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CAL and MAGI PDZ Protein Regulation of CRFR1 and 5-HT2AR Trafficking and Signaling

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Abstract

PDZ (PSD95/Disc Large/Zona Occludens) domain-containing proteins are scaffolding proteins that play important roles in regulating the activity of G protein-coupled receptors. Corticotropin Releasing Factor Receptor 1 (CRFR1) and Serotonin 2A Receptor (5-HT$_{2A}$R) are two GPCRs that are commonly associated with mental disorders. Both receptors also contain a class I PDZ-binding motif at the carboxyl terminal tail. In the first chapter, we investigate the effects of CAL (CFTR-associated ligand) on regulating the trafficking and signaling of CRFR1. We demonstrate a role for CAL in inhibiting CRFR1 endocytosis, cell surface expression, and CRF-mediated ERK1/2 signaling via the CRFR1 PDZ-binding motif. Additionally, CAL can elicit its effects on CRFR1 by mediating the post-translational glycosylation of the nascent receptor at the Golgi apparatus. The second and third chapters focus on the MAGI (MAGUK with inverted orientation PDZ) proteins; MAGI-1, MAGI-2 and MAGI-3. We observe distinct functions for the MAGI proteins in regulating the trafficking and signaling of CRFR1 compared to that of 5-HT$_{2A}$R. MAGI proteins can mediate CRFR1 endocytosis via regulating β-arrestin recruitment to the receptor. No effect is observed on the basal plasma membrane expression of CRFR1 or CRF-stimulated cAMP formation in response to overexpression or knockdown of MAGI proteins. On the other hand, MAGI proteins regulate 5-HT$_{2A}$R-stimulated inositol phosphate accumulation as well as surface expression levels but do not have an effect on the internalization of the receptor. MAGI proteins can also mediate ERK1/2 signaling activated by both CRFR1 and 5-HT$_{2A}$R. We propose a compensatory mechanism of regulation between MAGI-1, MAGI-2 and MAGI-3 based on their similar functional roles. Our work characterizes the interactions between two different GPCRs and four PDZ proteins, further confirming the importance of this diverse family of scaffolding proteins and providing possible targets for specific drug design.

Keywords

Co-Authorship Statement

In Chapter 2, Dr. Henry A. Dunn and I collaborated on the flow cytometry experiments in Figure 3. Dr. Cornelia Walther contributed the CAL-YFP construct. Dr. Stephen S.G. Ferguson and I collaborated on the experimental design and the preparation of the manuscript.

In Chapter 3, Dr. Henry A. Dunn and I collaborated on the flow cytometry experiments in Figure 6. Dr. Stephen S.G. Ferguson and I collaborated on the experimental design and the preparation of the manuscript.

In Chapter 4, Dr. Henry A. Dunn and I collaborated on data analysis in Figures 2, 4 and 5. Dr. Stephen S.G. Ferguson and I collaborated on the experimental design and the preparation of the manuscript.

Dedication

To My Wonderful Family…

My lovely parents, Hanan Abuhantash and Mahmoud Hammad,

Thank you for giving me all the love and encouragement to be where I am today…

My great siblings, May, Hammad, Mohammad and Shahed,

Thank you for inspiring me each in your own way…

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List of Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
5-HT$_{2A}$R	5-HT$_{2A}$ receptor
5-HT$_{2C}$R	5-HT$_{2C}$ receptor
7TMRs	seven Transmembrane Domain Receptors
AC	Adenylyl Cyclase
ACTH	adrenocorticotropin hormone
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride
AT$_1$R	angiotensin II type 1 receptor
ANOVA	analysis of variance
β$_1$-AR	β1-adrenergic receptor
BAI-1	brain-specific angiogenesis inhibitor-1
CAL	cystic fibrosis transmembrane conductance regulator-associated ligand
CaMK	calmodulin kinase-like
cAMP	cyclic adenosine monophosphate
CNS	Central Nervous System
CRF	corticotropin-releasing factor
CRFR1	CRF receptor 1
CRFR2	CRF receptor 2
DAG	diacylglycerol
DLGs	discs larges
ECLs	extracellular loops
EPAC	Exchange protein directly activated by cyclic AMP
ERGIC	ER-Golgi Intermediate Compartment
ERK1/2	extracellular signal-regulated kinase
ESCRT	endosomal sorting complex required for transport
FIG	fused in glioblastoma
GAPs	GTPase-activating proteins
GASPs	GPCR-associated sorting proteins
GEF	guanine nucleotide exchange factor
GIP	GPCR-interacting protein

GIPC	Gα-Binding Protein Interacting Protein Carboxyl-Terminus
GK	guanylate kinase-like
GOPC	Golgi-associated PDZ and coiled-coil motif-containing protein
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
HA	hemagglutinin
HBSS	HEPES-buffered saline solution
HEK 293	human embryonic kidney 293
HPA	Hypothalamo-Pituitary-Adrenal Axis
ICLs	intracellular loops
ILVs	intraluminal vesicles
IP3	inositol-1,4,5-trisphosphate
LPA$_2$R	lysophosphatidic acid-activated receptor subtype-2
MAGI	MAGUK with Inverted orientation PDZ
MAGUK	Membrane Associated Guanylate Kinase
MAPK	mitogen activated protein kinase
PAR	protease activated receptor
PDZ	PSD95/ Disc Large/ Zona Occludens
PIP2	phosphatidylinositol-4,5-bisphosphate
PIST	PDZ domain protein interacting specifically with TC10
PLCβ	phospholipase Cβ
PNGase F	Peptide N-Glycosidases F
PTB	phosphotyrosine binding
PTEN	protein phosphatase and tensin homolog
PTH1R	parathyroid hormone 1 receptor
PVN	paraventricular nucleus
RA	Ras-associated
RAMPs	receptor activity modifying proteins
RGS	regulators of G-protein signaling
SAP97	synapse-associated protein 97
SH2	Src-homology
shRNA	short hairpin RNA

SNX27	Sorting Nexin 27
SSRIs	Selective Serotonin Reuptake Inhibitors
SSTR5	Somatostatin Receptor Subtype 5
TGN	trans-Golgi network
V2R	vasopressin V2 receptor
$VPAC_1$	Vasoactive Intestinal Polypeptide Type 1 receptor/Pituitary Adenylate Cyclase-activating peptide

Chapter 1

1 Introduction

1.1 G Protein-Coupled Receptors

G Protein-coupled Receptors (GPCRs) represent the largest receptor superfamily and regulate numerous physiological responses. These include neurotransmission, immune function, reproduction, cardiac function, metabolism, and homeostasis (Gurevich and Gurevich, 2008). The number of GPCRs in the human genome is estimated to be 800 to 1000 and more than 40% of the currently prescribed drugs target GPCRs (Marchese et al., 1999; Urs et al., 2014). GPCRs share a similar structure that consists of seven transmembrane α helical domains connected by three extracellular loops (ECLs) and three intracellular loops (ICLs), an extracellular N-terminal domain and an intracellular C-terminal domain and they are also known as seven Transmembrane Domain Receptors (7TMRs). The extracellular and transmembrane regions of the receptor are involved in ligand binding while the intracellular domains are important for signal transduction and for feedback modulation of receptor function (Marchese et al., 1999). GPCRs can be bound and activated by a wide number of ligands such as photons of light, odorants, hormones, neurotransmitters, peptides, chemokines, amino acids and Ca^{2+} ions to name a few (Gurevich and Gurevich, 2008). GPCRs mediate their effects by coupling to a variety of heterotrimeric guanine nucleotide-binding proteins (G proteins). G proteins have two states; the inactive (GDP bound) and the active (GTP bound). Ligand binding to a receptor results in the exchange between GDP and GTP. This active G protein (GTP bound) dissociates into Gα subunit and Gβγ subunit. These dissociated subunits can mediate several downstream effectors such as adenylyl cyclases (AC), GPCR kinases (GRKs) and phospholipases and that ultimately leads to the activation of different signaling pathways.

The compounds that bind GPCRs can be classified into four classes based on their effects. 1) Full agonists which by binding the receptor stabilize the active conformation and result in maximal receptor activity, 2) partial agonists which bind the receptor without causing maximal activity 3) inverse agonists which decrease the constitutive or

spontaneous activity of the receptor by stabilizing the inactive conformation of the receptor and 4) full antagonists block agonists and inverse agonists activity either by binding to the same binding site as the endogenous ligand (competitive) or binding to an allosteric site (non-competitive) (Luttrell, 2008; Prazeres and Martins, 2015).

GPCRs are commonly classified into five major phylogenetic families. Those are rhodopsin (Class A), secretin (Class B), glutamate (Class C), adhesion and frizzled (Krishnan and Schioth, 2015; Prazeres and Martins, 2015).

1.2   G Protein-Coupled Receptor Trafficking

1.2.1   Anterograde Pathway (GPCR Biosynthesis)

Anterograde trafficking (forward trafficking) of GPCRs can be defined as the synthesis of GPCRs at the endoplasmic reticulum (ER) followed by their transport from the ER to the plasma membrane (Fig. 1.1). Therefore, it is an essential checkpoint in determining the level of expression of the functional receptors at the surface and this in turn dictates the strength of the signal initiated by the receptor. This process is mediated by multiple factors including ER chaperones, Rab GTPases, receptor activity modifying proteins (RAMPs), exit motifs encoded by the receptor sequence and post-translational modifications (Wu, 2012).

1.2.1.1   GPCR Synthesis at the Endoplasmic Reticulum

GPCRs are plasma membrane proteins and like many other membrane proteins, they are synthesized via ribosomes at the membranes of the rough endoplasmic reticulum (ER). They undergo the first set of post-translational modifications at the ER and this facilitates their proper folding and maturation (Achour et al., 2008). Key factors are involved in regulating this process including molecular chaperones such as Bip, calnexin, calreticulin, ERp57, HSP70, PDI and GRP94. The interactions that occur between the newly synthesized polypeptides and those ER-proteins slow the folding process, prevent aggregation and stabilize the intermediate forms of proteins (Dupré et al., 2012).
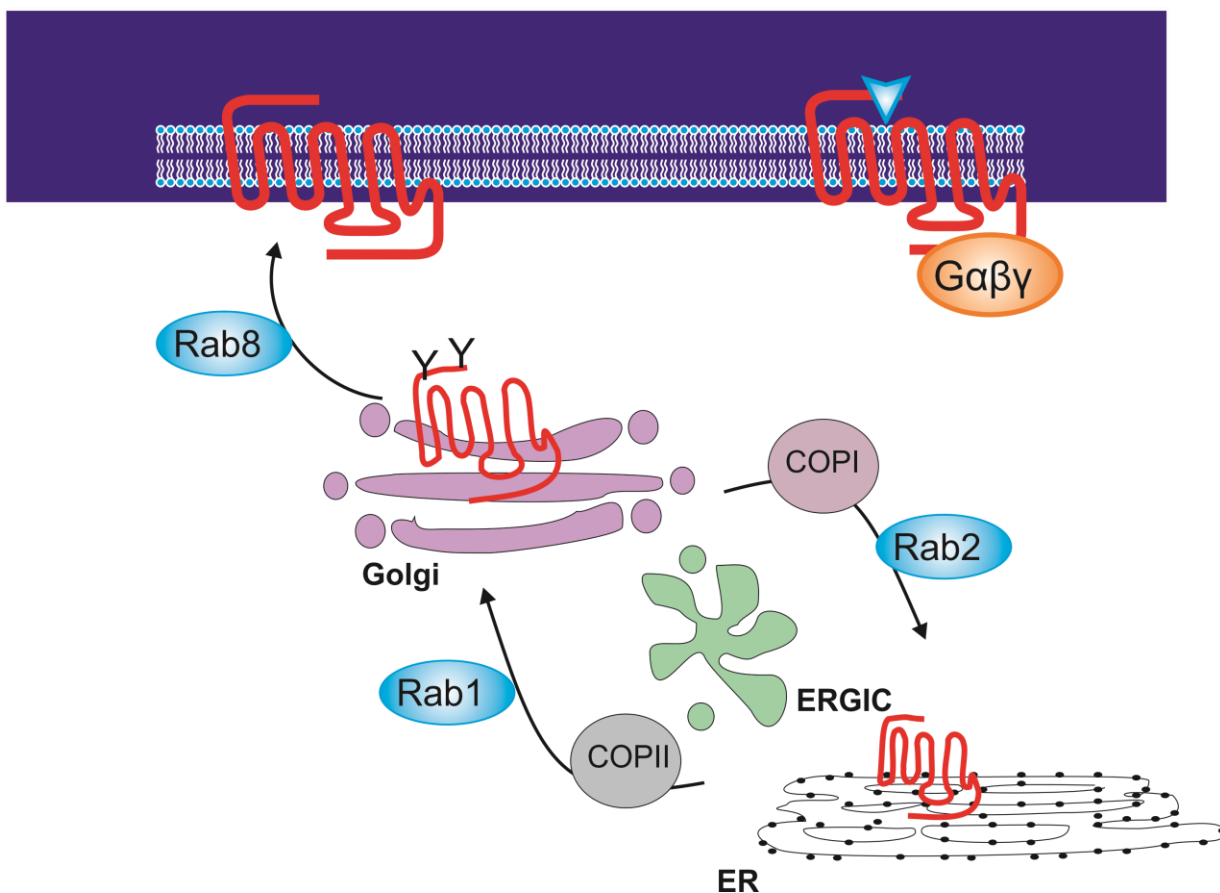
Figure 1.1: Schematic representation of the Anterograde (Forward) Trafficking of a GPCR: The receptor is synthesized at the Endoplasmic Reticulum (ER) then transported via COPII vesicles to the Golgi apparatus. The receptor undergoes post-translational modifications to achieve proper folding before trafficking to the plasma membrane. Immature forms of the receptor are transported back from the Golgi apparatus to ER via COPI vesicles. Different Rab GTPases are involved in regulating the steps in the pathway.

In addition, in order for the receptor to form its native conformation, enzymatic processes are required to catalyze disulfide-bond formation or peptidyl-prolyl cis–trans isomerization. These ER processes represent a crucial quality control system to ensure proper folding of proteins and any misfolded polypeptides are sent for the proteasome for degradation (ER-associated degradation) (Achour et al., 2008; Jean-Alphonse and Hanyaloglu, 2011).

1.2.1.2   GPCR Trafficking from ER to Golgi Apparatus

Once the receptor is mature and properly folded, it traffics from the ER exit sites to the Golgi complex via COPII-coated vesicles. The interaction of GPCRs with the components of the COPII vesicles is mediated by ER export motifs expressed at their carboxyl-terminal tails. These motifs consist of either di-acidic residues (Asp-x-Glu, where x = any amino acid), pairs of aromatic residues (Phe-Phe, Tyr-Tyr or Phe-Tyr) or bulky hydrophobic residues (Leu-Leu or Ile-Leu) (Achour et al., 2008). This transport of the receptor through the ER-Golgi Intermediate Compartment (ERGIC) is facilitated by many different regulator proteins including COPI, Rab GTPases and ARF GTPases. Studies on GPCRs transport suggest that Rab1 plays a role in anterograde trafficking from ER to Golgi, while Rab2 regulate the retrograde trafficking from the Golgi apparatus back to the ER and Rab6 is important for GPCRs transport through the Golgi cisternae (Appenzeller-Herzog and Hauri, 2006; Achour et al., 2008; Wu, 2012). Similar to Rab GTPases, ARF GTPases are also important and different members play different roles at distinct cellular compartments. ARF1 plays a role in both anterograde and retrograde trafficking by recruitment of the COPII complex proteins (Appenzeller-Herzog and Hauri, 2006; Wu, 2012).

1.2.1.3   GPCR Trafficking from Golgi to the Plasma Membrane

The trans-Golgi network (TGN) is known to function as the sorting center for newly synthesized proteins where their final destination is determined between the plasma membrane, lysosomes or endosomes. The vesicles that regulate post-Golgi transport are believed to be clathrin-coated vesicles. The vesicles that are involved in protein transport

from the Golgi apparatus to lysosomes are composed of clathrin and the adaptor proteins GGAs (Golgi-associated, γ-adaptin homologous, ARF-interacting proteins). GGAs have four main domains to facilitate their function. An N-terminal VHS domain that recognizes the sorting signals on the carboxyl-terminal tail of the protein being transported, a GAT domain that interacts with ARF1, a hinge domain that recruits clathrin and the C-terminal GAE domain that binds to accessory proteins (Wu, 2012). On the other hand, the vesicles regulating cargo transport from the Golgi apparatus to the plasma membrane are unknown and it has been suggested that they could be large pleiomorphic carriers. However, studies show that post-Golgi trafficking to the plasma membrane is also highly regulated by Rab GTPases and conserved sorting motifs. Rab8 is important for the transport of transmembrane proteins and GPCRs specifically from the Golgi apparatus to the plasma membrane. It has been previously shown that using Rab8 mutants or knocking down Rab8 expression results in reduced surface expression of the adrenergic receptors α_{2B}-AR and β_2-AR and retention of the receptors in the intracellular compartments (Dong et al., 2010; Wang and Wu, 2012). As for motifs, studies show an importance for the di-leucine (LL) residues at the carboxyl-terminal tail of certain family A GPCRs in plasma membrane expression (Schulein et al., 1998; Duvernay et al., 2004; Carrel et al., 2006; Sawyer et al., 2010). It has been shown that this motif is highly conserved in α_{2B}-AR, α_{1B}-AR, β_2-AR, angiotensin II type 1 receptor (AT$_1$R), serotonin 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors, vasopressin V2 receptor, and M1-muscarinc receptor and mutations lead to the retention of these receptors intracellularly and therefore reduced cell surface levels. In addition to the conserved motifs at the carboxyl-terminal end of GPCRs, studies illustrate an important role for the N-terminal end in GPCRs trafficking. Reports show that mutations of the N-terminal YS motif in some adrenergic receptors results in their retention in the Golgi (Wu, 2012).

1.2.2   The Life Cycle of G Protein-Coupled Receptors

1.2.2.1   GPCR Desensitization

GPCR desensitization is defined as the reduced strength of receptor activity or responsiveness. The process is mainly initiated after a ligand binds to a receptor to activate

it. The mechanisms underlying GPCR signal attenuation include G-protein uncoupling from the phosphorylated receptor, receptor internalization to the intercellular compartments and receptor degradation. Depending on the receptor structure as well as the cellular environment, desensitization can vary from reduced activity to complete loss of signaling. G protein uncoupling occurs once the receptor is phosphorylated either by second messenger-dependent kinases (PKA and PKC) or by G protein-coupled receptor kinases (GRKs). These enzymes commonly phosphorylate threonine and serine residues on the intracellular loop or the carboxyl-terminal tail of the receptor (Ferguson, 2001). In addition, different GRKs can phosphorylate different sites on the receptor resulting in a bar code that dictates the fate of the receptor (Lefkowitz and Shenoy, 2005).

1.2.2.2   GPCR Endocytosis

GPCR endocytosis is an important process for the regulation of receptor levels at the plasma membrane and hence receptor activity. Different GPCR subtypes undergo internalization via various mechanisms. The classical common pathway for internalization requires the binding of an agonist to the receptor resulting in a conformational change that leads to receptor phosphorylation by GRKs and β-arrestin recruitment (Ferguson and Caron, 1998) (Fig. 1.2). When β-arrestin binds the receptor, it uncouples G proteins and dissociates the heterotrimeric G protein complex from the receptor. β-arrestin binding also results in the recruitment of adaptor proteins AP-2 and clathrin. Studies showed that the carboxyl-terminal end of β-arrestin binds the amino-terminal globular region of the clathrin heavy chain and the β2-adaptin subunit of the AP-2 heterotetromeric complex (Ferguson and Caron, 1998; Ferguson, 2001; Sorkin and von Zastrow, 2009). The β2-adaptin subunit is important for the formation of the clathrin coat and the targeting of the receptor to the coated pits. These clathrin-coated pits are vesicular structures that are covered on the inner cytoplasmic surface by clathrin triskelions, which consist of three clathrin heavy chains and three clathrin light chains assembled into the clathrin lattice. The pits contain the receptor and the ligand and pinch off from the plasma membrane by a process that is mediated by the GTPase dynamin (Ferguson and Caron, 1998; Ferguson, 2001; Sorkin and von Zastrow, 2009). These vesicles are targeted to the endosomal compartment where they either recycle back to the plasma membrane or traffic to the lysosomes for degradation
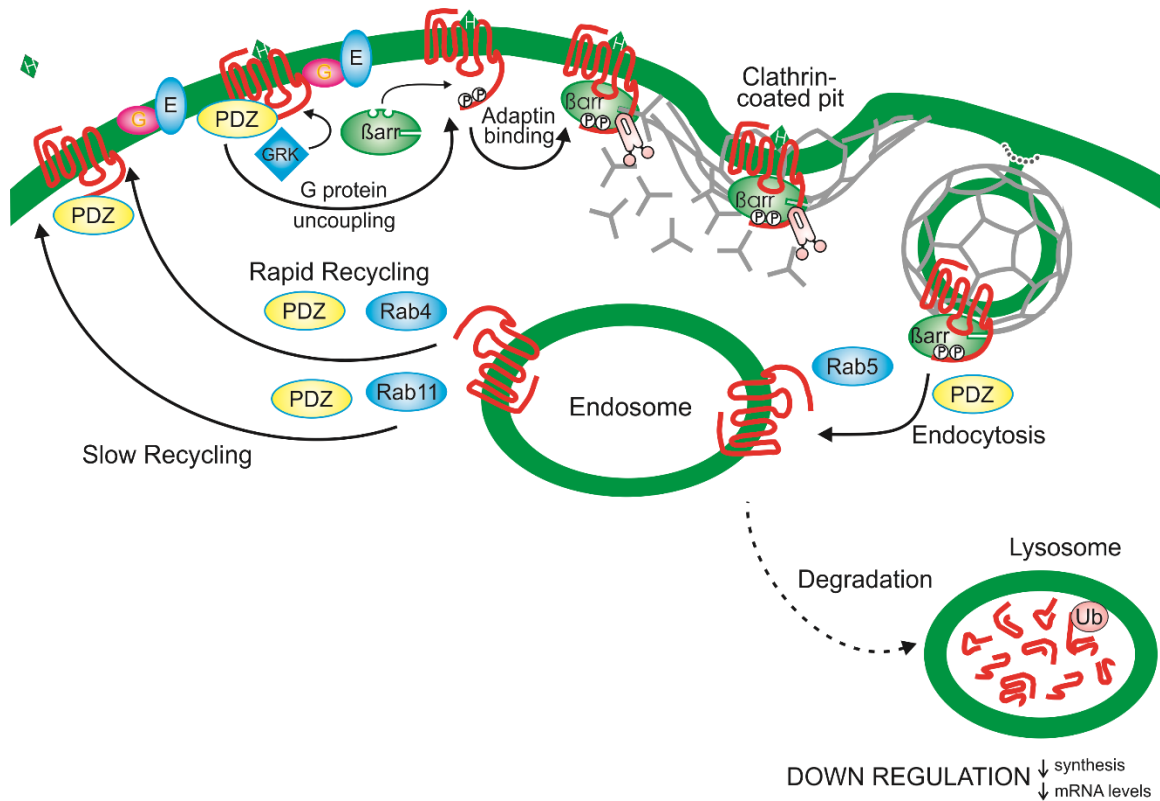
Figure 1.2: Schematic representation of the life cycle of a GPCR: Upon activation of the receptor with a ligand, it is phosphorylated by GRK. This phosphorylation results in the recruitment of β-arrestin and the initiation of endocytosis via clathrin-coated pits. The receptor is then internalized to the early endosomes and its fate is determined between either recycling back to the plasma membrane or getting transported to lysosomes for degradation. Rab GTPases as well as PDZ proteins are key modulators of the steps in this pathway. Modified from (Ferguson and Caron, 1998).

depending on certain motifs usually at the cytoplasmic tail of a GPCR (Sorkin and von Zastrow, 2009).

Although there is data supporting this mechanism for many receptors, there is also evidence for alternate steps and mechanisms for endocytosis. Despite the importance of GRKs and specially GRK2 for initiating the internalization process, some reports showed that phosphorylation by second messenger kinases such as PKA and PKC can also result in β-arrestin recruitment and G protein uncoupling. Furthermore, some studies illustrate that having sufficient levels of β-arrestin 1 and β-arrestin 2 could eliminate the requirement for receptor phosphorylation (Ferguson and Caron, 1998; Ferguson, 2001).

1.2.2.3   GPCR Recycling

One of the pathways that the receptor can go through after endocytosis is to recycle from the endosomes to the plasma membrane. Studies show that Rab GTPases play an important role in regulating this process. While Rab1 and Rab2 are the Rab proteins that control the anterograde and retrograde trafficking of GPCRs, Rab4 and Rab11 are involved in receptors recycling (Zerial and McBride, 2001). This reflects the specificity of the members of the small Rab GTPases family and their distinct distribution among the intracellular compartments. Rab4, Rab5 and Rab11 are expressed in the endosomal compartment. Furthermore, studies show that Rab5 regulates the transport of clathrin-coated vesicles from the plasma membrane to the endosomes and facilitates tethering and fusion of the vesicles to the early endosomes (Zerial and McBride, 2001). Rab4 and Rab11 on the other hand have a role in sorting and recycling either through the early endosomes (Rab4) or the perinuclear recycling endosomes (Rab11). In addition, recycling via Rab4-containing endosomes is rapid while Rab11-containing endosomes facilitate the slower recycling mechanism (Zerial and McBride, 2001).

1.2.2.4   GPCR Degradation

The other common fate for GPCRs following endocytosis is degradation in the lysosomes. When the early endosomes mature to multivesicular bodies (MVBs) and late endosomes, their cargo of internalized receptors is contained in the intraluminal vesicles

(ILVs) which are formed by ESCRT (endosomal sorting complex required for transport) complexes (Soldati and Schliwa, 2006; von Zastrow and Williams, 2012). Late endosomes and MVBs are then fused to the lysosomes that contain proteolytic enzymes to breakdown receptors. Similar to the anterograde pathway, certain motifs and post-translational modifications are important for initiating degradation (Sorkin and von Zastrow, 2009). However, the most common process that marks receptors for degradation is ubiquitination. The process consists of three key steps; First E1 (ubiquitin-activating enzyme) activates a ubiquitin molecule in an ATP-dependent step, then E2 (ubiquitin-conjugating enzyme) receives the active ubiquitin and transfers it to E3 (ubiquitin ligase) which would recognize the protein and label it with the ubiquitin molecule (Sorkin and von Zastrow, 2009; Alonso and Friedman, 2013).

1.3   G Protein-Coupled Receptor Signaling

1.3.1   G Protein-Dependent Signaling Pathways

The classical view of GPCRs signaling involves coupling to G proteins as their name suggests. Upon agonist binding, the GDP on the $G\alpha$ subunit is displaced by a GTP and this results in the functional disassociation of the heterotrimeric G protein ($G\alpha$ and $G\beta\gamma$). This would then allow each of the subunits to activate different effectors including ion channels and enzymes and eventually multiple signaling pathways. GTPase-activating proteins (GAPs) then bind $G\alpha$ resulting in the hydrolysis of GTP to GDP, which deactivates $G\alpha$ and causes disengagement of the effector (Gilman, 1987; Hepler and Gilman, 1992). Finally, $G\alpha$ reassociates with $G\beta\gamma$. Therefore, upon activation, the receptor acts as a guanine nucleotide exchange factor (GEF) for the heterotrimeric G protein. G proteins signaling can be classified into 4 major categories based on the G protein subtype that the receptor couples to; $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$ and $G_{12/13}$.

1.3.1.1   cAMP Signaling Pathway

The cAMP (cyclic adenosine monophosphate) signaling pathway is a downstream pathway that is mediated when a GPCR couples to either the stimulatory- or inhibitory-regulatory G proteins ($G\alpha_s$ and $G\alpha_i$) (Kristiansen, 2004). The effector that the G proteins

bind to in this case is adenylyl cyclase (AC), an enzyme that synthesizes cAMP from ATP. cAMP would in turn activates PKA as well as certain cAMP-regulated guanine nucleotide exchange factors, such as EPAC1 and EPAC2. There are 10 isoforms of AC and all of which can be stimulated by $G\alpha_s$. However only some of them can be stimulated by $G\beta\gamma$ or inhibited by $G\alpha_i$. The activity of AC can be also modulated through phosphorylation by PKA and PKC (Luttrell, 2008).

1.3.1.2   IP3 Signaling Pathway

The inositol 1,4,5-trisphosphate (IP3) signaling pathway is the downstream pathway that is mediated when a GPCR couples to $G\alpha_{q/11}$ subunit (Kristiansen, 2004). The main effector for $G\alpha_q$ is phospholipase Cβ (PLCβ), a membrane-bound enzyme that converts phosphatidylinositol-4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and IP3. IP3 binds to IP3 receptor in the ER and this results in the release of Ca^{2+} from intracellular stores, while DAG activates PKC. There are four known isoforms for PLCβ, PLCβ1-4 and they are known to be regulated by the Gα subunits as well as the G$\beta\gamma$ subunits of $G\alpha_{q/11}$. In fact, it has been suggested that certain isoforms (PLCβ2 and PLCβ3) are more sensitive to G$\beta\gamma$ regulation than others (PLCβ1) (Luttrell, 2008).

1.3.1.3   Rho Activation Signaling Pathway

The Rho activation signaling pathway is a less common pathway and it is mediated when the GPCR couples to $G_{12/13}$ subunit. $G_{12/13}$ is implicated in the regulation of Ras homology (Rho) guanine nucleotide exchange factors (RhoGEFs) which is considered to function as a link between the heterotrimeric G protein and small GTPase pathways. $G_{12/13}$ can control cytoskeletal rearrangement and affect cellular growth (Luttrell, 2008).

1.3.2   G Protein-Independent Signaling Pathways

GPCRs can signal via G-protein independent mechanisms and the best characterized example is β-arrestin-mediated signaling via Src and mitogen-activated kinase pathways (Naor et al., 2000; Kristiansen, 2004; Lefkowitz and Shenoy, 2005). Stimulation of many GPCRs leads to the activation of mitogen-activated protein (MAP)

kinases. There are three classes of MAP kinase, the extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase/Stress-activated protein kinases (JNK/SAPK) and p38/HOG1 MAP kinases (Naor et al., 2000; Belcheva and Coscia, 2002; Lefkowitz and Shenoy, 2005). MAP kinases are regulated via a series of modular kinase cascades. Each cascade is composed of three kinases that successively phosphorylate and activate the downstream component. β-arrestin acts as a multifunctional scaffold protein and helps assemble the MAP kinase modules with a GPCR to target it to the endosome of the cell (Lefkowitz, 1998; Ferguson, 2001; Lefkowitz et al., 2006). It is important to mention that this is a divergent pathway that can also be activated by multiple intracellular intermediates such as Ca^{2+}, Ras, PLC, proline-rich tyrosine kinase 2, cAMP, c-Src, and PI3K. For example, the activation of $G\alpha_s$ and $G\alpha_q$ via PKA and PKC leads to the phosphorylation of Ras. Ras would then activate Raf to phosphorylate MEK which subsequently phosphorylate ERK (Luttrell et al., 1999; Belcheva and Coscia, 2002; Kristiansen, 2004).

1.4   Regulation of GPCR Trafficking and Signaling

The various steps in the trafficking and signaling pathways of GPCRs are very efficiently regulated by two main factors; post-translational modifications and modulatory proteins.

1.4.1   GPCR Regulation by Post-Translational Modifications

Post-translational modifications serve as a method to label or mark the receptor particularly for the trafficking between cellular compartments by the addition of specific chemical groups or molecules on certain residues of the receptor. The four major types of post-translational modifications that have been well-characterized are glycosylation, phosphorylation, palmitoylation, and ubiquitination (Alonso and Friedman, 2013). These processes have distinct roles in regulating the expression and function of GPCRs.

1.4.1.1   Glycosylation

There are two forms of glycosylation; N- linked glycosylation and O-linked glycosylation. N-glycosylation is the addition of oligosaccharide to the nitrogen in the side-

chain amid of asparagine residues and it is the more common way of GPCRs glycosylation. Approximately 90% of all GPCRs are glycosylated on their extracellular domains at conserved consensus sites Asn-x-Ser/Thr (where x = any amino acid except proline). This process usually starts at the ER and is completed during the transport through the Golgi. O-Glycosylation on the other hand is the transfer of N-acetyl-galactosamine to a serine or threonine residue on the target protein (Qiu and Law, 2011). Studies with the β_2-AR and opioid receptors demonstrate an importance of glycosylation in receptor expression at the cell surface (McLawhon et al., 1983; Stiles et al., 1984; Law et al., 1985; George et al., 1986). Studies with the protease activated receptors (PARs) point to an effect for glycosylation on receptor signaling (Grimsey et al., 2011). It is suggested that inhibiting PAR1 glycosylation can enhance its signaling either due to the lack of the bulky sugars allowing for more efficient interaction of the newly generated tethered ligand with the second extracellular loop or a change in conformation that provide more flexibility of the ECL2 domain and more efficient G protein coupling (Grimsey et al., 2011). Receptor glycosylation is studied using different methods including monitoring the mobility of receptors on SDS-PAGE, enzymatic deglycosylation and site-directed mutagenesis. Multiple glycosidases have been characterized and shown to cleave the glycan chain differently (Spiro, 2002). Those include neuraminidases that cleave terminal N-aceylneuraminic acid, α-mannosidases that cleave mannose, endoglycosidases H that cleave asparagine-linked mannose rich oligosaccharides, Peptide N-Glycosidases F (PNGase F) which remove all types of N-linked glycosylation and O-glycosidase that cleaves O-linked glycan chains (Qiu and Law, 2011).

1.4.1.2   Phosphorylation

As explained earlier, GPCRs are phosphorylated by GRKs upon agonist activation. GPCRs can also be phosphorylated by protein kinase C (PKC), protein kinase A (PKA), casein kinases and receptor tyrosine kinases (Ferguson, 2001). Receptor phosphorylation occurs mainly on serine or threonine residues within the carboxyl-terminal tail or third intracellular loop. Studies with β_2-AR demonstrate the importance of GPCRs phosphorylation in regulating desensitization and internalization. Receptor phosphorylation promotes the association of arrestins to the activated receptor. This not

only uncouples the receptor from G protein to stop signaling (desensitization) but also recruits the receptor to the endocytic machinery (internalization) (Ferguson, 2001). Therefore, phosphorylation is an important factor in determining the fate of the receptor. In addition, studies on phosphorylation of the opioid receptors show an effect for this modification on the activity of adenylyl cyclase (Zhang et al., 2009). This suggests that post-translational modifications can play important roles in regulating receptor signaling in addition to the role they play in regulating trafficking. Studies on PAR1 illustrate that phosphorylation sites can be very specific in terms of regulating different functions meaning that different residues can differentially regulate association with clathrin adaptors that facilitate receptor internalization versus uncoupling from G protein signaling (Grimsey et al., 2011). Receptor phosphorylation is commonly studied by [^{32}P] orthophosphate labeling, mass spectrometry and phospho-specific antibodies.

1.4.1.3   Palmitoylation

Palmitoylation involves the attachment of palmitate to cysteine residues on the carboxyl-terminal tail or intracellular loop of the receptor through a thioester linkage. It is a covalent lipid modification that is observed with many GPCRs including rhodopsin, β_2-AR and the opioid receptors MOR and DOR (Qiu and Law, 2011). It usually occurs very early during protein synthesis. Therefore, it is important for receptor maturation and hence for receptor expression on the cell surface. However, there is evidence indicating that the receptor can undergo palmitoylation and depalmitoylation cycles in an agonist-dependent manner but the role of these processes is still unknown (Qanbar and Bouvier, 2003). There are multiple methods used to assess receptor palmitoylation such as metabolic labeling with [^{3}H] palmitate followed by immunoprecipitation, fatty acyl exchange labeling, SDS-PAGE and fluorography.

1.4.1.4   Ubiquitination

As described in the GPCR degradation section, ubiquitination is an important label that drives the receptor towards the degradation pathway. Furthermore, ubiquitination is important during the biosynthetic pathway to identify misfolded proteins and send them

for proteasomal degradation (ER-associated degradation) (Achour et al., 2008; Jean-Alphonse and Hanyaloglu, 2011). Ubiquitination involves the covalent addition of ubiquitin to the lysine side chains of the receptor which is facilitated by three enzymes; E1, ubiquitin-activating enzyme, E2, ubiquitin-carrying enzyme and E3, ubiquitin ligase (Sorkin and von Zastrow, 2009; Alonso and Friedman, 2013; Marchese and Trejo, 2013). First, ubiquitin is activated by E1 and transferred to the active site of the enzyme in an ATP-dependent process. Then, the ubiquitin molecule is passed on to E2 by a transthiolation reaction. Finally, E3 recognizes the target protein and label it with ubiquitin (Alonso and Friedman, 2013; Marchese and Trejo, 2013). Ubiquitination of GPCRs mainly occurs on the lysine residues of the carboxyl-terminal tail but studies show ubiquitin sites on the first cytoplasmic loop for μ-opioid receptor and on the third intracellular loop for the vasopressin V2 receptor (V2R) and β_2-AR receptor. Ubiquitin is a highly conserved 76-amino acid polypeptide and since it contains lysine residues, the peptide itself can be ubiquitinated forming polyubiquitin chains. In addition to its role in targeting the receptor to the late endosomes for degradation in the lysosomes, ubiquitination can serve as a quality control step for newly synthesized receptors resulting in this case in their proteosomal degradation. Studies with the DOR, MOR and thyrotropin-releasing hormone receptor show that when the receptor is misfolded, it gets retranslocated to the ER where it is labeled with ubiquitin to be degraded by the proteasome (Chaturvedi et al., 2001; Petaja-Repo et al., 2001; Shenoy, 2007). GPCRs can also be deubiquitinated by deubiquitination enzymes 130 and this leads to their rescue from degradation and an increase in their cell surface expression levels (Sorkin and von Zastrow, 2009). Furthermore, studies on PAR1 illustrate the importance for ubiquitination in the constitutive internalization of the receptor (Alonso and Friedman, 2013). In addition to regulating GPCRs trafficking, ubiquitination can indirectly regulate GPCRs signaling by regulating GRKs, MAPKs and β-arrestins (Alonso and Friedman, 2013; Marchese and Trejo, 2013). Ubiquitination is commonly studied by protease inhibitors and ubiquitin antibodies.

In addition to the previous main post-translational modifications that GPCRs undergo, recent studies are exploring two more processes; isoprenylation and sumoylation (Qiu and Law, 2011). Isoprenylation is a lipid modification of proteins characterized by

the addition of an isoprenoid moiety to the sulfhydryl group of a cysteine residue via a thioether bond. It has been suggested that isoprenylation and palmitoylation may collectively regulate GPCRs function (Miggin et al., 2003). Isoprenylation of prostacyclin receptors is suggested to be important for G-protein coupling, as well as receptor internalization (Miggin et al., 2002). Sumoylation is the covalent attachment of SUMO (an ubiquitin-like peptide) to lysine on the target receptor but unlike ubiquitin, it is not used to label the proteins for degradation. It appears to be important for transcriptional regulation, protein stability and receptor trafficking. Studies suggest that metabotropic glutamate receptors contains a sumoylation motif and can undergo this modification (Qiu and Law, 2011; Tang et al., 2005). However, further characterization of both isoprenylation and sumoylation is still required to understand their effects on regulating GPCRs activity.

1.4.2   GPCR Regulation by Modulator Proteins

Many intracellular and transmembrane proteins associate with GPCRs to regulate their processing in the endoplasmic reticulum, trafficking to the cell surface, compartmentalization to the plasma membrane microdomains, endocytosis and trafficking between intracellular membrane compartments. Examples include β-arrestins, receptor activity-modifying proteins (RAMPs), PSD95/Disc Large/Zona Occludens (PDZ) domain-containing proteins, small GTPases, regulators of G-protein signaling (RGS), GPCR-associated sorting proteins (GASPs), molecular chaperones, Homer, GRKs and protein phosphatases (Magalhaes et al., 2012).

1.5 PDZ Domain-Containing Proteins

PDZ domains comprise ~90 residues and PDZ domain-containing proteins are mainly known to be scaffolding proteins. They modulate protein interactions by recognizing short amino acid motifs usually at the carboxyl-terminal end of their target proteins (Lee and Zheng, 2010; Romero et al., 2011). They are found on more than 100 proteins in the human genome and there is usually more than one domain on the same protein to enable them to bind multiple proteins simultaneously and form functional molecular complexes. These complexes can regulate multiple biological processes such as

transport, ion channel signaling, protein trafficking and other signal transduction systems (Dunn and Ferguson, 2015). PDZ domains contain a conserved sequence that consists of glycine-leucine-glycine-phenylalanine and forms the globular structure of the domain that recognizes the PDZ-binding motif. PDZ motifs are usually classified into three classes. Class I is identified by their final three–amino acid sequence which consists of S/T-x-φ, where x symbolizes any amino acid and φ symbolizes any hydrophobic amino acid. The most common hydrophobic amino acids are found to be valine, isoleucine, or leucine (Songyang et al., 1997; Bezprozvanny and Maximov, 2001; Sheng and Sala, 2001; Vaccaro and Dente, 2002). Class II is characterized by having φ-x-φ as the final three amino acids. Class III is characterized by having Ψ-x-Ψ as the final three amino acids, where Ψ symbolizes any acidic amino acid residue (Sheng and Sala, 2001).

PDZ domain-containing proteins also contain multiple other modular protein interaction domains such as: Src-homology 2 (SH2), SH3, phosphotyrosine binding (PTB), WW motifs, guanylate kinase-like (GK), calmodulin kinase-like (CaMK) and Ras-associated (RA) domains (Harris and Lim, 2001; Magalhaes et al., 2012) (Fig. 1.3). Some of the recent studies show that PDZ proteins have distinct functions depending on the receptor being investigated (Table 1). A good example for that is MUPP1, which can enhance GABA$_B$ receptor signaling (Balasubramanian et al., 2007), uncouple the melatonin-1 receptor from Gα_i and facilitate the dephosphorylation (Guillaume et al., 2008) and resensitization of the 5-HT$_{2C}$R (Parker et al., 2003). Other studies show that unique PDZ proteins can regulate different pathways of the same receptor. For example, MAGI-2 has been shown to promote β_1-AR internalization while MAGI-3 seems to selectively attenuate β_1-AR signaling via ERK1/2 (He et al., 2006).
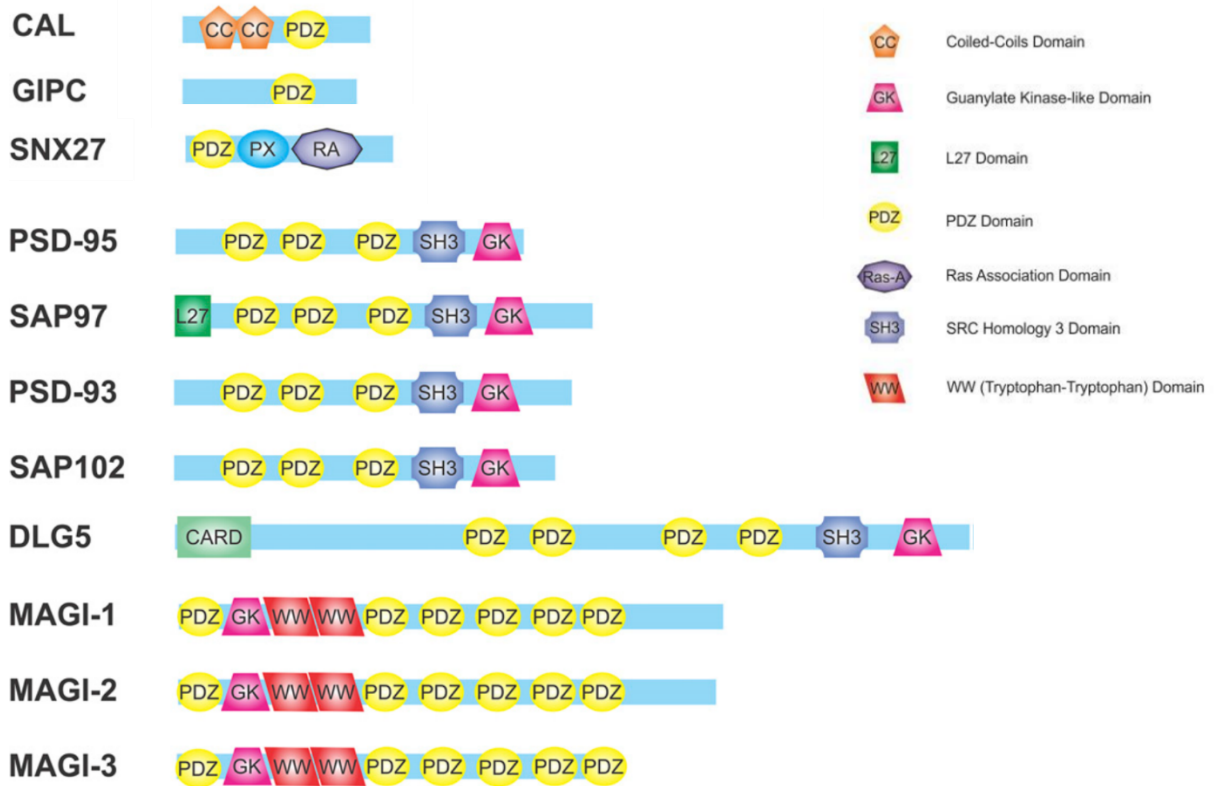
Figure 1.3: PDZ proteins that regulate Golgi sorting and MAGUKs: PDZ domain-containing proteins possess multiple domains that facilitate their function, binding to their target and subcellular localization. (Dunn and Ferguson, 2015).

Table 1: PDZ proteins and their role in GPCRs regulation (The table is part of tables published in Dunn and Ferguson, 2015)

PDZ protein	Effect on GPCR	Receptor
CAL/GOPC	↓ Membrane localization	β_1-AR, SSTR5
	↓ Recycling	β_1-AR
	↑ Golgi localization	SSTR5
	↓ ERK	mGluR1, β_1-AR
GIPC	↓ Gα_i coupling	Dopamine 3 receptor
	↑ Protein kinase B, ↑ Trafficking to early endosome	LPA$_1$R
	↓ ERK	β_1-AR
	↑ Endosome/Golgi localization	D$_2$R, dopamine 3 receptor
	↑ Membrane stability	Human LH receptor
SNX27	↑ Recycling	β_2-AR, β_1-AR, SSTR5
MAGI-2	↓ endocytosis	VPAC1
	↑ endocytosis	β_1-AR
	↑ Golgi localization	mGluR1a
	↓ cAMP	VPAC$_1$
MAGI-3	↑ ERK	BAI-1, LPA$_2$R
	↑ Rho	LPA$_2$R
	↓ ERK	β_1-AR, β_2-AR
SAP97	↑ Inositol 1,4,5-trisphosphate, ↑ ERK	5-HT$_{2A}$R
	↑ ERK	CRFR1, CRFR2
	↓ Endocytosis	CRFR1, 5-HT$_{2A}$R
PSD-95	↑ Inositol 1,4,5-trisphosphate	5-HT$_{2A}$R
	↑ cfos, ↑ Desensitization of Ca^{2+}, ↑ Endocytosis	5-HT$_{2C}$R
	↑ Membrane localization	GPR30
	↓ or = cAMP, ↑ Recycling, ↑ Endocytosis	D$_1$R
	↓ Endocytosis	β_1-AR, 5-HT$_{2A}$R
	↓ Endocytosis, ↓ β-arrestin recruitment	CRFR1
SAP102	↑ ERK, ↓ Mobility	A$_{2A}$R

1.5.1 Golgi- Associated PDZ Proteins (PDZ Proteins that Regulate Golgi Trafficking and Sorting)

1.5.1.1 CFTR-Associated Ligand (CAL)

The Golgi-localized cystic fibrosis transmembrane conductance regulator-associated ligand (CAL) is a single PDZ domain-containing protein with two coiled-coil motifs and two evolutionally conserved regions which are required for its Golgi localization (Charest et al., 2001; Yao et al., 2001; Cheng et al., 2002). CAL is also known as GOPC (Golgi-associated PDZ and coiled-coil motif-containing protein), PIST (PDZ domain protein interacting specifically with TC10) and FIG (fused in glioblastoma). CAL overexpression reduces cell surface expression of the β_1-AR and mGluR1a by retaining the receptor inside the cell via a direct interaction (He et al., 2004; Zhang et al., 2008). CAL also interacts with mGluR5a and stabilizes receptor expression by a mechanism that is proposed to involve the inhibition of ubiquitination-dependent degradation (Cheng et al., 2010). CAL also contributes to the regulation of somatostatin receptor recycling and degradation (Wente et al., 2005; Bauch et al., 2014).

1.5.1.2 GAIP-Interacting Protein (GIPC)

Another PDZ-binding protein involved in regulating the trafficking and sorting from Golgi is the Gα-Binding Protein Interacting Protein Carboxyl-Terminus (GIPC) (Liu et al., 2001). Similar to CAL/GOPC, GIPC contains one PDZ domain. GIPC is also known as Tax interaction protein 2 (TIP-2) and Synectin (Dunn and Ferguson, 2015). GIPC facilitates the trafficking of D_2R to the endosomes and Golgi (Jeanneteau et al., 2004). Similarly it has been found to enhance the trafficking of LPA_1R to the early endosomes and promote the receptor activation of the protein kinase B signaling pathway (Varsano et al., 2012). GIPC is also shown to regulate the trafficking of the human luteinizing hormone receptor and stabilize its expression at the plasma membrane (Hirakawa et al., 2003). In addition, GIPC plays a role in regulating the signaling of β_1-AR via the MAPK pathway but has no effect on the $G\alpha_s$ pathway since GIPC can reduce β_1-AR-stimulated ERK1/2 phosphorylation but does not interfere with cAMP formation (Hu et al., 2003). However,

GIPC prevents the coupling of Dopamine 3 receptor to $G\alpha_i$ to reduce signaling through the G protein-dependent pathway. (Jeanneteau et al., 2004; Dunn and Ferguson, 2015).

1.5.1.3 Sorting Nexin 27 (SNX27)

Sorting Nexin 27 (SNX27) contains one amino terminal PDZ domain, a Phox homology domain and a Ras-associating domain (Joubert et al., 2004). SNX27 can drive multiple GPCRs to the recycling pathway and therefore prevent their degradation. These include β_2-AR, β_1-AR and SSTR5 (Lauffer et al., 2010; Temkin et al., 2011; Nakagawa and Asahi, 2013; Bauch et al., 2014; Dunn and Ferguson, 2015). One of the mechanisms suggested for this role in recycling is that SNX27 can target the receptor to the retromer tubule by interacting with the endosomal WASH complex (Temkin et al., 2011).

1.5.2 Membrane-Associated Guanylate-like kinases (MAGUKs)

Membrane Associated Guanylate Kinase (MAGUK) family proteins are synaptic scaffolding proteins within a structured protein network responsible for the spatial organization of the presynaptic and postsynaptic compartments. They play a crucial role in the formation and function of synapses in the Central Nervous System (CNS) by regulating multiple aspects of synapse physiology such as synaptogenesis, receptor trafficking, synaptic function, and plasticity (Funke et al., 2005; Feng and Zhang, 2009; Yamagata and Sanes, 2010). MAGUKs are well-conserved throughout evolution and are widely-expressed in the brain and periphery. They include multiple subfamilies including membrane palmitoylated proteins (MPPs), zona occludens (ZO), caspase recruitment domain-containing MAGUK protein (CARMA), discs larges (DLGs) and MAGUK with inverted orientation PDZ proteins (MAGIs) (Oliva et al., 2012). Generally, these proteins contain multiple domains that control their function and facilitate their interactions with their targets. The two common domains among all members are PDZ domains and guanylate kinase (GK) domains (Oliva et al., 2012). An important target for the MAGUKs scaffolding proteins is the G protein-coupled receptors (GPCRs) family (Dunn and Ferguson, 2015). This protein-protein interaction between the receptor and MAGUKs results in the regulation of GPCRs function. MAGUK proteins play a key role in mediating

the subcellular localization, trafficking, cell surface expression and signal transduction of multiple GPCRs and different proteins have both overlapping and distinct roles in the regulation of GPCR activity (Harris and Lim, 2001; Lee and Zheng, 2010; Romero et al., 2011; Magalhaes et al., 2012). One of the important subfamilies of MAGUKs is MAGIs. It consists of three members; MAGI-1, MAGI-2 and MAGI-3. All three proteins share a similar structure containing one GK domain, two tryptophan tryptophan (WW) domains and six PDZ domains (te Velthuis et al., 2007; Oliva et al., 2012; Dunn and Ferguson, 2015).

1.5.2.1   MAGUK with Inverted orientation PDZ (MAGIs)

MAGI-1, also known as BAP-1 (BAI-1-associated protein 1), has 7 isoforms that are widely expressed in different tissues. Isoform 1, isoform 2 and isoform 6 are highly expressed in colon, kidney, lung, liver, and pancreas. Isoform 5 is predominantly expressed in brain and heart. Isoform 3 and isoform 4 are highly expressed in pancreas and brain (Dobrosotskaya et al., 1997; Laura et al., 2002). MAGI-1 interacts with BAI-1 (brain-specific angiogenesis inhibitor-1), a family B GPCR that functions as an adhesion molecule (Shiratsuchi et al., 1998). Therefore, it is suggested that MAGI-1 might play an important role in the organization of membrane proteins and cytoskeleton by transmitting signals related to cell- cell or cell- matrix interactions through BAI-1 (Shiratsuchi et al., 1998; Stetak et al., 2009; Stephenson et al., 2013). MAGI-1 also regulates AMPA receptor activity and modulate behavioral plasticity (Emtage et al., 2009).

MAGI-2, also known as S-SCAM (synaptic scaffolding molecule), is specifically expressed in the brain and has 2 isoforms (Wood et al., 1998). Previous studies illustrate an association between MAGI-2 and β_1-AR that is enhanced upon agonist stimulation (Xu et al., 2001). The study also demonstrate an increase in agonist-induced internalization of β_1-AR when MAGI-2 is co-expressed but no effect on cAMP generation induced by isoproterenol stimulation. Alternatively, a study of the effects of MAGI-2 on vasoactive intestinal polypeptide type1 (VPAC$_1$) receptor demonstrate an opposite function, where MAGI-2 overexpression results in the inhibition of VPAC$_1$-mediated cAMP production and agonist-induced VPAC$_1$ internalization (Gee et al., 2009).

MAGI-3 is widely expressed in different tissues. It has been shown to interact with β_2-AR and reduce signaling via the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway (Yang et al., 2010). MAGI-3 can also associate with lysophosphatidic acid-activated receptor subtype-2 (LPA$_2$R) and contrary to the effect observed with β_2-AR, knockdown of MAGI-3 results in a decrease of receptor-mediated ERK1/2 signaling (Zhang et al., 2007). The study also reports a significant reduction in Rho activation upon knockdown of MAGI-3.

1.5.2.2   Discs Large (DLGs)

This subfamily of MAGUKs contain five proteins (DLGs 1-4) also known as SAP97 (DLG1), PSD-93 (DLG2), SAP102 (DLG3) and PSD-95 (DLG4) as well as a Discs Large Homolog 5 (DLG5). All members contain an SH3 domain, a GK domain and three PDZ domains (with the exception of DLG5 which has 4 PDZ domains). All PDZ domains are type I domains (they bind class I PDZ binding motif consisting of T/S-x-φ) (Oliva et al., 2012). Similar to MAGIs, DLGs are widely expressed in the central nervous system and have been shown to regulate synaptic function (Dunn and Ferguson, 2015).

SAP97 (DLG1) regulates the trafficking and signaling of different members of the GPCRs family. It can antagonize the agonist-induced internalization of both CRFR1 and 5-HT$_{2A}$R when overexpressed. Furthermore, it plays an essential role in regulating the MAPK pathway for multiple GPCRs including CRFR1, CRFR2 and 5-HT$_{2A}$R. The fact that CRFR2 does not possess a PDZ-binding motif suggests that this role for SAP97 in regulating ERK1/2 activation does not require an interaction with the receptor and could be a global role for the regulation of MAPK pathway (Dunn et al., 2013). SAP97 can also enhance 5-HT-induced IP3 formation which illustrates how one PDZ-binding protein can regulate G protein-dependent as well as G protein-independent signaling pathways activated by the same receptor. In addition, SAP97 facilitates the recycling of β_1-AR and this emphasizes the involvement of PDZ proteins at different steps of the GPCRs life cycle.

PSD-95 (DLG4) has been shown to regulate a number of GPCRs. It has been shown to antagonize the internalization of both β_1-AR and 5-HT$_{2A}$R (Hu et al., 2000; Xia et al., 2003). It also has an enhancement effect on Gα_q signaling mediated by 5-HT$_{2A}$R.

Interestingly, PSD-95 has been shown to have an opposite effect on $5\text{-HT}_{2C}\text{R}$ since it can enhance the internalization of the receptor and decrease its cell surface expression (Gavarini et al., 2006). There are reports of an association between PSD-95 and $D_1\text{R}$ resulting in an increase in internalization and reduction in cAMP formation by the receptor (Zhang et al., 2007). Other studies suggest that PSD-95 can enhance the recycling of $D_1\text{R}$ without affecting its signaling via $G\alpha_s$ (Sun et al., 2009). A recent study has illustrated the effects of PSD-95 in regulating CRFR1 function. Similar to the effects observed with β_1-AR and $5\text{-HT}_{2A}\text{R}$, PSD-95 can reduce the endocytosis of CRFR1 (Dunn et al., 2016). The study also provide a mechanism for this regulation presented by a negative effect of PSD-95 on β-arrestin recruitment. The role of PSD-93 (DLG2) is not fully characterized yet. Reports indicate that PSD-93 and PSD-95 can compensate for one another and hence it is suggested they have similar functions (Sun and Turrigiano, 2011).

SAP102 (DLG3) is another understudied member of the DLGs subfamily. It has an effect on the adenosine A_{2A} receptor since it can regulate the mobility of the receptor and enhance ERK1/2 activation by the receptor (Thurner et al., 2014). Further studies are required to fully assess and understand the function of SAP102 in regulating GPCRs.

DLG5 has some differences in topology compared to the other four members of the subfamily. It contains four PDZ domains as opposed to three domains in the other proteins. It also contains a caspase activation and recruitment domain (CARD) at the amino terminal that is not present in any of the other DLGs (de Mendoza et al., 2010). There are no reports that suggest a direct role for DLG5 in regulating GPCRs function. However, it can scaffold PKC isoforms which might be a mechanism for mediating GPCRs signaling.

1.5.3   PDZ Proteins Network

The previous section highlighted the role that PDZ domain-containing proteins have in regulating different functions of GPCRs including their internalization, recycling, degradation and signaling. However, another important aspect that should always be considered when studying PDZ proteins is the interplay between the different proteins themselves. Bearing in mind the similar domains that PDZ proteins possess and their overlapping functions, there is a possibility for these proteins to either have

"complementary" effects or "competitive" effects. This also provides a possible explanation for the observation that one receptor can be mediated by multiple PDZ proteins.

A recent example that illustrates a complementary effect is the regulation of the somatostatin receptor subtype 5 (SSTR5) by PDZ proteins (Bauch et al., 2014). The trafficking of the receptor was studied and it appears that CAL, SNX27 and NHERF1 are involved in mediating the different steps to determine the subcellular distribution and membrane availability of the receptor. The data demonstrate that the receptor's interaction with SNX27 can drive the trafficking pathway away from lysosomal targeting and towards the recycling pathway (Bauch et al., 2014). It also shows that while CAL overexpression leads to the retention of SSTR5 in the Golgi, NHERF1 can reverse this effect and is able to release the receptor and enhance its trafficking to the cell surface. The study also suggests that the protein interacting with C kinase 1 (PICK1), a fourth PDZ protein, and SNX27 act antagonistically to mediate receptor recycling. This is an example showing that PDZ proteins can perhaps sequentially perform their function depending on the stage of receptor trafficking. Another study on β_1-AR demonstrated that PSD-95 can antagonize the intracellular retention of β_1-AR upon CAL overexpression and restore plasma membrane expression (Koliwer et al., 2015).

A similar observation is reported between CAL and two other PDZ proteins when the transmembrane protein Cadherin 23 is investigated (Xu et al., 2010). Overexpression of CAL can lead to the retention of Cadherin 23 in the transgolgi network and hence to a reduction in plasma membrane expression. This effect is reversed by MAGI-1 as well as harmonin.

In addition to the regulation of trafficking, the interplay between PDZ proteins can be important for regulation of GPCR signaling. It has been shown that NHERF2 and MAGI-3 can compete for binding to LPA$_2$R and this can bias the signaling of the receptor (Lee et al., 2011). The study shows that MAGI-3 can increase the coupling of G$\alpha_{12/13}$ to LPA$_2$R and hence decrease PLC activation while NHERF2 promotes the coupling of Gα_q to the receptor resulting in an enhancement in PLC activity. Therefore, MAGI-3 and

NHERF2 exhibit opposite effects on LPA-induced PLC activity by differentially regulating G protein coupling.

A similar role of PDZ proteins in regulating GPCRs signaling resulting in bias from one pathway to another was reported with the Parathairoid Hormone receptor 1 (PTH1R). The study showed that NHERF1 can bias signaling from activating adenylyl cyclase to activating PLCβ. In addition, NHERF1 was shown to interfere with β-arrestin recruitment to PTH1R and hence reduce receptor internalization (Mahon et al., 2002).

An example for a compensation effect between PDZ proteins is between PSD-93 and PSD-95 (Sun and Turrigiano, 2011). Double knockout studies show a functional compensation between PSD-95 and PSD-93 during scaling up in young cortical neurons.

1.6   G Protein-Coupled Receptors and Disease

In addition to being involved in regulating all physiological processes from neurotransmission and cardiovascular function to inflammation, metabolism and endocrine function, dysregulation or dysfunction of GPCRs has been associated with a broad range of pathological conditions. This is not surprising given their regulation of all physiological processes as well as their wide distribution in the different organs of the body. These conditions include neurological and cardiovascular disorders, renal and pulmonary diseases, immunological diseases, pain and cancer.

One important example that has been well studied would be heart diseases. There are approximately 200 members of the GPCR superfamily that have been shown to regulate heart function (cardiac GPCRs) (Salazar et al., 2007). However, most of the drugs used to treat heart problems act on the angiotensin and adrenergic receptors. These available drugs, although improving the quality of life for patients, still have their limitations since they show major side effects in some cases due to off target effects or the ubiquitous distribution of the receptors in organs other than the heart. To produce better drugs with fewer side effects, further characterization of GPCRs, their assembly into signaling complexes, their interacting partners as well as their trafficking from the endoplasmic reticulum to the cell surface is required to establish a complete understanding of the key factors in the processes.

The example that is going to be the focus for this project is psychiatric disorders. The role of GPCRs in regulating neurotransmission and the function of the central nervous system has been well documented. The two families that demonstrate crucial effects on synaptic transmission, synapse formation, axon guidance and development of neuronal circuits are Rhodopsin and Glutamate. Rhodopsin family GPCRs have this role because they are the main molecular targets for various neurotransmitters such as serotonin, dopamine, acetylcholine, histamine, adrenaline and norepinephrine and neuropeptides such as somatostatin, melanocortins, neuropeptide Y and neuropeptide FF. The same reason applies for Glutamate receptors being the target for glutamate which represent the principal excitatory neurotransmitter of the CNS.

A common disorder that has been associated with mGluR activation is Fragile X mental retardation (FMR) where it has been suggested that FMR results from unbalanced activation of Group I mGluRs and impaired synaptic maturation. mGluRs have also been associated with dementia and Alzhiemer's disease (Bear, 2005; Yan et al., 2005).

As for the rhodopsin family, the 5-HT_{2C} receptor has been shown to play an essential role in the regulation of mood and alteration of its functional activity has been implicated in the etiology of anxio-depressive states (Millan et al., 2005). Therefore, it has been targeted by antidepressants as a therapeutic approach. $5\text{-HT}_{2C}R$ is not the only 5-HT-activated receptor to be implicated in mood disorders and have antidepressants actions. Dopamine signaling via D1 and D2 receptors plays a key role in schizophrenia and blocking dopamine D2 receptors with any antipsychotic drug is thought to alleviate positive symptoms, but not sufficiently address negative symptoms and cognitive deficits (Meltzer et al., 1989). The A_{2a} receptor plays an important role in modulation of dopaminergic and glutamatergic neurotransmission and has been used as a target for the treatment of drug addiction, anxiety and depression (Komatsu, 2015).

Recently, some attention is being directed toward the role of adhesion GPCRs in regulating neurotransmission (Stephenson et al., 2014). For example, studies show effects of GPR56 on regulating brain development and brain-specific angiogenesis inhibitor (BAI) subfamily in mediating synaptogenesis. Another examples is GPR88 since the knockout

mouse model for this receptor shows abnormal behaviors associated with schizophrenia such as disrupted sensorimotor gating and impaired learning and bipolar disorder (Logue et al., 2009; Quintana et al., 2012; Komatsu, 2015).

1.6.1   Mental Disorders

Depression and Anxiety are co-morbid disorders that associate with stressful events (Anisman et al., 2008). According to Health Canada and Statistics Canada, 10-15% of Canadians are affected by depression and anxiety, and females are at higher risk since the number of women affected is doubled compared to men. Also, anxiety and depression are the leading causes of disability in females and the second cause generally just after ischemic heart disease (Nemeroff and Owens, 2004). The causes of depression and anxiety vary to include genetic determinants and environmental factors, but early life experiences and the effect of the environment seem to be more crucial, especially in the progression of the disease. There are some drugs available for the relief of symptoms associated with depression and anxiety. Despite being regularly prescribed to patients, these drugs still have limitations with the onset of action, response, remission rates, and side-effect profiles, especially that they are only effective in 50% of patients. These medications include serotonin 2A receptor ($5-HT_{2A}R$) antagonists, CRF receptor 1 (CRFR1) antagonists, selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants and monoamine oxidase inhibitors (Marek et al., 2003; Holtzheimer and Nemeroff, 2006).

Two of the major molecules implicated in these disorders are the corticotropin-releasing factor (CRF) and serotonin (5-HT). CRF is known to regulate physiological responses in anxiety states and behavioral mechanisms of defense and it can trigger some changes in the other neurotransmitter systems such as serotonergic signaling (Macri et al., 2007). Serotonin is also important in the central nervous system (CNS) and it modulates the etiology of anxiety and the response to anxiolytic drugs via its different receptors (Holmes et al., 2003; Holsboer, 2003). Different members of the serotonin receptors family are used as targets for the treatment of brain-related disorders such as schizophrenia, depression, anxiety, eating disorders and obesity (Roth et al., 2004). These two neurotransmitters elicit their effects via acting on their corresponding G protein-coupled

receptors. CRF activates two receptors, CRFR1 and CRFR2 while serotonin receptors form a bigger family with 15 members. Among these GPCRs, 5-HT$_{2A}$R and CRFR1 have been directly associated with anxiety behaviours (Magalhaes et al., 2010). Also, the role of 5-HT$_{2A}$R and CRFR1 in modulating anxiety and depression is clear given that the currently available drugs act directly or indirectly on these receptors.

1.6.2 Targeting Protein- Protein Interactions as a Pharmacological Tool

An increasing number of neurological and psychiatric disorders have been found to result from an alteration in the interaction between a GPCR and GPCR-interacting protein. This can provide new tools for therapeutic intervention. Many studies that investigated the association between GPCRs and diseases conclude that sometimes it is these interactions between the receptor and a specific protein that could be causing the problem (Bockaert et al., 2010). For example, it has been demonstrated that the interaction of Homer protein with mGluRs is associated with schizophrenia and anxiety (Norton et al., 2003; Szumlinski et al., 2005). Similarly, the interaction of Shank proteins and specifically Shank3 with mGluRs and somatostatin SSTR2 receptor is associated with autism (Durand et al., 2007). In addition, the interaction between mGluR7 and PICK1 (protein interacting with C kinase 1) is implicated with neurological disorders like epilepsy and in fact, *in vivo* studies showed that injecting rats and mice with a cell permeable mimetic peptide to specifically disrupt this interaction display a phenotype characteristic of absence epilepsy (Bockaert et al., 2010).

Serotonin receptors were the focus of many similar studies as well. It is suggested that targeting the interaction between 5-HT$_{2C}$ receptor and PTEN (protein phosphatase and tensin homolog) might be an effective strategy for the treatment of addiction-related behaviors, with perhaps less pronounced side effects than 5-HT$_{2C}$ agonists (Ji et al., 2006). Other proteins with important associations with the serotonin receptors are the PDZ domain-binding proteins. Proteins like PSD-95 and MAGUK p55 subfamily member 3 (MPP3) can mediate receptors desensitization and internalization (Gavarini et al., 2006). Therefore, targeting 5-HTR-PDZ protein interactions can be an attractive strategy to develop new drugs that may improve the therapeutic efficacy of antidepressants. P11 (also

called S100A10 or calpactin 1 light chain or annexin 2 light chain) is another important interactor with 5-HT receptors (Svenningsson et al., 2006; Warner-Schmidt et al., 2009). The interaction between p11 and 5-HT$_{1B}$ receptors is implicated in the response of Parkinson's disease patients to L-DOPA treatment. It is also suggested to be involved in the anxiolytic effects of 5-HT$_{1B}$ and 5-HT$_4$ receptors (Bockaert et al., 2010).

1.7   Corticotropin Releasing Factor Receptors

This family consists of two Class B GPCRs; CRFR1 and CRFR2. They are both activated by CRF and although they share 71% sequence homology, CRFR1 has ten-fold higher affinity for CRF than CRFR2 (Dautzenberg and Hauger, 2002). CRF is released in response to stress and it regulates the secretion of adrenocorticotropin (ACTH) from the anterior pituitary gland. In addition to anxiety and depression, CRF receptors are also known to regulate cardiovascular and inflammatory processes since they were shown to play a role in cardio-protection against ischemia reperfusion injury (Brar et al., 2004) and in the regulation of IL-18 expression.

The tissue distribution of CRFR1 and CRFR2 is also different. CRFR1 is expressed widely in the brain (neocortex and cerebellum) and pituitary, while CRFR2 is usually found in the heart and skeletal muscle (Chalmers et al., 1995; Dautzenberg and Hauger, 2002). Also, CRFR1 has a PDZ-binding motif on the carboxyl-terminal tail composed of three amino acids; threonine, alanine and valine (TAV), while CRFR2 does not (Myers et al., 1998; Chen et al., 2005). Upon CRF activation, both receptors mainly signal via coupling to Gα_s to activate adenylyl cyclase resulting in the formation of cAMP. Interestingly, this cAMP pathway plays a central role in amplifying and diversifying the signaling pathways activated by CRF receptors. cAMP formation results in the activation of PKA and this leads to the activation of diverse downstream signaling molecules including membrane guanylyl cyclase, the NF-kB transcription factor, glycogen synthase kinase-3 and the Wnt/β-catenin pathway, ERK1/2, tyrosine hydroxylase and ion channels (Haug and Storm, 2000; Aggelidou et al., 2002; Kovalovsky et al., 2002; Zhao and Karalis, 2002; Facci et al., 2003; Nemoto et al., 2005; Khattak et al., 2010). One of these important signaling pathways is the MAPK pathway. Studies show that PKA activation can have opposite effects on

ERK1/2 signaling depending on the tissues or type of cells investigated. For example, in the hippocampus, neuronal cells or pituitary, PKA activation leads to enhanced ERK1/2 phosphorylation, while in the myometrium, endometrial adenocarcinoma and breast cancer, PKA activation via CRF results in the inhibition of ERK activity (Elliott-Hunt et al., 2002; Kovalovsky et al., 2002; Graziani et al., 2007) (Fig. 1.4).

1.8 Serotonin Receptors

This family consists of 7 receptor types and 15 subtypes that belong to Class A GPCRs (except for 5-HT$_3$Rs which are ligand-gated ion channels). Serotonin receptors are involved in the modulation of sleep-wake cycles, appetite, mood, memory, breathing, cognition and sexual behavior (McCorvy and Roth, 2015). Serotonin has been the focus of many pharmacological studies for the treatment of neuropsychiatric disorders with drugs that target either the serotonin receptors or serotonin reuptake. Examples include serotonin-selective reuptake inhibitors (SSRIs), dual serotonin-norepinephrine reuptake inhibitor (SNRI) and partial agonist or antagonist for the serotonin receptors.

The 5-HT$_1$ subfamily consists of 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$ and 5-HT$_{1F}$ and mainly couples to Gα_i resulting in the inhibition of the activity of adenylyl cyclase and reduction in cAMP formation. 5-HT$_{1A}$R has been implicated in anxiety and depression while the other subtypes have been associated with migraine.

5-HT$_2$ subfamily consists of three subtypes; 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$. The family mainly couples to Gα_q for the activation of phospholipase C (PLC) which hydrolyzes the phosphatidylinositol diphosphate releasing diacylglycerol (DAG), protein kinase C (PKC) and inositol triphosphate (IP3). This also leads to the release of Ca^{2+} from intracellular stores (Roth et al., 2004; Gray and Roth, 2001). 5-HT$_{2A}$R has been suggested to be a good target for the treatment of a number of mental illnesses including schizophrenia, depression, and Tourette's syndrome given its high levels in the cortex (Meltzer and Roth, 2013). Similarly, 5-HT$_{2C}$R is ubiquitously distributed in the CNS and has been shown to regulate dopamine release upon treatment with antidepressants
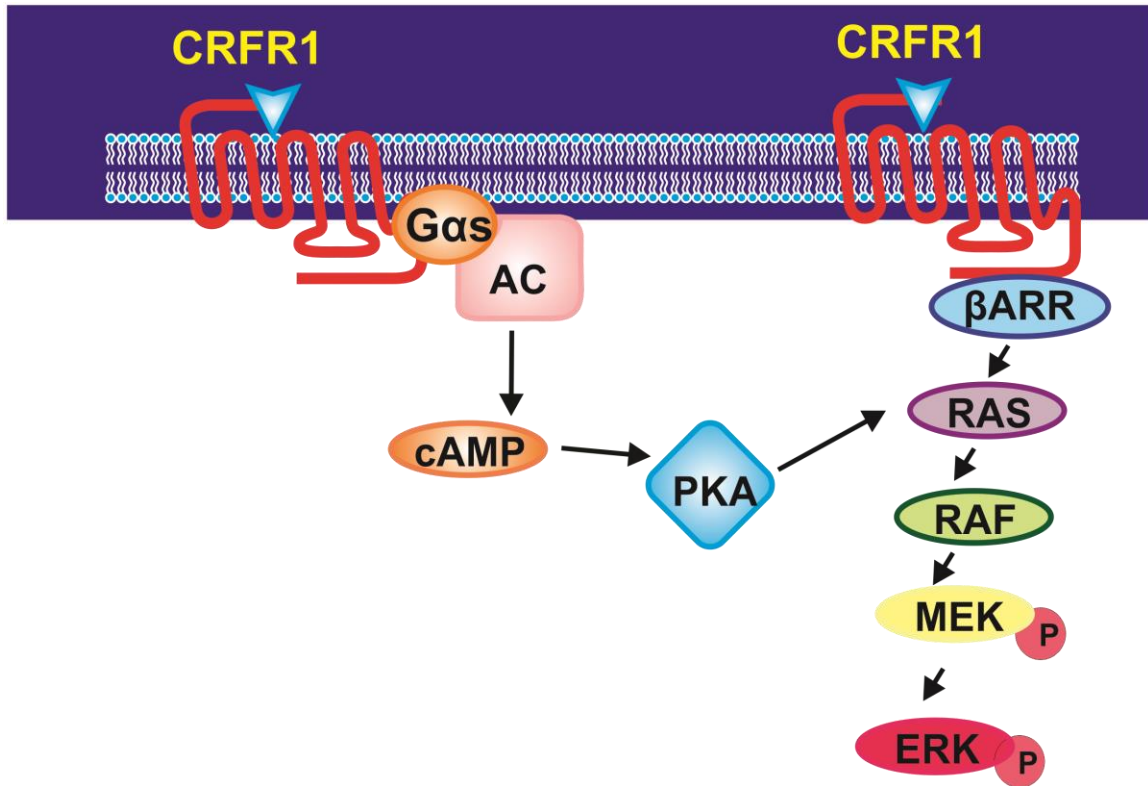
Figure 1.4: Signaling pathways activated by CRFR1: CRFR1 couples mainly to $G\alpha_s$ to stimulate Adenylyl Cyclase (AC) leading to the formation of cAMP. It can also signal via the MAPK pathway to activate ERK1/2 phosphorylation.

(Mengod et al., 1996; Millan et al., 2005). Little is known about the 5-HT$_{2B}$R other than being unselectively activated by 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor ligands. There is also some evidence to support that 5-HT$_{2B}$ activation can result in cardiac hypertrophy.

5-HT$_4$ receptors couple to Gα_s to activate AC leading to cAMP release. They are expressed in different tissues and therefore their activation can result in variable effects such as long-term potentiation (LTP) in hippocampus and peristalsis in the gut (McCorvy and Roth, 2015). It has been suggested that blocking the receptor with selective 5-HT$_4$R antagonists can be useful in the treatment of atrial fibrillation, irritable bowel syndrome, and urinary incontinence.

Similar to 5-HT$_1$, 5-HT$_5$ receptors couple to Gα_i. The family has two subtypes; 5-HT$_{5A}$ and 5-HT$_{5B}$ that have been associated with psychiatric disorders but have not been used as pharmacological targets (Thomas, 2006). Both 5-HT$_6$ and 5-HT$_7$ receptors, are Gα_s-coupled and expressed in the CNS (thalamus, hypothalamus and hippocampus) and peripheral tissues. Similar to the rest of serotonin receptors, they have been proposed as targets for the treatment of depression (McCorvy and Roth, 2015).

Among these serotonin receptor subtypes, the most ubiquitous and extensively studied serotonin receptors in the human brain are the 5-HT$_{1A}$R and 5-HT$_{2A}$R. These receptors have been shown to have potential roles in different neuropsychiatric diseases such as Alzheimer's disease, Schizophrenia, depression and suicide (Burnet et al., 1995). This project particularly focuses on 5-HT$_{2A}$R which is expressed in the neocortex (mainly prefrontal, parietal, and somatosensory cortex). It is involved in the modulation of mood and perception (Hall et al., 2000). Similar to the CRFR1, the 5-HT$_{2A}$R has a PDZ-binding motif at the carboxyl-terminal tail formed by three amino acids; serine, cysteine and valine (SCV). Furthermore, in addition to coupling to Gα_q for the activation of PLC, it can activate the MAPK pathway resulting in ERK1/2 phosphorylation (Fig. 1.5).
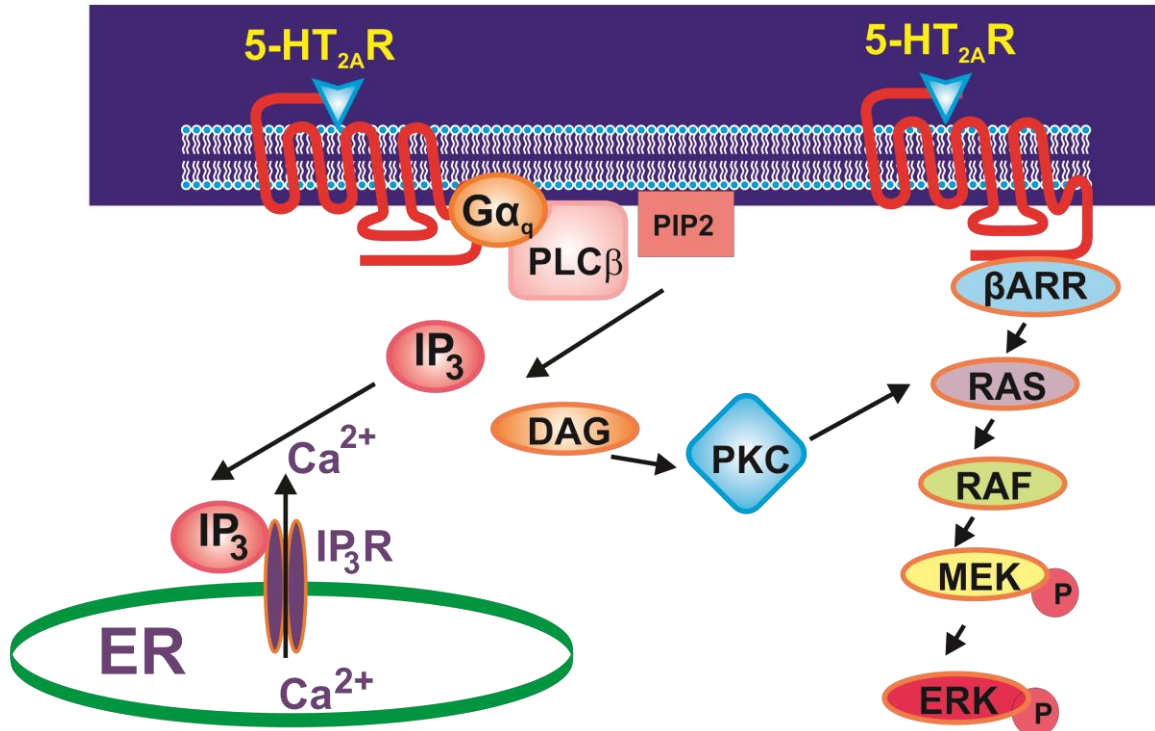
Figure 1.5: Signaling pathways activated by 5-HT₂ₐR: 5-HT₂ₐR couples mainly to $G\alpha_q$ to stimulate phospholipase Cβ (PLCβ) leading to the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). IP3 results in the release of Ca^{2+} from intracellular stores while DAG activates PKC. It can also signal via the MAPK pathway to activate ERK1/2 phosphorylation.

1.9 Corticosteroid-Serotonin Interactions

The studies on stress-related disorders such as depression have revealed that the two main systems involved are the Hypothalamo-Pituitary-Adrenal (HPA) Axis and the monoaminergic system and in particular the serotonergic (5-hyroxytryptamine) system. Studies show cross-regulation of the HPA axis and 5-HT system under normal physiological conditions, as well as pathological conditions.

1.9.1 Hypothalamo-Pituitary-Adrenal (HPA) Axis

The Hypothalamo-Pituitary-Adrenal (HPA) Axis is a neuroendocrine system that is responsible for regulating the stress response to restore homeostasis (Macri et al., 2007; Pompili et al., 2010). When the body is exposed to a stressor, the paraventricular nucleus (PVN) of the hypothalamus releases corticotrpoin-releasing hormone (CRH) and arginine-vasopressin (AVP). CRH and AVP activate the anterior pituitary gland to release adrenocorticotropin hormone (ACTH). ACTH which is secreted into the circulation stimulates the adrenal cortex to produce glucocorticoids. These are represented by the secretion of cortisol in human and corticosteroids in rodents. These hormones can control the response via a negative feedback mechanism by activating glucocorticoid receptor (GR) in the adrenal cortex, anterior pituitary gland, hypothalamus and hippocampus (Lanfumey et al., 2008; Pompili et al., 2010). Many reports demonstrate the effect that the serotonin system has on the release of corticosteroids. One example is the increase in ACTH and cortisol levels upon administration of $5\text{-}HT_{1A}$ agonists. Other studies show that 5-HT acts via synaptic contacts between the serotonergic terminals and CRH-containing cells in the PVN (Liposits et al., 1987). This is demonstrated when the activation of $5\text{-}HT_{1A}$ receptors on the PVN results in the secretion of ACTH (Lanfumey et al., 2008).

1.9.2 Serotonergic System

The serotonergic network is one of the most important systems in the brain, since the serotonergic neurons project to all parts of the CNS (Macri et al., 2007; Lanfumey et al., 2008; Pompili et al., 2010). One of these main structures is the hippocampus which receives many 5-HT projections from the neurons in the raphe nucleus. Therefore, different

5-HT receptor types are expressed in the hippocampus. The serotonin system can regulate variable functions such as food intake, learning and memory, thermoregulation, locomotion, cardiovascular function, sleep, endocrine regulation and sexual behavior. Studies demonstrate that the activation of HPA axis can result in a significant increase in the synthesis and production of 5-HT.

1.10   Hypothesis and Objectives

The previous section highlighted that there is plenty of evidence to support the interaction between the HPA axis and the serotonergic system. Interestingly, a recent study from our laboratory illustrates a crosstalk between the two systems at the receptor level. The study demonstrates a sensitization effect of $5\text{-HT}_{2A}R$ signaling upon pretreatment with CRF (Magalhaes et al., 2010). The study also proposes that this phenomena is dependent on the internalization and trafficking pathways of both receptors and suggests the involvement of a PDZ protein as a key regulator for this crosstalk. The role of PDZ proteins in regulating GPCRs function has been the focus of many studies in the last decade which confirms their significance and the important roles they play in that field. However, many interactions remain to be characterized to gain better understanding of the role of PDZ proteins in regulating GPCRs activity. Achieving complete characterization of PDZ-GPCRs interactions can provide an interesting pharmacological tool to develop more specific drugs. Targeting protein-protein interactions that are involved in pathological conditions is a challenging, yet very attractive, concept and focusing on the domains that mediate these interactions (such as PDZ domains) should be the priority. Such drugs will be very selective compared to agonists/antagonists of receptors and will therefore have fewer side effects. In order to be able to assess the effects of different PDZ proteins on the crosstalk between CRFR1 and $5\text{-HT}_{2A}R$, we would have to first study these effects on each receptor separately. The PDZ proteins that this project focuses on are CAL, MAGI-1, MAGI-2 and MAGI-3. The MAGI proteins have been shown to regulate synaptic function and have effects on learning and memory. CAL on the other hand has not been shown to be associated with mental disorders, but it is expressed in multiple regions of the brain including the cortex, hypothalamus and hippocampus which suggests a role that still needs to be explored.

Our **HYPOTHESIS** is that MAGI and CAL PDZ domain-containing proteins can differentially regulate the activity of GPCRs. The objectives are to determine the role of our PDZ proteins of interest in regulating the trafficking and signaling of CRFR1 and 5-$HT_{2A}R$. The three main aims for this project are:

1. **To determine the role of CAL in regulating the trafficking and signaling of CRFR1 and the mechanism for this regulation**

2. **To determine the role of MAGI proteins in regulating the trafficking and signaling of CRFR1 and the mechanism for this regulation**

3. **To determine the role of MAGI proteins in regulating the trafficking and signaling of 5-$HT_{2A}R$ and the mechanism for this regulation**

We use Human Embryonic Kidney 293 (HEK 293) cells to test the effects of overexpressing or knocking down the PDZ proteins of interest. We use co-immunoprecipitation and immunofluorescent microscopy to assess the interactions between the receptors and PDZ proteins. We employ flow cytometry assays to assess the trafficking and internalization of receptors. We examine the effects of PDZ proteins on GPCR signaling by BRET-based assays for cAMP formation, scintillation proximity assays for IP formation and western blotting for ERK1/2 activation.

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Chapter 2

2 Role of CAL in regulating CRFR1 activity

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2.1   Introduction

Corticotropin-releasing factor (CRF) is a 41 amino acid polypeptide that is secreted from the paraventricular nucleus of the hypothalamus in response to stress subsequently leading to the activation of the hypothalamic-pituitary-adrenal axis (HPA axis) (Vale et al., 1981; Arzt and Holsboer, 2006). CRF activates two Class B G protein-coupled receptor (GPCR) subtypes, CRFR1 and CRFR2, which are widely expressed in the brain (neocortex and cerebellum) and pituitary (Chalmers et al., 1995; Palchaudhuri et al., 1998). CRF can regulate the endocrine response to stress by triggering the anterior pituitary gland to release adrenocorticotropin hormone (ACTH) into circulation. ACTH then stimulates the secretion of glucocorticoids from the adrenal cortex. Glucocorticoids regulate various functions both in the central nervous system (CNS) and at the periphery (Lanfumey et al., 2008). In addition, studies have shown a link between pathophysiological changes in the CRF system and various neuropsychiatric disorders such as major depression, panic disorder, anorexia nervosa, and Alzheimer's disease (Behan et al., 1996; Lanfumey et al., 2008). CRFRs are primarily coupled to $G\alpha_s$ for the activation of adenylyl cyclase leading to the formation of cyclic adenosine monophosphate (cAMP) (Chalmers et al., 1996) as well as the mitogen activated protein kinase (MAPK) signaling pathway (Kageyama et al., 2007).

GPCRs can interact with a wide range of different proteins that control the trafficking, cell surface expression and signal transduction. Examples of these proteins include: β-arrestins, GPCR-associated sorting proteins (GASPs), small GTPases and PSD95/Disc Large/Zona Occludens (PDZ) proteins (Magalhaes et al., 2012). Many GPCRs, including CRFR1, encode a short class I PDZ domain recognition motif (S/T-x-ϕ, where ϕ is any aliphatic amino acid residue) at the end of the carboxyl-terminal tail. PDZ proteins are comprised of one or more 90 amino acid residue protein interaction domains that are thought to scaffold signaling complexes (Lee and Zheng, 2010; Romero et al., 2011). PDZ proteins function to regulate the subcellular localization, trafficking and signal transduction of multiple GPCRs and different PDZ proteins have both overlapping and distinct roles in the regulation of GPCR activity (Harris and Lim, 2001; Lee and Zheng, 2010; Romero et al., 2011; Magalhaes et al., 2012).

It is now recognized that PDZ proteins have distinct functions depending upon the particular GPCR with which they are associated. For example, MUPP1 interactions enhance $GABA_B$ receptor signaling (Balasubramanian et al., 2007), whereas MUPP1 functions to uncouple the melatonin-1 receptor from $G\alpha_i$ (Guillaume et al., 2008) and facilitates resensitization of the $5\text{-}HT_{2C}R$ (Parker et al., 2003). In the case of the β1-adrenergic receptor ($β_1$-AR), MAGI-2 has been shown to promote $β_1$-AR internalization, whereas MAGI-3 selectively attenuates $β_1$-AR-mediated ERK1/2 signaling (Xu et al., 2001; He et al., 2006). In contrast, PSD-95 inhibits the internalization of $β_1$-AR and facilitates the formation of $β_1$-AR heterodimer with N-methyl-D-aspartate receptors (Hu et al., 2000). SAP97 functions to antagonize the internalization of both CRFR1 and $5\text{-}HT_{2A}R$, whereas PDZK1 reduces the extent of $5\text{-}HT_{2A}R$ internalization, but has no significant effect on the endocytosis of CRFR1 (Dunn et al., 2013; Dunn et al., 2014; Walther et al., 2015). Although SAP97 regulates GPCR ERK1/2 signaling in a receptor-interaction independent manner, PDZK1 overexpression increases CRFR1- but not $5\text{-}HT_{2A}R$-mediated ERK1/2 activity.

The Golgi-localized cystic fibrosis transmembrane conductance regulator-associated ligand (CAL) is a single PDZ domain-containing protein with two coiled-coil motifs and two evolutionarily conserved regions which are required for its Golgi localization (Charest et al., 2001; Yao et al., 2001; Cheng et al., 2002). CAL is also known as GOPC, PIST and FIG. CAL overexpression reduces the cell surface expression of the $β_1$-AR and mGluR1a by retaining the receptor inside the cell via a direct interaction (He et al., 2004; Zhang et al., 2008). CAL interacts with mGluR5a and stabilizes receptor expression by a mechanism that is proposed to involve the inhibition of ubiquitination-dependent degradation (Cheng et al., 2010). CAL also contributes to the regulation of somatostatin receptor recycling and degradation (Wente et al., 2005; Bauch et al., 2014).

In the present study, we have identified CAL as a protein that interacts with CRFR1. We find that CAL overexpression leads to the retention of CRFR1 in the Golgi apparatus and alters the post-translational N-glycosylation status of the receptor, suggesting that CAL negatively regulates the anterograde transport of CRFR1 from the ER-Golgi to the plasma membrane surface. We also find that CAL knockdown enhances ERK1/2 phosphorylation

in response to CRFR1 activation, but that CAL expression does not influence cAMP signaling. Taken together, our results indicate that CAL interactions with CRFR1 play an important role in regulating the maturation and cell surface expression of CRFR1.

2.2   Materials and Methods

2.2.1   Materials

Protein G beads were purchased from GE Healthcare (Oakville, ON, Canada). CRF was purchased from R&D Systems (Minneapolis, MN, USA). HA peroxidase high affinity antibody was purchased from Roche (Mississauga, ON, Canada). Rabbit anti-GFP antibody was obtained from Invitrogen/Life Technologies (Burlington, ON, Canada). ECL Western blotting detection reagents were purchased from Bio-Rad (Mississauga, ON, Canada). Alexa Fluor 647 anti-mouse IgG antibody was purchased from Invitrogen/Molecular Probes (Burlington, ON, Canada). Rabbit CAL antibody was purchased from Abcam (Toronto, ON, Canada). Rabbit anti-phospho-p44/42 MAP kinase (Thr202/Tyr402), and rabbit anti-p44/42 MAP kinase antibodies were obtained from Cell Signaling Technology (Pickering, ON, Canada). Mouse anti-HA antibody, endoglycosidases F and H and all other biochemical reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.2.2   Plasmids

HA-CRFR1 and HA-CRFR1-ΔTAV were described previously (Holmes et al., 2006; Magalhaes et al., 2010). Human CAL was kindly provided by Dr. Randy Hall (Emory University, School of Medicine). The CAL cDNA was subcloned into the expression vector pEYFP-N1. CAL siRNA (Hs_GOPC_3) GCUGCAGCUUCAUGCUAAATT was purchased from Qiagen (Toronto, ON, Canada). For the negative controls, we used Silencer Negative Control #1 AM4635 AGUACUGCUUACGAUACGGTT from Invitrogen (Burlington, ON, Canada). The exchange proteins directly activated by cAMP biosensor (EPAC) was a gift from Drs. Ali Salahpour (University of Toronto) and Marc Caron (Duke University) (Barak et al., 2008). TGN-CFP was kindly provided by Dr. Stephen Pasternak (University of Western Ontario). Site-directed mutagenesis was used to introduce N to Q in the following constructs: CRFR1 N38Q, CRFR1 N78Q, CRFR1 N90Q and CRFR1 N98Q and mutations were confirmed by sequencing.

2.2.3   Cell culture and transfection

Human embryonic kidney (HEK 293) cells were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Cells were plated on 10-cm dishes 24h prior to transfection. All experiments were performed on 75–80% confluent plates. Transfections were performed using calcium phosphate protocol except for siRNA transfections which were performed using Lipofictamine 2000 following manufacturer's instructions. Transfections were performed with 1 µg of each construct. Empty pcDNA3.1 vector was used to equalize the total amount of plasmid cDNA used to transfect cells. 18h post transfection, cells were washed with phosphate-buffered saline (PBS) and resuspended with trypsin, 0.25% EDTA. Experiments were performed approximately 48h after transfection except for knockdown experiments which were performed 72h post transfection since this protocol resulted in the maximum knockdown of CAL.

2.2.4   Co-immunoprecipitation

24h after transfection, HEK 293 cells were seeded onto 10-cm dishes. Cells were starved with HBSS for 1h at 37 °C then stimulated with 100 nM CRF agonist for 30 min. Cells were then lysed in 500 µL lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10 µg/ml leupeptin, and 2.5 µg/ml aprotinin) for 20 min on a rocking platform at 4 °C. Samples were collected into 1.5 ml Eppendorf tubes and centrifuged at 15,000 g for 15 min at 4 °C to pellet insoluble material. A Bradford protein assay was performed, and 150 µg of protein was incubated for 2–4h at 4 °C with protein G-Sepharose and mouse anti-HA antibody (1:50). Beads were washed three times with cold lysis buffer and incubated overnight at room temperature in 3xSDS loading buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and western blots were then performed with the indicated antibodies (rabbit anti-GFP, 1:10000), (HA-POD, 1:1000).

2.2.5   Confocal microscopy

HEK 293 cells were co-transfected with HA-CRFR1, CAL-YFP and TGN-CFP in 10-cm dishes and 24h after transfection, cells were reseeded onto glass coverslips. 24h later, cells were washed with HBSS, fixed with 4% paraformaldehyde in PBS, and permeabilized with

0.5% Triton X-100 in PBS for 5 min at room temperature. After rinsing with PBS, cells were blocked with 5% donkey serum in PBS for 1h at room temperature then labeled with mouse HA antibody (1:1000) for 1h at room temperature. The reaction proceeded by incubation with a secondary antibody, donkey anti-mouse Alexa Fluor 647 (1:1000) for 1h at room temperature. Coverslips were mounted on slides using Pro- Long Gold Antifade Reagent. 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.

2.2.6   Receptor endocytosis

Following transfection, HEK 293 cells were re-seeded into 12- well plates. Cells were serum-starved in HBSS for 1h at 37 °C then stimulated for 0, 30 or 60 min with 500 nM CRF in HBSS at 37 °C. Cells were washed with cold HBSS and incubated with mouse anti- HA antibody (1:1000) for 1h on ice. Cells were washed with cold HBSS then labeled with Alexa Fluor 647 donkey anti-mouse IgG (1:1000) for 1h on ice in the dark. Cells were washed with cold HBSS and treated with 5 mM EDTA in PBS for 5 min on ice. Cells were collected and transferred to flow cytometry tubes containing 4% formaldehyde in PBS. Samples were run on a FACS Calibur cytometer using BD Cell Quest Pro software (BD Biosciences, Mississauga, ON, Canada) until 10,000 cells were counted. The geometric mean of fluorescence was determined using Flow Jo analysis software and was representative of the expression levels of the receptor on the plasma membrane (BD Biosciences, Mississauga, ON, Canada).

2.2.7   Bioluminescent Resonance Energy Transfer-based biosensor cAMP assay

HEK 293 cells were co-transfected with HA-CRFR1 (WT or ΔTAV) and either pcDNA3.1, CAL, scrambled (SCR) siRNA or CAL siRNA as well as 2 μg of an EPAC (exchange proteins directly activated by cAMP) construct in 10-cm dishes. 48h post-transfection, cells were re-seeded into 96-well plate (~10,000 cells/well) and left for another 24h. On the following day, cells were serum-starved for 1h in induction buffer (200 μM isobutyl-1-methylxanthine (IBMX) in HBSS). Coelenterazine was then added to the wells at a final

concentration of 5 μM. Cells were then stimulated with increasing concentrations of CRF peptide for 10 min. The plate was then read by a Victor Plate Reader (Perkin-Elmer) and the BRET signal was determined by calculating the ratio of the light emitted at 505 to 555 nm to the light emitted at 465 to 505 nm.

2.2.8   ERK phosphorylation

Following 48h of transfection, HEK 293 cells were re-seeded into 6- well plates. Cells were serum starved for 1h at 37 °C in HBSS and then stimulated with 500 nM CRF agonist for 0, 5 and 15 min. Cells were lysed with lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10 μg/ml leupeptin, and 2.5 μg/ml aprotinin) and phosphatase inhibitors (10 mM NaF and 500 μM Na_3VO_4) for 20 min on a rocking platform at 4 °C. Samples were collected into 1.5 ml Eppendorf tubes and centrifuged at 15,000 g for 15 min at 4 °C to pellet insoluble material. A Bradford protein assay was performed, and 30–50 μg of protein was incubated overnight at room temperature in 3xSDS loading buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and western blot analysis was performed for ERK1/2 (rabbit anti-p44/42MAPK, 1:1000), phospho-ERK1/2 (rabbit anti-phospho-p44/42 MAPK, 1:1000), CAL-YFP (rabbit CAL, 1:1000), HA-CRFR1 (HA-POD, 1:1000), and actin.

2.2.9   Western blot analysis

75–90 μl of the samples from the different assays which is equivalent to about 30–50 μg of protein was diluted in β-mercaptoethanol containing 3x loading buffer and then applied to 10% SDS-PAGE (30% acrylamide mix, 1.5 M tris-HCl, 20% SDS, 10% ammonium persulfate and TEMED). Separated proteins were then transferred to nitrocellulose membranes and membranes were then blocked in 10% milk in TBS for 1h. Membranes were then blotted overnight by incubation with the appropriate antibody at 4 °C. 24h later membranes were washed at least three times with 1X TBS with 0.05% Tween 20, and then incubated with a horseradish peroxidase- conjugated secondary antibody (1:10,000) for 1h. Membranes were finally washed again with 1X TBS with 0.05% Tween 20 three times before being developed using a BioRad chemiluminescence system.

2.2.10   Statistical analysis

All measurements are represented as mean ± SEM. Comparisons were performed using one way analysis of variance test (ANOVA) followed by Bonferroni's or Dunn's Multiple comparisons test to determine significance. The symbol * indicates P values less than 0.05 and is considered to be significant.

2.3   Results

2.3.1   CAL interacts with CRFR1 in a PDZ-binding motif-dependent manner

CAL is a PDZ domain-containing protein that was previously shown to interact with the β_1-AR and Group I mGluRs (He et al., 2004; Zhang et al., 2008; Cheng et al., 2010). We previously identified CAL as one of several PDZ domain-containing proteins that interact with a GST fusion of the CRFR1 carboxyl-terminal tail (Dunn et al., 2013). To further validate this potential interaction between CAL and CRFR1, we performed co-immunoprecipitation experiments with CAL-YFP and either wild-type HA-tagged CRFR1 (HA-CRFR1) or a HA-tagged CRFR1 mutant that lacked a class I PDZ-binding motif (HA-CRFR1-ΔTAV) in transiently transfected HEK 293 cells. We found that CAL-YFP was co-immunoprecipitated with HA-CRFR1, but not CRFR1-ΔTAV and that the interaction of CAL-YFP with HA-CRFR1 was not increased following agonist treatment with 100 nM CRF (Fig. 2.1A and 2.1B). We also observed that CAL overexpression resulted in a shift in the molecular weight of CRFR1 and this will be discussed in section 2.3.6.

2.3.2   HA-CRFR1 co-localizes with CAL-YFP in the Golgi apparatus

To further address whether CAL forms a complex with CRFR1, we assessed whether the two proteins could be co-localized in HEK 293 cells in a PDZ motif-dependent manner. In cells transfected to express HA-CRFR1 and YFP as a control, the HA-CRFR1 labeled with Alexa Fluor 647 primary mouse anti-HA antibody in permeabilized cells showed predominant localization at the cell surface with some co-localization with Golgi marker TGN-CFP (Fig. 2.2A). In HEK 293 cells transfected with HA-CRFR1 and CAL-YFP, CAL-YFP fluorescence was extensively co-localized with TGN-CFP consistent with previous reports where CAL was localized to the Golgi apparatus and could be used as a marker for this organelle (Fig. 2.2B) (Charest et al., 2001; Yao et al., 2001). In permeabilized HEK 293 cells, HA-CRFR1 immunofluorescence was co-localized with CAL-YFP with extensive co-localization in the Golgi (Fig. 2.2B). Similar to what was observed for HA-CRFR1, HA-CRFR1-ΔTAV immunofluorescence was localized at the cell surface of HEK 293 cells in YFP transfected cells (Fig. 2.2C), but unlike what was
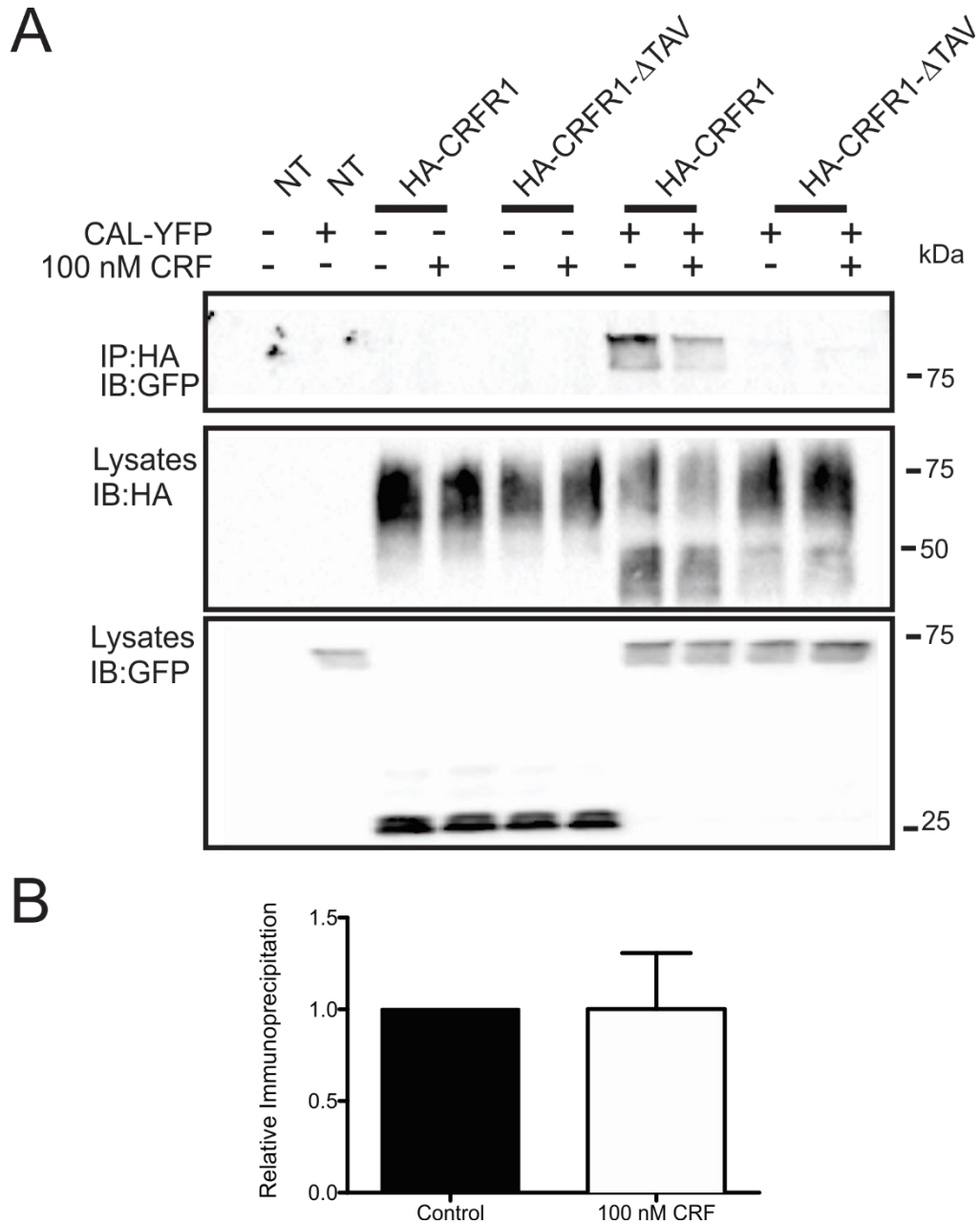
Figure 2.1: CAL-YFP co-immunoprecipitates with HA-CRFR1 in a PDZ-binding motif-dependent manner: HEK 293 cells were co-transfected with HA-CRFR1 (WT or ΔTAV) and YFP or CAL-YFP. A) Representative immunoblot of CAL-YFP co-immunoprecipitated (IP) with HA-CRFR1 but not HA-CRFR1-ΔTAV which lacks the PDZ-binding motif. SDS-PAGE was used to analyze samples and immunoblots were performed with rabbit anti-GFP. B) Effect of CRF treatment was quantified using densitometry and had no significant difference on the amount of CAL-YFP co-immunoprecipitated with HA-CRFR1. Data are representative of 5 independent experiments. NT: non-transfected, IB: immunoblot.
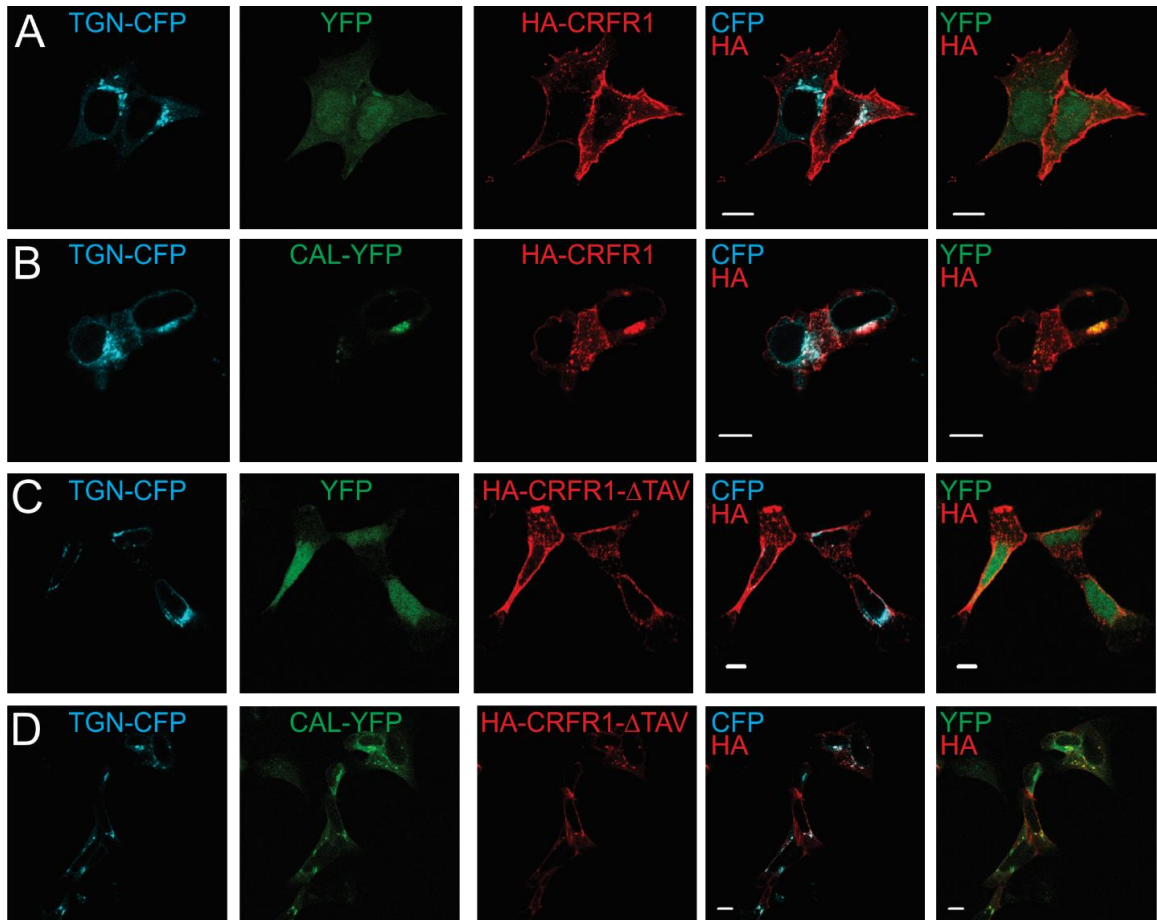
Figure 2.2: CAL-YFP co-localizes with HA-CRFR1 in the Golgi in a PDZ-binding motif-dependent manner: A) Representative confocal microscopy image of fixed HEK 293 cells transfected with TGN-CFP (blue), YFP (green), and HA-CRFR1 (red). B) Representative confocal microscopy image of fixed HEK 293 cells transfected with TGN-CFP (blue), CAL-YFP (green), and HA-CRFR1 (red). C) Representative confocal microscopy image of fixed HEK 293 cells transfected with TGN-CFP (blue), YFP (green), and HA-CRFR1-ΔTAV (red). D) Representative confocal microscopy image of fixed HEK 293 cells transfected with TGN-CFP (blue), CAL-YFP (green), and HA-CRFR1-ΔTAV (red). Scale bar=10 μM. Images are representative of 20 cells from five independent experiments.

observed for HA-CRFR1 it was not extensively localized to the Golgi to co-localize with CAL-YFP (Fig. 2.2D). In contrast, HA-CRFR1-ΔTAV immunofluorescence remained predominantly localized to the cell surface. Taken together these observations support the concept that CAL forms a complex with CRFR1 resulting in the accumulation of the receptor in the Golgi.

2.3.3   Effect of CAL expression on agonist-stimulated CRFR1 endocytosis

To determine whether CAL contributed to the regulation of CRFR1 endocytosis, we employed flow cytometry to assess the loss of cell surface HA-CRFR1 immunofluorescence. We found that CAL overexpression attenuated agonist-stimulated HA-CRFR1 internalization following treatment with 500 nM CRF for 30 and 60 min (Fig. 2.3A). The internalization of the mutant HA-CRFR1-ΔTAV was not altered by CAL overexpression (Fig. 2.3B). Thus, CAL overexpression attenuated CRFR1 endocytosis in a PDZ motif-dependent manner.

2.3.4   Effect of CAL expression on cAMP signaling

PDZ domain-containing proteins were previously shown to have the potential to regulate cAMP signaling of some GPCRs (Wang et al., 2007; Magalhaes et al., 2012). Therefore, we tested whether CAL contributed to the regulation of CRFR1- mediated cAMP formation. In HEK 293 cells transfected with either HA-CRFR1 or HA-CRFR1-ΔTAV, with or without CAL, and treated with increasing concentrations of CRF for 10 min, there was no significant change in the maximum efficacy for CRF-stimulated cAMP signaling (Fig. 2.4A and 2.4B). In addition we assessed whether the attenuation of CAL expression might alter CRFR1 cAMP signaling. We found that transfection of HEK 293 cells with CAL siRNA previously demonstrated to knockdown CAL expression (Cheng et al., 2010) had no effect on either HA-CRFR1- or HA-CRFR1-ΔTAV- stimulated cAMP signaling in response to the treatment of transfected HEK 293 cells with increasing concentrations of CRF (Fig. 2.4C and 2.4D). These results suggest that CAL does not contribute to the regulation of CRFR1 activation of the cAMP pathway.
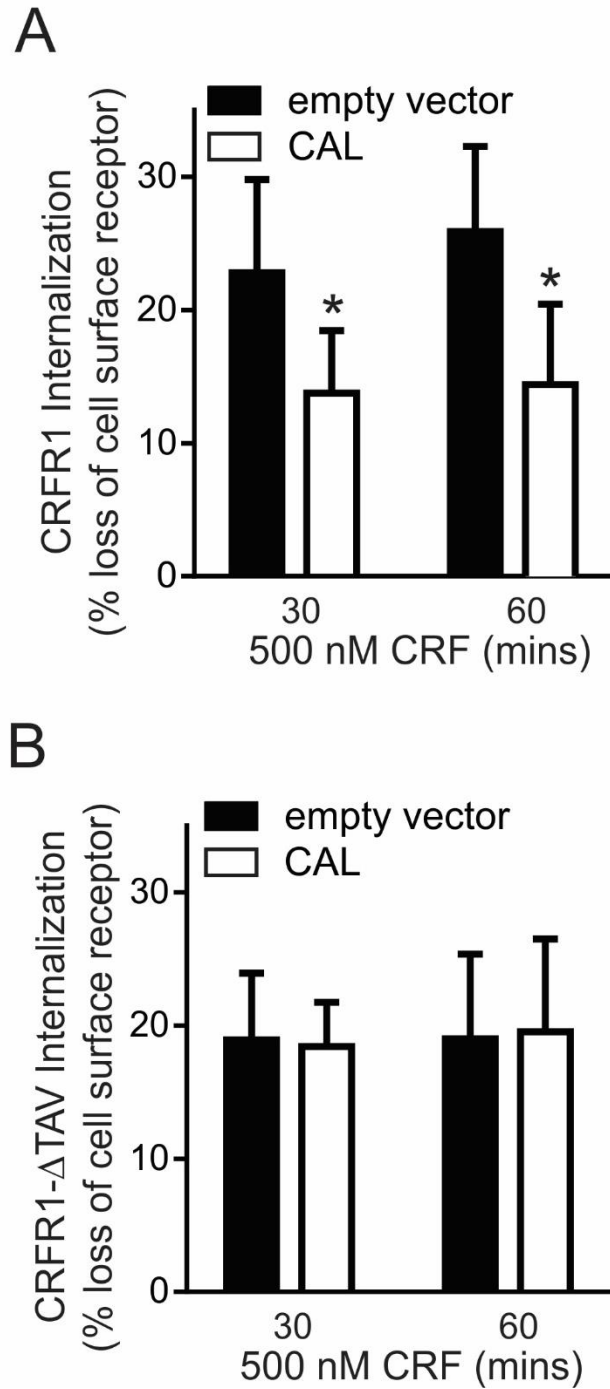
Figure 2.3: Effect of CAL expression on agonist-stimulated CRFR1 endocytosis: Agonist stimulated internalization of either A) HA-CRFR1 or B) HA-CRFR1-ΔTAV in the presence and absence of CAL overexpression following 500 nM CRF treatment for either 30 or 60 min. The data represent the mean ± SEM of 4 independent experiments. *P < 0.05 versus empty vector control.
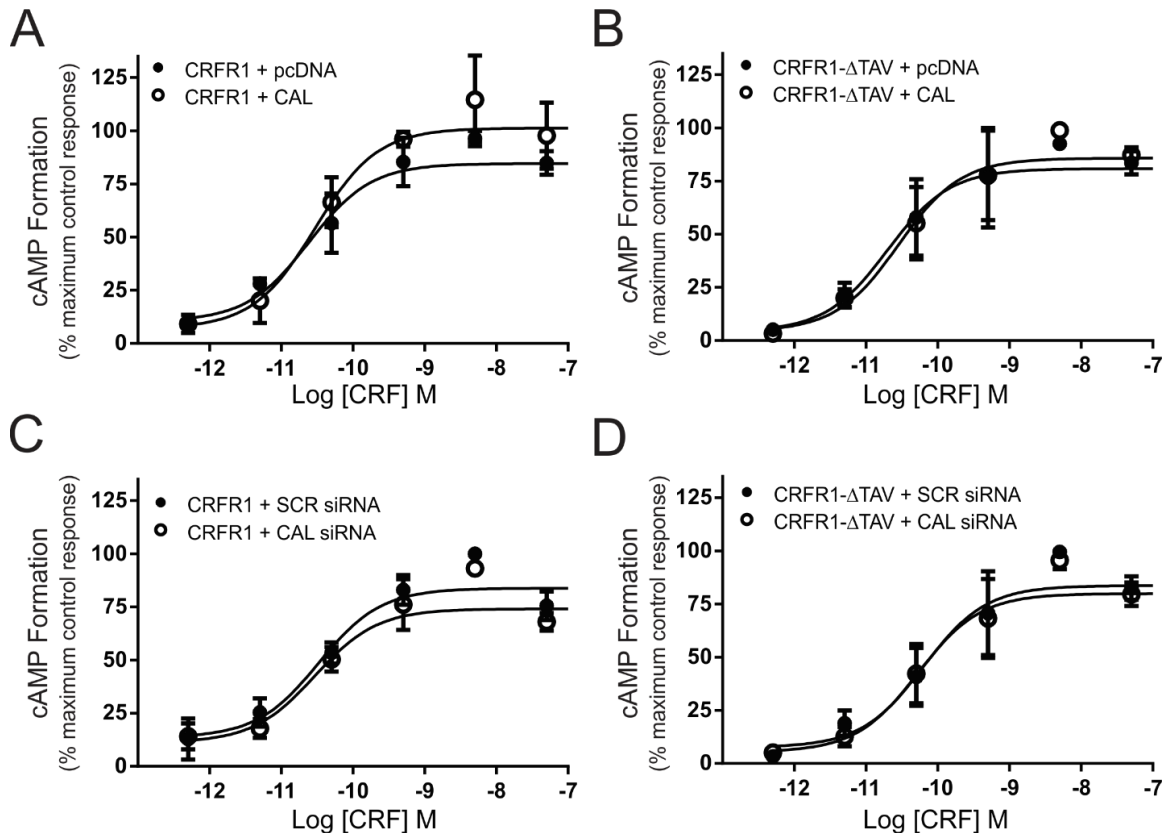
Figure 2.4: CAL does not regulate CRFR1-mediated cAMP formation: Dose response for CRF stimulated cAMP formation assessed using a BRET-based cAMP biosensor assay. A) CRFR1- and B) CRFR1-ΔTAV-mediated cAMP formation in either plasmid vector or CAL transfected HEK 293 cells. C) CRFR1- and D) CRFR1-ΔTAV-mediated cAMP formation in either scrambled or CAL siRNA transfected HEK 293 cells. The data represent the mean ± SEM of 3–5 independent experiments.

2.3.5   Effect of CAL expression on ERK1/2 signaling

In addition to being coupled to the $G\alpha_s$-dependent activation of adenylyl cyclase, agonist activation of CRFR1 leads to increased ERK1/2 phosphorylation (Kageyama et al., 2007; Dunn et al., 2013; Dunn et al., 2014). Therefore, we assessed whether either CAL-YFP overexpression or siRNA-mediated knockdown of endogenous CAL would alter CRFR1-mediated ERK1/2 phosphorylation in a PDZ interaction- dependent manner in response to 500 nM CRF treatment for 5 and 15 min. CAL overexpression resulted in a statistically significant reduction in ERK1/2 phosphorylation and siRNA knockdown of endogenously expressed CAL increased HA-CRFR1- stimulated ERK1/2 phosphorylation following the activation of HA-CRFR1 for 5 and 15 min with 500 nM CRF (Fig. 2.5A). In contrast, neither CAL overexpression nor CAL knockdown affected HA-CRFR1-ΔTAV-stimulated ERK1/2 phosphorylation (Fig. 2.5B). Taken together, these data suggested that CAL negatively regulates CRFR1-mediated ERK1/2 signaling via a PDZ binding motif interaction-dependent signaling mechanism.

2.3.6   Effect of CAL on CRFR1 glycosylation and degradation

CAL overexpression consistently altered the molecular migration of HA-CRFR1, but not HA-CRFR1-ΔTAV by SDS-PAGE (Fig. 2.1 and Fig. 2.5). To further characterize this CAL expression-dependent change in the molecular mass of the HA-CRFR1, we co-transfected HEK 293 cells with either HA-CRFR1 or HA-CRFR1-ΔTAV along with increasing cDNA concentrations of CAL and then performed western blotting on HEK 293 cell lysates for receptor expression (Fig. 2.6A). We found that, even at the lowest levels of CAL overexpression, the molecular mass of a substantial proportion of HA-CRFR1 decreased as evidenced by an increased mobility on the SDS-PAGE gel, suggesting that this represented an immature, non-glycosylated form of the receptor (Fig. 2.6A). In contrast, CAL overexpression did not alter the mobility of the HA-CRFR1-ΔTAV on an SDS PAGE gel (Fig. 2.6A). Densitometric analysis of the gels revealed that there was a significant reduction in the higher molecular mass HA-CRFR1 receptor band following CAL overexpression, whereas, CAL overexpression did not alter the proportion of the higher molecular mass HA-CRFR-ΔTAV band (Fig. 2.6B). To ascertain that the lower
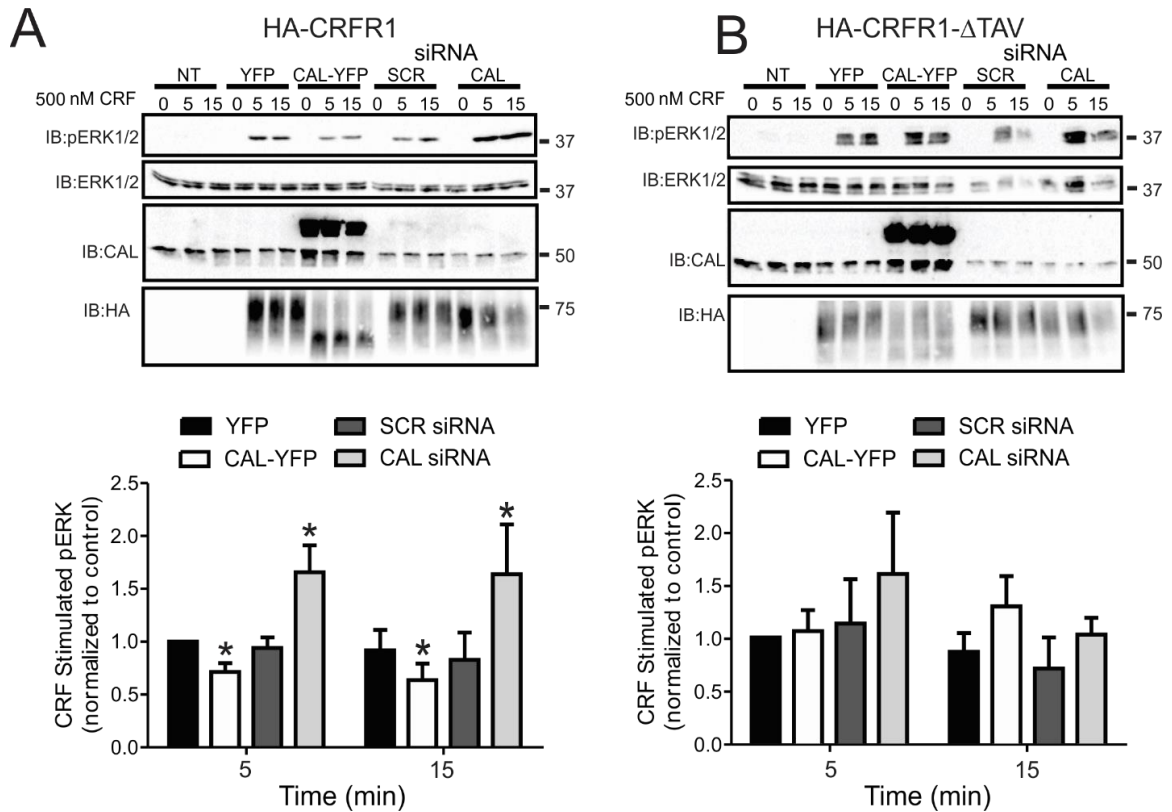
Figure 2.5: CAL negatively regulates HA-CRFR1-mediated ERK1/2 phosphorylation: A) Representative immunoblot showing ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 5, and 15 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with HA-CRFR1 along with either YFP, CAL-YFP, scrambled (SCR) siRNA or CAL siRNA. Also shown are corresponding immunoblots for total ERK1/2, CAL and HA-CRFR1 protein expression. Shown below is the densitometric analysis of ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 5, and 15 min in non-transfected (NT) HEK 293 cell, and HEK 293 cells transfected with HA-CRFR1 along with either YFP, CAL-YFP, scrambled (SCR) siRNA or CAL siRNA. The data represent the mean ± SEM of six independent experiments. * P < 0.05 versus SCR siRNA treated cells. B) Representative immunoblot showing ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 5, and 15 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with HA-CRFR1-ΔTAV along with either YFP, CAL-YFP, scrambled (SCR) siRNA or CAL siRNA. Also shown are corresponding immunoblots for total ERK1/2, CAL and HA-CRFR1-ΔTAV protein expression. Shown below is the densitometric analysis of ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 5, and 15 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with HA-CRFR1-ΔTAV along with either YFP, CAL-YFP, scrambled (SCR) siRNA or CAL siRNA. The data represent the mean ± SEM of 6 independent experiments. * P < 0.05 versus SCR siRNA treated cells.

molecular mass species of the HA-CRFR1 construct was not the consequence of increased receptor degradation following CAL overexpression, we stimulated HA-CRFR1 expressing cells with 100 nM CRF for 0, 2, 4 or 6 h in the presence of scrambled siRNA, CAL or CAL siRNA. However, we did not observe any time-dependent change in the fraction of the lower molecular mass HA-CRFR1 species in CAL overexpressing cells (Fig. 2.6C). Consistent with the hypothesis that CAL interactions with HA-CRFR1, but not CRFR1-ΔTAV, affected the maturation of the glycosylation state of the receptor, both endoglycosidase F (1h) and H (3h) treatment reduced the molecular mass of the lower molecular weight HA-CRFR1 species (Fig. 2.6D). Taken together, these results indicated that PDZ motif-dependent interactions with the CRFR1 altered CRFR1 glycosylation. The amino-terminal domain of CRFR1 has five potential N-linked glycosylation sites at positions N38, N45, N78, N90 and N98 (Assil and Abou-Samra, 2001). Therefore, we generated point mutations where potentially glycosylated asparagine residues were replaced with glutamine, HA-CRFR1-N38Q, HA-CRFR1-N78Q, HA-CRFR1-N90Q and HA-CRFR1-N98Q. When expressed with YFP in HEK 293 cells HA-CRFR1-N38Q and HA-CRFR1-N78Q exhibited a wild-type pattern of protein expression (Fig. 2.7). However, when transfected with YFP, HA-CRFR1-N90Q and HA-CRFR1-N98Q had a mobility pattern on SDS-PAGE that was similar to what was observed following CAL-YFP overexpression with HA-CRFR1 (Fig. 2.7). However, overexpression of CAL-YFP resulted in the appearance of a lower molecular mass species for the wild-type, as well as the N38Q and N78Q CRFR1 mutants (Fig. 2.7). CAL-YFP overexpression resulted in nearly the complete loss of upper molecular mass protein band for the HA-CRFR1-N90Q and HACRFR1-N98Q mutants (Fig. 2.7). Taken together, CRFR1 N90 and N98 appear to be the primary residues that undergo receptor glycosylation.
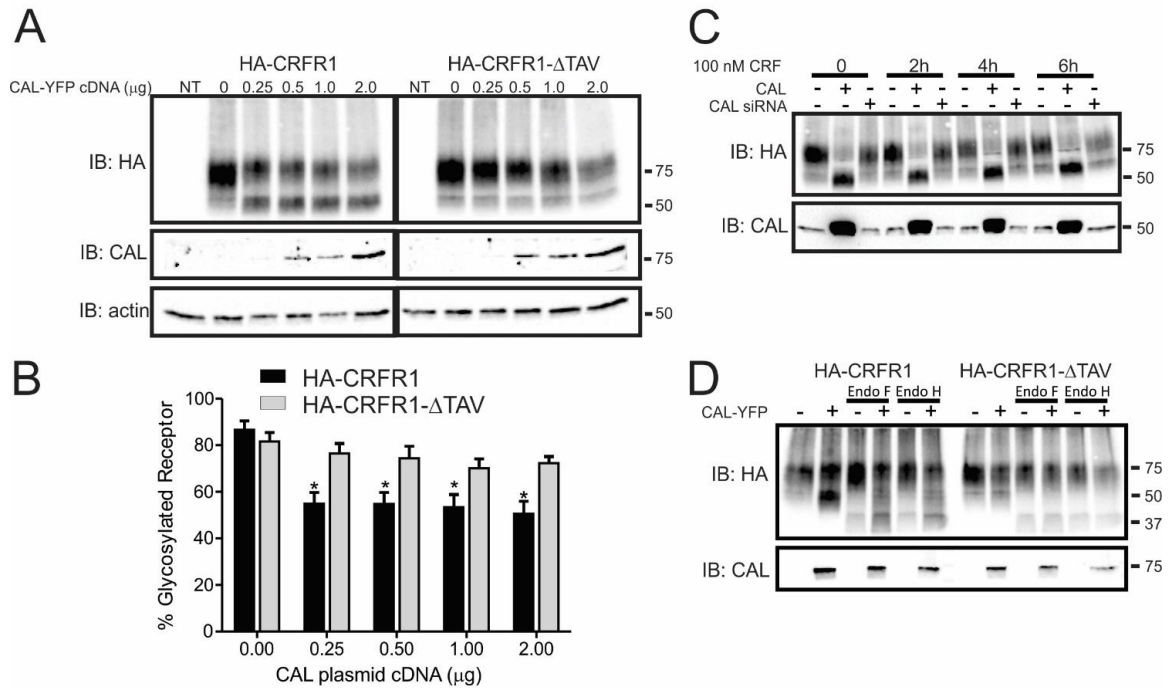
Figure 2.6: Effect of CAL on CRFR1 glycosylation and degradation: A) Representative immunoblot of 1 µg HA-CRFR1 and HA-CRFR1-ΔTAV cell lysates from transfected HEK 293 cells immunoblotted with HA antibody. Cells were co-transfected with increasing amounts of plasmid cDNA encoding CAL (0, 0.25, 0.5, 1, and 2 µg plasmid cDNA). Shown below are representative immunoblots for CAL and actin expression. B) Densitometric analysis of the relative amount of the upper molecular mass CRFR1 immunoreactive band compared to total receptor immunoreactivity. The data represent the mean ± SEM of 5 independent experiments. * $P < 0.05$ versus cells expressing receptor alone. C) Representative immunoblot (n= 3) of 1 µg HA-CRFR1 cell lysates from transfected HEK 293 cells immunoblotted with HA-antibody following CRF treatment 0, 2, 4, 6 h, in cells transfected with scrambled siRNA, CAL, or CAL siRNA. Shown below is a representative immunoblot for CAL expression. D) Representative immunoblot (n= 5) of 1 µg HA-CRFR1 and HA-CRFR1-ΔTAV cell lysates from transfected HEK 293 cells immunoblotted with HA-antibody. Cells were co-transfected with and without plasmid cDNA encoding CAL-YFP and treated with and without endoglycosidase (Endo) F and H for 60 and 180 min, respectively. Shown below is representative immunoblot for CAL expression.
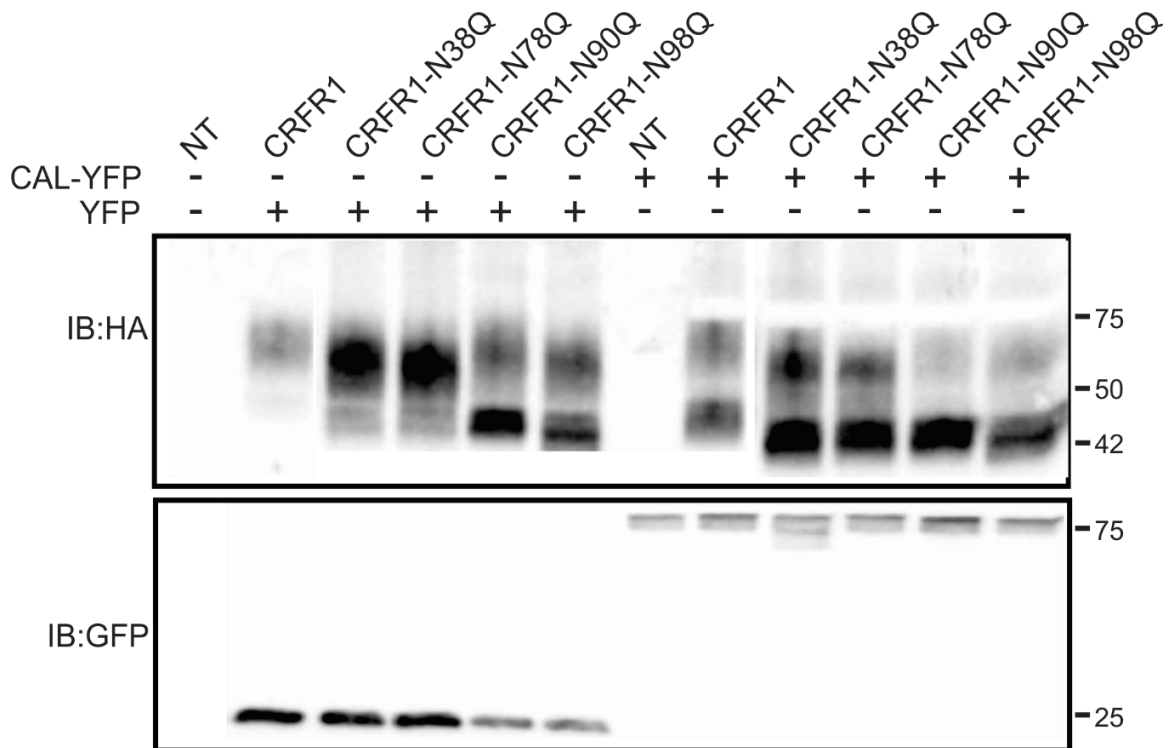
Figure 2.7: Glycosylation of HA-CRFR1-N38Q, -N78Q, -N90Q and -N98Q mutants in the absence and presence of CAL: Shown is a representative immunoblot of 1 µg HA-CRFR1, HA-CRFR1-N38Q, HA-CRFR1-N78Q, HA-CRFR1-N90Q and HA-CRFR1-N98Q cell lysates from HEK 293 cells co-transfected with either YFP or CAL-YFP. Blots are representative of four independent experiments.

2.3.7 Subcellular localization and signaling of CRFR1 glycosylation mutants

To examine whether the glycosylation status of the CRFR1 was important for cell surface expression, we assessed the subcellular localization of both the HA-CRFR1-N78Q mutant that exhibited a wild-type glycosylation pattern, and the HA-CRFR1-N90Q mutant that exhibited altered glycosylation in the presence and absence of CAL-YFP by immunofluorescence microscopy. We found that the HA-CRFR1-N78Q mutant was expressed at the cell surface of HEK 293 cells and was not co-localized with TGN-CFP Golgi marker in YFP expressing cells (Fig. 2.8A), but was retained in the Golgi following CAL-YFP overexpression (Fig. 2.8B). In contrast, the HA-CRFR1-N90Q mutant exhibited extensive co-localization with TGN-CFP Golgi marker in both the absence and presence of CAL-YFP (Fig. 2.8C and 2.8D). Consistent with this observation, the cell surface expression of both the HA-CRFR1-N90Q and HA-CRFR1-N98Q mutants was reduced to the same extent as what was observed for the HA-CRFR1 in the presence of CAL-YFP while the cell surface expression of the HA-CRFR1-N38Q and HA-CRFR1-N78Q was similar to the HA-CRFR1 (presented as the dotted line) (Fig. 2.9A). The reduction in cell surface expression was not sufficient to impair G protein coupling but all glycosylation-deficient mutations decreased the efficacy of CRF treatment for CRFR1-stimulated cAMP formation (Fig. 2.9B). Thus, a fully glycosylated CRFR1 was required for appropriate cell surface expression of the CRFR1 and mutation of any glycosylation site reduces the efficacy for CRF-stimulated cAMP formation. This unique effect on cAMP formation was not observed with the over-expression of CAL which suggests that introducing mutations and their effect on the structure and folding of the receptor has a more prominent effect than CAL interaction with the receptor. In addition to cAMP formation, we have also assessed ERK1/2 signaling by the glycosylation-deficient mutants. Our data showed that both CRFR1-N90Q and CRFR1-N98Q have altered ERK1/2 signaling illustrated as a reduction in phosphorylation that is about 50% less compared to CRF-stimulated ERK1/2 phosphorylation by CRFR1 (Fig. 2.9C).
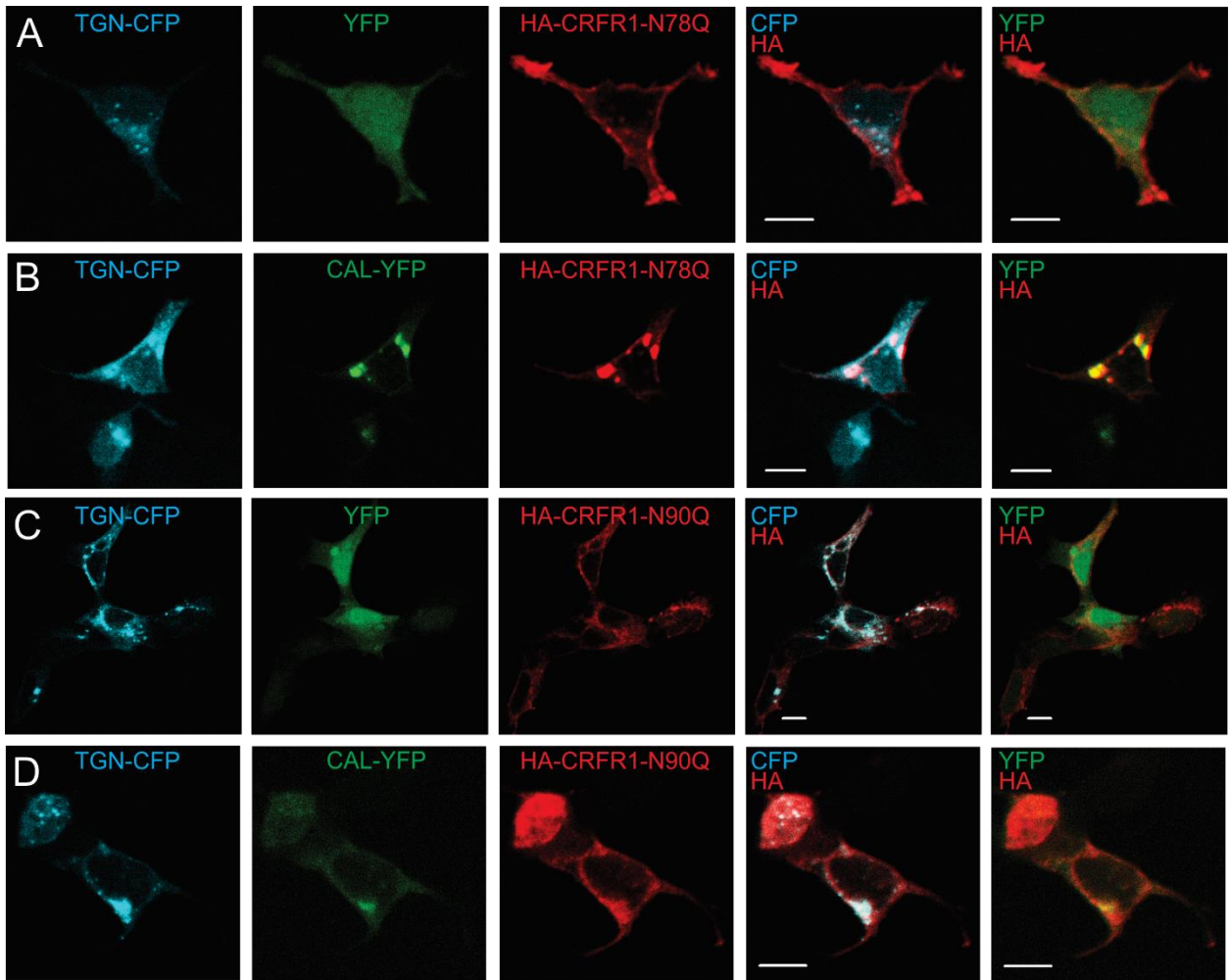
Figure 2.8: Subcellular localization of HA-CRFR1 glycosylation site mutants in the presence and absence of CAL: A) Representative confocal microscopy image of fixed HEK 293 cells transfected with TGN-CFP (blue), YFP (green), and HA-CRFR1-N78Q (red). B) Representative confocal microscopy image of fixed HEK 293 cells transfected with TGN-CFP (blue), CAL-YFP (green), and HA-CRFR1-N78Q (red). C) Representative confocal microscopy image of fixed HEK 293 cells transfected with TGN-CFP (blue), YFP (green), and HA-CRFR1-N90Q (red). D) Representative confocal microscopy image of fixed HEK 293 cells transfected with TGN-CFP (blue), CAL-YFP (green), and HA-CRFR1-N90Q (red). Scale bar= 10 μM. Images are representative of 12 cells from three independent experiments.
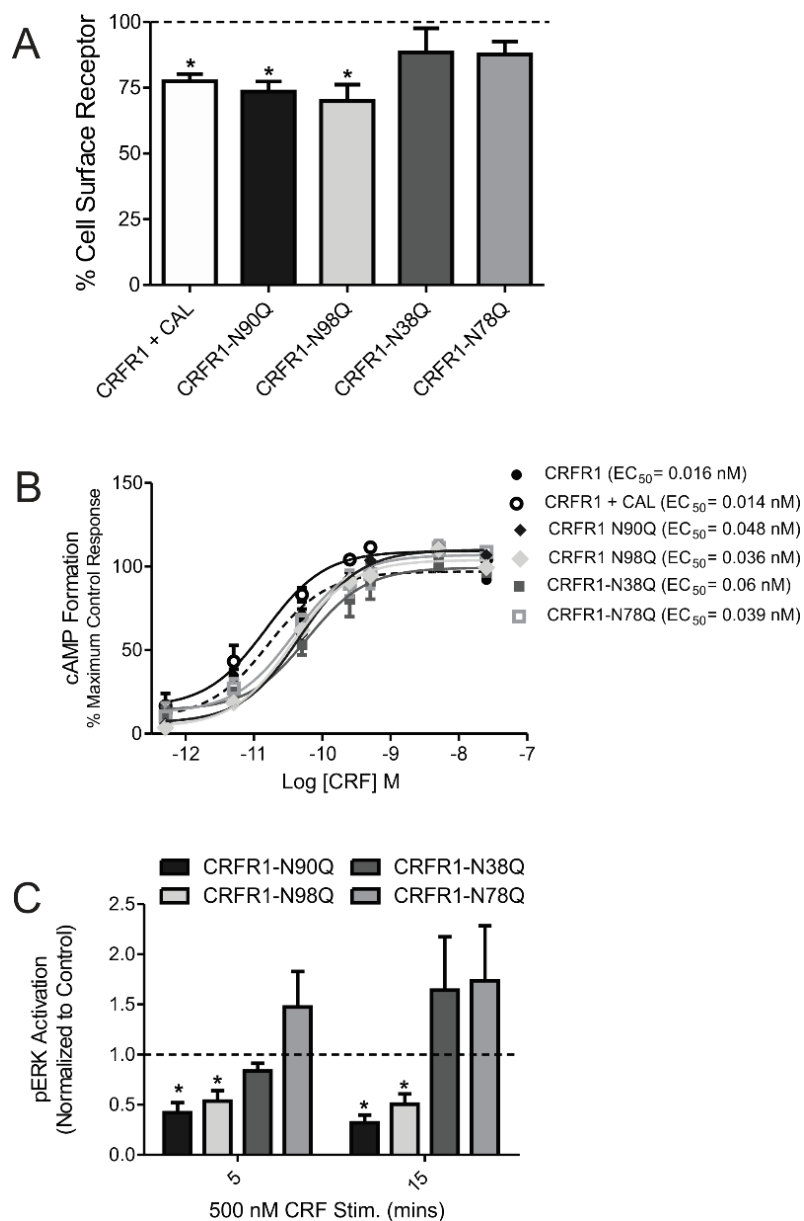
Figure 2.9: Cell surface expression, cAMP signaling and ERK1/2 signaling of CRFR1 glycosylation site mutants: A) Cell surface expression in HEK 293 cells expressing either HA-CRFR1 along with CAL, HA-CRFR1-N90Q, HA-CRFR1-N98Q, HA-CRFR1-N38Q or HA-CRFR1-N78Q expressed as measured by flow cytometry. Data is normalized to HA-CRFR1 expression (as illustrated by the dotted line). The data represent the mean ± SEM of 5 independent experiments. * $P < 0.05$ versus HA-CRFR1 cell surface expression. B) Dose-response for CRF-mediated cAMP activation in HEK 293 cells expressing either HA-CRFR1, HA-CRFR1 along with CAL, HA-CRFR1-N90Q, HA-CRFR1-N98Q, HA-CRFR1-N38Q or HA-CRFR1-N78Q. The data represent the mean ± SEM of 5 independent experiments. C) Densitometric analysis of ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 5, and 15 min in HEK 293 cells transfected with HA-CRFR1, HA-CRFR1-N90Q, HA-CRFR1-N98Q, HA-CRFR1-N38Q or HA-CRFR1-N78Q. The data represent the mean ± SEM of three independent experiments. * $P < 0.05$ versus cells transfected with HA-CRFR1.

On the other hand, ERK1/2 signaling of both CRFR1-N38Q and CRFR1-N78Q was similar to that of CRFR1. This data is consistent with the mobility patterns observed in Fig. 2.7 where both CRFR1-N90Q and CRFR1-N98Q had a similar pattern to CAL overexpression with the receptor and CAL overexpression also reduced ERK1/2 phosphorylation. This confirms the importance of CRFR1 glycosylation specifically of resides Asn90 and Asn98 for proper maturation and signaling. It also supports our hypothesis that CAL's interaction with CRFR1 and the effects it has on receptor trafficking and signaling is the result of its role in mediating glycosylation.

2.4 Discussion

We previously reported that CAL is one of a series of CRFR1- interacting PDZ proteins identified in a proteomic screen, but the functional role of this interaction has not been characterized (Dunn et al., 2013). In this study, we have identified the role of CAL in the regulation of the CRFR1 function as a protein that negatively regulates cell surface CRFR1 expression. We propose that CAL can regulate cell surface expression by modulating the post-translational modification involving the glycosylation of amino-terminal Asn residues which is required for proper maturation and folding of GPCRs (Wheatley and Hawtin, 1999; Duvernay et al., 2005). This suggests an important role for CAL in the anterograde trafficking of transmembrane proteins through the ER-Golgi complex. We also demonstrate a negative functional effect of CAL on CRFR1-stimulated ERK1/2 signaling in the absence of alterations in CRFR1-mediated cAMP formation. Mutagenesis analysis of putative sites of glycosylation revealed active glycosylation at Asn residues 90 and 98 and that mutation of these residues reduced cell surface CRFR1 expression and decreased the efficacy for CRF-stimulated cAMP formation.

The role of PDZ proteins is generally considered to be limited to scaffolding molecular complexes, but these proteins have started to emerge as important regulators of GPCR expression and signaling (Magalhaes et al., 2012). CAL has been shown to associate with and regulate the activity of a number of other GPCRs including: the β_1-AR (He et al., 2004), metabotropic glutamate receptor 1 and 5 (mGluR1a and mGluR5) (Zhang et al., 2008; Cheng et al., 2010) and the somatostatin receptor subtype 5 (SSTR5) (Wente et al., 2005; Bauch et al., 2014). A common finding in both our current study and previous reports examining the role of CAL in regulating GPCR function is that the overexpression of CAL results in the intracellular retention of the receptors in the ER-Golgi resulting in the reduction of receptor expression at the plasma membrane and this has been shown in multiple different cell lines. Moreover, CAL appears to play a role in the ER-Golgi retention not only of GPCRs, but also of other transmembrane proteins such as the CFTR (Cheng et al., 2002) and the voltage-gated potassium channel ($K_{V10.1}$) (Herrmann et al., 2013). However, the mechanism for this regulation is still not clear, especially for GPCRs. It has been suggested that CAL can reduce cell surface expression of CFTR by enhancing

their ubiquitination, thereby targeting them for lysosomal degradation. In contrast, opposite conclusions were drawn with regards to the mechanism by which CAL regulates mGluR5. In the case of mGluR5 it is suggested that CAL may stabilize the receptor by inhibiting its ubiquitination-dependent degradation process. This is not the case for CRFR1 as we have found that CAL overexpression does not contribute to increased CRFR1 degradation following CRF stimulation for up to 6h.

We find that CAL overexpression results in the appearance of a lower molecular mass HA-immunoreactive protein band that is not prominently observed in the absence of CAL expression. We have hypothesized that this band represents an immature partially glycosylated form of the CRFR1. Similar conclusions have been previously made regarding the effect of CAL overexpression on the glycosylation status of SSTR5 (Wente et al., 2005; Bauch et al., 2014). GPCR maturation is associated with post-translational modification of the receptors that can result in the glycosylation of Asn residues localized on the extracellular surfaces of the receptors. In the present study, we find that the introduction of point mutations at Asn90 and Asn98, results in impaired CRFR1 glycosylation that is indistinguishable from the effect of overexpressing CAL. We show that the addition of polysaccharide chains at either of these two residues is important for cell surface CRFR1 expression and the efficacy for CRF-stimulated cAMP formation. These results are in contradiction with a previous study of CRFR1 and the parathyroid hormone receptor (PTH) glycosylation (Assil and Abou-Samra, 2001). In the previous study, altered CRFR1 function required the mutation of at least three N-glycosylation sites and suggested that the loss of either one or two polysaccharide chains is well-tolerated (Assil and Abou-Samra, 2001). It is likely that the observed differences between these studies may be attributed to differences in the methodologies used to measure cell surface expression and cAMP formation and the cell types used.

CAL has been shown to interact with Golgi proteins involved in sorting such as Syntaxin 6 or Golgin160 (Herrmann et al., 2013). In addition, CAL can associate with many transmembrane proteins and retain them in the transgolgi network (Cheng et al., 2002; Herrmann et al., 2013). This suggests that CAL may play an important role as either an ER-Golgi sorting molecule or contribute directly to the ER-Golgi quality control process

to ensure that only properly folded terminally glycosylated proteins exit the ER-Golgi complex to traffic to the plasma membrane. If the protein is still not ready, CAL retains it to allow for other scaffolding proteins to bind and complete the folding process. This can also be used to explain CAL's role in mediating glycosylation. There is currently no known sorting mechanism for glycosyltransferases which are the key enzymes in the addition of glycans to proteins (Moremen et al., 2012). Given the ubiquitous distribution of CAL throughout the different Golgi compartments and its potential role as a sorting molecule, we can speculate that it is involved in the vesicular transport and sorting of glycosyltransferases and hence mediate the glycosylation of transmembrane proteins.

A very recent paper has demonstrated that CAL interacts with the β_1-AR (Koliwer et al., 2015). This interaction with the β_1-AR appears to play an important role in regulating the internalization and recycling of the β_1-AR and is proposed that this occurs as the consequence of an effect of CAL on the endosomal degradation of the receptor following endocytosis (Koliwer et al., 2015). The authors also demonstrate that β_1-AR expression levels are decreased in the hippocampus of CAL knockout mice. Therefore, based on this study and our own data showing that CAL is predominantly localized to the Golgi apparatus, we propose that CAL may mediate its effects on GPCR function by serving as a GPCR sorting protein via two distinct mechanisms. Specifically, by acting as a regulator of post-translational GPCR modifications involved in receptor glycosylation, as well as functioning as a regulator of endosomal degradation.

Our data show that CAL overexpression can block agonist-stimulated endocytosis. We are suggesting that CAL can mediate post-translational modifications and we have provided evidence for it regulating the glycosylation of CRFR1. We can speculate that it can mediate other modifications that might be important for receptor internalization like phosphorylation. In addition, other PDZ proteins were shown to regulate the recruitment of β-arrestin to GPCRs which is a key step in receptor endocytosis (Dunn and Ferguson, 2015). It has been shown that NHERF1 can enhance β-arrestin recruitment to the chemokine receptor CCR5 (Hammad et al., 2010) while PSD95 blocks β-arrestin recruitment to 5-HT$_{2A}$R (Schmid and Bohn, 2010). Therefore, it is possible that CAL

interferes with β-arrestin recruitment to the receptor and further studies are needed to investigate those mechanisms.

In addition to its role in CRFR1 trafficking, we find that CAL overexpression affects CRFR1-dependent activation of the ERK1/2 signaling pathway. Specifically, CAL expression significantly attenuates CRFR1- mediated ERK1/2 phosphorylation in a manner that is dependent upon an intact CRFR1 PDZ-binding motif. Similar results have been observed for mGluR1a, where CAL overexpression reduces mGluR1a-stimulated ERK1/2 phosphorylation (Zhang et al., 2008). Thus, it will be important in the future to examine the role of CAL interactions with CRFR1 and other GPCRs that encode PDZ-binding motifs in the central nervous system. PCR analysis of mouse tissue revealed that CAL is ubiquitously distributed in many different regions of the brain (cerebellum, cortex, hippocampus and hypothalamus) as well as the heart, kidney, lungs, testis and spinal cord (Duvernay et al., 2005). CAL-deficient mice are infertile so it has been suggested that CAL is important in the early stages of development.

We have reported previously that other PDZ proteins, SAP97 and PDZK1 are also important for the regulation of CRFR-mediated activation of the ERK1/2 signal transduction pathway. Unlike CAL, both SAP97 and PDZK1 facilitate CRFR1-mediated ERK1/2 phosphorylation and SAP97 contributes to GPCR-mediated ERK1/2 activation in the absence of PDZ binding motif interactions (Dunn et al., 2013; Dunn et al., 2014; Walther et al., 2015). Thus, CAL functions differently than both SAP97 and PDZK1 to antagonize CRFR1-mediated activation of ERK1/2 signaling and CAL siRNA increases CRFR1-stimulated ERK1/2 phosphorylation. Thus, it appears that, while a number of PDZ proteins contribute to the regulation of CRFR1 ERK1/2 signaling they have opposing effects and mediate their actions by distinct mechanisms. The precise mechanism by which CAL regulates the activation of the ERK1/2 pathway remains to be determined, but it does not appear to involve reduced cell surface expression of CRFR1, as cAMP formation is not altered following CAL overexpression.

2.5   Conclusion

In conclusion, we find that CAL regulates N-glycosylation and cell surface expression of CRFR1 as well as negatively regulates CRFR1- mediated ERK1/2 phosphorylation. This adds CRFR1 to a growing list of CAL-interacting GPCRs. The effects of CAL on CRFR1 glycosylation, cell surface expression and ERK1/2 signaling are all dependent upon an intact PDZ binding motif. Taken together, these observations and previous studies examining CAL function suggest that CAL represents another important PDZ domain containing protein that contributes to distinct aspects of GPCR trafficking and signaling.

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Chapter 3

3 Role of MAGI proteins in regulating CRFR1 activity

A version of this chapter has been accepted for publication in the Journal of Molecular Signaling

Hammad MM, Dunn HA, and Ferguson SS (2016) MAGI proteins regulate the trafficking and signaling of corticotropin-releasing factor receptor 1 via a compensatory mechanism

3.1   Introduction

Membrane-associated guanylate kinase (MAGUK) family proteins are synaptic scaffolding proteins within a structured protein network responsible for the spatial organization of the presynaptic and postsynaptic compartments. They play a crucial role in the formation and function of synapses in the central nervous system (CNS) by regulating multiple aspects of synapse physiology such as synaptogenesis, receptor trafficking, synaptic function, and plasticity (Funke et al., 2005; Feng and Zhang, 2009; Yamagata and Sanes, 2010). MAGUKs are well-conserved throughout evolution and are widely-expressed in the brain and periphery. They include multiple subfamilies including membrane palmitoylated proteins (MPPs), zona occludens (ZO), caspase recruitment domain-containing MAGUK protein (CARMA), discs large (DLGs) and MAGUK with inverted orientation PSD-95/Disc Large/Zona Occludens (PDZ) (MAGIs) proteins (Oliva et al., 2012). Generally, these proteins contain multiple domains that control their function and facilitate their interactions with their targets. The two common domains among all members are PDZ domains and the guanylate kinase (GK) domain. (Oliva et al., 2012). An important target for the MAGUKs scaffolding proteins is the G protein-coupled receptors (GPCRs) family (Dunn and Ferguson, 2015). Many GPCRs encode a short class I PDZ-binding motif (S/T-x-ϕ, where ϕ is any aliphatic amino acid residue) at the end of their carboxyl-terminal tail that is recognized by the PDZ domain of the MAGUK proteins. This protein-protein interaction between the receptor and MAGUKs results in the regulation of GPCR function. MAGUK proteins play a key role in mediating the subcellular localization, trafficking, cell surface expression and signal transduction of multiple GPCRs and different proteins have both overlapping and distinct roles in the regulation of GPCR activity (Harris and Lim, 2001; Lee and Zheng, 2010; Romero et al., 2011; Magalhaes et al., 2012). One of the important subfamilies of MAGUKs is the membrane-associated guanylate kinase with inverted orientation (MAGI) protein subfamily (Dunn and Ferguson, 2015), which consists of three members; MAGI-1, MAGI-2 and MAGI-3. All three proteins share a similar structure containing one guanylate kinase-like (GK) domain, two tryptophan tryptophan (WW) domains and six PDZ domains. MAGI proteins, particularly MAGI-2 and MAGI-3, have been shown to regulate the trafficking and signaling of multiple GPCRs.

MAGI-1, also known as BAP-1 (BAI-1-associated protein 1), has 7 isoforms that are widely expressed in different tissues. Isoform 1, isoform 2 and isoform 6 are highly expressed in colon, kidney, lung, liver, and pancreas. Isoform 5 is predominantly expressed in brain and heart. Isoform 3 and isoform 4 are highly expressed in pancreas and brain (Dobrosotskaya et al., 1997; Laura et al., 2002). MAGI-1 interacts with BAI-1 (brain-specific angiogenesis inhibitor-1), a family B GPCR that functions as an adhesion molecule. Therefore, it is suggested that MAGI-1 might play an important role in the organization of membrane proteins and cytoskeleton by transmitting signals related to cell-cell or cell- matrix interactions through BAI-1 (Shiratsuchi et al., 1998; Stetak et al., 2009; Stephenson et al., 2014). MAGI-1 also regulates AMPA receptor activity and modulate behavioral plasticity (Emtage et al., 2009).

MAGI-2, also known as S-SCAM (synaptic scaffolding molecule), is specifically expressed in the brain and has 2 isoforms (Wood et al., 1998). A previous study has illustrated an association between MAGI-2 and β_1-AR that is enhanced upon agonist stimulation (Xu et al., 2001). The study also reports an increase in agonist-induced internalization of β_1-AR when MAGI-2 is co-expressed, but no effect on cAMP generation induced by isoproterenol stimulation. In contrast, MAGI-2 has been shown to inhibit both vasoactive intestinal polypeptide type 1 (VPAC$_1$) receptor-mediated cAMP production and internalization (Gee et al., 2009).

MAGI-3 is also widely expressed in different tissues. It has been shown to interact with β_2-AR and reduce signaling via the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway (Yang et al., 2010). MAGI-3 also associates with lysophosphatidic acid-activated receptor subtype-2 (LPA$_2$R) and opposite to the effect that is observed for the β_2-AR, knockdown of MAGI-3 results in a decrease in LPA$_2$R-mediated ERK 1/2 signaling (Zhang et al., 2007). The study also reports a significant reduction in Rho activation upon knockdown of MAGI-3.

The corticotropin releasing factor receptor 1 (CRFR1) is activated by corticotropin-releasing factor (CRF), a 41 amino acid neuropeptide secreted from the paraventricular nucleus of the hypothalamus in response to stress. Release of CRF can lead to the activation of the hypothalamic-pituitary-adrenal axis (HPA axis) (Vale et al., 1981; Arzt and Holsboer, 2006). CRF can also bind to and activate CRFR2, however the affinity of CRF

for CRFR1 is much higher than that for CRFR2. Both CRF receptors are widely expressed in the brain (neocortex and cerebellum) and pituitary (Chalmers et al., 1995; Palchaudhuri et al., 1998). A number of studies have elucidated a link between the pathophysiological changes in the CRF system and various neuropsychiatric disorders such as major depression, panic disorder, anorexia nervosa, and Alzheimer's disease (Behan et al., 1996; Lanfumey et al., 2008). CRFRs can primarily couple to Gα_s for the activation of adenylyl cyclase leading to the formation of cyclic adenosine monophosphate (cAMP) (Chalmers et al., 1996) as well as the activation of the mitogen-activated protein kinase (MAPK) signaling pathway (Kageyama et al., 2007). CRFR1 encodes a PDZ-binding motif at the carboxyl-terminal tail presented by the last three amino acids, threonine - alanine - valine (TAV). Our laboratory has reports of the interaction between CRFR1 and multiple PDZ domain-containing proteins including SAP97, PSD-95, CAL and PDZK1. These studies showed that both SAP97 and CAL function to antagonize the internalization of CRFR1 (Dunn et al., 2013; Hammad et al., 2015). Interestingly, the two proteins had opposite effects on CRF-stimulated ERK1/2 signaling where knockdown of SAP97 suppressed ERK activation while knockdown of CAL resulted in a significant enhancement of this activation. CAL, a Golgi PDZ protein, seems to regulate CRFR1 function via mediating the post-translational modifications since it prevents the glycosylation of the receptor in the Golgi. On the other hand, SAP97 regulation of ERK signaling is a general regulatory mechanism that is independent from the receptor-PDZ protein interaction. This only further confirms the distinct functions of PDZ proteins depending on the particular GPCR with which they are associated. PDZK1 had no significant effect on the endocytosis of CRFR1 but enhanced ERK1/2 signaling (Walther et al., 2015).

A PDZ overlay assay previously performed utilizing the carboxyl-terminal tail of the CRFR1 suggests an interaction between the first PDZ domain of all three MAGI proteins with the receptor (Dunn et al., 2013). Therefore, we have further investigated these interactions and examined the effects of the MAGI family members on CRFR1 signaling and trafficking. We find that all three MAGI proteins as MAGUK proteins can interact with CRFR1 via the class I PDZ-binding motif. We find that MAGI proteins can regulate the endocytosis of CRFR1 by mediating β-arrestin recruitment upon stimulation with CRF. We also demonstrate that siRNA knockdown of MAGI proteins can result in an

enhancement in ERK1/2 signaling. Furthermore, we show that knocking down one of the MAGI proteins results in the upregulation of the expression levels of the other members of the MAGI subfamily suggesting a compensatory mechanism for regulation. Taken together, our results indicate that MAGI proteins interactions with CRFR1 play an important role in regulating CRFR1 function.

3.2   Materials and Methods

3.2.1   Materials

Protein G beads were purchased from GE Healthcare (Oakville, ON, Canada). CRF was purchased from R&D Systems (Minneapolis, MN). HA peroxidase high affinity antibody was purchased from Roche (Mississauga, ON, Canada). Rabbit anti-GFP antibody was obtained from Invitrogen/Life Technologies (Burlington, ON, Canada). MAGI-1, MAGI-2 and MAGI-3 antibodies were purchased from Thermo Fisher (Burlington, ON, Canada). ECL Western blotting detection reagents were purchased from Biorad (Mississauga, ON, Canada). Mouse anti-HA antibody and all other biochemical reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.2.2   Plasmids

HA-CRFR1, CRFR1-YFP and HA-CRFR1-ΔTAV were described previously (Holmes et al., 2006; Magalhaes et al., 2010). His-MAGI-1, HA-MAGI-2 and His-MAGI-3 were kindly provided by Dr. Randy Hall (Emory University, School of Medicine). MAGI-1 siRNA, MAGI-2 siRNA and MAGI-3 siRNA were purchased from Thermo Fisher (Burlington, ON, Canada). For the negative control, we used Silencer Negative Control #1 AM4635 AGUACUGCUUACGAUACGGTT from Thermo Fisher (Burlington, ON, Canada). The exchange proteins directly activated by cAMP biosensor (EPAC) was a gift from Drs. Ali Salahpour (University of Toronto) and Marc Caron (Duke University) (Barak et al., 2008).

3.2.3   Cell Culture and Transfection

Human embryonic kidney (HEK 293) cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Cells were plated on 10-cm dishes 24h prior to transfection. All experiments were performed on 75-80% confluent plates. Transfections were performed using calcium phosphate protocol except for siRNA transfections which were performed using Lipofectamine 2000 following manufacturer's instructions. Transfections were performed with 1 µg of each construct. Empty pcDNA3.1 vector was used to equalize the total amount of plasmid cDNA used to transfect cells. 18h

post-transfection, cells were washed with phosphate-buffered saline (PBS) and resuspended with trypsin, 0.25% EDTA. Experiments were performed approximately 48h after transfection except for knockdown experiments which were performed 72h post transfection since this protocol resulted in the maximum knockdown of the different MAGI proteins.

3.2.4   Co-immunoprecipitation

24h after transfection, HEK 293 cells were seeded onto 10-cm dishes. Cells were starved with HBSS for 1h at 37°C then stimulated with 100 nM CRF agonist for 30 min. Cells were then lysed in 500 µL lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10 µg/ml leupeptin, and 2.5 µg/ml aprotinin) for 20 min on a rocking platform at 4°C. Samples were collected into 1.5 ml Eppendorf tubes and centrifuged at 15,000 g for 15 min at 4°C to pellet insoluble material. A Bradford protein assay was performed and 300 µg of protein was incubated overnight at 4°C with protein G-Sepharose and mouse anti-HA antibody (1:50). Beads were washed three times with cold lysis buffer and incubated overnight at room temperature in 3xSDS loading buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and western blots were then performed with the indicated antibodies (rabbit anti-GFP, 1:10000), (HA-POD, 1:1000), (rabbit anti-MAGI-3, 1:1000), (rabbit anti-V5, 1:1000).

3.2.5   Bioluminescent Resonance Energy Transfer-based biosensor cAMP Assay

HEK 293 cells were co-transfected with 1 µg HA-CRFR1 (WT or ΔTAV) and either 1µg pcDNA3.1, MAGI-1, MAGI-2, MAGI-3, 80 pmoles scrambled (SCR) siRNA or siRNA against MAGI-1, MAGI-2 or MAGI-3 as well as 2 µg of an EPAC (exchange proteins directly activated by cAMP) construct in 10-cm dishes. 24h post-transfection, cells were reseeded into 96-well plate (~10,000 cells/well) and left for another 24h. On the following day, cells were serum-starved for 1h in induction buffer (200 µM isobutyl-1-methylxanthine (IBMX) in HBSS). Coelenterazine was then added to the wells at a final concentration of 5 µM. Cells were then stimulated with increasing concentrations of CRF

peptide for 10 min. The plate was then read by a Victor Plate Reader (Perkin-Elmer) and the BRET signal was determined by calculating the ratio of the light emitted at 505 to 555 nm to the light emitted at 465 to 505 nm.

3.2.6   Receptor Endocytosis

Following transfection, HEK 293 cells were re-seeded into 12-well plates. Cells were serum-starved in HBSS for 1h at 37°C then stimulated for 0 or 30 min with 500 nM CRF in HBSS at 37 °C. Cells were washed with cold HBSS and incubated with mouse anti-HA antibody (1:1000) for 1h on ice. Cells were washed with cold HBSS then labeled with Alexa Fluor 647 donkey anti-mouse IgG (1:1000) for 1h on ice in the dark. Cells were washed with cold HBSS and treated with 5mM EDTA in PBS for 5 min on ice. Cells were collected and transferred to flow cytometry tubes containing 4% formaldehyde in PBS. Samples were run on a FACS Calibur cytometer using BD Cell Quest Pro software (BD Biosciences, Mississauga, ON) until 10,000 cells were counted. The geometric mean of fluorescence was determined using Flow Jo analysis software and was representative of the expression levels of the receptor on the plasma membrane (BD Biosciences, Mississauga, ON).

3.2.7   Western Blot Analysis

75-90 µl of the samples from the different assays which is equivalent to about 50-70 µg of protein was diluted in β-mercaptoethanol-containing 3xSDS loading buffer and then applied to 10% SDS-PAGE (30% acrylamide mix, 1.5 M tris-HCl, 20% SDS, 10% ammonium persulfate and TEMED). Separated proteins were then transferred to nitrocellulose membranes and membranes were then blocked in 10% milk in TBS for 1h. Membranes were then blotted overnight by incubation with the appropriate antibody at 4°C. 24h later, membranes were washed at least three times with 1X TBS with 0.05% Tween 20, and then incubated with a horseradish peroxidase-conjugated secondary antibody (1:10000) for 1h. Membranes were finally washed again with 1X TBS with 0.05% Tween 20 three times before being developed using a BioRad chemiluminescence system.

3.2.8   Bioluminescent Resonance Energy Transfer

Cells were co-transfected with the indicated cDNA using Lipofictamine 2000. β-arrestin was tagged with Renilla luciferase (Rluc) and used as the energy donor while CRFR1 was tagged with YFP and used as the energy acceptor. 72h after transfection, cells were starved with HBSS for 1h at 37°C. The reaction was then started by the addition of Coelenterazine at a final concentration of 5 μM followed by increasing concentrations of CRF. Signal was collected on a Synergy Neo2 plate reader (Thermo Fisher) using 460/40-nm (luciferase) and 540/25-nm (YFP) band pass filters. Whether or not BRET occurred was determined by calculating the ratio of the light passed by the 540/25 filter to that passed by the 460/40 filter. This ratio is referred to as the BRET ratio.

3.2.9   Statistical Analysis

All measurements are represented as mean ± SEM. Comparisons were performed using one way analysis of variance test (ANOVA) followed by Bonferroni's or Dunn's Multiple comparisons test to determine significance. * indicate P values less than 0.05 and is considered to be significant.

3.3   Results

3.3.1   MAGI proteins interact with CRFR1 via the PDZ-binding motif independent of CRF activation

The different members of the MAGI subfamily of proteins were previously shown to interact with different GPCRs. MAGI-2 was shown to interact with β_1-AR, mGluR1a and VPAC$_1$ while MAGI-3 was shown to interact with β_1-AR, β_2-AR, BAI-1 and LPA$_2$R. We previously identified the first PDZ domains of MAGI-1, MAGI-2 and MAGI-3 as positive hits among several PDZ domains that interact with a GST fusion of the CRFR1 carboxyl-terminal tail (Dunn et al., 2013). To further validate this interaction between MAGI proteins and CRFR1, we performed co-immunoprecipitation experiments with His-MAGI-1 or His-MAGI-3 and either wild-type HA-tagged CRFR1 (HA-CRFR1) or HA-tagged CRFR1 mutant that lacked the PDZ-binding motif (HA-CRFR1-ΔTAV) in transiently transfected HEK 293 cells. We found that His-MAGI-1 and His-MAGI-3 co-immunoprecipitated with HA-CRFR1 but not CRFR1-ΔTAV (Fig. 3.1A and 3.1C) and that the interaction with HA-CRFR1 was not altered following agonist treatment with 100 nM CRF for 30 min (Fig. 3.1B and 3.1D). Similarly, MAGI-2 was previously shown to interact with CRFR1 via the carboxyl terminal tail by another group (Bender et al., 2015).

3.3.2   Effect of MAGI proteins expression on cAMP signaling

PDZ domain-containing proteins were previously shown to have the potential to regulate cAMP signaling of some GPCRs (Magalhaes et al., 2012; Dunn and Ferguson, 2015) and MAGI-2 in particular was shown to reduce cAMP formation upon activation of VPAC$_1$ (Gee et al., 2009). Therefore, we tested whether MAGI proteins contributed to the regulation of CRFR1-mediated cAMP formation. In HEK 293 cells transfected with either HA-CRFR1 or HA-CRFR1-ΔTAV, with or without MAGI-1, MAGI-2 or MAGI-3, there were no significant changes in the maximum efficacy for cAMP signaling in response to treatment with increasing concentrations of CRF for 10 min (Fig. 3.2A-C). In addition, we examined whether the attenuation of MAGI proteins expression could alter CRFR1-mediated cAMP signaling. We found that transfection of HEK 293 cells with siRNA directed to knockdown MAGI-1, MAGI-2 or MAGI-3 had no effect on either HA-CRFR1-
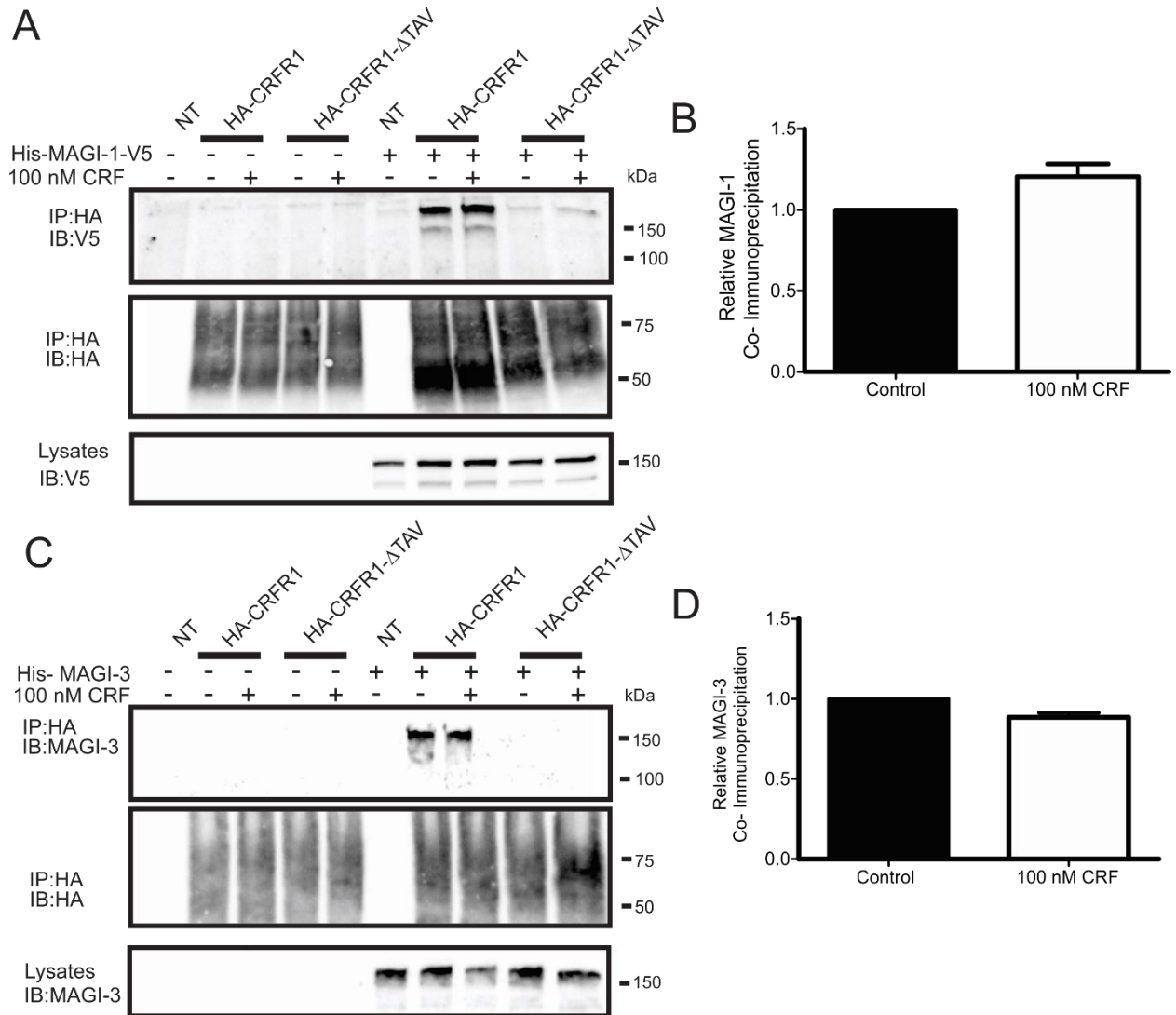
Figure 3.1: MAGI proteins co-immunoprecipitate with HA-CRFR1 in a PDZ-binding motif-dependent manner: HEK 293 cells were co-transfected with HA-CRFR1 (WT or ΔTAV) and pcDNA or His-MAGI-1-V5. **(A)** Representative immunoblot of His-MAGI-1-V5 co-immunoprecipitated (IP) with HA-CRFR1 but not HA-CRFR1-ΔTAV which lacks the PDZ-binding motif. SDS-PAGE was used to analyze samples and immunoblots were performed with rabbit anti-V5. **(B)** Effect of CRF treatment was quantified using densitometry and had no significant difference on the amount of MAGI-1 co-immunoprecipitated with HA-CRFR1. HEK 293 cells were co-transfected with HA-CRFR1 (WT or ΔTAV) and pcDNA or His-MAGI-3. **(C)** Representative immunoblot of His-MAGI-3 co-immunoprecipitated (IP) with HA-CRFR1 but not HA-CRFR1-ΔTAV which lacks the PDZ-binding motif. SDS-PAGE was used to analyze samples and immunoblots were performed with rabbit anti-MAGI-3. **(D)** Effect of CRF treatment was quantified using densitometry and had no significant difference on the amount of His-MAGI-3 co-immunoprecipitated with HA-CRFR1. Data are representative of three independent experiments.
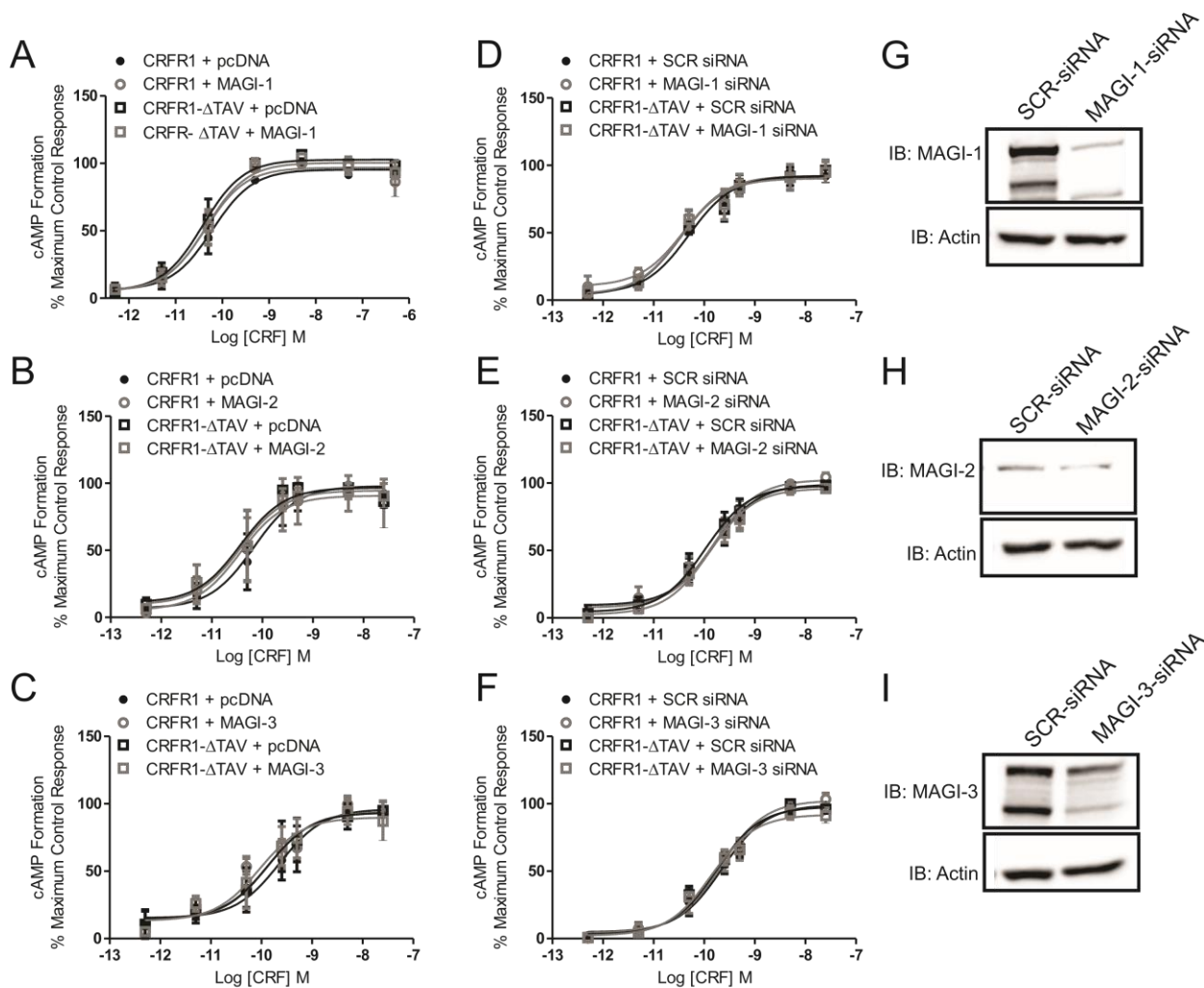
Figure 3.2: MAGI proteins do not regulate CRFR1-mediated cAMP formation: Dose response for CRF-stimulated cAMP formation assessed using a BRET-based cAMP biosensor assay. CRFR1- and CRFR1-ΔTAV- mediated cAMP formation in either plasmid vector or MAGI-1 (A), MAGI-2 (B) or MAGI-3 (C) transfected HEK 293 cells. CRFR1- and CRFR1-ΔTAV- mediated cAMP formation in either MAGI-1 siRNA (D), MAGI-2 siRNA (E) or MAGI-3 siRNA (F) transfected HEK 293 cells. Shown are representative immunoblots showing siRNA knockdown of (G) MAGI-1, (H) MAGI-2, and (I) MAGI-3 with 80 pmol siRNA for 72 hours. The data represent the mean ± SEM of at least three independent experiments.

or HA-CRFR1-ΔTAV- stimulated cAMP signaling in response to treatment with increasing concentrations of CRF for 10 min (Fig. 3.2D-F). These results suggested that MAGI proteins did not contribute to the regulation of CRFR1 activation of the cAMP pathway. We validated the knockdown of MAGI proteins by co-transfecting HEK 293 cells with MAGI proteins cDNA (MAGI-1, MAGI-2 or MAGI-3) and 80 pmoles of either SCR siRNA or MAGI-siRNA. We observed 90% knockdown with MAGI-1 siRNA (Fig. 3.2G), 50% knockdown with MAGI-2 siRNA (Fig. 3.2H) and 65% knockdown with MAGI-3 siRNA (Fig. 3.2I).

3.3.3   Effect of MAGI proteins on cell surface expression

A previous study from our laboratory illustrated an effect of CAL, a Golgi PDZ protein, on cell surface expression of CRFR1 (Hammad et al., 2015). Therefore, we used flow cytometry to measure the plasma membrane expression of CRFR1 upon overexpression as well as knockdown of MAGI proteins. While overexpression of MAGI proteins had no effect on receptor expression levels (Fig. 3.3A-C), knockdown of certain MAGI proteins altered CRFR1 membrane expression. Our data show that knockdown of MAGI-1 resulted in an enhancement in CRFR1 expression at the plasma membrane (Fig. 3.3D). Knockdown of MAGI-2 and MAGI-3 on the hand had no effect on CRFR1 expression levels (Fig. 3.3E-F). This illustrated the importance of MAGI proteins in regulating receptor expression and reflects distinguished effects among the members of the subfamily.

3.3.4   Effect of MAGI proteins on CRF-mediated ERK1/2 signaling

MAGI-3 was previously shown to regulate ERK1/2 signaling of other GPCRs in a distinctive manner as it was shown to enhance ERK1/2 activation in the case of LPA$_2$R and BAI-1 and inhibit β-AR-stimulated ERK1/2 phosphorylation (He et al., 2006; Zhang et al., 2007). Since CRFR1 was shown to have the ability to activate the MAPK cascade, we assessed the effects of MAGI proteins overexpression on CRFR1-mediated ERK1/2 phosphorylation. We found that the overexpression of MAGI-1, MAGI-2 and MAGI-3 did not have an effect on ERK1/2 phosphorylation (Fig. 3.4). Interestingly, when we knockdown endogenous expression of MAGI-1, MAGI-2 or MAGI-3, we observed a significant increase in ERK1/2 phosphorylation in response to stimulation of CRFR1
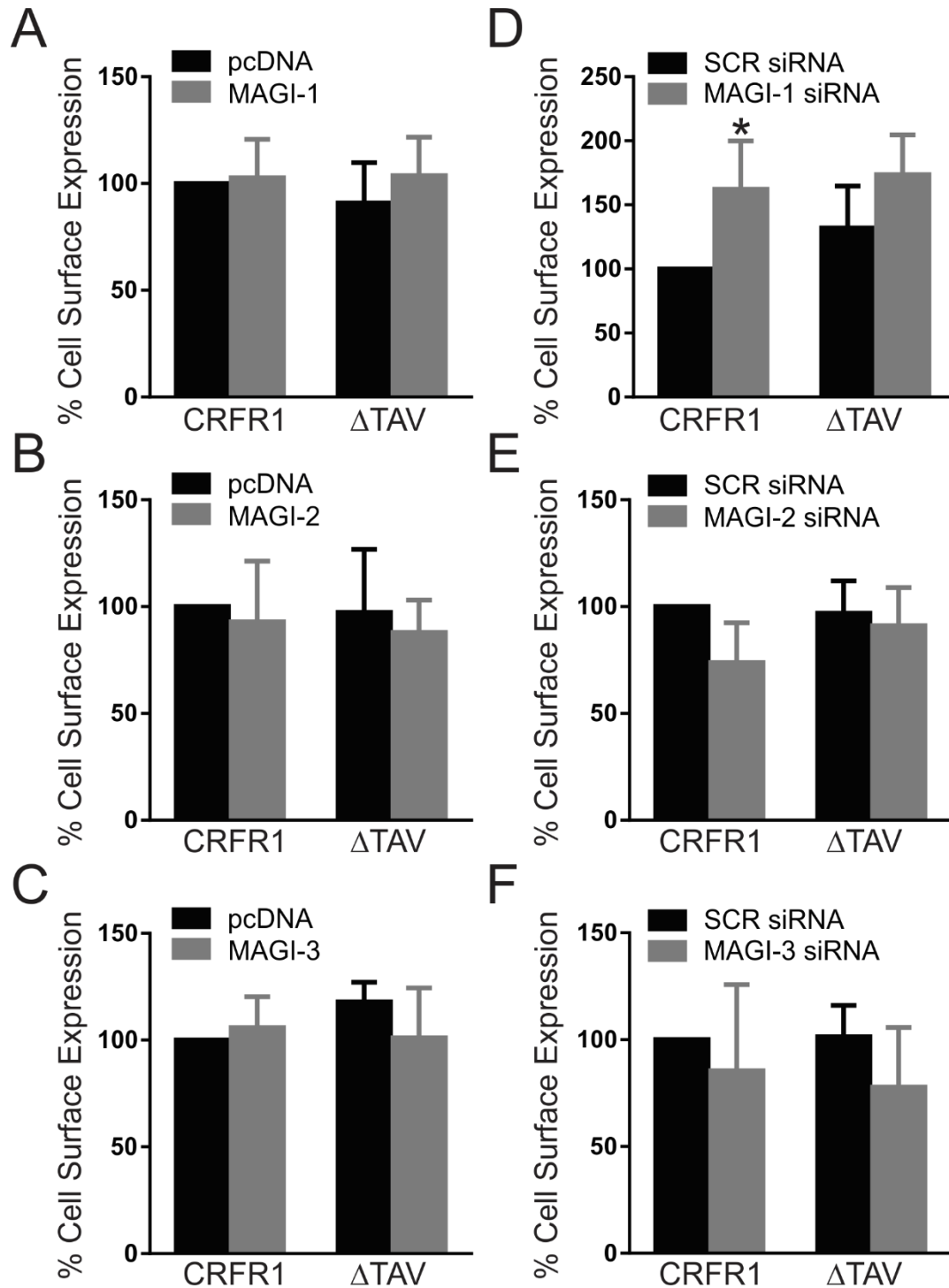
Figure 3.3: Effect of MAGI proteins on cell surface expression: Cell surface expression in HEK 293 cells expressing either HA-CRFR1 or HA-CRFR1-ΔTAV along with MAGI-1 **(A)**, MAGI-2 **(B)**, MAGI-3 **(C)**, MAGI-1 siRNA **(D)**, MAGI-2 siRNA **(E)** or MAGI-3 siRNA **(F)** expressed as measured by flow cytometry. Data is normalized to HA-CRFR1 expression in the control pcDNA or SCR siRNA. The data represent the mean ± SEM of four independent experiments. * P < 0.05 versus HA-CRFR1 cell surface expression.

Figure 3.4: Effect of MAGI proteins overexpression on CRFR1-mediated ERK1/2 signaling: (A) Representative immunoblot showing ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 2, 5, and 15 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with HA-CRFR1 along with either pcDNA or His-MAGI-1. Also shown are corresponding immunoblots for total ERK1/2, MAGI-1 and HA-CRFR1 protein expression. **(B)** Densitometric analysis of ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 2, 5, and 15 min. The data represent the mean ± SEM of four independent experiments. **(C)** Representative immunoblot showing ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 2, 5, and 15 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with HA-CRFR1 along with either pcDNA or MAGI-2. Also shown are corresponding immunoblots for total ERK1/2, MAGI-2 and HA-CRFR1 protein expression. **(D)** Densitometric analysis of ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 2, 5, and 15 min. The data represent the mean ± SEM of six independent experiments. **(E)** Representative immunoblot showing ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 2, 5, and 15 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with HA-CRFR1 along with either pcDNA or MAGI-3. Also shown are corresponding immunoblots for total ERK1/2, MAGI-3 and HA-CRFR1 protein expression. **(F)** Densitometric analysis of ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 2, 5, and 15 min. The data represent the mean ± SEM of six independent experiments.
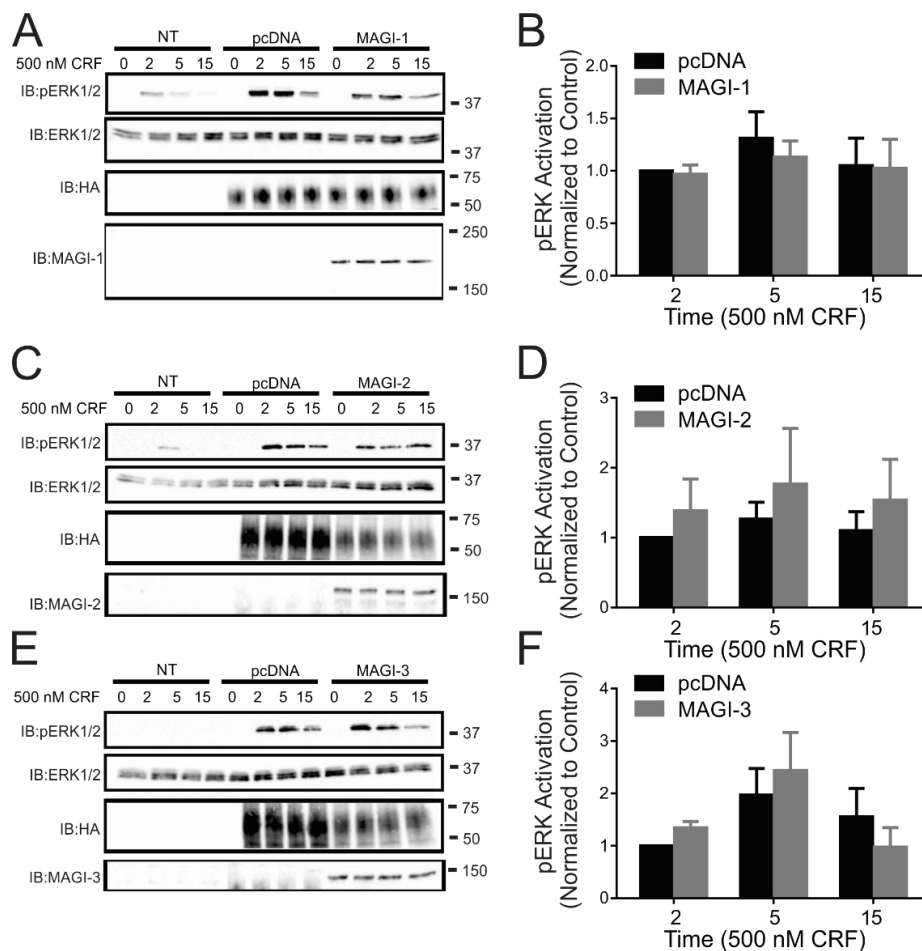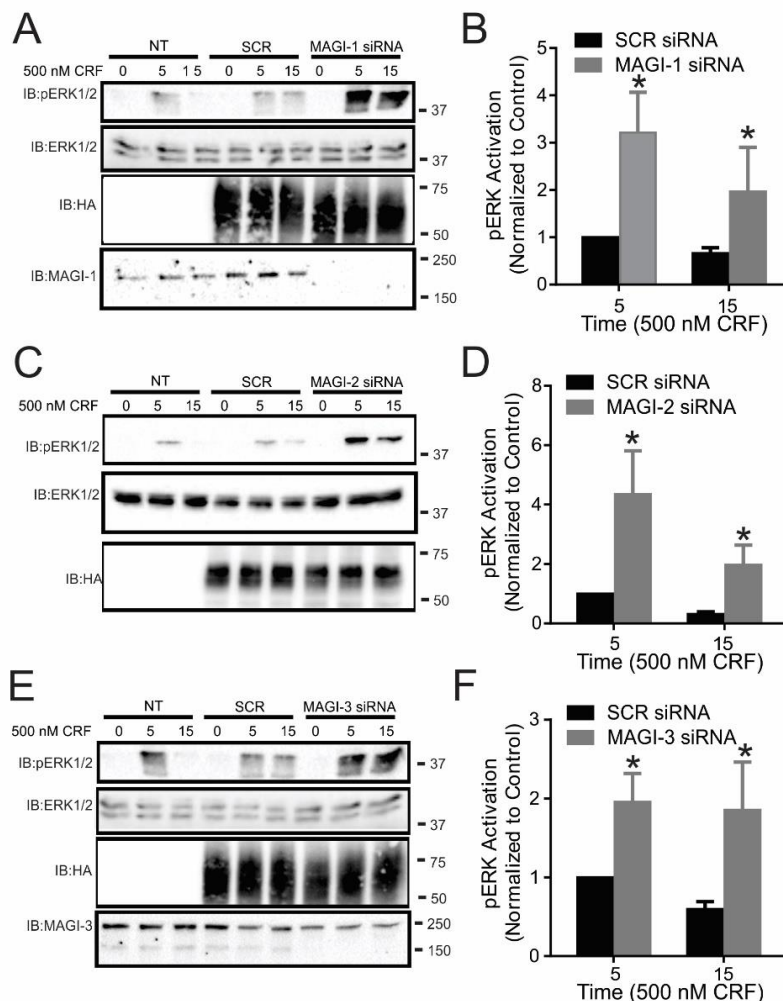
Figure 3.5: Effect of MAGI proteins knockdown on CRF-mediated ERK1/2 signaling:
(A) Representative immunoblot showing ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 5, and 15 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with HA-CRFR1 along with either SCR siRNA or MAGI-1 siRNA. Also shown are corresponding immunoblots for total ERK1/2, MAGI-1 and HA-CRFR1 protein expression. **(B)** Densitometric analysis of ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 5, and 15 min. The data represent the mean ± SEM of four independent experiments. **(C)** Representative immunoblot showing ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 5, and 15 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with HA-CRFR1 along with either SCR siRNA or MAGI-2 siRNA. Also shown are corresponding immunoblots for total ERK1/2 and HA-CRFR1 protein expression. The antibody was not sensitive enough to blot for endogenous MAGI-2. **(D)** Densitometric analysis of ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 5, and 15 min. **(E)** Representative immunoblot showing ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 5, and 15 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with HA-CRFR1 along with either SCR siRNA or MAGI-3 siRNA. Also shown are corresponding immunoblots for total ERK1/2, MAGI-3 and HA-CRFR1 protein expression. **(F)** Densitometric analysis of ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 5, and 15 min. The data represent the mean ± SEM of four independent experiments. * $P < 0.05$ versus SCR siRNA control.

with 500 nM CRF for 5 or 15 minutes (Fig. 3.5).

3.3.5 Effect of MAGI proteins expression on agonist-stimulated CRFR1 endocytosis

Our laboratory previously reported that other members of the MAGUK family such as SAP97 and PSD-95, as well as the Golgi PDZ protein CAL could regulate CRF-induced CRFR1 internalization (Dunn et al., 2013; Hammad et al., 2015; Dunn et al., 2016). To determine whether MAGI proteins also contributed to the regulation of CRFR1 endocytosis, we utilized flow cytometry to assess the loss of cell surface HA-CRFR1 by immunofluorescence. We found that the overexpression of MAGI-1, MAGI-2 and MAGI-3 each attenuated agonist-stimulated HA-CRFR1 internalization following treatment with 500 nM CRF for 30 min (Fig. 3.6A-C). However, although the internalization of the mutant HA-CRFR1-ΔTAV was not altered by either MAGI-2 or MAGI-3, the overexpression of MAGI-1 significantly reduced HA-CRFR1-ΔTAV (Fig. 3.6A-C). In addition, we assessed the effect of knockdown of endogenous MAGI proteins on agonist-stimulated CRFR1 internalization. We observe that all MAGI siRNAs attenuated the internalization of CRFR1 (Fig. 3.6D-F) and that MAGI-1 siRNA had the same effect on CRFR1-ΔTAV internalization (Fig. 3.6D). Thus, MAGI proteins could regulate CRFR1 endocytosis.

3.3.6 MAGI proteins can regulate CRFR1 endocytosis by mediating β-arrestin recruitment to the receptor

The primary pathway contributing to CRFR1 internalization was previously shown to be β-arrestin-dependent (Holmes et al., 2006). Because of the effect of MAGI proteins on the regulation of CRFR1 endocytosis observed in Fig. 3.6, we decided to study the effect of MAGI proteins on β-arrestin recruitment to CRFR1. To do this, we employed a BRET assay to investigate this interaction between rLuc-tagged β-arrestin2 and YFP-tagged CRFR1. The overexpression of all MAGI proteins resulted in a right-ward shift in the CRF dose response curve for β-arrestin2 recruitment (Fig. 3.7A). There was also a significant reduction in the maximal response for β-arrestin recruitment at 500 nM CRF with time (Fig. 3.7B). We also examined the effect of knockdown of MAGI proteins on β-arrestin2 recruitment and similar results were observed, with a right-ward shift in the does response
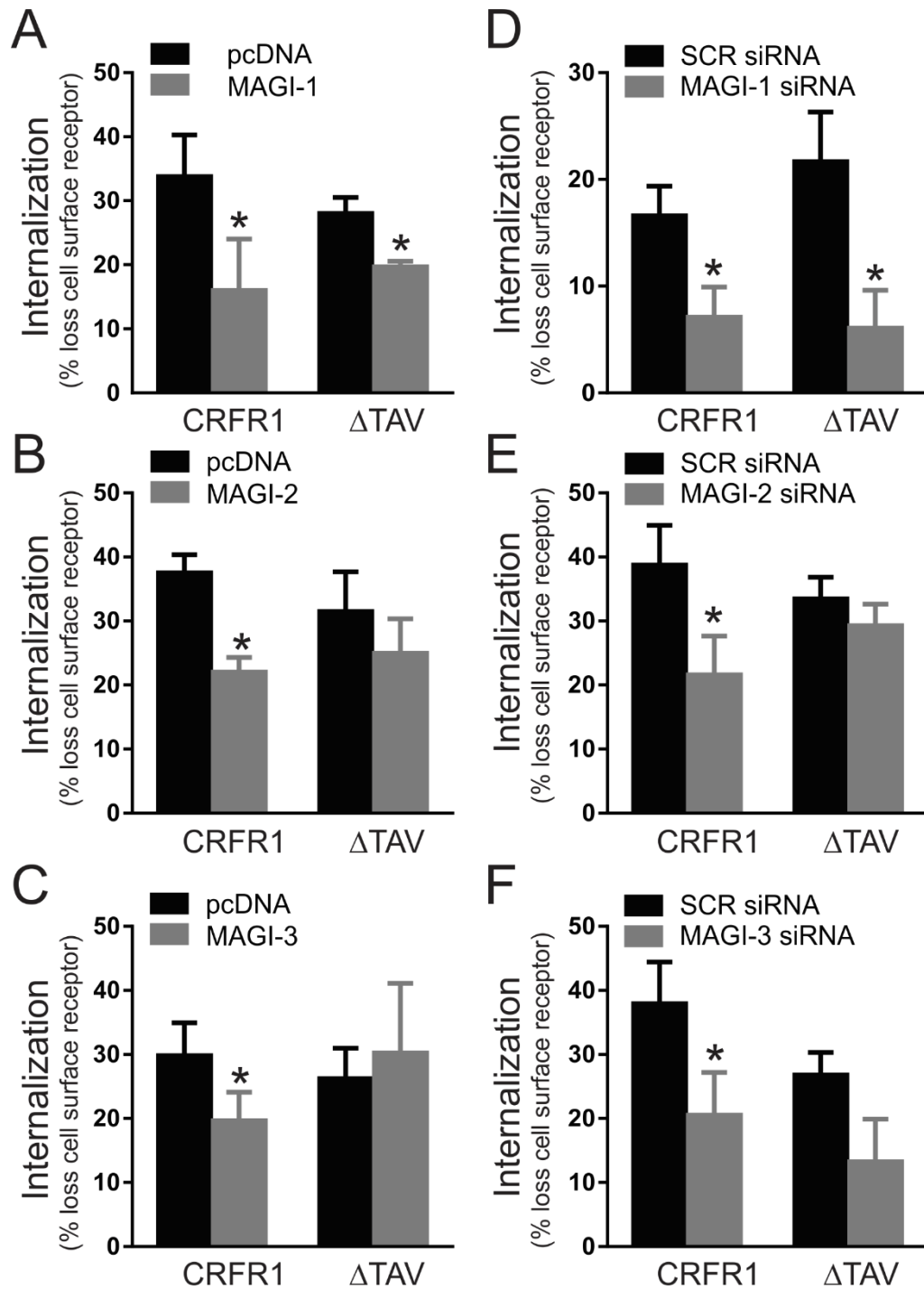
Figure 3.6: Effect of MAGI proteins expression on agonist-stimulated CRFR1 endocytosis: Agonist-stimulated internalization of either HA-CRFR1 or HA-CRFR1-ΔTAV following 500 nM CRF treatment for 30 min in the presence of either MAGI-1 (**A**), MAGI-2 (**B**), MAGI-3 (**C**), MAGI-1 siRNA (**D**), MAGI-2 siRNA (**E**), or MAGI-3 siRNA (**F**). The data represent the mean ± SEM of at least three independent experiments. * P < 0.05 versus empty vector or SCR siRNA control.
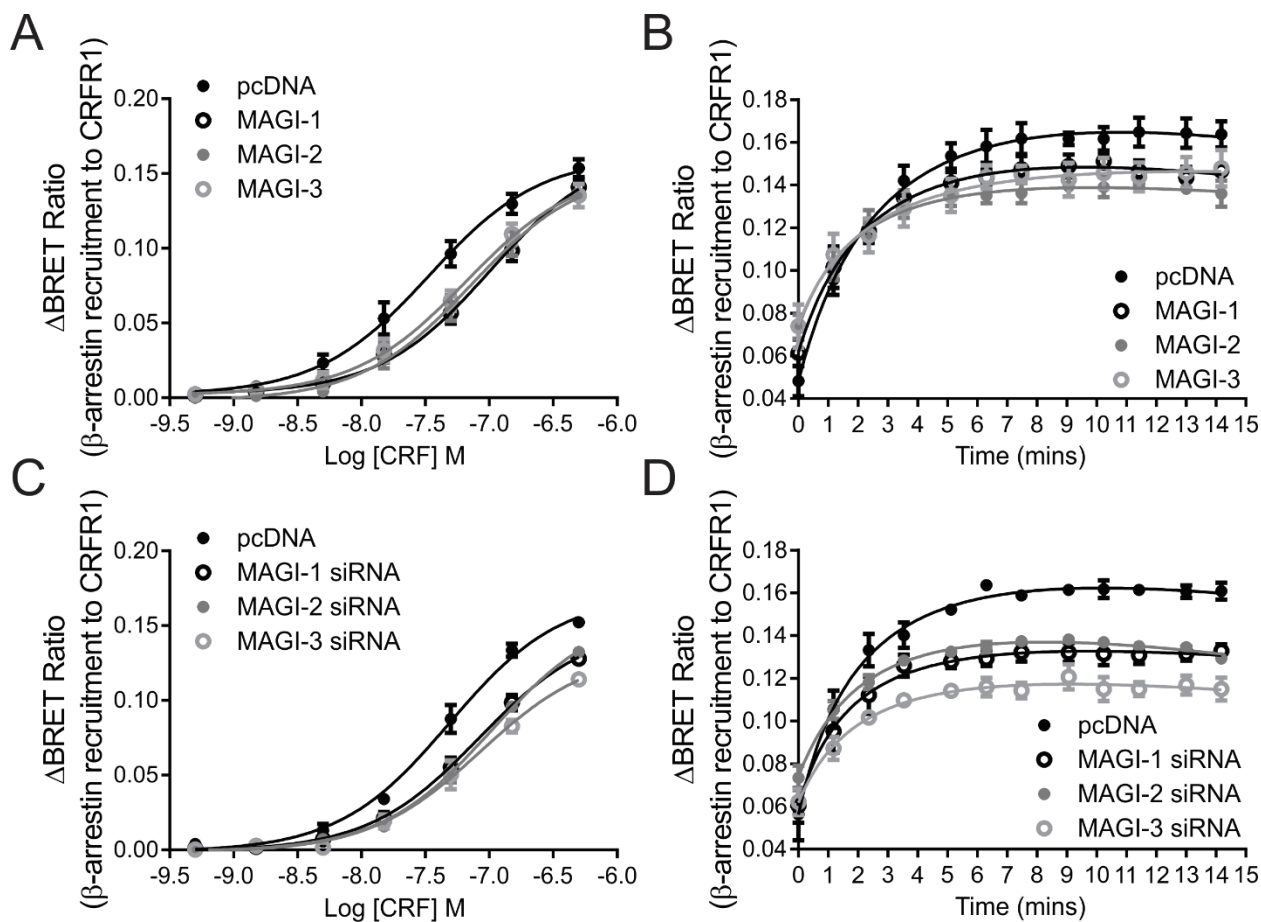
Figure 3.7: Effect of MAGI proteins expression on β-arrestin recruitment to CRFR1: BRET assays measured between β-arrestin-rLuc and CRFR1-YFP in the presence of MAGI-1, MAGI-2 or MAGI-3 **(A, B)** or MAGI-1 siRNA, MAGI-2 siRNA or MAGI-3 siRNA **(C, D). A** and **C** show dose responses of the interaction while **B** and **D** show the interaction as a function of time upon stimulation with 500 nM CRF.

curve and significant reduction in the maximal response, with MAGI-3 exhibiting the most prominent effect (Fig. 3.7C and 3.7D). Taken together, these data suggested that MAGI proteins are regulating CRFR1 endocytosis by mediating β-arrestin recruitment to CRFR1.

3.3.7 Knockdown of one MAGI proteins can lead to the upregulation in the expression level of the others

The results we obtained from our internalization assays and β-arrestin recruitment assays were surprising in terms of having similar effects upon overexpression and knockdown of MAGI proteins. Therefore, we hypothesized that the expression levels of different members of the MAGI subfamily of proteins might be changing to compensate for the loss of another family member. In order to test this, we assessed the expression levels of each MAGI protein upon knockdown of the other MAGI proteins. We observed a significant up-regulation of MAGI-1 expression levels upon knockdown of MAGI-3 (Fig. 3.8A). Similarly, there was a significant up-regulation of MAGI-3 expression levels when MAGI-1 is knocked down (Fig. 3.8B). This suggested a compensatory effect between MAGI-1 and MAGI-3 that could explain the similar results observed for CRFR1 internalization and β-arrestin2 recruitment following either MAGI protein overexpression or knockdown.
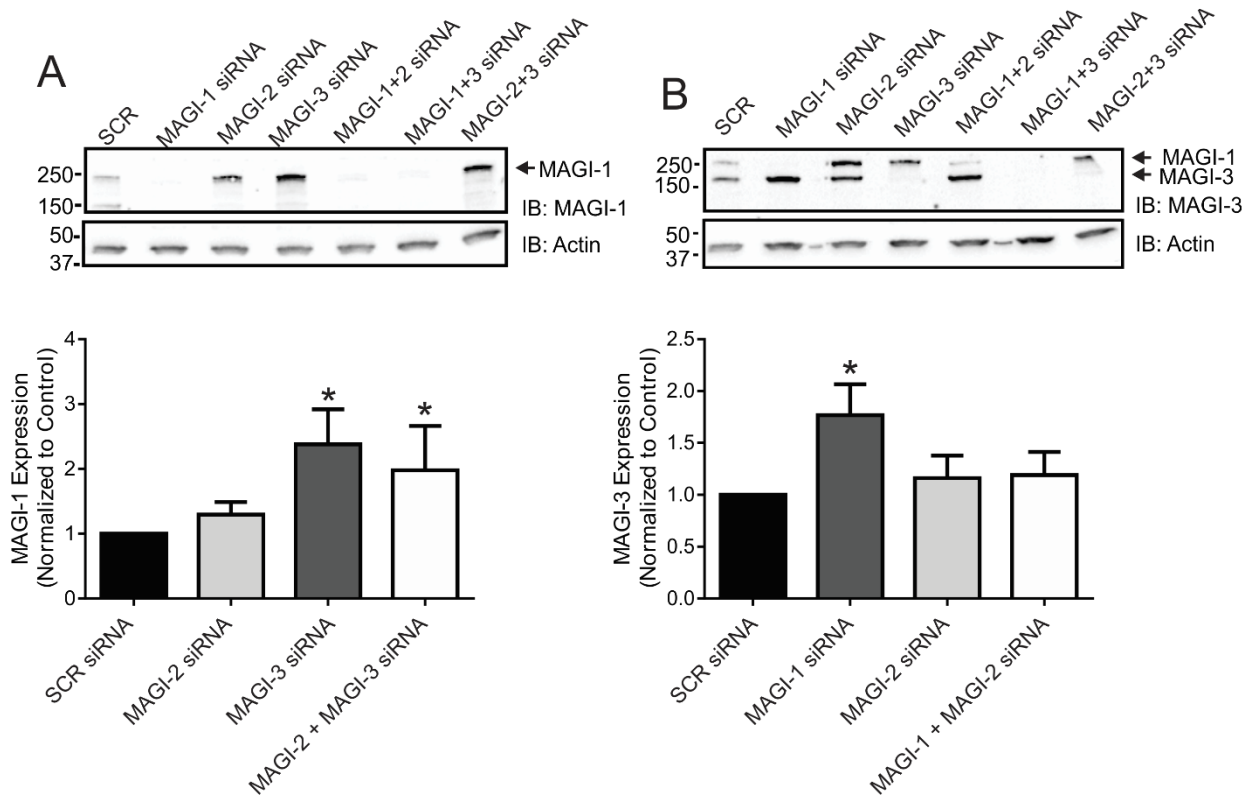
Figure 3.8: Knockdown of one MAGI protein results in an increase of expression of the other MAGI proteins: (A) Representative immunoblot showing the levels of MAGI-1 in HEK 293 cells transfected with siRNA combinations as labeled. Densitometric analysis shows MAGI-1 levels after normalization to actin as a loading control. **(B)** Representative immunoblot showing the levels of MAGI-3 in HEK 293 cells transfected with siRNA combinations as labeled. Densitometric analysis shows MAGI-3 levels after normalization to actin as a loading control. The data represent the mean ± SEM of at least eight independent experiments. * $P < 0.05$ versus SCR siRNA control.

3.4   Discussion

We have previously identified all three members of the MAGI subfamily as CRFR1-interacting PDZ proteins in the PDZ overlay assay (Dunn et al., 2013). Despite similarities in their molecular topology, the members of the MAGI subfamily, specifically MAGI-2 and MAGI-3, are known to have distinct function with respect to their functional regulation of different GPCRs, but their role in regulating CRFR1 activity has not been investigated (Xu et al., 2001; Sugi et al., 2007; Zhang et al., 2007; Gee et al., 2009; Yang et al., 2010). We find that all three MAGI proteins are co-immunoprecipitated with CRFR1 in a PDZ motif dependent manner and that either overexpression or knockdown of each of the MAGI proteins is able to negatively regulate CRFR1 endocytosis with MAGI-1 overexpression or knockdown negatively regulating CRFR1 endocytosis in a PDZ motif-independent manner. This antagonism of CRFR1 endocytosis that is mediated by either MAGI protein overexpression or knockdown appears to involve the antagonism of β-arrestin recruitment to the receptor. In the case of siRNA-mediated knockdown of individual MAGI proteins there appears to be a compensatory up-regulation of the expression of other MAGI protein subtypes. In addition we find that while the overexpression of each of the MAGI proteins does not influence CRFR1-stimulated ERK1/2 phosphorylation, siRNA knockdown of each of the MAGIs results in a significant increase in CRFR1-mediated ERK1/2 phosphorylation. Taken together, our results indicate that MAGI protein interactions with CRFR1 play an important role in regulating CRFR1 function.

MAGUK proteins comprise a subfamily of scaffolding proteins that are defined by having PDZ, SH3 and GK domains with an emerging role in synapse formation and function. DLG (discs large) subfamily (mainly SAP97 and PSD-95) have been the focus of many studies and are shown to play key roles in synaptogensis, the creation of neural circuits, synaptic transmission, memory and learning, as well as glutamate receptor clustering and trafficking (Stetak et al., 2009). On the other hand, few studies have looked at the MAGI subfamily of MAGUK proteins, although they are abundantly expressed in the brain. Current understanding of MAGI protein function in the brain suggests a role on memory and learning, but the mechanism is still unknown (Funke et al., 2005; Stetak et al.,

2009; Oliva et al., 2012). In terms of the MAGUKs role in regulating GPCRs function, studies from our laboratory illustrate distinct roles for SAP97 and PSD-95 in regulating CRFR1 trafficking and signaling (Dunn et al., 2013; Dunn et al., 2016). This is an intriguing observation because of the overlapping sequence and structural similarities of SAP97 and PSD-95. This lead to the hypothesis that MAGI proteins would also elicit diverse effects on receptor physiology. Although previous studies have looked at the effects of MAGI proteins on regulating GPCRs function, none of these previous reports have compared the relative function of all three MAGI proteins in regulating the activity of an individual GPCR, despite evidence that many GPCRs interact with all three MAGIs (Xu et al., 2001; He et al., 2006; Sugi et al., 2007; Zhang et al., 2007; Gee et al., 2009; Yang et al., 2010; Dunn and Ferguson, 2015). For example, MAGI-2 interacts with the β_1-AR and enhances its internalization, whereas it also interacts with $VPAC_1$ to regulate its intracellular localization in epithelial cells and inhibit agonist-induced internalization (Xu et al., 2001; He et al., 2006; Gee et al., 2009). MAGI-3 on the other hand is found to antagonize ERK1/2 activation by both the β_1-AR and the β_2-AR (Zhang et al., 2007; Yang et al., 2010; He et al., 2006). We find here that the overexpression of any of the MAGI proteins reduces CRF-stimulated CRFR1 internalization, which is similar to what is observed for $VPAC_1$, but opposite to what is observed for the β_1-AR. Interestingly, this effect is dependent on an intact CRFR1 PDZ-binding motif for MAGI-2 and MAGI-3, but not MAGI-1. This suggests that MAGI-1 may associate with CRFR1 via other interactions than those mediated by the PDZ domain. An alternative possibility would be that MAGI-1 is antagonizing the formation of protein complexes required for GPCR endocytosis. Further studies on receptors that do not harbor a PDZ-binding motif such as CRFR2 would be required to further investigate this role of MAGI-1. It will also be important to study the effect of MAGI-1 on the ESCRT complex and its components.

As is the case for many GPCRs, the primary internalization pathway utilized by the CRFR1 involves β-arrestin-dependent endocytosis via clathrin-coated pits (Ferguson et al., 1998; Ferguson, 2001; Holmes et al., 2006; von Zastrow and Williams, 2012). The observation that each of the MAGI proteins antagonize CRFR1 endocytosis suggested the possibility that MAGI proteins may play a role in regulating β-arrestin recruitment to the receptor. Consistent with this hypothesis, the overexpression of all of the MAGIs resulted

in the antagonism of β-arrestin2 recruitment to the receptor as measured by BRET assay as assessed by a right-ward shift in the CRF dose response curve and a blunting of the maximal translocation response. Surprisingly, when the same assay is repeated with siRNA against MAGI-1, MAGI-2 or MAGI-3 to knockdown the endogenous proteins, we still observe a right-ward shift in the dose response curve and an even more prominent reduction in the maximal response. This result is opposite to what we had anticipated. Therefore, we investigated whether changes in the expression levels of other MAGI protein subtypes is compensating for the loss of expression of the MAGI protein knocked down by siRNA. This concept of compensation is not entirely novel to the field of PDZ domain- containing proteins, as it is suggested that PSD-95 and PSD-93 can replace one another to regulate GPCR function in a similar manner (Sun and Turrigiano, 2011). Interestingly, we observe a compensatory up-regulation of MAGI-1 expression upon MAGI-3 knockdown and an up-regulation of MAGI-3 expression following MAGI-1 protein knockdown. Thus, compensatory MAGI protein expression provides a plausible mechanism by which knocking down individual MAGI proteins attenuates CRFR1 endocytosis and impairs β-arrestin2 recruitment. However, this mechanism may not be entirely sufficient to explain our results, as although MAGI-2 knockdown resulted in increased MAGI-1 and MAGI-3 protein expression in the representative blots shown in Fig. 3.8, this result is not reliably reproduced when compared to MAGI-1 and MAGI-3 knockdown. Additional work would be necessary to further assess this interplay between the MAGI subfamily members.

We have previously reported the interaction of other PDZ domain- containing proteins with CRFR1 and the consequences of that on receptor endocytosis. We have shown that the overexpression of SAP97 antagonizes agonist-stimulated CRFR1 internalization, whereas single hairpin (shRNA) knockdown of endogenous SAP97 in HEK 293 cells results in increased agonist-stimulated CRFR1 endocytosis and that PSD-95 elicits the same effects on CRFR1 endocytosis (Dunn et al., 2015; Dunn et al., 2016). Similarly, we also find that CAL negatively regulates CRFR1 endocytosis when overexpressed (Hammad et al., 2015). Despite seemingly having similar effects, the mechanism of GPCR regulation by these PDZ proteins is vastly different. SAP97 and PSD-95 are suggested to regulate the internalization by affecting β-arrestin recruitment to

CRFR1, while CAL acts as a major sorting protein at different subcellular levels and by modifying the post-translational modifications that the receptor undergoes at the Golgi. Thus, the MAGI proteins employ a similar "MAGUK-like" mechanism to regulate CRFR1 endocytosis via the attenuation of β-arrestin recruitment. However, their collective function is further complicated by a compensatory mechanism involving changes in protein expression of other subtypes. Future studies will examine this potential phenomenon and how it may affect other GPCRs function.

In addition to the role that MAGI proteins play in regulating the trafficking of CRFR1, we find that they have an important effect on the regulation of CRFR1 signaling, specifically via the MAPK pathway, but not G protein-mediated cAMP formation. While the overexpression of each of the MAGI proteins has no effect on CRFR1-mediated ERK1/2 phosphorylation, siRNA knockdown of all of the MAGIs results in a significant increase in the extent of ERK1/2 phosphorylation induced by CRFR1 activation. Interestingly, a study on LPA_2R showed opposite effects on ERK1/2 activation where MAGI-3 knockdown reduced the signal. However, similar to our study, the LPA_2R study which was performed on SW480 cells reported no difference in ERK activity upon overexpression of MAGI-3 suggesting that different tissues and cell lines express endogenous MAGI proteins in high levels. Previous experiments examining the regulation of CRFR1-mediated ERK1/2 activation by two other MAGUK family members, SAP97 and PSD-95 yielded different results (Dunn et al., 2013; Dunn et al., 2016). Whereas, SAP97 expression significantly enhanced CRFR1-stimulated ERK1/2 phosphorylation, PSD-95 overexpression had no effect on ERK1/2 phosphorylation induced by CRFR1 activation. In contrast, the observed increase in CRFR1-mediated ERK1/2 phosphorylation following siRNA knockdown of each of the MAGI proteins is similar to what we have previously observed following siRNA knockdown of PDZK1 (Walther et al., 2015). The mechanism by which siRNA knockdown of an individual MAGI protein results in compensatory changes in the expression levels of other MAGI proteins to attenuate CRFR1 endocytosis without compensating for CRFR1-mediated ERK1/2 activation is unclear. However, these results might suggest that the observed increase in ERK1/2 phosphorylation is mediated by a β-arrestin-independent pathway as CRFR1/β-arrestin

interactions are predominantly regulated by GRK5 phosphorylation, which is thought to preferentially allow activation of ERK1/2 via the β-arrestin-mediated pathway (Holmes et al., 2006; Zidar et al., 2009).

Now that we have a better understanding for the role of MAGI proteins in regulating CRFR1 function in HEK293 cells, the next step would be to asses these interactions *in vivo*. RT-PCR in mouse tissue showed that the MAGI proteins are expressed in different regions of the brain including the cortex, thalamus and cerebellum (Sugnet et al., 2006). We would predict that MAGI proteins are expressed in CRF-containing neurons and would regulate CRFR1 physiology. Some knockout mouse models of MAGI proteins are available (MAGI-1 and MAGI-2) and have exhibited some renal deficits (Ihara et al., 2014). This reflects the importance of MAGI proteins in periphery. In addition, MAGI-2 knockout mice show abnormal elongation of dendritic spines indicating a possible role for MAGI-2 during morphogenesis of neurons. (Iida et al., 2007). Furthermore, studies on C. elegans demonstrate a role for MAGI-1 in regulating the clustering of ionotropic glutamate receptors in certain neurons that are associated with learning and memory (Stetak et al., 2009). Therefore, further characterization of MAGI proteins knockout models is required to determine the role of this subfamily in the CNS.

In summary, the current study completes a series of investigations examining the role of PDZ proteins in regulating the expression, trafficking and signaling of CRFR1. We find that PDZ proteins play an overlapping but distinguishable role in regulating the post-translation modification, ER-Golgi trafficking, endocytosis and signaling of CRFR1 (Dunn et al., 2013; Hammad et al., 2015; Walther et al., 2015; Dunn et al., 2016). Although each of these proteins had a similar effect on CRFR1 endocytosis, as they each, with the exception of PDZK1, function to antagonize CRFR1 internalization, they exhibit pleiotropic effects on the regulation of CRFR1-mediated ERK1/2 activity. These observations indicate that there is likely no redundancy of function for PDZ proteins in the regulation of GPCR activity *in vivo*, and suggest that in a cellular context these proteins may interchangeably interact with GPCRs to differentially regulate the recruitment of signaling complexes required for their activation of mitogenic signaling pathways.

3.5   Conclusion

In conclusion, our data demonstrate an interaction between all three MAGI proteins, MAGI-1, MAGI-2 and MAGI-3 with CRFR1 that leads to the regulation of receptor activity. We illustrate that all MAGI proteins can regulate the internalization of the receptor by mediating β-arrestin recruitment. We also provide evidence that MAGI proteins can regulate CRFR1 signaling via the MAPK pathway but not the G protein-dependent pathways presented by cAMP formation. We also suggest a compensatory effect of regulation among the members of the MAGI subfamily. Taken together, these observations along with our previous studies on CRFR1 confirm the distinctive functions of PDZ domain-containing proteins in regulating GPCRs function.

3.6 References

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Chapter 4

4   Role of MAGI proteins in regulating 5-HT$_{2A}$R activity

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4.1   Introduction

The family of serotonin receptors (5-HTRs) consists of 7 receptor types and 15 subtypes that belong to Class A G protein-coupled receptors (GPCRs), except for 5-HT$_3$Rs which are ligand-gated ion channels (Gray and Roth, 2001; Lanfumey et al., 2008). 5-HTRs contribute to the modulation of various biological processes including sleep-wake cycles, appetite, mood, memory, breathing, cognition and sexual behavior (McCorvy and Roth, 2015). Serotonin has been the focus of many pharmacological studies for the treatment of neuropsychiatric disorders with drugs that target either the serotonin receptors or serotonin uptake (Kroeze and Roth, 1998; Roth et al., 2004). Examples include SSRIs (serotonin-selective reuptake inhibitors), SNRIs (dual serotonin-norepinephrine reuptake inhibitors) and partial agonists or antagonists for the 5-HTRs. Among the different serotonin receptor subtypes, the most ubiquitous and extensively studied 5-HTRs in the human brain are the 5-HT$_{1A}$R and 5-HT$_{2A}$R. These receptors have been shown to have potential roles in different neuropsychiatric diseases such as Alzheimer's disease, schizophrenia, depression and suicide (Burnet et al., 1995; Roth et al., 2004).

The 5-HT$_2$R subfamily consists of three subtypes: 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$. The family is primarily coupled to Gα_q leading to the activation of phospholipase Cβ (PLCβ) which hydrolyzes the phosphatidylinositol diphosphate (PIP2) to release diacylglycerol (DAG) and inositol triphosphate (IP3) (Gray and Roth, 2001; Roth et al., 2004). 5-HT$_2$R coupling to Gα_q ultimately culminates in the release of Ca^{2+} from intracellular stores and the subsequent activation of protein kinase C. In addition to activating Gα_q-dependent cell signaling, 5-HTRs can activate the MAPK pathway resulting in the extracellular signal-regulated kinase 1 and 2 (ERK1/2) phosphorylation. 5-HT$_{2A}$R also encodes a class I PDZ-binding motif at the distal end of its carboxyl-terminal tail that consists of three amino acids; serine, cysteine and valine (SCV). It was shown to be regulated by multiple PSD-95/Disc Large/Zona Occludens (PDZ) domain-containing proteins such as SAP97, PSD-95, PDZK1 and MUPP1 (Xia et al., 2003; Jones et al., 2009; Jones et al., 2009; Dunn et al., 2014; Walther et al., 2015). SAP97 and PSD-95 belong to the membrane associated guanylate kinase (MAGUK) subfamily of PDZ proteins (Funke et al., 2005; te Velthuis et al., 2007; Oliva et al., 2012).

The MAGUK family of proteins are synaptic scaffolding proteins that belong to a protein network responsible for the spatial organization of the presynaptic and postsynaptic compartments. One of their primary functions is to regulate the formation of synapses in the central nervous system (Funke et al., 2005; Zhang et al., 2009; Yamagata and Sanes, 2010). MAGUKs are widely-expressed in the brain and periphery and they consist of multiple subfamilies including membrane palmitoylated proteins (MPPs), zona occludens (ZO), caspase recruitment domain containing MAGUK protein (CARMA), discs larges (DLGs) and MAGUK with inverted orientation PDZ proteins (MAGIs) (Oliva et al., 2012). As scaffolding proteins, they contain multiple domains to control their function and facilitate their interactions with their targets. The two common domains among all members are PDZ domains and guanylate kinase (GK) domains (Oliva et al., 2012). One of the important targets for MAGUKs are GPCRs (Dunn and Ferguson, 2015). The protein-protein interaction between GPCRs and MAGUKs have crucial effects on the regulation of GPCR activity (reviewed by Dunn and Ferguson, 2015). MAGUK proteins play a central role in mediating the subcellular localization, trafficking, cell surface expression and signal transduction of multiple GPCRs and different proteins have both overlapping and distinct roles in the regulation of GPCR activity (Harris and Lim, 2001; Lee and Zheng, 2010; Romero et al., 2011; Magalhaes et al., 2012). MAGI protein represent an important subfamily of MAGUK proteins and is comprised of three members: MAGI-1, MAGI-2 and MAGI-3. All three proteins share a similar structure containing one guanylate kinase-like (GK) domain, two tryptophan tryptophan (WW) domains and six PDZ domains (te Velthuis et al., 2007; Oliva et al., 2012; Dunn and Ferguson, 2015).

MAGI-1 is also called BAP-1 (BAI-1-associated protein 1) and has 7 different splice variants that exhibit distinct but overlapping tissue distributions (Dobrosotskaya et al., 1997; Laura et al., 2002). MAGI-2 or S-SCAM (synaptic scaffolding molecule) has two alternative splice variants and is brain-specific, whereas MAGI-3 exhibits a widespread expression pattern. MAGI-1 through its interaction with the brain-specific angiogenesis inhibitor-1 (BAI-1) is proposed to contribute to regulating the cytoskeletal organization and membrane protein distribution, likely by relaying signals via BAI-1 that control both cell- cell and cell- matrix interactions (Shiratsuchi et al., 1998; Stetak et al., 2009; Stephenson et al., 2013). MAGI-2 interacts with β_1-AR in an agonist-dependent

manner to facilitate β_1-AR endocytosis without altering cAMP responses (Xu et al., 2001) In contrast, MAGI-2 negatively regulates the internalization and $G\alpha_s$-coupling of the vasoactive intestinal polypeptide type 1 receptor (VPAC$_1$R) (Gee et al., 2009). MAGI-3 overexpression inhibits β_2-AR-mediated signaling via the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway, whereas a loss of MAGI-3 expression antagonizes lysophosphatidic acid-activated receptor subtype-2 (LPA$_2$R)-stimulated ERK1/2 phosphorylation (Yang et al., 2010). We previously identified all three MAGI protein subtypes as 5-HT$_{2A}$R-interacting proteins using a 96 well PDZ protein array. In the present study we confirm the interaction of each of the MAGI proteins with 5-HT$_{2A}$R and investigate their role in regulating 5-HT$_{2A}$R cell surface expression, trafficking and signaling.

4.2   Materials and Methods

4.2.1   Materials

MAGI-1, MAGI-2 and MAGI-3 antibodies were purchased from Thermo Fisher (Burlington, ON, Canada). Rabbit anti-phospho-p44/42 MAPK (Thr-202/Tyr-402) and rabbit anti-p44/42 MAPK antibodies were obtained from Cell Signaling Technology (Pickering, ON, Canada). Rabbit anti-PLCβ3 was purchased from abcam (Toronto, ON, Canada). ECL Western blotting detection reagents were purchased from Biorad (Mississauga, ON, Canada). Rabbit anti-Flag antibody and all other biochemical reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada).

4.2.2   Plasmids

FLAG-tagged 5-HT$_{2A}$R and 5-HT$_{2A}$R-ΔSCV constructs were described previously (Magalhaes et al., 2010). His-MAGI-1, HA-MAGI-2 and His-MAGI-3 were kindly provided by Dr. Randy Hall (Emory University, School of Medicine). PLCβ3 was kindly provided by Dr. Alan Smrcka (University of Rochester Medical Center). MAGI-1 siRNA, MAGI-2 siRNA and MAGI-3 siRNA were purchased from Thermo Fisher (Burlington, ON, Canada). For the negative controls, we used Silencer Negative Control #1 AM4635 AGUACUGCUUACGAUACGGTT from Invitrogen (Burlington, ON, Canada).

4.2.3   Cell Culture and Transfection

Human embryonic kidney (HEK 293) cells were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Cells were plated on 10-cm dishes 24h prior to transfection. All experiments were performed on 75-80% confluent plates. Transfections were performed using calcium phosphate protocol except for siRNA transfections which were performed using Lipofictamine 2000 following manufacturer's instructions. Transfections were performed with 2 μg of each construct. Empty pcDNA3.1 vector was used to equalize the total amount of plasmid cDNA used to transfect cells. 18h post-transfection, cells were washed with phosphate-buffered saline (PBS) and resuspended with trypsin, 0.25% EDTA. Experiments were performed approximately 48h after transfection except for knockdown experiments which were performed 72h post

transfection since this protocol resulted in the maximum knockdown of the different MAGI proteins.

4.2.4   Co-immunoprecipitation

24h after transfection, HEK 293 cells were seeded onto 10-cm dishes. Cells were starved in HBSS lacking serum for 1h at 37°C then stimulated with 10 µM 5-HT for 30 mins. Cells were then lysed in 500 µL lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10 µg/ml leupeptin, and 2.5 µg/ml aprotinin) for 20 min on a rocking platform at 4°C. Samples were collected into 1.5 ml Eppendorf tubes and centrifuged at 15,000 g for 15 min at 4°C to pellet insoluble material. A Bradford protein assay was performed and 300 µg of protein was incubated overnight at 4°C with 40 µl Flag-immunoprecipitation beads. Beads were washed three times with cold lysis buffer and incubated overnight at room temperature in 3xSDS loading buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and western blots were then performed with the indicated antibodies (rabbit anti- Flag, 1:1000), (rabbit anti-MAGI-2, 1:1000), (rabbit anti-MAGI-3, 1:1000), (rabbit anti- V5, 1:1000), (rabbit anti-PLCβ3, 1:1000).

4.2.5   Measurement of Inositol Phosphate Formation

HEK 293 cells were transiently co-transfected with 2 µg Flag-5-HT$_{2A}$R or Flag-5-HT$_{2A}$R-ΔSCV with 2 µg of MAGI-1, MAGI-2 or MAGI-3. 48h post-transfection cells were incubated overnight in inositol-free Dulbecco's modified Eagle's medium with 1 µCi/ml myo-[^{3}H]-Inositol. For all experiments cells were incubated for 1h in warm HBSS and were then incubated with 10 mM LiCl alone for 10 minutes followed by increasing doses of 5-HT in LiCl for 30 minutes. Cells were placed on ice and the reaction was stopped with 500 µl of perchloric acid and was neutralized with 400 µl of 0.72 M KOH, 0.6 M KHCO$_3$. Total cellular [^{3}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 [^{3}H] inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system.

Alternatively for siRNA experiments, HEK 293 cells were transiently transfected with 2 µg Flag-5-HT$_{2A}$R or Flag-5-HT$_{2A}$R-ΔSCV with either MAGI-1, MAGI-2 or MAGI-3 siRNA as described in the figure legends. 24h post-transfection, cells were reseeded into 96-well poly-lysine coated plates. Cells were left to recover for another 24h and were then incubated overnight in inositol-free Dulbecco's modified Eagle's medium with 1 µCi/well myo-[^{3}H]-Inositol. On the next day, cells were incubated for 1h in HBSS and were then incubated with 15 mM LiCl for 15 minutes followed by increasing doses of 5-HT in LiCl for 30 minutes. Cells were then lysed with 50 mM formic acid. 30 µl of cell lysates were mixed with 75 µl of 2.67 mg/ml of SPA beads diluted in water (RNA binding YSi beads, GE Healthcare) in a 96-well/plate and agitated at 4°C for 3h. The radioactivity was counted with a Wallac MicroBeta luminescence counter (PerkinElmer).

4.2.6   Receptor Endocytosis

Following transfection, HEK 293 cells were re-seeded into 12-well plates. Cells were serum-starved in HBSS lacking serum for 1h at 37°C then stimulated for 0 or 30 min with 10 µM 5-HT in HBSS at 37 °C. Cells were washed with cold HBSS and incubated with mouse anti-Flag antibody (1:1000) for 1h on ice. Cells were washed with cold HBSS then labeled with Alexa Fluor 647 donkey anti-mouse IgG (1:1000) for 1h on ice in the dark. Cells were washed with cold HBSS and treated with 5 mM EDTA in PBS for 5 min on ice. Cells were collected and transferred to flow cytometry tubes containing 4% formaldehyde in PBS. Samples were run on a FACS Calibur cytometer using BD Cell Quest Pro software (BD Biosciences, Mississauga, ON) until 10,000 cells were counted. The geometric mean of fluorescence was determined using Flow Jo analysis software and was representative of the expression levels of the receptor on the plasma membrane (BD Biosciences, Mississauga, ON).

4.2.7   Western Blot Analysis

75-90 µl of the samples from the different assays which is equivalent to about 50-70 µg of protein was diluted in β-mercaptoethanol-containing 3xSDS loading buffer and then applied to 10% SDS-PAGE (30% acrylamide mix, 1.5 M tris-HCl, 20% SDS, 10% ammonium persulfate and TEMED). Separated proteins were then transferred to

nitrocellulose membranes and membranes were then blocked in 10% milk in TBS for 1h. Membranes were then blotted overnight by incubation with the appropriate antibody at 4°C. 24h later membranes were washed at least three times with 1X TBS with 0.05% Tween 20, and then incubated with a horseradish peroxidase- conjugated secondary antibody (1:10,000) for 1h. Membranes were finally washed again with 1X TBS with 0.05% Tween 20 three times before being developed using a BioRad chemiluminescence system.

4.2.8   ERK Phosphorylation

Following 48h of transfection, HEK 293 cells were re-seeded into 6-well plates. Cells were serum starved overnight in DMEM. On the day of the experiment, cells were starved for 1h at 37°C in HBSS and then stimulated with 10 µM 5-HT agonist for 0, 5 and 10 min. Cells were lysed with lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10 µg/ml leupeptin, and 2.5 µg/ml aprotinin) and phosphatase inhibitors (10 mM NaF and 500 µM Na_3VO_4) for 20 min on a rocking platform at 4°C. Samples were collected into 1.5 ml Eppendorf tubes and centrifuged at 15,000 g for 15 min at 4°C to pellet insoluble material. A Bradford protein assay was performed, and 30–50 µg of protein was incubated overnight at room temperature in 3xSDS loading buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and western blot analysis was performed for ERK1/2 (rabbit anti-p44/42 MAPK, 1:1000), phospho-ERK1/2 (rabbit anti-phospho-p44/42 MAPK, 1:1000), MAGI antibodies (1:1000), Flag-5-HT_{2A}R (rabbit anti-Flag, 1:1000).

4.2.9   Statistical Analysis

All measurements are represented as mean ± SEM. Comparisons were performed using one way analysis of variance test (ANOVA) followed by Bonferroni's or Dunn's Multiple comparisons test to determine significance. * indicate P values less than 0.05 and is considered to be significant.

4.3   Results

4.3.1   MAGI proteins co-immunoprecipitate with Flag-5-HT$_{2A}$R

All the MAGI subfamily proteins (MAGI-1, 2 and 3) were identified using a PDZ domain proteomic array as potential 5-HT$_{2A}$R-interacting proteins (unpublished data). To validate this interaction we assessed whether V5-His-MAGI-1, HA-MAGI-2 and V5-His-MAGI-3 were co-immunoprecipitated with either Flag-tagged 5-HT$_{2A}$R (Flag-5-HT$_{2A}$R) or a Flag-5-HT$_{2A}$R construct lacking the PDZ binding motif (Flag-5-HT$_{2A}$R-ΔSCV). We found that all of the MAGI proteins were effectively co-immunopreciptated with Flag-5-HT$_{2A}$R, but not Flag-5-HT$_{2A}$R-ΔSCV (Fig. 4.1A-C). However, the interaction between Flag-5-HT$_{2A}$R and each of the MAGI proteins was not altered by agonist activation of the receptor (Fig. 4.1D).

4.3.2   Effect of MAGI proteins on agonist-stimulated 5-HT$_{2A}$R endocytosis

MAGI proteins were previously shown to regulate the endocytosis of other GPCRs, such as VPAC$_1$ and β$_1$-AR (Xu et al., 2001; Gee et al., 2009). In addition, the internalization of both CRFR1 and 5-HT$_{2A}$R was shown to be mediated by the MAGUK protein SAP97 (Dunn et al., 2013; Dunn et al., 2014). Therefore, we assessed the effects of MAGI protein overexpression and knockdown on both Flag-5-HT$_{2A}$R and Flag-5-HT$_{2A}$R-ΔSCV internalization by flow cytometry. We found that the overexpression of each of the MAGI proteins had no effect on either Flag-5-HT$_{2A}$R or Flag-5-HT$_{2A}$R-ΔSCV internalization following agonist treatment with 10 μM 5-HT for 30 min (Fig. 4.2A-C). In contrast, while MAGI-2 siRNA knockdown did not alter either Flag-5-HT$_{2A}$R or Flag-5-HT$_{2A}$R-ΔSCV endocytosis, MAGI-3 knockdown significantly decreased Flag-5-HT$_{2A}$R internalization (Fig 4.2D and 4.2E). Thus, MAGI protein expression could differentially regulate 5-HT$_{2A}$R endocytosis.
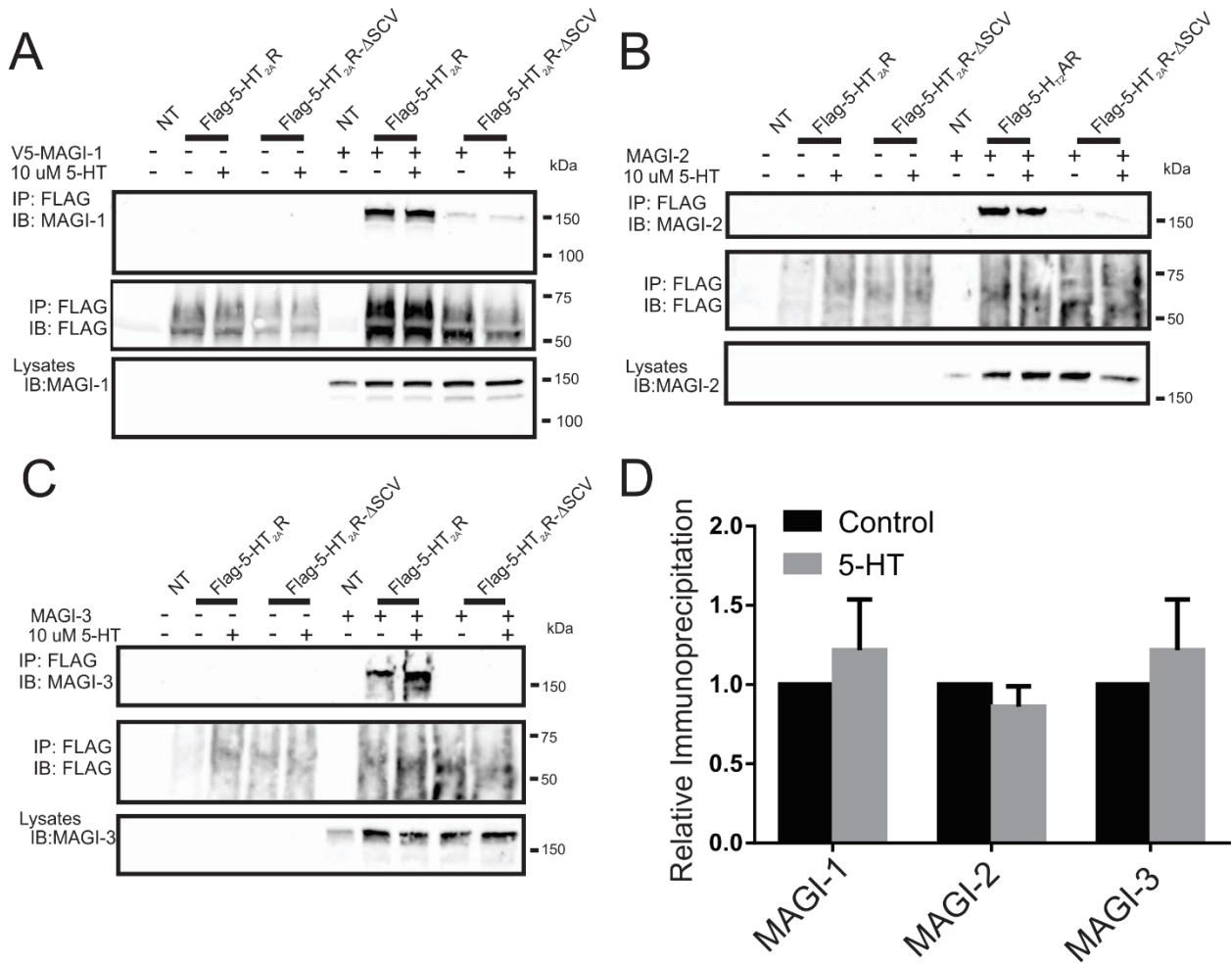
Figure 4.1: MAGI proteins co-immunoprecipitate with Flag-5-HT$_{2A}$R in a PDZ-binding motif-dependent manner and an agonist-independent manner: HEK 293 cells were co-transfected with Flag-5-HT$_{2A}$R (WT or ΔSCV) and pcDNA3.1 along with MAGI-1, MAGI-2 or MAGI-3. Shown are representative immunoblots for the co-immunoprecipitation (IP) of **(A)** MAGI-1, **(B)** MAGI-2, and **(C)** MAGI-3 with Flag-5-HT$_{2A}$R, but not Flag-5-HT$_{2A}$R-ΔSCV which lacks the PDZ-binding motif. **(D)** Densitometric quantification of changes in agonist-stimulated (10 μM 5-HT) co-immunoprecipitation of MAGI-1, MAGI-2 or MAGI-3 with Flag-5-HT$_{2A}$R. Data are representative of 3-4 independent experiments. NT: non-transfected.
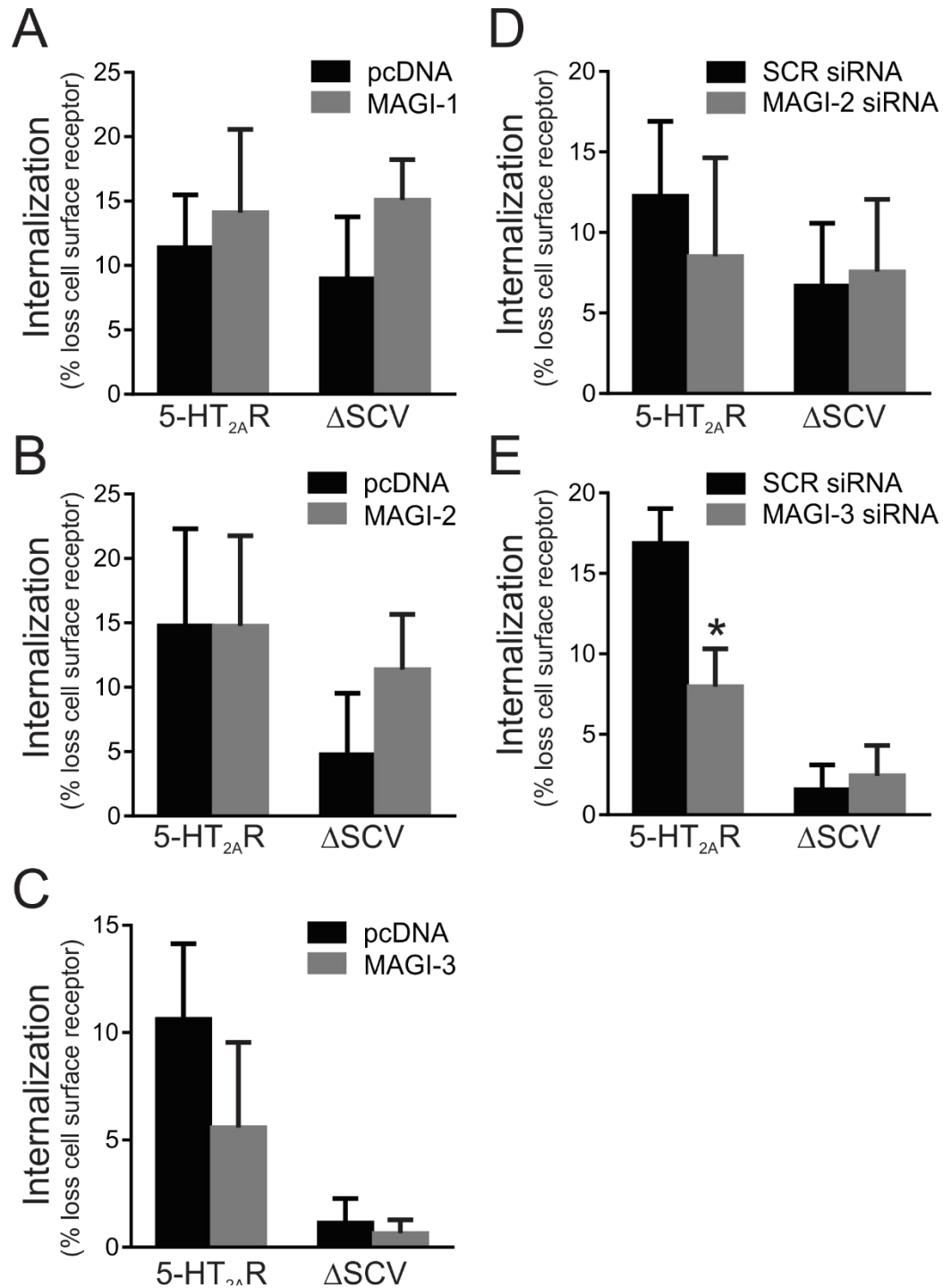
Figure 4.2: Effect of MAGI proteins on 5-HT$_{2A}$R endocytosis: Agonist-stimulated internalization of Flag-5-HT$_{2A}$R and Flag-5-HT$_{2A}$R-ΔSCV in response to 10 μM 5-HT for 30 min in HEK 293 cells upon overexpression of either **(A)** MAGI-1, **(B)** MAGI-2 or **(C)** MAGI-3. Agonist-stimulated internalization of Flag-5-HT$_{2A}$R and Flag-5-HT$_{2A}$R-ΔSCV in response to 10 μM 5-HT for 30 min in HEK 293 cells upon siRNA knockdown of either **(D)** MAGI-2 or **(E)** MAGI-3. ΔSCV, Flag-5-HT$_{2A}$R-ΔSCV. * P < 0.05 versus pcDNA or scrambled siRNA peptide (SCR) control.

4.3.3   MAGI proteins modulate 5-HT$_{2A}$R-stimulated IP formation

5-HT$_{2A}$R can couple to G$\alpha_{q/11}$ protein resulting in the activation of PLC and the formation of DAG and IP3. Both SAP97 and PSD-95 regulate 5-HT-mediated IP formation where knockdown of SAP97 reduces IP signaling and overexpression of PSD-95 enhances it (Xia et al., 2003; Dunn et al., 2014). Therefore, we examined whether either the overexpression or knockdown of MAGI proteins can alter 5-HT-stimulated IP formation in either Flag-5-HT$_{2A}$R or Flag-5-HT$_{2A}$R-ΔSCV expressing HEK 293 cells. We found that the overexpression of each of the MAGI proteins significantly increased the maximum response for 5-HT-stimulated IP formation mediated by both Flag-5-HT$_{2A}$R and Flag-5-HT$_{2A}$R-ΔSCV, compared with cells transfected with receptor alone (Fig. 4.3A-C). In contrast, we found that whereas siRNA knockdown of endogenous MAGI-1 or -3 had no effect upon Flag-5-HT$_{2A}$R-stimulated IP formation, knockdown of MAGI-2 resulted in a reduction of Flag-5-HT$_{2A}$R-induced IP formation significantly (Fig. 4.3D). The siRNA knockdown of either MAGI-2, or -3 resulted in a significant reduction of Flag-5-HT$_{2A}$R-ΔSCV-mediated IP formation (Fig. 4.3E). This suggested that this effect does not require a direct interaction between the receptor and the MAGI proteins, because each of the MAGI proteins did not immunoprecipitate with the truncated receptor mutant.

4.3.4   MAGI proteins can enhance cell surface trafficking of 5-HT$_{2A}$R to the plasma membrane

MAGUK proteins were previously shown to regulate the cell surface expression of the CRFR1 (Dunn et al., 2013; Dunn et al., 2016). Therefore, we examined whether either the overexpression or the siRNA knockdown of MAGI-1, -2 or -3 altered the cell surface expression of 5-HT$_{2A}$R. We found that the overexpression of MAGI-1 did not alter cell surface expression of either Flag-5-HT$_{2A}$R or Flag-5-HT$_{2A}$R-ΔSCV (Fig. 4.4A). In contrast, the overexpression of both MAGI-2 and MAGI-3 increased the cell surface expression of Flag-5-HT$_{2A}$R, whereas MAGI-3 also increased the plasma membrane expression of Flag-5-HT$_{2A}$R-ΔSCV (Fig. 4.4B and 4.4C). The siRNA-mediated knockdown of both MAGI-2 and MAGI-3 did not alter the relative cell surface expression of either Flag-5-HT$_{2A}$R or Flag-5-HT$_{2A}$R-ΔSCV (Fig. 4.4D and 4.4E).
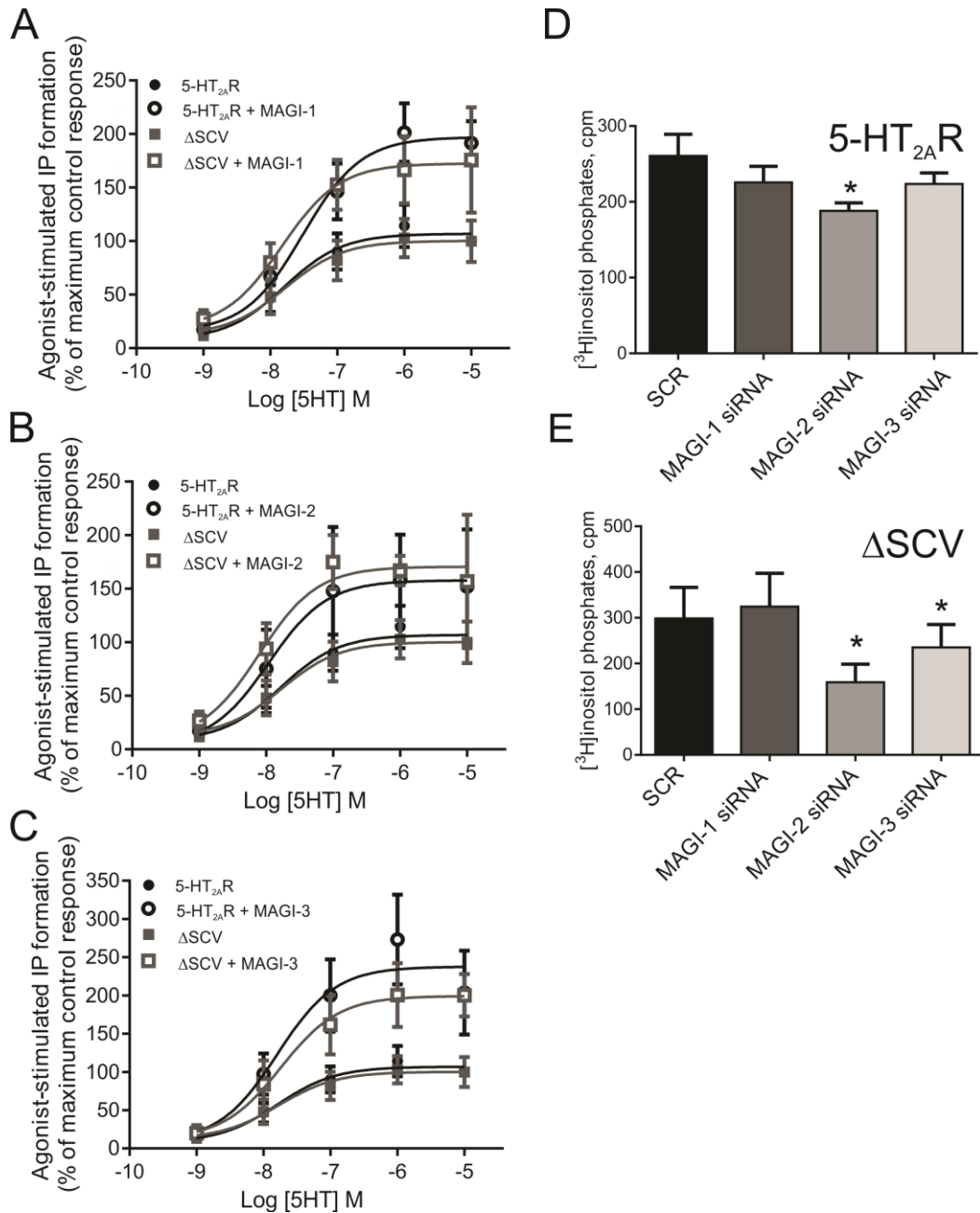
Figure 4.3: MAGI proteins regulate 5-HT₂AR-stimulated IP formation in PDZ motif-independent manner: Dose response curves for 5-HT-stimulated (30 min) inositol phosphate (IP) formation in HEK 293 cells co-transfected with either Flag-5-HT$_{2A}$R or Flag-5-HT$_{2A}$R-ΔSCV along with either **(A)** MAGI-1, **(B)** MAGI-2 or **(C)** MAGI-3. Inositol phosphate formation in cells expressing either **(D)** Flag-5-HT$_{2A}$R or **(E)** Flag-5-HT$_{2A}$R-ΔSCV along with MAGI-1 siRNA, MAGI-2 siRNA or MAGI-3 siRNA and treated with 10 μM 5-HT for 30 min. * P < 0.05 versus scramble (SCR) peptide treated control. ΔSCV, Flag-5-HT$_{2A}$R-ΔSCV.
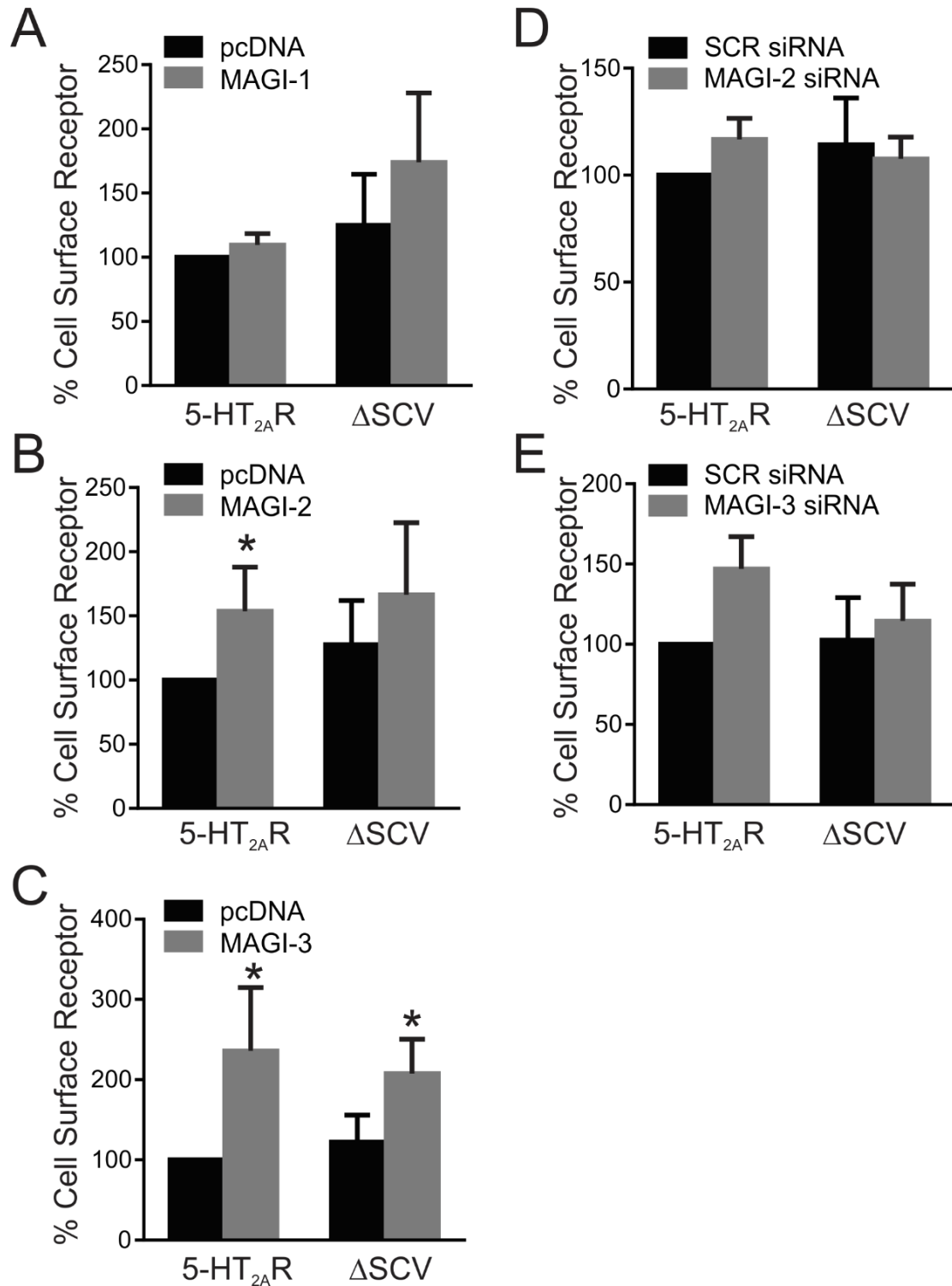
Figure 4.4: Effect of MAGI proteins on the trafficking of 5-HT$_{2A}$R to the plasma membrane: Cell surface expression of Flag-5-HT$_{2A}$R and Flag-5-HT$_{2A}$R-ΔSCV at basal level following the overexpression of MAGI-1 (**A**) or MAGI-2 (**B**) or MAGI-3 (**C**) or the knockdown of MAGI-2 (**D**) or MAGI-3 (**E**). ΔSCV, Flag-5-HT$_{2A}$R-ΔSCV. * P < 0.05 versus pcDNA or scrambled peptide (SCR) control.

4.3.5   MAGI-3 can enhance the recruitment of PLCβ3 to 5-HT$_{2A}$R

Overexpression of MAGI proteins appeared to significantly enhance 5-HT$_{2A}$R signaling via Gα$_{q/11}$ pathway for which the key effector enzyme is PLCβ. It was previously reported that MAGI-3 could interact directly with PLCβ3 to alter LPA$_2$ receptor-stimulated IP formation (Lee et al., 2011). Therefore, we assessed by co-immunoprecipitation whether PLCβ3 could be co-immumoprecipitated with both Flag-5-HT$_{2A}$R and Flag-5-HT$_{2A}$R-ΔSCV and whether this was influenced either by agonist treatment or by MAGI-3 overexpression. We found that PLCβ3 could be co-immunoprecipitated with Flag-5-HT$_{2A}$R and that 5-HT treatment (10 μM) for 30 min increased the association with the receptor (Fig. 4.5A and 4.5B). In contrast, PLCβ3 co-immunoprecipitation with Flag-5-HT$_{2A}$R-ΔSCV was enhanced when compared to Flag-5-HT$_{2A}$R but did not exhibit agonist regulation. Interestingly, we observe a stronger interaction between the Flag-5-HT$_{2A}$R and PLCβ3 in the presence of MAGI-3, but not Flag-5-HT$_{2A}$R-ΔSCV. This could provide another possible mechanism to explain the enhancement in 5-HT-stimulated IP formation.

4.3.6   Effect of MAGI proteins on regulating 5-HT-mediated ERK1/2 phosphorylation

MAGI proteins were previously shown to have distinct effects on ERK1/2 signaling since it has been reported that MAGI-3 can enhance BAI-1- and LPA$_2$R- mediated ERK1/2 phosphorylation and attenuate the same pathway if the β$_1$- and β$_2$- adrenergic receptors are stimulated (He et al., 2006; Zhang et al., 2007; Yang et al., 2010; Stephenson et al., 2013). Therefore, we assessed whether MAGI overexpression or siRNA-mediated knockdown of endogenous MAGI proteins would alter Flag-5-HT$_{2A}$R-mediated ERK1/2 phosphorylation in response to 10 μM 5-HT treatment for 5, 10 and 30 min. We found that the overexpression of MAGI-1, MAGI-2 or MAGI-3 had no effect on ERK1/2 signaling (Fig. 4.6A-F). However, when siRNA was used to silence the expression of endogenous MAGI proteins, we found a significant increase in the signal following stimulation with 10 μM 5-HT for 5, 10 and 30 min for MAGI-1 (Fig. 4.6A and 4.6B), 5 and 10 min for MAGI-2 (Fig 4.6C and 4.6D), but only 5 min for MAGI-3 (Fig. 4.6E and 4.6F). When ERK1/2 phosphorylation was tested in response to Flag-5-HT$_{2A}$R-ΔSCV activation in the presence
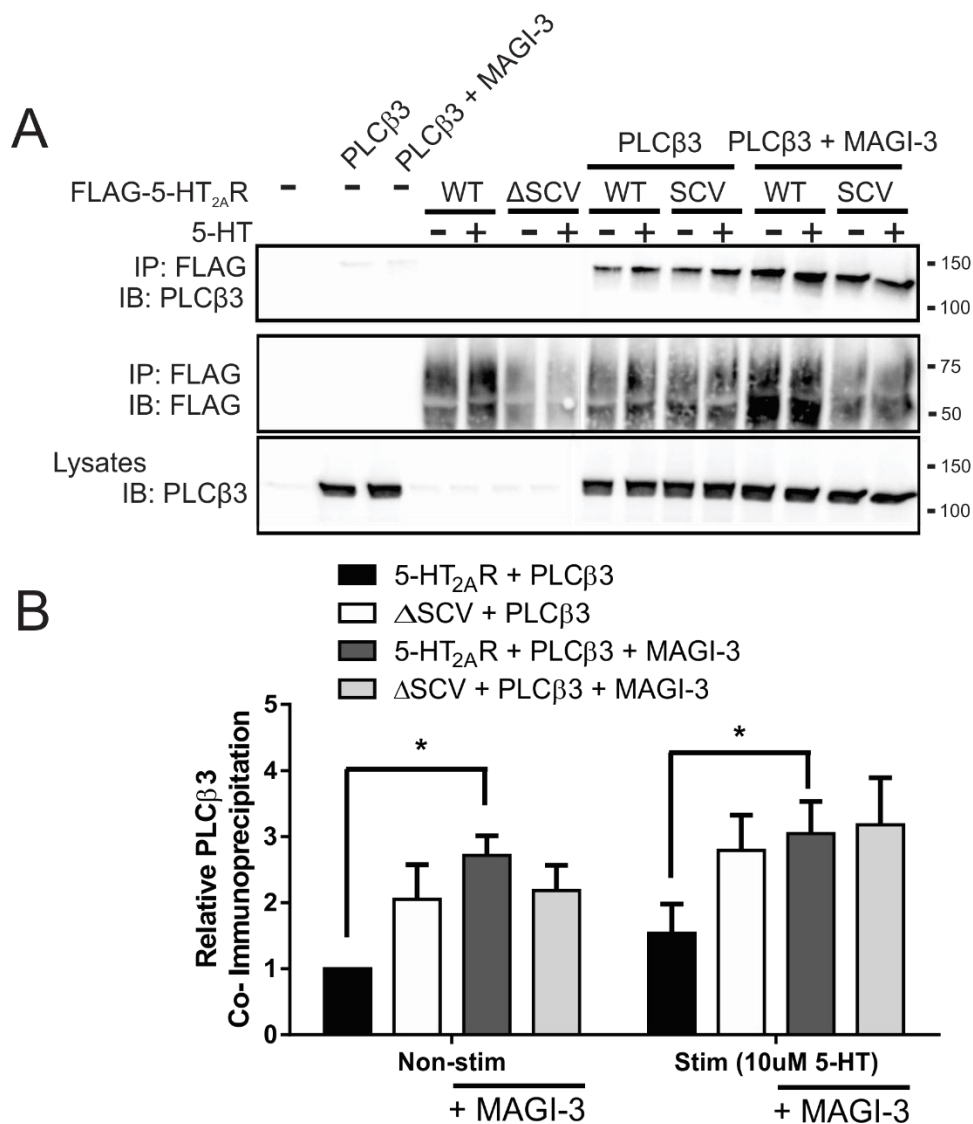
Figure 4.5: MAGI-3 can enhance the association of PLCβ3 with 5-HT₂ₐR: A) HEK 293 cells were co-transfected with Flag-5-HT₂ₐR (WT or ΔSCV) and PLCβ3 or MAGI-3 + PLCβ3. Representative immunoblot of PLCβ3 co-immunoprecipitated (IP) with both Flag-5-HT₂ₐR and Flag-5-HT₂ₐR-ΔSCV. SDS-PAGE was used to analyze samples and immunoblots were performed with rabbit anti-PLCβ3. **B)** Densitometric analysis of the effect of MAGI-3 co-transfection. Data are representative of four independent experiments. * P < 0.05 versus 5-HT₂ₐR + PLCβ3.

of overexpressed MAGI proteins no change in phosphorylation was observed (Fig.4.7A-F). In contrast, siRNA knockdown of MAGI-1 increased Flag-5-HT$_{2A}$R-ΔSCV-stimulated ERK1/2 phosphorylation following 5 and 10 min 5-HT treatment (Fig. 4.7A and 4.7B) and MAGI-2 knockdown increased ERK1/2 phosphorylation in response to 10 min 5-HT treatment (Fig. 4.7C and 4.7D). MAGI-3 knockdown had no effect on Flag-5-HT$_{2A}$R-ΔSCV activated ERK1/2 phosphorylation (Fig. 4.7E and 4.7F).
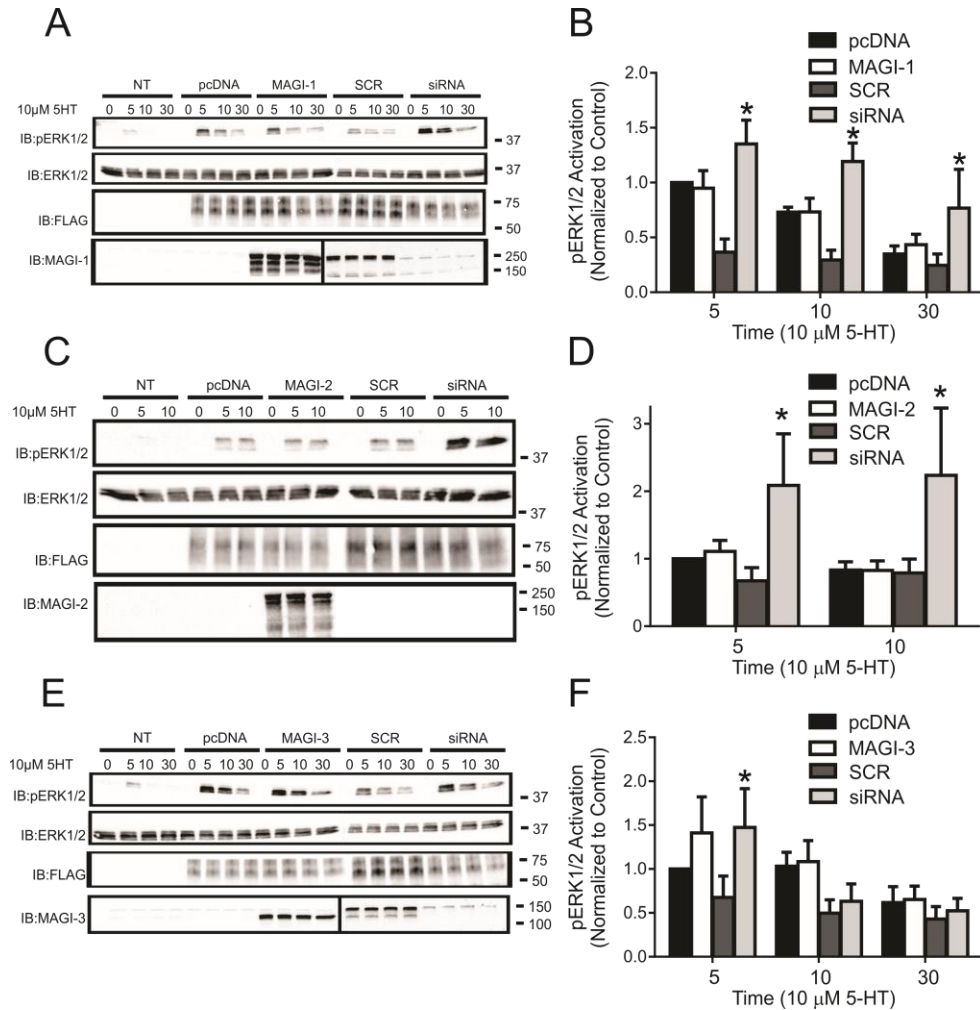
Figure 4.6: Effect of MAGI proteins on 5-HT$_{2A}$R-mediated ERK1/2 signaling: (A) Representative immunoblot showing ERK1/2 phosphorylation in response to 10 µM treatment for 0, 5, 10, and 30 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with Flag-5-HT$_{2A}$R along with either pcDNA, MAGI-1, SCR siRNA or MAGI-1 siRNA. Also shown are corresponding immunoblots for total ERK1/2, MAGI-1 and Flag-5-HT$_{2A}$R protein expression. (B) Densitometric analysis of ERK1/2 phosphorylation in response to 10 µM treatment for 0, 5, 10, and 30 min. The data represent the mean ± SEM of four independent experiments. (C) Representative immunoblot showing ERK1/2 phosphorylation in response to 10 µM treatment for 0, 5, and 10 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with Flag-5-HT$_{2A}$R along with either pcDNA, MAGI-2, SCR siRNA or MAGI-2 siRNA. Also shown are corresponding immunoblots for total ERK1/2, MAGI-2 and Flag-5-HT$_{2A}$R protein expression. (D) Densitometric analysis of ERK1/2 phosphorylation in response to 10 µM treatment for 0, 5, and 10 min. The data represent the mean ± SEM of four independent experiments. E) Representative immunoblot showing ERK1/2 phosphorylation in response to 10 µM treatment for 0, 5, 10, and 30 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with Flag-5-HT$_{2A}$R along with either pcDNA, MAGI-3, SCR siRNA or MAGI-3 siRNA. Also shown are corresponding immunoblots for total ERK1/2, MAGI-3 and Flag-5-HT$_{2A}$R protein expression. (F) Densitometric analysis of ERK1/2 phosphorylation in response to 10 µM treatment for 0, 5, 10, and 30 min. The data represent the mean ± SEM of four independent experiments. * P < 0.05 versus pcDNA or scramble peptide (SCR) control.
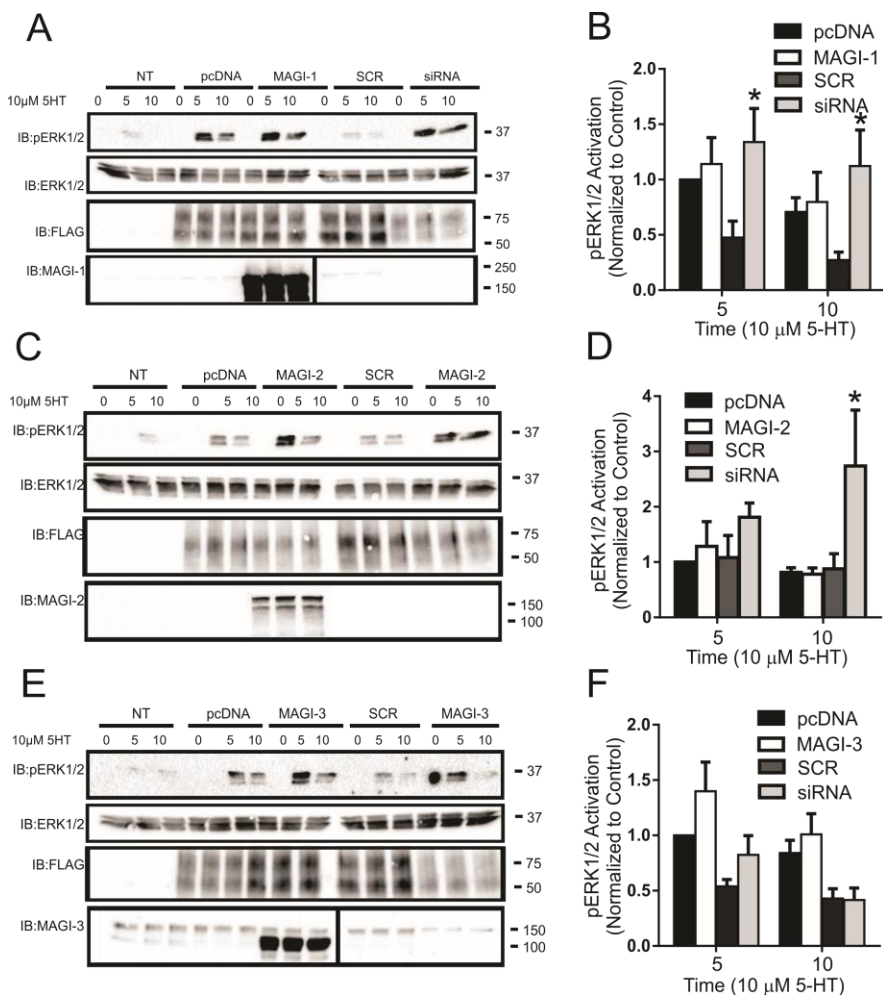
Figure 4.7: Effect of MAGI proteins on 5-HT$_{2A}$R-ΔSCV-mediated ERK1/2 signaling: (A) Representative immunoblot showing ERK1/2 phosphorylation in response to 10 µM treatment for 0, 5, and 10 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with Flag-5-HT$_{2A}$R-ΔSCV along with either pcDNA, MAGI-1, SCR siRNA or MAGI-1 siRNA. Also shown are corresponding immunoblots for total ERK1/2, MAGI-1 and Flag-5-HT$_{2A}$R-ΔSCV protein expression. (B) Densitometric analysis of ERK1/2 phosphorylation in response to 10 µM treatment for 0, 5, and 10 min. The data represent the mean ± SEM of four independent experiments. (C) Representative immunoblot showing ERK1/2 phosphorylation in response to 10 µM treatment for 0, 5, and 10 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with Flag-5-HT$_{2A}$R-ΔSCV along with either pcDNA, MAGI-2, SCR siRNA or MAGI-2 siRNA. Also shown are corresponding immunoblots for total ERK1/2, MAGI-2 and Flag-5-HT$_{2A}$R-ΔSCV protein expression. (D) Densitometric analysis of ERK1/2 phosphorylation in response to 10 µM treatment for 0, 5, and 10 min. The data represent the mean ± SEM of four independent experiments. E) Representative immunoblot showing ERK1/2 phosphorylation in response to 10 µM treatment for 0, 5, and 10 min in non-transfected (NT) HEK 293 cells, and HEK 293 cells transfected with Flag-5-HT$_{2A}$R-ΔSCV along with either pcDNA, MAGI-3, SCR siRNA or MAGI-3 siRNA. Also shown are corresponding immunoblots for total ERK1/2, MAGI-3 and Flag-5-HT$_{2A}$R-ΔSCV protein expression. (F) Densitometric analysis of ERK1/2 phosphorylation in response to 10 µM treatment for 0, 5, and 10 min. The data represent the mean ± SEM of four independent experiments. * P < 0.05 versus pcDNA or scramble peptide (SCR) control.

4.4   Discussion

Recent studies have illustrated the importance of PDZ domain-containing proteins in the regulation of GPCR activity (Dunn and Ferguson, 2015). Furthermore, studies on the 5-HT$_{2A}$R demonstrated the roles of MAGUKs such as PSD-95 (Xia et al., 2003) and SAP97 (Dunn et al., 2014) and other PDZ proteins including MUPP1 (Jones et al., 2009) and PDZK1 (Walther et al., 2015) in mediating the trafficking and signaling of the receptor. In a PDZ overlay assay performed in our laboratory, we found that all three MAGI proteins can associate with the carboxyl-terminal tail of 5-HT$_{2A}$R (unpublished data). Therefore, we decided to further characterize this interaction and determine its functional consequences on the signaling and trafficking of 5-HT$_{2A}$R. Despite the similarity in sequence and domain structure of these proteins, we hypothesized that they would exhibit distinct functions. We find that MAGI proteins regulate both IP3 and ERK1/2 signaling downstream of the activation of the 5-HT$_{2A}$R. We propose that the observed increase in 5-HT-mediated IP3 signaling is mediated at least in part by the regulated recruitment of PLCβ to the receptor. We also show that silencing the expression of endogenous MAGI proteins using siRNA can enhance 5-HT-mediated ERK1/2 signaling.

MAGI-2 has been shown to play an important role in regulating the trafficking and signaling of multiple receptors including β$_1$-AR and VPAC$_1$ (Xu et al., 2001; Gee et al., 2009). In the current study, we were able to show an effect of MAGI-2 knockdown on regulating ERK1/2 activation. This is the first report to implicate MAGI-2 with the MAPK pathway. Our data also showed that MAGI-2 can regulate IP formation where overexpression resulted in an enhancement of the signal and knockdown reduced it. This is the first report of MAGI-2 regulation of a Gα$_q$-dependent pathway as well, however MAGI-2 was previously shown to regulate Gα$_s$-mediated cAMP formation by VPAC$_1$ (Gee et al., 2009). Despite previous reports indicating an effect of MAGI-2 overexpression on GPCR internalization where it had a positive effect on β$_1$-AR (Xu et al., 2001) and a negative effect on VPAC$_1$ (Gee et al., 2009), our observations indicated that MAGI-2 did not affect the endocytosis of 5-HT$_{2A}$R. However, we observed a significant increase in receptor expression levels upon MAGI-2 overexpression. Therefore, it seems that MAGI-2 is involved in regulating both the signaling and trafficking of 5-HT$_{2A}$R.

MAGI-3 has also been shown to regulate the signaling of a number of GPCRs including LPA$_2$R and β-adrenergic receptors (Zhang et al., 2007; Yang et al., 2010; Lee et al., 2011). Our current study showed that MAGI-3 can regulate both G protein-dependent as well as G protein- independent signaling pathways. Our data demonstrated that MAGI-3 can regulate IP formation in a PDZ motif-independent manner. This is illustrated by the enhancement in IP formation mediated by both 5-HT$_{2A}$R and 5-HT$_{2A}$R-ΔSCV upon overexpression of MAGI-3 and the reduction in this effect upon MAGI-3 knockdown. The effect of MAGI-3 knockdown was statistically different only for the 5-HT$_{2A}$R-ΔSCV-tranfected cells despite a trend for reduction with the 5-HT$_{2A}$R-transfected cells suggesting that the assay needs to be repeated for more conclusive statistical data. Interestingly, opposite effects were previously observed when LPA-induced IP formation was studied where MAGI-3 overexpression resulted in a decrease in IP formation and MAGI-3 knockdown led to an enhancement in this signaling pathway (Lee et al., 2011). We showed that MAGI-3 could also mediate the MAPK cascade. While overexpression of MAGI-3 did not have an effect on ERK1/2 activation, knockdown led to an enhancement in ERK1/2 signaling. Interestingly, this effect is opposite to what was reported with LPA$_2$R where MAGI-3 knockdown reduced ERK activity (Zhang et al., 2007). However, similar to our study, the LPA$_2$R study which was performed on SW480 cells reported no difference in ERK activity upon overexpression of MAGI-3 suggesting that different tissues and cell lines express endogenous MAGI-3 in high levels (Zhang et al., 2007). Nonetheless, studies on both β$_1$-AR and β$_2$-AR showed that MAGI-3 overexpression could lead to a decrease in ERK1/2 signaling (He et al., 2006; Yang et al., 2010). It is noteworthy to mention that MAGI-3 was also shown to regulate Gα$_{12/13}$-mediated RhoA activation by LPA$_2$R (Zhang et al., 2007). Taken together, our data and previous studies confirmed the importance of MAGI-3 in regulating GPCR signaling via multiple pathways and the distinct effects it elicits based on the receptor being investigated. In addition to its role on signaling, we also illustrated a role for MAGI-3 on 5-HT$_{2A}$R internalization where MAGI-3 knockdown resulted in a significant reduction in the internalization of the receptor. This is the first report of a role for MAGI-3 in receptor endocytosis and as mentioned previously, MAGI-2 was usually the MAGI protein responsible for this regulation for other receptors (Xu et al., 2001; Gee et al., 2009). This emphasized the importance of investigating all PDZ

proteins that interact with a receptor to gain complete understanding of how they regulate its function.

To our knowledge, no previous reports studied the functional role of MAGI-1/GPCR interactions. MAGI-1 was first characterized as a BAI-1 (Brain-specific angiogenesis inhibitor 1) interacting protein and has been shown to co-immunoprecipitate with $VPAC_1$, but more studies are required to investigate the role it plays in regulating GPCRs. Data from our current study suggest that MAGI-1 seems to be more important for regulation of $5\text{-}HT_{2A}R$ signaling as opposed to its trafficking. We demonstrate that MAGI-1 overexpression can increase IP formation, while its knockdown can enhance ERK1/2 activation.

We proposed two mechanisms of regulation for $5\text{-}HT_{2A}R$ signaling by MAGI proteins. First, the increase in receptor expression level at the plasma membrane could partly explain the enhancement in IP formation upon overexpression. However, this would not apply to MAGI-1 which despite enhancing IP signaling had no effect on cell surface levels. It also does not completely explain the fact that this effect on IP formation did not require an intact PDZ motif, while the effect on receptor expression level was PDZ-dependent for MAGI-2 and PDZ-independent for MAGI-3. Second, the other mechanism we presented here involved the effect of MAGIs on PLCβ3 recruitment to the receptor. We specifically tested MAGI-3, since it has been shown to interact with PLCβ3 (Lee et al., 2011). We found that MAGI-3 overexpression can increase the association between $5\text{-}HT_{2A}R$ and PLCβ3. However, although the representative blot in Fig. 4.5A shows this enhancement with $5\text{-}HT_{2A}R\text{-}\Delta SCV$, it was not a reproducible effect and therefore, we could not conclude that it was PDZ-independent. These mechanisms might also provide a general MAGUK regulation of IP signaling since both SAP97 and PSD-95 were shown previously to mediate IP formation (Xia et al., 2003; Dunn et al., 2014). Another possibility that can be explored is to examine whether MAGI proteins can affect the coupling of $G\alpha_q$ to $5\text{-}HT_{2A}R$ in a PDZ motif-independent manner. This is particularly important because MAGI-3 has been shown to associate with different G proteins and has been suggested to bias the coupling of different G proteins to LPA_2R (Lee et al., 2011).

The mechanism of regulation of the MAPK cascade remains unknown and further studies are required to understand it. However, one speculation would be an effect of the MAGI proteins on the recruitment of β-arrestin to the receptor. The activation of MAP kinase pathway by GPCRs can be mediated by both arrestin- dependent and independent pathways and previous studies showed a role for multiple PDZ proteins in mediating β-arrestin recruitment either by enhancing it or by interfering with it (Hu et al., 2002; Hammad et al., 2010).

Now that we have demonstrated the distinct roles of MAGI proteins in regulating the function of the 5-HT$_{2A}$R in HEK 293 cells, it would be important to further examine this association and its physiological consequences *in vivo*. RT-PCR in mouse tissue revealed that MAGI proteins are expressed in different regions of the brain including the cortex, thalamus and cerebellum (Sugnet et al., 2006). We would predict that MAGI proteins are expressed in serotonergic neurons where they co-localize with 5-HT$_{2A}$R but their effects need to be explored. In light of the involvement of 5-HT$_{2A}$R in multiple mental disorders such as schizophrenia and anxiety, it will also be important to study how 5-HT$_{2A}$R- MAGIs interactions contribute to using the receptor as a pharmacological target (Gray and Roth, 2001; Roth et al., 2004). In fact, a study showed that PSD-95, another MAGUK protein, is essential for the actions of atypical antipsychotics on 5-HT$_2$Rs. Therefore, a better understanding of PDZ protein regulation of 5-HT$_{2A}$R function *in vivo* is vital.

In conclusion, in this study, we compare the function of three PDZ proteins in regulating 5-HT$_{2A}$R activity. This is unique because previous studies have focused on one MAGI protein based on the strength of interaction or tissue distribution despite reporting the interaction with at least two of the MAGI proteins (Xu et al., 2001; Zhang et al., 2007; Gee et al., 2009). It will be important to further investigate the dynamics between the three MAGI proteins to determine if they have synergistic or competitive effects with one another and one way to do so could be simultaneous overexpression and knockdown of the MAGI proteins followed by an assessment of the receptor function.

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Chapter 5

5 Discussion

5.1 Summary

Previous research from our laboratory illustrated a crosstalk between CRFR1 and 5-HT$_{2A}$R (Magalhaes et al., 2010). Magalhaes *et. al* showed that the activation of CRFR1 mediates the signaling of 5-HT$_{2A}$R since pretreatment with CRF enhances 5-HT$_{2A}$R-mediated inositol phosphate formation. This effect was abolished when mutant forms of the receptors that lack the PDZ motif are used indicating that intact PDZ motifs are required for the crosstalk to occur and suggested a role for PDZ proteins in regulating the process. They also reported that this effect was dependent on receptor internalization and recycling by showing that using dominant negative forms of Rab4, Rab5 or dynamin could lead to blockade of the crosstalk. Moreover, infusion of CRF peptide (1.5 μg) into the medial prefrontal cortex of mice followed by the intraperitoneal administration of the 5-HT$_2$R-selective agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) (0.15 mg/kg) results in enhanced 5-HT$_2$ receptor-stimulated anxiety-related behaviors. Our hypothesis was that a PDZ-binding protein was recruited for the CRFR1-mediated sensitization of 5-HT$_{2A}$R signaling and it could enhance the expression of both receptors by regulating the internalization and recycling of CRFR1/5-HT$_{2A}$R complexes. To better understand the molecular mechanism behind this crosstalk between receptors and to be able to use this phenomenon in the development of therapeutic agents, the overall objective of this study was to determine the PDZ protein responsible for CRFR1-mediated sensitization of 5-HT$_{2A}$ receptor signaling.

In order to find PDZ domain-containing proteins that would be good candidates for this regulation, a screen of 96 PDZ domains with the carboxyl-terminal tails of 5-HT$_{2A}$R and CRFR1 was performed (Dunn et al., 2013). This resulted in a sub-group of common PDZ proteins that interacts with both receptors. These were SAP97, PDS-95, MAGI-1 MAGI-2, MAGI-3, CAL, PDZK1, MUPP1 and PTPN13. The proteins I chose to work with for my thesis projects were CAL, MAGI-1, MAGI-2 and MAGI-3. These proteins have been previously shown to regulate the trafficking and signaling of some members of

the GPCRs family (Dunn and Ferguson, 2015). However, their role in regulating CRFR1 and 5-HT$_{2A}$R was yet to be determined. It was essential to characterize the functional roles of the interaction between the PDZ proteins and the two receptors separately before we could assess the effects on the crosstalk regulation. Therefore, the three specific objectives of this thesis were:

1. **To determine the role of CAL in regulating the trafficking and signaling of CRFR1 and the mechanism for this regulation**

2. **To determine the role of MAGI proteins in regulating the trafficking and signaling of CRFR1 and the mechanism for this regulation**

3. **To determine the role of MAGI proteins in regulating the trafficking and signaling of 5-HT$_{2A}$R and the mechanism for this regulation**

In the second chapter of this thesis, the interaction of CAL with CRFR1 is characterized and shown to be dependent on the PDZ-binding motif of the receptor. CAL is shown to localize with CRFR1 at the Golgi and is demonstrated to have a role in inhibiting CRFR1 endocytosis, cell surface expression, and CRF-mediated ERK1/2 signaling via the CRFR1 PDZ-binding motif (Hammad et al., 2015). CAL does not have an effect on CRF-mediated cAMP formation. Interestingly and contrary to studies where the mechanism of CAL regulation of receptor expression is attributed to an effect of CAL on degradation (Cheng et al., 2010; Koliwer et al., 2015), our work demonstrates that CAL can elicit its effects on CRFR1 by mediating the post-translational glycosylation of the nascent receptor at the Golgi. By characterizing glycosylation-deficient mutants of CRFR1 with a point mutation on the asparagine residues, we find that the effects that CAL has on CRFR1 expression are comparable to the receptor expression levels of CRFR1 mutants. In addition, the cellular distribution of the glycosylation-deficient mutants reveals that they are mainly expressed in the Golgi. Similar localization of CRFR1 is observed upon overexpression with CAL since a big portion of the receptor is retained intracellularly in the Golgi. We also find that the signaling of the mutants via the MAPK pathway is similar to that of CRFR1 in the presence of CAL. Although multiple PDZ proteins were shown to

regulate GPCRs, mediating post-translational modifications seems to be exclusive to CAL (Hammad et al., 2015; Dunn and Ferguson, 2015). It is also unique to show an effect for a PDZ- binding protein during the early biosynthesis pathway as opposed to the later stages during the life cycle of a GPCR.

Data presented in the third and fourth chapters illustrate distinct functions for the MAGI subfamily of proteins in regulating the trafficking and signaling of CRFR1 compared to that of 5-HT$_{2A}$R. Furthermore, we present different mechanisms by which MAGI proteins can elicit their effects. In terms of their effect on trafficking and internalization, we show that MAGI proteins can regulate CRFR1 endocytosis, but have no effect on 5-HT$_{2A}$R internalization. We also show that MAGI proteins can mediate their effect on CRFR1 internalization by interfering with β-arrestin2 recruitment to the receptor, a key step in the endocytic pathway. We also show that while no effect is observed on the basal plasma membrane expression of CRFR1 in response to overexpression or knockdown of MAGI proteins, there is a trend for an increase in expression levels of 5-HT$_{2A}$R when MAGI proteins are overexpressed. The results from investigating the trafficking and internalization reflect the specificity and distinct functions of the proteins in regulating the receptors. Our findings from studying the effects on the signaling pathways are also very intriguing. CRFR1 and 5-HT$_{2A}$R differ in the G protein subtypes that they couple to, but they both have the ability to activate the ERK1/2 signaling pathway. There is no effect of MAGI proteins on Gα$_s$-mediated cAMP upon activation of CRFR1. On the other hand, 5-HT-stimulated IP accumulation via Gα$_q$ coupling appears to be enhanced by MAGI proteins. There are two possible mechanisms for this regulation of IP3 formation. It could be in part due to the increase in receptor expression levels at the plasma membrane but the more appealing mechanism is the enhancement of PLCβ3 association with the receptor when MAGI proteins are overexpressed. Studying signaling via the MAPK pathway, we find that knockdown of the endogenous MAGI proteins results in an enhancement in both CRF-mediated and 5-HT-mediated activation of ERK1/2 signaling. Taken together, the data from the third and fourth chapters suggest that MAGI proteins play important roles in regulating the trafficking of CRFR1 and the signaling of 5-HT$_{2A}$R.

5.2 Regulation of GPCRs Trafficking by MAGI and CAL Proteins

Data presented in this thesis illustrate an important role for PDZ domain-containing MAGI and CAL proteins in regulating the trafficking of CRFR1 and 5-HT$_{2A}$R (Fig. 5.1). The effect on CRFR1 is prominent upon studying agonist-induced internalization of the receptor. The main effect that is reported here is a decrease in CRFR1 internalization when CAL is overexpressed and when levels of MAGI proteins are altered either by overexpression or siRNA-mediated knockdown. An effect on 5-HT$_{2A}$R plasma membrane expression levels is observed following altered MAGI protein expression. Although not significant for all the MAGI transfection conditions, there is a trend towards an enhanced surface receptor levels. The reason for the big variation in the flow measurement of 5-HT-mediated endocytosis, as well as surface receptor levels could be attributed to the nature of the receptor which exhibits constitutive internalization activity and exhibits poor dependence on β-arrestins for internalization (Albert and Tiberi, 2001; Bhatnagar et al., 2001; Gray et al., 2001; Bhattacharyya et al., 2002). Therefore, it might be worth repeating the assays with a more sensitive approach to arrive at more conclusive results. Interestingly, previous work from our laboratory demonstrates an effect of other PDZ proteins on the trafficking of CRFR1 and 5-HT$_{2A}$R. These include SAP97, PSD-95 and PDZK1. SAP97 and PSD-95 function to reduce the internalization of both CRFR1 and 5-HT$_{2A}$R (Dunn et al., 2013; Dunn et al., 2014; Dunn et al., 2016). PDZK1 also reduces 5-HT$_{2A}$R internalization, but has no effect on CRFR1 internalization (Walther et al., 2015). Other groups show a role for MAGI-2 in regulating GPCR trafficking, where it reduces VPAC$_1$ internalization (Gee et al., 2009), enhances β$_1$-AR internalization (Xu et al., 2001) and facilitates localization of mGluR1 to the Golgi (Sugi et al., 2007). MAGI-1 and MAGI-3 have not been shown to affect GPCRs trafficking prior to the studies presented in this thesis.

Another aspect of GPCRs trafficking is the regulation of CRFR1 surface expression by CAL. We show that overexpression of CAL results in the retention of CRFR1 intracellularly in the Golgi. Previous studies show a similar effect of CAL on other GPCRs such as mGluRs, SSTR5 and β$_1$-AR, as well as other transmembrane proteins including
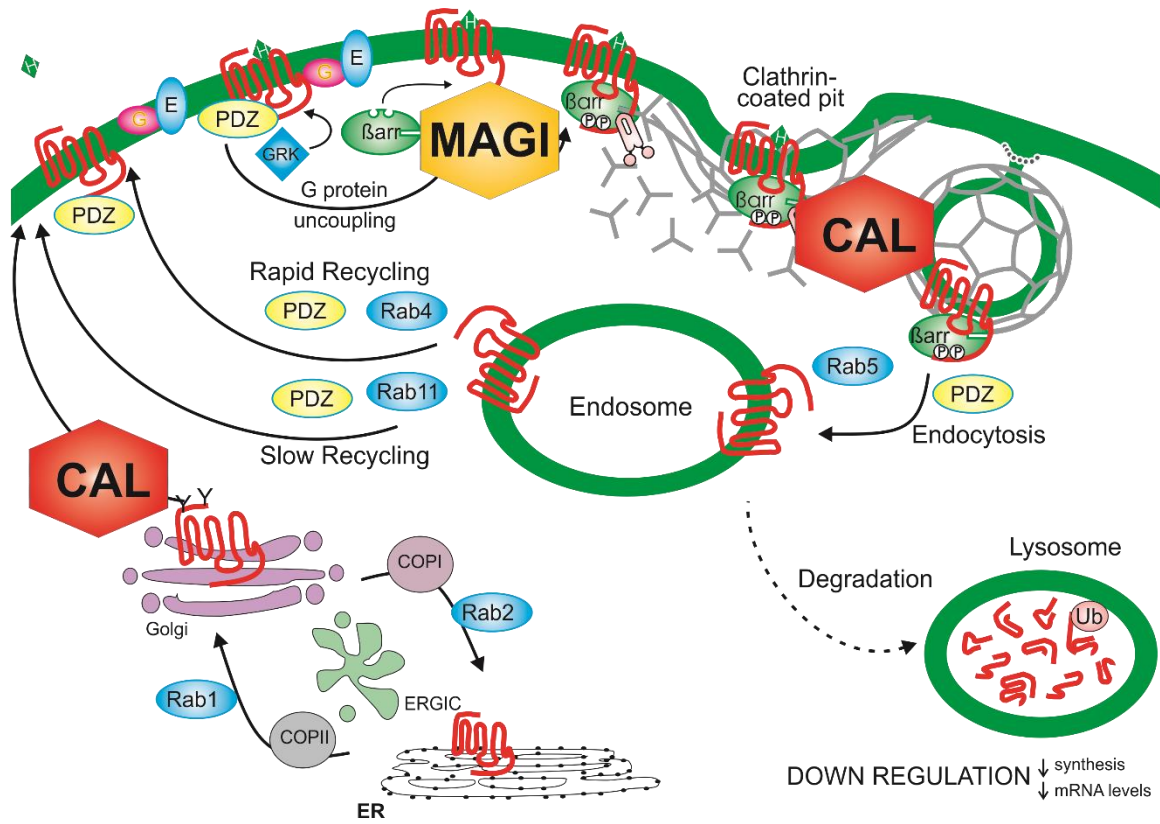
Figure 5.1: A schematic representation of the processes in GPCRs endocytosis and biosynthesis summarizing the findings of this thesis in terms of the role of MAGI and CAL proteins in regulating these functions.

CFTR and the voltage-gated potassium channel $K_{V10.1}$ (Cheng et al., 2002; Wente et al., 2005; Zhang et al., 2008; Cheng et al., 2010; Herrmann et al., 2013; Koliwer et al., 2015). It has been previously suggested that CAL might be playing a role as a sorting molecule, but the mechanism was believed to be via ubiquitination and degradation (Cheng et al., 2010; Koliwer et al., 2015). However, we show here that at least for the regulation of CRFR1, CAL contributes to the regulation of post-translational glycosylation and to the assessment of the maturation state of the receptor and whether it has achieved proper folding to be transported from the Golgi and expressed at the plasma membrane.

5.3 Regulation of GPCRs Signaling by MAGI and CAL Proteins

Data presented in the previous chapters demonstrate distinct roles for MAGI and CAL proteins in regulating both G protein-dependent as well as G protein-independent signaling pathways (Fig. 5.2). Although MAGI-2 has previously been shown to reduce cAMP signaling mediated by $VPAC_1$, it does not seem to affect $G\alpha_s$-mediated signaling by CRFR1 and neither did MAGI-1, MAGI-3 nor CAL. However, the MAGI proteins do have a significant effect on $G\alpha_q$-mediated IP3 formation as a result of $5\text{-HT}_{2A}R$ activation. It is of interest to observe an effect of the MAGI proteins on one G protein sub-type, but not another and confirms the selectivity of their effect on G protein signaling pathways. Previous studies from our laboratory have shown an effect of SAP97 and PDZK1 on 5-HT-induced IP3 formation where knockdown of endogenous expression of the PDZ proteins results in a reduction in $G\alpha_q$-mediated signaling by $5\text{-HT}_{2A}R$ (Dunn et al., 2014; Walther et al., 2015). However, a noticeable difference among the previous studies with SAP97 and PDZK1 and the data reported here is that the effects shown here with MAGI proteins were observed with both $5\text{-HT}_{2A}R$ and $5\text{-HT}_{2A}R\text{-}\Delta SCV$, while SAP97 and PDZK1 could only elicit their functional effects on $5\text{-HT}_{2A}R$. This most likely suggests that the effects of SAP97 and PDZK1 on $5\text{-HT}_{2A}R$ signaling are PDZ motif-dependent and the effects of MAGIs on $5\text{-HT}_{2A}R$ signaling are PDZ motif-independent. Other examples of PDZ domain-containing proteins that are known to regulate $G\alpha_q$ signaling include NHERF1, which was demonstrated to increase coupling and activation of $G\alpha_q$ by parathyroid 1 receptor (PTH1R), and NHERF2 which increases secondary messenger IP3
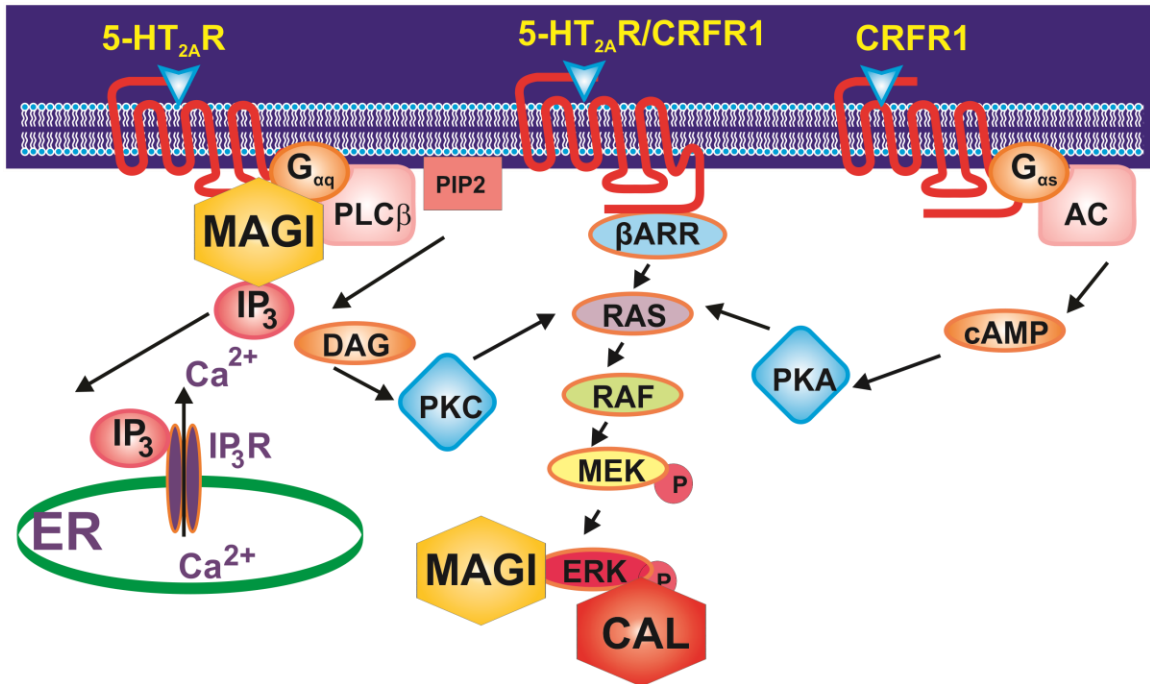
Figure 5.2: A schematic representation of the signaling pathways activated by CRFR1 and 5-HT$_{2A}$R and the involvement of MAGI and CAL proteins in regulating these pathways as reported in this thesis.

accumulation via LPA$_2$R (Oh et al., 2004; Wang et al., 2010).

It is also essential to study the effects of our PDZ proteins of interest on the MAPK pathway, which represents a signaling pathway shared by both CRFR1 and 5-HT$_{2A}$R. Interestingly, our data show that MAGI proteins can regulate ERK1/2 activation by both CRFR1 and 5-HT$_{2A}$R in a similar manner. We find that the overexpression of MAGI proteins do not alter the extent of ERK1/2 phosphorylation stimulated by the activation of either the CRFR1 or the 5-HT$_{2A}$R. On the other hand, siRNA-mediated knockdown of endogenous MAGI proteins in HEK 293 cells results in a significant enhancement in MAPK activation by the CRFR1 and the 5-HT$_{2A}$R. The only other MAGI protein to exhibit an effect on ERK1/2 signaling is MAGI-3. MAGI-3 has been reported to show distinctive effects on the MAPK pathway depending on the receptor being investigated (He et al., 2006; Zhang et al., 2007; Yang et al., 2010; Stephenson et al., 2013). MAGI-3 expression results in an enhancement in signaling for BAI-1 and LPA$_2$R and a reduction in signaling for the adrenergic receptors. Our laboratory has previously investigated the role of SAP97 in regulating GPCR-mediated ERK1/2 signaling (Dunn et al., 2013). This work demonstrates a general role for SAP97 in regulating the MAPK signaling pathway independently from the PDZ motif, since the effects are observed with CRFR1-ΔTAV as well as CRFR2. Interestingly, SAP97 has an opposite effect to MAGI proteins in regulating ERK1/2 phosphorylation by both CRFR1 and 5-HT$_{2A}$R, as the knockdown of endogenous SAP97 results in a reduction in ERK1/2 activation.

In this thesis, we also study the effects of CAL on CRFR1-mediated ERK1/2 signaling and demonstrate that CAL can negatively regulate this pathway. Our data indicate that CAL overexpression results in a significant attenuation of ERK1/2 activation, while CAL siRNA knockdown of endogenous levels of the CAL protein results in enhanced CRFR1-mediated ERK1/2 signaling (Hammad et al., 2015). Similarly, CAL overexpression reduces ERK1/2 activation by mGluR1 (He et al., 2004; Zhang et al., 2008) and β$_1$-AR (He et al., 2004). The effect of CAL on regulating 5-HT-mediated ERK1/2 signaling remains to be determined.

5.4 Mechanisms of GPCRs Regulation by MAGI and CAL Proteins

The various mechanisms underlying the functional regulation of PDZ motif expressing GPCRs examined in the present thesis highlight the specificity and uniqueness of this group of scaffolding proteins. While CAL mediates its effects via regulation of post-translational glycosylation, MAGI proteins perform their functions by regulating receptor interactions with key elements such as β-arrestin and PLCβ. This confirms the importance of PDZ domain-containing proteins in regulating GPCRs functions and given that more than 50% of pharmaceutical drugs directly or indirectly act on GPCRs, proper characterization of PDZ protein-receptor interactions is essential.

Despite some differences in the extent of their effects on different aspects of CRFR1 and 5-HT$_{2A}$R activity, it is fair to say that MAGIs 1, 2 and 3 share some similar effects on the trafficking and signaling of CRFR1 and 5-HT$_{2A}$R. Therefore, we further investigated their ability to compensate for one another and provided evidence for a possible compensatory protein expression mechanism of up-regulation between MAGI-1 and MAGI-3 based on their similar functional roles, when the expression of either protein is altered by siRNA knockdown. This is not the first report to suggest either compensatory or complimentary effects of PDZ proteins. It has been previously documented that PSD-93 and PSD-95 can compensate for one another (Sun and Turrigiano, 2011) and regulate GPCRs function similarly.

5.5 MAGI and CAL Proteins in Disease

CAL-deficient mice are infertile and it has been suggested that they can present a unique model for human globozoospermia, an inherited infertility syndrome in humans where the acrosome is lost. The acrosome is a unique organelle that plays an important role at the site of sperm-zona pellucida binding during the fertilization process and has been reported to be completely lost in CAL knockout mouse models (Yao et al., 2002). It is therefore suggested that CAL plays a role in the fusion of transport vesicles to the acrosome. PCR analysis of mouse tissue reveals that CAL is ubiquitously distributed in many different regions of the brain (cerebellum, cortex, hippocampus and hypothalamus)

as well as the heart, kidney, lungs, testis and spinal cord (Charest et al., 2001; Cheng et al., 2002; Herrmann et al., 2013). However, other than spermatogenesis, no overt abnormalities are noted in CAL knockout mice (Yao et al., 2002). A study shows that β_1-AR expression levels are decreased in the hippocampus of CAL knockout mice (Koliwer et al., 2015). Given its wide distribution in multiple areas of the brain as well as its confirmed role as an essential sorting protein, further examination is required to understand the role of CAL and whether it has any implications for mental illnesses.

MAGI proteins are important scaffolding proteins and have been implicated in various physiological functions (Funke et al., 2005; Oliva et al., 2012). MAGI proteins are suggested to be involved in learning and memory. All MAGI proteins are also believed to be involved in cancer cell signaling via their interaction with PTEN, a tumor suppressor gene (Zaric et al., 2012; Matsuda et al., 2013). MAGIs expression level is correlated with the cancer cell invasion where their levels were shown to be reduced in different cancer types (Matsuda et al., 2013). PCR results performed in our laboratory indicate that MAGIs 1, 2 and 3 are expressed in the human brain. RT-PCR in mouse tissues show that MAGI-1 is expressed in the colon, kidney, lung, liver, and pancreas and different areas of the brain including the cerebellum, cortex and thalamus. MAGI-2 is expressed in the cortex and thalamus as well as the kidneys and testis. MAGI-3 is very widely distributed in the cerebellum, cortex and thalamus as well as heart, kidney, colon and lung (Wu et al., 2000; Sugnet et al., 2006).

MAGI-1 expression is decreased in cancers and this correlates with poor prognosis, suggesting MAGI-1 as a novel prognostic marker for cancer (Zaric et al., 2012). On the other hand, overexpression of MAGI-1 induces stabilization of E-cadherin and β-catenin localization at cell-cell junctions, enhances actin stress fiber and focal adhesion formation, increases cell adhesion to matrix proteins and suppresses anchoragein dependent growth and migration. In fact, MAGI-1 overexpression suppresses subcutaneous primary tumor growth and attenuates spontaneous lung metastasis in experimental colon cancer (Pleasance et al., 2010). MAGI-1 deficient mice are suggested to have renal problems but further characterization of this model is required.

MAGI-2 has been found to localize to the postsynaptic density area of the spine and bind to N-methyl-D-aspartate receptor (Hirao et al., 1998; Iida et al., 2007; Sumita et al., 2007). Furthermore, MAGI-2 has been shown to be localized to both excitatory and inhibitory synapses. Taken together, this data suggest a neuronal role for MAGI-2. In fact, MAGI-2 knockout mice models show abnormal elongation of dendritic spines, indicating a possible role for MAGI-2 during morphogenesis of neurons (Iida et al., 2007). MAGI-2 has also been shown to be expressed in the kidneys and was recently found to play a critical role in the formation and maintenance of the glomerular filtration barrier (Ihara et al., 2014). It is reported that the renal function of excretion of waste matter was entirely lost in MAGI-2 knockout mice and this caused neonatal lethality. MAGI- 2 gene has been shown to undergo rearrangement in the genome of a melanoma cell line and prostate cancer (Berger et al., 2011).

MAGI-3 has been shown to interact with Lysophosphatidic Acid (LPA) receptor type 2 and regulate its signaling pathways. LPA is a potent inducer of colon cancer and LPA receptor type 2 is overexpressed in colon tumors (Zhang et al., 2007). No reports are published about a knockout model of MAGI-3.

5.6 Future Directions

Now that we have a better understanding for the role of MAGI proteins in regulating both CRFR1 and 5-HT$_{2A}$R, the next step would be to assess whether MAGI proteins are involved in regulating the crosstalk between the receptors. The best way to test this is by performing IP3 assays in cells transfected with 5-HT$_{2A}$R and CRFR1 and either overexpressed MAGI proteins or siRNA against the MAGI proteins. We could then pretreat cells with CRF and measure IP3 formation to examine the effects of MAGI proteins on the enhancement of signaling that was observed in Magalhaes *et al.* (2010). We would expect that overexpression of MAGI proteins will result in further enhancement in IP3 formation, because it had this effect even without CRF pretreatment. Given the similar function of the three MAGI proteins and the apparent compensation mechanism governing that function, one should also consider the possibility that a certain combination of overexpression or knockdown might be involved and therefore, these conditions would have to be tested as well.

The effect of CAL on the signaling and trafficking of 5-HT$_{2A}$R remains unknown. CAL is one of the PDZ proteins that was detected as an interacting protein in the PDZ blot overlay assay performed with the carboxyl-terminal tail of 5-HT$_{2A}$R to screen 96 PDZ domains. Despite containing one PDZ domain, it is still a candidate regulator of the crosstalk between CRFR1 and 5-HT$_{2A}$R because of its ability to self-dimerize via the coiled coil motif. Given that CAL overexpression results in the reduction of expression of CRFR1 at the cell surface, it would be unlikely for CAL to be required for CRFR1-mediated 5-HT$_{2A}$R sensitization. In fact, we could speculate that maybe a knockdown of CAL would facilitate this crosstalk since CAL seems to be retaining receptors in the intracellular compartments as reported by us for CRFR1 and by others for mGluRs, β_1-AR and SSTR5. This would suggest that it is indeed a group of PDZ proteins that collectively work in regulating the crosstalk as opposed to one PDZ protein.

Our studies confirm the importance of the family of PDZ domain-containing proteins in regulating GPCRs activity. We illustrated the distinct function of the MAGI subfamily of proteins in regulating the trafficking and signaling of CRF1 and 5-HT$_{2A}$ receptors. We also showed a unique mechanism of regulation for CAL in mediating post-translational modifications of the receptors. Considering the fact that more than 40% of currently available pharmaceuticals act directly or indirectly on GPCRs, it is essential to fully characterize their interacting partners and the consequences such interactions have on receptor function. Multi-domain-containing proteins such as PDZ proteins present exciting models since it would be possible to target either their interaction with the receptor, their PDZ domains, the PDZ motifs of the receptor or allosteric sites on the proteins to change their conformation. Therefore, the findings from these studies can be utilized in the design of new pharmacological tools for the treatment of diseases including mental disorders.

5.7   References

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Curriculum Vitae

<u>Maha M. Hammad</u>

<u>Education</u>

September 2012 to August 2016: <u>Graduate Studies</u>
- Schulich School of Medicine & Dentistry - Western University, Canada
- PhD in Physiology and Pharmacology
- Thesis Title: CAL and MAGI PDZ protein regulation of CRFR1 and 5-HT$_{2A}$R trafficking and signaling

September 2008 to August 2010: <u>Graduate studies</u>
- Faculty of Medicine - Dalhousie University, Canada
- Master of Science in Pharmacology
- Thesis Title: CHARACTERIZATION OF THE ANGIOTENSIN TYPE 1 RECEPTOR AND THE BETA2 ADRENERGIC RECEPTOR PROPERTIES: THE INVOLVEMENT OF ARRESTIN2, RAB1 AND SOME MOLECULAR CHAPERONES IN THE ASSEMBLY AND TRAFFICKING OF GPCRS
- Defense: July 21st 2010
- Convocation: October 16th 2010

September 2004 to June 2008: <u>Undergraduate studies</u>
- Faculty of Science - Kuwait University, Kuwait
- Bachelor of Science (Distinction with Class Honors)
- Major: Biochemistry   Minor: Molecular Biology
- GPA: 3.78   mGPA: 3.69

<u>Work Experience</u>
- Teaching Assistant at Western University
  Course: Cellular and Molecular Neurobiology
  Jan. 2013 - Apr. 2013

- Teaching Assistant at Western University
  Course: Cellular and Molecular Neurobiology
  Jan. 2014 - Apr. 2014

- Research Assistant at the Biochemistry and Molecular Biology Unit at Dasman Diabetes Institute
  Dec. 2010 - July 2012

Publications

Maha M. Hammad, Henry A. Dunn, and Stephen S.G. Ferguson. *MAGI proteins regulate the trafficking and signaling of corticotropin-releasing factor receptor 1 via a compensatory mechanism*. **Accepted in** *Molecular Signaling, Aug. 2016*

Maha M. Hammad, Henry A. Dunn, Cornelia Walther, and Stephen S.G. Ferguson. *The Role of Cystic Fibrosis Transmembrane Conductance Regulator-Associated Ligand (CAL) in the Regulation of the Trafficking and Signaling of Corticotropin Releasing Factor Receptor 1*. ***Cellular Signaling, 2015***

Baturcam E, Abubaker J, Tiss A, Abu-Farha M, Khadir A, Al-Ghimlas F, Al-Khairi I, Cherian P, Elkum N, **Hammad M**, John J, Kavalakatt S, Lehe C, Warsame S, Behbehani K, Dermime S, Dehbi M. *Physical Exercise Reduces the Expression of RANTES and Its CCR5 Receptor in the Adipose Tissue of Obese Humans. **Mediators Inflamm. 2014***

Abu-Farha M, Tiss A, Abubaker J, Khadir A, Al-Ghimlas F, Al-Khairi I, Baturcam E, Cherian P, Elkum N, **Hammad M**, John J, Kavalakatt S, Warsame S, Behbehani K, Dermime S, Dehbi M. *Proteomics analysis of human obesity reveals the epigenetic factor HDAC4 as a potential target for obesity. **PLoS One***. ***2013***

Abubaker J, Tiss A, Abu-Farha M, Al-Ghimlas F, Al-Khairi I, Baturcam E, Cherian P, Elkum N**, Hammad M**, John J, Kavalakatt S, Khadir A, Warsame S, Dermime S, Behbehani K, Dehbi M. *DNAJB3/HSP-40 Cochaperone Is Downregulated in Obese Humans and Is Restored by Physical Exercise*. ***PLoS One***. ***2013***

Dupré DJ, **Hammad MM**, Holland P, and Wertman J. *Role of chaperones in G protein coupled receptor signalling complex assembly. In GPCR Signalling Complexes – Synthesis, Assembly, Trafficking and Specificity **Subcell Biochem 2012***

Hammad, Maha, Kuang, Yi-Qun; Morse, Alexa; Dupré, Denis. *Rab1 interacts directly with the beta2-adrenergic receptor to regulate receptor anterograde trafficking **Biol Chem. 2012***

Hammad MM, Kuang YQ, Yan R, Allen H, and Dupré DJ. *NA+/H+ Exchanger Regulatory Factor-1 is Involved in Chemokine Receptor Homodimer CCR5 Internalization and Signal Transduction, but Does Not Affect CXCR4 Homodimer OR CXCR4-CCR5 Heterodimer*. ***J Biol Chem***. ***2010***

Hammad MM and Dupré DJ. *Chaperones Contribute to G protein Couples Receptor Oligomerization, but do not Participate in Assembly of the G Protein with the Receptor Signalling Complex. **J Mol Signal. 2010***

Conferences and Meetings

- GPCR Workshop, Kona, Hawaii, December 2015

- 16th Annual Joint Meeting of the Great Lakes GPCR Retreat and Club des Récepteurs à Sept Domaines Transmembranaires du Québec, HockleyVally, Canada, October 2015

- Robarts Research Retreat, London, Ontario, June 2015

- Gordon Research Conference - Molecular Pharmacology, Ventura, CA, February 2015

- 15th annual joint meeting of the Great Lakes GPCR Retreat and le Club des Recepteurs a Sept Domaines Transmembranaires du Québec, Bromont (Quebec), Canada, October 2014

- London Health Research Day, London, Ontario, March 2014

- 6th International Conference on Drug Discovery and Therapy, Dubai, UAE, February 2014

- Phys/Pharm Research Day, Western University, London, Ontario, Nov. 2013

- 14th annual joint meeting of the Great Lakes GPCR Retreat and le Club des Recepteurs a Sept Domaines Transmembranaires du Québec, Cleveland, Ohio, October 2013

- 13th annual joint meeting of the Great Lakes GPCR Retreat and le Club des Recepteurs a Sept Domaines Transmembranaires du Québec, London, Ontario, October 2012

- Poster Day Competition, Faculty of Medicine, Dalhousie University, Nova Scotia, May 2010

- 10th Annual Joint Meeting of the Great Lakes GPCR Retreat and le Club des Recepteurs a Sept Domaines Transmembranaires in Rochester, New York, October 2009

- Poster day Competition, Faculty of Medicine, Dalhousie University, Nova Scotia, May 2009

- Scientific Poster day in the Faculty of Science, Kuwait University, Kuwait, May 2008

Oral Presentations:

- *The Role of PDZ Domain-Containing MAGI Proteins in the Regulation of GPCRs Trafficking and Signaling.* Neuroscience Work In Progress-Department of Cellular and Molecular Medicine –University of Ottawa, Apr. 4- 2016

- *Characterization of CRF and 5-HT$_{2A}$ Receptors Function: The Involvement of PDZ Domain-Containing Proteins CAL and MAGIs in the Regulation of Trafficking and Signaling of GPCRs.* Department of Physiology and Pharmacology Seminar Series, University of Western Ontario, Feb. 29- 2016

Poster Presentations:

Title: Role of cystic fibrosis transmembrane conductance regulator-associated ligand (CAL) in regulating the trafficking and signaling of corticotropin- releasing factor receptor 1
Authors: <u>**Maha M. Hammad,**</u> Henry A. Dunn, and Stephen S.G. Ferguson
Meeting: GPCR Workshop, Kona Hawaii. Dec. 1- 5, 2015

Title: The role of MAGI proteins in the regulation of Corticotropin Releasing Factor1 and Serotonin 2A receptors function
Authors: <u>**Maha M. Hammad,**</u> Henry A. Dunn, and Stephen S.G. Ferguson
Meeting: 16th Annual Joint Meeting of the Great Lakes GPCR Retreat and Club des Récepteurs à Sept Domaines Transmembranaires du Québec, HockleyVally, Canada. Oct. 22-24, 2015

Title: The Role of CAL in the Regulation of the trafficking and Signaling of Corticotropin Releasing Factor Receptor1
Authors: <u>**Maha M. Hammad,**</u> Henry A. Dunn, Cornelia Walther, and Stephen S.G. Ferguson
Meeting: Gordon Research Conference, Ventura, CA. Feb. 1-6, 2015

Title: The role of CAL in the regulation of corticotropin-releasing factor receptor 1 function
Authors: <u>**Maha M. Hammad,**</u> Cornelia Walther, Henry A. Dunn, and Stephen S.G. Ferguson
Meeting: 15th Annual Joint Meeting of the Great Lakes GPCR Retreat and Club des Récepteurs à Sept Domaines Transmembranaires du Québec, Bromont (Quebec), Canada. October 2-4, 2014
Meeting: Phys/Pharm Research Day, Western University, London, Ontario, Nov. 4, 2014

Title: The role of PDZ proteins in the regulation of the signaling of CRF1 and 5HT2A receptors
Authors: <u>**Maha M. Hammad**</u>, Cornelia Walther, Henry A. Dunn, and Stephen S.G. Ferguson
Meeting: 6th International Conference on Drug Discovery and Therapy, Dubai, UAE. Feb.10- 12, 2014

Meeting: Phys/Pharm Research Day, Western University, London, Ontario. Nov. 4, 2013
Meeting: London Health Research Day, London, Ontario. March 18, 2014

Title: The role of PDZ proteins in regulating the corticotropin releasing factor 1 and the serotonin 2A receptors
Authors: **Maha M. Hammad,** Cornelia Walther, Henry A. Dunn, and Stephen S.G. Ferguson
Meeting: 14th annual joint meeting of the Great Lakes GPCR Retreat and le Club des Recepteurs a Sept Domaines Transmembranaires du Québec, Cleveland, Ohio. October 2013
Title: Multiplexed analysis of inflammatory, metabolic and stress markers in obese subjects before and after a defined exercise program
Authors: Baturcam E, Abubakr J, Abu-Farha M, Al-Arouj M, Al-Ghimlas F, Al-Khairi I, Al-Mass A, Al-Mudhaf D, Bennakhi A, Cherian P, **Hammad M**, John J, Kavalakatt S, Khadir A, Tiss A, Warsame S, Dermime S, Dehbi M
Meeting: 10th Joint Meeting of International Cytokine Society and International Society for Interferon and Cytokine Research. Sept. 2012

Title: Association of obesity with down-regulation of heat shock protein 40 expression and evidence that exercise retrieves its normal expression
Authors: Abubakr J, Abu-Farha M, Al-Arouj M, Al-Ghimlas F, Al-Khairi I, Al-Mudhaf D, Baturcam E, Bennakhi A, Cherian P, **Hammad M**, John J, Kavalakatt S, Khadir A, Tiss A, Warsame S, Dermime S, Dehbi M
Meeting: BMC Proceedings Washington, DC. May 29-30, 2012

Title: Increased expression of TLR-2 and 4 in monocytes of obese individuals: association with the induction and progression of insulin resistance
Authors: Almass A, Ahmad R, Al-Ghawas D, Hasan A, Atizado VL, **Hammad MM**, Lehe C, Zghoul N, Al-Ghimlas F, AlKandari J, Al-Yousef A, Al-Arouj M, Bennakhi A, Dermime S
Meeting: Annual Congress of the British Society for Immunology, Liverpool, UK. Dec. 5-8, 2011

Title: Peripheral blood mononuclear cells (PBMCs) and adipose tissue from overweight and obese individuals express significant high levels of matrix metallopeptidase 9 (MMP-9)
Authors: Ahmad R, Al-Ghawas D, Al-Saleh S, Almass A, Hasan A, Atizado VL, **Hammad MM**, Lehe C, Zghoul N, Al-Ghimlas F, AlKandari JR, Al-Yousef A, Al-Sabah S, Al-Arouj M, Bennakhi A, Dermime S
Meeting: Annual Congress of the British Society for Immunology, Liverpool, UK. December 5-8, 2011

Title: Different Roles for Chaperones and Scaffolds in GPCR Homo and Heterodimeric Receptor Complexes Assembly and Functions
Authors: Dupré DJ, **Hammad MM**, Allen H, and Yan R.
Meeting: Bit-PepCon Life Sciences 3rd Annual Conference, Beijing, China

Title: EBP50/NHERF1 Regulates CCR5 Homodimers, but not CXCR4 homo or CXCR4-CCR5 Heterodimers
Authors: **Hammad MM**, Yan R, Allen H, and Dupré DJ
Meeting: The 10th Annual Joint Meeting of the Great Lakes G Protein-Coupled Receptor Retreat and le Club des Récepteurs à Sept Domaines Transmembranaires – Rochester, New York. October 15-18, 2009

Title: Characterization of Assembly, Trafficking and Signaling of Homo and Heterodimeric G Protein Coupled Receptors
Authors: Allen H, **Hammad MM**, Yan R, and Dupré DJ
Meeting: The 8th European Symposium of The Protein Society – Zurich, Switzerland. June 14-18, 2009

Title: The Specificity of Signaling of β2-Adrenergic/Angiotensin Heterodimeric Receptor Complex
Authors: **Hammad MM**, Morse A, and Dupré DJ
Meeting: Graduate Student Research Day Poster Competition– Faculty of Medicine at Dalhousie. May 2009

<u>Awards and Scholarships</u>

- *Doctoral Research Award from Canadian Institute of Health Research*
  Sept. 2014 - Aug. 2017

- *"Image of the month" from the Confocal Facility at Robarts Research Institute*
  July 2015

- *Second place poster prize in the 6th International Conference on Drug Discovery and Therapy*
  Dubai, UAE, Feb.10-12, 2014

- *Second place poster prize for the Neuroscience category in the Phys/Pharm Research Day*
  Western University, Nov. 4, 2013

- *Focus on Stroke Doctoral Research Award from Heart and Stroke Foundation*
  Sept. 2013 - Aug. 2016
  Award terminated in Aug. 2014 after receiving the Doctoral Award from CIHR

- *Dean's PhD Graduate Stipend award from Schulich School of Medicine & Dentistry*
  Western University from Sept. 2012 - Aug. 2016
  Award terminated in Aug. 2013 after receiving the FOS Doctoral Award from HSF

- *Student Research Award from the Nova Scotia Health Research Foundation (NSHRF)*
  Sept. 2009 - Aug. 2010

- *Stipend Scholarship from the Department of Pharmacology, Dalhousie University*
  Sept. 2008 - Aug. 2010

- Conference Travel Grant from the Faculty of Graduate Studies, Dalhousie University
  Oct. 2009

- First Place Prize in the Scientific Poster Day, Faculty of Science, Kuwait
  May 2008

- Dean's Honor List from Faculty of Science, Kuwait University
  Winter Semester 2004-05, Winter Semester 2005-06, Fall Semester 2006-07 and Fall Semester 2007-08

- Highest GPA award in the Biochemistry Department, Kuwait University
  2005 and 2006