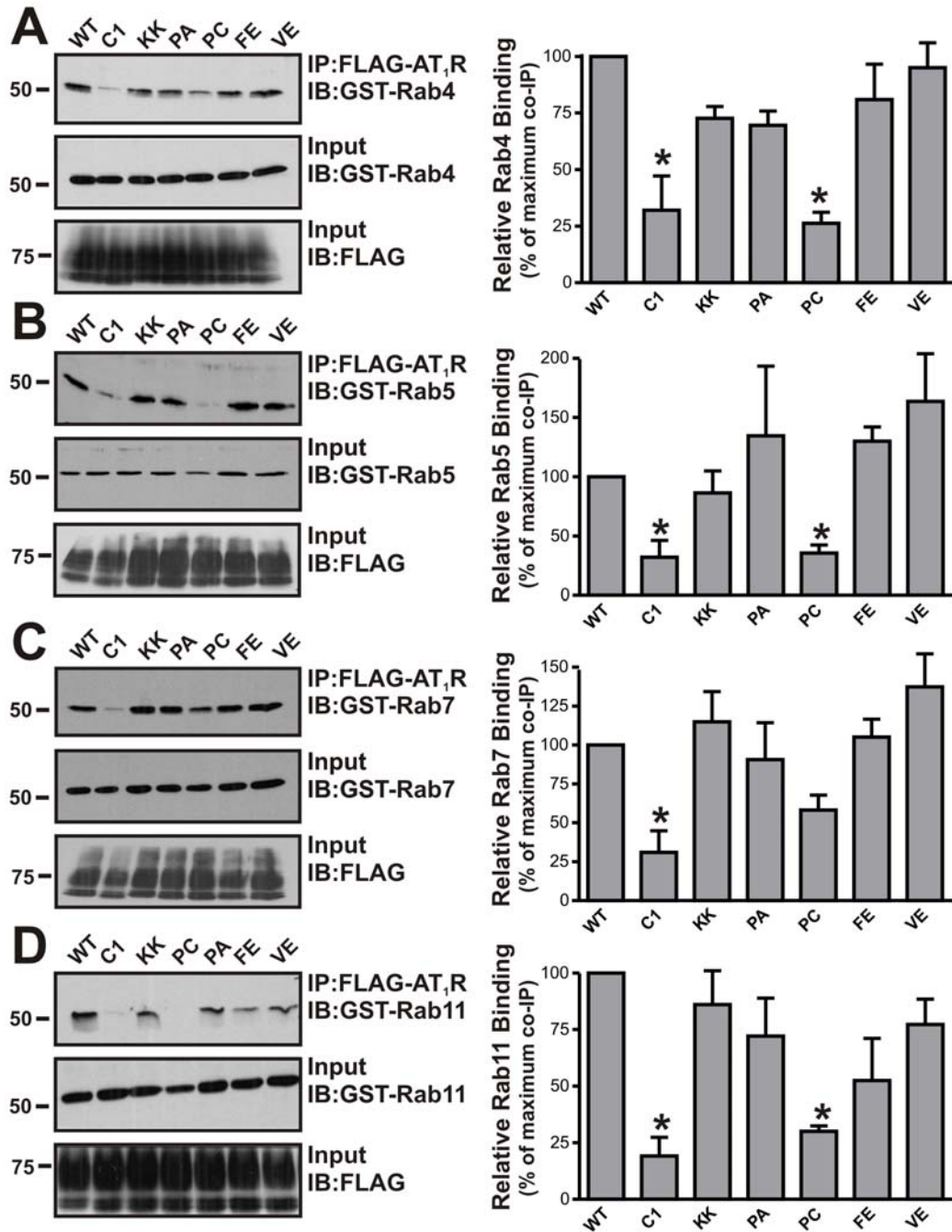


A2 receptor wild-type Rab11 interacted with the FLAG-AT<sub>1</sub>R, but both constitutively active Rab11-Q70L and dominant-negative Rab11-S25N mutants did not effectively interact with FLAG-AT<sub>1</sub>R (Figure 2.2C). This observation suggests that GTP hydrolysis is required for Rab11 binding to the AT<sub>1</sub>R. Taken together, the data indicated that Rab4, Rab5, Rab7 and Rab11 each bind to the AT<sub>1</sub>R but that the association of each of the Rab GTPases was mediated by different activation states of the GTPases.

### **2.3.2. Identification of the AT<sub>1</sub>R Rab GTPase binding site**

Previously, we demonstrated that the deletion of the last 10 amino acid residues of the rat AT<sub>1A</sub>R C-tail (AT<sub>1A</sub>R-C1) resulted in a loss of AT<sub>1A</sub>R colocalization with GFP-Rab5a (Dale et al., 2004). Therefore, we tested whether the deletion of the distal 10 amino acid residues of the human AT<sub>1</sub>R would result in both the loss of Rab5 binding, as well as a loss of Rab4, Rab7 and Rab11 binding to a human FLAG-AT<sub>1</sub>R-C1 construct. We found that the deletion of the last 10 amino acid residues resulted in a significant decrease in Rab4, Rab5, Rab7 and Rab11 protein that was co-immunoprecipitated with the FLAG-AT<sub>1</sub>R-C1 mutant (Figure 2.3A-D). Therefore, we examined which amino acid residues localized with the distal AT<sub>1</sub>R C-tail sequence KKPAPCFEVE were required for Rab4, Rab5, Rab7 and Rab11 binding to the receptor by performing alanine scanning mutagenesis of pairs of amino acid residues (Figure 2.3A). We found that Rab4, Rab5, Rab7 and Rab11 binding to FLAG-AT<sub>1</sub>R-KK, FLAG-AT<sub>1</sub>R-PA, FLAG-AT<sub>1</sub>R-FE and FLAG-AT<sub>1</sub>R-VE mutant receptors was unaffected by alanine substitutions at the corresponding residues (Figure 2.3A-D). In contrast, Rab4, Rab5, and Rab11 were not co-immunoprecipitated effectively with the FLAG-AT<sub>1</sub>R-PC alanine substitution mutant (Figure 2.3A, 2.3B and 2.3D). Although Rab7 binding to the FLAG-AT<sub>1</sub>R-PC alanine

**Figure 2.3. Identification of the Rab GTPase binding site within the AT<sub>1</sub>R carboxyl-terminal tail.** (A) Representative immunoblot showing the co-immunoprecipitation of Rab4 with either the wild-type AT<sub>1</sub>R (WT) or AT<sub>1</sub>R-C1 (1-349), AT<sub>1</sub>R-K350A/K351A (KK), AT<sub>1</sub>R-P352A/A353G (PA), AT<sub>1</sub>R-P354A/C355A (PC), AT<sub>1</sub>R-F356A/E357A (FE), and AT<sub>1</sub>R-V358A/E359A (VE) mutants from HEK 293 cells. (B) Representative immunoblot showing the co-immunoprecipitation of Rab5 with either the wild-type AT<sub>1</sub>R (WT) or AT<sub>1</sub>R mutants from HEK 293 cells. (C) Representative immunoblot showing the co-immunoprecipitation of Rab7 with either the wild-type AT<sub>1</sub>R (WT) or AT<sub>1</sub>R mutants from HEK 293 cells. (D) Representative immunoblot showing the co-immunoprecipitation of Rab11 with either the wild-type AT<sub>1</sub>R (WT) or AT<sub>1</sub>R mutants from HEK 293 cells. Data represents the mean  $\pm$  SD of 3-5 independent experiments. Data were normalized for both individual Rab protein expression levels and wild-type Rab protein binding to the AT<sub>1</sub>R in each experiment. \*p< 0.05 compared to wild-type Rab co-immunoprecipitated with the AT<sub>1</sub>R.



substitution mutant was reduced, binding was not statistically significantly different from control (Figure 2.3C). None of the alanine substitutions to the AT<sub>1</sub>R C-tail affected the coupling of the AT<sub>1</sub>R to the activation of IP<sub>3</sub> formation (Figure 2.4). Taken together, the data suggested that proline residue 354 and Cysteine residue 355 played an important role in the binding of the Rab4, Rab5, Rab7 and Rab11 GTPases to the AT<sub>1</sub>R and that each of these different Rab GTPases bind to the same site on the receptor.

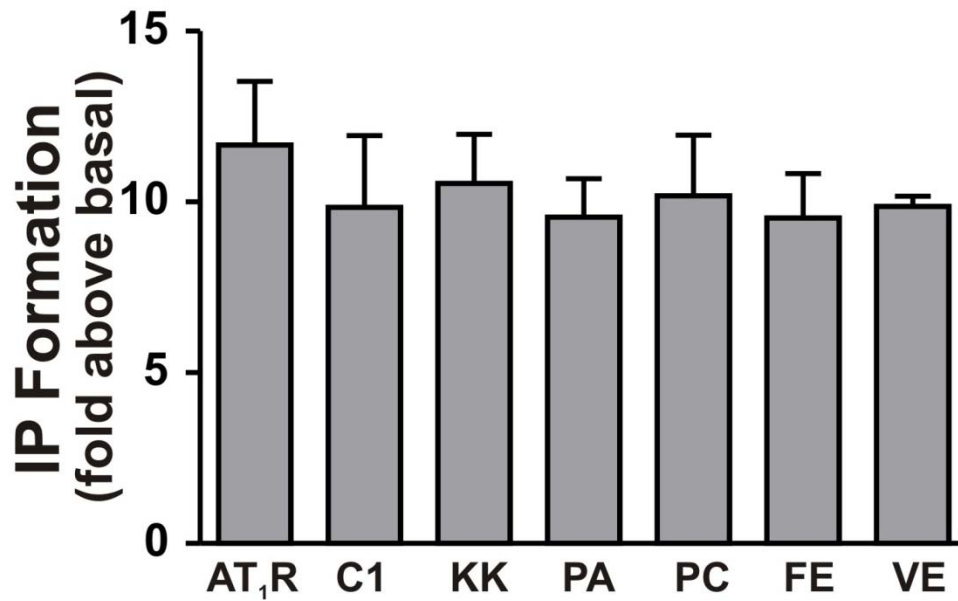
### **2.3.3. Rab GTPases compete with each other for association with AT<sub>1</sub>R**

Because Rab4, Rab5, and Rab11 interact with an overlapping site in the AT<sub>1</sub>R C-tail and the overexpression of constitutively active Rab7 and Rab11 was previously shown to alter the intracellular trafficking of the receptor (Dale et al., 2004), we examined whether Rab GTPases compete for binding to the AT<sub>1</sub>R. We found that the co-immunoprecipitation of GST-Rab5 with the FLAG-AT<sub>1</sub>R could be antagonized by the overexpression of increasing amounts of HA-Rab11 protein (Figure 2.5A). Moreover, despite the fact that GST-Rab4 was apparently a weak FLAG-AT<sub>1</sub>R-interacting protein, the overexpression of HA-Rab4 effectively prevented GST-Rab11 co-immunoprecipitation with FLAG-AT<sub>1</sub>R in an expression-dependent manner (Figure 2.5B). Unexpectedly, increasing expression levels of HA-Rab11 did not result in the attenuation of GST-Rab4 binding to FLAG-AT<sub>1</sub>R (Figure 2.5C).

### **2.3.4. Rab4 but not Rab11 affects the phosphorylation state and desensitization of AT<sub>1</sub>R signalling**

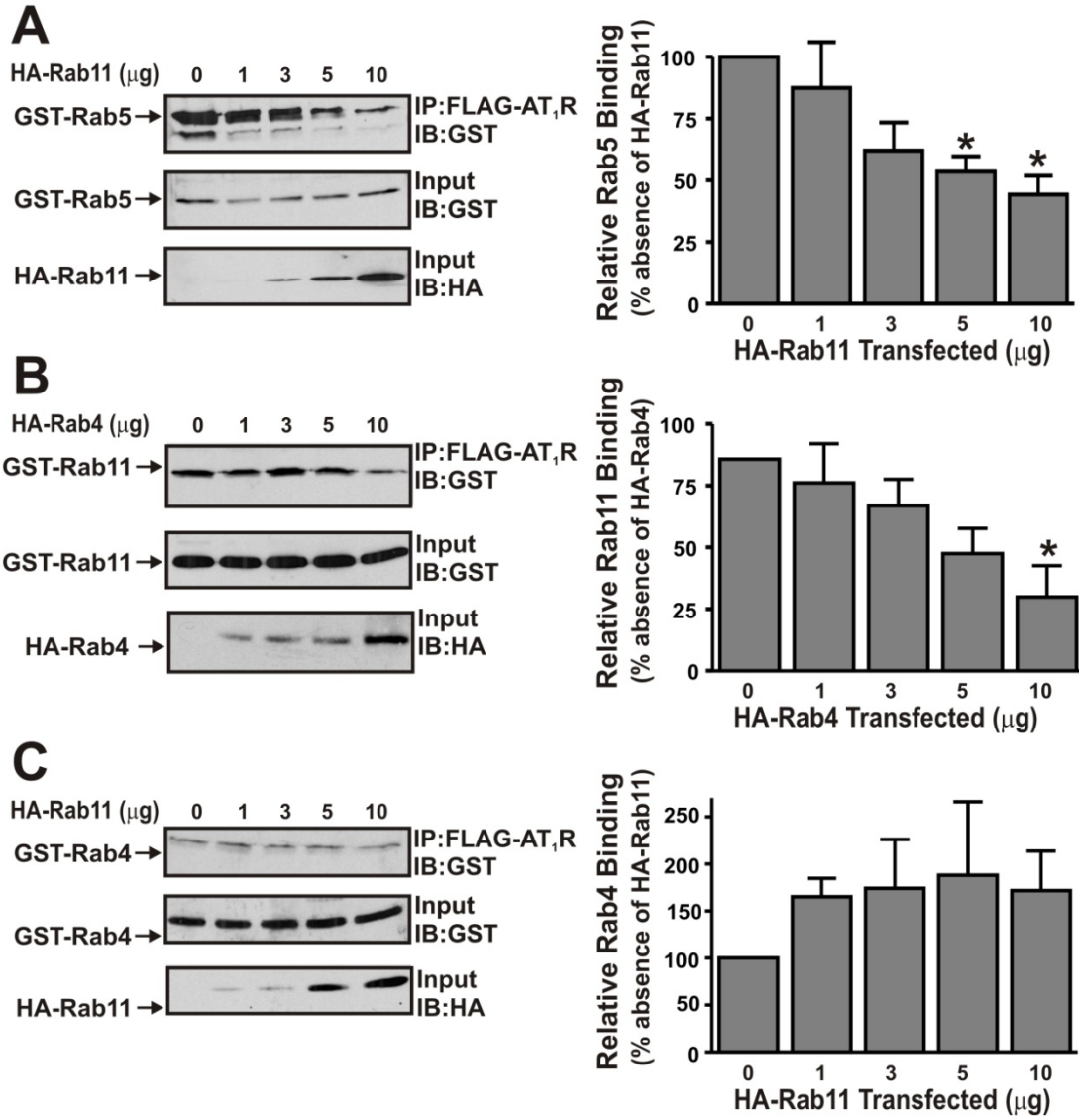
Because Rab 4, Rab5 and Rab11 GTPases appeared to compete for a common binding site on the carboxyl-terminal tail of the AT<sub>1</sub>R, we examined whether the overexpression of wild-type, dominant-negative and constitutively active Rab4, Rab5 and





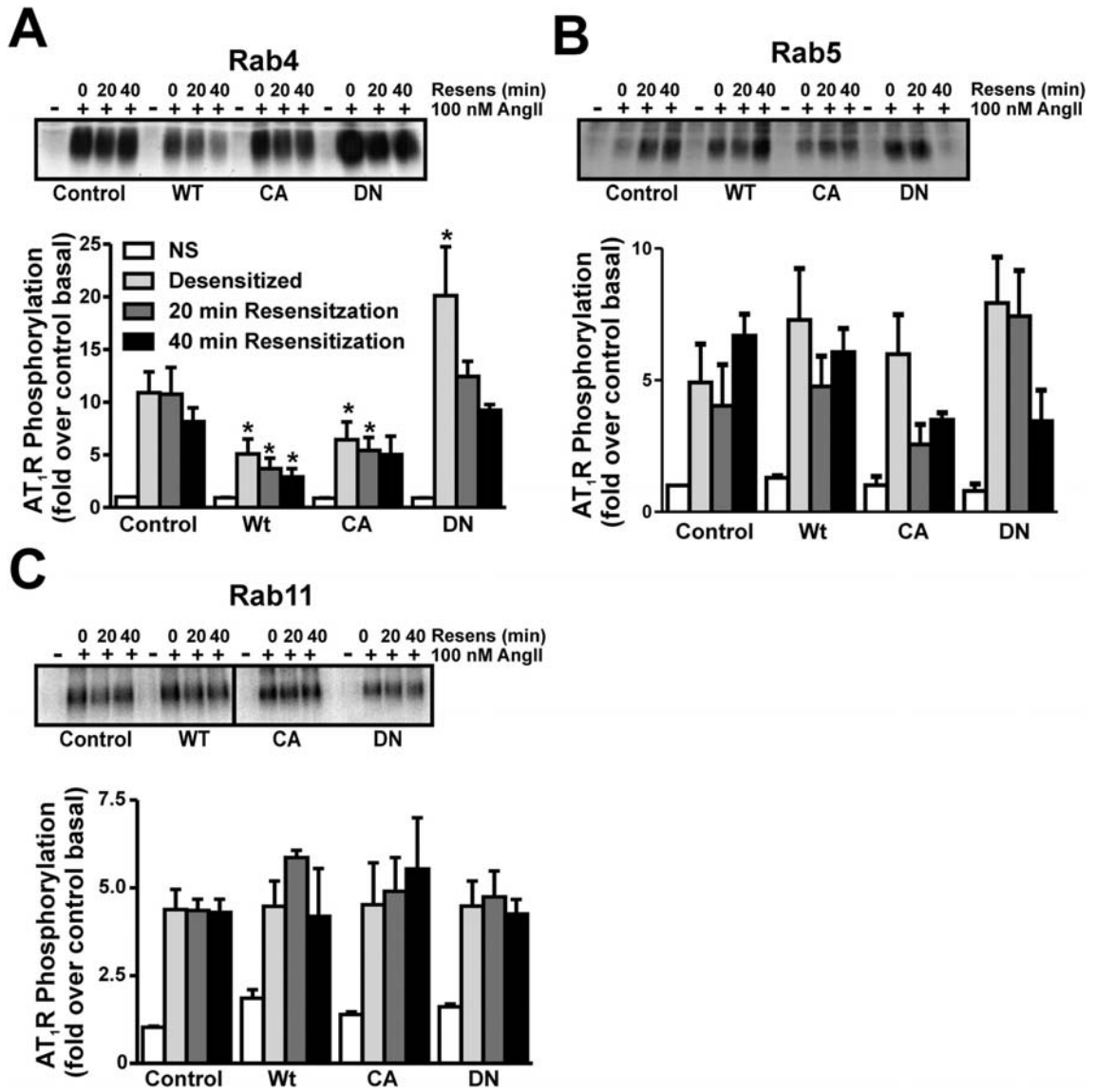
**Figure 2.4. Agonist-stimulated AT<sub>1</sub>R inositol phosphate formation.** Shown is agonist-stimulated (100 nM AngII, 10 min) inositol phosphate formation mediated by either the wild-type FLAG-AT<sub>1</sub>R (WT) or FLAG-AT<sub>1</sub>R-C1 (1-349), FLAG-AT<sub>1</sub>R-K350A/K351A (KK), FLAG-AT<sub>1</sub>R-P352A/A353G (PA), FLAG-AT<sub>1</sub>R-P354A/C355A (PC), FLAG-AT<sub>1</sub>R-F356A/E357A (FE), and FLAG-AT<sub>1</sub>R-V358A/E359A (VE) mutants from HEK 293 cells. Data represents the mean  $\pm$  SD of 3 independent experiments.

**Figure 2.5. Competition between Rab GTPases for co-immunoprecipitation with FLAG-AT<sub>1</sub>R.** (A) Representative immunoblots and densitometric analysis of the co-immunoprecipitation of GST-Rab5 with FLAG-AT<sub>1</sub>R in the absence or presence of increasing amounts of HA-Rab11. \*p< 0.05 compared GST-Rab5 co-immunoprecipitated with the AT<sub>1</sub>R in the absence of HA-Rab11. (B) Representative immunoblots and densitometric analysis of the co-immunoprecipitation of GST-Rab11 with FLAG-AT<sub>1</sub>R in the absence or presence of increasing amounts of HA-Rab4. \*p< 0.05 compared to GST-Rab11 co-immunoprecipitated with the AT<sub>1</sub>R in the absence of HA-Rab4. (C) Representative immunoblots and densitometric analysis of the co-immunoprecipitation of GST-Rab4 with FLAG-AT<sub>1</sub>R in the absence or presence of increasing amounts of HA-Rab11. \*p< 0.05 compared to GST-Rab4 co-immunoprecipitated with the AT<sub>1</sub>R in the absence of HA-Rab11. Data represents the mean ± SD of 3-5 independent experiments. Data were normalized for both GST-Rab protein expression levels and GST-Rab protein binding to the AT<sub>1</sub>R in absence of HA-Rab.



Rab11 mutants might lead to altered AT<sub>1</sub>R phosphorylation and dephosphorylation. Consistent with previous studies (Opperman et al., 1996; Anborgh et al., 2000), agonist-stimulation of the AT<sub>1</sub>R for 10 min effectively promoted the phosphorylation of the AT<sub>1</sub>R (Figure 2.6A-C). However, when agonist was washed out for 20 and 40 min, no dephosphorylation of the AT<sub>1</sub>R was observed under control conditions (Figure 2.6A-C). In contrast, the overexpression of either wild-type Rab4 or constitutively active Rab4-Q67L significantly reduced the extent of agonist-stimulated AT<sub>1</sub>R phosphorylation (Figure 2.6A). Consistent with a role of Rab4 in promoting AT<sub>1</sub>R dephosphorylation, overexpression of a dominant-negative Rab4-S22N mutant resulted in a significant increase in agonist-stimulated AT<sub>1</sub>R phosphorylation, which was reduced to phosphorylation levels observed in control cells following agonist washout (Figure 2.6A). The overexpression of wild-type Rab5 had no effect on either AT<sub>1</sub>R phosphorylation or dephosphorylation (Figure 2.6B). However, the overexpression of either constitutively active Rab5-Q79L or dominant-negative Rab5-S34N appeared to result in a trend towards increased dephosphorylation of the receptor the results did not reach statistical significance. The extent of agonist-stimulated AT<sub>1</sub>R phosphorylation when compared to control cells was not altered by the overexpression of either wild-type, dominant-negative Rab11-S25N or constitutively active Rab11-Q67L and none of the Rab11 proteins resulted in AT<sub>1</sub>R dephosphorylation following agonist washout (Figure 2.6C). Given that wild-type Rab4 and constitutively active Rab4-Q67L lead to decreased AT<sub>1</sub>R phosphorylation, we examined whether the expression of either wild-type or dominant-negative Rab4, Rab5 and Rab11 would alter the desensitization and resensitization of the AT<sub>1</sub>R. To assess AT<sub>1</sub>R desensitization, cells were pretreated with 100 nM AngII for 3

**Figure 2.6. Whole cell phosphorylation of AT<sub>1</sub>R in the presence and absence of wild-type and mutant Rab4 and Rab11.** (A) Representative autoradiograph and densitometric analysis of AT<sub>1</sub>R phosphorylation in absence (control) and presence of wild-type Rab4 (WT), constitutively active Rab4-Q67L (CA), and dominant-negative Rab4-S22N (DN) mutants. HEK 293 cells expressing FLAG-AT<sub>1</sub>R were treated with 100 nM AngII for 10 min, washed and allowed to recover for 0 (desensitization), 20 (resensitized) and 40 (resensitized) min. Data represents the mean  $\pm$  SD of 6 independent experiments. \*p< 0.05 compared to corresponding control. (B) Representative autoradiograph and densitometric analysis of AT<sub>1</sub>R phosphorylation in absence (control) and presence of wild-type Rab5 (WT), constitutively active Rab5-Q79L (CA), and dominant-negative Rab4-S34N (DN) mutants. Data represents the mean  $\pm$  SD of 4 independent experiments. \*p< 0.05 compared to corresponding control. (C) Representative autoradiograph and densitometric analysis of AT<sub>1</sub>R phosphorylation in absence (control) and presence of wild-type Rab11 (WT), constitutively active Rab11-Q70L (CA), and dominant-negative Rab11-S25N (DN) mutants. Data represents the mean  $\pm$  SD of 4 independent experiments. \*p< 0.05 compared to corresponding control.



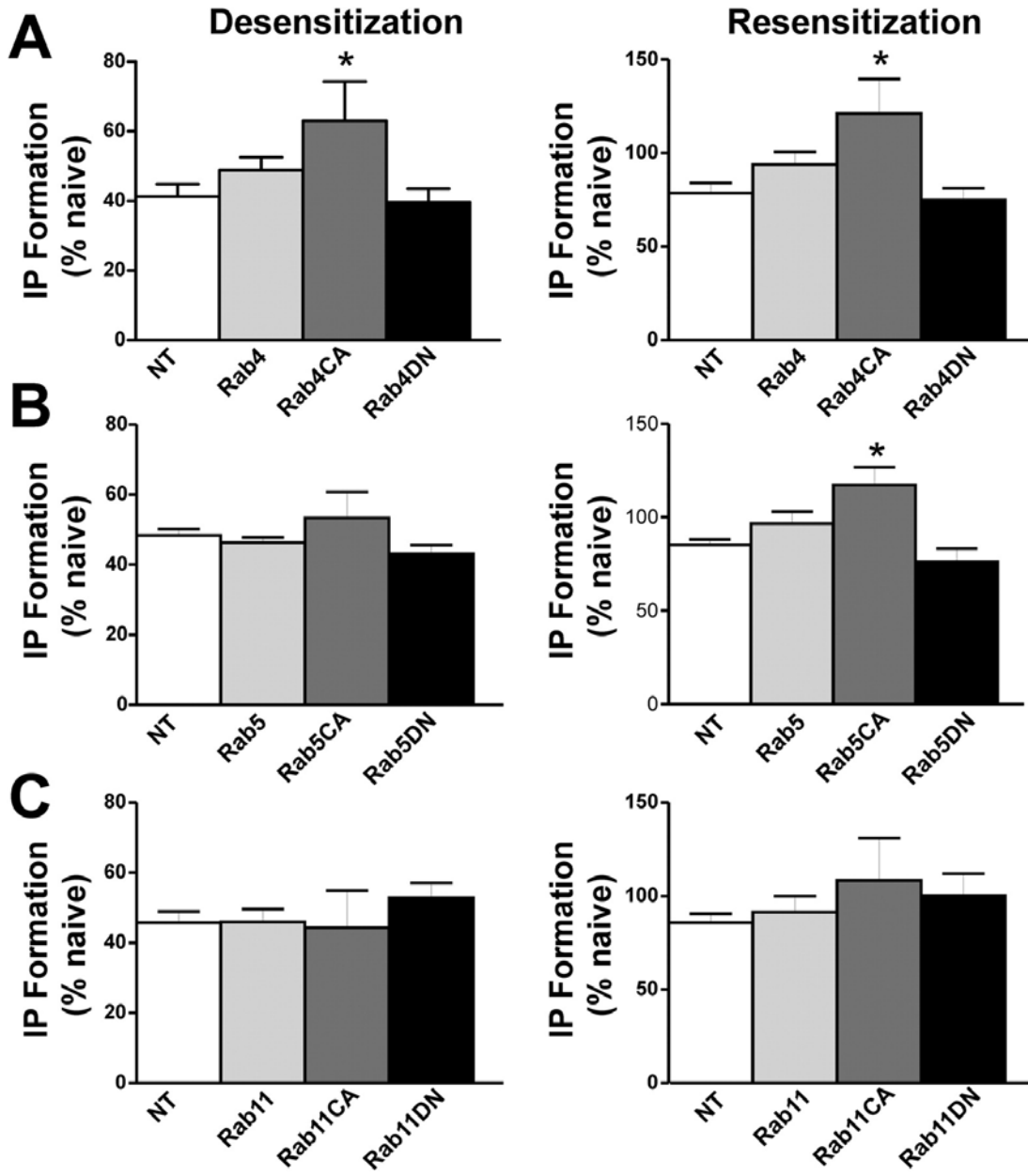
min in HBSS lacking LiCl (desensitizing stimulus), washed and then treated with and without AngII for 10 min in HBSS containing LiCl. Receptor resensitization of AT<sub>1</sub>R-mediated IP<sub>3</sub> responses was measured in the same way except that cells were allowed to recover in the absence of agonist for 30 min prior to being subjected to a second round of agonist treatment. The pretreatment of control cells (desensitizing stimulus) reduced AT<sub>1</sub>R-stimulated IP<sub>3</sub> formation to between  $41 \pm 4\%$  and  $48 \pm 2\%$  of control (naïve) response when cells were exposed to a subsequent 10 min exposure to AngII (Figure 2.7A-C). The overexpression of constitutively active Rab4-Q67L significantly reduced the extent of AT<sub>1</sub>R desensitization and increased the extent of AT<sub>1</sub>R resensitization (Figure 2.7A). The overexpression of the constitutively active Rab5-Q67L mutant did not alter AT<sub>1</sub>R desensitization, but facilitated the resensitization response (Figure 2.7B). None of the other Rab constructs had any effect on AT<sub>1</sub>R desensitization and resensitization. Taken together these results indicate that Rab4 binding, but not Rab11 binding, to the AT<sub>1</sub>R carboxyl-terminal tail alters the phosphorylation status of the AT<sub>1</sub>R leading to reduced AT<sub>1</sub>R desensitization.

## 2.4. DISCUSSION

In the present study we have investigated whether multiple Rab GTPases might associate with the carboxyl-terminal tail of the AT<sub>1</sub>R tail and influence the activity and function of the receptor. We find that Rab4, Rab5, Rab7 and Rab11 each exhibit the capacity to bind to the distal 10 amino acids of the AT<sub>1</sub>R carboxyl-terminal tail and can compete with one another for binding. Previously, we demonstrated that the AT<sub>1</sub>R preferentially associated with the GDP-bound form of Rab5. We show here that the AT<sub>1</sub>R does not distinguish between GDP- and GTP-bound forms of Rab4, binds

**Figure 2.7. Desensitization and resensitization of AT<sub>1</sub>R-mediated inositol phosphate formation.** (A) HEK 293 cells transfected with FLAG-AT<sub>1</sub>R with empty pEBG vector (NT), wild-type Rab4 (WT), constitutively active Rab4-Q67L (CA), and dominant-negative Rab4-S22N (DN) mutants. (B) HEK 293 cells transfected with FLAG-AT<sub>1</sub>R with empty pEBG vector (NT), wild-type Rab5 (WT), constitutively active Rab5-Q79L (CA), and dominant-negative Rab4-S34N (DN) mutants. (C) HEK 293 cells transfected with FLAG-AT<sub>1</sub>R with empty pEBG vector (NT), wild-type Rab11 (WT), constitutively active Rab11-Q70L (CA), and dominant-negative Rab11-S25N (DN) mutants. Transfected cells were treated either with or without 100 nM AngII for 3 min in the absence of LiCl (desensitizing stimulus) and then either washed and subjected to a second treatment of 100 nM AngII for 10 min in the presence of LiCl (desensitized) or washed and allowed to recover for 30 min prior to a second treatment of 100 nM AngII for 10 min in the presence of LiCl (resensitized). Data were normalized for protein expression and basal IP<sub>3</sub> formation and desensitized and resensitized IP<sub>3</sub> responses compared to naive control cells that were not subjected to desensitizing stimulus. Data are representative of 5 independent experiments. \*p < 0.05 compared to corresponding control.





preferentially to GTP-bound Rab7 and interacts with wild-type Rab11 and does not associate with either constitutively active or dominant negative Rab11 mutants. We have also identified two amino acid residues (proline 354 and Cysteine 355) within the Rab binding domain of the AT<sub>1</sub>R carboxyl-terminal tail that are essential for the association of Rab4, Rab5 and Rab11 but not Rab7. The association of different Rab GTPases with the AT<sub>1</sub>R carboxyl-terminal tail has different functional outcomes, with Rab5 promoting the retention of the AT<sub>1</sub>R in early endosomes (Seachrist et al., 2002), Rab7 facilitating the trafficking of the AT<sub>1</sub>R to lysosomes (Dale et al., 2004) and Rab4 promoting the dephosphorylation and resensitization of the receptor. Taken together, our data indicate that the association of different Rab GTPases with the carboxyl-terminal tail domain of the AT<sub>1</sub>R may regulate different functional outcomes for AT<sub>1</sub>R signalling in tissues that may express differing levels of each of the relevant Rab GTPases as the overexpression of a constitutively active Rab4-Q67L mutant decreases AT<sub>1</sub>R desensitization, while facilitating resensitization.

In the current study, we have demonstrated that the domain required for Rab GTPase interactions with AT<sub>1</sub>R are identical for Rab4, Rab5, and Rab11. Previously, we identified that the Rab5 binding domain resides within the distal 10 amino acids of AT<sub>1</sub>R carboxyl-terminal tail and that deletion of this motif resulted in altered AT<sub>1</sub>R trafficking to lysosomes as opposed to the retention of the receptor in early endosomes (Dale et al., 2004). We have further defined the critical residues required for Rab GTPase binding to the AT<sub>1</sub>R and show that proline 354 and Cysteine 355 are essential for binding Rab4, Rab5, and Rab11. Previously, it has been shown that the dephosphorylation and resensitization of the  $\beta_2$ AR occurs as the receptor transits between the Rab5-positive

early endosome and the Rab4-positive rapid recycling endosome (Seachrist et al., 2000). Moreover, it has been reported that phosphorylated  $\mu$ -opioid receptor is preferentially recycled through Rab4-positive endosomes (Wang et al., 2008). We find here, that the overexpression of a constitutively active Rab4-Q67L mutant decreases both AT<sub>1</sub>R phosphorylation and desensitization, while promoting the resensitization of the receptor. Thus, these data are consistent with the hypothesis that the Rab4-positive recycling endosome functions as the compartment in which GPCR dephosphorylation is mediated by phosphatases.

Several GPCRs have now been reported to associate with Rab GTPases including the  $\beta_2$ AR, thromboxane A<sub>2</sub> receptor and prostacyclin receptor (Hamelin et al., 2005; Parent et al., 2009; Reid et al., 2010). However, the residues that we have identified to be essential for Rab GTPase binding to the AT<sub>1</sub>R are not conserved in any of these GPCRs. Rab11 binding to the thromboxane A<sub>2</sub> receptor is mediated by residues 335-345 that are localized within the central region of the thromboxane A<sub>2</sub> receptor carboxyl-terminal tail and Rab11 binds  $\alpha$ -helix 8 at the proximal end of the prostacyclin receptor. In contrast, Rab11 binding to the  $\beta_2$ AR involves a bipartite binding motif, with arginine 333 and lysine 348 representing the essential amino acid residues mediating Rab11 binding to the receptor (Parent et al., 2009). Thus, to date there is no clearly defined consensus motif for Rab GTPase association with GPCRs. However, previous work from our laboratory using yeast two hybrid screen suggest that the regional of the AT<sub>1A</sub>R carboxyl-terminal tail that is proximal to the seventh transmembrane spanning domain of the AT<sub>1A</sub>R may also be involved in Rab5 binding (Seachrist et al., 2002). Thus, the fact that we do not observe complete loss of binding of the Rab GTPases to the carboxyl-terminal tail of the

receptor suggests that secondary residues within the membrane proximal domain of the receptor likely also contribute in part to Rab protein binding.

Rab GTPases not only influence the intracellular trafficking and recycling of GPCRs by directly interacting with these vesicular cargo proteins, but Rab GTPases also indirectly influence the trafficking of receptors between intracellular compartments as a consequence of their intrinsic activity. Following their internalization, many GPCRs have been shown to either recycle to the cell surface via the Rab4-mediated rapid pathway directly from sorting endosomes or via the Rab11-mediated slow pathway from perinuclear recycling endosomes. The recycling of other GPCRs, including the corticotrophin releasing factor receptor 1, somatostatin-3 receptor, vasopressin V2 receptor, neurokinin-1 receptor, chemokine CXCR2, m4 muscarinic acetylcholine receptor and protease receptor, are also differentially regulated by Rab4 and Rab11 (Innamorati et al., 2001; Kreuzer et al., 2001; Schmidlin et al., 2001; Signoret et al., 2001; Fan et al., 2002; Volpicelli et al., 2002; Roosterman et al., 2003; Holmes et al., 2006). Thus, potential alterations in individual Rab GTPase protein expression may have profound effects on GPCR activity. This could occur as the consequence of either direct competition for GPCR binding or by increasing the relative efficiency of the intracellular trafficking and membrane fusion of vesicular compartments within the cell that is regulated by the Rab GTPase. Rab GTPase protein expression and activity has been demonstrated to be regulated by a number of different signals. First, Rab1, Rab4 and Rab6 protein expression is altered in dilated cardiomyopathy model of heart failure and overexpression of Rab4 in the heart leads to altered  $\beta_2$ AR desensitization and resensitization (Wu et al., 2001; Odley et al., 2004). Second, parasitic infection of

cardiomyocytes *in vitro* with the protozoan *Tyranosoma cruzi* results in the downregulation of both Rab7 and Rab11 protein expression (Batista et al., 2006). Finally, insulin is able to stimulate GTP-loading of Rab11 in cardiomyocytes indicating the potential of Rab GTPases to serve as substrates for GPCR activated kinases such as phosphatidylinositol 3-kinase (Schwenk and Eckel, 2007). Thus, taken together alterations in Rab GTPase expression and activity have the potential to both directly and indirectly influence GPCR signalling under both physiological and pathophysiological conditions suggesting that these proteins may represent targets for the treatment of cardiovascular-related diseases.

In HEK 293 cells, the AT<sub>1</sub>R is internalized to and retained in early endosomes, where it remains phosphorylated and does not recycle to the plasma membrane (Anborgh et al., 2000; Seachrist et al., 2002; Dale et al., 2004). We find that the overexpression of different Rab GTPases can specifically alter the intracellular trafficking fate of the AT<sub>1</sub>R with Rab7 overexpression favouring the trafficking of the receptor to lysosomes and Rab4 overexpression favouring the dephosphorylation of the receptor. In contrast, although Rab11 effectively interacts with the AT<sub>1</sub>R, the interaction of the wild-type Rab11 does not influence the dephosphorylation of the receptor, although it can promote plasma membrane recycling (Dale et al., 2004). Interestingly, Rab4 is able to effectively displace Rab11 binding to the AT<sub>1</sub>R, despite the observation that Rab11 is more effectively co-immunoprecipitated with the receptor. Therefore, even small differences in Rab4 expression may lead to profound changes in AT<sub>1</sub>R activity. However, Rab binding to the AT<sub>1</sub>R, if competitive, should be reciprocal and Rab11 protein expressed at sufficiently high levels should be able to compete for binding. It is possible that in our

experiments we have not achieved Rab11 expression that can displace Rab4 from the receptor at complimentary expression levels. Moreover, the overexpression of one Rab protein may shift the receptor from one cellular compartment to another that is not available to the competing Rab protein. It is also possible that Rab GTPases selectively bind to different receptor sites depending upon their activation state, since wild-type Rab7 does not bind to the receptor as effectively as Rab7-Q70L and wild-type Rab7 binding is not significantly impaired when the AT<sub>1</sub>R C-tail is truncated. This may explain why we previously observed that truncation of the AT<sub>1</sub>R C-tail resulted in the targeting of the receptor to endosomes (Dale et al., 2004). Nevertheless, depending on the complement of Rab GTPases expressed in different tissue and cell types, it is likely that the AT<sub>1</sub>R will exhibit differences in its functional regulation ranging from prolonged desensitization associated with impaired dephosphorylation and resensitization to rapid resensitization associated with receptor dephosphorylation.

To date, few GPCRs, including the AT<sub>1</sub>R,  $\beta_2$ AR, thromboxane A<sub>2</sub> receptor and prostacyclin receptor have been shown to directly associate with members of the Rab family. Emerging evidence suggests that these interactions are critical to proper trafficking and regulation of these receptors. Understanding the role of Rabs in the regulation of GPCR redistribution into different intracellular compartments will serve to improve our understanding of the molecular and physiological consequences of GPCR signalling. It is now evident that multiple small GTP-binding proteins, including Rabs interact with GPCRs and future studies should reveal whether GPCRs either interact with or regulate additional components of the intracellular trafficking machinery.

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## CHAPTER 3

### **RAB8 MODULATES METABOTROPIC GLUTAMATE RECEPTOR SUBTYPE 1 INTRACELLULAR TRAFFICKING AND SIGNALLING IN A PKC-DEPENDENT MANNER<sup>1</sup>**

<sup>1</sup>A version of this chapter has been submitted for publication to the Journal of Neuroscience

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FMR contributed figures 3.4A and 3.5A. JLE completed all other experiments

### 3.1. INTRODUCTION

Glutamate is the primary excitatory neurotransmitter in the brain and its actions are mediated through two types of receptors: ionotropic glutamate receptors that are ligand-gated cation channels and metabotropic glutamate receptors (mGluR) that are G protein-coupled receptors (GPCRs) (Olney, 1994; Dingledine et al., 1999; Pin et al., 2003). mGluRs play an important role in processes underlying learning and memory, neuronal development, and neurodegeneration (Nakanishi, 1994; Pin et al., 1994; Pin and Duvoisin, 1995; Conn and Pin, 1997; Dale et al., 2002). mGluRs are categorized into three subclasses based on sequence homology and G protein coupling specificity. Group I mGluRs include mGluR1 and mGluR5, which are coupled through  $G_{\alpha q}$  to the activation of phospholipase C, which in turn catalyzes the formation of inositol 1,4,5 trisphosphate and diacylglycerol, which mediate the release of intracellular calcium stores and the activation of protein kinase C (PKC) (Conn and Pin, 1997; Dhimi and Ferguson, 2006; Niswender and Conn, 2010).

The GPCR desensitization paradigm involves receptor phosphorylation by either second messenger-dependent protein kinases or G protein-coupled receptor kinases (GRKs) followed by  $\beta$ -arrestin binding, which functions to uncouple the receptor from the G protein and targets GPCRs for clathrin-mediated endocytosis (Krupnick and Benovic, 1998; Ferguson, 2001). However, Group I mGluR desensitization and endocytosis is mediated by GRK2 in a phosphorylation-independent manner and does not require  $\beta$ -arrestin (Ferguson, 2001; Dhimi et al., 2002; Dhimi et al., 2004; Dhimi et al., 2005; Dhimi and Ferguson, 2006; Ferguson, 2007; Ribeiro et al., 2011). PKC also contributes to the regulation of glutamate-induced mGluR1a internalization whereas

constitutive mGluR1a internalization is mediated by RalA and phospholipase D2 (Mundell et al., 2003; Bhattacharya et al., 2004; Mundell et al., 2004).

Optineurin, a protein we have previously shown to contribute to the attenuation of mGluR1a signalling, has also been shown to be a Rab8 effector protein (Hattula and Peränen, 2000; Anborgh et al., 2005). Rab8 is a member of the Rab GTPase family of small G proteins that are involved in regulating the trafficking, docking and fusion of vesicles between intracellular membrane compartments (Zerial and McBride 2001; Seachrist and Ferguson, 2003; Gaborik and Hunyady, 2004). Rab8 is localized to the Golgi apparatus, intracellular membrane vesicles and membrane ruffles and is involved in trafficking of basolateral proteins in polarized epithelial cells, neurite outgrowth, delivery and recycling of the ionotropic glutamate AMPA receptors at synapses and has recently been shown to directly associate with the  $\alpha_{2B}$ - and  $\beta_2$ -adrenergic receptors (ARs) (Deretic, 1997; Gerges et al., 2004; Gerges et al., 2005; Brown et al., 2007; Ng and Tang, 2008; Dong et al., 2010). Since Rab8 has been shown to interact with optineurin, in the present study we have investigated whether Rab8 contributes to the regulation of mGluR1a desensitization and endocytosis. We report that Rab8 interacts with mGluR1a in an agonist-regulated manner to antagonize mGluR1a endocytosis and coordinates the attenuation of mGluR1a-stimulated IP3 formation and release of  $Ca^{2+}$  from intracellular stores in PKC-regulated manner.

## **3.2. EXPERIMENTAL PROCEDURES**

### **3.2.1. Materials**

*myo*-[ $^3H$ ]-Inositol was acquired from PerkinElmer Life Sciences (Waltham, MA). Dowex 1-X8 (formate form) resin 200 - 400 mesh was purchased from BioRad

(Mississauga, ON, Canada). Goat anti-glutathione-S-transferase (GST) antibodies as well as ECL Western blotting detection reagents were purchased from GE Healthcare (Oakville, ON, Canada). Horseradish peroxidase-conjugated anti-rabbit and anti-goat IgG secondary antibody was obtained from BioRad (Mississauga, ON, Canada). Anti-Gαq/11 rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-mGluR1a rabbit polyclonal antibody was obtained from Upstate (Lake Placid, NY, USA). Rabbit polyclonal phospho-p44/44 MAP kinase (Thr202/Tyr402), p44/44 MAP kinase antibodies were obtained from Cell Signalling Technology (Pickering, ON, Canada). Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 568 donkey anti-rabbit IgG, Zenon Rabbit Alexa Fluor 555 and Fluo-4 AM ester calcium indicators were purchased from Invitrogen/Molecular Probes (Burlington, ON, Canada). Rabbit anti-FLAG antibody, M2 anti-FLAG agarose and all other biochemical reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada).

### **3.2.2. Cell Culture**

Human embryonic kidney (HEK) 293 cells were maintained in Eagle's minimal essential medium supplemented with 8% (v/v) heat inactivated fetal bovine serum (Invitrogen, Burlington, ON) and 50 µg/ml gentamicin. Cells seeded in 100 mm dishes were transfected using a modified calcium phosphate method as described previously (Ferguson and Caron, 2004). Following transfection (18 h), the cells were incubated with fresh medium and allowed to recover for 24 hrs for co-immunoprecipitation studies. Otherwise, they were allowed to recover for 6-8 hrs and re-seeded into 24-well dishes and then grown an additional 18 hrs prior to experimentation.

### **3.2.3. Primary Hippocampal Neuronal Culture**

Hippocampi from embryonic day 18 CD-1 mice were processed, as described previously (Xie et al., 2000) and maintained in Neurobasal medium supplemented with B27, glutamax and pen/strep. Neurons were transfected at DIV 7-10 using a modified calcium phosphate technique and imaged 24 hr later.

### **3.2.4. Co-Immunoprecipitation**

HEK 293 cells were transiently transfected with the cDNAs as described in the *Figure Legends*. Following transfection, the cells were incubated for 15 minutes in HEPES balanced salt solution (HBSS) at 37°C with or without 30 µM quisqualate. The cells were then placed on ice, washed two times with ice-cold phosphate-buffered saline (PBS) and lysed with cold-lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 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 15000 x g for 15 min at 4°C to pellet insoluble material. Cleared supernatant containing 250 µg protein was incubated with 25 µL of FLAG M2-affinity beads for 1h rotating at 4°C to immunoprecipitate FLAG-mGluR1a. Following incubation, the beads were washed twice with PBS, and proteins were solubilized in a 3X SDS sample buffer containing 2-mercaptoethanol (BME). Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted to identify co-immunoprecipitated GST-tagged Rab8 protein using a primary goat anti-GST antibody (1:1000 dilution) followed by a horseradish peroxidase-conjugated secondary anti-goat (1:2500 dilution). Receptor and Rab8 protein expression was determined by

immunoblotting 10  $\mu$ g of protein from each cell lysate used for immunoprecipitation. Proteins were detected by chemiluminescence.

### **3.2.5. Biotinylation Assay**

HEK 293 cells were transiently transfected with the cDNAs as described in the *Figure Legends*. For cell surface biotinylation 48 hours post transfection cells were incubated in 37°C HBSS for 1 hour and then treated vehicle or 30  $\mu$ M quisqualate. Cells were then placed on ice, washed in ice-cold HBSS and cell surface proteins labelled with 1.5 mg/ml biotin for 1 hour and biotin was subsequently quenched with 100 mM glycine for 30 min. For internalization experiments, cells were labelled with biotin on ice, quenched and then cells were treated with vehicle or 30  $\mu$ M quisqualate for the times indicated in the *Figure Legends* to induce mGluR1a internalization. Cell surface biotin was stripped from the cells using 150 mM MesNa. The cells were then placed on ice, washed two times with ice-cold phosphate-buffered saline (PBS) and lysed with cold-lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10  $\mu$ g/ml leupeptin, and 5  $\mu$ g/ml aprotinin). The lysates were placed on a rocking platform for 15 min at 4°C and centrifuged at 15000 x g for 15 min at 4°C to pellet insoluble material. Cleared supernatant containing 250  $\mu$ g protein was incubated with 35  $\mu$ L of neutravidin-affinity beads for 1h rotating at 4°C to immunoprecipitate biotin-labeled proteins. Following incubation, the beads were washed twice with PBS, and proteins were solubilized in a 3X SDS sample buffer containing BME. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted to identify biotinylated mGluR1a proteins using a primary rabbit anti mGluR1a antibody (1:1000 dilution) followed by a horseradish peroxidase-conjugated

secondary anti-rabbit (1:10000 dilution). Receptor and Rab8 protein expression was determined by immunoblotting 10 µg of protein from each cell lysate used for biotinylation. Proteins were detected by chemiluminescence.

### **3.2.6. Inositol Phosphate Formation**

HEK 293 cells were transiently transfected with the cDNAs as described in the *Figure Legends*. 48 hours post-transfection cells were incubated overnight in inositol- and glutamate-free DMEM with 100 µCi/mL *myo*-[<sup>3</sup>H]-inositol. For PKC inhibition experiments, cells were washed twice and incubated for one hour in 37°C HBSS then preincubated for 10 min at 37°C with either DMSO (control) alone or with 1 µM bisindolymaleimide I or 5 µM chelerythrine chloride. For all other experiments cells were incubated for one hour in 37°C HBSS and were then incubated with 10 mM LiCl alone for 10 min followed by 30 µM quisqualate treatment for 30 min. Cells were placed on ice and the reaction was stopped with 500 µL of perchloric acid and neutralized with 400 µl of 0.72 M KOH, 0.6 M KHCO<sub>3</sub>. Total cellular [<sup>3</sup>H]-inositol incorporation was determined in 50 µl of cell lysate. Total inositol phosphate was purified by anion exchange chromatography using Dowex 1-X8 (formate form) 200 - 400 mesh anion exchange resin and [<sup>3</sup>H]-inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system.

### **3.2.7. ERK Activation**

HEK 293 cells were transiently transfected with FLAG-mGluR1 and pEGFP (control) or GFP-Rab8. 48 hours post-transfection cells were serum starved overnight in glutamate-free DMEM and stimulated for 0, 1, 5 or 15 min with 30 µM quisqualate. Cells were lysed and proteins were solubilized in a 3X SDS sample buffer containing BME.



Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted to identify phosphorylated and total extracellular regulated kinase (ERK1/2) antibody (1:1000 dilution) followed by a horseradish peroxidase-conjugated secondary anti-Rabbit antibody (1:10,000 dilution). Receptor and Rab8 protein expression was determined by immunoblotting 10 µg of protein from each cell lysate. Proteins were detected by chemiluminescence.

### **3.2.8. Confocal Microscopy**

Confocal microscopy was performed using a Zeiss LSM510 META laser scanning confocal microscope equipped with a Zeiss 63X, 1.4 numerical aperture, oil immersion lens (North York, ON, Canada). HEK293 cells expressing GFP-Rab8 and FLAG-mGluR1a were serum starved for 1 hr at 37°C in HBSS (116 mM NaCl, 20 mM HEPES, 11 mM glucose, 5 mM NaHCO<sub>3</sub>, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4). HEK293 cells were prelabelled with Alexa Fluor 568-conjugated anti-FLAG polyclonal rabbit antibody. Cells were then treated with 30 µM quisqualate and live cells imaged over a 30 min time period at 37°C. Primary mouse hippocampal neurons were transiently transfected with FLAG-mGluR1a, fixed with periodate lysine paraformaldehyde and permeabilized with 0.1% Triton X-100. Receptor was labelled with rabbit polyclonal anti-FLAG and endogenous Rab8 labelled with mouse monoclonal anti-Rab8a antibody. Colocalization studies were performed using dual excitation (488, 543 nm) and emission (band pass 505-530 nm and long pass 560 nm for Alexa Fluor 488 and 568, respectively) filter sets.

### **3.2.9. Calcium Imaging**

DIV 7-10 hippocampal neurons were transiently transfected with either empty pEGFP (control) or GFP-Rab8 and imaged 24 hr later. Transfected cells were identified via confocal microscopy at 488nm excitation and band pass 505-530 emission after which cells were loaded with 10  $\mu$ M fluo-4 for 30 min at room temperature and imaged using the same parameters. Cells were stimulated with 100  $\mu$ M DHPG and 1  $\mu$ M ionomycin.

### **3.2.10. Statistical Analysis**

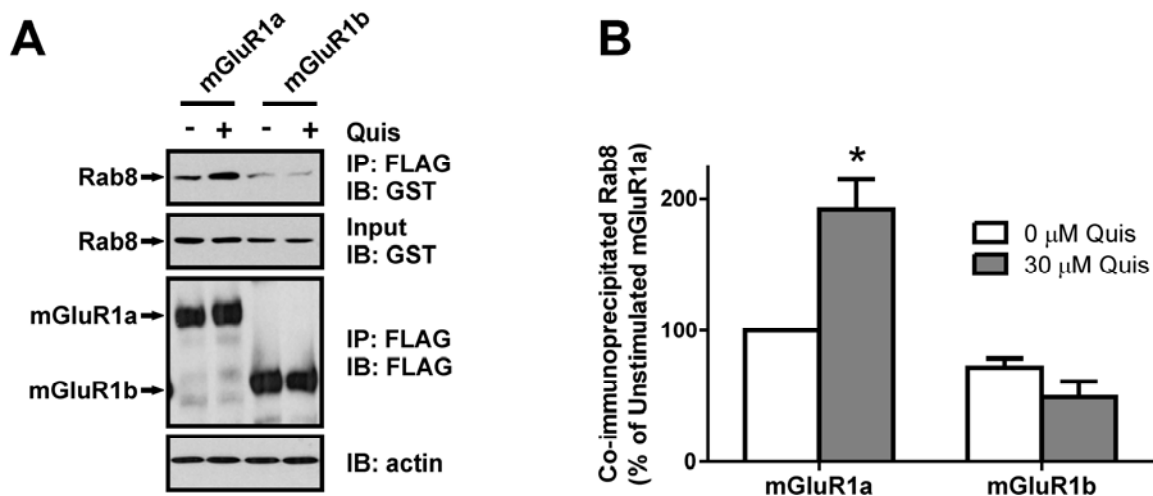
Densitometric data were normalized first for protein expression and the control value was set to 100, with all other values displayed as percentage thereof. One-way analysis of variance test (ANOVA) was performed to determine significance, followed by a post-hoc Tukey multiple comparison test or Bonferroni's multiple comparisons test to determine which means were significantly different ( $p < 0.05$ ) from one another.

### 3.3. RESULTS

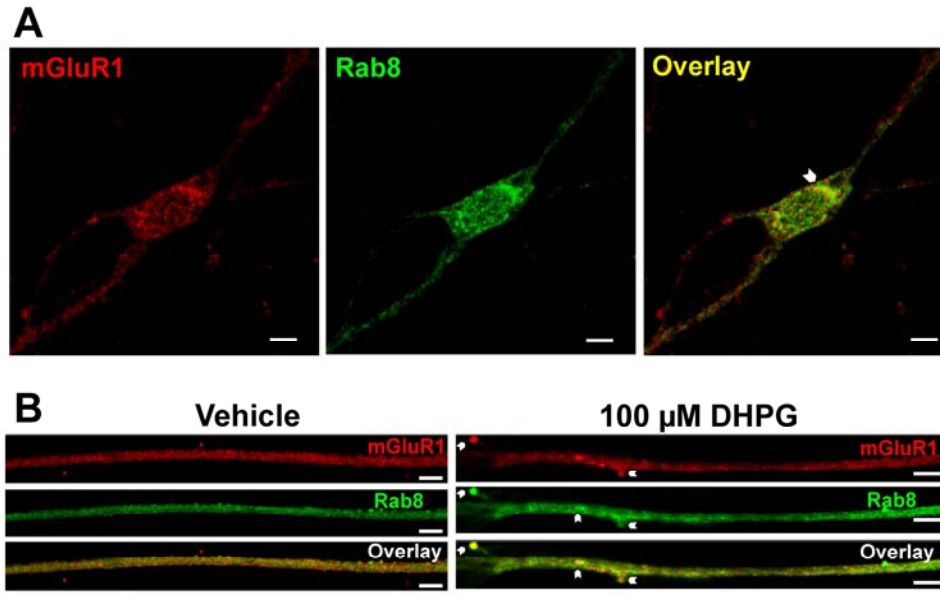
#### 3.3.1. Agonist-stimulated Rab8 interaction with the mGluR1a C-tail

Previously, we demonstrated that the Rab8 effector optineurin was an mGluR1a interacting protein by yeast two hybrid and that it played a role in antagonizing mGluR1a G protein signalling (Anborgh et al., 2005). Therefore, in the present study, we investigated whether Rab8 might also interact with mGluR1a. To do this, HEK 293 cells were transiently transfected with FLAG-tagged mGluR1a or FLAG-mGluR1b and GST-tagged Rab8 and the co-immunoprecipitation of GST-Rab8 with either FLAG-mGluR1a or FLAG-mGluR1b was assessed. We found that GST-Rab8 was co-immunoprecipitated with FLAG-mGluR1a in the absence of agonist, but that this association was increased two fold ( $92 \pm 23\%$ ) in response to 30  $\mu$ M quisqualate treatment (Figure 3.1A and 3.1B). In contrast, GST-Rab8 co-immunoprecipitation with Flag-mGluR1b (which lacks an extended intracellular C-tail) was reduced to  $72 \pm 7\%$  of control FLAG-mGluR1a immunoprecipitation (Figure 3.1A and 3.1B). Agonist treatment did not increase GST-Rab8 co-immunoprecipitation with FLAG-mGluR1b (Figure 3.1A and 3.1B). Therefore, agonist-dependent increases in Rab8 association with mGluR1a required interactions with the extended mGluR1a C-tail.

To determine whether endogenous Rab8 colocalizes with FLAG-mGluR1a, primary hippocampal neurons (DIV 14) were fixed and labelled for FLAG-mGluR1a and endogenous Rab8 protein distribution and imaged using confocal microscopy. Interestingly, while FLAG-mGluR1a and Rab8 share partial overlapping populations in the neuronal cell body (Figure 3.2A), they show little colocalization in neuronal projections in the absence of agonist (Figure 3.2B). However, following the treatment of



**Figure 3.1. Agonist-dependent co-immunoprecipitation of Rab8 with mGluR1a.** (A) Representative immunoblot and (B) densitometric analysis showing the co-immunoprecipitation of GST-Rab8 with the FLAG-mGluR1a and FLAG-mGluR1b in the absence (-) and presence (+) of 30  $\mu$ M quisqualate (Quis) treatment for 15 min. HEK 293 cells were transiently transfected with 1  $\mu$ g of plasmid cDNA encoding either FLAG-mGluR1a or FLAG-mGluR1b along with 2  $\mu$ g of plasmid cDNA encoding GST-Rab8. 48 h post-transfection cells were stimulated and lysates were collected and FLAG-mGluR1 was immunoprecipitated. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted for FLAG-mGluR1 and GST-Rab8. Data represents the mean  $\pm$  SD of 3 independent experiments. Data were normalized for both GST-Rab protein expression levels and FLAG-mGluR immunoprecipitation and normalized to GST-Rab protein binding to the mGluR1a in absence of agonist. \* $p$  < 0.05 compared to GST-Rab8 co-immunoprecipitated with the mGluR1a in the absence of agonist.

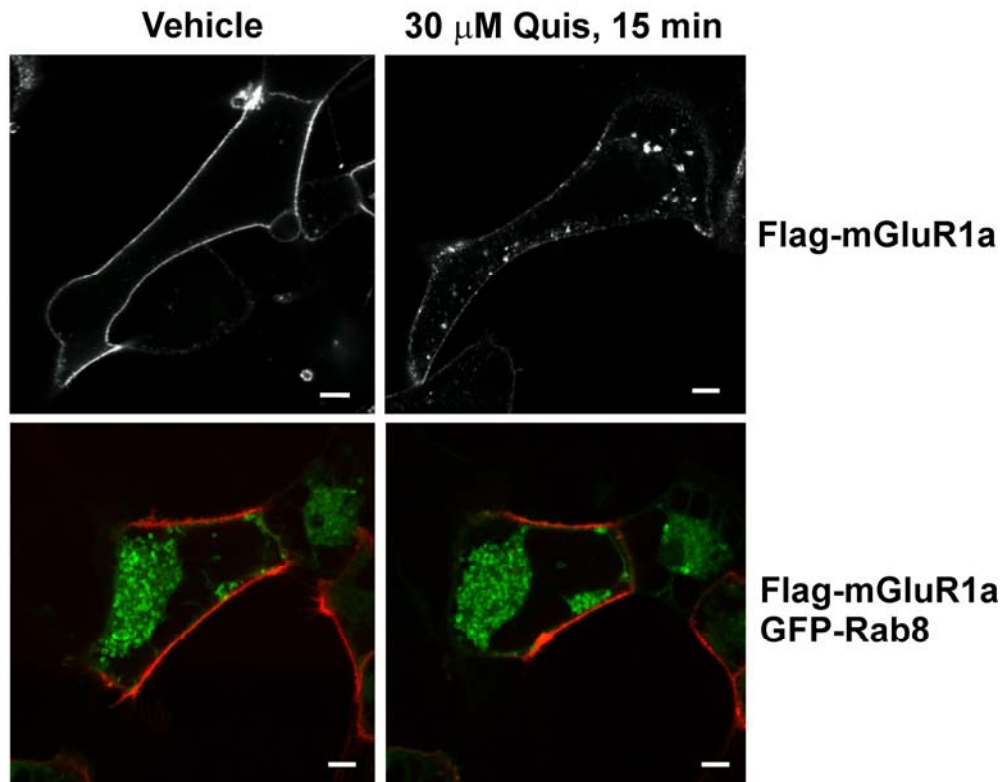


**Figure 3.2. Colocalization of mGluR1 and Rab8 in primary hippocampal neurons.** (A) Representative micrographs showing localization of endogenous mGluR1 and Rab8 protein in neuronal cell body. (B) Representative micrographs showing localization of mGluR1 and Rab8 in neuronal projections. DIV 14 neurons were treated with and without 100  $\mu$ M DHPG for 30 min, fixed and labelled for endogenous mGluR1a (red) and Rab8 (green). Arrows highlight colocalization. Bars represent 5  $\mu$ m. Images represent 3 independent experiments.

hippocampal neurons with 100  $\mu$ M (S)-3,5-dihydroxyphenylglycine (DHPG), both FLAG-mGluR1a and Rab8 immunofluorescence exhibited colocalization in the spines of these projections (Figure 3.2B). Thus, agonist treatment appeared to increase FLAG-mGluR1a and endogenous Rab8 association in primary cells.

### **3.3.2. Rab8 antagonizes mGluR1a endocytosis to increase cell surface expression**

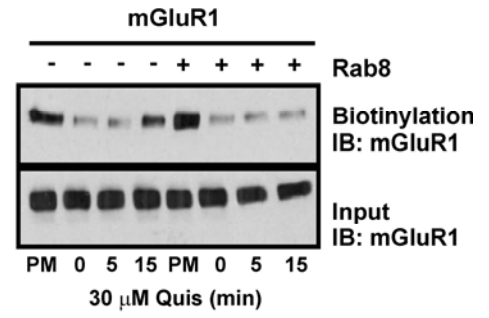
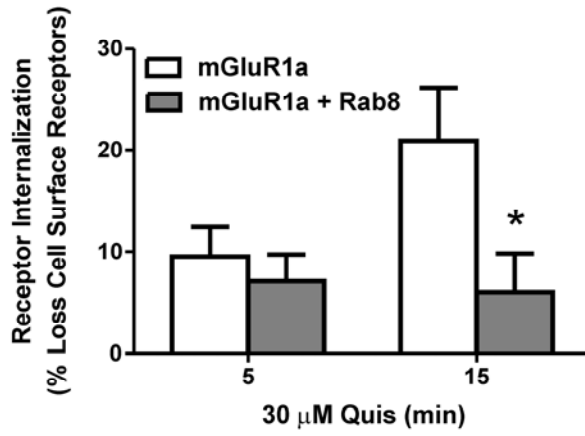
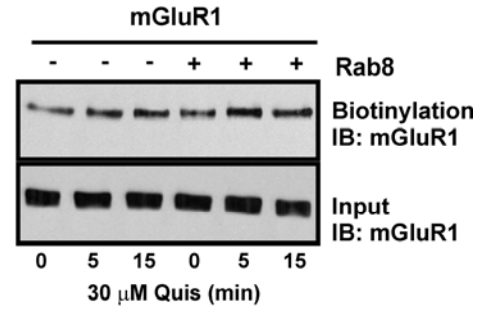
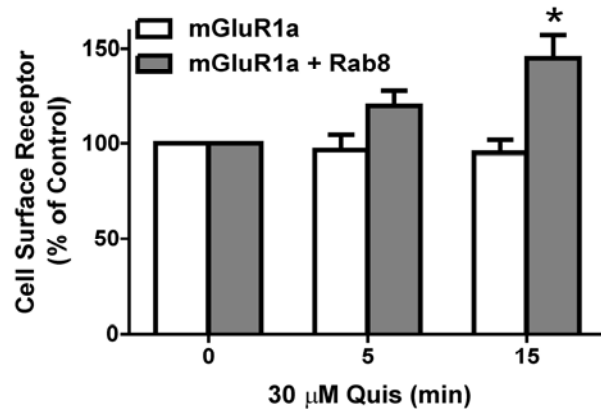
Rab8 has been shown to differentially regulate the trafficking of the  $\alpha_{2B}$ AR and  $\beta_2$ AR and contributes to the regulation of the synaptic delivery and recycling of the ionotropic glutamate AMPA receptor (Deretic, 1997; Gerges et al., 2004; Brown et al., 2007; Ng and Tang, 2008; Dong et al., 2010). Therefore, we investigated whether Rab8 overexpression would affect FLAG-mGluR1a intracellular trafficking in HEK 293 cells. Initial experiments using confocal microscopy determined whether Rab8 overexpression resulted in an alteration of cell-surface FLAG-mGluR1a in live HEK 293 cells labelled with Zenon 555 Alexa Fluor-labelled primary mouse FLAG monoclonal antibody. Control cells expressing FLAG-mGluR1a alone showed steady receptor internalization upon treatment with 30  $\mu$ M quisqualate (Figure 3.3, upper panels). However, in cells overexpressing GFP-Rab8 FLAG-mGluR1a internalization was not observed (Figure 3.3, lower panels). To quantify the extent of FLAG-mGluR1a internalization in the absence and presence of Rab8, we tested FLAG-mGluR1a endocytosis using a cell surface biotinylation assay following 5 and 15 min exposures to 30  $\mu$ M quisqualate. We found that following the 15 min exposure of FLAG-mGluR1a to agonist that FLAG-mGluR1a internalization was significantly reduced in HEK 293 cells overexpressing Rab8, when compared to control cells (Figure 3.4A). When we assessed the overall cell surface expression of FLAG-mGluR1a, we found that the fraction of



**Figure 3.3. Live cell imaging of mGluR1a endocytosis in the absence and presence of Rab8.** Representative confocal micrograph showing internalization of FLAG-mGluR1a in the presence and absence GFP-Rab8. Live HEK 293 cells transfected 1  $\mu$ g of plasmid cDNA encoding FLAG-mGluR1a either with (lower panels) or without (upper panels) 2  $\mu$ g of plasmid cDNA encoding GFP-Rab8 were labelled with Zenon AlexaFluor 555 on ice, warmed to 37°C and stimulated with 30  $\mu$ M Quis for 15 min. Bars represent 5  $\mu$ m. Data representative of 3 independent experiments.

**Figure 3.4. Effect of Rab8 on mGluR1a endocytosis and cell surface expression.** **(A)** Representative immunoblot and densitometric analysis of internalized biotinylation of FLAG-mGluR1a. HEK 293 cells transiently expressing 1  $\mu$ g FLAG-mGluR1a and 2  $\mu$ g either pEBG (control) or GST-Rab8 placed on ice and cell surface proteins were biotinylated. Cells were then stimulated with 30  $\mu$ M Quis for 0, 5 or 15 min to induce internalization and cell surface biotin stripped away. Biotin-labelled proteins were collected with neutravidin-conjugated beads, separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted for mGluR1a. Data were normalized for protein expression. Data are representative of 6 independent experiments. \* $p < 0.05$  compared to corresponding control. **(B)** Representative immunoblot and densitometric analysis of cell surface biotinylation of FLAG-mGluR1a. HEK 293 cells transiently expressing 1  $\mu$ g FLAG-mGluR1a and 2  $\mu$ g either pEBG (control) or GST-Rab8 were stimulated with 30  $\mu$ M Quis for 0, 5 or 15 min, placed on ice and cell surface proteins were biotinylated. Biotin-labelled proteins were collected with neutravidin-conjugated beads, separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted for mGluR1a. Data represent changes in cell surface mGluR1 expression. Data were normalized for protein expression and are representative of 6 independent experiments. \* $p < 0.05$  compared to corresponding control.



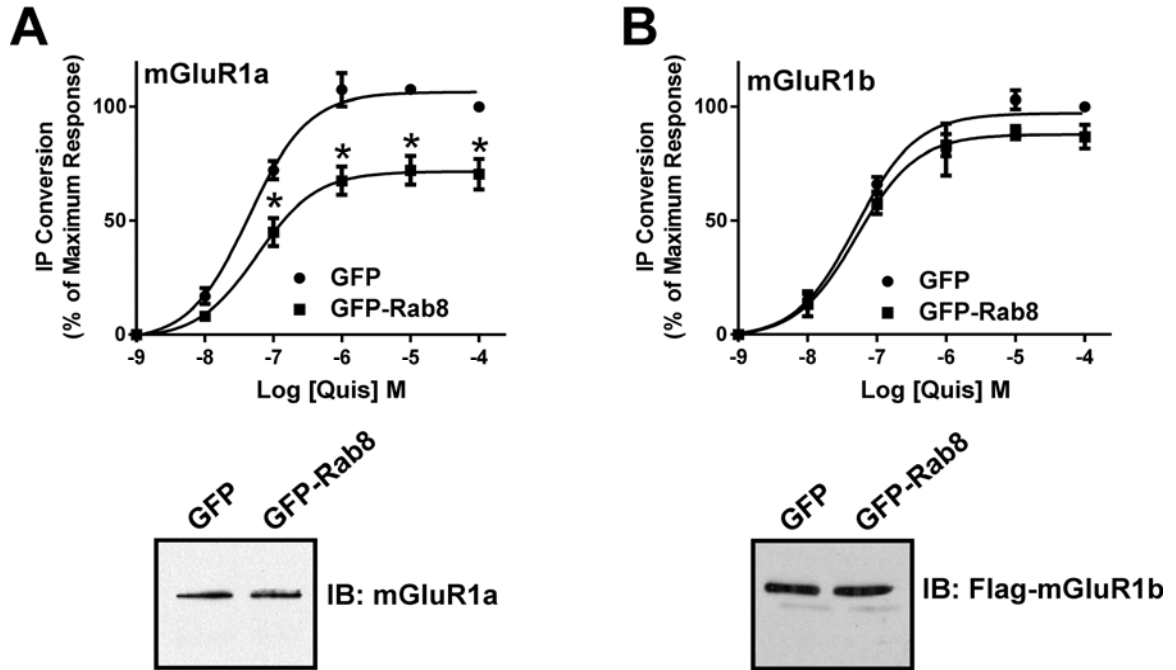
**A****B**

FLAG-mGluR1a at the cell surface following 15 min agonist treatment was significantly greater than in control cells (Figure 3.4B). Therefore, Rab8 appeared to prevent mGluR1a endocytosis and increase cell surface mGluR1a expression.

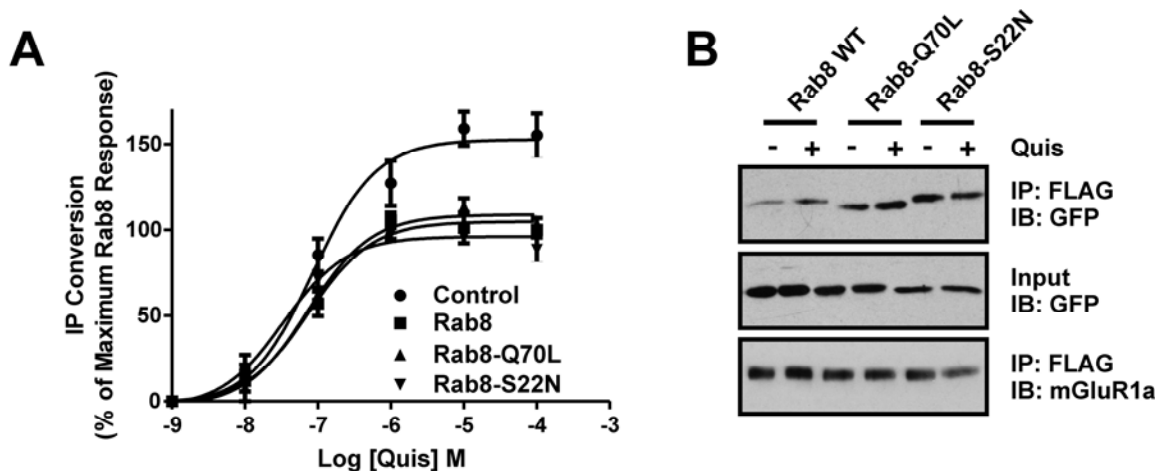
### **3.3.3. Rab8 antagonizes mGluR1a inositol phosphate and calcium signalling**

The Rab8 effector molecule, optineurin is known to contribute to phosphorylation-independent G protein-uncoupling and desensitization of mGluR1a (Anborgh et al., 2005). Therefore, we assessed whether Rab8 overexpression would result in altered FLAG-mGluR1a- and FLAG-mGluR1b-stimulated inositol phosphate (IP) formation in HEK 293 cells. We found that the over-expression of Rab8 resulted in a significant reduction in the maximal response for quisqualate-stimulated IP formation in FLAG-mGluR1a expressing cells reducing the maximum response to  $70 \pm 7\%$  when compared to control FLAG-mGluR1a expressing cells (Figure 3.5A). However, Rab8 overexpression had no effect on the maximal response for quisqualate-stimulated IP formation in FLAG-mGluR1b expressing cells (Figure 3.5B). Thus, consistent with what was observed for agonist-stimulated association of Rab8 with mGluR1a versus mGluR1b, Rab8 overexpression selectively attenuated mGluR1a and not mGluR1b G protein signalling.

To determine whether Rab8 GDP for GTP exchange was required for the attenuation of FLAG-mGluR1a-stimulated IP formation, we assessed whether FLAG-mGluR1a-stimulated IP formation would be inhibited in the presence of either dominant-negative (Rab8-S22N) or constitutively active (Rab8-Q70L) proteins. We found that the overexpression of wild-type Rab8, Rab8-S22N and Rab8-Q70L resulted in a reduction of FLAG-mGluR1a stimulated IP formation to a similar extent (Figure 3.6A). To determine



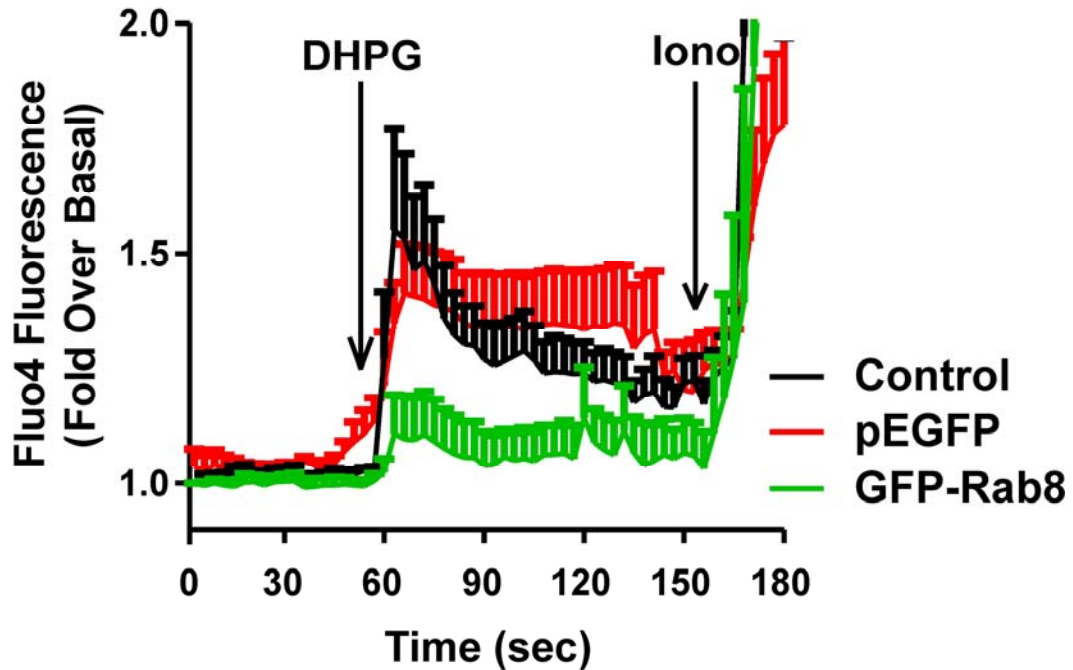
**Figure 3.5. Effect of Rab8 on mGluR1a- and mGluR1b-mediated inositol phosphate (IP) formation.** Inositol phosphate formation in HEK 293 cells transfected with 1  $\mu$ g plasmid cDNA encoding (A) FLAG-mGluR1a or (B) FLAG-mGluR1b with 2  $\mu$ g empty pEGFP vector or GFP-Rab8. Transfected cells were treated for 10 min with 10 mM LiCl and stimulated with 30  $\mu$ M Quis for 30 min in the presence of LiCl. Data were normalized for protein expression and basal IP formation. Lower panels show relative mGluR1a and mGluR1b protein expression in GFP and GFP-Rab8 transfected cells. Data are representative of 3-5 independent experiments. \* $p < 0.05$  compared to corresponding control.



**Figure 3.6. Effect of Rab8 nucleotide binding mutants on mGluR1a-mediated inositol phosphate formation.** (A) Inositol phosphate (IP) formation in HEK 293 cells transfected with 1  $\mu$ g of plasmid cDNA encoding FLAG-mGluR1a with 2  $\mu$ g of plasmid cDNA encoding GFP-tagged Rab8 wild type (WT), constitutively active Rab8-Q67L (CA), and dominant-negative Rab8-S34N (DN) mutants. Transfected cells were treated for 10 min with 10 mM LiCl and stimulated with 30  $\mu$ M quisqualate (Quis) for 30 min in the presence of LiCl. Data were normalized for protein expression and basal IP formation. (B) Representative immunoblot showing the co-immunoprecipitation of GFP-tagged Rab8WT, Rab8CA and Rab8DN with the FLAG-mGluR1a in the absence (-) and presence (+) of 30  $\mu$ M Quis treatment for 15 min. HEK 293 cells were transiently transfected with 1  $\mu$ g of plasmid cDNA encoding FLAG-mGluR1a along with 2  $\mu$ g of plasmid cDNA encoding GFP-tagged Rab8WT, Rab8CA or Rab8DN. 48 h post-transfection cells were stimulated and lysates were collected, separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted for mGluR1a and GFP-Rab8. Data represents 3-5 independent experiments.

whether Rab8 associated with the receptor in a nucleotide-specific manner, we also examined the agonist-stimulated co-immunoprecipitation of wild-type Rab8, Rab8-S22N and Rab8-Q70L with FLAG-mGluR1a. Although FLAG-mGluR1a interactions with both wild type Rab8 and constitutively active Rab8-Q70L were increased upon agonist stimulation, agonist did not regulate the association of dominant-negative Rab8-S22N with the receptor (Figure 3.6B). Thus, although Rab8 associated with mGluR1a in a GTP-dependent manner, the Rab8-dependent attenuation of mGluR1a G protein signalling was independent of the nucleotide-binding state of the GTPase.

Because we found that Rab8 significantly attenuated FLAG-mGluR1a-mediated IP formation in HEK 293 cells, we investigated whether Rab8 overexpression might also attenuate DHPG-stimulated  $\text{Ca}^{2+}$  release mediated by endogenous mGluR1 expressed in primary hippocampal neurons. To assess this, primary mouse hippocampal neurons (DIV7-10) were transiently transfected with either pEGFP (control) or GFP-Rab8. Neurons were imaged to identify cells that were transfected with GFP protein, and GFP protein positive cells were then subsequently loaded with the calcium indicator fluo-4 AM and the same cell imaged for DHPG-mediated calcium release as evidence by an increase in cellular fluorescence. The treatment of hippocampal neurons with 100  $\mu\text{M}$  DHPG resulted in an increase in Fluo-4 fluorescence in both untransfected and pEGFP transfected neurons as expected (Figure 3.7). However, in GFP-Rab8 positive neurons DHPG-stimulated increases in intracellular  $\text{Ca}^{2+}$  concentration, as measured by increased Fluo-4 fluorescence, was attenuated (Figure 3.7). All of the cells exhibited similar responses to challenge with ionomycin indicating that GFP-Rab8 overexpression was not causing a generalized defect in  $\text{Ca}^{2+}$  regulation (Figure 3.7). In order to investigate

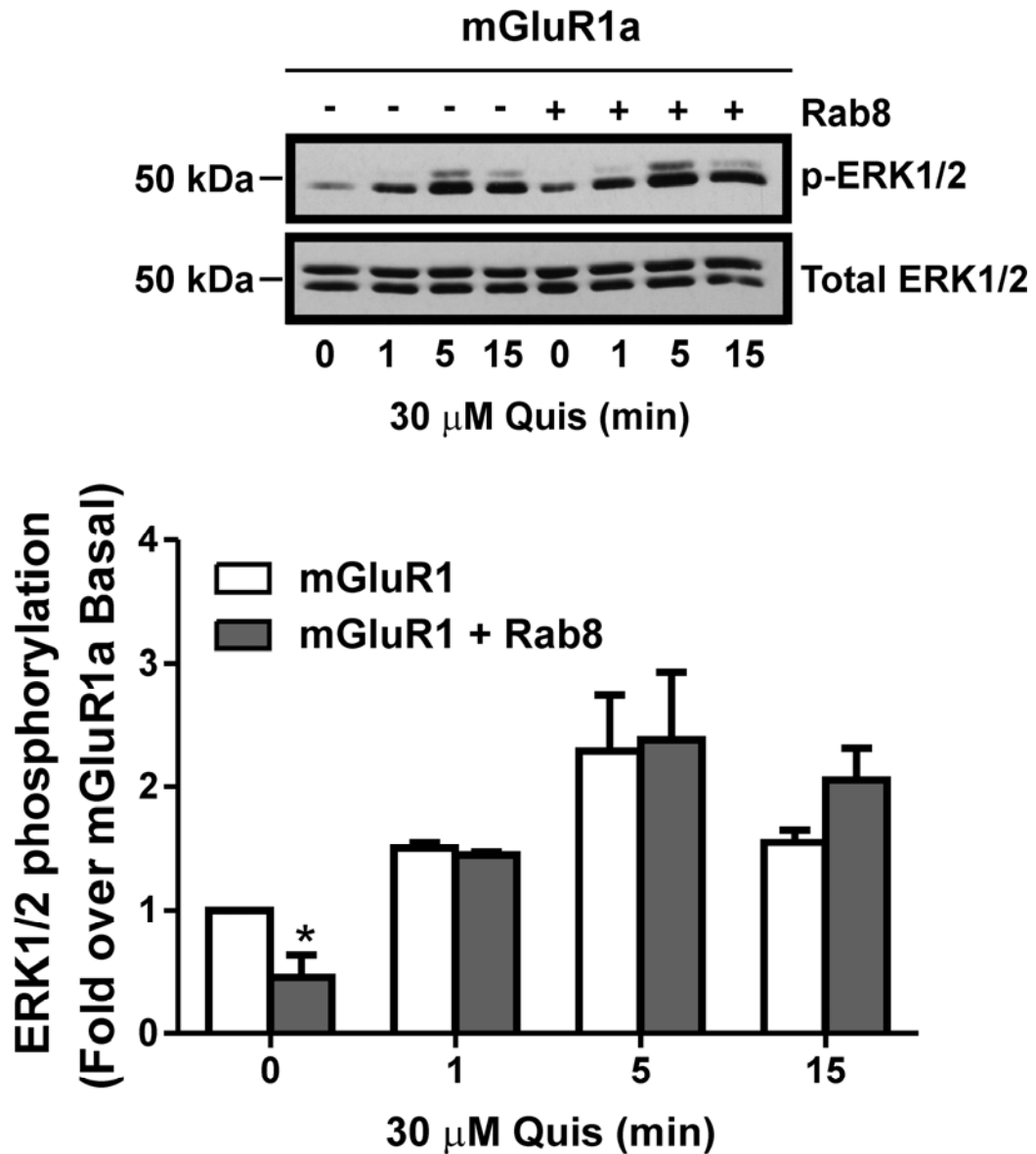


**Figure 3.7. Effect of Rab8 on DHPG-stimulated  $\text{Ca}^{2+}$ -release in hippocampal neurons.** Fluorescent intensity analysis of  $\text{Ca}^{2+}$  release as represented by the fluorescent calcium indicator Fluo-4. DIV7-10 primary mouse hippocampal neurons were transiently transfected with 10  $\mu\text{g}$  of plasmid cDNA encoding either pEGFP (control) or GFP-Rab8. Twenty four hours after transfection neurons were imaged to identify transfected cells, after which they were loaded with 5  $\mu\text{M}$  of the calcium indicator Fluo-4 AM for 30 min at room temperature and the same field of view was imaged for calcium release with the addition of 100  $\mu\text{M}$  DHPG followed by 10  $\mu\text{M}$  ionomycin (iono). Data representative of 3 independent experiments.

whether Rab8 overexpression also affected other aspects of mGluR1a signalling, we examined FLAG-mGluR1a-stimulated ERK1/2 phosphorylation in HEK 293 cells (Figure 3.8). We found that agonist-stimulated FLAG-mGluR1a-mediated ERK1/2 phosphorylation was not affected by the overexpression of Rab8, but that basal ERK1/2 phosphorylation was decreased in cells expressing both FLAG-mGluR1a and Rab8 (Figure 3.8). Therefore, Rab8 appeared to selectively regulate FLAG-mGluR1a-stimulated IP formation in HEK 293 and  $\text{Ca}^{2+}$  release in response to the activation of endogenous mGluR1 in primary mouse hippocampal neurons.

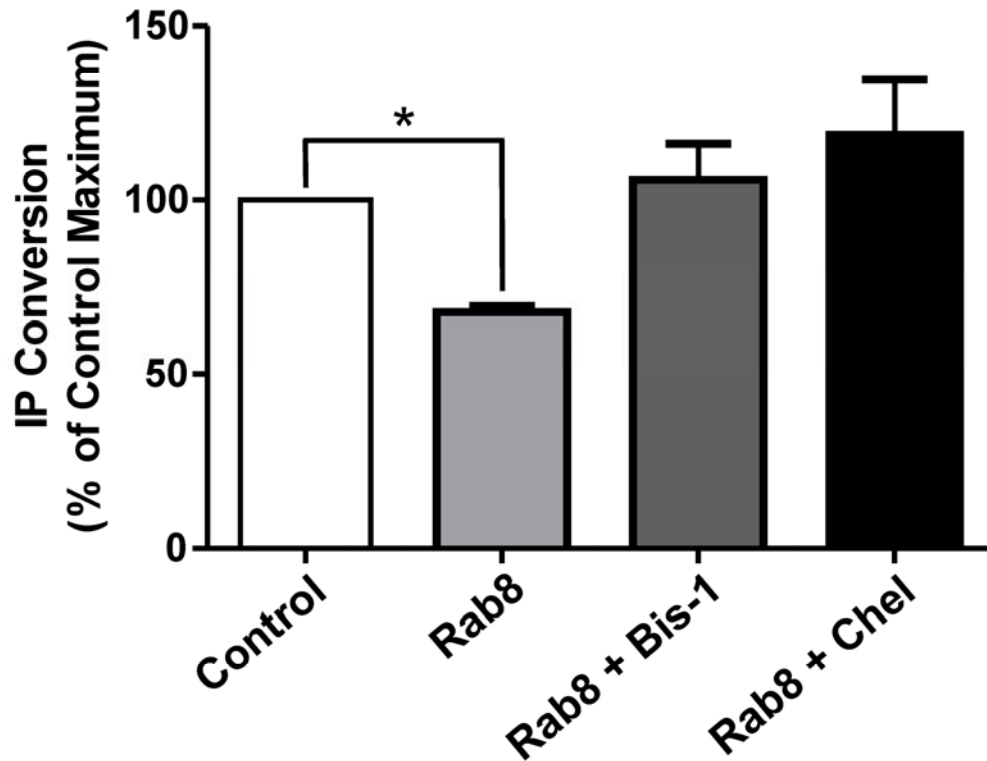
#### **3.3.4. Rab8 decrease of mGluR1a mediated IP3 signalling is PKC-dependent**

Second messenger dependent kinases, such as PKC, can contribute to the desensitization of agonist activated group 1 mGluRs (Schoepp and Johnson, 1988; Herrero et al., 1994; Desai et al., 1999; Francesconi and Duvoisin, 2000; Ferguson, 2001). Because we found that Rab8 expression causes a significant decrease in FLAG-mGluR1a-mediated IP formation, we sought to determine whether PKC-mediated desensitization of mGluR1a signalling might be influenced by Rab8 expression. To test this, HEK 293 cells were pretreated with one of two PKC inhibitors, bisindolylmaleimide-1 (Bis-1) or chelerythrine chloride. We found that the pretreatment of HEK 293 cells with either 1  $\mu\text{M}$  Bis-1 or 5  $\mu\text{M}$  chelerythrine chloride for 10 minutes prevented the Rab8-mediated uncoupling of mGluR1a-stimulated IP formation to DMSO control levels (Figure 3.9). This observation suggested a role for PKC-mediated phosphorylation in the observed Rab8-dependent attenuation of FLAG-mGluR1a IP signalling.



**Figure 3.8. Effect of Rab8 on mGluR1a-stimulated ERK1/2 phosphorylation.** Shown is a representative immunoblot and densitometric analysis of p42/44 (ERK1/2) phosphorylation. HEK 293 cells were transiently transfected with 1  $\mu$ g of plasmid cDNA encoding FLAG-mGluR1a along with 2  $\mu$ g of plasmid cDNA encoding either pEGFP (control) or GFP-Rab8. Cells were stimulated for 0, 1, 5 or 15 min with 30  $\mu$ M Quis, lysates were collected, separated by SDS-PAGE and transferred to nitrocellulose and phosphorylated ERK1/2 was detected by immunoblot. Data were normalized for total ERK expression. Data represents the mean  $\pm$  SD of 3-5 independent experiments.





**Figure 3.9. PKC inhibition prevents Rab8-dependent attenuation of mGluR1a-mediated IP formation.** HEK 293 cells were transfected with 1  $\mu\text{g}$  of plasmid cDNA encoding FLAG-mGluR1a along with 2  $\mu\text{g}$  empty pEGFP vector (Control) or GFP-Rab8. Cells were pre-incubated for 10 min with either DMSO alone or with either 1  $\mu\text{M}$  bisindolymaleimide I or 5  $\mu\text{M}$  chelerythrine chloride followed by 10 mM LiCl for 10 min and then 10  $\mu\text{M}$  quisqualate for 30 min. Data were normalized for protein expression and basal IP formation. Data are representative of 5 independent experiments. \* $p < 0.05$  compared to control.

### 3.4. DISCUSSION

To date only a few Rab isoforms including Rab3, Rab8 and Rab23 have been shown to be enriched in the brain and play a role in neurons (Evans et al., 2003; Geppert et al., 1997; Ng and Tang, 2008). Rab8 is essential in several areas of polarized neuronal transport as well as in plasma membrane trafficking in epithelial cells and Rab8 siRNA knockdown prevents maturation of hippocampal neurons in culture (Huber et al., 1995; van Ijzendoorn et al., 2003; Ng and Tang, 2008). Studies now link Rab8 to a variety of different human diseases, including polycystic kidney disease, microvillus inclusion disease and Bardet-Biedl syndrome, emphasizing the physiological importance of protein trafficking in human disease (Nachury et al., 2007; Ng and Tang, 2008). Here we show that Rab8 associates with mGluR1a, but not its alternatively spliced variant, mGluR1b, which lacks an extended carboxyl-terminal tail suggesting that Rab8 binds to the carboxyl-terminal tail of mGluR1a in an agonist-regulated manner. Other Rabs, including Rab4, Rab5, Rab7 and Rab11 have previously been reported to associate with the carboxyl-terminal tail of the angiotensin II type 1 receptor and other GPCRs (Anborgh et al., 2000; Seachrist et al., 2002; Dale et al., 2004; Hamelin et al., 2005; Parent et al., 2009; Reid et al., 2010; Esseltine et al., 2011). Additionally, Rab8 has recently been shown to associate with different regions of the  $\alpha_{2B}$ AR and  $\beta_2$ AR carboxyl-terminal tails and differentially modulates their trafficking to the cell surface from the trans-Golgi network (TGN) (Dong et al., 2010). We find that the association of Rab8 contributes to attenuated mGluR1a endocytosis, increased cell surface expression and functions to uncouple mGluR1a from G protein signalling by a mechanism that requires PKC activity.

Agonist-activated mGluR1a preferentially binds Rab8 wild type and GTP bound constitutively active mutant Rab8Q70L, but not GDP bound dominant negative mutant Rab8-S22N. This differs from the  $\alpha_{2B}$ AR and  $\beta_2$ AR, which were recently shown to preferentially bind the GDP bound dominant negative mutant Rab8-S22N (Dong et al., 2010). However, although this study did not investigate whether agonist activation regulated Rab8 interactions with the  $\alpha_{2B}$ AR and  $\beta_2$ AR, Rab8 knockdown resulted in attenuated cell surface expression of the  $\alpha_{2B}$ AR. Rab8 also has documented roles in the intracellular trafficking of other receptors such as the transferrin receptor, and has been shown to drive synaptic delivery of ionotropic glutamate AMPA receptors and their insertion into synaptic membranes (Henry and Sheff, 2008; Brown et al., 2007). However, to date, most studies have focused on the role of Rab8 in regulating receptor delivery to the plasma membrane from areas such as the TGN or recycling endosomes (Deretic, 1997; Gerges et al., 2004; Gerges et al., 2005; Brown et al., 2007; Dong et al., 2010). We present data here implicating Rab8 in the inhibition of mGluR1a internalization. Similar to our findings, the Rab8 effector molecule optineurin is also implicated in attenuating the internalization of transferrin receptors (Nagabhushana et al., 2010; Park et al., 2010). Rab5 has a well documented role in facilitating endocytosis of a number of receptors including the angiotensin II type 1 receptor and  $\beta_2$ AR (Seachrist et al., 2000; Seachrist et al., 2002; Dale et al., 2004). However, to our knowledge, Rab8 represents the first example of a wild-type Rab protein that contributes to the attenuation of receptor endocytosis.

Interestingly, we find that Rab8 overexpression leads to an increase in cell surface mGluR1a expression. This increase in cell surface expression might be explained by one

of two mechanisms: 1) Rab8 overexpression increases cell surface expression by either reducing constitutive mGluR1a internalization or agonist-stimulated internalization of mGluR1a as the consequence of the release of endogenous glutamate into the media by HEK 293 cells. 2) Rab8 overexpression facilitates the movement of receptors from the TGN to the plasma membrane. This would be similar to what was observed for the  $\alpha_{2B}AR$ , where Rab8 knockdown led to impaired TGN to plasma membrane transport of the receptor (Dong et al., 2010). In hippocampal neurons, endogenous Rab8 along with the mGluR1a redistribute to spine regions after agonist stimulation, suggesting that Rab8 may regulate the localization of mGluR1a to synapse. This might be similar to the reported role of Rab8 in the neuronal trafficking and insertion of the ionotropic AMPA-type glutamate receptor at synapses (Gerges et al., 2004, Gerges et al., 2005).

Our laboratory has previously reported that the Rab8 effector molecule, optineurin associates with mGluR1a resulting in attenuated mGluR1a-stimulated IP formation (Anborgh et al., 2005). In the present study, we find that Rab8 also contributes to the regulation of mGluR1a G protein signalling. We find that Rab8 overexpression specifically contributes to the uncoupling of mGluR1a-, but not mGluR1b-stimulated IP formation. This inability of Rab8 to regulate mGluR1b signalling is correlated with the observations that mGluR1b lacks an extended carboxyl-terminal tail, and that Rab8 does not effectively associate with this mGluR1 variant. Therefore, the association between Rab8 and mGluR1a appears to be crucial for the ability of Rab8 to regulate mGluR1a signalling. We also show that mGluR1a displays no difference in Rab8-mediated IP signal attenuation when co-expressed with Rab8 nucleotide binding mutants, indicating

that Rab8 nucleotide-binding status does not play a role in regulating mGluR1a signalling.

Rab8-mediated attenuation of mGluR1a signalling is reversed by PKC inhibition, suggesting that Rab8 modulates PKC-dependent desensitization of mGluR1a. Similar to the decrease in IP formation found in HEK293 cells following Rab8 overexpression, we also find that GFP-Rab8 overexpression in primary mouse hippocampal neurons significantly reduces intracellular  $\text{Ca}^{2+}$  release in response to the activation of endogenously expressed group I mGluRs with DHPG. This role for PKC in Rab8-mediated attenuation of mGluR1a signalling is likely dependent upon PKC-dependent mGluR1a phosphorylation (Hermans and Challis, 2001).

Dominant negative or depletion of Rab8 by siRNA significantly attenuates ERK1/2 activation by the  $\alpha_{2B}$ AR, but not the  $\beta_2$ AR and inhibits plasma membrane delivery of  $\alpha_{2B}$ AR from the TGN (Dong et al., 2010). We find here, that although Rab8 overexpression blocks second messenger formation in response to mGluR1a, agonist-stimulated ERK1/2 phosphorylation is unaltered in the presence of the GTPase. However, we did find basal ERK1/2 phosphorylation in mGluR1a expressing cells was significantly attenuated in cells co-expressing Rab8. Thus, Rab8 may selectively contribute to the regulation of agonist-stimulated G protein-signalling, as opposed to regulating G protein-independent signalling mediated by the association of other proteins with mGluR1a such as Pyk2 (Nicodemo et al., 2010). However, the diminished basal ERK1/2 activity may be associated with attenuated basal mGluR1a endocytosis.

In summary, our results establish a novel role for Rab8 in the regulation of mGluR1a endocytosis and signalling. In contrast to what has previously been shown for

other GPCRs and Rabs, this is the first report of a Rab GTPase inhibiting GPCR endocytosis, while simultaneously attenuating receptor signalling. This opens a new and exciting avenue of research to improve our understanding of the molecular and physiological consequences of Rab GTPase-mediated regulation of GPCR signalling.

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## CHAPTER 4.

### **Naturally Occurring Pathological Mutations in Group I Metabotropic Glutamate Receptor Alter Receptor Intracellular Localization and Signalling<sup>1</sup>**

<sup>1</sup>A version of this chapter has been submitted for publication in *Molecular Pharmacology*

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Figures 4.7 and 4.8B were contributed by MDW, MEL and IHW (Eli Lilly and Company). All other experiments were performed by JLE

#### 4.1. INTRODUCTION

Metabotropic glutamate receptors mediate the actions of the excitatory neurotransmitter, glutamate. These class C receptors are characterised by a large extracellular amino-terminal glutamate binding region comprised of 2 globular domains, which form a distinctive “venus fly trap” (VFT) (Conn and Pin, 1997). The specific amino-terminal glutamate binding region has been identified as a stretch of 24 amino acids whose mutations affect glutamate affinity (Dhami and Ferguson, 2006; O’Hara et al., 1993). Adjacent to the VFT domain is a 70 amino acid Cysteine-rich domain required for allosteric coupling between the VFT and the transmembrane domains (Huang et al., 2011). This region also participates in receptor dimerization via the formation of a Cysteine bridge between receptor pairs, the disruption of which leads to receptor loss of function (Rondard et al., 2008; Romano et al., 2001; Romano et al., 1996).

The second intracellular loop of mGluR is involved in G protein coupling and selectivity (Pin et al., 1994). Group 1 mGluRs (mGluR1 and mGluR5) primarily couple through G $\alpha_q$  and also activate G protein-independent signal transduction pathways including mitogen activated kinases (Gerber et al., 2007; Nicodemo et al., 2010). Group I mGluR-mediated ERK1/2 activation can occur via PKC,  $\beta$ -arrestin or through the non-receptor tyrosine kinases Src and Pyk2 (Emery et al., 2010; Nicodemo et al., 2010; Thandi et al., 2002).

Receptor activation is quickly followed by signal desensitization, a tightly regulated process essential to prevent aberrant signalling or chronic receptor overstimulation (Ferguson, 2001a). Desensitization of mGluRs is complex as mGluR1 desensitization includes phosphorylation-dependent and -independent mechanisms

(Ferguson, 2007; Dhami and Ferguson, 2006; Dhami et al., 2005, Dhami et al., 2004; Dhami et al., 2002). GRK2-mediated mGluR desensitization is phosphorylation-independent, whereby the GRK2 RGS homology domain associates with lysine residues 691 and 692 in the receptor second intracellular loop as well as the  $G\alpha_{q/11}$  subunit of the heterotrimeric G protein (Dale et al., 2000; Dhami et al., 2005; Dhami and Ferguson, 2006; Ferguson, 2007). Meanwhile, second messenger-dependent kinases such as PKC mediate phosphorylation-dependent mGluR desensitization in the receptor second intracellular loop as well as carboxyl-terminal tail (Francesconi and Duvoisin, 2000; Ciruela et al., 1999). Threonine 695 within the second intracellular loop is a target for PKC phosphorylation and mutation at this residue specifically disrupts PKC-mediated receptor desensitization (Francesconi and Duvoisin, 2000; Medler and Bruch, 1999).

GPCR C-tails are responsible for association with many regulatory proteins involved in protein scaffolding and/or transport. For example, the Homer family of proteins is comprised of 3 family members each encoding multiple splice variants (Shiraishi-Yamaguchi and Furuichi, 2007). Homers associate with PPxxFR motif in the C-tails of mGluR1/5 via their amino-terminal ENA/VASP homology domains and regulate the subcellular distribution, plasma membrane target and signalling of mGluR1/5 (Ango et al., 2002; Ciruela et al., 1999; Coutinho et al., 2001; Roche et al., 1999; Tadokoro et al., 1999). Homer proteins link mGluR1/5 to the activation of IP3 receptors, ERK1/2 phosphorylation and the modulation of ion channel activity (Mao et al., 2005; Kammermeier et al., 2000; Yamamoto et al., 2005; Ango et al., 2011).

Recently, genetic screening studies of multiple tumour types has identified several naturally occurring pathological mutations in the ligand binding and intracellular

regulatory domains of mGluR1a (Kan et al., 2010; Parsons et al., 2008; Sjoblom et al., 2006; Wood et al., 2007). In the present study, we have examined the effect of eight mutations identified in lung adenocarcinoma and squamous cell cancer including: mutations in the orthosteric glutamate binding region (D44E and A168) and Cysteine-rich region of the amino-terminal domain (R375G and G396V), intracellular loop 2 (R684C, G696W and G668V) and Homer binding motif (P1148L) on mGluR1a signalling. We find that a subset of these mutations result in altered mGluR1a-stimulated G protein-coupling, biased ERK1/2 phosphorylation, intracellular retention in the endoplasmic reticulum (ER), as well as lost Homer binding that are associated with altered subcellular localization of mGluR1a

## **4.2. EXPERIMENTAL PROCEDURES**

### **4.2.1. Materials**

*myo*-(<sup>3</sup>H)Inositol was acquired from PerkinElmer Life Sciences (Waltham, MA). Dowex 1-X8 (formate form) resin 200–400 mesh was purchased from BioRad (Mississauga, ON, Canada). Normal donkey serum was purchased from Jackson ImmunoResearch (West Grove, PA, USA). ECL Western blotting detection reagents were purchased from GE Healthcare (Oakville, ON, Canada). Horseradish peroxidase-conjugated anti-rabbit and anti-goat IgG secondary antibody were obtained from BioRad (Mississauga, ON, Canada) and anti-mGluR1 rabbit polyclonal antibody purchased from Upstate (Lake Placid, NY, USA). Rabbit polyclonal phospho-p42/44 MAP kinase (Thr202/Tyr402), p42/44 MAP kinase antibodies were obtained from Cell Signaling Technology (Pickering, ON, Canada). Alexa Fluor 488 donkey anti-Rabbit IgG, Alexa Fluor 568 donkey anti-rabbit IgG and Zenon Rabbit Alexa Fluor 555 were purchased

from Invitrogen/Molecular Probes (Burlington, ON, Canada). Rabbit anti-FLAG antibody, M2 anti-FLAG agarose and all other biochemical reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada).

#### **4.2.2. Cell Culture**

Human embryonic kidney (HEK) 293 cells were maintained in Eagle's minimal essential medium supplemented with 8% (v/v) heat inactivated fetal bovine serum (Invitrogen, Burlington, ON) and 50 µg/ml gentamicin. Cells seeded in 100 mm dishes were transfected using a modified calcium phosphate method as described previously (Ferguson and Caron, 2004). Following transfection (18 h), the cells were incubated with fresh medium and allowed to recover for 24 hrs for co-immunoprecipitation studies. Otherwise, they were allowed to recover for 6-8 hrs and re-seeded into 12- well or 24- well dishes and then grown an additional 18 hrs prior to experimentation.

#### **4.2.3. ERK Activation and Immuno Blotting**

HEK 293 cells were transiently transfected with the cDNAs described in the *Figure Legends*. Forty-eight hours post-transfection cells were serum starved overnight in glutamine-free DMEM and stimulated for the indicated times with 30 µM quisqualate. The cells were then placed on ice, washed two times with ice-cold phosphate-buffered saline (PBS) and lysed with cold-lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10 µg/ml leupeptin, and 5 µg/ml aprotinin) and phosphatase inhibitors (10 mM NaF, 5 µM Na<sub>3</sub>VO<sub>4</sub>). The cells were placed on a rocking platform for 15 min at 4°C and centrifuged at 15000 x g for 15 min at 4°C to pellet insoluble material. Cell extracts were solubilized in a 3X SDS sample buffer containing 2-mercaptoethanol (BME). Samples were separated by SDS-



PAGE, transferred to a nitrocellulose membrane and immunoblotted to identify phosphorylated (active) and total p42/44 (ERK1/2) (1:1000 dilution, Cell Signalling) followed by a horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:10000, BioRad). Receptor protein expression was determined by immunoblotting 10 µg of protein from each cell lysate. Proteins were detected using chemiluminescence with the ECL kit from GE Healthcare.

#### **4.2.4. Co-immunoprecipitation**

HEK 293 cells were transiently transfected with cDNAs as described in the *Figure Legends*. Following transfection, the cells were incubated for 15 min in HEPES balanced salt solution (HBSS) at 37°C with or without 30 µM quisqualate. The cells were then placed on ice, washed two times with ice-cold phosphate-buffered saline (PBS) and lysed with cold-lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 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 15000 x g for 15 min at 4°C to pellet insoluble material. Cleared supernatant containing 250 µg protein was incubated with 25 µL of FLAG M2-affinity beads for 1h rotating at 4°C to immunoprecipitate FLAG-mGluR1a. Following incubation, the beads were washed twice with PBS, and proteins were solubilized in a 3X SDS sample buffer containing 2-mercaptoethanol (BME). Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted to identify co-immunoprecipitated YFP-tagged GRK2 protein using a primary mouse anti-GFP antibody (1:1000 dilution) followed by a horseradish peroxidase-conjugated secondary anti-mouse antibody (1:2500 dilution). Receptor and GRK2 protein expression was

determined by immunoblotting 10 µg of protein from each cell lysate used for immunoprecipitation. Proteins were detected by chemiluminescence.

#### **4.2.5. Measurement of inositol phosphate formation**

HEK 293 cells were transiently transfected with cDNAs as described in the *Figure Legend*. Forty-eight hours post-transfection cells were incubated overnight in inositol- and glutamine-free DMEM with 100 µCi/mL *myo*-[<sup>3</sup>H]-Inositol. For all experiments cells were incubated for one hour in warm HBSS (116 mM NaCl, 20 mM HEPES, 11 mM glucose, 5 mM NaHCO<sub>3</sub>, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) and were then incubated with 10 mM LiCl alone for 10 min followed by 30 µM quisqualic acid in LiCl for 30 min. Cells were placed on ice and the reaction was stopped with 500 µL of perchloric acid and neutralized with 400 µl of 0.72 M KOH, 0.6 M KHCO<sub>3</sub>. Total cellular [<sup>3</sup>H]-inositol incorporation was determined in 50 µl of cell lysate. Total inositol phosphate was purified by anion exchange chromatography using Dowex 1-X8 (formate form) 200-400 mesh anion exchange resin and [<sup>3</sup>H]-inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system.

#### **4.2.6. Confocal microscopy**

Confocal microscopy was performed using a Zeiss LSM- 510 META laser scanning confocal microscope equipped with a Zeiss 63X, 1.4 numerical aperture, oil immersion lens (North York, ON, Canada). For live cell imaging, HEK 293 cells expressing FLAG-mGluR1a constructs were serum starved for 1 hr at 37°C in HBSS. HEK 293 cells were pre-labelled with Zenon Alexa Fluor 568-conjugated with anti-FLAG polyclonal rabbit antibody (Invitrogen). Cells were then either kept on ice or

stimulated with 30  $\mu$ M quisqualate for 30 min at 37°C. For fixed cell imaging, cells were washed three times at room temperature PBS, fixed for 10 min at room temperature with Periodate-Lysine-Paraformaldehyde (PLP) fixative (McLean and Nakone, 1974) followed by 10 min permeabilization with 0.01% Triton X-100. Cells were blocked with 3% normal donkey serum (Jackson ImmunoResearch) and labelled with Rabbit anti-FLAG polyclonal rabbit antibody followed by donkey anti-rabbit Alexa Fluor 488 antibody. Endoplasmic reticulum was labelled with red fluorescence protein (RFP)-fused lysine-aspartate-glutamate-leucine (KDEL) ER retention sequence. Colocalization studies were performed using dual excitation (488, 543 nm) and emission (band pass 505-530 nm and long pass 560 nm for Alexa Fluor 488 and 568, respectively) filter sets.

#### **4.2.7. Flow Cytometry**

HEK 293 cells were transiently transfected with the cDNAs described in the *Figure Legends*. Forty-eight hours after transfection cells were placed on ice and washed in ice-cold HBSS. Flag-tagged mGluR1a constructs were labelled with primary rabbit anti-Flag antibody (1:500) followed by secondary goat anti-rabbit AlexaFluor-488 antibody (1:500). Cells were incubated for 10 min in 5  $\mu$ M EDTA, gently removed from dish by pipetting and fixed in 3.6% formaldehyde final concentration. Cell surface mean fluorescence was assessed by flow cytometry.

#### **4.2.8. Statistical Analysis**

Densitometric data were normalized first for protein expression and the control value was set to 100, with all other values displayed as percentage thereof. One-way analysis of variance test (ANOVA) was performed to determine significance, followed by a post-hoc Tukey multiple comparison test or Bonferroni's multiple comparisons test to

determine which means were significantly different ( $p < 0.05$ ) from one another.

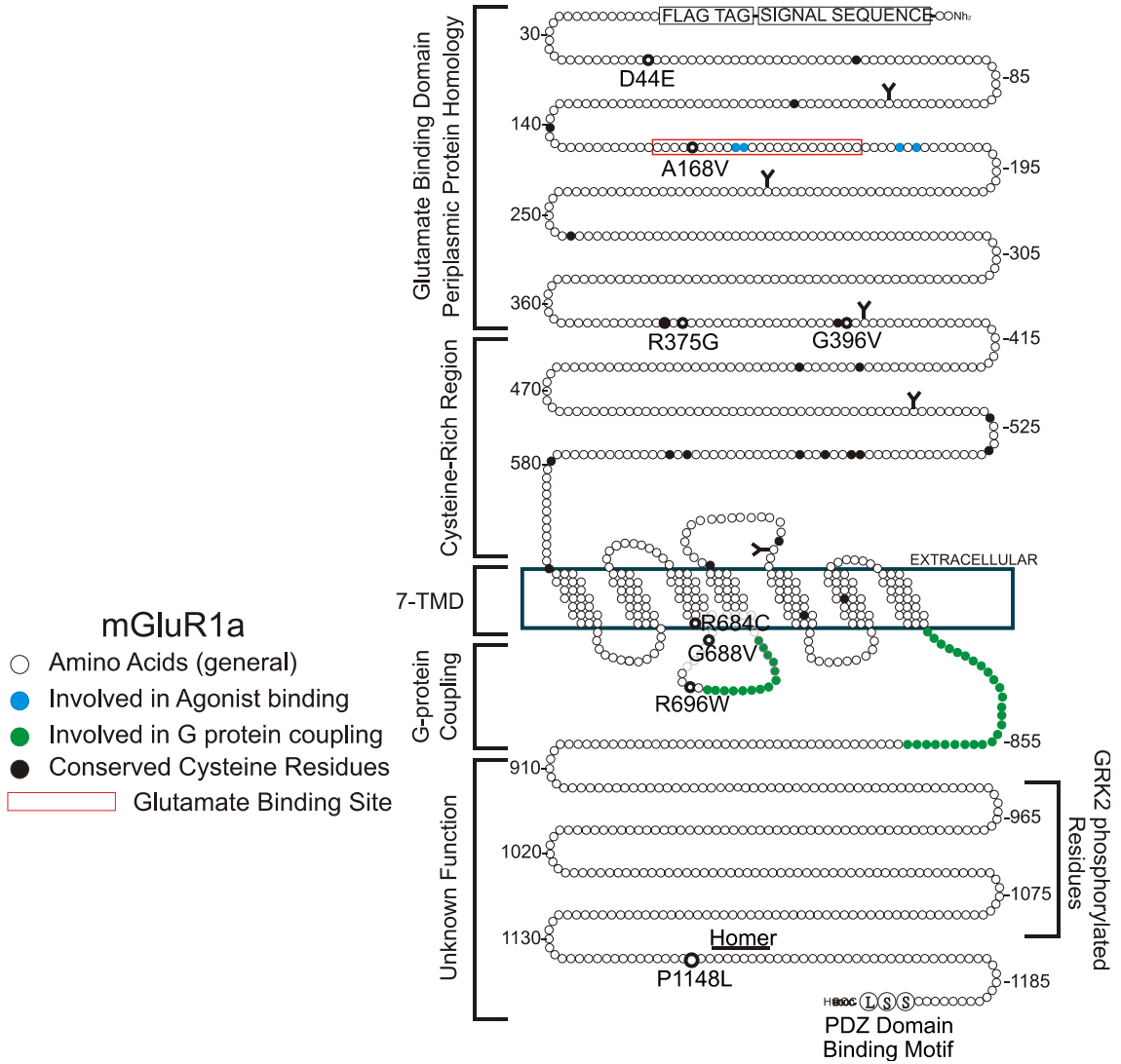
### **4.3. RESULTS**

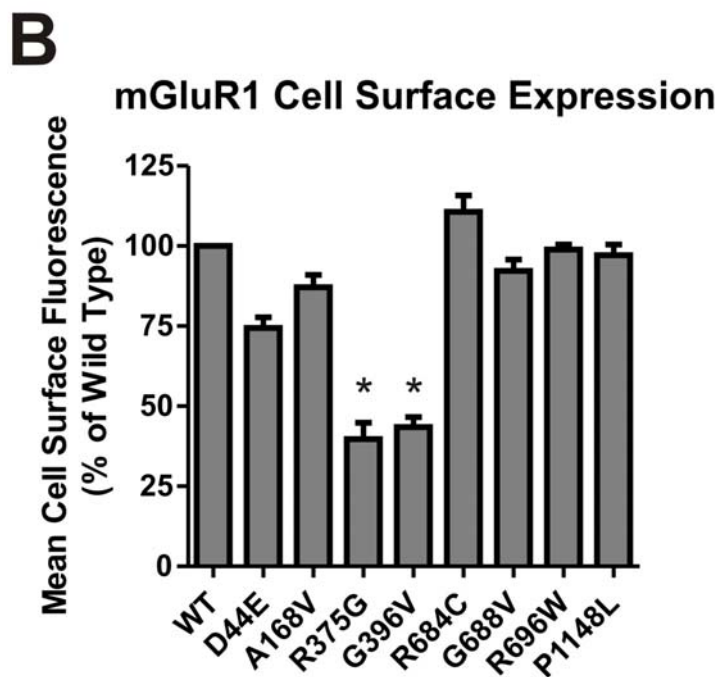
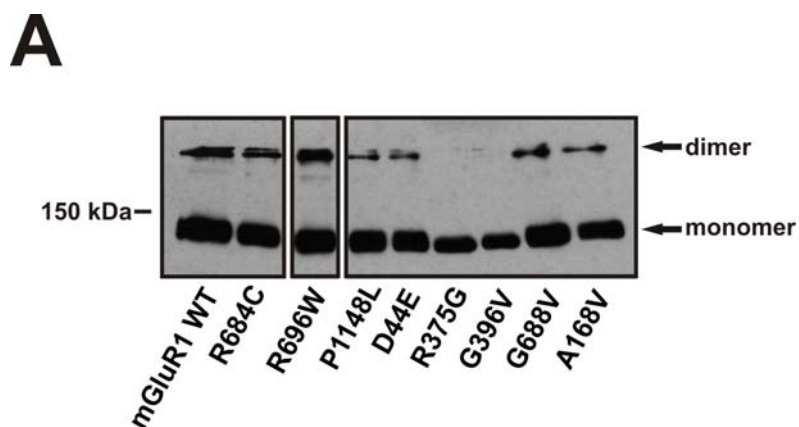
#### **4.3.1. Mutations in the cysteine-rich domain contribute to receptor dimerization and cell surface expression**

Eight naturally occurring mGluR1 mutations were previously identified in variety of tumour types with four mutations localized to the amino-terminal domain of mGluR1 (D44E, A168V, R375G and G396V) three mutations localized to the mGluR1 second intracellular loop (R684C, G688V and R696W) and one mutation localized to the Homer binding motif (P1148L) (Figure 4.1) (Kan et al., 2010; Parsons et al., 2008; Sjoblom et al., 2006; Wood et al., 2007). Initial experiments examined whether the cell surface expression and dimerization of FLAG-mGluR1a was affected by each of the mutations introduced into the coding sequence for mGluR1a. Immunoblot analysis demonstrated that FLAG-mGluR1 mutations R375G and G396V, which are localized at the border of the glutamate binding domain and cysteine-rich regions, exhibited a reduction in dimer formation (Figure 4.2A). Consistent with a loss of mGluR1a dimerization, cell surface expression of FLAG-mGluR1a-R375A and -G396A exhibited reduced cell surface expression as assessed by flow cytometry; with cell surface expression reduced to  $39.7 \pm 5.1\%$  and  $43.4 \pm 3.2\%$  of wild type mGluR1a control transfected cells (Figure 4.2B). The reduction in cell surface FLAG-mGluR1a-R375A and -G396A expression was associated with an increased retention of both receptor mutants in the ER as demonstrated by increased colocalization with the ER marker construct KDEL-GFP (Figure 4.3). Thus, both the R375A and -G396A mutations result in a significant reduction of mGluR1a expression at the cell surface as a consequence of ER retention.

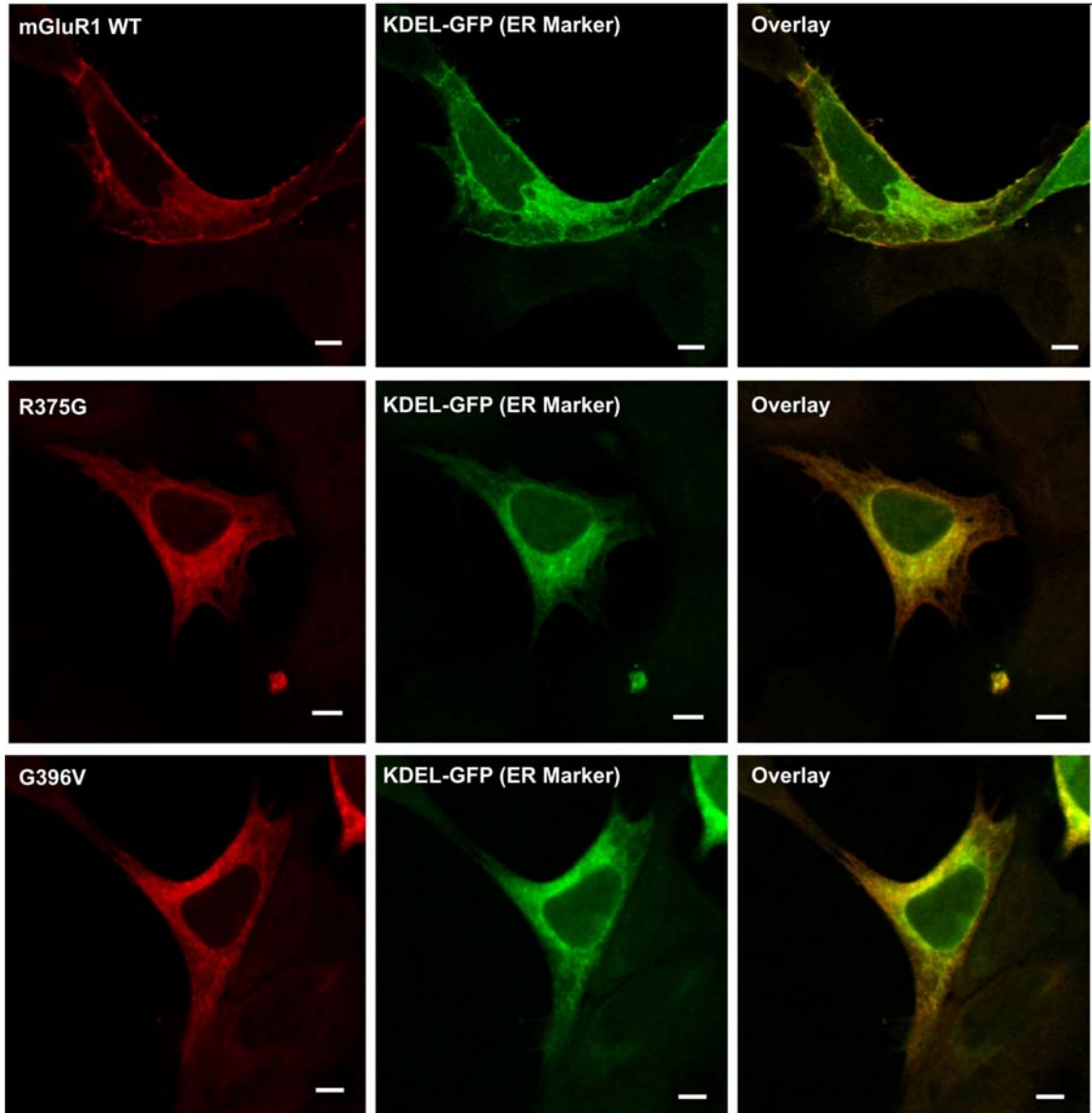
**Figure 4.1. Eight naturally occurring single nucleotide polymorphisms in mGluR1a.** Eight mutations have been identified within the coding sequence for mGluR1a including: A168V, a mutation in the orthosteric glutamate binding region identified in lung adenocarcinoma; two mGluR1a variants in the cysteine-rich region, R375G (identified in squamous cell carcinoma) and G396V (identified in lung adenocarcinoma); three mutations in the second intracellular loop including: the glioblastoma mutation, R684C, the squamous cell carcinoma mutation, G688V, and the colorectal cancer G696W mutation located close to the putative PKC phosphorylation site T695A. A colorectal cancer mutation is located within the Homer binding region in the carboxyl-terminal mGluR1, P1148L.

# Metabotropic Glutamate Receptor 1a





**Figure 4.2. Cell surface expression and intracellular localization of mGluR1a variants.** (A) Representative immunoblot of HEK 293 cells expressing 2  $\mu$ g Flag-tagged mGluR1 constructs highlighting the monomeric (lower) and dimeric (upper) species of the receptor. (B) HEK 293 cells expressing 2  $\mu$ g Flag-tagged mGluR1a constructs were immunolabelled and cell surface mean fluorescence was assessed by flow cytometry. Data were normalized for total protein expression and represent the standard error of the mean of four independent experiments. \*,  $p < 0.001$  compared to wild type.



**Figure 4.3. mGluR1 variants localize to endoplasmic reticulum.** Representative confocal micrographs illustrating receptor intracellular localization (red) and colocalization with the endoplasmic reticulum (green). HEK293 cells expressing 2 μg Flag-tagged mGluR1 constructs were fixed and immunolabelled for total cellular complement of receptor. Images are representative of 3 independent experiments. Bars represent 5 μm.



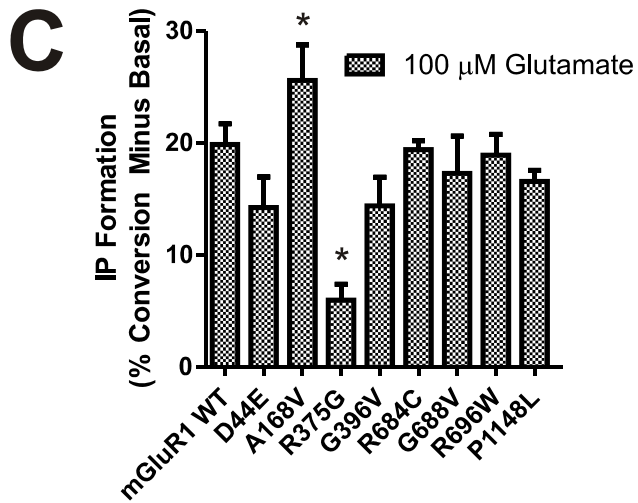
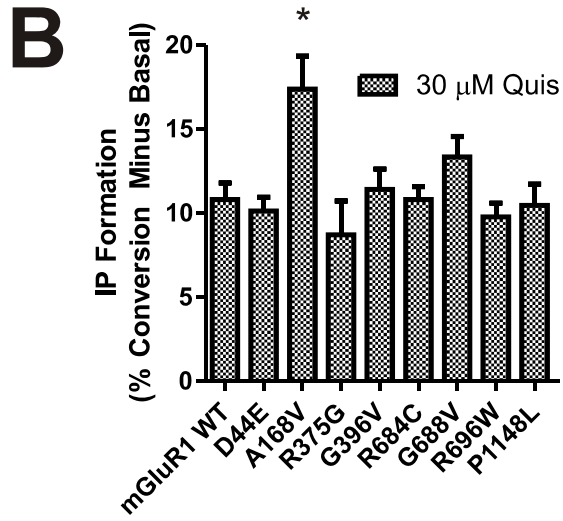
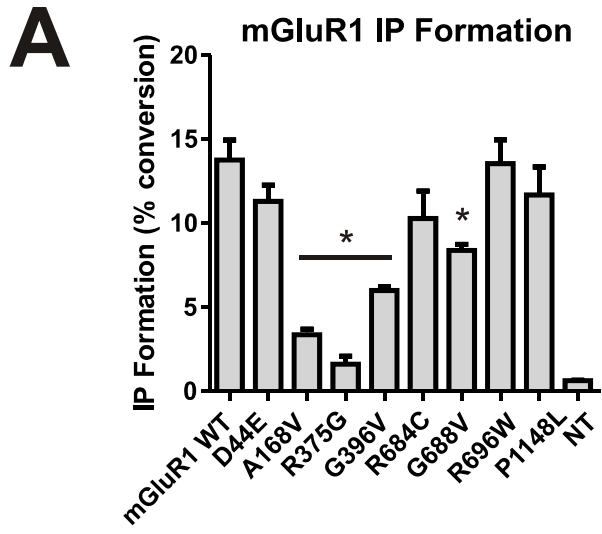
#### **4.3.2. Mutations in the amino-terminus exhibit altered basal and agonist-activated activity**

Group I mGluRs activate both IP3 and ERK signal transduction cascades and exhibit high basal activity (Dale et al., 2000). Interestingly, three of the four amino-terminal mGluR1 variants we examined (A168V, R375G, G396V) showed significantly reduced basal IP3 formation (in the absence of agonist) compared to wild type and FLAG-mGluR1a-R375G exhibited basal IP3 formation that was indistinguishable from non-transfected cells (Figure 4.4A). Quisqualate-mediated activation of FLAG-mGluR1a-A168V (a site located within the glutamate binding region) resulted in a  $63 \pm 17\%$  increase in IP3 formation when compared to control FLAG-mGluR1a transfected cells (Figure 4.4B). Quisqualate-stimulated FLAG-mGluR1a-R375G IP3 formation was comparable to FLAG-mGluR1a transfected cells, despite reduced cell surface expression. However, glutamate-stimulated FLAG-mGluR1a-R375G IP3 formation was reduced to  $29 \pm 4\%$  of FLAG-mGluR1a transfected cells (Figure 4.4C). Therefore, activation of this mutant by glutamate and quisqualate resulted in divergent signalling patterns.

#### **4.3.3. R375G displays functional selectivity toward ERK1/2**

To examine whether the mGluR1a mutations associated with various cancer cell lines also affected other mGluR1a-activated cell signalling pathways, we examined whether the mutations influenced the ability of mGluR1a to stimulate ERK1/2 phosphorylation in HEK293 cells. Similarly to what we found for IP3 formation, all four amino-terminal mutations also exhibited decreased basal ERK1/2 phosphorylation, but still displayed a statistically significant increase in ERK1/2 activation in response to

**Figure 4.4. Mutations in mGluR1 alter basal and agonist-activated inositol phosphate formation.** (A) Basal IP3 formation in HEK 293 cells transiently transfected with 2  $\mu$ g Flag-tagged mGluR1a constructs. Cells were labelled overnight with *myo*-( $^3$ H)Inositol in glutamine-free DMEM, incubated for 10 min with 10 mM LiCl followed by (B) 30  $\mu$ M quisqualic acid or (C) 100  $\mu$ M glutamate for 30 min. Total cellular ( $^3$ H)inositol was collected and purified by anion exchange chromatography and [ $^3$ H]-inositol phosphate formation was determined by liquid scintillation. Data were normalized for protein expression and represent the standard error of the mean of four independent experiments. \*,  $p < 0.01$  compared to wild type with the same treatment.

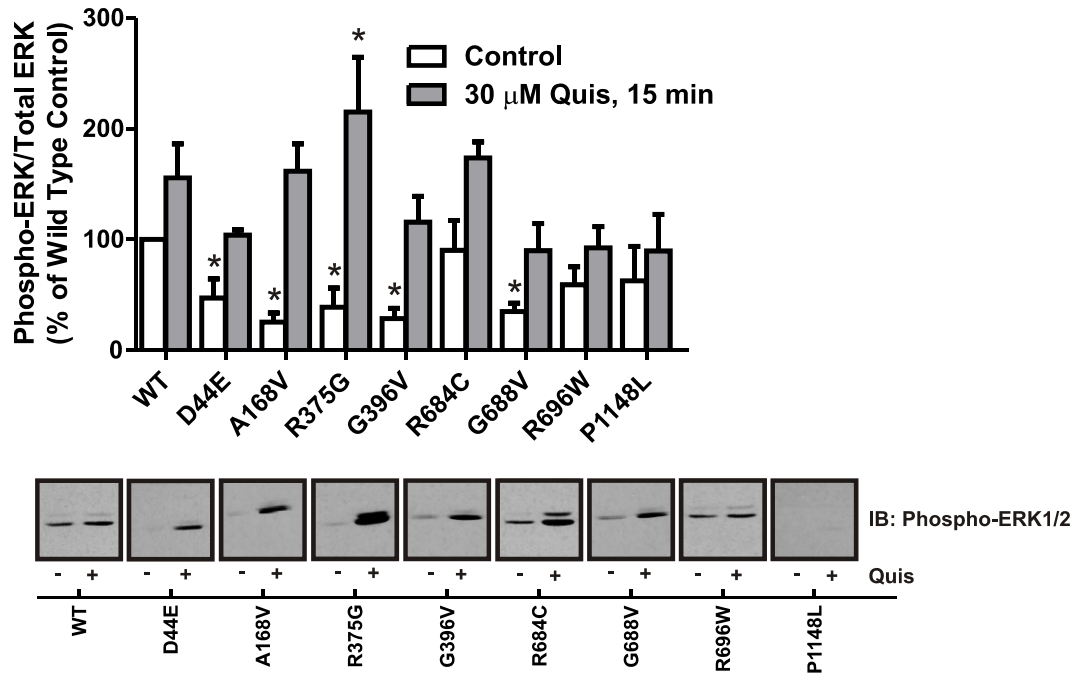
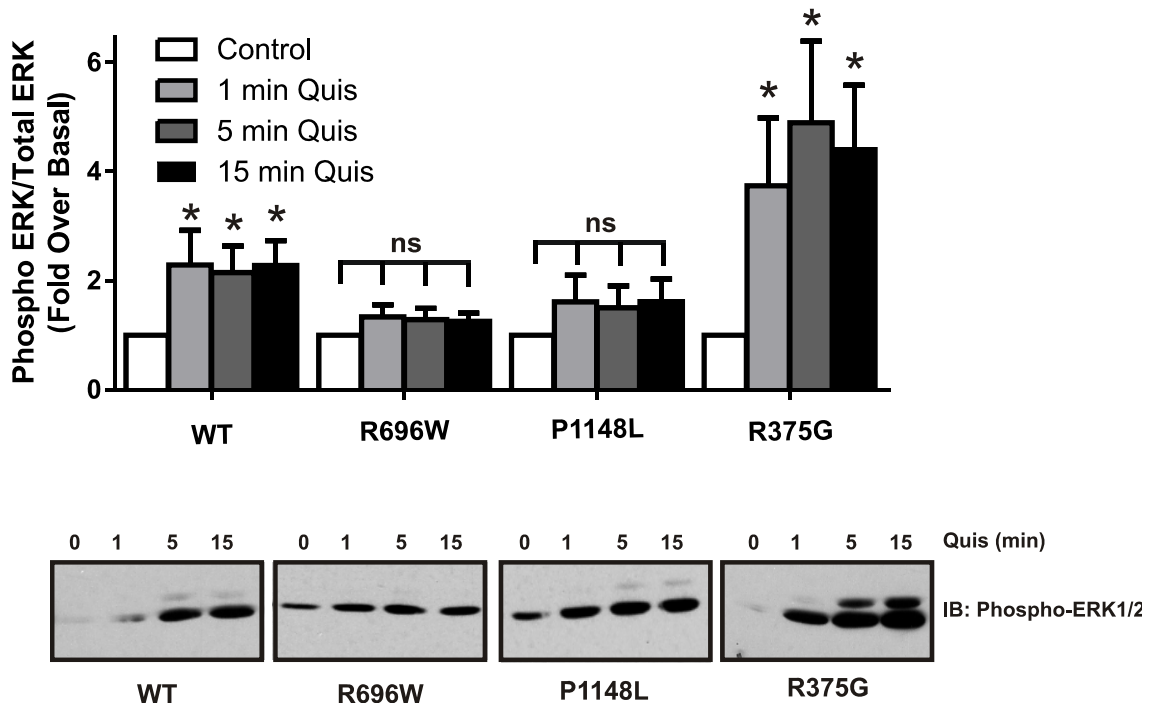


quisqualate treatment (Figure 4.5A). Interestingly, although FLAG-mGluR1a-R375G demonstrated decreased basal ERK1/2 phosphorylation, quisqualate-mediated ERK1/2 phosphorylation in FLAG-mGluR1-R375G expressing cells resulted in significantly increased ERK1/2 phosphorylation following 1, 5 and 10 min agonist stimulation when compared to wild-type FLAG-mGluR1a (Figure 4.5B). In contrast, ERK1/2 phosphorylation in response to the activation of either FLAG-mGluR1a-R696W or -P1148L was significantly reduced when compared to wild-type FLAG-mGluR1a. Therefore, the R375G mutant appeared to be biased towards the activation of the ERK1/2 pathway, whereas the R696W and P1148L mutations were biased for G protein-mediated signalling.

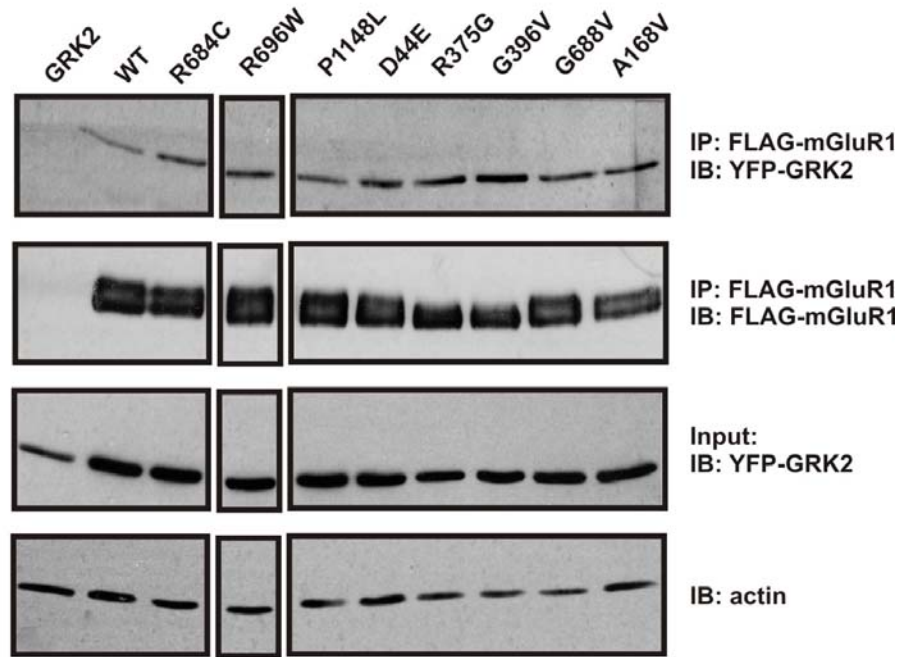
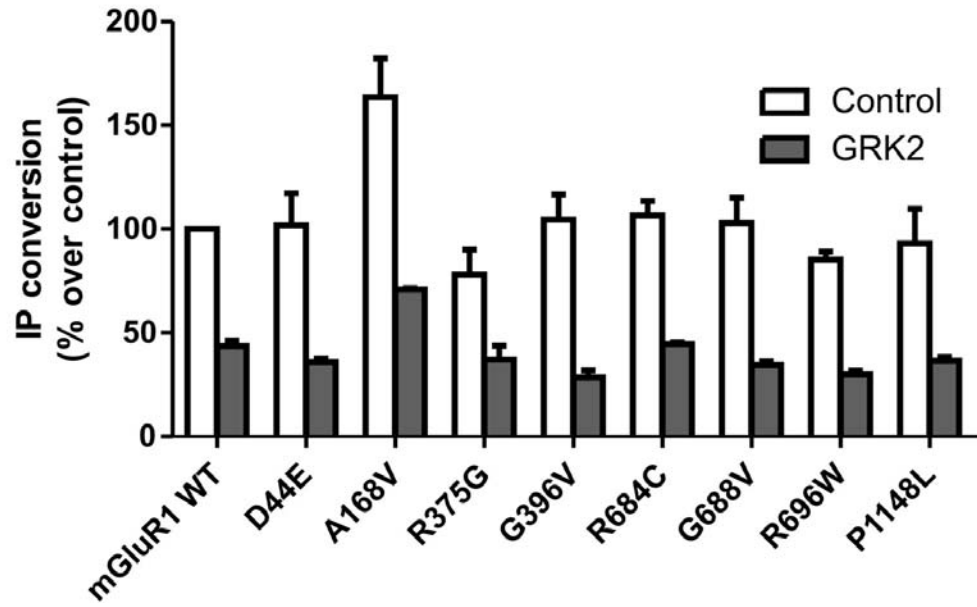
#### **4.3.4. GRK2 binding to mGluR1a mutants**

Several of the identified mGluR1a mutations are localized to the second intracellular loop domain of the receptor, including R684C, G688V and G696W, a domain that is important for GRK2 binding and phosphorylation-independent desensitization of the receptor. We find that association of GRK2 with these FLAG-mGluR1a variants was unchanged compared to wild-type FLAG-mGluR1a as determined by co-immunoprecipitation (Figure 4.6A). Additionally, these mGluR1 second intracellular loop mutations all continue to be desensitized by GRK2 overexpression (Figure 4.6B). Interestingly, although R696W showed no alterations in IP3 formation, agonist-activation of this mutant failed to induce ERK1/2 phosphorylation (Figure 4.5B). Therefore, mutation in this region does not alter GRK2 association or GRK2-mediated IP3 desensitization, but showed reduced receptor-mediated ERK1/2 activity.

**Figure 4.5. Changes in mGluR1-mediated ERK1/2 activation.** HEK 293 cells were transiently transfected with 2  $\mu$ g FLAG-mGluR1a constructs. Forty-eight hours post-transfection cells were serum starved overnight in glutamine-free DMEM and stimulated for **(A)** 15 min or **(B)** 0, 1, 5 or 15 min with 30  $\mu$ M quisqualate. Cell lysates were collected, subjected to SDS-page and immunoblotted for phosphorylated and total p42/44 (ERK1/2). Data were normalized for protein expression and represent the standard error of the mean for 3-5 independent experiments. ns, not significant from unstimulated. \*,  $p < 0.05$  compared to wild type.

**A****B**

**Figure 4.6. Effect of mGluR1a mutations on GRK2 binding and receptor desensitization.** (A) HEK 293 cells were transiently transfected with 2  $\mu$ g Flag-tagged mGluR1 constructs along with 1  $\mu$ g GFP-GRK2. Forty-eight hours after transfection cells were lysed and FLAG-mGluR1a was immunoprecipitated. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted to identify co-immunoprecipitated YFP-tagged GRK2 protein. Receptor and GRK2 protein expression was determined by immunoblotting 10  $\mu$ g of protein from each cell lysate used for immunoprecipitation. (B) HEK 293 cells were transiently transfected with 2  $\mu$ g Flag-tagged mGluR1 constructs along with 1  $\mu$ g empty pEGFP or GFP-GRK2. Cells were labelled overnight with *myo*-( $^3$ H)inositol in glutamine-free DMEM, incubated for 10 min with 10 mM LiCl followed by 30  $\mu$ M quisqualate for 30 min. Total cellular [ $^3$ H]-inositol was collected and purified by anion exchange chromatography and ( $^3$ H)inositol phosphate formation was determined by liquid scintillation. Data were normalized for protein expression and represent the standard error of the mean of three independent experiments.

**A****B**

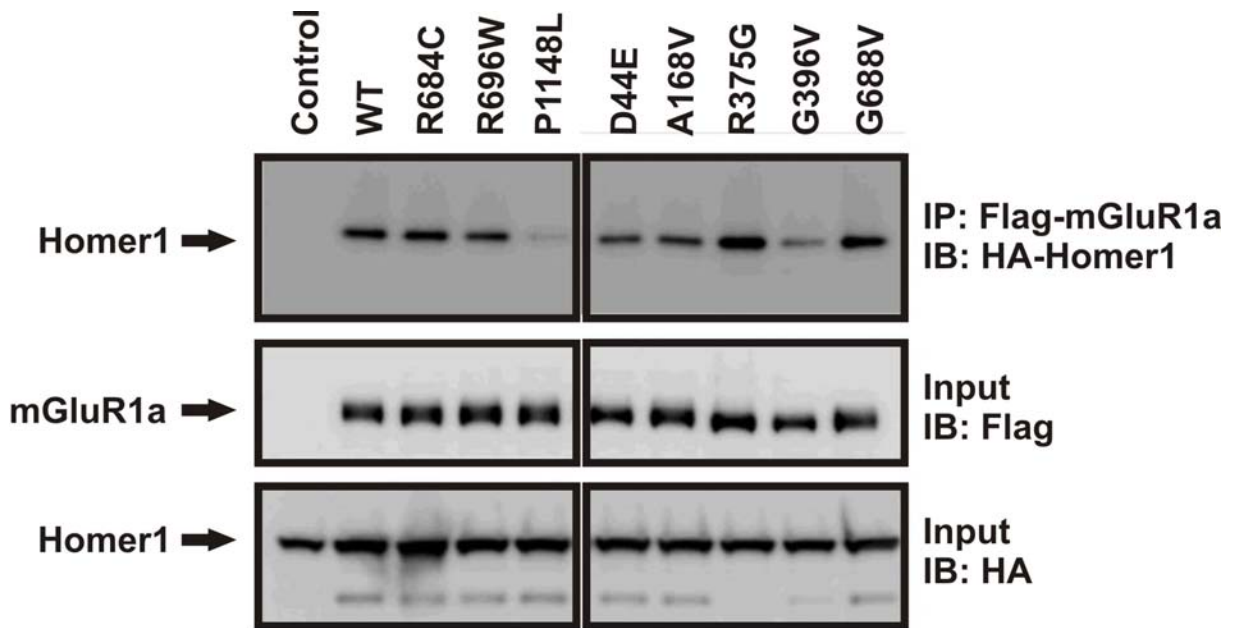


#### **4.3.5. Attenuated Homer binding to FLAG-mGluR1a P1148L**

The carboxyl-terminus mGluR1a encodes a Homer binding motif and the association of Homers with mGluR1a has been shown to modulate both the subcellular localization and signalling of mGluR1a (Brakeman et al., 1997; Wood et al., 2007; Kammermeier, 2008; Bertaso et al., 2010; Ronesi et al., 2008, 2012). The introduction of the P1148L mutations into the C-tail of FLAG-mGluR1a did not affect IP3 formation in response to agonist activation of the receptor (Figure 4.4), but diminished agonist-stimulated ERK1/2 phosphorylation and resulted in a loss of Homer 1b binding to the receptor (Figure 4.5B and 4.7). Co-immunoprecipitation experiments show this mutant exhibits decreased association with Homer 1 compared to wild type mGluR1 (Figure 4.7). Moreover, the P1148L mutation resulted in altered subcellular localization of mGluR1a to membrane ruffles, multinucleated cells and increased filopodia formation in HEK 293 cells, an effect that was not observed for the wild-type receptor (Figure 4.8).

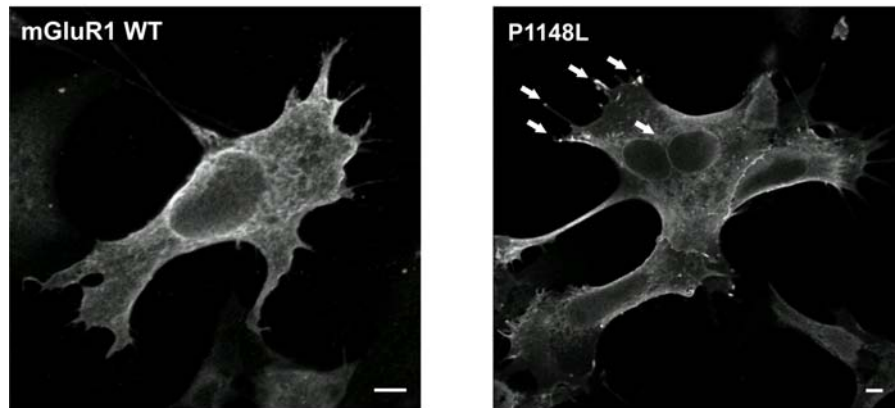
#### **4.4. DISCUSSION**

In the present study, we have characterised the intracellular localization, signalling, association with regulatory molecules and cellular morphology of eight previously unstudied naturally occurring mGluR1 mutations (Kan et al., 2010; Parsons et al., 2008; Sjoblom et al., 2006; Wood et al., 2007). A unique characteristic of class C GPCRs is their constitutive dimerization at the cell surface (Kniazeff et al., 2011). Group I mGluRs have been shown to homodimerize through covalent linkage via disulphide bonding at cysteine 140 within the large extracellular amino-terminal domain, although mutation of this residue is not sufficient to inhibit dimerization (Kniazeff et al., 2004; Ray and Hauschild, 2000; Robbins et al., 1999). Here we identify two mGluR1a variants,

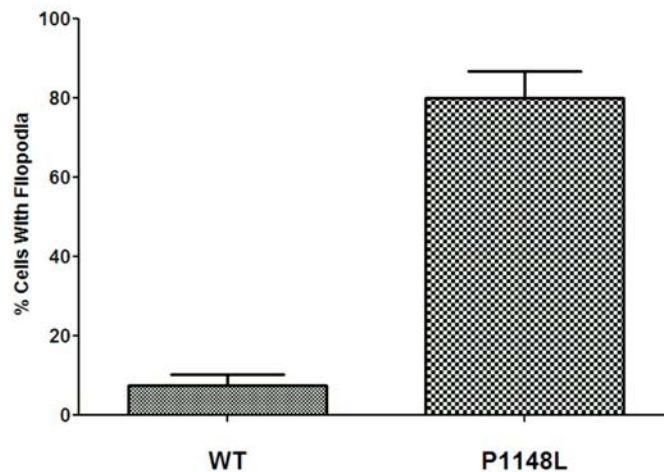


**Figure 4.7. mGluR1 P1148L is deficient in Homer1 binding.** HEK293 cells were co-transfected with HA-Homer1 and empty vector ('-'), or indicated FLAG-tagged mGluR1 constructs. Anti-FLAG was used to immunoprecipitate ('IP') mGluR1 from resultant lysates. 48h post-transfection, co-immunoprecipitated proteins and total lysates resolved by SDS-PAGE were immunoblotted ('IB') with anti-FLAG and anti-HA antibodies.

**A**



**B**



**Figure 4.8. Homer binding mutant mGluR1 P1148L promotes filopodia formation.**

(A) Representative confocal micrographs illustrating receptor localization and cell morphology. HEK293 cells expressing 2  $\mu$ g Flag-tagged mGluR1 constructs were fixed and immunolabelled for total cellular complement of receptor. Images are representative of 3 independent experiments. Bars represent 5  $\mu$ m. (B) Graph scoring the number of cells displaying filopodia. NIH-3T3 cells transiently expressing Flag-mGluR1 wild type or P1148L were fixed and immunolabelled for total cellular complement of the receptor and number cells displaying filopodia were counted per field of view. 100 cells from three separate experiments were counted randomly and a data represent the average standard error of the mean of three independent experiments.

R375G and G396V, which migrate on a western blot exclusively at the size of the monomer (~140 kDa). The amino-terminal region of these mutations is consistent with other studies, which report covalent linkages are not essential for dimerization of all Group I mGluRs, suggesting that multiple interactions are involved in the dimerization of these receptors (Sato et al., 2003; Romano et al., 2001, 1996). However, it is becoming clear that proper receptor targeting and function of many GPCRs is dependent on dimer formation (Milligan, 2010). For example, the GABA<sub>B</sub> receptor requires both GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits in order to reach the plasma membrane as well as activate G proteins (White et al., 1998; Duthey et al., 2002; Pin et al., 2004). Heterodimerization of GABA<sub>B</sub> receptor subunits masks an ER retention sequence in the carboxyl-terminus of GABA<sub>B</sub> (Margeta-Mitrovic et al., 2000). Additionally, heterodimerization between the  $\alpha_{1D}$ - and  $\alpha_{1B}$ -adrenoceptors, was shown to be necessary for the proper cell-surface expression of the  $\alpha_{1D}$ AR subtype (Hague et al., 2006). Dimerization is also essential for receptor-G protein coupling. GABA<sub>B1</sub> requires co-expression with GABA<sub>B2</sub> in order to couple functionally to the G protein signalling cascade (Galvez et al., 2001; Margeta-Mitrovic et al., 2000). This is not just due to plasma membrane localization as a mutant form of GABA<sub>B1</sub> that lacks its ER retention signal and can reach the cell surface on its own still requires GABA<sub>B2</sub> for functional activity (Margeta-Mitrovic et al., 2000). Our data would support the concept that dimer formation may contribute to appropriate cell surface expression of mGluR1a, as two amino-terminal mutants (R375G and G396V) result in ER retention of mGluR1a and reduced cell surface expression. We also find that these mGluR1a mutants, exhibit significantly reduced basal inositol phosphate formation. However, this reduction in constitutive mGluR1a signalling may be independent of cell

surface expression as another amino-terminal mutation (A168V), which displays the same cell surface expression as wild type, also results in attenuated basal activity.

Group I mGluRs exhibit significant basal G protein activation (Dale et al., 2000). However, three amino-terminal mGluR1a mutations, A168V, R375G and G396V as well as one second intracellular loop mutant (G688V) exhibited a loss of basal activity in HEK 293 cells. The reduction in basal activity for the amino-terminal mGluR1a mutations may be the consequence of altered affinity for glutamate that may be released from the HEK 293 cells to feedback on the receptor. However, inconsistent with this notion is the observation that the mGluR1a-A168V mutant exhibits increased activation of IP3 formation in response to agonist treatment. The intracellular loop mutation G688V also resulted in decreased basal mGluR1a activity without affecting agonist-stimulated responses. However, the rationale for the observed reduction in basal activity of the mGluR1a-G688V variant remains to be determined.

It is well established that mGluRs activate downstream mitogenic pathways such as the ERK1/2 signalling cascade that contributes to alterations in cell proliferation (Rozenfurt, 2007). Group I mGluRs activate ERK1/2 in both calcium-dependent and -independent manners, the latter involving G $\beta\gamma$  and non-receptor tyrosine kinases such as Src and Pyk2 (Nicodemo et al., 2010). We have identified three mGluR1a mutants with altered ERK activation including mGluR1 R375G, which exhibits markedly increased ERK1/2 phosphorylation upon quisqualate stimulation. Although this mutation does not affect quisqualate-stimulated IP3 formation, glutamate-mediated IP3 formation is significantly impaired for the R375G mutant. Taken together, these observations suggest

that mutations localized to the amino-terminal domain of mGluR1a may bias receptor signalling towards the ERK1/2 pathway.

Receptor modulation by regulatory proteins such as GRK2 represents a major mechanism controlling the magnitude and duration of GPCR signal transduction. GRK2-mediated attenuation of mGluR1a signalling occurs as the consequence of the concomitant association of the kinase with the second intracellular loop domain of the receptor and  $G\alpha_{q/11}$  (Dhami et al., 2004; Dhami et al., 2005; Dhami and Ferguson, 2006; Ferguson, 2007). We investigated here, the effect of three intracellular loop 2 mutations on the association of GRK2 with mGluR1a. We find that none of the mutations exhibits reduced GRK2 association and that GRK2 overexpression results in normal attenuation of G protein signalling in response to the activation of mGluR1a second intracellular loop mutants. Interestingly, we also find that ERK1/2 activation in response to activation of mGluR1a-R696W is lost, suggesting that this residue may contribute to the regulation of ERK1/2 signalling by the receptor. Our previous studies have shown that Pyk2 binds to the second intracellular loop domain of mGluR1a and may directly contribute to the activation of ERK1/2 phosphorylation (Nicodemo et al., 2010). Future studies will be required to assess whether this mutation affects Pyk2 binding to mGluR1a.

Of the many regulatory proteins that interact with Group I mGluRs, the Homer family of proteins are predominantly featured as they are synaptically localized and couple Group I mGluRs to the activation of a variety of ion channels at the synapse (Ango et al., 2002; Ciruela et al., 1999; Coutinho et al., 2001; Roche et al., 1999; Tadokoro et al., 1999). Here we show that the naturally occurring mGluR1 variant (P1148L) does not associate with Homer1b, is uncoupled from the activation of ERK1/2

phosphorylation and causes enhanced multinucleation and filopodia formation in HEK 293 cells. The loss of agonist-stimulated ERK1/2 phosphorylation by the mGluR1a-P1148L mutant is consistent with recent reports that Homer 1a may contribute in part to the coupling of mGluR1a to the activation of ERK1/2 (Mao et al., 2005; Mao et al., 2008). The mechanism by which the P1148L mutation leads to increased multinucleation and filopodia formation is unclear, but likely relevant to the association of this mutation with a cancer cell line.

In this study, we characterised the signalling and intracellular localization of eight somatic mutations in mGluR1 identified in genome-wide screens of various cancerous tissue samples. We identified two mutations involved in dimerization and plasma membrane targeting as well as several mutations differentially affecting IP3 formation and MAPK signalling of this receptor. For example, we demonstrate that the R375G mutant preferentially couples to ERK1/2 activation while exhibiting decreased basal and glutamate-activated inositol phosphate production. Additionally, the A168V mutant displays decreased basal and increased agonist-mediated IP3 formation. These alterations in the mGluR1a activity may contribute, at least in part, to the phenotype of the cancer cell lines in which they were identified. This study sheds new light on the functionality of different regions of the receptor as well as further defining the residues involved in mGluR1 signalling and the role of mGluR1 signalling in pathologies and may provide an exciting opportunity for developing new mGluR1-targeted treatments.

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**CHAPTER 5.**  
**DISCUSSION**

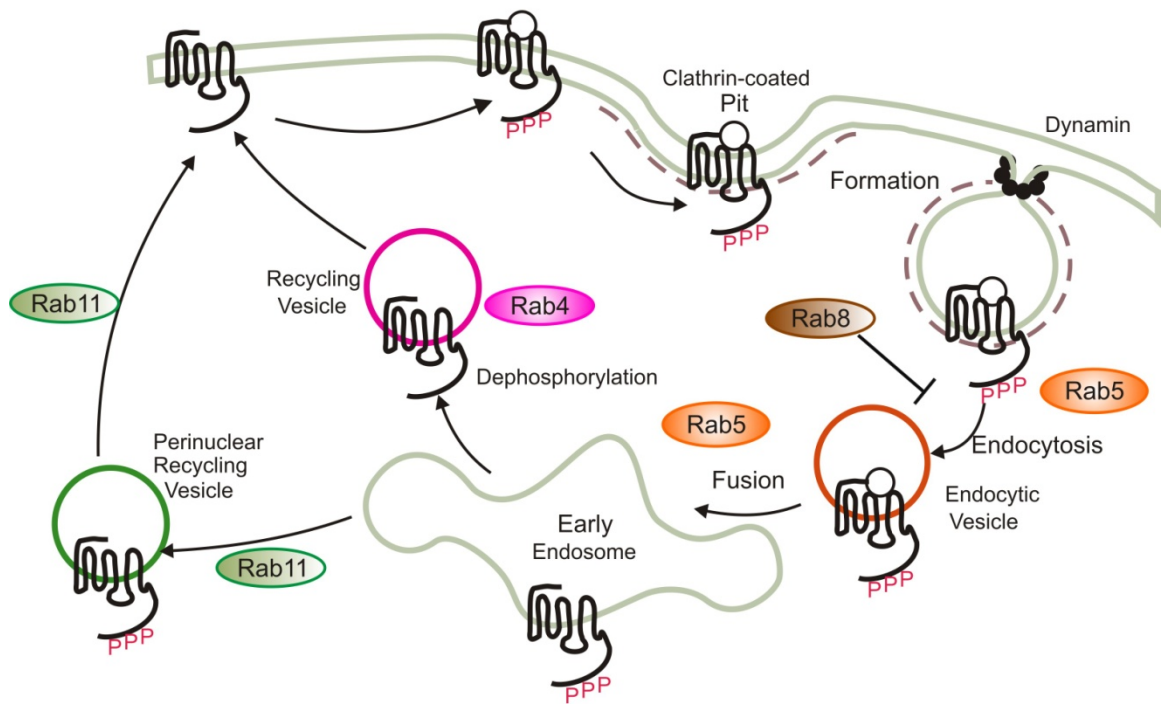
## 5.1. SUMMARY

The aim of this thesis was to elucidate the role that receptor trafficking plays in signal transduction by addressing the following three questions:

5. Do multiple Rab proteins associate with AT<sub>1</sub>R to alter receptor desensitization or resensitization?
6. Does Rab8 associate with mGluR1 to modulate its intracellular trafficking and signalling?
7. What alterations do previously uncharacterized single nucleotide polymorphisms in mGluR1 exhibit in intracellular localization and signal transduction?

The data presented in chapters 2, 3 and 4 summarize my findings aimed at answering these important questions pertaining to the role intracellular trafficking plays in GPCR signal transduction (Figure 5.1). In chapter 2 we learned that, in addition to the previously published Rab5a, other Rab GTPases including Rab4, Rab7 and Rab11 associate with overlapping residues of the angiotensin II type 1 receptor C-tail and compete with each other to regulate receptor phosphorylation, desensitization and resensitization. Dale et al., (2004) showed that overexpression of constitutively active Rab7 and Rab11 mutants could override Rab5a-mediated AT<sub>1A</sub>R retention in early endosomes, and divert the receptor through Rab7 or Rab11 pathways, suggesting that other Rabs may compete with each other for AT<sub>1A</sub>R trafficking. Here we found that overexpression of Rab4 and Rab11 can each uncouple Rab5 from AT<sub>1</sub>R. Additionally, Rab4 can cause Rab11 to dissociate from AT<sub>1</sub>R. However, Rab11 was unable to uncouple Rab4 from the receptor. Additionally, Rab4, but not Rab11, facilitates the dephosphorylation of AT<sub>1</sub>R, thus decreasing the desensitization and enhancing the

## GPCR Trafficking by Rab GTPases

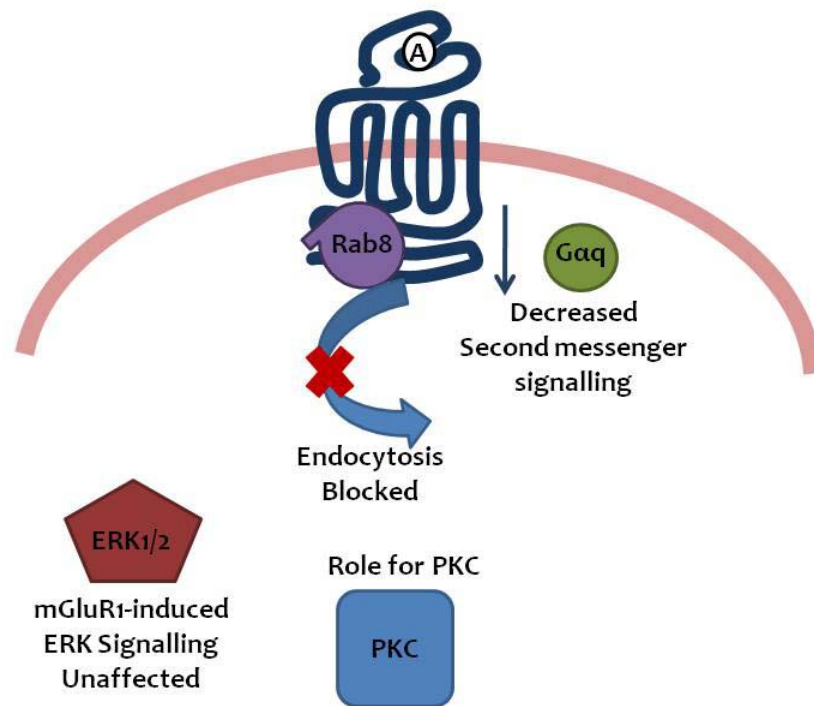


**Figure 5.1. Newly identified roles of Rab family members in GPCR endocytic trafficking and signalling.** Although Rab5 is well known to participate in clathrin-mediated endocytosis, we find here that Rab8 blocks mGluR1a endocytosis. In addition to mediating the “fast” recycling route, Rab4, but not Rab11, facilitates dephosphorylation of AT<sub>1</sub>R, thus decreasing desensitization and facilitating resensitization.



resensitization of the receptor.  $\beta$ 2AR dephosphorylation and resensitization occurs as the receptor transits between the Rab5-positive early endosome and the Rab4-positive rapid recycling endosome while dephosphorylated CRF1 $\alpha$  receptor colocalized with Rab4 but not with Rab5 (Seachrist et al., 2000; Holmes et al., 2004). Moreover, it has been reported that phosphorylated  $\mu$ -opioid receptor is preferentially recycled through Rab4-positive endosomes (Wang et al., 2008). The dephosphorylation of these and other receptors within Rab4 positive recycling vesicles identifies this compartment as a key region of GPCR resensitization. The data presented in chapter 2 support the theory that in addition to regulating receptor trafficking, the association of different Rab GTPases may regulate different functional outcomes for AT<sub>1</sub>R signalling.

In chapter 3 we investigated the role of the previously typified exocytic Rab8 in the regulation of the metabotropic glutamate receptor 1 (Figure 5.2). We found that through association with the long C-tail isoform mGluR1a, Rab8 expression resulted in decreased internalization and attenuated IP3 signalling in HEK293 cells and Ca<sup>2+</sup> signalling in neurons. Multiple Rabs have been shown to be involved in both endocytic and exocytic events. However, this is the first time a Rab has been shown to inhibit internalization. A consequence of this Rab8-mediated internalization attenuation is increased overall cell surface mGluR1 expression. Surprisingly, the increased cell surface expression did not translate into increased receptor signalling, but instead resulted in a PKC-dependent attenuation of signalling via increased phosphorylation. In chapter 3, we present a novel role for Rab8 in attenuating mGluR1a internalization and signalling, which opens a new and exciting avenue of research into the role of Rabs in GPCR regulation.



**Figure 5.2. Rab8 coordinates mGluR1a intracellular trafficking and signal transduction.** Rab8 associates with mGluR1a upon agonist activation and attenuates receptor internalization, leading to increased mGluR1a plasma membrane expression. While attenuating mGluR-mediated IP3 and calcium signalling, Rab8 has no effect on mGluR-mediated ERK1/2 phosphorylation.

Finally, in chapter 4 we look at the subcellular localization and signal transduction of eight previously uncharacterised mGluR1 variants identified in various cancerous tissues. These include four mutations within the amino-terminus, three within the second intracellular loop and one located within the Homer binding region in the carboxyl-terminal tail. We find that two mutants in close proximity in the amino-terminus, R375G and G396V do not appear to form dimers, exhibit significantly lower plasma membrane expression compared to wild type mGluR1 and co-localize considerably within the ER with an ER marker GFP-KDEL. We show that these mutants along with other amino-terminal mutants exhibit significantly reduced basal IP3 formation as well as decreased basal ERK1/2 phosphorylation compared to wild type mGluR1a. Interestingly, the mGluR1a A168VA mutant, located in the glutamate binding region, displays significantly reduced basal signalling. However, it also displays significantly increased agonist-induced IP3 formation. R375G, on the other hand displays biased agonism and functional selectivity. Quisqualate-induced IP3 formation of the R375G variant shows little difference from wild type mGluR1a, but R375G exhibits significantly attenuated response to the natural ligand glutamate. Consequently, the activation of this receptor variant by different ligands appears to result in divergent signalling patterns. Additionally, mGluR1a R375G also exhibits functionally selective signal transduction in response to quisqualate. Although quisqualate-activated mGluR1a-R375G shows no alterations in IP3 formation compared to wild type, quisqualate treatment induces significantly higher ERK1/2 activation. This finding is especially dramatic considering that this mutant displays only 40% cell surface expression when compared with wild type receptor. We also identified two mGluR1a mutations, which no longer associate with

regulatory molecules. GRK2 remains associated with mutations in the second intracellular loop and GRK2 overexpression continues to mediate mGluR1a desensitization. Additionally, P1148L fails to associate with Homer 1b, is impaired in the activation of ERK1/2 phosphorylation and appears to stimulate significant changes in cellular morphology including increased filopodia formation and multinucleation. The findings presented in chapter 4 opens exciting, previously undocumented roles for mGluR1a regions involved in agonist binding and activity including biased agonism or functional selectivity. Taken together, our findings support the important role that intracellular trafficking and localization plays in receptor function and signal transduction.

## **5.2. CONTRIBUTIONS TO THE FIELD OF GPCR RESEARCH**

In recent years, studies from our laboratory and others have provided increasingly convincing evidence that GPCR trafficking actively affects signal transduction and indeed, receptor signal transduction can modulate the activity of proteins controlling the trafficking vesicular cargo (Smythe, 2002). Thus GPCRs represent unique cargo proteins that contribute to the regulation of their own intracellular trafficking. Our research presented here regarding Rab-mediated GPCR alterations in signalling further supports this idea that intracellular trafficking of GPCRs not only passively participates in receptor signalling, but can actively alter receptor signalling.

Seachrist et al. (2002) demonstrated that AT<sub>1A</sub>R activation causes GTP loading of Rab5a and upon agonist activation, is endocytosed in a Rab5a-dependent manner and sequestered to large, hollow Rab5a-positive early endosomes. Dale et al. (2004) further went to show that concurrent overexpression of either Rab7 or Rab11 overcomes this

Rab5a-mediated AT<sub>1A</sub>R retention in early endosomes and redirects the receptor to either Rab7-positive late endosomes or Rab11-positive recycling endosomes, respectively. This led to the question we posed in chapter 2: do other Rab GTPases also associate with AT<sub>1R</sub>? If so, do they compete with each other for trafficking of the receptor? Interestingly, we found that Rab4, Rab5, Rab7 and Rab11 all associate with the same amino acid residues of the AT<sub>1R</sub> C-tail and compete with each other for association. Internalized AT<sub>1R</sub> usually does not readily dephosphorylate nor recycle and the Rab11 “slow” pathway has previously been shown to regulate this receptor’s recycling (Anborgh et al., 2000; Hunyady et al., 2002; Dale et al., 2004). However, Li et al., 2008 provided evidence to suggest that in addition to Rab11, the so-called “fast” Rab4 pathway can also regulate AT<sub>1R</sub> recycling. Therefore, we sought to determine what recycling pathway AT<sub>1R</sub> prefers, and the consequences thereof. We found that indeed AT<sub>1R</sub> binds more to Rab11 than Rab4. However, Rab4 can cause dissociation of Rab11 from the receptor but Rab11 is unable to displace Rab4. Additionally, Rab4 expression results in a significant decrease in agonist-mediated AT<sub>1R</sub> phosphorylation and thus significantly decreased receptor desensitization, as well as enhanced receptor resensitization. Therefore, although AT<sub>1R</sub> normally associates with the Rab11-mediated slow recycling pathway, when presented the opportunity, it can alter its trafficking patterns in favour of Rab4-mediated fast recycling, thus altering its activity.

Rab GTPases have well-documented roles in receptor endocytosis and exocytosis. Rab5a participates in clathrin-mediated endocytosis of a number of receptors, including AT<sub>1R</sub>,  $\beta_2$ AR, CRF among others and Rab4, Rab11, Rab8 are known to facilitate recycling of GPCRs, including the corticotropin releasing factor receptor 1, somatostatin-

3 receptor, vasopressin V2 receptor, neurokinin-1 receptor, chemokine CXCR2, m4 muscarinic acetylcholine receptor and protease activated receptor, are also differentially regulated by Rab4 and Rab11 (Innamorati et al., 2001; Kreuzer et al., 2001; Schmidlin et al., 2001; Signoret et al., 2001; Fan et al., 2002; Volpicelli et al., 2002; Roosterman et al., 2003; Holmes et al., 2006; Deretic, 1997; Seachrist et al., 2000; Seachrist et al., 2002; Trischler et al., 1999). Rab8, specifically was recently shown to differentially regulate the TGN to plasma membrane trafficking of  $\alpha_2$ AR and  $\beta_2$ AR and has also been shown to coordinate with Rab11 to regulate the insertion of AMPA-type glutamate receptors into spines of hippocampal neurons (Brown et al., 2007; Dong et al., 2010; Gerges et al., 2004). Interestingly, we uncovered a novel role for Rab8 in the attenuation of internalization of mGluR1a resulting in an increased plasma membrane receptor occupancy. To our knowledge, this is the first example of a Rab protein blocking internalization of a receptor and this finding opens up the possibility of Rabs managing yet another trafficking pathway with which to participate in receptor regulation. Contrary to other reports, which indicate that Rab8 expression facilitates receptor signalling by increased cell surface expression, we found that Rab8-mediated increases in mGluR1a plasma membrane expression resulted in decreased receptor signalling both in HEK 293 cells as well as hippocampal neurons. However, our results are consistent with the role of the Rab8 effector molecule optineurin, which was also found to contribute to mGluR1a desensitization (Anborgh et al., 2005). This study uncovers a novel role of a Rab GTPase in negatively regulating receptor endocytosis and intracellular signal transduction.

The signal transduction of the prototypic mGluR1a has been extensively characterised, including mutagenesis of many putative regulatory and signalling-related

regions of the receptor. However, recent high-throughput genetic screening studies have identified several naturally occurring, previously uncharacterised mGluR1 mutations. In chapter 4 we examined these naturally occurring pathological mutations of mGluR1 and found them to play a substantial role in signal transduction and cellular localization. We have studied three mutations in the amino terminus, which display decreased basal signalling, a mutant in the second intracellular loop, which no longer associates with GRK2, yet is still desensitized by it, as well as one mutation in the carboxyl-terminal tail, which no longer associates with the regulatory protein Homer1, displays altered receptor localization, increased filipodia formation and defective ERK1/2 activation. One mutation in the Cysteine-rich region of the amino terminus emerged as a significant player in intracellular trafficking as well as signalling. Specifically, mGluR1 R375G simultaneously exhibited decreased basal IP3 formation while significantly increased agonist-activated ERK1/2 formation. These mutations further our understanding of mGluR1 signal transduction and intracellular localization and provide the possibility of studying previously uncharacterised amino acid residues for the purpose of generating novel ligands with functional selectivity.

### **5.3. PHYSIOLOGICAL RELEVANCE OF TRAFFIC-DEPENDENT SIGNALLING OF GPCRS**

#### **5.3.1. Deregulated Trafficking and Disease**

The majority of pharmaceutical interventions specifically target GPCRs due to their critical involvement in all physiological systems and the contribution of receptor perturbations to multiple diseases and disorders such as obesity and diabetes, hypertension, cancer and neurodegenerative diseases such as Alzheimer's and Huntington's diseases among others (Ahren, 2009; Lappano and Maggiolini, 2012;

Johnson and Liggett, 2011; Ribeiro et al., 2011; Thathiah and De Strooper, 2011). Mutations in multiple GPCRs, which alter receptor signalling can cause or contribute to disease progression, but it is now becoming apparent that disruption of trafficking pathways can also contribute to deregulation of GPCR signalling and influence disease formation. Because many GPCR ligands do not readily cross the plasma membrane, receptor trafficking to and from the plasma membrane is crucial to cellular responsiveness. For example, a naturally occurring loss of function mutation in the vasopressin receptor is associated with hereditary nephrogenic diabetes insipidus (Rochdi et al., 2010; Barak et al., 2001). This mutant is constitutively phosphorylated and sequestered in arrestin-positive endocytic vesicles. However, disrupting the receptor-arrestin complex restores plasma membrane localization and signalling. Altered endocytosis and enhanced receptor activity has been linked to Alzheimer's disease. Inhibition of dynamin-dependent endocytosis has been shown to increase A $\beta$  secretion and a natural variant in the delta-opioid receptor containing phenylalanine at position 27 rather than Cysteine matures more efficiently and has higher stability at the plasma membrane (Chyung and Selkoe, 2003; Sarajarvi et al., 2011). Interestingly, this variant also enhances  $\beta$  and  $\gamma$ -secretase activity leading to amyloid precursor protein accumulation. Therefore, in addition to rapid and effective signal desensitization, the endocytosis and intracellular trafficking of GPCRs can spatially and temporally determine receptor signal transduction and play a role in disease pathology.

### **5.3.2. Consequence of Altered Rab Protein Expression and Function**

As discussed above, the role Rabs play in signal transduction is becoming more evident, especially the direct regulation of target proteins by Rabs, including Rab-



mediated phosphorylation, ubiquitination and palmitoylation of target proteins. Rabs and their effector proteins are often associated with diseases, including pathogen-induced as well as inherited dysfunctions and are also associated with multiple neurodegenerative disorders. For example, neuronal cell death in Parkinson's disease correlates with Rab5a-specific endocytosis of  $\alpha$ -synuclein and dominant-negative Rab5 reduces neuronal cell death due to incomplete endocytosis of  $\alpha$ -synuclein (Sung et al., 2001). Rab7 mutations impair GTP hydrolysis and contribute to the hereditary neurological disorder Charcot-Marie-Tooth disease type 2B (Cogli et al., 2009). As noted above, Rab8 may play a role in the etiology of Huntington's disease via association with mutant huntingtin (htt) and optineurin as mutant htt prevents post-Golgi trafficking by disrupting the Rab8/optineurin complex (del Toro et al., 2009). However, Rab11 may also contribute to the pathology of Huntington's disease. Rab11-dependent vesicle formation in fibroblasts is impaired in Huntington patients and Rab11DN-expressing adult mouse brains display similar neurodegeneration to the HD mutant mouse model (Li et al., 2009a, b). It is now apparent that coordination of Rab proteins and their effectors can play a significant part in the onset and development of pathologies and learning more about the role they play in normal and diseased states may lead to better treatment options.

#### **5.4. FUTURE DIRECTIONS**

The novel discoveries presented in this thesis highlight a number of interesting questions to pursue in the future. Primarily, the precise details of the molecular mechanisms involved in the crosstalk and signal transduction cascades between Rab small G proteins and other GPCRs remain to be elucidated. Additionally, we have shown that Rab4 influences the phosphorylation state of AT<sub>1</sub>R. How exactly does Rab4 regulate

the phosphorylation/dephosphorylation of this receptor? Does it block kinase-mediated phosphorylation or does it recruit phosphatases to dephosphorylate the receptor? Does Rab4 function by itself or does it work in conjunction with an effector molecule? We also show that different Rabs compete with each other for association with the receptor and that some Rabs are more effective at displacing others. Therefore, a greater understanding of how to manipulate the Rab-mediated receptor trafficking may enable us to influence receptor signalling. Specifically, if receptors were to be found in cells or tissues endogenously expressing higher levels of different Rabs, would their intracellular trafficking and signalling patterns differ? It would be extremely interesting to look at different GPCRs in endogenous tissues or cells displaying divergent Rab expression patterns to discover whether this significantly alters signal transduction. Further, can we alter the response of certain cells or tissues to stimulus by manipulating the complement of Rabs? In chapter 2 we learned that Rab4, but not Rab11 alters AT<sub>1</sub>R agonist-induced phosphorylation, thus modifying receptor desensitization and resensitization. However, we know that as a Class B receptor, AT<sub>1</sub>R does not usually become dephosphorylated and recycled back to the cell surface. Therefore, this raises the question of whether AT<sub>1</sub>R activity might be manipulated in either whole tissues or whole animals by altering the expression patterns of Rab GTPases. It would also be very exciting, for example, to engineer a transgenic mouse expressing dominant negative Rab4 in vascular smooth muscle cell, to determine whether these animals exhibit differences in vascular tone, contraction both under basal conditions and after stimulation with vasoconstrictors such as AngII. We could further study the signal transduction in isolated cells to identify any altered or aberrant signalling in these cells compared to wild type litter mates.

Investigating the complement of Rabs in different tissue types or diseased tissue and the influence on GPCR regulation would grant us greater understanding to the physiological role Rabs play in signal transduction.

In Chapter 3 we reported how Rab8 modulates the trafficking and signalling of mGluR1a. We need to first isolate all of the players in this regulatory pathway. For example, we know that PKC is involved, as PKC inhibition reverses the Rab8-mediated effect on mGluR1a signalling. However, are there other second messenger-dependent kinases involved or does PKC exert its effect through regulation of some downstream target? Additionally, we identified for the first time a Rab protein that blocks receptor internalization. It is also unclear what the specific mechanism underlying the Rab8-mediated inhibition of mGluR1a signalling is and what the specific purpose of this inhibition may achieve. The consequence of Rab8-mediated attenuation of mGluR1a endocytosis is increased receptor localization at the plasma membrane and the concurrent reduction in mGluR1a-mediated signalling. Our laboratory has shown that mGluR1a also associates with the Rab8 effector molecule, optineurin (Anborgh et al., 2005) and we have found here that Rab8 itself plays a similar role to optineurin in the desensitization of mGluR1. Therefore, it is of interest to determine whether Rab8-mediated attenuation of mGluR1a signalling is either independent of optineurin or works in concert with optineurin. The glaucoma-associated optineurin E50K mutant continues to associate with mGluR1a, however does not associate with Rab8. Presumably, if Rab8 works in concert with optineurin, then expression of this mutant would inhibit the Rab8-mediated mGluR1a desensitization. Conversely, if Rab8 works independently of optineurin, there should be no effect of expressing with mutant on mGluR1a signalling. There are several

other molecules involved in the desensitization and endocytosis of mGluR1, including GRK2, phospholipase D2 and RalA among others (Dhami et al., 2005, 2004, 2002; Bhattacharya et al., 2004). In the future it will be of interest to determine if any of these molecules play a role in Rab8-modulation of mGluR1a trafficking and signal transduction.

It would be most interesting to engineer a Rab8 conditional knockout, or knockdown mouse (as Rab8 null mice have been reported to be lethal 3 weeks after birth, (Sato et al., 2007) in order to determine whether these animals display altered behaviour such as deficiencies in learning or memory, motor skills, or other behavioural characteristics. In addition to being a Rab8 effector molecule, optineurin is also a well known htt binding protein. Huntington's disease is a neurodegenerative disorder caused by an abnormal polyglutamine expansion in the amino-terminus of the huntingtin protein, leading to cell dysfunction and neuronal death (Ribeiro et al., 2011). Optineurin colocalizes with htt in the Golgi apparatus where it participates in post-Golgi trafficking and Rab8 coordinates with optineurin to mediate the post-Golgi transport of proteins (del Toro et al., 2009). Although wild-type htt does not contribute to optineurin-mediated mGluR desensitization, mutant htt synergistically increases optineurin-mediated mGluR desensitization in HEK 293 cells as well as in a knock-in mouse model of HD (HdhQ111/Q111) (Anborgh et al., 2005). As was found for Rab8-mediated desensitization of mGluR signalling, the attenuation of mGluR5 signalling observed in HdhQ111/Q111 mice is PKC dependent (Ribeiro et al., 2010). Therefore, it would be most interesting to further elucidate the role that Rab8 plays in the etiology of HD. Specifically, does Rab8 complex with the receptor, optineurin and htt? Does it

preferentially associate with wild type or mutant htt? Finally, it would be exciting to generating a cross between our mutant HdhQ111/Q111 mice with Rab8 knock-down mice to see if we reverse mGluR desensitization in this model and alter the Huntington phenotype.

To our knowledge, this is the first example of a Rab protein blocking internalization of a receptor and this finding opens up the possibility of Rabs managing yet another aspect of receptor trafficking with which to participate in receptor regulation. It would be of significant interest to determine whether other Rabs serve a similar purpose, or whether Rab8 itself inhibits internalization of other receptors. Our findings suggest that, although Rab8 expression leads to an overall increase in cell surface receptors, phosphorylation of those receptors by second messenger-dependent kinases is increased. Thus, Rab may either function to block mGluR1a internalization ultimately to prevent mGluR1a dephosphorylation and resensitization or Rab8 association may block other regulatory molecules from accessing the receptor.

In Chapter 4 we examined several previously uncharacterised naturally occurring pathological mutations of mGluR1a, which we found played a substantial role in modulating the signal transduction and cellular localization of mGluR1a. We first need to delineate the regulatory molecules involved in the altered signal transduction pathways. For example, we know that mGluR1a-G696W no longer associates with GRK2, but is still uncoupled following GRK2 overexpression. Therefore, this begs the question as to just how GRK2 continues to exert its effect on mGluR1a activity. The most probable explanation is that GRK2 continues to associate with  $G\alpha_{q/11}$  through its RGS domain and that GRK2 overexpression mediated uncoupling of mGluR1a signalling is the

consequence of its selective interaction with  $G\alpha_{q/11}$ . A good way to test this would be to repeat the experiment with kinase-dead GRK2 mutants as well as GRK2 mutants deficient in  $G\alpha_{q/11}$  binding. We also found that this mGluR1a mutant does not activate ERK1/2. It would also be of value to determine whether the association of other downstream signalling molecules, such as Pyk2 (Nicodemo et al., 2010) are also affected by the mutation, and of course whether this affects mGluR1a signalling via these proteins. In addition, GRK2 overexpression reverses mGluR1a-mediated cell death in HEK 293 cells (Dale et al., 2000). Thus, it is possible that the mGluR1a-G696W mutant may be associated with increased apoptotic cell death.

Homer proteins are post-synaptic density proteins with known functions in receptor trafficking and calcium homeostasis. We have shown that mGluR1a-P1148L no longer associates with Homer 1b, and although IP3 signalling is unaffected, consistent with other reports, mGluR1a-P1148L can no longer activate ERK1/2 phosphorylation. We also found evidence that mGluR1a-P1148L intracellular trafficking and cell morphology is altered. What are the physiological consequences of this altered phenotype? For example, it would be interesting to test whether the increased filopodia formation of mGluR1a-P1178L results in an increase in cell motility.

One mutation in the Cysteine-rich region of the amino terminus emerged as a significant player in intracellular trafficking as well as signalling. Specifically, mGluR1a-R375G activation by quisqualate resulted in divergent signal transduction outcomes, the differential activation of G protein versus ERK1/2 signalling. This “biased agonism” now represents an important target for drug design and focuses on the development of compounds that can stabilize different receptor conformations, thus encouraging signal

transduction through one pathway or another. It would, therefore, be beneficial to study the conformation that this receptor assumes in the presence and absence of different agonist binding. Additionally, R375G simultaneously exhibited unchanged quisqualate-induced IP3 formation as well as significantly increased agonist-activated ERK1/2 formation. We should probe into the consequences of this functionally selective signal transduction to see whether we can manipulate the signal patterns of this receptor variant. For example, increased ERK1/2 activity of mGluR1a-R375G may lead to a change in cell cycle regulation, apoptosis or growth.

As these mGluR1a mutants were originally isolated from human tissue, and specifically diseased tissue, the altered signal transduction portfolios of each raise interesting and potentially crucial questions as to the *in vivo* roles these mGluR1a mutants may play in pathology. To that end, creating transgenic animal models of at least mGluR1a-R375G and -P1148L would enable us to study the signal transduction and intracellular trafficking of these variants in various endogenous cells and tissues as well as to study the organism as a whole to look at development, behaviour and pathologies.

The studies that originally isolated these mutations in mGluR1a also highlighted mutations in a number of other GPCRs, including mGluR3,  $\alpha_{1a}$ AR, luteinizing hormone receptor, as well as regulatory molecules GRK1 and Homer2 and downstream effector molecules, PLC $\beta$ , PLD2 and small G proteins such as Ral, RalGDS, Rab5c, Rab3a (Kan et al., 2010; Parsons et al., 2008; Sjoblom et al., 2006; Wood et al., 2007). It would be beneficial to study the mutations in these other molecules, and how they interact with each other to determine their altered signal transduction and intracellular localization mechanisms.

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EDUCATION

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- **Ph.D. in Physiology and Pharmacology**  
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- **BSc in Biology, Honors Genetics**  
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RESEARCH EXPERIENCE

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- Ph.D. Candidate, Department of Physiology and Pharmacology** **2006-Present**  
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Supervised by Dr. Stephen S.G. Ferguson, The Robarts Research Institute
- Summer Student Researcher** **2005-2006**  
The Robarts Research Institute. Stephen S.G. Ferguson Lab
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TEACHING

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- Teaching assistant** for Physiology 314a/3140, "Cellular Physiology" **2007-2010**  
The University of Western Ontario.
- Tutor and Examination Proctor.** **2006**  
The University of Western Ontario, Biology 281b, "Genetics"
- Supervision**
- Assisted in the training and education of 2 Master's students, 1 PhD student and 2 Post Doctoral Fellows in the Ferguson Lab.
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**AWARDS AND FELLOWSHIPS**

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**Scholarships**

- Ontario Graduate Scholarship in Science and Technology **2010**
- Ontario Graduate Scholarship **2009**
- Heart and Stroke Foundation of Ontario Master's Studentship **2007-2009**
- Canadian Institutes for Health Research Strategic Training Program in Vascular Research **2006-2008**
- Ontario Graduate Scholarship (Declined) **2007**
- Schulich School of Medicine and Dentistry Graduate Scholarship **2006-2011**

**Travel Awards**

- Fifth Canadian Neuroscience Meeting. Quebec City, Quebec, Canada **2011**
- Second annual Molecular Pharmacology of G Protein-Coupled Receptors Meeting. Monash University, Melbourne, Australia. **2010**
- First annual School of Translational Science. AC Camargo Cancer Hospital. Sao Paulo, Brazil. **2009**
- Ninth annual joint meeting of Great Lakes G Protein-Coupled Receptor Retreat and le Club des Recepteurs a Sept Domaines Transmembranaires. Montreal, Quebec, Canada **2008**

**PUBLICATIONS**

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1. **Jessica L. Esseltine**, Lianne B. Dale and Stephen S.G. Ferguson. (2011) Rab GTPases Bind at a Common Site within the Angiotensin II Type I Receptor Carboxyl-Terminal Tail: Evidence that Rab4 Regulates Receptor Phosphorylation, Desensitization, and Resensitization. *Mol Pharm* **79**; 179-84.

**SUBMITTED MANUSCRIPTS**

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1. **Jessica L. Esseltine**, Melinda D. Willard, Mary E. Lajiness, Isabella H. Wulur, Thomas D. Barber, and Stephen S. G. Ferguson. Group I Metabotropic Glutamate Receptor Mutations in Cancer Alter Receptor Intracellular Localization and Signalling. *Mol Pharm*, Submitted. **(In Collaboration with Eli Lilly and Company)**.
2. **Jessica L. Esseltine**, Fabiola Ribeiro and Stephen S.G. Ferguson. Rab8 modulates Metabotropic Glutamate Receptor Signalling and Intracellular Trafficking. *J. Neurosci*. Submitted.

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ORAL PRESENTATIONS

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1. **Jessica Esseltine**, Fabiola Ribeiro, and Stephen Ferguson. Rab8 modulates Metabotropic Glutamate Receptor Signalling and Intracellular Trafficking. *Invited trainee oral presentation* and poster presentation at the annual Molecular Pharmacology of G Protein-Coupled Receptors meeting, Melbourne Australia. December 2-4, 2010.
2. **Jessica Esseltine**, Fabiola Ribeiro, and Stephen Ferguson. Rab8 modulates Metabotropic Glutamate Receptor Signalling and Intracellular Trafficking. *Invited trainee oral presentation* and poster presentation at the 13<sup>th</sup> annual joint meeting of Great Lakes G Protein-Coupled Receptor Retreat and le Club des Recepteurs a Sept Domaines Transmembranaires October 27-29, 2011.
3. **Jessica L. Esseltine**, Robert Gros, Hubert H. M. Van Tol, Natalie Lavine, and Stephen S. G. Ferguson. Role of G Protein-coupled Receptor Kinase 2 in Regulating Vascular Function. *Invited trainee oral presentation* and poster presentation at the 9th annual joint meeting of Great Lakes G Protein-Coupled Receptor Retreat and le Club des Recepteurs a Sept Domaines Transmembranaires , October 16-18, 2008.
4. **Esseltine, J.**, Farmakovski, N., Tarnowski, H., Prtenjaca, A., and Hill, K. Development of a Murine Single-Cell Mutation Load Assay. *Oral presentation* at 19th Annual Ontario Biology Day, March 19, 2006. (B.Sc. work).

ABSTRACTS

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1. Melinda D. Willard, **Jessica L. Esseltine**, Mary E. Lajiness, Isabella H. Wulur, Thomas D. Barber, and Stephen S. G. Ferguson. Group I Metabotropic Glutamate Receptor Mutations in Cancer Alter Receptor Intracellular Localization and Signalling. Poster presentation at the annual American Association for Cancer Research meeting March 31-April 4, 2012.
2. **Jessica L. Esseltine**, Melinda D. Willard, Mary E. Lajiness, Isabella H. Wulur, Thomas D. Barber, and Stephen S. G. Ferguson. Group I Metabotropic Glutamate Receptor Mutations in Cancer Alter Receptor Intracellular Localization and Signalling. Poster presentation at the 13th annual joint meeting of Great Lakes G Protein-Coupled Receptor Retreat and le Club des Recepteurs a Sept Domaines Transmembranaires , October 27-29, 2011.

3. Melinda D. Willard, **Jessica L. Esseltine**, Mary E. Lajiness, Isabella H. Wulur, Thomas D. Barber, and Stephen S. G. Ferguson. Group I Metabotropic Glutamate Receptor Mutations in Cancer Alter Receptor Intracellular Localization and Signalling. Poster presentation at the annual American Society of Human Genetics meeting October 11-15, 2011.
4. **Jessica L. Esseltine**, Fabiola Ribeiro, Lianne Dale and Stephen Ferguson. Rab8 modulates Metabotropic Glutamate Receptor Signalling and Intracellular Trafficking. Annual Physiology and Pharmacology Research Day. ***Awarded first place in student poster presentation signal transduction category.*** November 9, 2010
5. **Jessica L. Esseltine**, Dale, LB. and Ferguson, SSG. Regulation of Angiotensin II Type 1 Receptor by RabGTPases. ***Awarded best integration of physical sciences in biology*** at the Canadian Institutes for Health Research strategic training program poster competition, April 8, 2008
6. **Jessica L. Esseltine**, Dale, L., and Ferguson, SSG. Regulation of Angiotensin II Type 1 Receptor by RabGTPases. Poster presentation at the 8th annual joint meeting of Great Lakes G Protein-Coupled Receptor Retreat and le Club des Recepteurs a Sept Domaines Transmembranaires , September 27-29, 2007.
7. Magalhaes, AC., Holmes, KD., **Esseltine, J.** and Ferguson, SSG. Modulation of 5HT receptor activity upon CRF stimulation. Poster presentation at the 8th annual joint meeting of Great Lakes G Protein-Coupled Receptor Retreat and le Club des Recepteurs a Sept Domaines Transmembranaires , September 27-29, 2007.
8. **Jessica L. Esseltine**, Dale, L., Magalhaes, AC. and Ferguson, SSG. Direct and Indirect Regulation of Angiotensin II Type 1 Receptor by RabGTPases. Poster Presentation at the First Joint Annual Research Day Heart and Stroke Foundation of Ontario, June 6, 2007.
9. **Jessica L. Esseltine**, Dale, L., Magalhaes, AC. and Ferguson, SSG. Direct and Indirect Regulation of Angiotensin II Type 1 Receptor by RabGTPases. Poster Presentation at the Robarts Research Institute Vascular and Cell Biology Joint Annual Retreat, March 28, 2007.
10. **Jessica L. Esseltine**, Dale, L., Magalhaes, AC. and Ferguson, SSG. Direct and Indirect Regulation of Angiotensin II Type 1 Receptor by RabGTPases. Poster Presentation at the Margaret Moffat Research Day, March 22, 2007.
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