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Regulation of CRFR1 and 5-HT2AR by PDZ Domain-Containing Proteins SAP97 and PSD-95

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree
in Physiology

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by

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Graduate Program in Physiology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

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Abstract

Previous studies identified a crosstalk mechanism whereby CRFR1 sensitized 5-HT_{2A}R-mediated signaling via interactions with PDZ domain-containing proteins: a mechanism that may underlie stress-induced anxiety and depression. This prompted an investigation into uncovering which PDZ domain-containing proteins could regulate the crosstalk between these two receptors, and how they could be regulated individually. In the current studies, a subset of PDZ domain-containing proteins were identified that may interact with CRFR1 and 5-HT_{2A}R. The focus narrowed to two candidates previously implicated in psychiatric disease: SAP97 and PSD-95. We confirmed SAP97 and PSD-95 as interacting partners of CRFR1 in adult mouse cortex via co-immunoprecipitation. Both proteins exhibited functional regulation of CRFR1 by antagonizing CRFR1 endocytosis in HEK293 cells, measured by flow cytometry. Additionally, PSD-95 suppressed β -arrestin2 recruitment, thereby providing a potential mechanism for antagonizing endocytosis. Although neither SAP97 nor PSD-95 appeared to play a significant role in CRFR1-mediated cAMP signaling, endogenous SAP97 was integral for CRF-mediated ERK1/2 phosphorylation in HEK293 and AtT20 cells. Despite extensive sequence homology between SAP97 and PSD-95, PSD-95 did not appear to play a significant role in CRF-mediated ERK1/2 phosphorylation. Thus, we begin to understand subtle signaling biases between these two proteins. As PSD-95 was already documented to regulate 5-HT_{2A}R, we investigated if SAP97 could play a role in regulating 5-HT_{2A}R function. The interaction between SAP97 and 5-HT_{2A}R was confirmed in adult mouse cortex. As was seen with CRFR1, SAP97 antagonized 5-HT_{2A}R endocytosis. Although SAP97 did not appear to significantly modulate G_s-coupled signaling via CRFR1, the endogenous expression of

SAP97 was integral for maximal G_q-coupled signaling via 5-HT_{2A}R. Endogenous SAP97 was also required for ERK1/2 phosphorylation, and this regulatory role appears to be downstream of receptor interactions. Finally, we were unable to prevent the CRFR1-mediated sensitization of 5-HT_{2A}R-mediated signaling by knocking down either SAP97 or PSD-95 using shRNA. Therefore, neither SAP97 nor PSD-95 appear to be exclusively involved in this heterologous crosstalk mechanism. Nevertheless, we have identified SAP97 and PSD-95 as novel regulators of CRFR1 function, and SAP97 as a novel regulator of 5-HT_{2A}R function. These functional interactions may be targeted for the treatment of CRFR1- and 5-HT_{2A}R-mediated mood disorders.

Keywords: GPCR, PDZ, CRFR1, 5-HT_{2A}R, PSD-95, SAP97, endocytosis, ERK, MAPK, phosphorylation, IP₃ signaling, G_q-coupling, anxiety, depression, schizophrenia

Co-Authorship Statement

In Chapter 2, Dr. Christina M. Godin and I collaborated with the cell culturing of HEK293 and AtT20 cells. Dr. Randy A. Hall (Emory) provided the materials to perform Figure 1. Dr. Cornelia Walther and I collaborated on Figure 8. Dr. Stephen S.G. Ferguson and I collaborated on the experimental design and the preparation of the manuscript.

In Chapter 3, Dr. Fabiana A. Caetano and Dr. Christina M. Godin provided the FL-5-HT_{2A}-rLuc-x-22-SCV construct for Figure 3. George Y. Yuan, Dr. Christina M. Godin, and I collaborated on Figure 3. Dr. Cornelia Walther and I collaborated on Figure 5 and Figure 7. Dr. Stephen S.G. Ferguson and I collaborated on the experimental design and the preparation of the manuscript.

In Chapter 4, Dr. Kevin Holmes and I collaborated on Figure 2. Harpreet S. Chahal, Ruchi Parikh and I collaborated on Figure 3. Harpreet S. Chahal and I collaborated on Figure 4 and Figure 5. George Y. Yuan and I collaborated on Figure 6. Dr. Fabiana A. Caetano and I collaborated on data that was not included in the final manuscript. Dr. Cornelia Walther contributed a YFP-PSD-95 construct that was not included in the final manuscript. Dr. Stephen S.G. Ferguson and I collaborated on the experimental design and the preparation of the manuscript.

Dedication

I dedicate this thesis to my father, Peter Kenneth Dunn, who taught me “brain before brawn” during bedtime stories, and emphasized I could do anything I put my mind to. I credit these early experiences with my infatuation with the brain and the mind. I still believe I can accomplish anything I put my mind to, and I hope that includes sailing the Atlantic Ocean, building my own house, brewing my own beer, and raising a loving family as you have. Cheers to you, Dad. I love you.

I dedicate this thesis to my mother, Carol Anne Dunn, whose love of biology led me to reconsider my business path in my final year of high school in pursuit of the medical sciences. My curiosities would never have been quenched had I not followed in your footsteps, and I am forever grateful I made that decision. Heather Rose, Hilary Anne and I are the luckiest children in the world to have you as our Mom for your everlasting love and unwavering support in pursuing whatever we desired, including my passion for music. I know I couldn’t have done anything in my life without you. I’ll love you forever and I’ll like you for always, too.

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Harpreet, I'll always remember our late nights in the lab when you weren't supposed to be there. I'm not sure I'd have made it this far without your support, both as a scientist and as a friend. Every time I was ready to give up, you'd show up unannounced in the middle of the night and surprise me with coffee. You kept me sharp with your deep intellectual thoughts and you kept me sane with your humour. I owe a special thanks to you.

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Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); 5-HT_{2A}R, 5-HT_{2A} receptor; 5-HT_{2C}R, 5-HT_{2C} receptor; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; ANOVA, analysis of variance; β 1AR, β 1-adrenergic receptor; CRF, corticotropin-releasing factor; CRFR1, CRF receptor 1; CRFR2, CRF receptor 2; ERK1/2, extracellular signal-regulated kinase; GIP, GPCR-interacting protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HA, hemagglutinin; HBSS, HEPES-buffered saline solution; HEK293, human embryonic kidney 293; PTH1R, parathyroid hormone 1 receptor; MAPK, mitogenactivated protein kinase; PDZ, PSD95/Zona Occludens/Disc Large; PTH1R, parathyroid hormone 1 receptor; SAP97, synapse-associated protein 97; shRNA, short hairpin RNA.

Chapter 1: Introduction

1.1 G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs) represent an expansive superfamily of transmembrane receptors that play an important role in regulating every aspect of mammalian physiology (Heng et al., 2013; Bockaert et al., 2010). Approximately 2% of the human genome encodes for GPCRs and over 40% of currently prescribed pharmaceuticals target these receptors for their desired consequence (Urs et al., 2014). Each of these GPCRs possess common characteristics, including an extracellular amino terminus, an intracellular carboxyl terminus, and seven transmembrane domains that are linked by three intracellular- and three extracellular-loops (Baldwin, 1993; Bockaert & Pin, 1999). Another highly conserved feature amongst the GPCR superfamily is the inclusion of two cysteine residues within the first- and second-extracellular loops (Bockaert & Pin, 1999). These residues are capable of forming a disulfide bond which is believed to be integral for stabilizing GPCRs in a finite number of conformations associated with various activation- and inactivation-states of the receptor (Bockaert & Pin, 1999).

The amino terminal, extracellular loops and portions of the transmembrane domains are essential for ligand-binding, the most common mechanism for activating GPCRs (Wess, 1997). There are a multitude of stimuli responsible for activating GPCRs, including: hormones, peptides, amino acid-residues, nucleotides, Ca^{2+} , odorants, and photons of light (Bockaert & Pin, 1999). Each individual GPCR has specific stimuli that can influence the receptor's function. Traditional pharmacology can describe these stimuli

as agonists or antagonists of GPCR signalling. However, recent advances have uncovered an increasing complexity in GPCR activation and inactivation (Kristiansen, 2004). Ligand binding can cause a conformational change in the GPCR leading to the exchange of GDP for GTP on the α -subunit of the heterotrimeric G protein (Kristiansen, 2004). The now “active” G protein dissociates from the receptor and splits into separate α - and $\beta\gamma$ -subunits, each of which contribute to the regulation of various aspects of GPCR-signalling (Kristiansen, 2004). The $G\alpha$ subunit will be of primary focus for the studies to follow.

1.2 G Protein-Coupled Receptor Signalling

The intracellular loops, carboxyl-terminal tail, and the intracellular face of some transmembrane domains are integral for G protein-coupling, as well as other important interactions with intracellular proteins such as G protein-coupled receptor kinases (GRKs), β -arrestins and membrane scaffolding proteins, such as PDZ domain-containing proteins (Wess, 1997; Lefkowitz, 1998; Kristiansen, 2004; Magalhaes et al., 2012). Each unique GPCR can initiate an assortment of signalling pathways which are influenced not only by different extracellular agonists, but also by a diversity of intracellular interacting proteins (Lefkowitz, 1998; Kristiansen, 2004). Perhaps the most important determinant of GPCR signalling is the subtype of α -subunit within the heterotrimeric G protein that is coupled to the receptor. Of these, the stimulative- and inhibitory-regulatory G proteins ($G\alpha_s$ and $G\alpha_i$) and $G\alpha_{q/11}$ appear most common and have best been described (Lefkowitz, 1998; Kristiansen, 2004) (Figure 1.1).

1.2.1 cAMP Signalling Pathway

The stimulatory- and inhibitory-regulatory G proteins (G_{α_s} and G_{α_i}) act inversely on the same signalling pathway (Lefkowitz, 1998; Kristiansen, 2004). Following receptor activation, G_{α_s} -coupled receptors activate adenylyl cyclase to increase the conversion of adenosine triphosphate (ATP) to cyclic-adenosine monophosphate (cAMP) (Lefkowitz, 1998) (Figure 1.1). cAMP acts as a secondary messenger in response to G_{α_s} -coupled receptor activation and subsequently binds the two regulatory subunits of protein kinase A (PKA). Once four cAMP molecules have bound, the regulatory subunits of PKA dissociate from the two catalytic subunits, thereby activating the kinase to phosphorylate a myriad of intracellular proteins, kinases, and transcription factors that ultimately leads to alterations in cellular activity (Wess, 1997; Lefkowitz, 1998; Kristiansen, 2004). This signal is terminated by the cAMP phosphodiesterase-mediated degradation of cAMP into 5'-AMP. Conversely, G_{α_i} -coupled receptors maintain adenylyl cyclase in the inactive state, inhibiting the buildup of cAMP and subsequent PKA activation (Lefkowitz, 1998; Kristiansen, 2004).

1.2.2 IP₃ Signalling Pathway

A distinctly different signalling pathway is mediated by $G_{\alpha_{q/11}}$ -coupled receptors. Once GDP is exchanged for GTP, $G_{\alpha_{q/11}}$ activates phospholipase C (PLC) which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) from the membrane into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) (Lefkowitz, 1998; Kristiansen, 2004) (Figure 1.1). These molecules act as the secondary messengers of $G_{\alpha_{q/11}}$ -coupled receptor

activation. IP_3 binds to IP_3 receptors located in the endoplasmic reticulum, causing the release of intracellular Ca^{2+} stores (Lefkowitz, 1998; Kristiansen, 2004). The concomitant increase in intracellular DAG and Ca^{2+} leads to the activation of protein kinase C (PKC) and Ca^{2+} -calmodulin kinase (CaMK) (Lefkowitz, 1998; Kristiansen, 2004). These kinases have many protein targets for phosphorylation including other kinases and transcription factors that mediate the biological effect at the cellular level (Lefkowitz, 1998; Kristiansen, 2004).

1.2.3 ERK/MAPK Signalling Pathway

Another divergent signalling pathway activated by GPCRs is the mitogen-activated protein kinase/extracellular signal-related kinase (ERK/MAPK). Interestingly, this pathway can be activated following either $\text{G}\alpha_s$ or $\text{G}\alpha_{q/11}$ activation via PKA and PKC respectively, as well as G protein-independent mechanisms (Luttrell et al., 1997). In the case of $\text{G}\alpha_s$ - or $\text{G}\alpha_{q/11}$ -mediated ERK1/2 signalling, PKA or PKC can directly phosphorylate the small GTPase Ras leading to the exchange of GDP for GTP (Luttrell et al., 1997). Once Ras is activated, it can subsequently activate Raf, thereby initiating a cascade of kinase phosphorylation where Raf phosphorylates MAP kinase kinase (MEK), and MEK phosphorylates ERK1/2 (Luttrell et al., 1997). Phosphorylated ERK1/2 can then go on to phosphorylate a number of different proteins, including a number

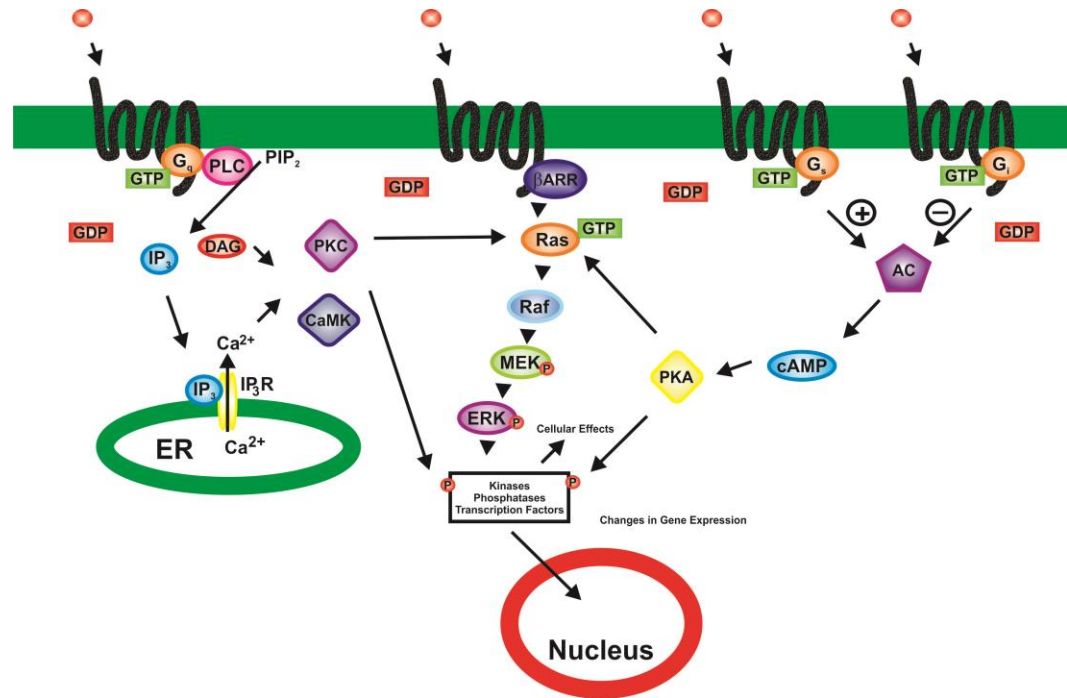


Figure 1.1. GPCR signalling pathways from left to right: G_q -coupled signalling, ERK signalling (example shown as G protein-independent, β arrestin-dependent), G_s -coupled signalling, and G_i -coupled signalling.

of intracellular proteins, kinases, and transcription factors to modulate cellular function (Luttrell et al., 1997) (Figure 1.1).

1.3 G Protein-Coupled Receptor Trafficking

1.3.1 Endocytosis of G Protein-Coupled Receptors

Following the agonist activation of GPCRs, there exists a series of events that result in the uncoupling or desensitization of GPCR signalling that are designed to terminate the GPCR signalling (Ferguson, 2001) (Figure 1.2). The most rapid of these processes involves GPCR phosphorylation by second messenger-dependent kinases that are activated downstream of G protein signalling, and by GRKs that phosphorylate the GPCR's carboxyl terminal tail and third intracellular loop to promote the recruitment of β -arrestins (Lohse et al., 1990; Lohse et al., 1992; Ferguson, 2001; Luttrell and Lefkowitz, 2002). β -Arrestin binding requires both agonist activation of the receptor and GRK phosphorylation (Ferguson, 2001). β -arrestins not only serve to sterically hinder the reassociation of GPCRs with the heterotrimeric G protein, but initiate GPCR endocytosis via their association with both clathrin and the β -adaptin subunit of the AP2 adaptor protein complex required for the formation of clathrin-coated pits (Ferguson, 2001; Luttrell and Lefkowitz, 2002). Clathrin forms a triskelion protein-structure which, when assembled, provides the intracellular lattice for endocytotic machinery like β -arrestins (Ferguson, 2001). Clathrin-independent and β -arrestin-independent endocytosis mechanisms have been described involving lipid rafts or caveolae. However, clathrin- and β -arrestin-dependent mechanisms are better described (Gonnord et al., 2012). This dense cluster of endocytotic proteins, including clathrin, AP-2, and β -arrestins, promotes the invagination of the membrane,

which ultimately pinches off intracellularly as a membrane-encapsulated endosome, a process known as receptor endocytosis or receptor internalization (Ferguson, 2001). This final fission from the rest of the cellular membrane is generally mediated by the large GTPase dynamin (Ferguson, 2001; Luttrell and Lefkowitz, 2002).

With the displacement of the heterotrimeric G protein and the removal of the receptor from the membrane, the GPCR-mediated signal prototypically terminates and becomes desensitized to further ligand activation. Notwithstanding, there have been recent indications that endosomal signalling may persist or initiate following endocytosis, whereby β -arrestins may act as scaffolds for ERK1/2 signalling proteins (Sorkin and von Zastrow, 2009). However, the physiological outcomes of these endosomal signalling mechanisms are poorly described and receptor endocytosis has prototypically been correlated with desensitization of traditional signalling pathways. Once the receptor is contained within the membrane of an early endosome, it can undergo a series of fates. These include: 1) dephosphorylation of the receptor in the early endosome, 2) sorting of endosomal contents to recycling pathways, 3) return of the receptor to the plasma membrane for further activation and/or 4) degradation via lysosomes (Pitcher et al., 1995; Zerial and McBride, 2001; Sorkin and von Zastrow, 2009; Grant and Donaldson, 2009).

1.3.2 G Protein-Coupled Receptor Recycling

Following receptor endocytosis, the receptor-containing endosome can undergo a series of fates ranging from receptor degradation to receptor recycling to the membrane. Interestingly, the intracellular trafficking of the receptor-containing endosome is highly

regulated by a collection of small Rab GTPases (Zerial and McBride, 2001). These proteins act as molecular switches that recruit the appropriate trafficking machinery for the endosomal stage and desired outcome by cycling between GDP- and GTP-bound states (Zerial and McBride, 2001). The fission of the endosome from the cell membrane aligns with the recruitment of Rab5, a marker of the early endosome (Zerial and McBride, 2001). Maturation of the endosome coincides with the dissociation of Rab5 and the initiation of divergent fates depending on endosomal contents and recruited trafficking machinery (Pitcher et al., 1995; Zerial and McBride, 2001; Sorkin and von Zastrow, 2009; Grant and Donaldson, 2009). For example, recruitment of Rab4 to the endosome initiates the formation of rapidly recycling endosomes and promotes GPCR dephosphorylation as the receptor transits from the early to the recycling endosome. GPCRs mobilized to rapidly recycling endosomes are trafficked back to the cell surface as fully functional resensitized receptors (Pitcher et al., 1995, Magalhaes et al., 2012). Another method of receptor recycling involves the Rab11 GTPase. Although the outcome is similar to Rab4-dependent recycling, this form of trafficking is mediated through the endosomal recycling compartment and has a greater latency (Sorkin and von Zastrow, 2009; Grant and Donaldson, 2009).

1.3.3 Lysosomal Degradation of G Protein-Coupled Receptors

In addition to recycling to the cell surface, GPCRs are also targeted for degradation in lysosomes (Sorkin and von Zastrow, 2009). This occurs when endosomes mature into multivesicular bodies and late endosomes (Sorkin and von Zastrow, 2009). The cargo destined for degradation is often targeted to the intraluminal vesicles via a process called

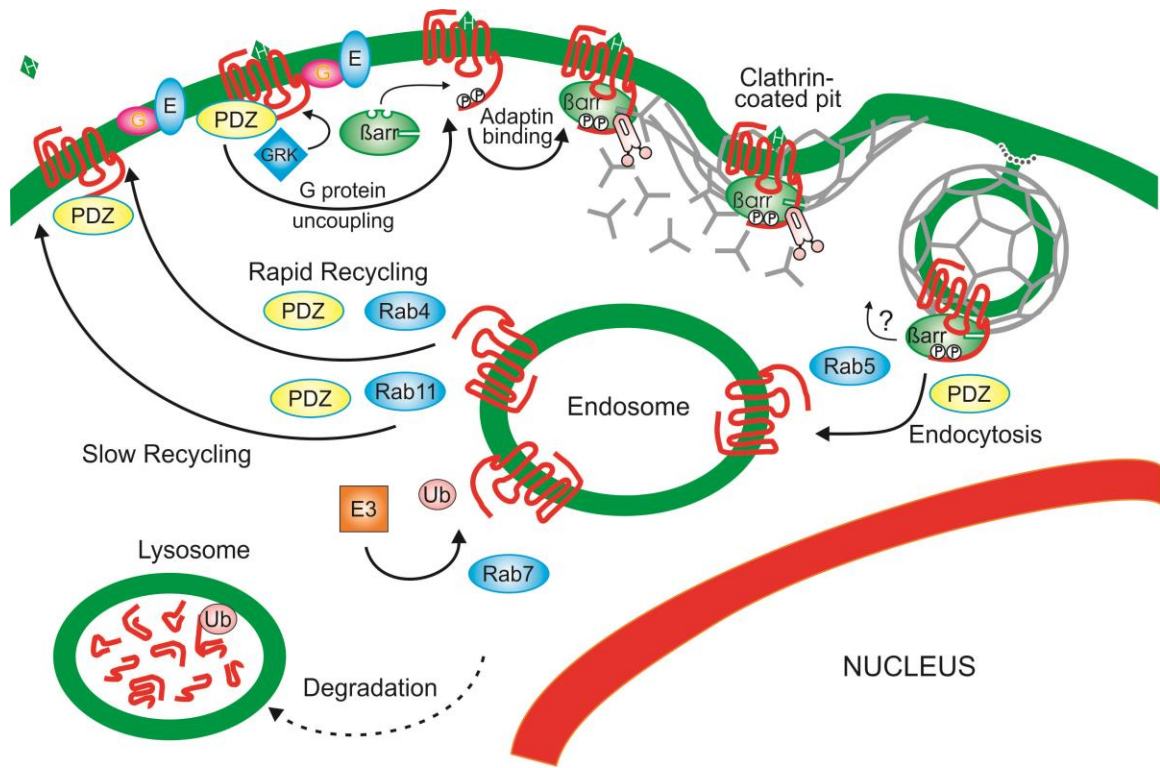


Figure 1.2. Mechanism of GPCR Trafficking and Sorting. PDZ signifies proposed roles for regulatory PDZ domain-containing proteins. G signifies G protein. E signifies G protein-dependent effector. E3 signifies ubiquitin ligase. Ub signifies Ubiquitin.

ubiquitination (Sorkin and von Zastrow, 2009). This post-translational modification is targeted to lysine residues by ubiquitin ligases and not only promotes receptor internalization, but delivers the cargo to the lysosomes (Sorkin and von Zastrow, 2009). After fusion of the intraluminal vesicles with the lysosome, the cargo is released into the lysosomal compartment where the proteins are met by a myriad of proteolytic enzymes for degradation (Sorkin and von Zastrow, 2009). This process involves the inclusion of Rab7 GTPase and trafficking machinery is removed and recycled to be used in another lysosomal degradation event (Sorkin and von Zastrow, 2009).

1.4 Organization & Structure of the Post-Synaptic Density

Within the central nervous system, GPCRs and ion channels are primarily targeted at the membrane of dendritic post-synaptic terminals in and around a region termed the post-synaptic density (PSD) (Zheng and Feng, 2009; Neubig and Siderovski, 2002; Magalhaes et al., 2012) (Figure 1.3). Each post-synaptic density is specifically organized such that dozens to hundreds of receptors are targeted to this specialized membrane domain via the interaction of scaffolding proteins with the receptors. These scaffolding proteins containing multiple protein-protein interaction domains that allow them to interact with a multitude of structural and signalling proteins holding them in close proximity with one another (Zheng and Feng, 2009). Of these scaffolding proteins, it is believed that PDZ (PSD-95, Disc large, Zona occludens-1) domain-containing proteins are the most abundant, and often provide direct contact with both GPCRs and ion channels at the post-synaptic

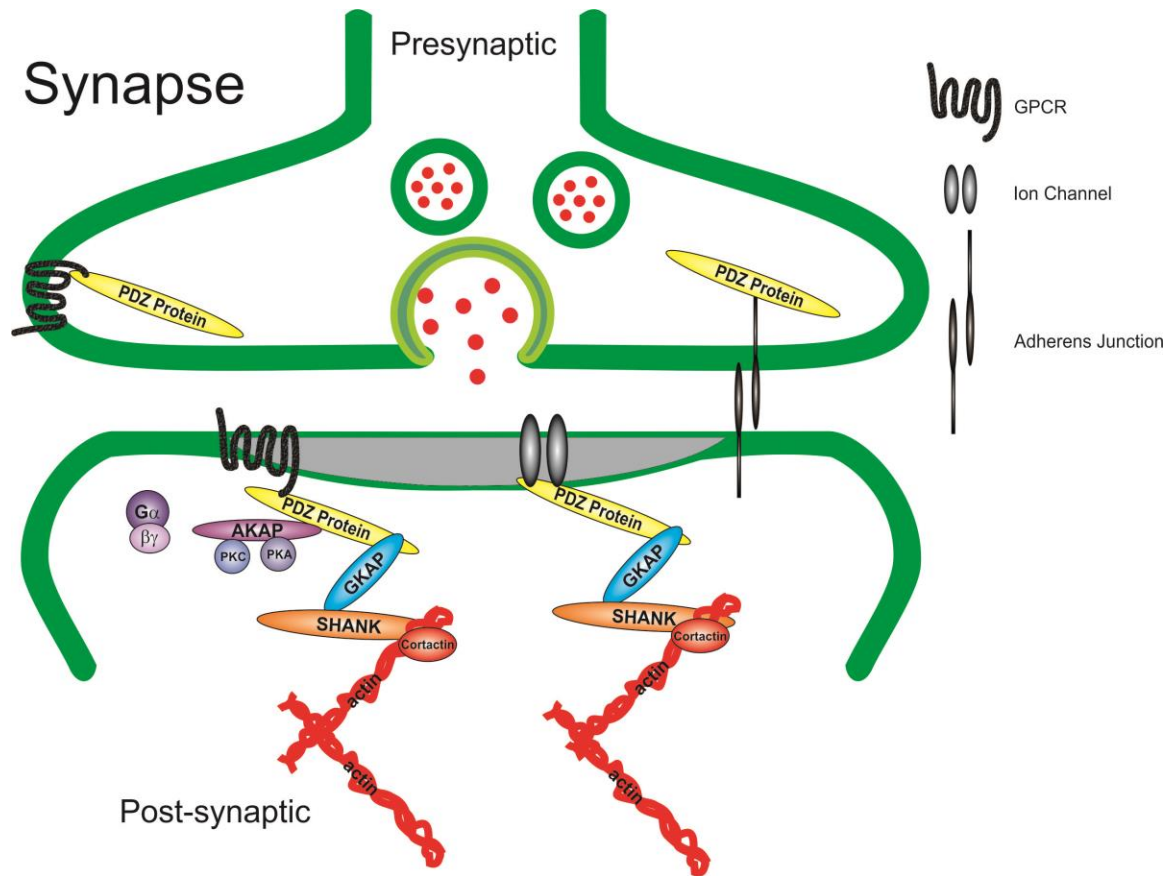


Figure 1.3. Simplified synapse to demonstrate the structural hierarchy of the postsynaptic density.

density (Cheng et al., 2006; Zheng and Feng, 2009). Structurally, PDZ domain-containing proteins make up the second layer of the post-synaptic density after the receptors, and are arranged perpendicularly such that their amino termini are attached to the membrane (Cheng et al., 2006; Zheng and Feng, 2009). A large subgroup of these second layer PDZ domain-containing proteins are known as PSD-95 family membrane-associated guanylyl kinase (MAGUK) proteins. These MAGUK proteins are characterized not only by the inclusion of one or multiple PDZ domains, but two additional protein-protein interaction domains termed the Src homology 3 (SH3) domain and the guanylyl kinase-like (GK or GuK) domain (Zheng and Feng, 2009). These domains are important for linking the second layer of PDZ domain-containing proteins to the third layer within the post-synaptic density which is abundantly occupied by Shank and guanylate kinase-associated protein family members (Cheng et al., 2006; Zheng and Feng, 2009). It is this layer that provides the majority of connections between the post-synaptic density and the actin cytoskeleton and completes a vast network of protein-protein interactions capable of accommodating a plethora of signalling molecules and enzymatic proteins (Cheng et al., 2006; Zheng and Feng, 2009).

1.5 PDZ Domains

Of the network of proteins that make up the post-synaptic density, PDZ domain-containing proteins appear to be most abundant and influential adaptor proteins involved in directly regulating GPCR activity (Cheng et al., 2006; Zheng and Feng, 2009; Magalhaes

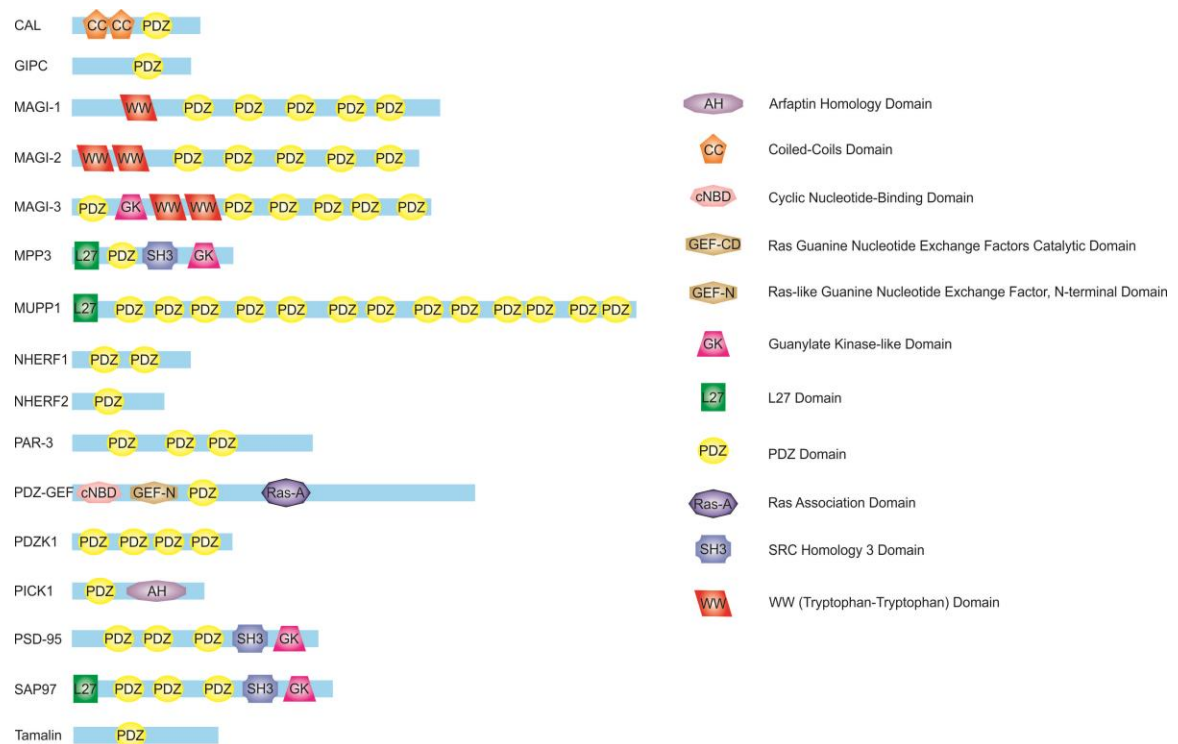


Figure 1.4. Comparison of conserved domains amongst PDZ domain-containing proteins, focussed predominantly on PDZ domain-containing proteins demonstrated to functionally regulate GPCRs. See also *Table 1.1*.

et al., 2012) (Figure 1.4). PDZ domains are approximately 80-90 amino acid residues in length and are the most common protein-protein interaction domain (Doyle et al., 1996; Zheng & Feng, 2009; Magalhaes et al., 2012). Although there are hundreds of unique PDZ domain sequences, they all contain a conserved glycine-leucine-glycine-phenylalanine (GLGF) sequence that provides the domain's folded, globular, cup-like structure that is capable of recognizing short, finger-like peptides (Harris and Lim, 2001). Because of this structure, PDZ domains appear best suited for binding the distal regions of receptor carboxyl terminal tails, labelled the PDZ-binding motif (Kornau et al., 1995; Niethammer et al., 1996; Harris & Lim, 2001; Magalhaes et al., 2012). Interestingly, additional studies have identified internal PDZ ligands that, like a carboxyl terminal tail, project outwardly from the protein (Xu et al., 1998; Hillier et al., 1999; Christopherson et al., 1999; Fouassier et al., 2000; Harris and Lim, 2001; Trejo, 2005). In this case the internal PDZ-binding motif is manifested as a sharply folded, finger-like projection (Xu et al., 1998; Hillier et al., 1999; Christopherson et al., 1999; Fouassier et al., 2000; Harris and Lim, 2001; Trejo, 2005).

1.6 PDZ-Binding Motifs

Although seemingly imperfect and biased against internal PDZ ligands, a simple classification system has evolved to identify potential PDZ-binding motifs on carboxyl terminal tails and help to predict potential PDZ domain-containing proteins that interact with them (Songyang et al., 1997; Bezprozvanny and Maximov, 2001; Sheng and Sala, 2001; Vaccaro and Dente, 2002). While there is some debate over how many classes of PDZ-binding motifs there are, it is most commonly limited to three classes (Sheng and

Sala, 2001; Magalhaes et al., 2012). Class I PDZ-binding motifs are the most commonly described class within the literature and are distinguished by their final 3 amino acid sequence of S/T-X- Φ , where X indicates any amino acid and Φ indicates any hydrophobic amino acid (Songyang et al., 1997; Bezprozvanny and Maximov, 2001; Sheng and Sala, 2001; Vaccaro and Dente, 2002). However, valine, isoleucine, or leucine appear to be most common of the hydrophobic amino acids that contribute to the formation of a Class I PDZ binding motif (Songyang et al., 1997; Bezprozvanny and Maximov, 2001; Sheng and Sala, 2001; Vaccaro and Dente, 2002). Class II and III PDZ-binding motifs are not as well characterized and show slightly more ambiguous sequences: with class II having its final 3 amino acids as Φ -X- Φ , and class III having Ψ -X- Φ , where Ψ represents any acidic amino acid residue (Sheng and Sala, 2001).

1.7 PSD-95 Family of PDZ Domain-Containing MAGUK Proteins

PSD-95 is perhaps the best described PDZ domain-containing protein and has been estimated to be one of the most abundant proteins within the post-synaptic density (Cheng et al., 2006). PSD-95 immunostaining has been extensively used as a marker to identify post-synaptic densities by immunofluorescence imaging. However, less attention has been drawn to the specific roles of PSD-95 both within the post-synaptic density and beyond the central nervous system at other cell junctions (Gardoni et al., 2009). Interestingly, it now appears that PSD-95 was merely the first identified of a small, structurally homologous group of PDZ domain-containing proteins that is currently termed

the PSD-95 family of MAGUK proteins (Gardoni et al., 2009). The members of this group include PSD-95, synapse-associated protein 97 (SAP97), and the lesser studied PSD-93 and synapse-associated protein 102 (SAP102) (Gardoni et al., 2009).

The hallmark of the PSD95 family of PDZ domain-containing MAGUKs lies within their protein-protein interaction domains. Although there is some minor variability at the distal amino termini of these proteins, all members of this family follow with three PDZ domains followed by an SH3 domain and a GK domain on the carboxyl termini (Gardoni et al., 2009). The abundance of these proteins within the post-synaptic density, coupled with these five protein-protein interaction domains, allows for an enormous potential for scaffolding numerous receptors, ion channels, signalling proteins and enzymes into a diverse and fluid signalling complex.

In addition to variations in sequence and the distal amino termini, the PSD-95 family of PDZ domain-containing MAGUKs also show differences within their subcellular localization in the brain (Gardoni et al., 2009). PSD-95 and PSD-93 exhibit high levels of palmitoylation on their amino termini which promotes their recruitment to the membrane of the post-synaptic density (El-Husseini et al., 2000; Kim and Sheng, 2004). SAP97 and SAP102 can also be found within the synapse. However they also show subcellular distribution within the axon, dendrites, and the cytosolic compartment (Kim and Sheng, 2004). Another point of difference between these proteins is their expression levels, both within cell types and during the various stages of development (Kim and Sheng, 2004).

The PSD-95 family of PDZ domain-containing MAGUK proteins play an essential role in human neurophysiology and development. This is demonstrated in mouse knockout studies, where PSD-95 and PSD-93 double-knockout mice exhibit severe deficiencies in

AMPA currents, and SAP97 knockout mice show neonatal lethality (Caruana and Bernstein, 2001; Howard et al., 2010). Of particular interest to the current study, PSD-95 has been shown to be essential for hallucinogenic and atypical antipsychotic action (Abbas et al., 2009). SAP102 has also demonstrated importance during early synaptic development (Kim and Sheng, 2004; Elias et al., 2006). The importance of these proteins is again highlighted by their diversity of binding partners, including: voltage-gated K^+ channels, glutamate-gated AMPA, NMDA, and kainite ion channels, GPCRs and a myriad of other important neuronal proteins (Kim et al., 1995; Kornau et al., 1995; Muller et al., 1996; Leonard et al., 1998; Garcia et al., 1998; Magalhaes et al., 2012; Gardoni et al., 2009). Comprehending both the specific and the redundant roles of the PSD-95 family of PDZ domain-containing MAGUK proteins promises to be an integral component for understanding human neurophysiology.

1.8 Regulation of G Protein-Coupled Receptors by PDZ Domain-Containing Proteins

PDZ domain-containing proteins represent an intriguing group of candidates for the development of new pharmaceuticals. Their ability to regulate GPCRs is becoming more apparent throughout the recent literature (Magalhaes et al., 2012), adding another dimension of GPCR function that only furthers the complexity GPCR signalling. However, it is this complexity that should aid in developing new pharmaceuticals with greater efficacy and fewer off-target effects. Each GPCR-PDZ functional relationship appears dependent on the receptor in question, the interacting PDZ domain-containing

protein, and the cell type in which this interaction takes places (Magalhaes et al., 2012). For example, the β_1 -adrenergic receptor (β_1 AR) contains a class I PDZ-binding motif on the distal end of its carboxyl terminal tail and has been one of the most documented GPCRs regulated by PDZ domain-containing proteins (Hu et al., 2000; Xu et al., 2001; Pak et al., 2002; Hu et al., 2003; He et al., 2004; He et al., 2006; Gardner et al., 2007). While the PDZ domain-containing proteins PSD-95 and SAP97 both appear to promote membrane localization of the β_1 AR, PSD-95 has been documented to do so by inhibiting receptor internalization, while SAP97 has been suggested to promote receptor recycling to the membrane (Hu et al., 2000; Gardner et al., 2007). Conversely, two other PDZ domain-containing proteins, MAGI-2 and Cal, have been demonstrated to suppress membrane localization of the β_1 AR (Hu et al., 2000; He et al., 2004). However, MAGI-2 prevents membrane stability by promoting receptor internalization into endocytic vesicles, while Cal traps receptor in the Golgi apparatus (Hu et al., 2000; He et al., 2004). The ability of PSD-95 to suppress β_1 AR internalization is also observed for the serotonin 2A receptor (5-HT_{2A}R). However, it appears to play the opposite role in promoting 5-HT_{2C}R internalization (Xia et al., 2003; Gavarini et al., 2006).

Albeit confusing, understanding each of these specified GPCR-PDZ functional relationships may lead to fine-tuned development of new pharmaceuticals that specifically target GPCR-PDZ interactions. A table of these documented GPCR-PDZ functional relationships is outlined in Table 1.1 and some trends are developing. For example, NHERF1 has demonstrated a clear importance for receptor trafficking and membrane localization by both inhibiting receptor endocytosis and, to a lesser extent, promoting receptor recycling (Rochdi and Parent, 2003; Wang et al., 2007; Wang et al., 2009; Li et

PDZ Protein	GPCR	Reported Effect on GPCR	Reference
CAL (PIST, GOPC, FIG)	β_1AR mGluR1a mGluR5a SSTR5	↓ membrane expression Agonist-induced intracellular co-localization with receptor, ↓ ERK activation ↑ mGluR5a protein expression, ↓ receptor ubiquitination Targets receptor to Golgi apparatus (agonist-independent), ↓ membrane-targeting/recycling	He <i>et al</i> , 2004 Zhang <i>et al</i> , 2008 Cheng <i>et al</i> , 2010 Wente <i>et al</i> , 2005
GIPC (TIP-2, Synectin)	β_1AR D₂ D₃ hLHR	↓ ERK activation, no effect on cAMP signalling Targets receptor to Golgi region ↓ signal transduction through G _i , targets receptor to Golgi region, ↓ D ₃ degradation Maintains receptor cell surface expression during hormone internalization	Hu <i>et al</i> , 2003 Jeanneteau <i>et al</i> , 2004 Jeanneteau <i>et al</i> , 2004 Hirakawa <i>et al</i> , 2003
MAGI-2 (S-SCAM, ARIP-1)	β_1AR VPAC1	↑ agonist-induced internalization, no effect on cAMP signalling ↓ cAMP signalling, ↓ agonist-induced internalization	Xu <i>et al</i> , 2001 Gee <i>et al</i> , 2009
MAGI-3	β_1AR β_2AR LPA₂	↓ ERK activation ↓ ERK activation ↑ ERK activation, ↑ RhoA activation	He <i>et al</i> , 2005 Yang <i>et al</i> , 2010 Zhang <i>et al</i> , 2007
MPP3	5-HT_{2c}	↑ membrane stability, ↓ desensitization	Gavarini <i>et al</i> , 2006
MUPP-1	5-HT_{2a} GABA_B MT₁ OR2AG1	↑ localization to cell surface ↑ Ca ²⁺ signalling ↑ coupling with G _i protein, ↑ inhibition of adenylyl cyclase activity Modulates Ca ²⁺ signalling	Jones <i>et al</i> , 2009 Balasubramanian <i>et al</i> , 2006 Guillaume <i>et al</i> , 2008 Dooley <i>et al</i> , 2009
NHERF1 (EBP50)	5-HT_{4a} β_2AR β_2AR hKOR PTH1R PTH1R PTH1R TPβ	Targets receptor to microvilli to interact with ezrin (potential role in cytoskeletal remodelling) Agonist-induced co-localization with receptor Regulates receptor sorting through ERM-binding domain interactions ↓ receptor down-regulation, ↑ recycling ↓ agonist-induced internalization, negligible effect on recycling and cAMP signalling ↑ coupling to, and activation of, G _q ↓ interaction with β -arrestin-2, ↓ desensitization Targets receptor to membrane regions in close proximity to cytoskeleton, ↑ cAMP signalling ↓ internalization	Joubert <i>et al</i> , 2004 Hall <i>et al</i> , 1998 Cao <i>et al</i> , 1999 Li <i>et al</i> , 2002 Wang <i>et al</i> , 2007 Wang <i>et al</i> , 2010 Wang <i>et al</i> , 2009 Wheeler <i>et al</i> , 2008 Rochdi <i>et al</i> , 2003
NHERF2 (E3KARP, SIP-1, TKA-1)	LPA₂ LPA₂ mGluR5a P2Y₁R PTH1R PTH1R PTH1R	↑ interaction with PLC β 3 ↑ IP ₃ signalling, ↑ ERK activation ↑ agonist-induced Ca ²⁺ signalling ↑ interaction with PLC β , ↑ agonist-induced Ca ²⁺ signalling ↑ interaction with, and activates, PLC β ↓ adenylyl cyclase activity through stimulation of inhibitory G proteins (G _{i/o} proteins). ↑ coupling to, and activation of, G _q and G _s ; ↓ coupling to, and activation of, G _s	Choi <i>et al</i> , 2010 Oh <i>et al</i> , 2004 Paquet <i>et al</i> , 2006 Fam <i>et al</i> , 2005 Mahon <i>et al</i> , 2002 Mahon <i>et al</i> , 2002 Wang <i>et al</i> , 2010
PAR-3	BK	↑ interaction with PLC β 1	Choi <i>et al</i> , 2010
PDZ-GEF (CNrasGEF, RA-GEF, Rap GEP)	β_1AR	Required for agonist-induced Ras activation via G _s -mediated cAMP signalling	Pak <i>et al</i> , 2002
PICK1	mGluR7a PrRP (GPR10)	↓ PKC α -mediated receptor phosphorylation ↑ intracellular clustering of receptor	Dev <i>et al</i> , 2000 Lin <i>et al</i> , 2001
PSD-95 (DLG4, SAP90)	5-HT_{2a} 5-HT_{2c} β_1AR D₁ D₁	↓ agonist-induced internalization, ↑ IP ₃ signalling ↑ constitutive and agonist-induced internalization, ↑ desensitization ↓ agonist-induced internalization, no effect on cAMP signalling ↑ constitutive internalization, ↓ cAMP signalling ↑ recycling, ↑ resensitization, no effect on G _s activation or cAMP signalling	Xia <i>et al</i> , 2003 Gavarini <i>et al</i> , 2006 Hu <i>et al</i> , 2000 Zhang <i>et al</i> , 2007 Sun <i>et al</i> , 2009
SAP97 (DLG1)	β_1AR	↑ PKA-mediated receptor phosphorylation, ↑ recycling	Gardner <i>et al</i> , 2007
Tamalin (GRASP)	mGluR1a	↑ membrane expression, ↑ complex formation with cytohesin-2 (GEF)	Kitano <i>et al</i> , 2002

Table 1. Functional interactions of PDZ domain-containing proteins and GPCRs. A version of this table is published in the *British Journal of Pharmacology*: Magalhaes AC, Dunn H and Ferguson SS (2012) Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins. *Br J Pharmacol* **165**:1717-1736.

al., 2002). Conversely, NHERF2 does not yet have a well-documented role in GPCR trafficking, but appears to have a significant role in Gαq-coupling, and subsequent PLC activation, IP₃ accumulation and Ca²⁺ release (Wang et al., 2010; Mahon et al., 2002; Choi et al., 2010; Fam et al., 2005; Oh et al., 2004; Paquet et al., 2006). MAGI2 has also demonstrated a role in the regulation of GPCR trafficking, whereas the role of MAGI3 has been described primarily in the context of ERK signalling (Xu et al., 2001; Gee et al., 2009; He et al., 2006; Zhang et al., 2007; Yang et al., 2010). Interestingly, PDZ domain-containing proteins Cal and GIPC both appear to be Golgi-associated proteins that have a role in post-translational modifications of GPCRs and regulating the forward trafficking of the receptor from the Golgi to the cell surface (Wente et al., 2005; Cheng et al., 2010; Jeanneteau et al., 2004). A schematic of proposed roles of PDZ domain-containing proteins in regulating GPCR activity is shown in Figure 1.2 and is based on the currently available evidence. For the studies to follow in Chapter 2, 3, and 4, PSD-95 and SAP97 will be the focus.

1.8.1 PSD-95 and SAP97 in Regulating G Protein-Coupled Receptor Function

Although the definitive roles for PSD-95 and SAP97 in GPCR regulation have yet to be clearly elucidated, there is a small body of work to make inferences upon the subcellular physiological processes they may contribute to (Magalhaes et al., 2012). PSD-95 has been demonstrated to decrease the agonist-induced endocytosis of both the 5HT_{2A}R and the β₁AR (Xia et al., 2003; Hu et al., 2000). While this interaction is capable of enhancing 5HT_{2A}R-mediated IP₃ signalling via Gαq, it had no effect on β₁AR-stimulated

cAMP signalling via $G\alpha_s$ (Xia et al., 2003; Hu et al., 2000). As mentioned previously, the role for PSD-95 in regulating 5HT_{2C}R appears to be in opposition to what is observed for β_1 AR, as PSD-95 increases both constitutive and agonist-mediated endocytosis of 5-HT_{2C}R leading to increased desensitization of 5-HT_{2C}R signalling (Gavarini et al., 2006). Interestingly, PSD-95 has been demonstrated to also increase both the constitutive endocytosis, recycling and resensitization of the dopamine D1 receptor (Zhang et al., 2007; Sun et al., 2009). However, there have been conflicting results regarding whether PSD-95 inhibits D1 receptor-mediated cAMP signalling (Zhang et al., 2007; Sun et al., 2009). Nevertheless, the potential role of PSD-95 in enhancing receptor recycling is mirrored by the ability of SAP97 to regulate the recycling of the β_1 AR (Gardner et al., 2007). Although there are some discrepancies in the functional characterization of PDZ domain-containing proteins, these studies suggest PSD-95 and SAP97 play a major role in regulating GPCR trafficking with a predisposition for promoting increased plasma membrane localization of GPCRs, and that this mechanism involves a reduction in GPCR internalization and an enhancement in GPCR recycling.

1.9 Mood Disorders and Psychiatric Disease

One of the least effective and minimally developed group of pharmaceuticals over the past half-century has been those used to treat mood disorders and psychiatric disease (Urs et al., 2014). This is undoubtedly due to our insufficient knowledge of the cellular and subcellular pathologies that are associated with these disorders (Urs et al., 2014). It is estimated that 25% of the population will be affected by some form of mental illness:

ranging from drug abuse, anxiety disorders, depression, bipolarism, schizophrenia and other psychiatric illnesses (WHO, 2001; Urs et al., 2014). A common misconception about mental illnesses, like depression, is the suggestion that patients could conquer the illness without medical intervention (Catapano and Manji, 2007; Fribourg et al., 2011). In fact, many studies have demonstrated that those suffering from mental illness frequently have pathological differences in their brain that could benefit from pharmacological intervention (Catapano and Manji, 2007; Fribourg et al., 2011). Although many of these disorders have similarities and co-morbidities, properly identifying the illness can heavily influence the treatment strategy.

1.9.1 Anxiety Disorders

Anxiety is a common emotional state exemplified by intensified emotional arousal and autonomic nervous system activity (Lowry et al., 2005). This complex emotion is believed to be prevalent in scenarios where the outcome of a particularly emotionally-salient event is undefined, and both positive and negative outcomes are possible (Lowry et al., 2005). It is the uncertainty and uncontrollability of the outcome associated with one's actions that leads to this heightened state of emotional arousal, often promoting the avoidance of the emotionally-salient scenario and the associated behaviours (Lowry et al., 2005). Although anxiety can be positive in heightening arousal levels when faced with difficult decisions of high emotional salience, anxiety disorders can prolong or sensitize the anxiety in seemingly mild scenarios. These disorders include phobias, panic disorders, separation anxiety, obsessive-compulsive disorder, situational anxiety, post-traumatic

stress disorder and general anxiety disorder. Although there are subtle differences amongst these disorders, they are united by the intense emotional and autonomic arousal under innocuous circumstances with perceivably undefined, uncontrolled, and/or unpredictable outcomes (Lowry et al., 2005).

1.9.2 Depressive Disorders

Anxiety disorders and major depressive disorders are often mentioned in the same sentence, often because of the comorbidity of these two forms of mental illness (Nesler et al., 2002; Lowry et al., 2005). Nonetheless, there are tangible differences between the two. In anxiety disorders, there exists a heightened state of arousal caused by the uncertainty of an outcome, where both positive and negative outcomes are possible (Lowry et al., 2005). Depression is instead characterized by a state of low energy, fatigue, and lowered concentration, with feelings of hopelessness, low self-esteem and general depressed mood (Nestler et al., 2002). Depressed individuals exhibit higher levels of irritability and have dysfunctions in their sleeping patterns, appetite, and metabolism (Nestler et al., 2002; Shelton, 2007). Furthermore, the hallmarks of the disease include negative rumination, a lack of motivation, the inability to experience pleasure (termed anhedonia) and often reoccurring thoughts of death and/or suicide (Nestler et al., 2002; Shelton, 2007). Of these characteristics, anhedonia may be the most distinguishing factor from general anxiety disorders. The inability to experience pleasure eliminates a potential positive or reinforcing outcome for the depressed individual's behaviours: which can lead to the observed avoidance behaviours and feelings of early defeat and hopelessness (Nestler et al., 2002; Shelton, 2007). Nevertheless, there is a high-degree of similarity between anxiety disorders and major depressive disorders, as well as comorbidity of the disorders.

Depressive disorders affect an astounding 15-20% of North Americans within their lifetime with a high rate of reoccurrence and therefore represents a serious threat to the health of the population (Nestler et al., 2002; Krishnan and Nestler, 2008). Not only do depressed individuals have a heightened risk of suicide, but prolonged depression can increase the probability of many other serious and chronic diseases, including coronary heart disease and type II diabetes (Evans et al., 2005; Krishnan and Nestler, 2008). Despite the prevalence and severity of major depressive disorders, the underlying pathology and treatment/prevention strategies remain poorly understood. The hypothesized molecular neurobiology of anxiety disorders, depressive disorders, and schizophrenia will be discussed in the “G Protein-Coupled Receptors Implicated in Mood Regulation and Psychiatric Disorders” section.

1.9.3 Schizophrenia

Schizophrenia is a serious psychiatric disorder that occurs in approximately 1% of the general population (Bromet and Fennig, 1999). The severity of schizophrenia can vary from one individual to another, with symptoms ranging from dulled emotion, lack of motivation, poor attention, debilitated memory, and dysfunction in cognitive control, to the inclusion of delusional beliefs, hallucinogenic experiences, and disorganized thinking (Lewis and Lieberman, 2000). Many of these symptoms could be mistaken for anxiety or depressive disorders in earlier life, however the severe symptoms of delusions and hallucinations often don't present until early adulthood (Lewis and Lieberman, 2000). Sadly, over 10% of individuals suffering from schizophrenia will commit suicide within

their lifetime, and there is a strong genetic component in the etiology of the disease (Lewis and Lieberman, 2000). Despite the genetic predisposition for schizophrenia, the comorbidity of schizophrenia and anxiety and depressive disorders suggests early experience with these disorders may increase the risk of the manifestation of the psychosis-based disease (Marcelis et al., 1998; Hartley et al., 2013). Although second generation atypical antipsychotics have become the preferred treatment for this disorder, efficacy of these drugs remains suboptimal and serious side-effects remain prevalent, such as extrapyramidal symptoms and tardive dyskinesia (Pierre, 2005; Lewis and Lieberman, 2000).

1.9.4 G Protein-Coupled Receptors Implicated in Mood Regulation and Psychiatric Disorders

GPCRs are targeted by over 30% of currently available pharmaceuticals because of their widespread regulation of human physiological processes (Urs et al., 2014). This regulatory role of GPCRs has additionally been implicated in the modulation of mood and the manifestation of psychiatric disease (Catapano and Manji, 2007). This notion is supported by the fact that 90% of GPCRs are located within the brain (Vassilatis et al., 2003). Interestingly, this regulation of mood is not limited to a single family of GPCRs (Catapano and Manji, 2007). For example, agonists of dopamine receptors (D_{1-4}) have demonstrated some value as antidepressants, whereas antagonizing these receptors could be utilized for the treatment of acute periods of mania (Sporn et al., 2000). Antidepressants have been shown to generally downregulate both α - and β -AR activity, with exception of

the α_1 AR, which appears to be up-regulated by antidepressants (Catapano and Manji, 2007). Additionally, antidepressants have been associated with down-regulation of cholinergic GPCRs, and cholinergic muscarinic receptor 2 polymorphisms have been implicated in depression (Ellis and Lenox, 1990; Comings et al., 2002). Although the majority of research on the glutamatergic system's role in mood regulation has focussed on the ionotropic AMPA and NMDA receptors, metabotropic glutamate receptor 2 (mGluR2) has been demonstrated to be up-regulated with treatment of atypical antipsychotics (Fribourg et al., 2011). Furthermore, GABA_B receptor agonists have been shown to induce acute depression and antagonism of neurokinin1 (NK1) receptors offer effects similar to antidepressants (Post et al., 1991; Gobbi and Blier, 2005).

Despite an obvious complexity and diversity of GPCRs involved in mood regulation, the serotonergic and corticotrophic systems are of primary focus to our studies. 5HT_{2A}R, 5-HT_{2C}R, and corticotropin-releasing factor receptor 1 (CRFR1) are thought to contribute to the manifestation of mood disorders, whereas 5HT_{1A}R and CRFR2 may play more of a preventative role (Fernandes et al., 1997; Mansbach et al., 1997; Meltzer et al., 2003; Bale and Vale, 2004; Catapano and Manji, 2007). The studies to follow in Chapters 2, 3, and 4 will focus primarily on 5HT_{2A}R and CRFR1.

1.9.4.1 Serotonin 2A Receptor (5-HT_{2A}R)

Serotonin, or 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter that has a large role in regulating mood and behaviour (Hale et al., 2012). Serotonin also contributes to the modulation of sleep cycles, circadian rhythms, and appetite. These

important physiological systems that are commonly dysregulated in mood disorders and psychiatric disease (Krishnan and Nestler, 2008; Hale et al., 2012). The vast majority of serotonergic neurons in the brain are densely clustered within the raphe nuclei of the reticular formation, within the brain stem (Bobillier, 1976; Hornung, 2003). These neurons project towards many cortical and subcortical regions of the brain, including important regions of the limbic system like the nucleus accumbens, amygdaloid structures, basal ganglia, hippocampus, and prefrontal cortex (Hornung, 2003; Lowry et al., 2005). These limbic regions have been heavily implicated in regulating many aspects of mood and behaviour, such as emotional salience, reward, punishment, memory, and decision-making (Hornung, 2003; Lowry et al., 2005).

Serotonin can act on a seven different receptor subtypes and, with the exception of the 5-HT₃R ion-channel, all of these receptors are GPCRs (Barnes, 2011). Interestingly, a number of these receptors have been implicated in contributing to the antipsychotic action of second generation atypical antipsychotics, however the 5-HT₂R subtype, primarily 5-HT_{2A}R and 5-HT_{2C}R appear to be of greatest significance (Meltzer et al., 2003; Catapano and Manji, 2007).

In a study of the post-mortem brains of suicide victims who had been known to be afflicted with a major depressive disorder, a significant enhancement in 5-HT_{2A}R and 5-HT_{2C}R expression levels was observed when compared to control brains (Catapano and Manji, 2007). Interestingly, a similar increase in 5-HT_{2A}R expression has been documented in a population of schizophrenics who had not been treated with second generation atypical antipsychotics, whereas those who had received this treatment saw a decrease in 5-HT_{2A}R expression (Fribourg et al., 2011). Furthermore, the efficacy of

atypical antipsychotics, such as clozapine, aripiprazole, and risperidone appears to be reliant on the compounds ability to suppress 5-HT_{2A}R-biased signalling of a 5-HT_{2A}R-mGluR2 heterodimer (Fribourg et al., 2011). Additionally, 5-HT_{2A}R knockout mice exhibit significantly lower anxiety-related behaviours when compared to control mice (Weisstaub et al., 2006). For these reasons, 5-HT_{2A}R and 5-HT_{2C}R have become primary targets for the treatment of a range of mental illnesses including anxiety disorders, major depressive disorders, eating disorders, obsessive-compulsive disorder, and schizophrenia (Gray and Roth, 2001; Roth and Xia., 2004; Catapano and Manji, 2007).

Although 5-HT₂Rs appear to be relevant for a continuum of mental illnesses, relatively little is known about the subcellular regulation of 5-HT₂Rs in the brain (Schmid et al., 2008; Abbas et al., 2009). Both the increased 5-HT_{2A}R expression levels in depressed patients, and the strong antagonism of 5-HT_{2A}R exhibited by the most efficacious atypical antipsychotics, drive the hypothesis that suppressing 5-HT_{2A}R expression and activity may be a pertinent strategy in mood regulation across a multitude of mental illness (Catapano and Manji, 2007; Fribourg et al., 2011). An emerging topic of the past decade has been the demonstration that 5-HT₂Rs are subject to the regulation of PDZ domain-containing proteins (Table 1). These proteins regulate specific receptor activities including membrane localization, receptor signalling, endocytosis, and desensitization (Table 1). In regards to the 5-HT_{2A}R and 5-HT_{2C}R, these signalling pathways primarily include IP₃ signalling and ERK/MAPK signalling (Raymond et al., 2009).

Perhaps the most important study exemplifying the importance of PDZ-regulation of 5-HT_{2A}R in mental illness demonstrated that atypical antipsychotic action via 5-HT_{2A}R

was dependent on the endogenous expression of PDZ domain-containing protein PSD-95 (Abbas et al., 2009). Although atypical antipsychotics represent the latest and most effective development in the treatment of psychoses-based psychiatric disease, the efficacy of these drugs remains suboptimal. Manipulating 5-HT_{2A}R interactions with specific PDZ domain-containing proteins may represent a viable strategy for the advancement of more effective psychiatric medications and mood regulation strategies. Although PSD-95 has previously been recognized as a regulator of 5-HT_{2A}R-mediated IP₃ signalling and receptor trafficking, an additional subset of PDZ domain-containing proteins have been identified that biochemically interact with 5-HT_{2A}R and may participate in regulating its function (Xia et al., 2003; Becamel et al., 2004; Abbas et al., 2009). Understanding how each of these PDZ domain-containing proteins may specifically influence 5-HT_{2A}R function may provide the opportunity to design new pharmacological intervention strategies to fine-tune 5-HT_{2A}R function and minimize the side-effects seen in current medications of mental illness.

1.9.4.2 Corticotropin-Releasing Factor Receptor 1 (CRFR1)

Corticotropin-releasing factor (CRF), also referred to as corticotropin-releasing hormone (CRH), is a neuropeptide commonly associated with its ability to initiate the hypothalamic-pituitary-adrenal (HPA) axis “stress response” (Tsigos and Choursos, 2002). This pathway is activated in response to stressors and leads to the release of CRF from the paraventricular nucleus of the hypothalamus (Tsigos and Choursos, 2002). CRF travels through the blood via the hypothalamic-hypophyseal portal system towards the anterior

pituitary. Following CRF stimulation, the anterior pituitary subsequently releases adrenocorticotrophic hormone (ACTH) into the bloodstream, which ultimately reaches the adrenal cortex of the adrenal glands located atop the kidneys (Tsigos and Choursos, 2002). This ACTH stimulation promotes the synthesis and release of cortisol and other glucocorticoids from the adrenal glands into the blood stream. Cortisol consequently circulates throughout the bloodstream and is capable of activating glucocorticoid receptors in the brain to regulate behaviour and liberate glucose from intracellular stores to provide energy for coping with the stressor (Tsigos and Choursos, 2002).

CRF's role in initiating the HPA axis stress response clearly demonstrates the neuropeptides importance in the body's physiological response to stress. However, emerging research suggests that an elevation of CRF in extra-hypothalamic brain regions can correlate with both anxiety disorders and depression (Austin et al., 2003). Apart from the paraventricular nucleus of the hypothalamus, CRF can also be synthesized and released from additional limbic regions such as the central amygdala and the bed nucleus of the stria terminalis (Swanson et al., 1983). Notably, increased levels of CRF in extra-hypothalamic brain regions have been observed in the post-mortem brains of depressed individuals who fell victim to suicide (Austin et al., 2003). Apart from the well-established role in stress, the CRF system has now become a major pharmacological target for the treatment of both anxiety disorders and depressive disorders.

CRF mediates its response via two different receptors, CRFR1 and CRFR2 both of which are GPCRs. Although these receptors have both been implicated in regulating mood and behaviour, CRFR1 has a ten-fold greater affinity for CRF neuropeptide and is expressed at higher levels throughout the brain (Bale and Vale, 2004). Additionally,

CRFR1 antagonists have demonstrated anxiolytic- and antidepressant-like effects: suggesting this receptor may be the most viable target for mood regulation and psychiatric treatment (Mansbach et al., 1997; Catapano and Manji, 2007). Analyzing all of the studies on knockout mice further reinforces CRFR1's role in anxiogenesis (Bale and Vale, 2004; Janssen and Kozicz, 2013). Although some inconsistencies have been documented depending on the type of knockout mouse, the majority of work demonstrates that CRFR1 knockout mice exhibit lowered-levels of anxiety-like behaviour, whereas CRFR2 knockout mice have heightened levels of anxiety- and depressive-like behaviour (Bale and Vale, 2004; Janssen and Kozicz, 2013). Although CRF-regulation of anxiety and depression is likely far more complicated than this model for receptor dualism, it is clear that CRFR1, and likely CRFR2, are viable targets for the treatment of mental illness.

Despite the promise of CRFR1 as a pharmacological target, very little is known about the regulation of CRFR1 function at the subcellular-level. CRFR1 is prototypically coupled to G α s and thereby signals primarily through the cAMP signalling pathway. However, it has additionally been demonstrated to activate the MAPK/ERK signalling pathway (Hauger et al., 2009). In terms of receptor trafficking, CRFR1 has been demonstrated to be phosphorylated by GRKs leading to the recruitment of β -arrestins and the initiation of receptor endocytosis (Holmes et al., 2006). CRFR1 colocalizes with the early endosomal small GTPase Rab5 during receptor internalization, and subsequently with Rab4 which initiates recycling of the receptor (Holmes et al., 2006).

Interestingly, a recent study in mouse cortical slices has demonstrated that CRFR1 is capable of enhancing the signalling of the 5-HT_{2A}R, another GPCR heavily involved in mood regulation and psychiatric pharmacology (see section 1.9.4.1) (Magalhaes et al.,

2010). Cotreatment of both CRF and DOI, a potent 5-HT_{2A}R agonist, was demonstrated to elicit a significant enhancement in anxiety-like behaviour in mice that was not observed with DOI alone (Magalhaes et al., 2010). Additionally, suicide victims with documented major depressive disorder have been observed with significantly enhanced CRF levels within serotonergic neurons of the raphe nuclei (Austin et al., 2003). Furthermore, CRF is capable of activating serotonergic efferents of the raphe nuclei that terminate in the medial prefrontal cortex (mPFC) and initiate anxiety-like behaviour (Meloni et al., 2008). Taken together, it is clear that the interaction of the CRF and serotonergic systems could play an integral role in mood regulation and the manifestation of psychiatric disease.

Some insight into potential CRFR1 regulation mechanisms was recently provided by the observation that the CRFR1 pre-activation enhances 5-HT_{2A}R-mediated IP₃ signalling and that this effect of CRFR1 on 5-HT_{2A}R signaling is dependent upon intact receptor PDZ-binding motifs (Magalhaes et al., 2010). Specifically, when deleted, the lack of a PDZ-binding motif in the distal carboxyl terminus of either the CRFR1 or 5-HT_{2A}R prevented the heterologous sensitization of 5-HT_{2A}R signaling by CRFR1 (Magalhaes et al., 2010). Interestingly, this phenomenon was also dependent upon effective receptor endocytosis and rapid recycling to the membrane, as blocking these processes abolished receptor crosstalk (Magalhaes et al., 2010). As PDZ domain-containing proteins appear integral in the regulation of GPCR trafficking, this finding provides further evidence for involvement of PDZ domain-containing proteins in the heterologous sensitization of 5-HT_{2A}R by CRFR1 (Magalhaes et al., 2010; Magalhaes et al., 2012). This suggests that not only could PDZ domain-containing proteins be important in regulating the crosstalk between CRFR1 and 5-HT_{2A}R, but PDZ interactions could foreseeably regulate CRFR1

function independently of 5-HT_{2A}R. The interaction of PDZ domain-containing protein PSD-95 with 5-HT_{2A}R has proved to be essential for the action of second generation atypical antipsychotics (Abbas et al., 2009). Therefore, it is plausible that PDZ protein-dependent regulation of CRFR1 may prove important in the manifestation of mental illness and the development of new pharmacological treatment strategies (Abbas et al., 2009).

1.9.5 Implications for PSD-95 and SAP97 in the Manifestation of Psychiatric Disease

PDZ domain-containing proteins have been implicated in regulating two important receptors involved in mood regulation: 5-HT_{2A}R and CRFR1 (Magalhaes et al., 2010). However, the specific PDZ domain-containing proteins that are involved in regulating 5-HT_{2A}R and CRFR1 remains relatively unknown. The first and only PDZ domain-containing protein documented to regulate 5-HT_{2A}R is PSD-95, and regulation of CRFR1 by PDZ domain-containing proteins has yet to be documented (Xia et al., 2003). Although the mechanisms for regulating mood-related receptors are yet to be understood, abnormalities in both PSD-95 and SAP97 have previously been implicated in psychiatric disease (Toyooka et al., 2002; Clinton et al., 2003; Clinton & Meador-Woodruff, 2004; Toro and Deakin, 2005; Clinton et al., 2006; Kristiansen, 2006; Tsai et al., 2007; Sato et al., 2008; Funk et al., 2009). In examining post-mortem brains of schizophrenic patients, a reduction of PSD-95 has been observed in the thalamus, hippocampus, and anterior cingulate cortex (Clinton & Meador-Woodruff, 2004; Toro and Deakin, 2005; Kristiansen, 2006; Funk et al., 2009). Conversely, other studies demonstrate that PSD-95 appears to be increased in the thalamus in elderly populations of schizophrenic patients (Clinton et al.,

2003; Clinton et al., 2006). This discrepancy is complicated by the method of determining changes in PSD-95 expression, mRNA transcript levels versus protein expression, as one study documented higher transcript levels associated with lower protein levels (Kristiansen, 2006). This suggests that despite potential increases in PSD-95 translation, increased PSD-95 protein degradation may prevail as the mechanism regulating functional PSD-95 protein available to regulate receptor activity at the synapse. Although the changes in SAP97 expression have not been examined in anxiety and depression, one study has reported that SAP97 expression is significantly lower in the prefrontal cortex of schizophrenic patients (Toyooka et al., 2002). Additionally, a variety of single nucleotide polymorphisms (SNPs) of the genes encoding PSD-95 and SAP97 have been associated with schizophrenia (Tsai et al., 2007; Sato et al., 2008). Most convincingly, the antipsychotic action of second generation atypical antipsychotics appears dependent on the endogenous expression of PSD-95 (Abbas et al., 2009). Taken together, it is clear that PSD-95 and SAP97 play an important role in the progression of psychiatric disease, and this role may be linked to their potential ability to regulate CRFR1 and 5-HT_{2A}R function.

1.10 Current Study Hypothesis, Objectives and Scientific Design

From the aforementioned evidence, CRFR1 and 5-HT_{2A}R appear to play a role in the manifestation of mental illness, and there is indication that these receptors may be regulated by PDZ domain-containing proteins. One of these studies indicated that the PDZ domain-containing protein PSD-95 was capable of regulating 5-HT_{2A}R trafficking and IP₃

signalling (Xia et al., 2003). Interestingly, PSD-95, and the similar structural homologue SAP97, have both been implicated in psychiatric disease. For this reason, it is possible that PSD-95 and SAP97 may regulate these receptors and this relationship may be of relevance for understanding, treating, and preventing mental illness. In addition, CRFR1 heterologously sensitizes 5-HT_{2A}R signalling in a PDZ protein interaction-dependent manner and synergistically increases 5-HT_{2A}R-mediated anxiety behaviour in mice (Magalhaes et al., 2010). Therefore, this thesis will test the hypothesis that the MAGUK family PDZ proteins, PSD-95 and SAP97, not only contribute to the regulation of CRFR1 and 5-HT_{2A}R activity, but that these interactions are involved in the sensitization of 5-HT_{2A}R signalling by CRFR1.

Because PSD-95 was already demonstrated to regulate 5-HT_{2A}R function (Xia et al., 2003), our objectives were as follows:

1. Evaluate whether SAP97 regulates CRFR1 signalling and trafficking
2. Evaluate whether SAP97 regulates 5-HT_{2A}R signalling and trafficking
 - Determine whether SAP97 is responsible for CRFR1-mediated sensitization of 5-HT_{2A}R signalling
3. Evaluate whether PSD-95 regulates CRFR1 signalling and trafficking
 - Determine whether PSD-95 is responsible for CRFR1-mediated sensitization of 5-HT_{2A}R signalling

To achieve these objectives, a number of experimental techniques were utilized. The interaction between PDZ domain-containing protein and receptor was determined via co-immunoprecipitation and immunofluorescent confocal microscopy utilizing HEK293 cells, rat neurons, or homogenized adult mouse

brain. To investigate the roles of PSD-95 or SAP97 in receptor trafficking, immunofluorescent confocal microscopy and flow cytometry were also employed. To determine PDZ protein effect on receptor signalling, a combination of cAMP assays, radiolabelled IP accumulation experiments, and western blot analysis of ERK1/2 phosphorylation were performed.

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Chapter 2: SAP97 Regulation of CRFR1

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Dunn HA, Walther C, Godin CM, Hall RA and Ferguson SS (2013) Role of SAP97 protein in the regulation of corticotropin-releasing factor receptor 1 endocytosis and extracellular signal-regulated kinase 1/2 signaling. *J Biol Chem* **288**:15023-15034.

2.1 Introduction

Corticotropin-releasing factor (CRF) is a neuropeptide that regulates the physiological response of the body to stress and initiates the hypothalamic-pituitary-adrenal axis stress response (Vale et al., 1981; Leonard, 2005). CRF mediates these responses by activating two distinct G protein-coupled receptors (GPCRs): CRF receptor 1 (CRFR1) and CRF receptor 2 (CRFR2) (Chalmers et al., 1996; Dautzenberg et al., 2002). In comparison to CRFR2, CRFR1 exhibits a significantly higher affinity for CRF and shows far greater expression within the brain and in the pituitary (Chalmers et al., 1995; Palchoudhuri et al., 1998). The dysregulation of the hypothalamic-pituitary-adrenal axis is thought to be involved in the onset of psychiatric diseases, such as depression, and CRFR1 antagonists have recently been shown to demonstrate anxiolytic- and antidepressant-like effects (Overstreet et al., 2004; Chaki et al., 2004). GPCRs, like CRFR1, and their associated GPCR-interacting Proteins (GIPs) have vast potential as pharmacological targets in the treatment of disease. Over 40% of modern pharmaceuticals target G protein-coupled receptors because of their widespread contributions to physiology (Marshall et al., 2001). For these reasons, CRFR1 represents an excellent candidate as a pharmacological target for the treatment of mood disorders, such as depression, and thus better understanding of the trafficking and signaling properties of CRFR1 may lead to the discovery of new pharmacological targets for mood regulation.

The physiological effects of GPCRs, like the CRFR1, are determined by the activation of receptor-mediated intracellular signaling pathways, and the intracellular trafficking of the receptor is responsible for determining the availability of receptor for agonist activation (Ferguson et al., 1998; von Zastrow et al., 2001; Brady et al., 2002).

CRFR1 has recently been shown to couple to, and activate, multiple different G proteins, including $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$, $G\alpha_o$, and $G\alpha_z$ (Grammatopoulos et al., 2001; Gutknecht et al., 2009). However, CRFR1 is thought to preferentially couple to $G\alpha_s$ leading to adenylyl cyclase activation and cAMP production, which functions to stimulate protein kinase A (Chen et al., 1986; Dautzenberg et al., 2002; Grammatopoulos et al., 2002; Hauger et al., 2006; Arzt et al., 2006). In addition, agonist stimulation of the CRFR1 results in the desensitization of CRFR1 signaling, as a consequence of both second-messenger-dependent protein kinase and G protein-coupled receptor kinase phosphorylation (Holmes et al., 2006; Oakley et al., 2007). In particular, GRK6 promotes β -arrestin recruitment to CRFR1 to facilitate CRFR1 endocytosis (Holmes et al., 2006). However, unlike what is observed for many GPCRs, CRFR1 stimulates the redistribution of β -arrestin2 to intracellular vesicles, but does not colocalize with β -arrestin2 in the intracellular compartment (Holmes et al., 2006).

Previous work from our laboratory has demonstrated a functional significance for the CRFR1 carboxyl terminal Class I (S/T-X- Φ -COOH, where Φ represents any aliphatic amino acid residue) PSD-95/Discs Large/Zona Occludens-1 (PDZ)-binding motif in the heterologous sensitization of serotonin 2A receptor (5-HT_{2A}R) signaling, suggesting a novel role for PDZ domain-containing proteins in the regulation of CRFR1 function (Magalhaes et al., 2010). Specifically, CRFR1 pre-activation selectively leads to increased 5-HT_{2A}R signaling in response to subsequent 5-HT treatment, which is dependent upon both intact CRFR1 and 5-HT_{2A}R carboxyl-terminal tail PDZ domain binding motifs. This CRFR1-dependent sensitization of 5-HT_{2A}R-signaling is also correlated with increased

anxiety responses in mice (Magalhaes et al., 2010). However, the identity of the specific PDZ protein(s) involved remains unknown.

We have investigated the capacity of the CRFR1 carboxyl-terminal tail to bind to a proteomic array of 96 Class I PDZ domains (Fam et al., 2005; He et al., 2006). We found that the CRFR1 carboxyl-terminal tail binds to a selective subset of Class I PDZ domains on the array. In the present study, we investigated the role of one of the positive CRFR1-interacting PDZ proteins identified in the screen that has been linked to the regulation of GPCR trafficking and signaling: synapse-associated protein 97 (SAP97; also known as DLG1). SAP97 has previously been identified as a GIP that binds to the 5-HT_{2A}R, 5-HT_{2C}R, β_1 -adrenergic receptor (β_1 AR), and somatostatin receptor subtype 1 and is reported to regulate β_1 AR recycling, as well as to couple somatostatin receptor subtype 1 activation to neurite outgrowth in development (Cai et al., 2008; Becamel et al., 2004; Gardner et al., 2007). We found that CRFR1 expression results in the PDZ binding motif-dependent redistribution of SAP97 from the cytoplasm to the plasma membrane and that SAP97 functions to negatively regulate CRFR1 endocytosis and is required for CRFR1-mediated activation of extracellular signal-regulated kinase (ERK1/2) phosphorylation, without affecting CRFR1-stimulated cAMP formation.

2.2 Experimental Procedures

Materials:

Goat anti-glutathione-S-transferase (GST) antibodies as well as ECL Western blotting detection reagents were purchased from GE Healthcare (Oakville, ON, Canada). Rabbit

anti-phospho-p44/42 MAP kinase (Thr202/Tyr402), and rabbit anti-p44/42 MAP kinase antibodies were obtained from Cell Signalling Technology (Pickering, ON, Canada). Rabbit anti-GFP antibody was obtained from Invitrogen/Life Technologies (Burlington, ON, Canada). Mouse anti-SAP97 antibody was obtained from Assay Designs/Enzo Life Sciences (Farmingdale, NY, USA). Alexa Fluor 647 anti-mouse IgG and Alexa Fluor 633 goat anti-mouse IgG Zenon antibodies were purchased from Invitrogen/Molecular Probes (Burlington, ON, Canada). cAMP GLO Assay was obtained from Promega (Madison, WI, USA). Mouse anti-HA antibody and all other biochemical reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Plasmid Constructs:

HA-tagged CRFR1 constructs (HA-CRFR1 and HA-CRFR1 Δ TAV) were described previously (Holmes et al., 2006; Magalhaes et al., 2010). The YFP-SAP97 and SAP97 single hairpin RNA (shRNA) constructs were graciously provided by Dr. Suleiman W. Bahouth (Neuroscience Institute, University of Tennessee Health Sciences Center) (Gardner et al., 2007). The YFP-SAP97 (rat isoform 2) was subcloned into the pEGFP1 vector. For the human siRNA studies (HEK293 cells), we used Silencer Validated siRNA (SAP97/DLG1) ID#146328 human NM_004087 GGAGAUCGUAUUAUAUCGGTT from Invitrogen (Burlington, ON, Canada). For the mouse siRNA studies (AtT20), we used Silencer Select siRNA (SAP97/DLG1) ID# s232370 mouse NM_007862 AUGACAAGCGUAAAAAGAATT from Invitrogen (Burlington, ON, Canada). For the negative controls, we used Silencer Negative Control #1 AM4635

AGUACUGCUUACGAUACGGTT from Invitrogen (Burlington, ON, Canada). The EPAC cAMP biosensor was the gift of Drs. Ali Salahpour (University of Toronto) and Marc Caron (Duke University) (Barak et al., 2008). The CRFR1 carboxyl-terminal tail was cloned into pGEX-4 with ECOR1/NotI.

Cell Culture and Transfection:

Human embryonic kidney (HEK 293) cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Cells were seeded on 10 cm dishes at 70-80% density 24 h prior to transfection. Transfection was performed using a modified calcium phosphate method, as described previously (Ferguson et al., 2004). Transfections were performed with 1 µg of each construct, with exception that 3 µg of plasmid cDNA was used for all shRNA constructs. Empty pcDNA3.1 vector was used to equalize the total amount of plasmid cDNA used to transfect cells. 18 h post-transfection, cells were washed with phosphate buffered saline (PBS) and re-suspended with Trypsin 0.25% EDTA.

For AtT20 cells, 3 mL aliquots of suspended AtT20 cells were transferred to T25 Nunc flasks 30 µL of Lipofectamine was incubated with 250 µL of Opti-MEM for 5 minutes at room temperature and then added to 250 µL of Opti-MEM and 80 pmol of either control or SAP97 siRNA. After 6 hours, cells were centrifuged at 13,000 g and transfection reagents were aspirated and replaced with 3 mL media. Cells were then reseeded for experimentation. 72 hours post-transfection, cells were spun down and media was aspirated and replaced with 3 mL HBSS for 1 hour at 37°C. Control and SAP97 siRNA samples were aliquoted into 1 mL samples and stimulated with 500 nM CRF for 0, 5, or 10 minutes. Cells were put on ice, spun down at 13,000 g at 4°C, HEPES-buffered saline

solution (HBSS) was aspirated and cells were lysed with 200 μ L of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10 μ g/ml leupeptin, and 5 μ g/ml aprotinin).

All experiments were conducted approximately 48 h after the initial transfection, with exception of transfections involving SAP97 shRNA, which were conducted 72 h after initial transfection to optimize the knockdown of endogenous SAP97, as confirmed by Western blotting.

PDZ blot overlay assay:

GST and GST-CRFR1 fusion proteins were generated by growing recombinant BL21 bacteria at 21°C to an A_{600} of 0.6-1.0. Cultures were induced for 3 hrs with 1mM IPTG, pelleted, resuspended in PBS containing protease inhibitors (1 mM AEBSF, 10 μ g/ml leupeptin, and 5 μ g/ml aprotinin) and lysed by mild sonication. The bacterial lysates were cleared of cellular debris by centrifugation and then applied to Glutathione Sepharose 4B overnight at 4°C. GST and GST-CRFR1 fusion proteins bound to the matrix were washed extensively in PBS-containing 0.3% Triton X-100. 100 nM of GST and GST-CRFR1 in blot buffer (2% nonfat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM Hepes, pH 7.4) were incubated with gridded nylon membranes that were spotted with His/S-tagged PDZ domain fusion proteins (1 μ g/bin) for 1 h at room temperature (Fam et al., 2005; He et al., 2006). The arrays were then washed three times with blot buffer, and incubated with a horseradish peroxidase-conjugated anti-GST antibody (1:3000). Interactions of the GST fusion proteins with the various PDZ domains were then visualized via chemiluminescence using the enhanced chemiluminescence kit from GE Healthcare.

Co-immunoprecipitation:

Transfected HEK 293 cells were seeded on 10 cm dishes the day before the experiment. Cells were serum-starved for 1 hour in HBSS, and dishes were treated with either HBSS alone or with 100 nM CRF agonist in HBSS for 30 min at 37°C. Cells were subsequently lysed in lysis buffer 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 Bronsted-Lowry protein assay was performed and 400 µg of protein was incubated for 1-2 h at 4°C with Protein G Sepharose and mouse anti-HA antibody (1:50). After incubation, beads were washed 3 times with cold lysis buffer and incubated overnight at room temperature in 3X SDS Loading Buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted to identify co-immunoprecipitated GFP-SAP97 (rabbit anti-GFP, 1:1000). An additional Western blot was performed to examine HA-CRFR1, HA-CRFR1 Δ TAV (mouse anti-HA, 1:1000) and GFP-SAP97 (rabbit anti-GFP, 1:1000) protein expression.

For the co-immunoprecipitation of endogenous proteins from cortical extracts, adult mouse brains were employed. Tissue was dissected and homogenized on ice in lysis buffer containing protease inhibitors. The particulate fraction was removed by centrifugation and 2 mg of supernatant protein was incubated with 5 µL/sample of either goat polyclonal anti-CRFR1 (CRF-RI (V14) sc-12381) or CRFR2 (CRF-RII (C-15) sc-20550) antibody from Santa Cruz (Santa Cruz, CA, USA) and protein G-sepharose beads by 2 hr rotation at 4°C. Afterwards, the beads were washed 2 times with lysis buffer and 1 time with PBS, and proteins were eluted in SDS-PAGE loading buffer by warming the

samples at 55°C for 5 min. Eluted samples were subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose membranes for immunoblotting with antibodies described in the *Figure Legends*.

Live HEK293 Cell Immunofluorescent Confocal Microscopy:

Following transfection, HEK 293 cells were re-seeded on 35-mm glass-bottom confocal dishes. Cells were serum-starved for 1 hour at 37°C in HBSS, then labeled with mouse anti-HA antibody (1:200) and Zenon Alexa Fluor 647 mouse IgG1 Labeling Kit (Invitrogen, Burlington, ON, Canada) at 4°C for 30 minutes. The cells were washed with HBSS and warmed to 37°C for live imaging using a heated stage. Confocal microscopy was performed on a Zeiss LSM-510 META laser scanning confocal microscope using a Zeiss 63X, 1.3 NA, oil immersion lens. Co-localization studies were performed using dual excitation (488nm, 633nm) and emission (band pass 505-550nm and long pass 650nm for YFP/GFP and Alexa Fluor 647, respectively) filter sets. The specificity of labeling and absence of signal crossover were established by examination of single-labeled samples. In receptor endocytosis experiments, the cells were additionally stimulated with 500 nM CRF agonist (Tocris) and specified cells were re-imaged at regular intervals for up to 60 minutes. Colocalization analysis was performed using Imaris 7.0 Colocalization module (bit-plane) to determine the colocalization of the brightest 2% of pixels in each channel, as described previously (Lorenzen et al., 2010).

Receptor Endocytosis:

Following transfection, HEK 293 cells were re-seeded into 12-well plates. Cells were serum-starved for 1 h at 37°C in HBSS and then stimulated for 30 minutes with or without 500 nM CRF in HBSS at 37°C or for the times indicated in the *Figure Legends*. Cells were washed with cold HBSS and treated with mouse anti-HA antibody (1:500) for 45 min on ice. Cells were washed with cold HBSS and additionally treated with Alexa Fluor 633 goat anti-mouse IgG (Invitrogen, Burlington, ON, Canada) (1:500) for 45 min on ice. Cells were washed with cold PBS and treated with 5 mM EDTA in PBS for 5 min on ice. Newly suspended HEK 293 cells were then transferred to flow cytometry tubes containing 4% formaldehyde in PBS. Samples were run on a FACSCalibur cytometer using BD CellQuest Pro software until 10,000 cells were counted. The geometric mean of fluorescence was determined using FlowJo analysis software, with less fluorescence corresponding to less CRFR1 on the membrane.

cAMP Assay:

The cAMP GLO Assay protocol was carried out as suggested by the manufacturer (Promega). Transfected HEK 293 cells were seeded into 96-well plate (~10,000 cells per well). Cells were incubated in induction buffer (HBSS with 500 μ M isobutyl-1-methylxanthine (IBMX) and increasing concentrations of CRF agonist for 30 min at 37 °C. Following stimulation, cells were solubilized with cAMP-GLO Lysis Buffer for 15 min with gentle shaking at 20–23 °C. cAMP-GLO detection solution containing protein kinase A was added for 20 min at 20–23 °C, followed by the addition of Kinase-Glo Reagent for 10 min. Each solution was carefully transferred to a white, opaque, 96-well plate and

Luminescence was measured using a Victor Plate Reader (Perkin-Elmer). SAP97 knockdown experiments were additionally performed using a BRET-based biosensor (EPAC) for cAMP and the protocol was adapted from Barak *et al.* (2008).

ERK Phosphorylation:

Following transfection, HEK 293 cells were re-seeded into 6-well plates. Cells were serum-starved for 1 hour at 37°C in HBSS and then stimulated with 500 nM CRF agonist for the duration of the described time-points. Cells were lysed with lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10 µg/ml leupeptin, and 5 µg/ml aprotinin) 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 Bronsted-Lowry protein assay was performed and 50 µg of protein was incubated overnight at room temperature in 3X SDS Loading Buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted for ERK1/2 (rabbit anti-p44/42 mitogen-activated protein kinase (MAPK), 1:1000), phospho-ERK1/2 (rabbit anti-phospho-p44/42 MAPK, 1:1000), SAP97 (mouse anti-SAP97, 1:1000), and HA-CRFR1 expression (mouse anti-HA, 1:1000), followed by a horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:10,000) or anti-mouse antibody (1:10,000) where appropriate. Proteins were detected using chemiluminescence with the enhanced chemiluminescence kit from GE Healthcare.

Statistical Analysis:

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

2.3 Results

Proteomic Analysis of CRFR1-interacting PDZ proteins. Previously, we demonstrated that the carboxyl-terminal tail class I CRFR1 PDZ binding motif was essential for CRFR1-mediated sensitization of 5-HT_{2A}R signaling (Magalhaes et al., 2010). Therefore, we utilized an array of 96 class I PDZ domains spotted on a gridded nylon membrane, as described previously (Fam et al., 2005; He et al., 2006), to identify potential CRFR1 interacting PDZ domain-containing proteins. The PDZ array was overlaid with 100 nM of either purified glutathione-S-transferase GST-CRFR1-carboxyl-terminal tail or GST (as a control). As can be observed in Fig. 1.1, a subset of PDZ proteins on the array exhibited binding to the GST-CRFR1-carboxyl-terminal tail. Specifically, we found that the CRFR1 carboxyl-terminal tail selectively bound to a discrete group of PDZ domain-containing proteins: MAGI-1 PDZ1, MAGI-2 PDZ1, MAGI-3 PDZ1, PSD95 PDZ 1&2, PSD95 PDZ3, CAL PDZ, SAP97 PDZ 1&2, PTPN13 PDZ 4&5, PDZK2 PDZ1 and MUPP1 PDZ 12.

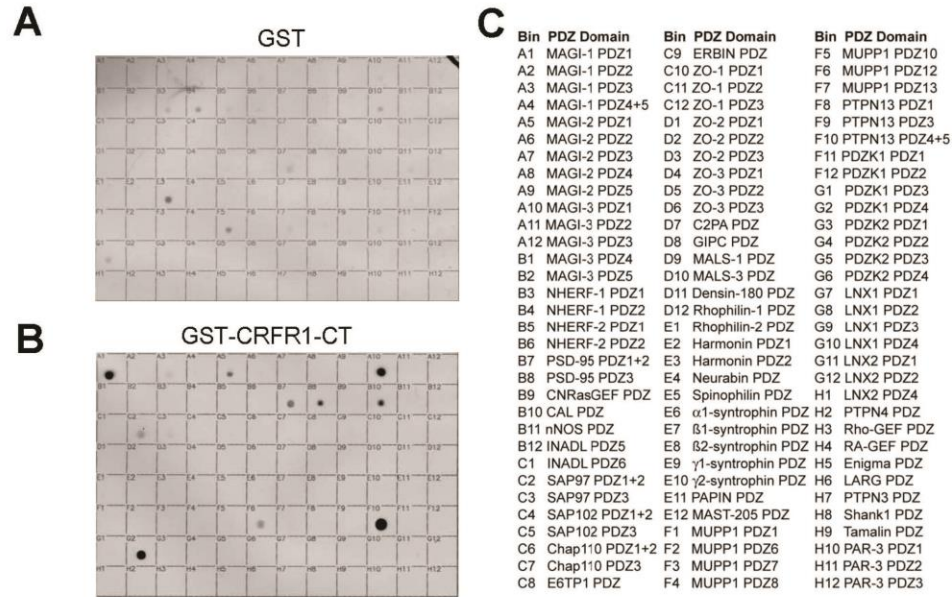


Figure 2.1: The CRFR1-CT binds to a specific subset of PDZ proteins. Equal amounts of purified His-tagged fusion proteins corresponding to a subset of PDZ domains were spotted on nylon membranes and overlaid with GST (A) versus GST-CRFR1-CT (B) to reveal specific CRFR1-CT binding to the spotted PDZ domains. (C) Identity of 96 distinct PDZ domains that were spotted on the nylon membranes. Data are representative of four independent experiments.

SAP97 is co-immunoprecipitated with CRFR1 in a PDZ-binding motif-dependent manner. SAP97 was one of the candidate CRFR1 binding proteins identified in the proteomic PDZ domain screen (Fig. 1.1). Since our previous data revealed that PDZ interactions were critical for cross-talk between CRFR1 and 5-HT_{2A}R (Magalhaes et al., 2010), and SAP97 was previously identified as an interacting partner of 5-HT_{2A}R function (Becamel et al., 2004), we focused on SAP97 in further experiments. First, we sought to confirm that SAP97 interacted with CRFR1 by co-immunoprecipitation. We found that GFP-SAP97 was co-immunoprecipitated with HA-CRFR1 from HEK 293 cells, but that this interaction was not increased by agonist activation of HA-CRFR1 with 100 nM CRF (Fig. 2.2A and B). The interaction was dependent upon an intact CRFR1 carboxyl-terminal PDZ binding motif, as the deletion of the last three critical amino acids (TAV) of the CRFR1 carboxyl-terminal tail prevented the co-immunoprecipitation of SAP97 with the subsequent HA-CRFR1-ΔTAV mutant (Fig. 2.2A and B). Furthermore, we found that endogenous SAP97 could be co-immunoprecipitated with CRFR1 from cortical mouse brain lysates (Fig. 2.C). Thus, an intact CRFR1 carboxyl-terminal PDZ binding motif was required for SAP97 interactions with the receptor.

SAP97 recruitment to the plasma membrane is dependent on the CRFR1 PDZ binding motif. When expressed alone in HEK 293 cells, GFP-SAP97 was diffusely localized throughout the cytoplasm and did not exhibit localization to the plasma membrane (data not shown). However, when GFP-SAP97 (green) was co-expressed with HA-CRFR1 (red), the GFP-SAP97 was predominantly localized with the receptor at the

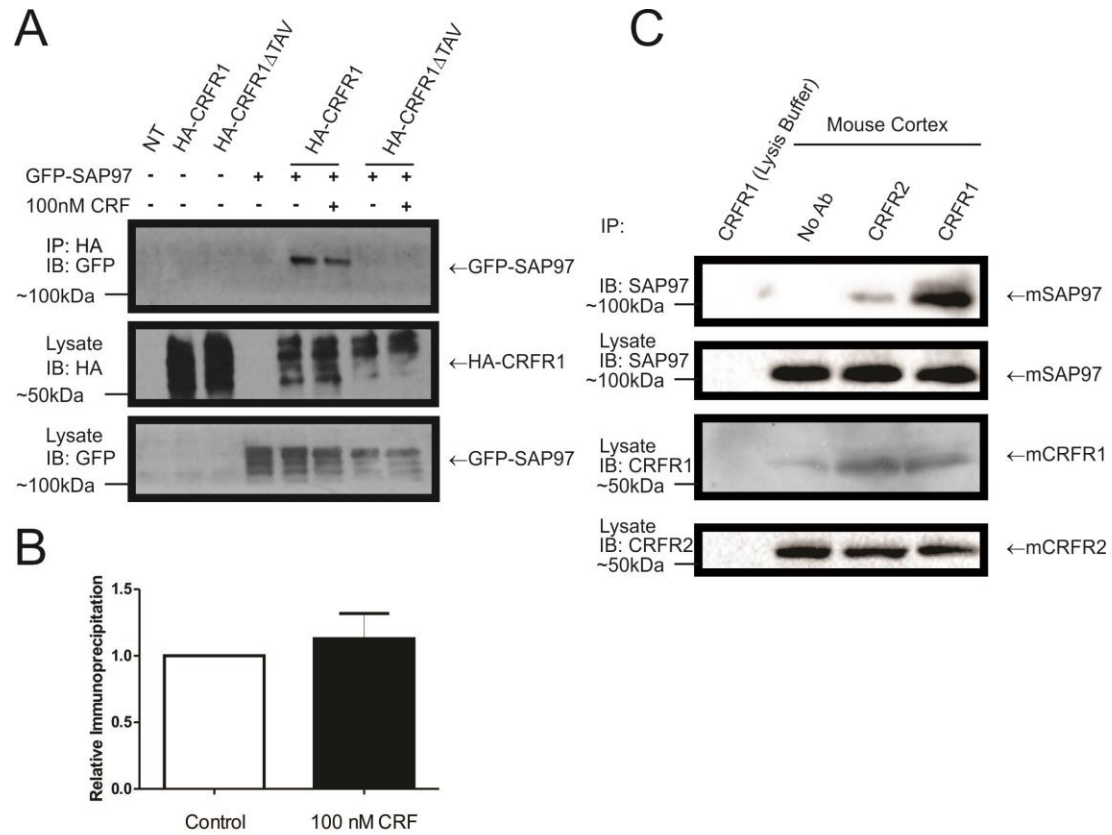


Figure 2.2: GFP-SAP97 co-immunoprecipitates with HA-CRFR1 in a PDZ-binding motif-dependent, CRF agonist-independent manner. (A) Representative immunoblot of SAP97 co-immunoprecipitated with HA-CRFR1 but not HA-CRFR1 Δ TAV. Transient transfections were performed in HEK 293 cells as labelled. Samples were run using SDS-PAGE and immunoblotted with rabbit anti-GFP. GFP-SAP97 co-immunoprecipitated with HA-CRFR1, but not HA-CRFR1 Δ TAV which lacks the PDZ-binding motif. (B) Effect of CRF treatment was quantified using densitometry and had no significant effect on the amount of GFP-SAP97 co-immunoprecipitated with HA-CRFR1. Data are representative of six independent experiments. (C) Representative immunoblot for endogenous SAP97 co-immunoprecipitated with endogenous CRFR1 but not CRFR2 from 2 mg of mouse cortical lysate. SAP97, CRFR1 and CRFR2 expression in 100 μ g of cortical lysate are shown below. Data are representative of three independent experiments.

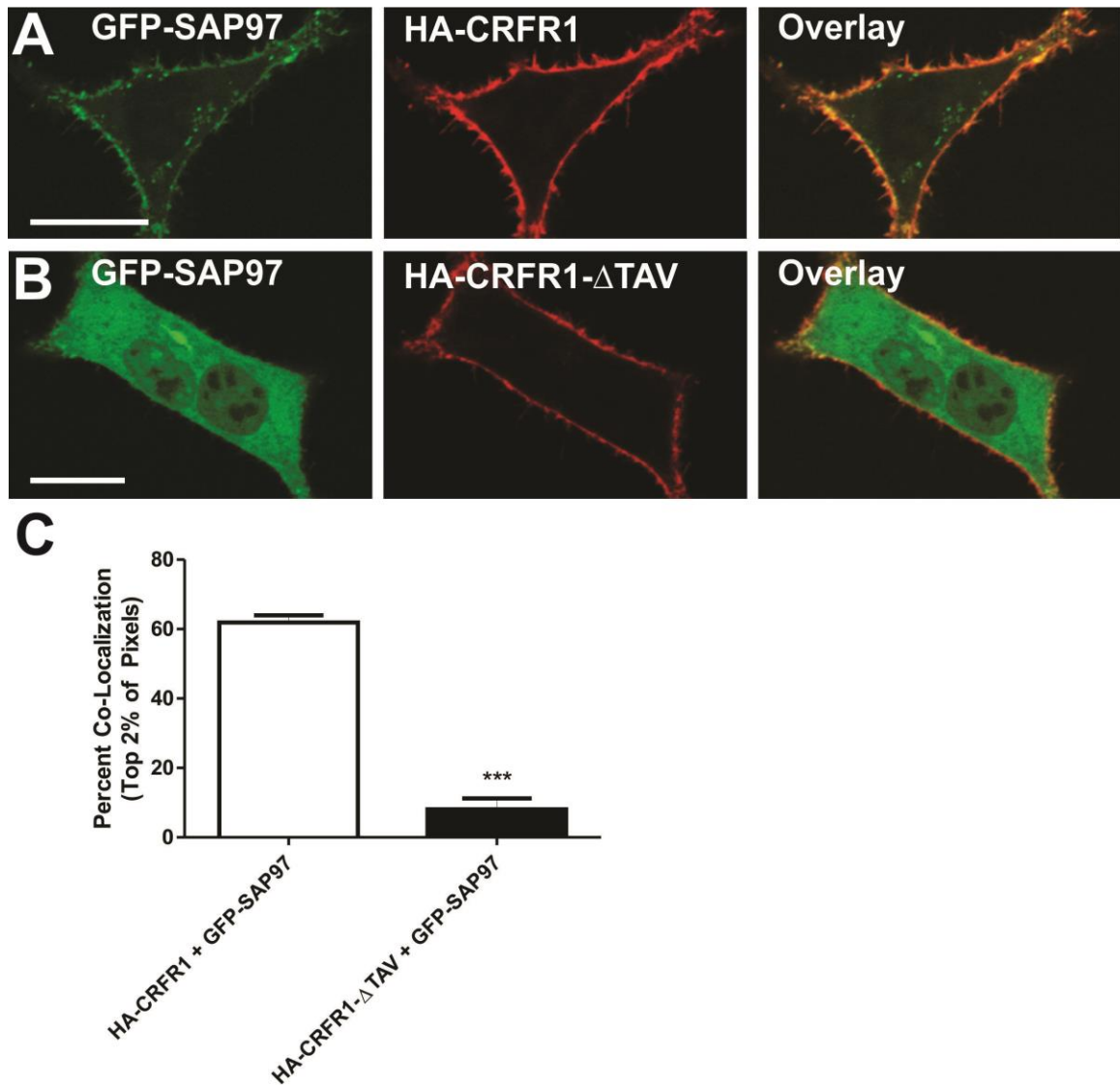


Figure 2.3: GFP-SAP97 co-localizes at the membrane with HA-CRFR1 in a PDZ-binding motif-dependent manner. (A) Representative confocal microscopy image demonstrating the colocalization of GFP-SAP97 (green) and cell surface HA-CRFR1 (red) labelled with Zenon Alexa Fluor 633-conjugated mouse HA antibody in live HEK 293 cells. (B) Representative confocal microscopy image demonstrating the colocalization of GFP-SAP97 (green) and cell surface HA-CRFR1-ΔTAV (red) labelled with Zenon Alexa Fluor 633-conjugated mouse HA antibody in live HEK 293 cells. Data are representative of 37 (HA-CRFR1) and 17 (HA-CRFR1-ΔTAV) cells.

plasma membrane (Fig. 2.3A). When the CRFR1 PDZ motif was deleted from the carboxyl-terminus of the receptor (TAV), the resulting HA-CRFR1- Δ TAV mutant did not promote the plasma membrane localization of GFP-SAP97 (Fig. 2.3B). We found that 62 ± 2 % of SAP97 was colocalized with HA-CRFR1 at the cell surface, whereas only 8 ± 3 % of SAP97 was colocalized with the HA-CRFR1- Δ TAV mutant (Fig. 2.3C). Thus, these data in combination with the co-immunoprecipitation data indicated that SAP97 interacts with CRFR1 in a cellular context and that this interaction is dependent upon the CRFR1 PDZ binding motif.

SAP97 antagonizes CRFR1 endocytosis in a PDZ motif-dependent manner. PDZ interactions have been reported to regulate the endocytosis and trafficking of a number of GPCRs (Ritter et al., 2009; Magalhaes et al., 2012). Therefore, we initially examined the effect of overexpressing GFP-SAP97 on the endocytosis of wild-type CRFR1 and the CRFR1 mutant lacking a PDZ binding motif (TAV). In cells expressing only wild-type CRFR1, agonist treatment for 30 min with 500 nM CRF at 37°C resulted in a 24 ± 4 % loss of cell surface HA-CRFR1 as measured by flow cytometry (Fig. 2.4A). However, co-expression of GFP-SAP97 led to a significant attenuation of HA-CRFR1 endocytosis (Fig. 2.4A). Unexpectedly, deletion of the CRFR1 PDZ binding motif resulted in a HA-CRFR1- Δ TAV mutant that was impaired in its endocytosis when compared with the internalization of the wild-type receptor (Fig. 4A). GFP-SAP97 overexpression did not further antagonize the internalization of the HA-CRFR1- Δ TAV mutant (Fig. 2.4A). To examine the role of endogenous SAP97 in the regulation of agonist-stimulated CRFR1 endocytosis in HEK

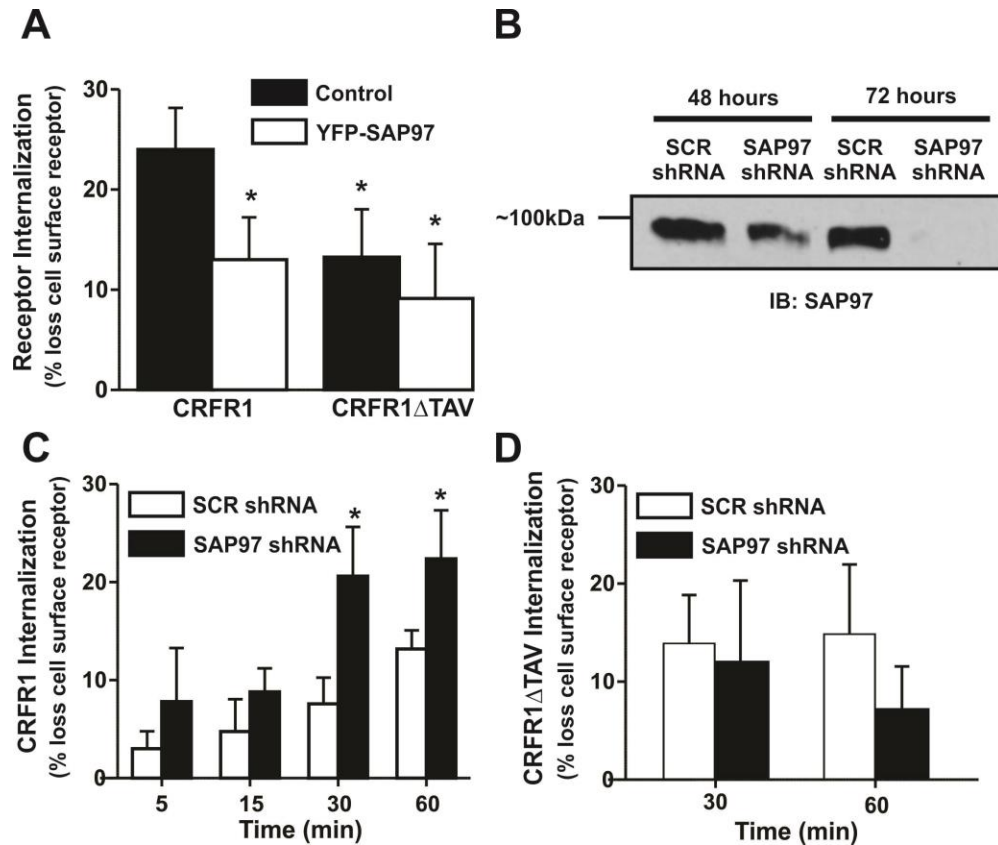


Figure 2.4: SAP97 antagonizes HA-CRFR1 endocytosis. (A) Agonist-stimulated internalization of either HA-CRFR1 or HA-CRFR1- Δ TAV in cells co-transfected with either GFP or GFP-SAP97. The internalization of HA tagged receptors labelled with Alexa Fluor-conjugated mouse anti-NA antibody was measured in cells treated with 500 nM CRF for 30 min and compared with vehicle treated control cells. The data represent the mean \pm SEM of nine independent experiments. * $P < 0.05$ versus control CRFR1 internalization. (B) Representative immunoblot of endogenous SAP97 protein expression in HEK 293 cells transfected with 3 μ g plasmid cDNA encoding either scrambled (SCR) or SAP97 shRNA at 48 and 72 h initial transfection. (C) Agonist stimulated (500 nM CRF) internalization of HA-CRFR1 in cells co-transfected with scrambled (SCR) and SAP shRNA at 5, 15, 30 and 60 min. The data represent the mean \pm SEM of five independent experiments. * $P < 0.05$ versus SCR shRNA treated cells. (D) Agonist stimulated (500 nM CRF) internalization of HA-CRFR1- Δ TAV in cells co-transfected with scrambled (SCR) and SAP shRNA at 30 and 60 min. The data represent the mean \pm SEM of four independent experiments.

293 cells, we transfected the cells with either scrambled shRNA or a shRNA SAP97 construct that was previously shown to knockdown SAP97 expression and tested CRFR1 internalization (Gardner et al., 2007). As shown in Fig. 2.4B, the SAP97 shRNA construct effectively knocked down the expression of endogenous SAP97 protein expression in HEK 293 cells 72 h post-transfection. Consequently, all subsequent shRNA experiments were performed 72 h after HEK 293 cell transfection. We found that shRNA knockdown of SAP97 significantly increased the maximal extent of HA-CRFR1 endocytosis following 30 and 60 minutes of agonist treatment with 500 nM CRF (Fig. 2.4C). In contrast knockdown of endogenous SAP97 expression did not influence the extent of HA-CRFR1- Δ TAV mutant internalization (Fig. 2.4D). Thus, taken together these data indicated that SAP97 antagonizes agonist-stimulated internalization of CRFR1, but that the CRFR1 PDZ binding motif is required for effective internalization of the receptor.

SAP97 co-localizes with CRFR1 during receptor endocytosis. The overexpression of GFP-SAP97 antagonized HA-CRFR1 endocytosis, but did not completely block the internalization of the receptor. Therefore, we examined whether internalized HA-CRFR1 was either internalized as a complex with GFP-SAP97 or whether a population of HA-CRFR1 was internalized independently of GFP-SAP97. To do this, HEK 293 cells were transfected with both HA-CRFR1 and GFP-SAP97 and the HA-CRFR1 was labeled with Alexa Fluor 633-conjugated monoclonal HA mouse antibody (1:1000 dilution) for 45 min on ice. Live labeled cells were then imaged by laser scanning confocal microscopy. Each cell was allowed to warm to 37°C and imaged prior to the addition of 500 nM CRF, then consecutively imaged every 30 s for 30 min. We found that, prior to agonist treatment,

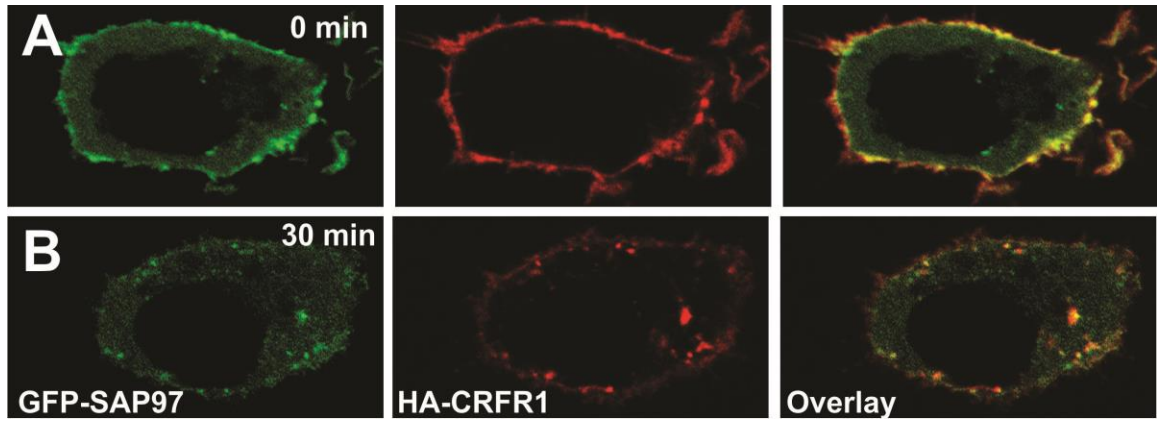


Figure 2.5: GFP-SAP97 exhibits limited endocytosis with HA-CRFR1. Live cell microscopic imaging of GFP-SAP97 (green) and HA-CRFR1 (red) labelled with Alexa Fluor 633-conjugated mouse anti-HA antibody (A) prior to and following (B) 500 nM activation for 30 min in a live HEK293 cell. Image is representative of 20 cells.

Alexa Fluor 633-conjugated mouse monoclonal HA antibody-labeled CRFR1 was colocalized with GFP-SAP97 at the cell surface (Fig. 2.5A). Upon CRF treatment, we observed limited internalization of HA-CRFR1 at 30 min stimulation with agonist, but the HA-CRFR1 that was internalized was co-localized with GFP-SAP97 in endocytic vesicles (Fig. 2.5B). This indicated that endocytosed CRFR1 redistributed GFP-SAP97 into the endosomal compartment, despite the role for SAP97 in antagonizing CRFR1 endocytosis.

SAP97 does not regulate CRFR1-mediated cAMP signaling: Because SAP97 overexpression antagonized CRFR1 internalization and SAP97 down-regulation enhanced CRFR1 endocytosis, we sought to determine whether SAP97 and/or the CRFR1 PDZ binding motif contributed to the regulation of CRFR1-mediated cAMP formation. In cells transfected with HA-CRFR1 with and without GFP-SAP97 and treated with increasing concentrations of CRF, there was no significant change in the maximum efficacy for CRF-stimulated cAMP formation (Fig. 2.6A). Similarly, deletion of the CRFR1 PDZ binding motif had no effect on the maximum efficacy for CRF-stimulated cAMP formation in response to the activation of either the wild-type CRFR1 or the CRFR1- Δ TAV mutant (Fig. 2.6B). Consistent with what was observed following GFP-SAP97 overexpression, SAP97 shRNA knockdown did not result in an increase in the maximum efficacy for CRF-stimulated cAMP formation by the CRFR1 (Fig. 2.6C). Thus, SAP97 did not appear to contribute to the regulation of CRFR1-stimulated cAMP production.

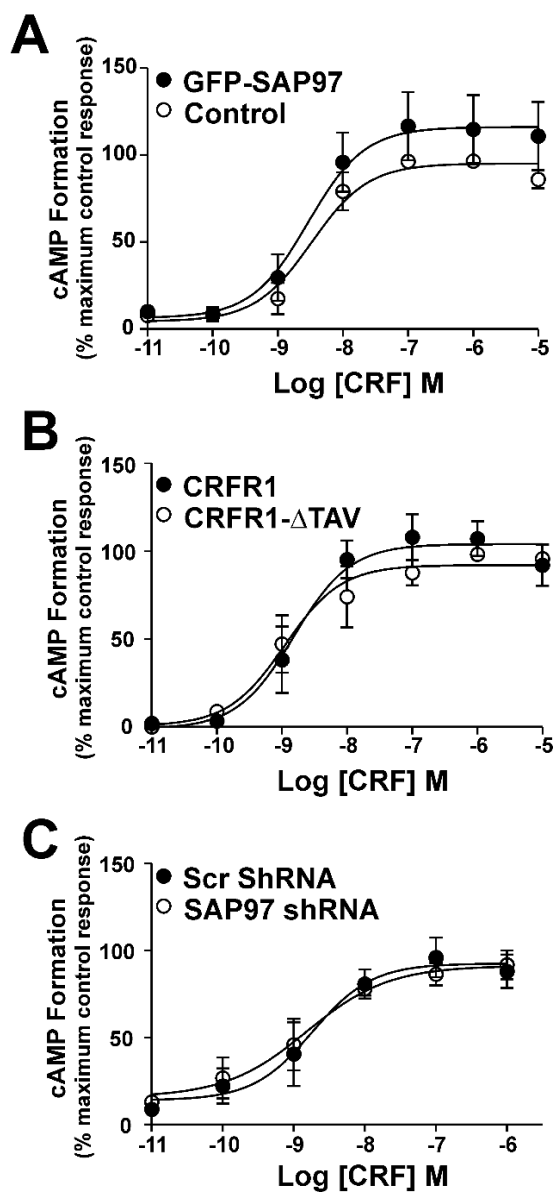


Figure 2.6: SAP97 does not regulate CRFR1-mediated cAMP formation. (A) CRFR1-mediated cAMP formation, as assessed by a cAMP GLO assay, following co-transfection with either GFP (control) or GFP-SAP97. The data represent the mean \pm SEM of seven independent experiments. (B) CRFR1- and CRFR1- Δ TAV-mediated cAMP formation, as assessed by a cAMP GLO assay. The data represent the mean \pm SEM of four independent experiments. (C) CRFR1-mediated cAMP formation as assessed by a cAMP GLO assay following co-transfection with either scrambled (SCR) or SAP97 shRNA. The data represent the mean \pm SEM of five independent experiments.

CRFR1-mediated ERK1/2 phosphorylation is dependent on endogenous SAP97

expression. Previous work has demonstrated CRFR1 can activate the MAPK signaling pathway, as evidenced by CRFR1-mediated ERK1/2 phosphorylation (Kageyama et al., 2007). Therefore, we examined whether endogenous SAP97 expression was required for CRFR1-mediated ERK1/2 phosphorylation. HEK 293 cells were transiently transfected with and without HA-CRFR1 along with either scrambled shRNA or SAP97 shRNA to knockdown SAP97 expression and ERK1/2 phosphorylation in response to 500 nM CRF for 0, 2, 5, 15 and 30 min was determined by densitometric analysis of immunoblots (2.7B). We found that the treatment of non-transfected HEK 293 cells with 500 nM CRF led to an increase in detectable ERK1/2 phosphorylation at 5 min, which was likely due to endogenous CRFR2 that is expressed in these cells (Fig. 2.7A and B) (Magalhaes et al., 2010). However, in cells transfected with HA-CRFR1 and scrambled shRNA, 500 nM CRF treatment resulted in a more robust and sustained activation of ERK1/2 phosphorylation (Fig. 2.7A and B). Knockdown of SAP97 protein expression led to an attenuation of CRFR1-mediated ERK1/2 phosphorylation following 500 nM CRF treatment, to levels that were comparable to those observed in non-transfected cells (Fig. 2.7A and B). The over-expression of GFP-SAP97 had no significant effect on ERK1/2 phosphorylation (data not shown). To examine whether SAP97 regulates ERK1/2 phosphorylation in response to the activation of endogenous CRFR1 activation, identical experiments were performed in AtT20 cells that express endogenous CRFR1 (Westendorf and Schonbrunn, 1985). AtT20 cells grow in suspension, thereby complicating transfection efficiency and determinations of ERK1/2 phosphorylation in this cell type. Nevertheless, following siRNA knockdown of endogenous SAP97, we observed a small but

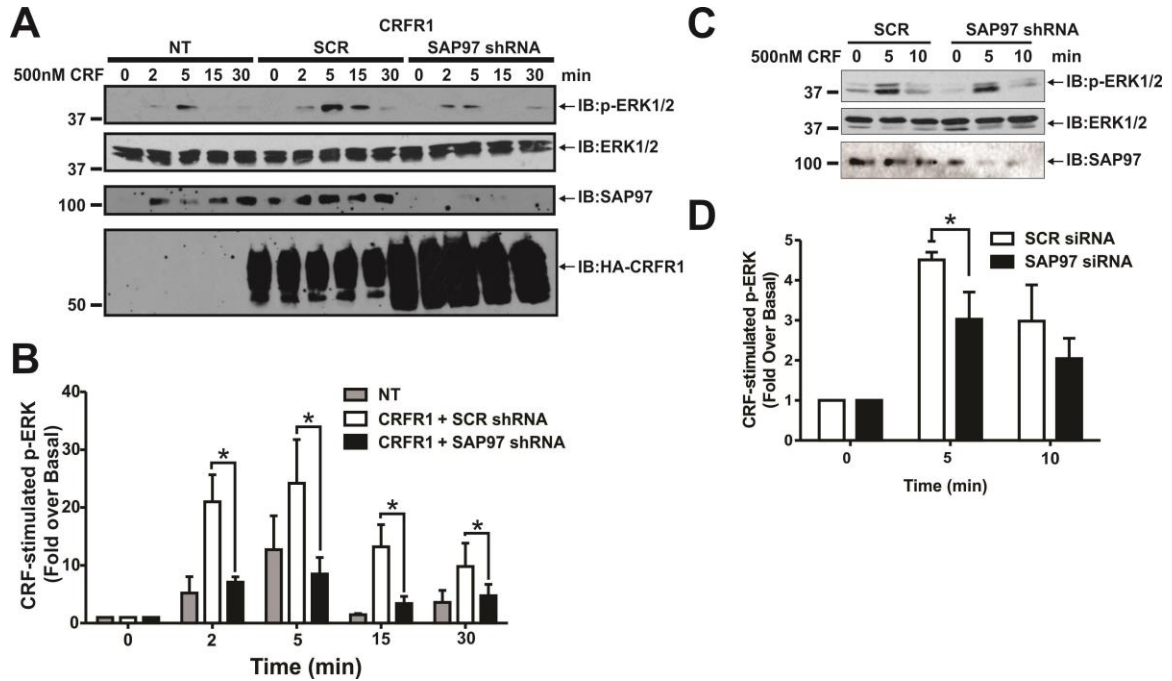


Figure 2.7: Knockdown of endogenous SAP97 suppresses HA-CRFR1-mediated ERK1/2 phosphorylation. (A) Representative immunoblot showing ERK1/2 phosphorylation in response 500 nM CRF treatment for 0, 5, 15 and 30 min in non-transfected (NT) HEK 293 cell, and HEK 293 cells transfected with HA-CRFR1 and either scrambled (SCR) or SAP97 shRNA. Shown in the panels below are corresponding immunoblots for total ERK1/2, SAP97 and HA-CRFR1 protein expression. (B) Densitometric analysis of ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 5, 15 and 30 min in non-transfected (NT) HEK 293 cell, and HEK 293 cells transfected with HA-CRFR1 and either scrambled (SCR) or SAP97 shRNA. The data represent the mean \pm SEM of four independent experiments. * $P < 0.05$ versus SCR shRNA treated cells. (C) Representative immunoblot showing ERK1/2 phosphorylation in response 500 nM CRF treatment for 0, 5, and 10 min in AtT20 cells transfected with either scrambled (SCR) or SAP97 siRNA. Shown in the panels below are corresponding immunoblots for total ERK1/2, and SAP97 protein expression. (D) Densitometric analysis of ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 5, and 10 min in AtT20 cells transfected with either scrambled (SCR) or SAP97 siRNA. The data represent the mean \pm SEM of four independent experiments. * $P < 0.05$ versus SCR shRNA treated cells.

significant attenuation of ERK1/2 phosphorylation following 5 min CRF stimulation (Fig. 2.7C and 7D).

To further examine the specificity of SAP97 with the CRFR1 PDZ binding motif we assessed whether deleting the motif altered CRFR1-stimulated ERK1/2 phosphorylation in the presence and absence of SAP97 expression. Surprisingly, we found that the deletion of the CRFR1 PDZ binding motif did not prevent ERK1/2 phosphorylation following agonist activation of CRFR1- Δ TAV mutant (Fig. 2.8A and B). Moreover, we found that shRNA knockdown of SAP97 reduced CRFR1- Δ TAV mutant ERK1/2 phosphorylation to the same extent to that was observed for the wild-type CRFR1 (Fig. 2.8A and B). To determine whether this effect of SAP97 was specific to CRFR1-mediated ERK1/2 activation we assessed whether SAP97 knockdown attenuated ERK1/2 phosphorylation following the stimulation of CRFR2, a GPCR that does not encode a PDZ binding motif. Again, we found that SAP97 knockdown resulted in a significant reduction of CRFR2-mediated ERK1/2 phosphorylation (Fig. 2.8C and 8D). Taken together, these data suggested that SAP97 is required for the activation of ERK1/2 signaling by the CRFR1, without modulating cAMP production, but does so via a mechanism that is independent of interactions with a PDZ binding motif.

2.4 Discussion

In a previous study, we found that PDZ protein interactions with CRFR1 play an important role in regulating CRFR1-dependent sensitization of 5-HT_{2A}R signaling (Magalhaes et al., 2010), prompting us to search for candidate PDZ proteins that interact

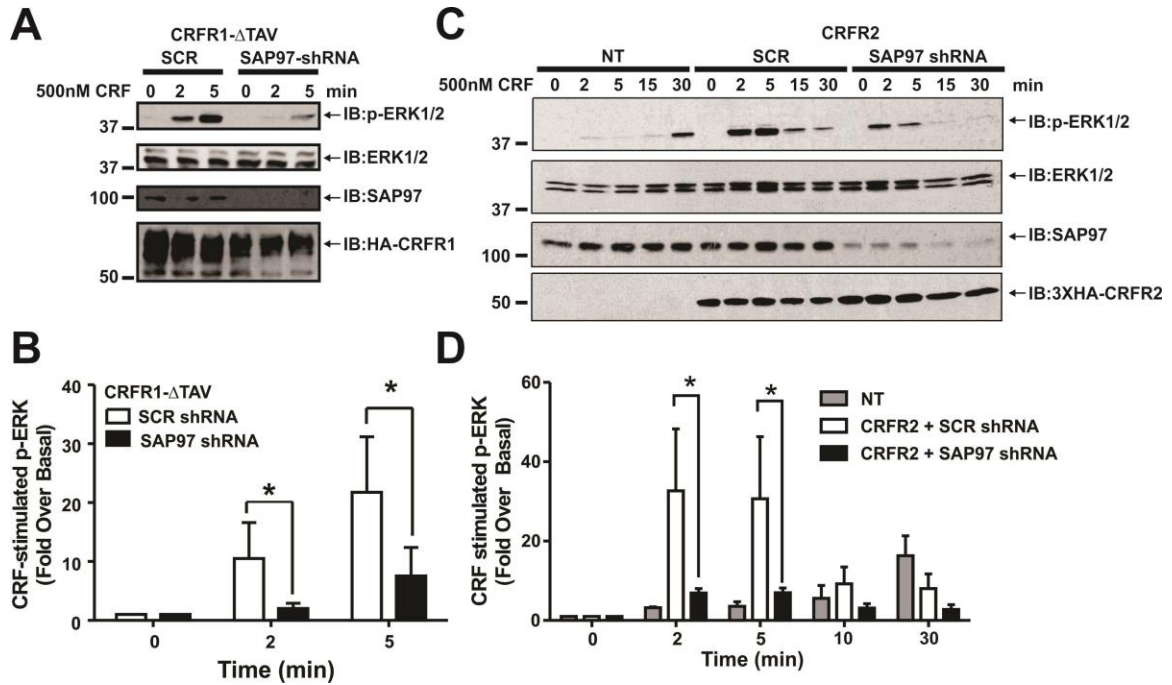


Figure 2.8: Knockdown of endogenous SAP97 suppresses HA-CRFR1-ΔTAV- and HA-CRFR2-mediated ERK1/2 phosphorylation. (A) Representative immunoblot showing ERK1/2 phosphorylation in response 500 nM CRF treatment for 0, 2, and 5 min in HEK 293 cells transfected with HA-CRFR1-ΔTAV and either scrambled (SCR) or SAP97 shRNA. Shown in the panels below are corresponding immunoblots for total ERK1/2, SAP97 and HA-CRFR1-ΔTAV protein expression. (B) Densitometric analysis of ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 2 and 5 min in HEK 293 cells transfected with HA-CRFR1-ΔTAV and either scrambled (SCR) or SAP97 shRNA. The data represent the mean \pm SEM of four independent experiments. * $P < 0.05$ versus SCR shRNA treated cells. (C) Representative immunoblot showing ERK1/2 phosphorylation in response 500 nM CRF treatment for 0, 5, 15 and 30 min in non-transfected (NT) HEK 293 cell, and HEK 293 cells transfected with HA-CRFR2 and either scrambled (SCR) or SAP97 shRNA. Shown in the panels below are corresponding immunoblots for total ERK1/2, SAP97 and HA-CRFR2 protein expression. (D) Densitometric analysis of ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 5, 15 and 30 min in non-transfected (NT) HEK 293 cell, and HEK 293 cells transfected with HA-CRFR2 and either scrambled (SCR) or SAP97 shRNA. The data represent the mean \pm SEM of three independent experiments. * $P < 0.05$ versus SCR shRNA treated cells.

with CRFR1. We found that a subset of Class I PDZ proteins, including SAP97, interact with the CRFR1 C-tail on a proteomic PDZ domain array. We further demonstrated that SAP97 interacts with the carboxyl-terminal CRFR1 PDZ binding motif resulting in recruitment of SAP97 to the cell surface as well as antagonism of CRFR1 endocytosis and promotion of CRFR1-stimulated ERK1/2 signaling but not cAMP production. CRFR1 represents the fifth GPCR to which SAP97 has been demonstrated to interact (Gardner et al., 2007; Cai et al., 2008; Becamel et al., 2004). SAP97 was previously shown to regulate the recycling of the β_1 AR and neurite outgrowth in response to somatostatin receptor activation (Gardner et al., 2007; Cai et al., 2008). In contrast to the positive effects of SAP97 on CRFR1-stimulated signaling to ERK1/2 observed in the present study, the interaction of the PDZ domain-containing protein MAGI-3 with the β_1 AR was shown to antagonize β_1 AR-dependent activation of ERK1/2 (He et al., 2006). Our studies indicate that SAP97 may be required for the activation of ERK1/2 phosphorylation by many GPCRs independent of PDZ domain interactions as a loss of SAP97 expression resulted in significantly reduced ERK1/2 phosphorylation following the activation of CRFR1- Δ TAV mutant and CRFR2 that does not encode a PDZ binding motif.

Previous to our current studies with CRFR1, SAP97 had been demonstrated to bind to the β_1 AR to regulate the recycling of the receptor (Gardner et al., 2007; Magalhaes et al., 2010). Interestingly, both receptors share a similar Class I carboxyl terminal PDZ motif, defined by S/T-X- Φ , where Φ represents any hydrophobic residue (Songyang et al., 1997; Beuming et al., 2005). Gardner et al. (2007) provided evidence that SAP97 may link the β_1 AR to A-Kinase Anchoring Protein 79/150 (AKAP79/150). This interaction was hypothesized to facilitate protein kinase A-mediated phosphorylation of Ser 312 of the

β_1 AR third intracellular loop, thereby promoting receptor recycling and resensitization (Gardner et al., 2004; Gardner et al., 2007). In support of this hypothesis, knock-down of endogenous SAP97 suppresses β_1 AR phosphorylation and recycling. Based on these previous data with the β_1 AR (Gardner et al., 2007), it might seem tempting to speculate that SAP97 may play a generalized role in regulating the activity of GPCRs that encode a Class I PDZ binding motifs. However, PDZ protein interactions with a variety of GPCRs have demonstrated PDZ protein-specific functions with respect to the regulation of GPCR activity. There are several examples to support this assertion. First, PSD-95 was reported to suppress 5HT_{2A}R receptor internalization (Xia et al., 2003) but yet promotes the internalization of the 5HT_{2C}R (Gavarini et al., 2006). Second, MAGI-2 overexpression was found to have no effect on β_1 AR-mediated cAMP signaling and promotes agonist-induced β_1 AR internalization (Xu et al., 2001), but was found to reduce vasoactive intestinal polypeptide type-1 receptor-mediated cAMP signaling and suppress agonist-induced receptor internalization (Gee et al., 2009). Finally, while MUPP1 enhances GABA_B receptor signaling, it functions to uncouple the melatonin-1 receptor from G α_i (Balasubramanian et al., 2007; Guillaume et al., 2008). Similarly, our current study demonstrates that SAP97 regulates CRFR1 trafficking, since over-expression of GFP-SAP97 prevents CRFR1 endocytosis and knockdown of endogenous SAP97 promotes CRFR1 endocytosis. However, due to the antagonism of CRFR1 endocytosis in the presence of SAP97, it is unlikely that SAP97 subserves the same recycling function for CRFR1 as it does for the β_1 AR. For this reason, discussions of PDZ protein functions in the regulation of GPCR activity should likely be prefaced by the GPCR with which a given PDZ protein is interacting.

Previous research has identified a number of proteins that appear to form complexes with CRFR1 at various stages of receptor activation and trafficking. It has been demonstrated that CRFR1 activation leads to β -arrestin1 and β -arrestin2 recruitment to the membrane where they co-localize and directly interact with CRFR1 (Rasmussen et al., 2004; Perry et al., 2005; Holmes et al., 2006). Additionally, CRFR1 has been shown to internalize to both Rab5-positive early endosomes and Rab4-positive recycling endosomes (Holmes et al., 2006). Previous work on the parathyroid hormone 1 receptor (PTH1R) and another PDZ domain-containing protein, NHERF1, demonstrated that the β -arrestin2 interaction with the PTH1R was prevented by the receptor's interaction with NHERF1 (Wang et al., 2009). Additionally, NHERF1 inhibited the uncoupling of PTH1R from G_{α_s} (Wang et al., 2009). It is plausible that SAP97 and/or one of the other CRFR1-interacting PDZ scaffolds identified in our screens could similarly dictate which proteins are associating with CRFR1 during different states of receptor activation and trafficking, thereby regulating the trafficking and signaling of CRFR1. Future studies will look to examine what role SAP97 and other CRFR1-interacting PDZ scaffolds might play in preventing CRFR1 recruitment of β -arrestin1/2. However, quantitative studies of potential SAP97-mediated antagonism of β -arrestin translocation to CRFR1 are technically challenging due to the fact that any carboxyl-terminal fusion of bioluminescent reporter proteins may disrupt PDZ protein interactions with the receptor.

Neither the overexpression of GFP-SAP97 nor the shRNA knockdown of endogenous SAP97 in our studies had a significant effect on the EC_{50} or maximal CRFR1-mediated cAMP accumulation. Additionally, deletion of the CRFR1 receptor PDZ-binding motif had no significant effect on CRFR1-mediated cAMP accumulation. These results

are consistent with previous research on the β_1 AR, where mutation of the PDZ-binding motif had no effect on β_1 AR-mediated cAMP accumulation (Gardner et al., 2007). Interestingly, PSD-95 has similar structural domains to SAP97 and has similarly been shown to suppress β_1 AR endocytosis and have no effect on β_1 AR-mediated cAMP accumulation (Hu et al., 2000).

Previous work has demonstrated CRFR1 can signal through the MAPK pathway, as evidenced by CRFR1-mediated ERK1/2 phosphorylation (Kageyama et al., 2007). Interestingly, shRNA knockdown of endogenous SAP97 significantly reduced ERK1/2 phosphorylation to levels comparable with cells lacking the overexpressed HA-CRFR1. Work from our lab has demonstrated that HEK 293 cells express endogenous functional CRFR2, as evidenced by stimulation of cAMP production at the high CRF concentrations necessary to activate CRFR2 (Holmes et al., 2006). Thus, the observed CRF-induced increases in ERK1/2 phosphorylation in non-transfected control cells in the present studies were expected. Knockdown of SAP97 would not be expected to alter CRFR2-mediated ERK1/2 phosphorylation, as the receptor does not possess a PDZ binding motif at its carboxyl-terminal tail. However, we found that SAP97 knockdown significantly impaired ERK1/2 phosphorylation in cells either overexpressing CRFR2 or a CRFR1- Δ TAV mutant suggesting that SAP97 may not only play a role in scaffolding ERK1/2 protein complexes, but may function to regulate GPCR-mediated ERK1/2 signaling independently of receptor interactions. Thus, our studies suggest a novel role for SAP97 in GPCR-mediated signaling, but further studies will be required to understand the precise role SAP97 plays in the regulation of ERK1/2 phosphorylation by GPCRs.

Although this is the first evidence of a role for SAP97 in the GPCR-mediated activation of ERK1/2 phosphorylation, previous research has demonstrated an ability of members of the MAPK family to phosphorylate SAP97, including ERK2 specifically (Sabio et al., 2005). This phosphorylation of SAP97 by MAPK family members was shown to lead to its dissociation from guanylate-kinase-associated protein and, consequently, the cytoskeleton (Sabio et al., 2005). Additionally, PSD-95 has been shown to be phosphorylated by MAPKs, including ERK2, in response to mitogens (Sabio et al., 2004). It is plausible that SAP97-dependent CRFR1-mediated ERK1/2 phosphorylation may participate in a negative feedback loop whereby “activated” pERK1/2 could slowly phosphorylate SAP97, leading to dissociation of a signaling complex that would lead to attenuated receptor-mediated ERK1/2 phosphorylation. The dissociation of this signaling complex could also disrupt cytoskeletal interactions and allow for relocalization of proteins through trafficking mechanisms. The mechanism by which SAP97 facilitates CRFR1-mediated activation of ERK1/2 remains to be determined. However, the demonstration that SAP97 knockdown significantly attenuates CRFR1-mediated ERK1/2 phosphorylation indicates that other GIPs scaffolded by GPCR protein interaction motifs, in addition to β -arrestins, play a role in dictating the intracellular signaling pathways activated by GPCRs. Thus, it may be that in addition to ligand-dependent stabilization of distinct “biased” GPCR activation states (Luttrell et al., 2010; Galandrin et al., 2007; Kenakin et al., 2007), the association of intracellular GIPs with GPCRs bias GPCR signal transduction. It is likely that these two events are not mutually exclusive but function interdependently, thereby complicating the outcome of ligand biased screening approaches for “biased” GPCR ligands.

In conclusion, we have presented the first evidence for SAP97 as a regulator of CRFR1 trafficking and signaling. Specifically, we have found that SAP97 interacts with the CRFR1 PDZ binding motif to antagonize CRFR1 endocytosis without affecting cAMP production, whereas the regulation of ERK1/2 phosphorylation by the receptor does not require PDZ motif interactions. This suggests that SAP97 may function to bias CRFR1 signaling toward the ERK1/2 pathway. This provides the potential for GIPs other than β -arrestins to mediate biased signaling of GPCRs. This interaction may be a pharmaceutical target for the regulation of CRFR1 function, and therefore has implications in the potential treatment of mood disorders, such as depression.

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Chapter 3: SAP97 Regulation of 5-HT_{2A}R

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3.1 Introduction

Serotonin (5-HT) is a monoamine neurotransmitter that regulates a wide range of higher neurological functions including the regulation of mood, behaviour, sleep, and appetite (Hale et al., 2012). The majority of serotonergic neurons in the brain project outwards from the raphe nuclei towards a multitude of brain regions, including many structures within the limbic system (Bobillier et al., 1976). Once released, serotonin activates a large subset of 5-HT receptors (5-HT_{1-7R}), all of which, with exception to 5-HT_{3R}, are G protein-coupled receptors (GPCRs) (Barnes, 2011). The 5-HT_{2AR} and 5-HT_{2CR} GPCRs are currently primary molecular targets for drugs used in the treatment of mood and behavioral disorders, such as: anxiety disorders, depression, schizophrenia, obsessive-compulsive disorder, and eating disorders (Gray and Roth, 2001; Roth and Xia., 2004; Catapano and Manji, 2007). The involvement of these specific 5-HT_{2Rs} in depression is highlighted by the examination of post-mortem human brain tissue from depressed individuals and victims of suicide (Catapano and Manji, 2007). These brains of these subjects are found to have significantly greater expression of both 5-HT_{2AR} and 5-HT_{2CR} when compared to their control counterparts. Untreated schizophrenics show a similar increase in 5-HT_{2AR} expression in the brain (Fribourg et al., 2011). Additionally, current antidepressants and atypical (second generation) anti-psychotics have demonstrated the ability to down-regulate 5-HT_{2AR} expression in the cortical brain (Catapano and Manji, 2007; Fribourg et al., 2011), suggesting an importance for 5-HT_{2AR} in the symptoms of depression and psychoses. In particular, pharmacological studies and knockout mice have demonstrated that 5-HT_{2AR} and 5-HT_{2CR} contribute to anxiety and are pharmacological targets for the treatment of anxiety (Weisstaub et al., 2006).

Although many studies have investigated the pharmacological regulation of 5-HT_{2A}R and 5-HT_{2C}R in psychiatric disorders, there is a limited number of studies that have focused on the contribution of C-terminal 5-HT_{2A}R and 5-HT_{2C}R PSD-95/Disc Large/Zona Occludens (PDZ) binding motif interactions with PDZ domain-containing proteins to 5-HT_{2R}-related disorders (Abbas et al., 2009; Jones et al., 2009; Magalhaes et al. 2010; Pichon et al., 2010; Wattiez et al., 2013). For example, PSD-95 regulates the actions of atypical antipsychotics on 5-HT_{2Rs}, and kalirin-7 mediates 5-HT_{2R}-mediated modulation of spine morphology (Abbas et al., 2009; Jones et al., 2009). In addition, disruption of 5-HT_{2A}R PDZ protein interactions enhances SSRI efficacy in neuropathic pain and alleviates mechanical sensitivity to inflammation (Pichon et al., 2010; Wattiez et al., 2013). Thus, the emerging understanding of the importance of PDZ domain-containing proteins in the regulation of 5-HT_{2A}R activity will be essential for our understanding of how the intracellular trafficking of 5-HT_{2A}R may contribute to the regulation of agonist-stimulated 5-HT_{2A}R cellular responses (Backstrom et al., 2000; Xia et al., 2003a; Xia et al., 2003b; Gavarini et al., 2006; Jones et al., 2009; Magalhaes et al. 2010; Pichon et al., 2010; Wattiez et al., 2013).

The 5-HT_{2A}R primarily signals via the activation of G $\alpha_{q/11}$ -mediated signaling pathways, leading to PLC activation and the accumulation of inositol phosphate (IP) and diacylglycerol resulting in the release of intracellular Ca²⁺ stores and the activation of protein kinase C. However, alternate signaling pathways have been identified, including the extracellular regulated protein kinase 1/2 (ERK1/2) signalling pathway that can be activated by both G protein-dependent and - independent mechanisms (Raymond et al., 2001; Magalhaes et al., 2012). Recently, studies from our laboratory have demonstrated

that the activation of the corticotropin releasing factor receptor 1 (CRFR1) enhances subsequent 5HT_{2A/C}R-mediated IP₃ signaling via a mechanism that is dependent upon PDZ protein-interactions with both receptors (Magalhaes et al., 2010). This suggests that one or more PPDZ domain-containing receptors interact with both CRFR1 and 5HT_{2A}R to positively modulate 5HT_{2A}R signaling.

Previously, SAP97 has been demonstrated to interact with both 5-HT_{2A}R and 5-HT_{2C}R (Becamel et al., 2002; Becamel et al., 2002), and we have shown that SAP97 interacts with and negatively regulates CRFR1 endocytosis, without affecting CRFR1-stimulated cAMP accumulation (Dunn et al., 2013). Therefore, in the present study, we have tested the hypothesis that 5-HT_{2A}R signaling and trafficking may also be modulated as a consequence of the interaction with SAP97 with the receptor. We find that similar to what we reported for CRFR1, SAP97 is involved in the regulation of 5-HT_{2A}R endocytosis and ERK1/2 activation. However, unlike what was previously observed for CRFR1 signaling, SAP97 expression is required for 5-HT_{2A}R G protein coupling, but is not involved in the observed CRFR1 pretreatment-dependent enhancement of 5-HT_{2A}R signaling.

3.2 Experimental Procedures

Materials:

ECL Western blotting detection reagents were purchased from GE Healthcare and BioRad. The Dowex 1-X8 (formate form) resin with 200-400 mesh was purchased from Bio-Rad (Mississauga, ON). Bovine serum albumin (BSA) was obtained from BioShop Canada Inc. (Mississauga, ON). Horseradish peroxidase-conjugated anti-rabbit IgG secondary

antibody was from BioRad (Mississauga, ON). Rabbit anti-phospho-p44/42 MAPK (Thr-202/Tyr-402) and rabbit anti-p44/42 MAPK antibodies were obtained from Cell Signaling Technology (Pickering, Ontario, Canada). Rabbit anti-GFP antibody was obtained from Invitrogen. Mouse anti-SAP97 antibody was obtained from Assay Designs/Enzo Life Sciences (Farmingdale, NY). Alexa Fluor 647 anti-rabbit IgG and Zenon antibody were purchased from Invitrogen (Burlington, ON, Canada). Rabbit anti-FLAG antibody and all other biochemical reagents were purchased from Sigma.

Plasmid Constructs:

FLAG-tagged 5-HT_{2A}R and 5-HT_{2A}R-ΔSCV constructs were described previously (Magalhaes et al., 2010). The GFP-SAP97 (rat isoform 2) construct and SAP97 shRNA were described previously and graciously provided by Dr. Suleiman W. Bahouth (Neuroscience Institute, University of Tennessee Health Sciences Center) (Gardner et al., 2006; Dunn et al., 2013). The GFP-SAP97 (rat isoform 2) construct was subcloned into peYFP-N1. A FLAG-tagged 5-HT_{2A}R *renilla luciferase* (rLuc) fusion protein (5-HT_{2A}R-x22-SCV) was custom synthesized by GenScript (Piscataway, NJ) to insert rLuc into the 5-HT_{2A}R C-terminal tail 22 amino acids upstream of the PDZ binding motif. The construct was digested HindIII/EcoRI from a pU57 plasmid and subcloned into a pcDNA3.1 plasmid expression vector digested with the same restriction enzymes.

Cell Culture and Transfection:

Human embryonic kidney (HEK 293) cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Cells were seeded on 10-cm dishes at 70–80% density 24 h prior to transfection. Transfection was performed using a modified calcium phosphate method, as described previously (Caron and Ferguson, 2004). Transfections were performed with 1 μ g of each construct, with exception that 3 μ g of plasmid cDNA was used for all shRNA constructs. Empty pcDNA3.1 vector was used to equalize the total amount of plasmid cDNA used to transfect cells. 18 h post-transfection, cells were washed with phosphate-buffered saline (PBS) and suspended with trypsin, 0.25% EDTA. All experiments were conducted 48 hours after the initial transfection, with the exception of transfections involving SAP97 shRNA, which were conducted 72 hours after initial transfection to optimize the knockdown of endogenous SAP97, as confirmed by Western blotting.

Co-immunoprecipitation:

Transfected HEK293 cells were seeded onto 10-cm dishes the day before the experiment. Cells were serum-starved for 1 hour in HBSS, and dishes were treated with either HBSS alone or with 10 μ M 5HT agonist in HBSS for 30 min at 37 °C. Cells were subsequently lysed in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors (1mM AEBSF, 10 μ g/ml leupeptin, and 5 μ g/ml aprotinin) for 20 minutes on a rocking platform at 4°C. Samples were collected into 1.5 ml Eppendorf tubes and centrifuged at 15,000 x g for 15 min at 4°C to pellet insoluble material. A Bronsted-Lowry protein assay was performed, and 400 μ g of protein was incubated for overnight at 4 °C with FLAG-immunoprecipitation beads from Sigma. After incubation, beads were

washed three times with cold lysis buffer and incubated overnight at room temperature in 3x SDS Loading Buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted to identify co-immunoprecipitated GFP-SAP97 (rabbit anti-GFP, 1:1000). An additional Western blot was performed to examine FLAG-5-HT_{2A}R/FLAG-5-HT_{2A}R-ΔSCR (rabbit anti-FLAG, 1:1000), and GFP-SAP97 (rabbit anti-GFP, 1:1000) protein expression. For the co-immunoprecipitation of endogenous proteins from cortical extracts, adult mouse brains were employed. Tissue was dissected and homogenized on ice in lysis buffer containing protease inhibitors. The particulate fraction was removed by centrifugation, and 2 mg of supernatant protein was incubated with 5 µl/sample of either goat polyclonal 5-HT_{2A}R or FLAG antibody from Santa Cruz Biotechnology (Santa Cruz, CA) and protein G Sepharose beads overnight at 4°C. Afterwards, the beads were washed three times with lysis buffer and proteins were eluted in 3x SDS-PAGE loading buffer by warming the samples at 55°C for 5 min. Eluted samples were subjected to SDS-PAGE, followed by transfer onto nitrocellulose membranes for immunoblotting with antibodies described in the *Figure Legends*.

Live Cell Confocal Microscopy:

Following transfection, HEK 293 cells were re-seeded onto 35 mm glass bottom confocal dishes. Cells were serum-starved for 1h at 37°C in HBSS and then labeled with rabbit anti-FLAG antibody (1:200) and Zenon Alexa Fluor 647 rabbit IgG1 labeling kit (Invitrogen) at 4°C for 30 min. The cells were washed with HBSS and warmed to 37°C for live imaging using a heated stage. Confocal microscopy was performed on a Zeiss LSM-510 META

laser scanning confocal microscope using a Zeiss 63x, 1.3 NA, oil immersion lens. Co-localization studies were performed using dual excitation (488 and 633 nm) and emission (band pass 505–550 nm and long pass 650 nm for YFP/GFP and Alexa Fluor 647, respectively) filter sets. The specificity of labeling and absence of signal crossover were established by examination of single-labeled samples.

Receptor Endocytosis:

Following transfection, HEK 293 cells were re-seeded into 12-well plates. Cells were serum-starved for 1h at 37°C in HBSS and then stimulated with or without 10 μ M 5HT in HBSS at 37°C for the times indicated in the figure legends. Cells were washed with cold HBSS and treated with mouse anti-FLAG antibody (1:1000) for 45 min on ice. Cells were washed with cold HBSS and additionally treated with Alexa Fluor 647 donkey anti-mouse IgG (Invitrogen) (1:1000) for 45 min on ice. Cells were washed with cold PBS and treated with 5mM EDTA in PBS for 5 min on ice. Newly suspended HEK 293 cells were then transferred to flow cytometry tubes containing 4% formaldehyde in PBS. Samples were run on a FACSCalibur cytometer using BD CellQuest Pro software until 10,000 cells were counted. The geometric mean of fluorescence was determined using FlowJo analysis software, with less fluorescence corresponding to less 5-HT_{2A}R on the membrane.

Measurement of inositol phosphate formation:

HEK 293 cells were transiently transfected with the cDNAs as described in the *Figure Legends*. 48 h post-transfection cells were incubated overnight in inositol- and glutamine-free DMEM with 100 μ Ci/ml *myo*-[³H]-Inositol. For all experiments cells were incubated

for 1 h in warm HBSS (116 mM NaCl, 20 mM HEPES, 11 mM glucose, 5 mM NaHCO₃, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, pH 7.4) and were then incubated with 10 mM LiCl alone for 10 min followed by 30 μ M quisqualate in LiCl for 30 min. Cells were placed on ice and the reaction was stopped with 500 μ l of perchloric acid and was neutralized with 400 μ l of 0.72 M KOH, 0.6 M KHCO₃. Total cellular [³H]-inositol incorporation was determined in 50 μ l of cell lysate. Total inositol phosphate was purified by anion exchange chromatography using Dowex 1-X8 (formate form) 200-400 mesh anion exchange resin and [³H]-inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system.

ERK Phosphorylation:

Following 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 10 μ M 5HT agonist for the duration of the described time points. Cells were lysed with lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors (1mM AEBSF, 10 μ g/ml leupeptin, and 5 μ g/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 x g for 15 minutes at 4 °C to pellet insoluble material. A Bronsted-Lowry protein assay was performed, and 50 μ g of protein was incubated overnight at room temperature in 3x SDS Loading Buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted for ERK1/2 (rabbit anti-p44/42 MAPK, 1:1000), phospho-ERK1/2 (rabbit anti-phospho-p44/42 MAPK, 1:1000), SAP97 (mouse anti-SAP97, 1:1000), and FLAG-5-HT_{2A}R expression (rabbit anti-FLAG,

1:1000), followed by a horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:10,000) or anti-mouse antibody (1:10,000) where appropriate. Proteins were detected using chemiluminescence with the enhanced chemiluminescence kit from GE Healthcare.

Statistical Analysis:

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

3.3 Results

SAP97 is co-immunoprecipitated with 5-HT_{2A}R in a PDZ-binding motif-dependent manner. Previously, we demonstrated that the class I carboxyl-terminal tail CRFR1 PDZ binding motif was essential for CRFR1-mediated sensitization of 5-HT_{2A}R signaling and that the PDZ domain-containing protein SAP97 interacted with CRFR1 to regulate its endocytosis and signaling (Magalhaes et al., 2010; Dunn et al., 2013). Therefore, we tested whether SAP97 might also be co-immunoprecipitated with 5-HT_{2A}R in a PDZ motif-dependent manner. We found that green fluorescent protein (GFP-SAP97) fusion protein was co-immunoprecipitated with a FLAG (FL) epitope-tagged 5-HT_{2A}R (FL-5-HT_{2A}R) from HEK 293 cells, but that agonist stimulation with 10 μ M 5-HT did not increase the

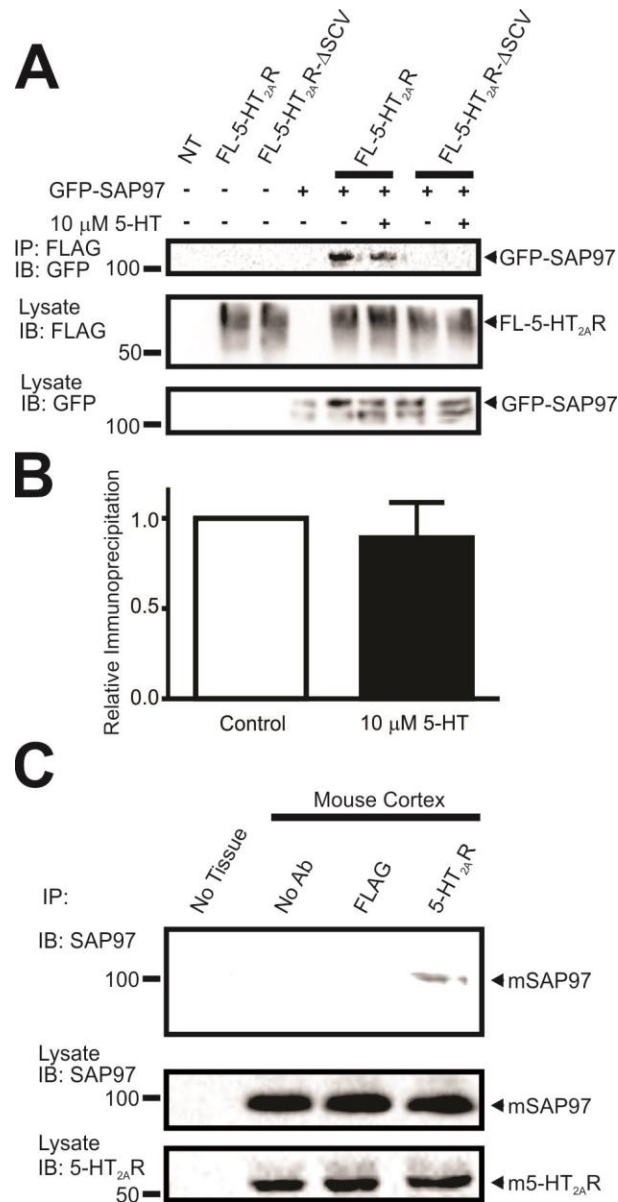


Figure 3.1: SAP97 co-immunoprecipitates with 5-HT_{2A}R in a PDZ-binding motif-dependent and agonist-independent manner: (A) Representative immunoblot of SAP97 co-immunoprecipitated with FL-5-HT_{2A}R but not FL-5-HT_{2A}R-ΔSCV, which lacks the PDZ-binding motif. Transient transfections with 1 μg of plasmid cDNA for each construct were performed in HEK 293 cells as labelled. Samples were run using SDS-PAGE and immunoblotted with rabbit anti-GFP. (B) Effect of 5HT treatment was quantified using densitometry and had no significant effect on the amount of YFP-SAP97 co-immunoprecipitated with FL-5-HT_{2A}R. Data are representative of the mean ± SEM of six independent experiments. (C) Representative immunoblot of endogenous SAP97 co-immunoprecipitated with 5-HT_{2A}R antibody from adult mouse cortex. Data are representative of three independent experiments.

association of GFP-SAP97 with FL-5-HT_{2A}R (Fig. 3.1A and B). This interaction was dependent on an intact PDZ binding motif at the end of the carboxyl-terminal tail, as the deletion of the last three critical amino acid residues (SCV) of the 5-HT_{2A}R carboxyl-terminal tail prevent GFP-SAP97 co-immunoprecipitation with the resulting FL-5-HT_{2A}R-ΔSCV mutant (Fig. 3.1A and B). We found that endogenous SAP97 could also be co-immunoprecipitated with endogenous 5-HT_{2A}R from cortical brain lysates (Fig. 3.1C). Thus, SAP97 interacts with 5-HT_{2A}R via the 5-HT_{2A}R carboxyl-terminal PDZ binding motif.

SAP97 colocalization with 5HT_{2A}R at the plasma membrane is dependent on an intact 5HT_{2A}R PDZ-binding motif. GFP-SAP97 when expressed alone in HEK 293 cells was diffusely localized throughout the cytoplasm and was not localized to the plasma membrane (Fig. 3.2A). However, when GFP-SAP97 was co-expressed with FL-5-HT_{2A}R in HEK293 cells, GFP-SAP97 immunofluorescence redistributed to the plasma membrane, where it colocalized with Alexa Fluor 647 labeled FL-5-HT_{2A}R (Fig. 3.2B). In contrast, deletion of the 5-HT_{2A}R carboxyl-terminal PDZ binding motif (SCV) resulted in a FL-5-HT_{2A}R-ΔSCV mutant that was unable to promote the redistribution of GFP-SAP97 from the cytosol to the plasma membrane (Fig. 3.2C). To further establish whether SAP-97 interacts with 5-HT_{2A}R, we prepared a FL-5-HT_{2A}R renilla luciferase (rLuc) fusion protein, where rLuc was inserted in frame into the 5-HT_{2A}R carboxyl-terminal tail 22 amino acid residues upstream of the SCV PDZ binding motif (FL-5-HT_{2A}R-rLuc-x22-SCV). We found that this construct was expressed at the cell surface of HEK 293 cells (Fig. 3.3A) and was coupled to the stimulation of inositol phosphate formation (Fig. 3.3B). The EC₅₀ for

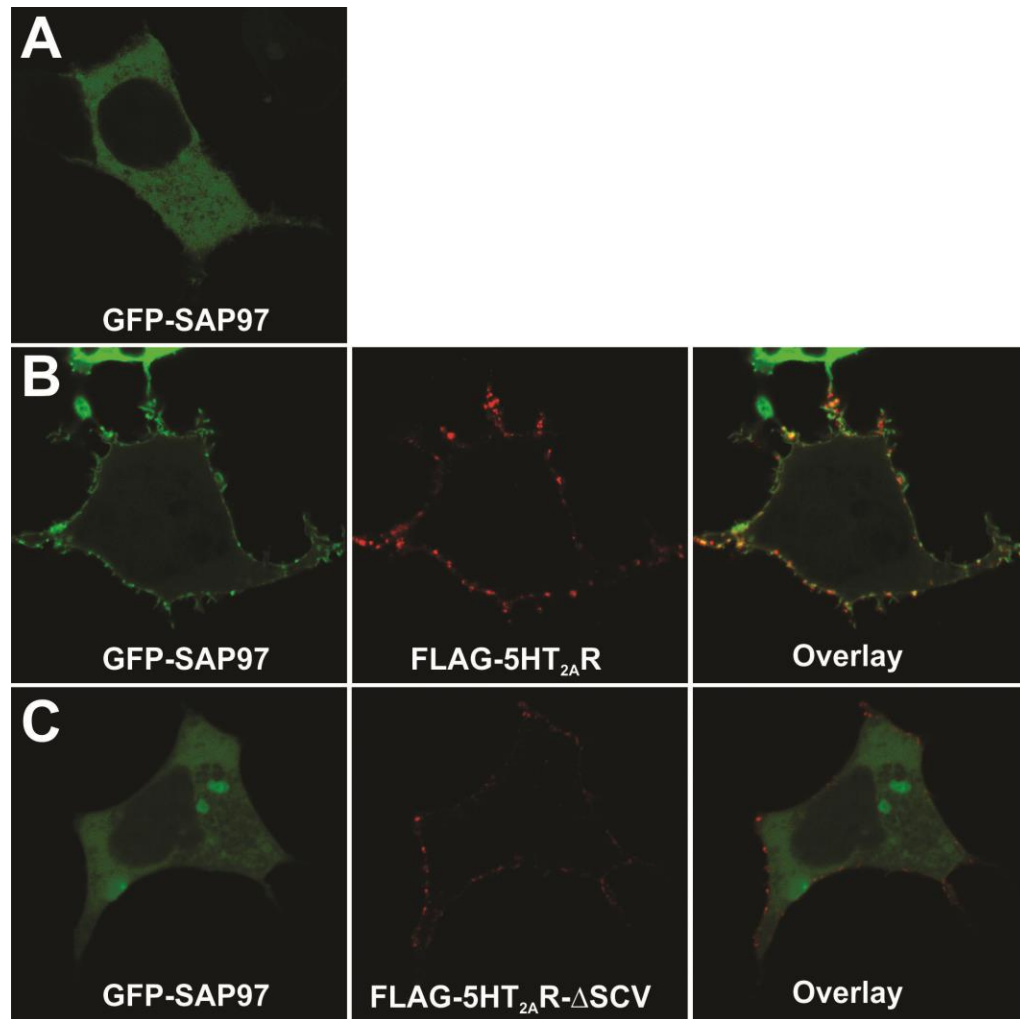


Figure 3.2: GFP-SAP97 co-localizes at the membrane with FL-5-HT_{2A}R in a PDZ-binding motif-dependent manner: (A) Representative confocal microscopy image demonstrating the subcellular localization of GFP-SAP97 (green) when expressed alone HEK 293 cells. (B) Representative confocal image demonstrating the colocalization of FLAG-tagged 5-HT_{2A}R (red) labelled with Zenon Alexa Fluor 633-conjugated mouse anti-FLAG antibody GFP-SAP97 (green). (C) Representative confocal image demonstrating the colocalization of FLAG-tagged 5-HT_{2A}R-ΔSCV (red) labelled with Zenon Alexa Fluor 633-conjugated mouse anti-FLAG antibody GFP-SAP97 (green). HEK 293 cells were transfected with 3 μg of receptor and 1 μg of GFP-SAP97 plasmid cDNA, respectively. Data are representative of over 30 cells.

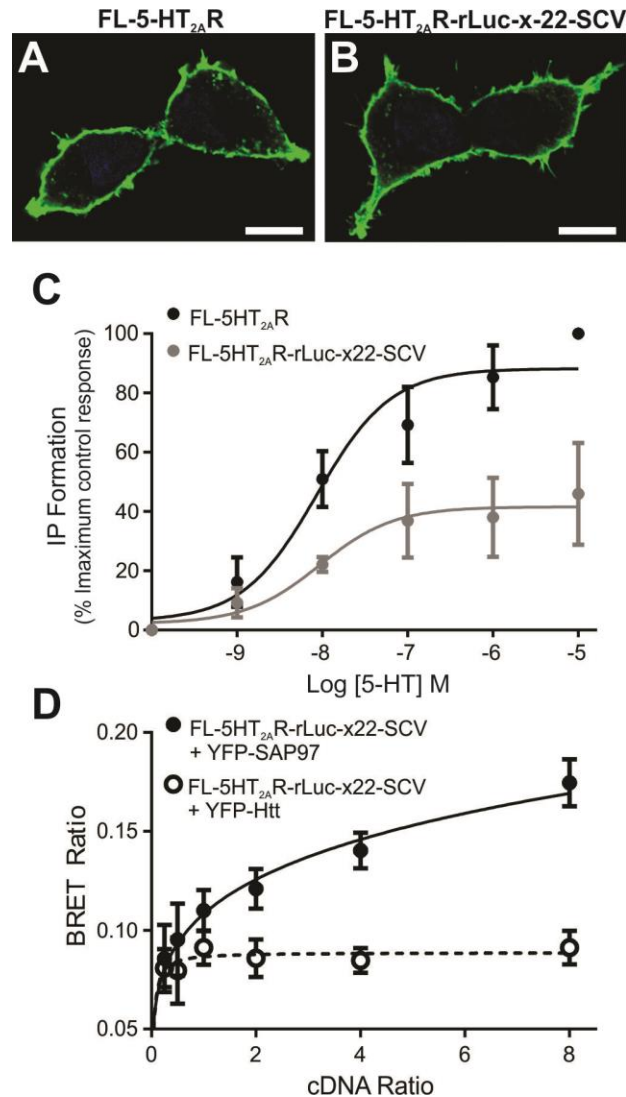


Figure 3.3: YFP-SAP97 directly interacts with 5-HT_{2A}R-rLuc and FL-5-HT_{2A}R -rLuc-x22-SCV. (A) Representative confocal microscopy image demonstrating surface expression of FL-5-HT_{2A}R (green) and (B) FL-5-HT_{2A}R-rLuc-x22-SCV (green) with mouse anti-FLAG followed by secondary mouse Alexa Fluor 488. (C) Dose response curves for 5-HT-mediated IP formation in response to treatment with increasing concentrations of 5-HT for 30 minutes in HEK 293 cells transfected with 1 µg of plasmid cDNA expressing FL-5-HT_{2A}R or 5-HT_{2A}R -rLuc-x22-SCV. The EC₅₀ for FL-5-HT_{2A}R -mediated IP formation was 8.6 nM and the EC₅₀ for FL-5-HT_{2A}R -Luc-x22-SCV-mediated IP formation was 9.3 nM. The data are representative of the mean ± S.E.M. of three independent experiments. (D) HEK 293 cells were transfected with 20 ng of plasmid cDNA expressing FL-5-HT_{2A}R -rLuc-x22-SCV cDNA with increasing ratios of plasmid cDNA expressing either YFP-SAP97 or YFP-Htt. Total cDNA was normalized with pcDNA3.1 and the BRET ratio determined. The data represent the mean ± S.E.M. of six independent experiments.

FL-5-HT_{2A}R mediated IP formation was 8.6 nM and the EC₅₀ for FL-5-HT_{2A}R-rLuc-x22-SCV mediated IP formation was 9.3 nM. Although the maximum efficacy for FL-5-HT_{2A}R-rLuc-x22-SCV stimulated IP formation was attenuated when compared with FL-5-HT_{2A}R, this was the consequence of reduced cell surface FL-5-HT_{2A}R-rLuc-x22-SCV expression (data not shown). When HEK 293 cells were transfected with a constant amount of FL-5-HT_{2A}R-rLuc-x22-SCV plasmid cDNA (1 µg) along with increasing ratios of plasmid cDNA expressing SAP97-YFP we observed an increasing, but saturable level of bioluminescence resonance energy transfer (BRET) (Fig. 3.3D). In contrast, co-expression of FL-5-HT_{2A}R-rLuc-x22-SCV with increasing ratios of wild-type huntingtin-YFP did not result in an increased BRET ratio (Fig. 3.3D). Thus, the immunofluorescence subcellular localization data, co-immunoprecipitation data, and BRET data all indicated that SAP97 interactions 5-HT_{2A}R were PDZ binding motif dependent.

SAP97 antagonizes the internalization of 5-HT_{2A}R. We previously demonstrated that SAP97 contributed to the regulation of agonist-stimulated CRFR1 internalization by attenuating the endocytosis of the receptor (Dunn et al., 2013). Therefore, we tested whether the overexpression of GFP-SAP97 or knockdown of endogenous SAP97 expression in HEK 293 cells would alter the agonist-stimulated internalization of FL-5-HT_{2A}R. In HEK 293 cells expressing FL-5-HT_{2A}R the overexpression of SAP97 reduced agonist-stimulated (30 min, 10 µM 5-HT) internalization of the receptor and SAP97 shRNA expression resulted in an increase in FL-5-HT_{2A}R endocytosis, as measured by a loss of cell surface FL-5-HT_{2A}R immunofluorescence (Fig.3.4). In contrast, deletion of the FL-5-HT_{2A}R PDZ binding motif resulted in a FL-5-HT_{2A}R-ΔSCV mutant that was

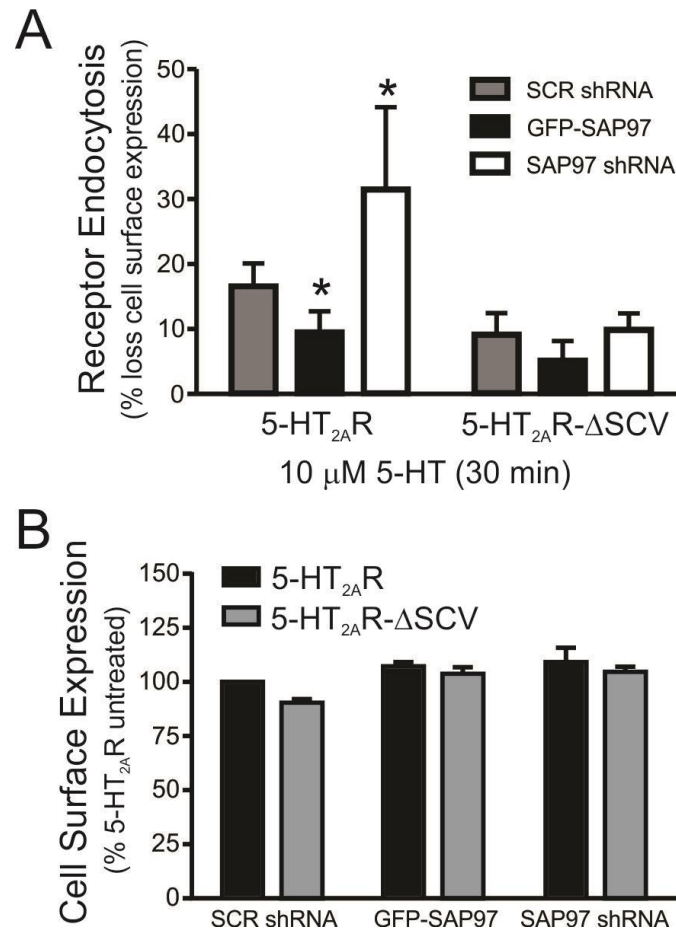


Figure 3.4: Role of SAP97 in the internalization of 5HT_{2A}R: (A) HEK 293 cells were transfected with 1 μg of plasmid cDNA expressing either FL-5-HT_{2A}R or FL-5-HT_{2A}R-ΔSCV along with either 3 μg of plasmid cDNA expressing scrambled shRNA (SCR), 1 μg of plasmid cDNA expressing GFP-SAP97 or 3 μg of plasmid cDNA expressing SAP97 shRNA. Shown is the loss of cell surface receptor following the treatment of cells with 10 μM 5-HT for 30 min, as assessed by flow cytometry. The data represents the mean ± SEM. (B) Basal levels of cell surface expression across various conditions examined in panel A, as measured by flow cytometry. The data represents the mean ± SEM.

impaired in its internalization when compared to the wild-type type receptor and neither GFP-SAP97 overexpression nor SAP97 shRNA expression altered the extent of FL-5-HT_{2A}R-ΔSCV endocytosis (Fig. 3.4A). Basal cell surface receptor was unchanged (Fig. 3.4B). Taken together, similar to what was observed for the CRFR1 (Dunn et al., 2013), FL-5-HT_{2A}R internalization was modulated by SAP97 expression, but normal internalization of the receptor was dependent upon an intact PDZ binding motif. This observation suggests that another PDZ protein may be integral for receptor endocytosis.

SAP97 regulates 5HT_{2A}R-mediated IP formation. We previously found that SAP97 did not contribute to the regulation of CRFR1-mediated cAMP signaling (Dunn et al., 2013). However, a structurally similar PDZ containing protein, PSD95, was previously demonstrated to enhance 5-HT_{2A}R-mediated inositol phosphate (IP) signaling (Xia et al., 2003b). Therefore, we tested whether either the overexpression of GFP-SAP97 or SAP97 shRNA treatment altered 5-HT-stimulated IP formation in FL-5-HT_{2A}R expressing HEK 293 cells. In HEK 293 cells transfected with FL-5-HT_{2A}R overexpression of GFP-SAP97 did not alter IP formation in response to increasing concentrations of 5-HT (Fig. 3.5A). However, shRNA knockdown of endogenous SAP97 significantly attenuated the maximum efficacy for 5-HT-stimulated IP formation in cells transfected FL-5-HT_{2A}R, when compared to cells treated with scrambled shRNA (Fig. 3.5A). In contrast, neither GFP-SAP97 overexpression nor SAP97 shRNA treatment altered the dose response for 5-HT-stimulated IP formation in cells expressing FL-5-HT_{2A}R-ΔSCV (Fig. 5B). Interestingly, when the dose response curves for 5-HT-stimulated IP formation in FL-5-HT_{2A}R and FL-5-HT_{2A}R-ΔSCV were compared, the maximum efficacy for FL-5-HT_{2A}R-

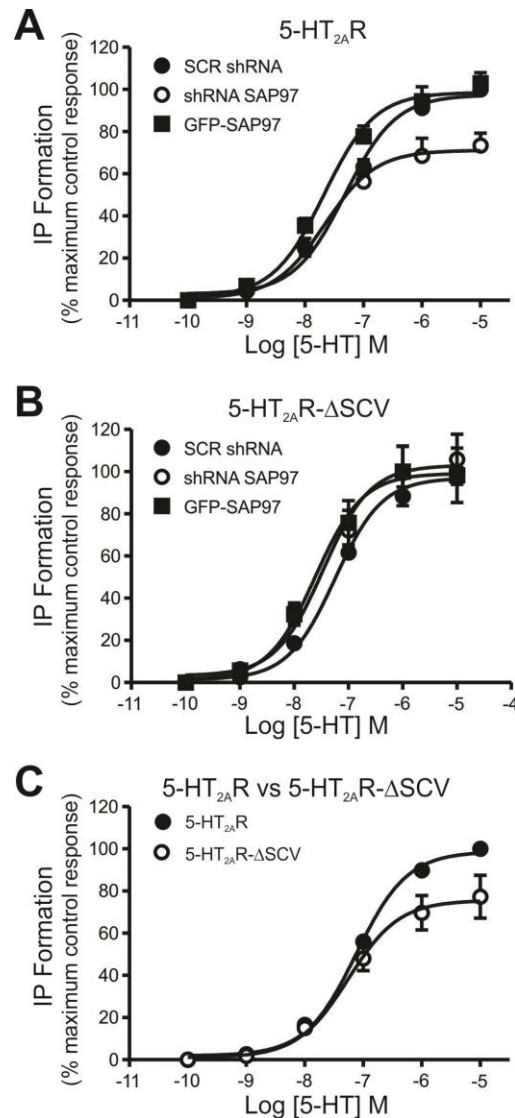


Figure 3.5: SAP97 plays an integral role in 5-HT_{2A}R-mediated IP₃-signaling. (A) Dose response curves for 5-HT_{2A}R-mediated IP formation in response to treatment with increasing concentrations of 5-HT for 30 min in cells transfected with 1 μg of plasmid cDNA expressing FL-5-HT_{2A}R along with either 3 μg of plasmid cDNA expressing scrambled shRNA (SCR), 1 μg of plasmid cDNA expressing GFP-SAP97 or 3 μg of plasmid cDNA expressing SAP97 shRNA. (B) Dose response curves for 5-HT_{2A}R-ΔSCV-mediated IP formation in response to treatment with increasing concentrations of 5-HT for 30 min in cells transfected with 1 μg of plasmid cDNA expressing FL-5-HT_{2A}R-ΔSCV along with either 3 μg of plasmid cDNA expressing scrambled shRNA (SCR), 1 μg of plasmid cDNA expressing or 3 μg of plasmid cDNA expressing SAP97 shRNA. (C) Comparison of the dose response curves for 5-HT_{2A}R- and 5-HT_{2A}R-ΔSCV-mediated IP formation in response to treatment with increasing concentrations of 5-HT for 30 min. Cells were transfected with 1 μg of plasmid cDNA expressing either FL-5-HT_{2A}R or FL-5-HT_{2A}R-ΔSCV. The data represent the mean ± SEM of six independent experiments.

Δ SCV-stimulated IP formation was found to be significantly attenuated when compared to wild-type FL-5-HT_{2A}R (Fig. 3.5C). These results indicated that endogenous SAP97 interactions with the 5-HT_{2A}R PDZ binding motif contribute to the regulation of 5-HT_{2A}R-mediated G $\alpha_{q/11}$ signaling.

SAP97 regulation 5HT_{2A}R-mediated ERK1/2 phosphorylation is PDZ binding motif-independent. We recently demonstrated that SAP97 regulated CRFR1-mediated ERK1/2 phosphorylation in a manner that was independent of SAP97 interactions with CRFR1 PDZ binding motif (Dunn et al., 2013). Therefore, we tested the effect of GFP-SAP97 overexpression and shRNA knockdown on ERK1/2 phosphorylation in response to 10 μ M 5-HT stimulation of the 5-HT_{2A}R for 0, 5 and 10 min. We found the overexpression of GFP-SAP97 did not significantly alter 5-HT-stimulated ERK1/2 phosphorylation in response to the activation of the 5-HT_{2A}R, but SAP97 shRNA treatment significantly attenuated 5-HT_{2A}R-mediated ERK1/2 phosphorylation (Fig. 3.6A). Similar to what we observed for a CRFR1 mutant receptor that lacked its carboxyl-terminal PDZ binding motif, ERK1/2 phosphorylation in response to the activation of the 5-HT_{2A}R- Δ SCV mutant was also unaffected by GFP-SAP97 overexpression, but was still significantly attenuated following the knockdown of SAP97 following SAP97 shRNA treatment (Fig. 3.6B). Taken together these results support a generalized role for SAP97 in the regulation of GPCR-mediated ERK1/2 phosphorylation that does not require PDZ domain interactions with GPCRs.

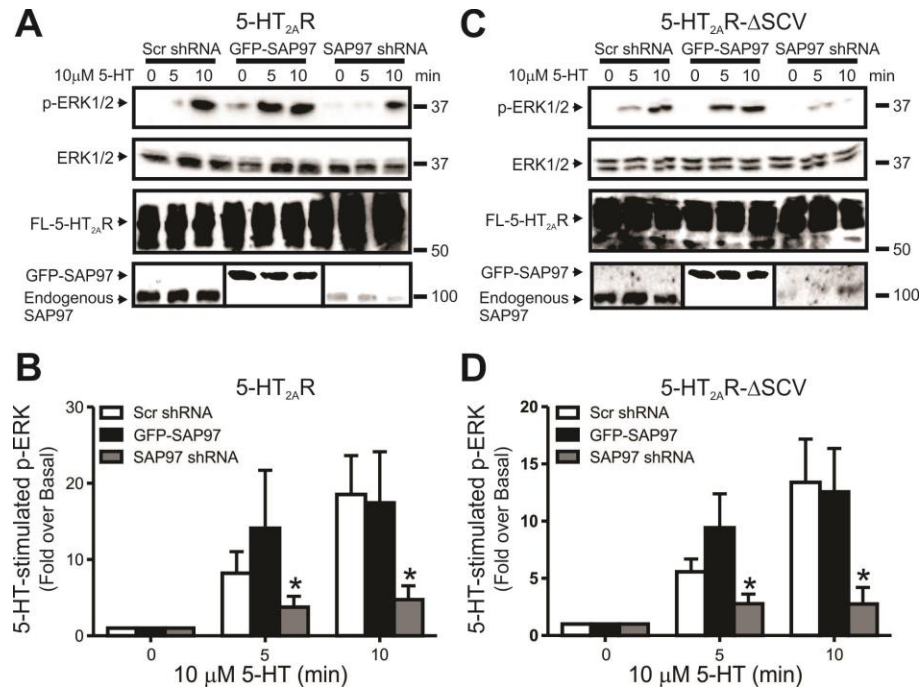


Figure 3.6: Endogenous SAP97 is required for 5HT_{2A}R-mediated ERK1/2 phosphorylation independent of PDZ-binding motif interactions: (A) Representative immunoblot showing ERK1/2 phosphorylation in response 10 μ M 5-HT treatment for 0, 5, 10 min in HEK 293 cells transfected with 1 μ g of plasmid cDNA encoding FL-5-HT_{2A}R 1 μ g of plasmid cDNA expressing FL-5HT_{2A}R along with either 3 μ g of plasmid cDNA expressing scrambled shRNA (SCR), 1 μ g of plasmid cDNA expressing GFP-SAP97 or 3 μ g of plasmid cDNA expressing SAP97 shRNA.. Shown in the panels below are corresponding immunoblots for total ERK1/2, FL-5-HT_{2A}R, GFP-SAP97, and endogenous SAP97 protein expression. Immunoblot of GFP-SAP97 overexpression is shown at a different exposure (as indicated by box) than the exposure for endogenous SAP97 treated with scrambled and SAP97 shRNA. (B) Densitometric analysis of ERK1/2 phosphorylation in response to 10 μ M 5HT treatment for 0, 5, and 10 min in HEK 293 cells transfected with FLAG-5HT_{2A}R and either scrambled shRNA, GFP-SAP97, or SAP97 shRNA. The data represent the mean \pm SEM of four independent experiments. (C) Representative immunoblot showing ERK1/2 phosphorylation in response 10 μ M 5-HT treatment for 0, 5, 10 min in HEK 293 cells transfected with 1 μ g of plasmid cDNA encoding FL-5-HT_{2A}R- Δ SCV 1 μ g of plasmid cDNA expressing FL-5HT_{2A}R along with either 3 μ g of plasmid cDNA expressing scrambled shRNA (SCR), 1 μ g of plasmid cDNA expressing GFP-SAP97 or 3 μ g of plasmid cDNA expressing SAP97 shRNA.. Shown in the panels below are corresponding immunoblots for total ERK1/2, FL-5-HT_{2A}R- Δ SCV, GFP-SAP97, and endogenous SAP97 protein expression. Immunoblot of GFP-SAP97 overexpression is shown at a different exposure (as indicated by box) than the exposure for endogenous SAP97 treated with scrambled and SAP97 shRNA. (D) Densitometric analysis of ERK1/2 phosphorylation in response to 10 μ M 5HT treatment for 0, 5, and 10 min in HEK 293 cells transfected with FLAG-5HT_{2A}R- Δ SCV and either scrambled shRNA, GFP-SAP97, or SAP97 shRNA. The data represent the mean \pm SEM of four independent experiments.

SAP97 is not exclusively involved in CRFR1-mediated enhancement of 5-HT_{2A}R-mediated IP formation. Our primary rationale for assessing the role of SAP97 in the regulation of CRFR1 and 5-HT_{2A}R signaling and endocytosis was to determine whether SAP97 was the PDZ protein underlying CRFR1-mediated sensitization of 5-HT_{2A}R signaling (Magalhaes et al., 2010). Therefore, we tested the effect of SAP97 shRNA-mediated knockdown on CRFR1-stimulated enhancement of 5-HT_{2A}R signaling. In HEK 293 cells transfected with CRFR1, 5-HT_{2A}R and scrambled shRNA, pretreatment with 500 nM CRF for 30 min resulted in an increase in the maximum efficacy for 5-HT-stimulated IP formation, when compared to cells that were not CRF pretreated (Fig. 3.7). As demonstrated in Fig. 5.5A, SAP97 shRNA treatment reduced the maximum efficacy for 5-HT-stimulated IP formation, when compared to 5-HT-stimulated IP responses in cells transfected with CRFR1, 5-HT_{2A}R and scrambled shRNA (Fig. 3.7). However, SAP97 shRNA down-regulation of SAP97 expression did not block the CRF pretreatment-induced increase in the maximum efficacy for 5-HT-stimulated IP formation CRFR1 and 5-HT_{2A}R expressing cells (Fig. 3.7). Taken together, these results indicated that, although SAP97 contributed to the regulation of 5-HT_{2A}R-mediated IP formation, it did not contribute to the CRFR1-mediated sensitization of 5-HT_{2A}R-signaling previously reported by Magalhaes and colleagues (2010).

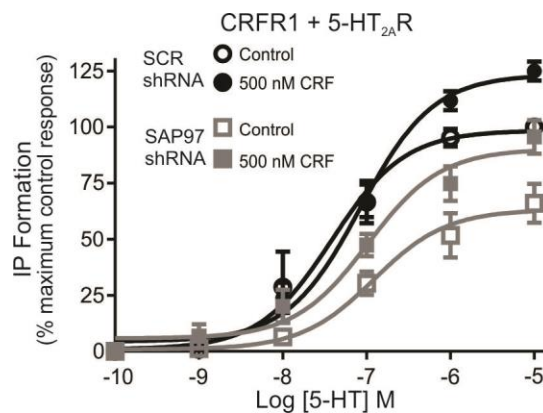


Figure 3.7: SAP97 shRNA knockdown does not prevent CRFR1-mediated enhancement of 5-HT_{2A}R-mediated signaling: 5-HT stimulated dose response curves for inositol phosphate (IP) formation in HEK 293 cells that are transfected with 1 μ g of plasmid cDNA encoding HA-CRFR1 and 1 μ g of plasmid cDNA encoding either FL-5-HT_{2A}R or FL-5-HT_{2A}R- Δ SCV along with 3 μ g of plasmid cDNA encoding either scrambled shRNA (SCR) or SAP97 shRNA. Transfected HEK 293 cells were treated either with or without 500 nM CRF for 30 min prior to being treated with increasing concentrations of 5-HT for 30 min. The data represent the mean \pm SEM of 5 independent experiments.

3.4 Discussion

We previously demonstrated that two GPCRs linked to the manifestation of mood disorders and psychiatric disease, CRFR1 and 5-HT_{2A}R, exhibit crosstalk that is dependent upon PDZ-binding motif interactions with an unknown PDZ protein that results in the heterologous sensitization of 5HT_{2A}R signaling (Magalhaes et al., 2010). This prompted us to screen for PDZ-containing proteins that may interact with both CRFR1 and 5-HT_{2A}R (Dunn et al., 2013) and which may also function as novel regulators of both CRFR1 and 5-HT_{2A}R trafficking and signaling. We show here, that similar to what we previously reported for CRFR1 (Dunn et al., 2013), SAP97 functions to antagonize 5-HT_{2A}R endocytosis. Thus, SAP97 appears to play a generalized and functionally consistent role in regulating the endocytosis of both the CRFR1 and 5-HT_{2A}R. Although SAP97 expression is essential for both CRFR1- and 5-HT_{2A}R-mediated activation of ERK1/2 phosphorylation, the role of SAP97 in regulating GPCR-dependent ERK1/2 signaling is independent of PDZ domain interactions with the receptors. Moreover, we find that the loss of SAP97 expression impairs 5-HT_{2A}R-stimulated IP signaling, but that PDZ domain containing protein SAP97 is not exclusively required for CRFR1-dependent heterologous sensitization of 5HT_{2A}R signaling (Magalhaes et al., 2010).

SAP97 has previously been reported to interact with several neurotransmitter receptors including: the β_1 -adrenergic receptor (β_1 AR), CRFR1, somatostatin receptor subtype 1, kainite receptor, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), N-methyl-D-aspartate receptor (NMDAR) and was identified as a 5-HT_{2A}R interacting protein in a proteomic screen (Leonard et al., 1998; Bassand et al., 1999; Metha et al., 2001; Sans et al., 2001; Rumbaugh et al., 2003; Bécamel et al., 2004; He et

al., 2006; Gardner et al., 2007; Cai et al., 2008; Dunn et al., 2013). Interestingly, each of these receptors have a class I PDZ-binding motif on the distal region of the carboxyl terminal tail. SAP97 plays a role in regulating either the cell surface localization, endocytosis, signaling, compartmentalization or recycling of each of these receptors proteins. For example, SAP97 regulates the cell surface expression of the AMPAR via its association with the AMPAR GluR1 subunit in the secretory pathway, but SAP97 is not involved in the NMDAR-mediated internalization of the receptor (Sans et al., 2001; Rumbaugh et al., 2003). SAP97 is also involved in the subcellular localization of kanite receptors (Metha et al., 2001) and in the case of the NMDAR, SAP97 modulates NMDAR-mediated CaMKII phosphorylation and NMDAR synaptic localization (Gardoni et al., 2003). It is also reported that SAP97 may mediate somatostatin receptor type 1-dependent regulation of synaptic growth cone dynamics (Cai et al., 2008). We have shown previously (Dunn et al., 2013) and in the present study that SAP97 interactions also contribute to the antagonism of agonist-induced endocytosis of both CRFR1 and 5-HT_{2A}R. Taken together, it appears that SAP97 may have a general role in promoting the membrane localization of transmembrane receptors that specifically encode class I PDZ-binding motifs. Future studies will be required to understand how PDZ proteins regulate the functional activity of GPCRs *in vivo* and contribute to the physiological regulation of GPCR-modulated animal behaviours and pathophysiological GPCR signaling.

Our previous studies have shown that SAP97 does not contribute to the regulation of G α_s -mediated cAMP signaling in response to CRFR1 activation (Dunn et al., 2013). In contrast, our current study now suggests that SAP97 expression facilitates the ability of the 5-HT_{2A}R to stimulate IP formation via the activation of G $\alpha_{q/11}$. Previous studies have

demonstrated that PSD95 and NHERF1 contribute to the regulation of IP signaling via the thromboxane A2 receptor (TP receptor) and 5-HT_{2A}R, respectively (Rochdi et al., 2002; Xia et al., 2003b). The association of PSD95 with 5-HT_{2A}R enhances 5-HT_{2A}R signal transduction similar to what we have observed for the structurally related PDZ protein SAP97 (Xia et al., 2003b). In contrast, activation of the TP receptor increases NHERF1 interactions with G $\alpha_{q/11}$, which in turn functions to impair G $\alpha_{q/11}$ /TP receptor interactions that are required for the activation of phospholipase C-mediated IP formation (Rochdi et al., 2002). This suggests that PDZ domain containing proteins may function to both positively and negatively regulate the interface between GPCRs and heterotrimeric G proteins such as G $\alpha_{q/11}$. Future studies will be required to determine the mechanism by which PSD95 and SAP97 function to enhance 5-HT_{2A}R signaling and whether this mechanism is either complementary to or antagonistic to the means by which NHERF1 antagonizes TP receptor signaling.

We previously demonstrated that CRF-mediated ERK1/2 phosphorylation is dependent upon the endogenous expression of SAP97 in both HEK293 and AtT20 cells (Dunn et al., 2013). This role for SAP97 in the regulation of ERK1/2 signaling is independent of the class I CRFR1 PDZ-binding motif, and SAP97 expression is required for CRFR2-mediated ERK1/2 phosphorylation, a receptor which, despite ~60% sequence homology with CRFR1, does not contain a class I PDZ-binding motif (Dunn et al., 2013). Our studies with 5-HT_{2A}R indicate that SAP97 plays a generalized role in regulating GPCR-dependent ERK1/2 signaling. Previous studies have demonstrated the ability of p38 MAPK family proteins to interact with and subsequently phosphorylate SAP97 following cellular stress responses resulting in disrupted SAP97 interactions with the actin

cytoskeleton (Sabio et al., 2005). The fact that SAP97 is a substrate for p38 MAPK suggests that it may also serve as a scaffold for the assembly of ERK signaling complexes. This provides a potential molecular mechanism whereby SAP97 might regulate ERK1/2 signaling downstream of SAP97/GPCR.

Of central importance to this study was to assess whether SAP97 represents the PDZ protein that is required for CRFR1-mediated sensitization of 5-HT_{2A}R signaling. Although SAP97 shRNA-dependent knockdown of SAP97 protein expression significantly attenuates 5-HT_{2A}R-mediated IP signaling, CRF pretreatment in the absence of SAP97 expression still evokes heterologous sensitization of 5-HT_{2A}R-mediated IP signaling. This suggests that SAP97 is either not involved in the crosstalk between CRFR1 and 5-HT_{2A}R, or that other PDZ proteins are also required and/or compensate for the loss of SAP97 expression.

In summary, in the current study we have characterized the role of the PDZ domain-containing protein SAP97 as a novel regulator of 5-HT_{2A}R trafficking and signaling and confirm a receptor-independent role for SAP97 in regulating ERK1/2 signaling. PDZ domain containing proteins of the PSD95/SAP97 family have an established link to the regulation and treatment of mood disorders and psychiatric disease that may implicate 5-HT_{2A}R signaling (Gray and Roth, 2001; Roth et al., 2004; Catapano and Manji, 2007). Further understanding of how PDZ proteins regulate CRFR1 and 5-HT_{2A}R function will be essential for understanding their contribution to the pathological cell signaling associated with psychiatric diseases and the development of new strategies to treat these diseases.

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Chapter 4: PSD-95 Regulation of CRFR1

A version of this manuscript has been submitted to the journal of *Molecular Pharmacology*:

Henry A. Dunn, Harpreet S. Chahal, Kevin D. Holmes, George Y. Yuan, Ruchi Parikh, Fabiana Caetano, Cornelia Walther and Stephen S. G. Ferguson (submitted). Role of PSD-95 in regulating CRFR1 trafficking and β -arrestin2 recruitment.

4.1 Introduction

Corticotropin-releasing factor (CRF) is a neuropeptide that is released from the hypothalamus during periods of stress and ultimately serves to increase blood-cortisol levels, subsequently leading to the production of energy to cope with the stressor (Emeric-Sauval, 1986). Once released, CRF can activate two receptor subtypes, CRF receptor 1 (CRFR1) and CRF receptor 2 (CRFR2), both of which are G protein-coupled receptors (GPCRs) (Chalmers et al., 1996; Dautzenberg & Hauger, 2002). Although both receptors appear to contribute to stress responses and mood-regulation (Laryea et al., 2012), CRFR1 has an order of magnitude higher affinity for CRF than CRFR2, and CRFR1 is also expressed at higher levels throughout the brain (Chalmers et al., 1995; Palchaudhuri et al., 1998). CRFR1 antagonists have demonstrated anxiolytic and antidepressant-like effects suggesting that CRFR1 is a viable target for mood-regulation (Overstreet & Griebel, 2004; Chaki et al., 2004). The link between stress, mood disorders, and psychiatric disease has long been hypothesized. Recent studies suggest a potential molecular mechanism whereby CRFR1 activation leads to enhanced 5-HT_{2A}R expression and signaling that leads to increased stress-induced anxiety and depression (Leonard, 2005; Magalhaes et al., 2010). 5HT_{2A}R has consistently been implicated in psychiatric disease and 5HT_{2A}R antagonists have shown limited success in alleviating the negative effects of depression and psychoses (Eison et al., 1991; Marek et al., 2005; Fribourg et al., 2011).

Although CRFR1 represents an intriguing target for regulating mood, the characterization of CRFR1-interacting proteins that influence its signal transduction and trafficking have not been extensively characterized. Recently, we have shown that the

PSD-95/Disc Large/Zona Occludens (PDZ) protein, SAP97, is a novel CRFR1-interacting protein that functions to reduce CRFR1 endocytosis, but also promotes CRFR1-mediated ERK1/2 signaling independently of receptor interactions (Dunn et al., 2013). Interestingly, PDZ interactions are also integral for CRFR1-mediated enhancement of 5-HT_{2A}R signaling (Magalhaes et al., 2010). However, SAP97 is not the PDZ domain-containing protein that is independently responsible for the heterologous sensitization of 5-HT_{2A}R signaling (Dunn et al., 2014).

PSD-95 is another PDZ domain-containing protein with structural similarities to SAP97 that we identified as a CRFR1-interacting protein in a screen for PDZ domains that bind to the CRFR1 C-tail (Dunn et al., 2013). SAP97 and PSD-95 have both been linked to psychiatric disease, specifically schizophrenia (Toyooka et al., 2002; Clinton et al., 2003; Sato et al., 2008; Clinton & Meador-Woodruff, 2004; Toro & Deakin, 2005; Kristiansen et al., 2006; Clinton et al., 2006; Funk et al., 2009; Chen et al., 2010; Balan et al., 2013). Additionally, hallucinogen and atypical antipsychotic actions of 5-HT_{2A}R are dependent upon the expression of PSD-95 (Abbas et al., 2009). Thus, PSD-95 represents a viable target in the regulation of CRFR1 function, as it has been previously demonstrated to regulate the function of other GPCRs, in addition to 5HT_{2A}R (Hu et al., 2000; Xu et al., 2001; Hu et al., 2002; Xia et al., 2003; Gavarini et al., 2006; Zhang et al., 2007; Sun et al., 2009, Abbas et al., 2009). With respect to 5-HT_{2A}R, which like CRFR1, encodes a class I PDZ-binding motif at the distal end of its carboxyl terminal tail, PSD-95 overexpression is shown to inhibit 5-HT_{2A}R endocytosis and to facilitate 5-HT_{2A}R-stimulated inositol phosphate (IP) formation (Xia et al., 2003; Abbas et al., 2009). Consequently, understanding the contribution of PSD-95 to the regulation of CRFR1 activity, as well as

the crosstalk between CRFR1 and 5-HT_{2A}R signaling, may provide significant insight into the development of new therapeutic strategies for mood disorders and psychiatric disease.

In this study, we identified PSD-95 as a CRFR1 interacting protein that colocalizes with CRFR1 at the cell surface of HEK 293 cells and in the dendrites of cortical neurons in a PDZ motif-dependent manner. Unlike SAP97, PSD-95 overexpression or knockdown does not affect either CRFR1-stimulated IP formation or ERK1/2 phosphorylation. However, similar to what is observed for SAP97, the overexpression of PSD-95 attenuates CRFR1 internalization and a reduction of PSD-95 expression increases CRFR1 internalization. PSD-95 modulates CRFR1 endocytosis by competing with β -arrestin for receptor binding, but PSD-95 is not required for CRFR1-dependent sensitization of 5-HT_{2A}R signaling. We conclude that although PSD-95 and SAP97 are structurally similar they play distinct roles in the regulation of CRFR1 and 5-HT_{2A}R signaling.

4.2 Experimental Procedures

Materials:

Goat anti-glutathione-S-transferase (GST) antibodies, as well as ECL Western blotting detection reagents were purchased from GE Healthcare (Oakville, ON, Canada). Rabbit anti-phospho-p44/42 MAP kinase (Thr202/Tyr402), and rabbit anti-p42/44 MAP kinase and rabbit PSD-95 antibodies were obtained from Cell Signalling Technology (Pickering, ON, Canada). Rabbit anti-GFP antibody was obtained from Invitrogen/Life Technologies (Burlington, ON, Canada). Alexa Fluor 647 anti-mouse IgG and Alexa Fluor 633 goat anti-mouse IgG Zenon antibodies were purchased from Invitrogen/Molecular Probes

(Burlington, ON, Canada). cAMP GLO Assay was obtained from Promega (Madison, WI, USA). Mouse anti-HA antibody and all other biochemical reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada). All other biochemical reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Plasmid Constructs:

HA-CRFR1 AND HA-CRFR1- Δ TAV were described previously (Dunn et al., 2013). GFP-PSD-95 was provided by Dr. Gregory Dekaban (Robarts Research Institute). The myc-PSD-95 construct was a gift from Dr. Richard Huganir (John's Hopkins). The PSD-95 shRNA construct was kindly provided by Dr. Roger Nicoll (UCSF). The EPAC BRET-based cAMP biosensor was the gift of Drs. Ali Salahpour (University of Toronto) and Marc Caron (Duke University) (Barak et al., 2008).

Cell Culture and Transfection:

Human embryonic kidney (HEK 293) cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Cells were seeded in 10 cm dishes at 70-80% density 24 h prior to transfection. All transfections (except for the β -arrestin2 recruitment experiments) were performed using a modified calcium phosphate method, as described previously (Ferguson and Caron, 2003). 18 hours post-transfection, cells were washed with phosphate-buffered saline (PBS) and resuspended with trypsin, 0.25% EDTA. These transfections were performed with 1 μ g of each construct, with exception that 3 μ g of plasmid cDNA was used for all shRNA constructs. Transfections for BRET-based β -arrestin2-recruitment assays utilized Lipofectamine 2000 reagent with each cDNA

transfected in units of 150 ng total cDNA. Empty pcDNA3.1 vector was used to equalize the total amount of plasmid cDNA used to transfect cells. All experiments were conducted 48 hours after the initial transfection, with the exception of transfections involving PSD-95 shRNA which were conducted 72 hours after initial transfection. Primary mouse cortical cultures were prepared from E18 embryos as described previously (Ribiero et al., 2009) and were maintained in Neurobasal medium containing B27 and N2 supplements as well as pen/strep and L-glutamine. Cells were plated on poly-L-lysine coated 50 mm glass coverslips in Neurobasal media for 5 h at 37°C and 5% CO₂ in a humidified incubator to permit cell attachment. Neurobasal media was subsequently replaced with culture media that was replenished every 3 days. Culture media consisted of neurobasal media supplemented with B-27, 0.5 U/ penicillin, 0.5 µg/ streptomycin, 10 µM MK-801, 25 mM KCl and 5 pg/glia1-derived neurotrophic factor. Primary neuronal cultures were astrocyte free and transfected using Lipofectamine 2000 following the manufacturer's instructions. The University of Western Ontario Animal Care Committee approved all animal procedures.

Co-immunoprecipitation:

Transfected HEK 293 cells were seeded into 10 cm dishes the day before the experiments. Cells were serum-starved for 1 h in HBSS, and dishes were treated with either HBSS alone or with 500 nM CRF agonist in HBSS for 30 min at 37°C. Cells were subsequently lysed in lysis buffer for 20 min on a rocking platform at 4°C. Samples were collected into 1.5 mL Eppendorf tubes and centrifuged at 15,000 x g for 15 min at 4°C to pellet insoluble material. A Bronsted-Lowry protein assay was performed, and 400 µg of protein was

incubated overnight at 4 °C with protein G-sepharose and mouse anti-HA antibody (1:50). After incubation, beads were washed three times with cold lysis buffer and incubated overnight at room temperature in 3x SDS loading buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted to identify co-immunoprecipitated GFP-PSD95 (rabbit anti-GFP, 1:1000). An additional Western blot was performed to examine HA-CRFR1, HA-CRFR1- Δ TAV (mouse anti-HA, 1:1000), and GFP-PSD95 (rabbit anti-GFP, 1:1000) protein expression. For the co-immunoprecipitation of endogenous proteins from cortical extracts, adult mouse brains were employed. Tissue was dissected and homogenized on ice in lysis buffer containing protease inhibitors. The particulate fraction was removed by centrifugation, and 2 mg of supernatant protein was incubated with 5 μ L/sample of either goat polyclonal CRFR1 or CRFR2 antibody from Santa Cruz Biotechnology (Santa Cruz, CA) and protein G-Sepharose beads overnight at 4°C. The beads were then washed three times with 1% triton-X lysis buffer and proteins were eluted in 3x SDS loading buffer by warming the samples at 55 °C for 5 min. Eluted samples were subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose membranes for immunoblotting with antibodies described in the figure legends.

Immunofluorescent confocal microscopy:

HEK293 cells grown on 35 mm confocal dishes or primary mouse cortical cultures grown on round 15 mm glass cover slips were fixed for 30 min in 4% formaldehyde in PBS at 48 h post transfection with 1 μ g of either HA-CRFR1 or HA-CRFR1- Δ TAV along with GFP-PSD-95. Cells were washed twice with PBS and then incubated for 20 min at room

temperature in PBS-TB (PBS containing 0.05% Triton X-100 and 3% BSA) to permeabilize cells and block non-specific sites. Cells were then incubated in PBS-TB containing anti-HA antibody (1:1000) labelled with Zenon Alexa Fluor 647 rabbit IgG1. Cells were then washed twice with PBS and stored at 4°C until imaged. Confocal microscopy was performed on a Zeiss LSM-510 META laser scanning confocal microscope using a Zeiss 63X, 1.3 NA, oil immersion lens. HEK 293 cells in 35 mm glass bottomed dishes and neuronal cultures maintained on cover slips were imaged using dual excitation (488, 543 nm) and emission (BP 505-530 nm and LP 585 nm for GFP and AlexaFluor 546, respectively) filter sets. Specificity of labeling and absence of signal crossover were established by examination of single-labeled samples.

cAMP Signaling:

Quantification of cAMP accumulation utilized a BRET-based cAMP biosensor that has previously been described (Barak et al., 2008; Dunn et al., 2013).

ERK Phosphorylation:

Following transfection, HEK 293 cells were reseeded into 6 well plates. Cells were serum starved for 1 hour at 37°C in HBSS and then stimulated with 500 nM CRF agonist for the duration of the described time points. Cells were lysed with lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors (1mM AEBSF, 10 µg/ml leupeptin, and 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 x g for 15 minutes at 4°C to pellet insoluble material. A Bronsted-Lowry protein assay was

performed, and 50 µg of protein was incubated overnight at room temperature in 3x SDS Loading Buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted for ERK1/2 (rabbit anti-p44/42 MAPK, 1:1000), phospho-ERK1/2 (rabbit anti-phospho-p42/44 MAPK, 1:1000), PSD-95 (rabbit anti-PSD-95, 1:500), GFP-PSD-95 (rabbit anti-GFP, 1:1000) and HA-CRFR1 expression (mouse anti-HA, 1:1000), followed by a horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:5000-1:10,000) or anti-mouse antibody (1:10,000) where appropriate. GFP-PSD95 blots were separated from endogenous PSD-95 blots because of the vast overexpression of GFP-PSD95 when compared with endogenous PSD-95 levels in HEK293 cells. Proteins were detected using chemiluminescence with the enhanced chemiluminescence kit from GE Healthcare (Mississauga, ON).

Receptor Endocytosis:

Following transfection, HEK 293 cells were reseeded into 12 well plates. Cells were serum-starved for 1 h at 37°C in HBSS and then stimulated for 30 min with or without 500 nM CRF in HBSS at 37°C. Cells were washed with cold HBSS and treated with mouse anti-HA antibody (1:1000) for 45 min on ice. Cells were washed with cold HBSS and additionally treated with Alexa Fluor 647 donkey anti-mouse IgG (Invitrogen) (1:1000) for 45 min on ice. Cells were washed again with cold PBS and treated with 5mM EDTA in PBS for 5 min on ice. Newly suspended HEK 293 cells were then transferred to flow cytometry tubes containing 4% formaldehyde in PBS. Samples were run on a FACSCalibur cytometer using BD CellQuest Pro software until 10,000 cells were counted (BD Biosciences, Mississauga, ON). The geometric mean of fluorescence was determined

using FlowJo analysis software (BD Biosciences, Mississauga, ON), with less fluorescence corresponding to less CRFR1 on the membrane.

β-Arrestin2 BRET:

HEK 293 cells were transfected using Lipofectamine 2000 with 20 ng of plasmid cDNA encoding HA-CRFR1-rLuc and β-arrestin2 (βarr2)-YFP and 60 ng of either pcDNA3.1, myc-PSD-95, SCR shRNA, or PSD-95 shRNA in 96 well plates along with empty vector for a total of 150 ng of transfected cDNA. Coelenterazine, was added to each well and light emission was measured from the luciferase donor and YFP acceptor at 460 nm and 535 nm wavelengths, respectively. The BRET ratio was defined as $[(\text{emission at 535 nm}) - (\text{emission at 460 nm}) \times Cf] / (\text{emission at 460 nm})$ where Cf corresponded to $(\text{emission at 535 nm} / \text{emission at 460 nm})$ for the control, Rluc expressed alone. Cells were then treated with 500nM CRF and the BRET ratio was determined over time.

Statistical Analysis:

Densitometric data were normalized first for protein expression, and the maximum value was set to 100, with all other values displayed as the percentage thereof for ERK1/2 phosphorylation analysis. One-way analysis of variance test was performed to determine significance, followed by either a post hoc Tukey multiple comparison test or Bonferroni's multiple comparisons test to determine which means were significantly different ($p < 0.05$) from one another.

4.3 Results

PSD-95 interacts with CRFR1 via the class I PDZ-binding motif. We previously identified SAP97, which is structurally similar to PSD-95 as a novel regulator of CRFR1 (Dunn et al., 2013). We found here, that GFP-PSD-95 was co-immunoprecipitated with HA-CRFR1, but not with the truncated HA-CRFR1- Δ TAV mutant which lacked a class I PDZ-binding motif on the distal region of the carboxyl terminal tail (Fig. 4.1A). Moreover, stimulation with 500 nM CRF for 30 minutes did not lead to a significant increase in GFP-PSD-95 co-immunoprecipitated with HA-CRFR1 (Fig. 4.1B). To determine whether endogenous CRFR1 and PSD-95 interact, lysates from adult mouse cortex were incubated with either a CRFR1- or CRFR2-specific antibody for receptor immunoprecipitation on protein G sepharose beads (Fig. 4.1C). We found that endogenous PSD-95 was co-immunoprecipitated with endogenous CRFR1, but not CRFR2, which has a disrupted PDZ-binding motif (TAAV) (Fig. 4.1C). Therefore, we concluded that endogenous PSD-95 interacts with CRFR1 in PDZ-binding motif-dependent manner.

Colocalization of PSD-95 with CRFR1. To confirm the biochemical interaction between CRFR1 and PSD-95, we investigated whether the two proteins colocalized with one another when transfected into either HEK 293 cells or mouse cortical neurons. As predicted by the co-immunoprecipitation data (Fig. 4.1), GFP-PSD-95 colocalized with HA-CRFR1 at the plasma membrane in HEK 293 cells (Fig. 4.2A). In contrast, GFP-PSD-95 was not colocalized at the plasma membrane with HA-CRFR1- Δ TAV (Fig. 4.2B). Because PSD-95 is a major component and marker of the post-synaptic density (Iasevoli et al., 2013), we hypothesized that HA-CRFR1 transfected into primary mouse cortical

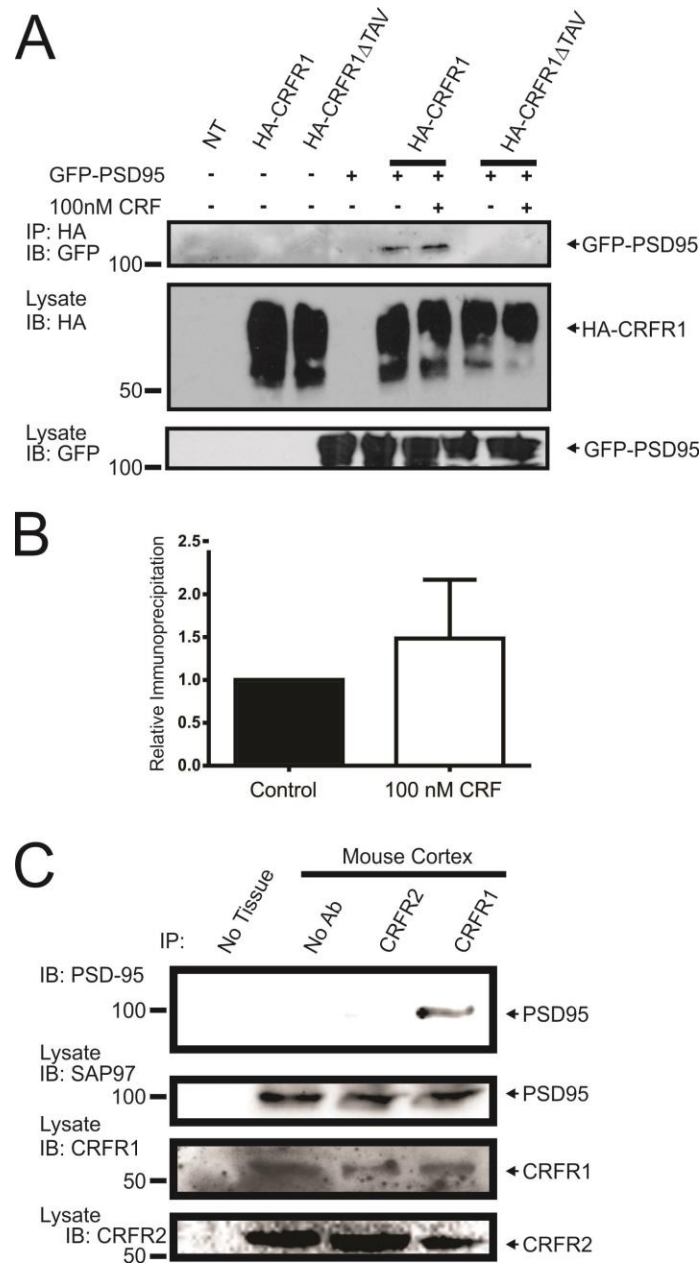


Figure 4.1: PSD-95 co-immunoprecipitates with CRFR1 in a PDZ-binding motif-dependent and agonist-independent manner. (A) Representative immunoblot of PSD-95 co-immunoprecipitated with CRFR1, but not CRFR1- Δ TAV, which lacks the PDZ-binding motif. Transient transfections were performed in HEK 293 cells as labelled. Samples were run using SDS-PAGE and immunoblotted with rabbit anti-GFP. (B) Effect of CRF treatment was quantified using densitometry and had no significant effect on the amount of GFP-PSD-95 co-immunoprecipitated with HA-CRFR1. Data represents the mean \pm SD of four independent experiments. (C) Representative immunoblot of endogenous PSD-95 co-immunoprecipitated with CRFR1 antibody, but not CRFR2 antibody, and appropriate cell lysates from adult mouse cortex. Data is representative of two independent experiments.

neurons may be targeted to the post-synaptic density via its interaction with PSD-95. Consistent with this hypothesis, HA-CRFR1 was colocalized with GFP-PSD-95 in cortical dendrites (Fig. 4.2C). However, transfected HA-CRFR1- Δ TAV was not colocalized with GFP-PSD-95, despite exhibiting punctate distribution in dendrites (Fig. 4.2D). Taken together these results suggested that the CRFR1 class I PDZ-binding motif was integral for its interaction with PSD-95 and the synaptic targeting of CRFR1.

PSD-95 does not regulate CRFR1-mediated cAMP signaling. Although PSD-95 was previously demonstrated to play a role in regulating $G\alpha_q$ -mediated IP formation (Xia et al., 2003), there are conflicting reports regarding the role of PSD-95 in the regulation of $G\alpha_s$ -mediated cAMP signaling (Hu et al., 2000; Zhang et al., 2007; Sun et al., 2009). We found that neither the overexpression of PSD-95, shRNA knockdown of endogenous PSD-95 expression nor the truncation of the CRFR1 C-tail (HA-CRFR1- Δ TAV) had a significant effect on CRFR1-mediated cAMP formation in response to increasing concentrations of CRF (Fig. 4.3A and B). Thus, our results were consistent with the idea that PSD-95 does not contribute to the regulation of $G\alpha_s$ -mediated cAMP signaling (Hu et al., 2000; Sun et al., 2009).

PSD-95 does not contribute to the regulation of CRFR1-mediated ERK1/2 signaling.

Previously, we demonstrated that SAP97, a PDZ protein that is structurally similar to PSD-95, was required for CRFR1- and 5-HT_{2A}R-stimulated ERK1/2 phosphorylation in a PDZ interaction-independent manner (Dunn et al., 2013; Dunn et al., 2014). Given the extensive sequence homology between SAP97 and PSD95, we hypothesized that PSD95 might also

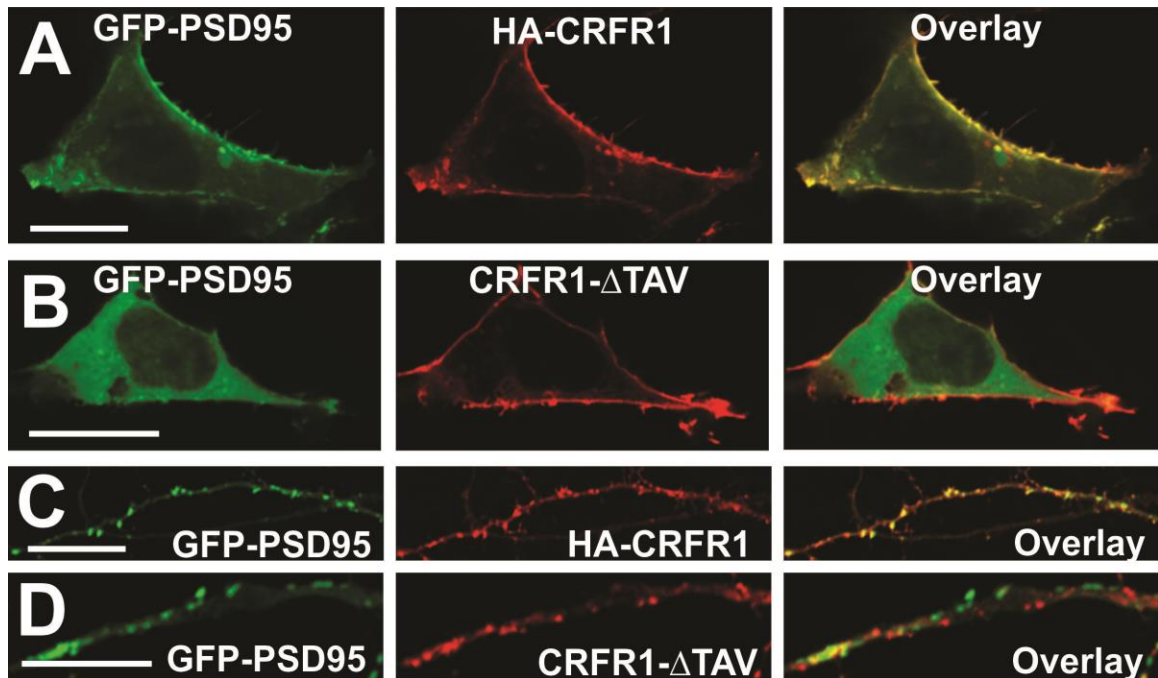


Figure 4.2: GFP-PSD-95 co-localizes at the membrane with HA-CRFR1 in a PDZ-binding motif-dependent manner. **A:** Representative confocal microscopy image demonstrating the colocalization of GFP-PSD-95 (green) and HA-CRFR1 (red) labelled with Alexa Fluor 633-conjugated secondary antibody in fixed and permeabilized HEK293 cells. **B:** Representative confocal microscopy image demonstrating the lack of colocalization of GFP-PSD-95 (green) and HA-CRFR1-ΔTAV (red) labelled with Alexa Fluor 633-conjugated secondary antibody in fixed and permeabilized HEK293 cells. **C:** Representative confocal microscopy image demonstrating the colocalization of GFP-PSD-95 (green) and HA-CRFR1 (red) labelled with Alexa Fluor 633-conjugated secondary antibody in fixed and permeabilized cultured embryonic mouse dendrites (E18). **D:** Representative confocal microscopy image demonstrating the lack of colocalization of GFP-PSD-95 (green) and HA-CRFR1-ΔTAV (red) labelled with Alexa Fluor 633-conjugated secondary antibody in fixed and permeabilized cultured embryonic mouse dendrites (E18). Images are representative of 4 independent experiments.

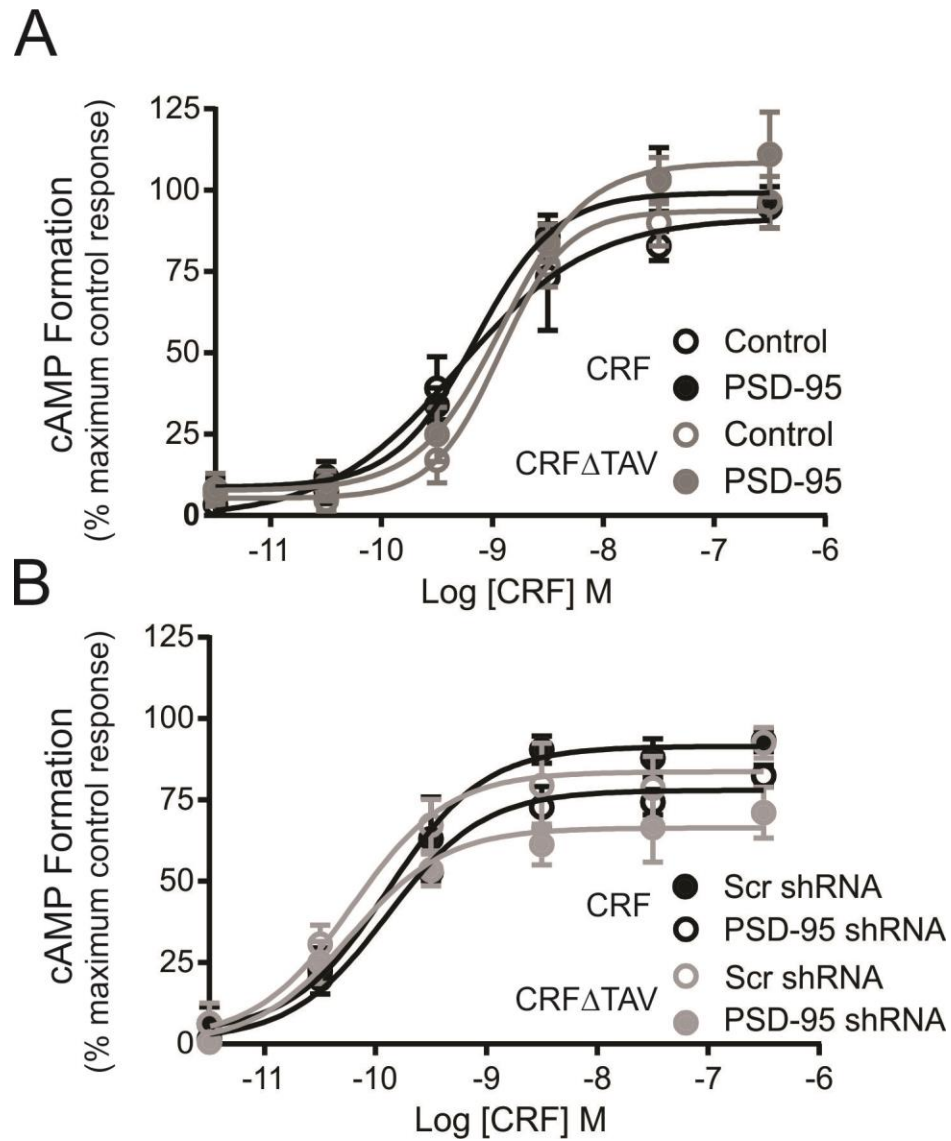


Figure 4.3: PSD-95 does not regulate CRFR1-mediated cAMP formation. (A) HA-CRFR1-mediated and HA-CRFR1- Δ TAV-mediated cAMP formation, as assessed by BRET-based cAMP biosensor (protocol adapted from Barak *et al.*, 2008.), following co-transfection with either pcDNA3.1 (control) or myc-PSD-95. The data represent the mean \pm SEM of four independent experiments. (B) HA-CRFR1-mediated and HA-CRFR1- Δ TAV-mediated cAMP formation following co-transfection with either scrambled shRNA (control) or PSD95 shRNA. The data represent the mean \pm SEM of four independent experiments.

be essential for CRFR1-mediated activation of ERK1/2 phosphorylation. However, we did not find that GFP-PSD-95 overexpression or shRNA knockdown of endogenous PSD-95 expression altered either HA-CRFR1- or HA-CRFR1- Δ TAV-stimulated ERK1/2 phosphorylation (Fig. 4.4A and B).

PSD-95 regulates CRFR1 endocytosis. PSD-95 was previously shown to inhibit β_1 -adrenergic receptor (β_1 AR) and 5-HT_{2A}AR endocytosis (Hu *et al.*, 2000; Xia *et al.*, 2003; He *et al.*, 2004), and its structurally related homologue SAP97 functioned to attenuate both CRFR1 and 5-HT_{2A}AR internalization (Dunn *et al.*, 2013; Dunn *et al.*, 2014). Therefore, we assessed whether PSD-95 was involved in the regulation of CRFR1 endocytosis. We found that overexpression of GFP-PSD-95 reduced CRF-stimulated HA-CRFR1 internalization and shRNA knockdown of endogenous PSD-95 expression increased HA-CRFR1 endocytosis (Fig. 4.5). The truncation of the CRFR1 C-tail attenuated HA-CRFR1 internalization and prevented PSD-95-dependent regulation of HA-CRFR1 internalization (Fig. 4.5). Taken together, these data provided further evidence for the importance of PDZ protein interactions in the endocytosis and trafficking of GPCRs.

PSD-95 competes for β -arrestin recruitment to CRFR1. We previously demonstrated that CRFR1 internalization was β -arrestin-dependent (Holmes *et al.*, 2006), and it was previously shown that GRK5-mediated phosphorylation of the β_1 AR prevented PSD-95 binding to the receptor (Hu *et al.*, 2002). Therefore, we hypothesized that PSD-95 interactions with CRFR1 might antagonize β -arrestin interactions with the CRFR1. When tested, we found that overexpression of myc-PSD-95 attenuated CRF-stimulated

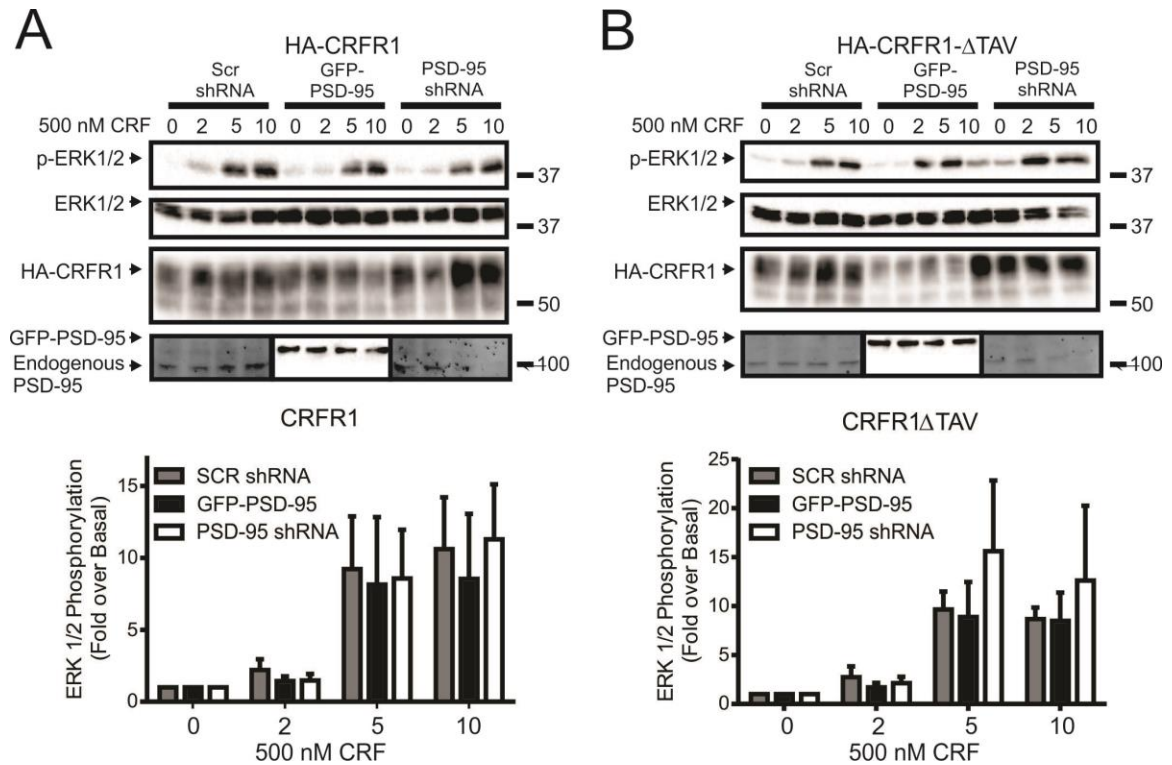


Figure 4.4: PSD-95 has no effect on CRFR1- or CRFR1-ΔTAV-mediated ERK1/2 phosphorylation.

(A) Representative immunoblot showing ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 2, 5, and 10 min in HEK 293 cells transfected with HA-CRFR1 and either scrambled shRNA, GFP-PSD-95, or PSD95 shRNA. Also shown is the total expression of ERK1/2, HA-CRFR1, GFP-PSD-95 and endogenous PSD-95. Densitometric analysis of ERK1/2 phosphorylation represents the mean \pm SEM of four independent experiments. (B) Representative immunoblot showing ERK1/2 phosphorylation in response to 500 nM CRF treatment for 0, 2, 5, and 10 min in HEK 293 cells transfected with HA-CRFR1-ΔTAV and either scrambled shRNA, GFP-PSD-95, or PSD95 shRNA. Also shown is the total expression of ERK1/2, HA-CRFR1, GFP-PSD-95 and endogenous PSD-95. Densitometric analysis of ERK1/2 phosphorylation represents the mean \pm SEM of four independent experiments.

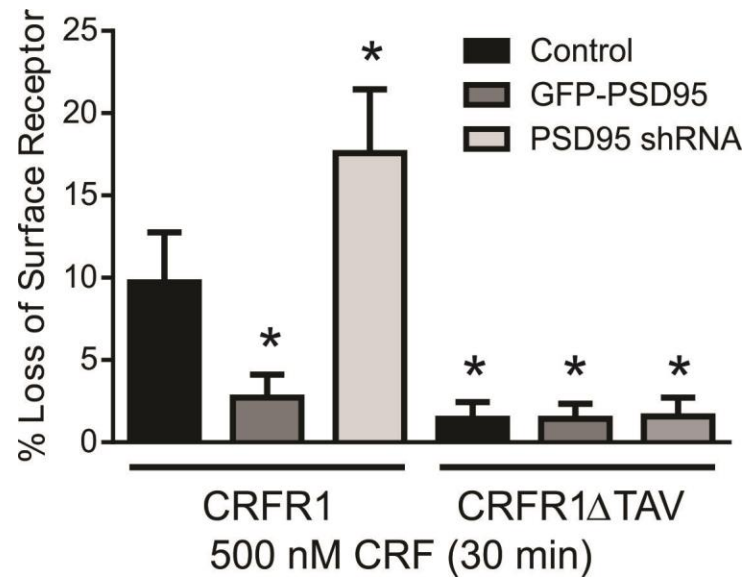


Figure 4.5: Role of PSD-95 on CRFR1 internalization. Agonist-stimulated internalization of HA-CRFR1 or HA-CRFR1ΔTAV in cells co-transfected with either control plasmid, GFP-PSD-95, or PSD-95 shRNA. The internalization of HA-tagged receptors labelled with Alexa Fluor 647-conjugated mouse anti-HA antibody was measured in cells treated with 500 nM CRF for 30 minutes and compared with vehicle treated control cells. The data represents the mean \pm SD of 6 independent experiments. * $p < 0.05$ versus control.

β -arrestin2 recruitment to HA-CRFR1 (Fig. 4.6A), and that shRNA knockdown increased β -arrestin2 recruitment to HA-CRFR1 (Fig. 4.6B). Thus, it appeared that PSD-95 antagonized CRFR1 internalization by preventing β -arrestin interactions required for the endocytosis of the receptor.

PSD-95 is not independently involved in CRFR1-mediated sensitization of 5-HT_{2A}R signaling. Previously, we demonstrated that CRFR1 preactivation sensitized 5-HT_{2A}R-stimulated IP formation via a mechanism that required intact CRFR1 and 5-HT_{2A}R PDZ binding motifs (Magalhaes et al., 2010). Therefore, we tested the effect of PSD-95 shRNA-mediated knockdown on CRFR1-stimulated enhancement of 5-HT_{2A}R signaling. In HEK 293 cells transfected with CRFR1, 5-HT_{2A}R and scrambled shRNA, pretreatment with 500 nM CRF for 30 min resulted in an increase in the maximum efficacy for 5-HT-stimulated IP formation, when compared to cells that were not CRF pretreated (Fig. 4.7). However, PSD-95 shRNA down-regulation of PSD-95 expression did not block the CRF pretreatment-induced increase in the maximum efficacy for 5-HT-stimulated IP formation in CRFR1 and 5-HT_{2A}R expressing cells (Fig. 7). Taken together, these results indicated that PSD-95 does not independently contribute to the heterologous sensitization of 5-HT_{2A}R signaling.

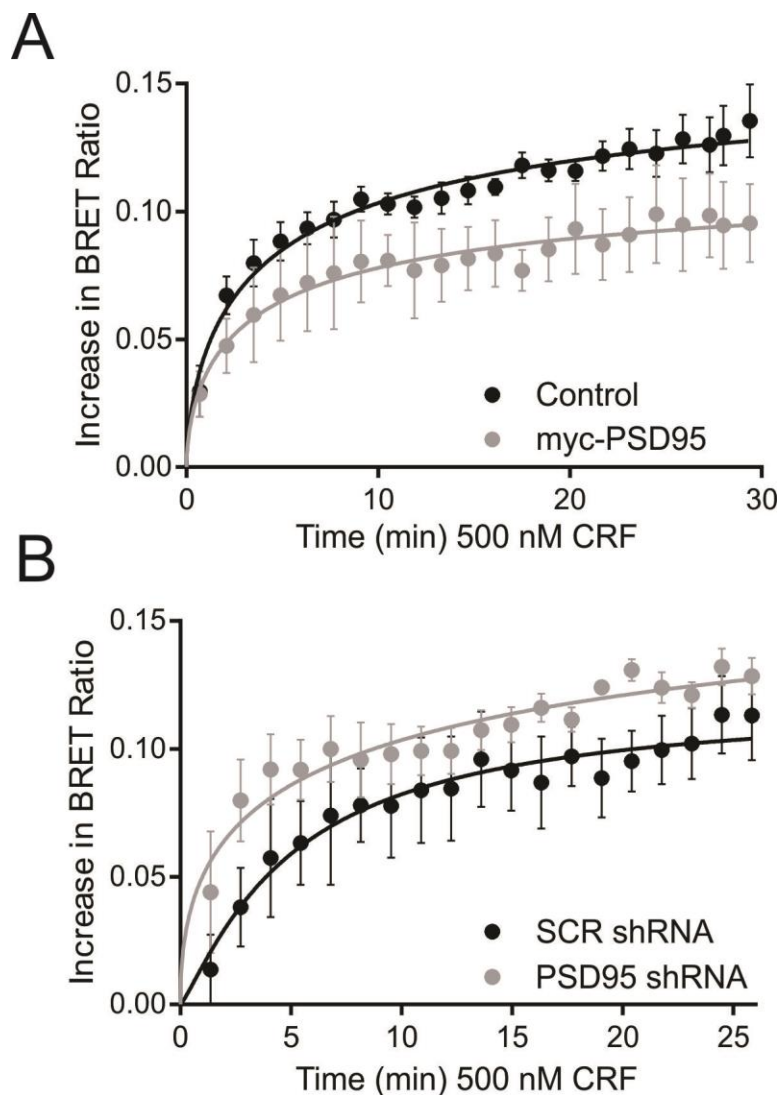


Figure 4.6: PSD-95 antagonizes β -arrestin2 recruitment to CRFR1. (A) Bioluminescent Resonance Energy Transfer (BRET) was employed to quantify the recruitment of β -arrestin2 to CRFR1 with control vector or myc-PSD95. An initial BRET ratio was calculated followed by 500nM CRF stimulation for the time shown in Figure. BRET ratios were repeatedly calculated following CRF stimulation and plotted over time. BRET ratios are expressed as values above the prestimulated basal reading. The data are representative of the mean \pm SD of 4 independent experiments, respectively. (B) BRET readings corresponding to the recruitment of β -arrestin2 to CRFR1 with control shRNA or PSD-95 shRNA. An initial BRET ratio was calculated followed by 500nM CRF stimulation. BRET ratios are expressed as values above the prestimulated basal reading. The data are representative of the mean \pm SD of 4 independent experiments.

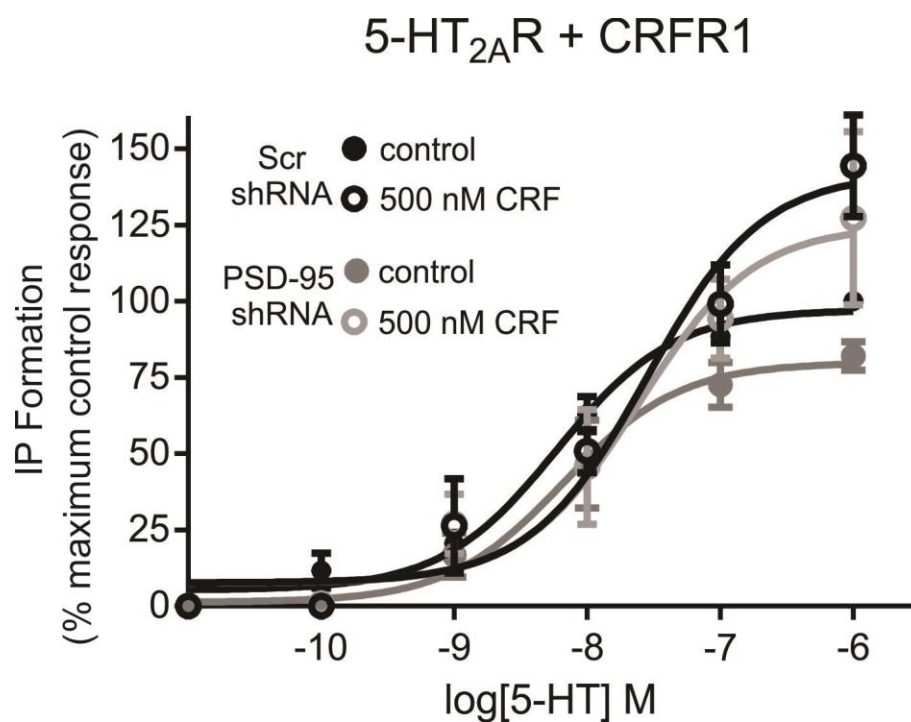


Figure 4.7: PSD-95 shRNA knockdown is insufficient to prevent CRFR1-mediated enhancement of 5-HT_{2A}R-mediated signaling. 5-HT stimulated dose response curves for inositol phosphate (IP) formation in HEK 293 cells that are transfected with 1 μ g of plasmid cDNA encoding HA-CRFR1 and 1 μ g of plasmid cDNA encoding FL-5-HT_{2A}R along with 3 μ g of plasmid cDNA encoding either scrambled shRNA (SCR) or PSD-95 shRNA. Transfected HEK 293 cells were treated either with or without 500 nM CRF for 30 min prior to being treated with increasing concentrations of 5-HT for 30 min. The data represent the mean \pm SEM of 4 independent experiments.

4.4 Discussion

We find here that PSD-95, like SAP97 is a MAGUK family PDZ protein that interacts with CRFR1 in the brain and regulates CRFR1 trafficking, but not CRFR1-mediated ERK1/2 signalling (Dunn et al., 2013). Thus, PSD-95 is a second documented CRFR1-interacting PDZ protein that contributes to the regulation of CRFR1 activity (Dunn et al., 2013). Our interest in the interaction of PDZ proteins with CRFR1 stems from our previous observation that intact PDZ binding motifs are required for CRFR1-mediated heterologous sensitization of 5-HT_{2A}R signaling in response to CRF pretreatment (Magalhaes et al., 2010). Although PSD-95 on its own does not appear to be the PDZ protein that is required for CRFR1-mediated sensitization of 5-HT_{2A}R signaling, it does contribute to the regulation of CRFR1 endocytosis. The mechanism by which PSD-95 attenuates CRFR1 endocytosis appears to involve its competition with β -arrestin proteins for receptor binding.

Although the regulatory activity of PSD-95 appears to differ depending upon the GPCRs examined, PSD-95 appears to regulate CRFR1 and β_1 AR activity in a similar manner (Hu et al., 2000; Xu et al., 2001; Xia et al., 2003; Gavarini et al., 2006; Zhang et al., 2007; Sun et al., 2009). In the case of both receptors, PSD-95 antagonizes receptor internalization, but does not alter G_{α_s} -mediated cAMP signaling (Hu et al., 2000). The antagonism of endocytosis appears to be a common feature of PSD-95 activity as it also regulates the internalization of the 5-HT_{2A}R (Hu *et al.*, 2000; Xia *et al.*, 2003; He *et al.*, 2004). Here, we provide evidence that the PSD-95 functions to antagonize the recruitment of β -arrestin2 to CRFR1. This provides a mechanism by which PSD-95 overexpression antagonizes CRFR1 internalization and PSD-95 knockdown increases CRFR1

internalization in response to agonist treatment. In agreement with this hypothesis, it has been reported that the recruitment of β -arrestin2 to 5-HT_{2A}R in mouse frontal cortex corresponds with the concomitant decrease in PSD-95 association with 5-HT_{2A}R (Schmid et al., 2010). Interestingly, the fact that GRK5 phosphorylation, which often precedes β -arrestin recruitment, disrupts PSD-95 interactions with the β_1 AR is also consistent with a PSD-95/ β -arrestin competition model (Hu et al., 2002). Taken together, it is plausible that PSD-95 may antagonize the internalization of any GPCR that encodes a class I PDZ-binding motif at the end of its C-tail by inhibiting β -arrestin2 recruitment.

We previously showed that SAP97, a MAGUK family PDZ protein, plays an essential role in regulating both CRFR1- and 5-HT_{2A}R ERK1/2 signaling, but that this occurs via a mechanism that was independent of receptor PDZ motif interactions (Dunn et al., 2013; Dunn et al., 2014). Because both SAP97 and PSD-95 share extensive sequence homology and are both substrates for ERK1/2 phosphorylation, we expected that PSD-95 would also contribute to the regulation of ERK1/2 activity (Sabio et al., 2004; Sabio et al., 2005; Cai et al., 2006). However, we found shRNA knockdown of PSD-95 in HEK 293 cells did not alter CRFR1-stimulated activation of ERK1/2 phosphorylation, suggesting that the regulation of ERK1/2 signaling was unique to SAP97. Although PSD-95 and SAP97 share sequence homology, SAP97 differed from PSD-95 in that it encodes an amino terminal L27 domain. Thus, it was possible that this L27 domain may have coupled SAP97 to a MAPK signaling complex that was integral for ERK1/2 phosphorylation, whereas PSD-95 would not associate with this complex. Future structure-function studies will be required in order to investigate the potential contribution of the SAP97 L27 domain to GPCR-mediated activation of ERK1/2 signaling.

In a previous study, we demonstrated a heterologous sensitization mechanism whereby CRFR1 is capable of enhancing 5-HT_{2A}R-mediated IP formation via a mechanism that requires receptor endocytosis and recycling as well as intact receptor PDZ-binding motifs (Magalhaes et al., 2010). This prompted us to screen a PDZ protein array for potential PDZ proteins that interact with both the CRFR1 and 5-HT_{2A}R C-tails and PSD-95 and SAP97 were identified as potential interacting proteins (Dunn et al., 2013). We have demonstrated that both PSD-95 and SAP97 interact with endogenous CRFR1 in brain homogenates and with overexpressed receptor in HEK 293 cells, but were unable to show that they are required for the heterologous sensitization of 5-HT_{2A}R signaling (Dunn et al., 2014). This is despite the fact that SAP97 appears to enhance G α_q -mediated signaling by the 5-HT_{2A}R (Dunn et al., 2014). A previous study has also demonstrated that the association of PSD95 with 5-HT_{2A}R enhanced 5-HT_{2A}R signal transduction (Xia et al., 2003). Thus, both SAP97 and PSD-95 facilitate 5-HT_{2A}R G α_q -mediated signaling by an unknown mechanism, but do not appear to be exclusively involved in the CRFR1-mediated sensitization of 5-HT_{2A}R-mediated signaling. Thus alternative CRFR1- and 5-HT_{2A}R-interacting proteins will have to be investigated.

Developing new strategies to manipulate CRFR1 function is of increasing importance as CRFR1 has been identified as a potential pharmacological target for the treatment of mood disorders and psychiatric disease (Overstreet & Griebel, 2004; Chaki et al., 2004). Interestingly, PSD-95 has previously been linked the psychiatric disorder schizophrenia (Ohnuma et al., 2000; Kristiansen et al., 2006), and the efficacy of atypical antipsychotics has demonstrated a dependence on the endogenous presence of PSD-95 (Abbas et al., 2009). Thus, PSD-95 is directly implicated as an important molecule

involved in mood regulation. In the current study, we have identified PSD-95 as a novel interacting partner of CRFR1 in the cerebral cortex that is capable of inhibiting CRFR1 internalization by antagonizing β -arrestin2 recruitment. Thus, it is possible that targeting of PSD-95 interactions with GPCRs may function to alter β -arrestin-mediated signaling downstream of GPCRs that encode PDZ binding motif and/or enhance their resensitization by increasing their rate of endocytosis.

4.5 References

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Chapter 5: Discussion

5.1 Summary

In the previous three chapters, the role of PDZ domain-containing proteins SAP97 and PSD95 in regulating GPCRs such as CRFR1 or 5-HT_{2A}R has been thoroughly documented. Because PSD-95 was already demonstrated to regulate 5-HT_{2A}R function (Xia et al., 2003), our objectives were as follows:

1. Evaluate whether SAP97 regulates CRFR1 signalling and trafficking
2. Evaluate whether SAP97 regulates 5-HT_{2A}R signalling and trafficking
 - Determine whether SAP97 is responsible for CRFR1-mediated sensitization of 5-HT_{2A}R signalling
3. Evaluate whether PSD-95 regulates CRFR1 signalling and trafficking
 - Determine whether PSD-95 is responsible for CRFR1-mediated sensitization of 5-HT_{2A}R signalling

In Chapter 2, SAP97 was identified as a novel interacting partner of CRFR1 in the brain capable of maintaining receptor surface expression following agonist activation by inhibiting receptor endocytosis (Dunn et al., 2013). Both the interaction and functional consequence of suppressing internalization were found to be dependent on the CRFR1 PDZ-binding motif on the distal tip of the carboxyl terminal tail (Dunn et al., 2013). Although this interaction had no effect on CRF-induced cAMP accumulation, the endogenous expression of SAP97 was integral for CRF-mediated ERK1/2 phosphorylation in HEK293 cells and pituitary adenoma AtT20 cells (Dunn et al., 2013). Interestingly, this

effect occurred independently of SAP97 interactions with receptor, as SAP97 knockdown reduced the phosphorylation of ERK1/2 by both a truncated CRFR1 Δ TAV and CRFR2 which lack type I PDZ-binding motifs (Dunn et al., 2013).

In Chapter 3, SAP97 was further demonstrated as an interacting partner of 5-HT_{2A}R in the brain and this interaction – via the CRFR1 PDZ-binding motif – was similarly capable of suppressing receptor endocytosis (Dunn et al., 2014). The endogenous expression of SAP97 in HEK293 cells was integral for 5HT-induced ERK1/2 phosphorylation via either 5-HT_{2A}R or the truncated 5-HT_{2A}R Δ SCV, which lacks the PDZ-binding motif on the distal tail of the receptor (Dunn et al., 2014). Interestingly, SAP97 did not appear to have a significant effect on CRF-induced cAMP accumulation; however, endogenous SAP97 expression and an intact 5-HT_{2A}R PDZ-binding motif were essential for maximal 5-HT-induced IP₃ signalling (Dunn et al., 2013; Dunn et al., 2014). Finally, knockdown of endogenous SAP97 was insufficient to abolish the previously described mechanism whereby CRF-pretreatment provides an enhancement of 5-HT_{2A}R-mediated IP₃ signalling (Magalhaes et al., 2010; Dunn et al., 2014). This suggested that SAP97 is either not involved, or not exclusively involved in the crosstalk between these two receptors.

In Chapter 4, the structurally similar PSD-95 was identified as a novel interacting partner of CRFR1 in the brain (Dunn et al., submitted). This interaction was further demonstrated by colocalization of CRFR1 with PSD95 in dendritic puncta in cultured rat neurons, and both the biochemical interaction and this colocalization was found to be dependent on an intact PDZ-binding motif (Dunn et al., submitted). Like SAP97, PSD-95 was demonstrated to play an inhibitory role in CRFR1 endocytosis and had no effect on

the truncated CRFR1 Δ TAV mutant that lacks the PDZ-binding motif interaction site (Dunn et al., 2013; Dunn et al., submitted). Interestingly, PSD-95 did not seem to play a significant role in either CRF-mediated cAMP signalling or ERK1/2 phosphorylation; however, PSD-95 appears to compete with β -arrestin2 binding to CRFR1, thereby providing a potential mechanism for PSD-95's ability to suppress receptor internalization.

5.2 PSD-95 and SAP97 in the Regulation of GPCR Trafficking

In the aforementioned chapters, SAP97 was demonstrated to suppress CRFR1 and 5-HT_{2A}R internalization (Dunn et al., 2013; Dunn et al., 2014). Similarly, the homologue PSD-95 was demonstrated to suppress CRFR1 internalization, and a previous study by another group has demonstrated that PSD-95 can also inhibit 5-HT_{2A}R internalization (Dunn et al., submitted; Xia et al., 2003). In this previous study, HEK293 cells transiently transfected with 5-HT_{2A}R and either empty plasmid or PSD-95 were treated with vehicle or 5-HT and examined using immunofluorescent confocal microscopy (Xia et al., 2003). Cells were then analyzed qualitatively to determine whether 5-HT_{2A}R internalization occurred, as characterized by the appearance of 5-HT_{2A}R-positive cytosolic vesicles following stimulation (Xia et al., 2003). In our studies, we attempted a more quantitative approach using surface immunofluorescent labeling of receptors for flow cytometry and arrived at a complimentary conclusion: overexpression of PSD-95 led to decreased CRFR1-endocytosis, and overexpression of SAP97 led to decreased CRFR1- and 5-HT_{2A}R-endocytosis (Dunn et al., 2013; Dunn et al., 2014; Dunn et al., submitted). Furthermore, we were able to knockdown endogenous SAP97 or PSD-95 in HEK293 cells and, in every case, receptor internalization was enhanced (Dunn et al., 2013; Dunn et al.,

2014; Dunn et al., submitted). Taken together, it is clear that SAP97 and PSD-95 work to maintain CRFR1 and 5-HT_{2A}R on the cell surface by inhibiting receptor internalization.

It is noteworthy that amongst the three independent studies presented in this thesis, truncation of the CRFR1 or 5-HT_{2A}R PDZ-binding motif led to dysfunctional receptor internalization. This is not the first time the PDZ-binding motif has been implicated as an essential motif for GPCR trafficking, as it was first identified as being essential for β_2 AR recycling (Cao et al., 1999). Since this study, many GPCRs have been identified to be regulated by PDZ domain-containing proteins via the receptor PDZ-binding motif (Magalhaes et al., 2012). In regards to PSD-95's regulation of GPCRs, the majority of studies have demonstrated PSD-95 to influence some aspect of GPCR trafficking via its interaction with the PDZ-binding motif (Magalhaes et al., 2012). PSD-95 appears to suppress the agonist-induced internalization of 5-HT_{2A}R, β_1 AR, and most recently CRFR1 (Xia et al., 2003; Hu et al., 2000; Dunn et al., submitted). Conversely, PSD-95 may promote constitutive internalization of 5-HT_{2C}R and D₁R (Gavarini et al., 2006; Zhang et al., 2007). A more recent study has now demonstrated that PSD-95 promotes the recycling of D₁R to the membrane (Sun et al., 2009). Prior to the current studies, SAP97 had similarly been identified to promote β_1 AR recycling (Gardner et al., 2007). Now it has been suggested that SAP97 can inhibit the internalization of CRFR1 and 5-HT_{2A}R (Dunn et al., 2013; Dunn et al., 2014). Although there are some discrepancies, the majority of the work on PSD-95 and SAP97 conclude that these proteins promote the membrane localization of GPCRs by either inhibiting receptor endocytosis or promoting receptor recycling. However, it remains plausible that the effects of these proteins on GPCR

trafficking may be specific to the GPCR in question, and the complimentary trafficking machinery available to a specific cell type.

5.2.1 PSD-95 Regulation of β -arrestin Recruitment

A potential mechanism whereby PSD-95, and foreseeably SAP97, may regulate GPCR endocytosis may involve modulating the recruitment β -arrestins. In Chapter 4, we demonstrated that overexpression of PSD-95 overexpression in HEK293 cells led to decreased β -arrestin2 recruitment to CRFR1 and decreased receptor internalization, whereas PSD-95 shRNA knockdown led to enhanced β -arrestin2 recruitment and increased CRFR1 internalization (Dunn et al, submitted). It is plausible that PSD-95 may prevent the recruitment of β -arrestins to a subset of GPCRs, thereby inhibiting β -arrestin-dependent receptor endocytosis. This hypothesis is supported by a previous study investigating the recruitment of β -arrestin2 to 5-HT_{2A}R (Schmid and Bohn, 2010). In this study, stimulation of 5-HT_{2A}R by an agonist led to the recruitment of β -arrestin2 and the dissociation of PSD-95 away from the receptor; however, PSD-95 did not dissociate in β -arrestin2 knockout mice (Schmid and Bohn, 2010). Together with the current study on CRFR1, it is plausible that PSD-95 and β -arrestins could compete for binding to certain GPCRs to influence the trafficking fate of the receptor, whereby PSD-95 would promote membrane localization and stabilization of the receptor, and β -arrestins would initiate the endocytotic process.

5.3 PSD-95 and SAP97 in the Regulation of GPCR-Mediated Signalling

In the current studies, we were unable to find a role for PSD-95 or SAP97 in the CRFR1-mediated activation of G α s, as measured by the accumulation of secondary messenger cAMP (Dunn et al., 2013; Dunn et al., submitted). In comparison, the β_1 AR shares a similar class I PDZ-binding motif to CRFR1 and neither PSD-95 nor other PDZ-domain-containing proteins MAGI-2 or GIPC demonstrated any significant role in β_1 AR-mediated cAMP signalling (Hu et al., 2000; Xu et al., 2001; Hu et al., 2003). Although PSD-95 was previously reported to inhibit D $_1$ R-mediated cAMP signaling, a more recent study has suggested that PSD-95 has no significant effect in D $_1$ R-mediated G α s activation or cAMP accumulation (Zhang et al., 2007; Sun et al., 2009). Apart from these studies, there have been some documentations of other PDZ-domain containing proteins having a regulatory role over GPCR-mediated signaling via G α s. However, few consistencies have been found between different receptors, and conflicting studies exist (Magalhaes et al., 2012). One piece of evidence suggesting minimal effect of PDZ interactions on cAMP signalling comes from Chapters 2 and 4 (Dunn et al., 2013; Dunn et al., submitted). When examining CRF-mediated cAMP accumulation, there was no significant differences observed between wild-type CRFR1 and the truncated CRFR1 Δ TAV mutant which lacks the PDZ-binding motif required for prototypical interactions with PDZ domain-containing proteins (Dunn et al., 2013; Dunn et al., submitted). If PDZ domain-containing proteins were important for CRF-mediated cAMP signalling, one might expect that simply truncating the PDZ-binding motif, thereby abolishing the ability of most PDZ domain-containing proteins to interact with the receptor, would have some effect on the

accumulation of cAMP. With all of the evidence considered, we conclude that neither PSD-95 nor SAP97 appear to play a significant, generalized role in GPCR-mediated cAMP signaling, and this may be consistent amongst other members of the PDZ domain-containing protein family.

Interestingly, we did observe a significant role for SAP97 in regulating 5-HT_{2A}R-mediated Gαq activation, as measured by the accumulation of the secondary messenger IP₃ (Dunn et al., 2014). This is supported by a previous study which demonstrated that overexpression of PSD-95 led to enhanced maximum efficacy of 5-HT_{2A}R-mediated IP₃ signalling (Xia et al., 2003). Although overexpression of SAP97 resulted in a negligible shift to the left in our dose-response curve, the knockdown of endogenous SAP97 led to a significant decrease in the maximum efficacy for IP₃ accumulation (Dunn et al., 2014). Furthermore, the truncated 5-HT_{2A}RΔSCV mutant which lacks the PDZ-binding motif showed a similar decrease in the maximum efficacy for IP₃ accumulation when compared to the wild-type 5-HT_{2A}R (Dunn et al., 2014). This result reinforces the importance of PSD-95, SAP97, and perhaps other PDZ domain-containing proteins in the promotion of 5-HT_{2A}R-mediated signalling via Gαq. Other PDZ domain-containing proteins that have been implicated in regulating Gαq signalling include NHERF1, which was demonstrated to increase coupling and activation of Gαq with parathyroid 1 receptor (PTH1R), and NHERF2 which increased secondary messenger IP₃ accumulation via LPA2R (Wang et al., 2010; Oh et al., 2004). Additionally, NHERF2 was shown to increase coupling of Gαq to PTH1R, while decreasing PTH1R coupling with Gas (Wang et al., 2010). In regards to PSD-95 and SAP97, it appears that these proteins are important for Gαq-coupled signalling via 5-HT_{2A}R, but not Gas-coupled signalling via CRFR1 (Xia et al., 2003; Dunn et al.,

2014; Dunn et al., 2013; Dunn et al., submitted). It is possible that PSD-95 and SAP97 may act similarly to NHERF2 in biasing GPCRs towards G α q-coupling while, in this case, providing minimal influence over G α s-coupling.

5.3.1 SAP97 in the Regulation of GPCR-Mediated ERK1/2 Signalling

A consistent and novel finding amongst the enclosed studies was the observation that SAP97 appears to be an integral protein for ERK1/2 phosphorylation (Dunn et al., 2013; Dunn et al., 2014). When using SAP97 shRNA in HEK293 cells, both CRFR1- and 5-HT_{2A}R-mediated ERK1/2 phosphorylation was significantly reduced when compared to control with scrambled shRNA (Dunn et al., 2013; Dunn et al., 2014). Additionally, using SAP97 siRNA in AtT20 cells, which endogenously express CRFR1, also led to a significant decrease in CRF-mediated ERK1/2 phosphorylation (Dunn et al., 2013). Surprisingly, SAP97 shRNA knockdown in HEK293 cells also led to significant inhibition of ERK1/2 phosphorylation via CRFR2, and via truncated mutants CRFR1 Δ TAV and 5-HT_{2A}R Δ SCV: all of which lack the class I PDZ-binding motif which is fundamental for SAP97's interaction (Dunn et al., 2013; Dunn et al., 2014). This unexpected finding suggests that SAP97's role in CRF- and 5-HT_{2A}R-mediated ERK1/2 phosphorylation goes beyond its interaction with the receptor and may indicate that SAP97 is an integral protein in MAPK signalling as a whole. Albeit beyond the scope of the current studies, SAP97's potential role in regulating general ERK1/2 phosphorylation is an exciting finding as MAPK signalling is fundamental in modulating cellular growth, proliferation, and apoptosis and understanding its regulation is relevant for the development of new cancer

treatments (Roberts and Der, 2007). If this hypothesis holds true, it may provide some reasoning behind why insertional mutations of the SAP97-encoding gene in transgenic mice exhibit growth retardation *in utero* and perinatal lethality (Caruana and Bernstein, 2001).

Although this is the first indication of SAP97 as an integral protein for GPCR-mediated ERK1/2 phosphorylation, previous studies have demonstrated proteins of the MAPK signalling pathway, like ERK2, to be capable of phosphorylating SAP97 (Sabio et al., 2005). As a consequence of this phosphorylation, SAP97 becomes disconnected from GKAP protein which indirectly links SAP97 to the cytoskeleton (Sabio et al., 2005). Taken together with the current studies, it is foreseeable that SAP97 could maintain CRFR1, 5-HT_{2A}R, and perhaps other GPCRs at the cell membrane to allow for agonist activation and the propagation of MAPK signalling, but also provide an unknown role in ERK1/2 phosphorylation independent of receptor interactions. One potential function could be scaffolding MAPK family members in close proximity similarly to what has been shown for β -arrestins (Tohgo et al., 2002). Upon ERK1/2 phosphorylation and activation, ERK1/2 or another MAPK family kinase could phosphorylate SAP97, thereby interrupting its interaction with the cytoskeleton and encouraging the trafficking or sorting of SAP97 and its bound receptor(s). This could hypothetically lead to receptor internalization and the preclusion of further agonist activation and MAPK signalling, consequently acting as a negative feedback mechanism for MAPK signalling. Future studies could examine what role SAP97 plays in regulating MAPK signalling and the potential interplay between ERK1/2 and SAP97.

Despite extensive structural homology between SAP97 and PSD-95, PSD-95 shRNA knockdown in HEK293 cells had no significant effect on CRFR1-mediated ERK1/2 phosphorylation in our study (Dunn et al., submitted). This could have multiple explanations. Firstly, it could be that SAP97 is exclusively involved in ERK1/2 phosphorylation when compared to PSD-95. The most obvious structural difference of SAP97 when compared to PSD-95 is the inclusion of an L27 protein-protein interaction domain on the distal amino terminal. It is possible that this L27 domain is essential for targeting SAP97 to the MAPK signalling pathway and perhaps providing a means for scaffolding these kinases in close proximity. Secondly, it is possible that the endogenous expression of SAP97 was capable of compensating for the loss of PSD-95, therefore no significant decrease in ERK1/2 phosphorylation was observed. This is supported by the observation that some PDZ domain-containing proteins can exhibit functional compensation as a consequence of downregulating PDZ domain-containing proteins of similar background (Misawa et al., 2001). Thirdly, the function of these PDZ domain-containing proteins may be cell-type-specific and dependent upon the complement of MAPK signalling proteins available. As we have only tested PSD-95's role on MAPK signalling in HEK293, other cell types may be considered.

5.4 Crosstalk between CRFR1 and 5-HT_{2A}R

The initial reasoning for investigating the potential for PDZ domain-containing proteins in the regulation of CRFR1 and 5-HT_{2A}R originated following the observation that preactivation of CRFR1 was capable of enhancing 5-HT_{2A}R-mediated IP₃ signalling,

and that this result occurs dependently upon both receptors containing intact PDZ-binding motifs (Magalhaes et al., 2010). This unidirectional relationship between CRFR1 and 5-HT_{2A}R has been proposed to be a potential molecular mechanism underlying stress-sensitive mood disorders such as anxiety disorders and depression (Magalhaes et al., 2010). We went on to perform a PDZ-binding array using the PDZ-binding motifs of CRFR1 and 5-HT_{2A}R with a subset of peptides containing known PDZ domains in search of a potential protein, or proteins, which may facilitate this crosstalk (Dunn et al., 2013). In doing so, we identified SAP97, PSD-95, MAGI1, MAGI2, MAGI3, CAL, PDZK1, MUPP1 and PTPN13 as possible candidates for regulating the crosstalk between CRFR1 and 5-HT_{2A}R, as well as each receptor's independent function (Dunn et al., 2013). The current studies focused on SAP97 and PSD-95.

Despite the shRNA knockdown of either SAP97 or PSD-95 in HEK293 cells, the CRFR1-mediated enhancement of 5-HT_{2A}R-mediated IP₃ signalling was still observed (Dunn et al., 2014; Dunn et al., submitted). This result has a few possible explanations. Firstly, it is possible that neither SAP97 nor PSD-95 participate in the CRFR1-mediated enhancement of 5-HT_{2A}R-mediated IP₃ signalling. In this case, we still have MAGI1, MAGI2, MAGI3, CAL, PDZK1, MUPP1 and PTPN13 to consider for future studies (Dunn et al., 2013). Secondly, it could be that the shRNA-mediated knockdown of PSD-95 and/or SAP97 via transient transfection wasn't sufficiently complete to abolish the receptor crosstalk. In this case, targeted knockout-animals may need to be explored. Thirdly, it could be that SAP97 and/or PSD-95 participate in the CRFR1-mediated enhancement of 5-HT_{2A}R signalling, but they are not exclusively involved. In this case, knocking down

one of these proteins could allow for another PDZ domain-containing protein to compensate for its loss.

Such a compensation mechanism amongst PDZ domain-containing proteins has been previously described in double-knockout mice for synaptic proteins MALS1 and MALS2 (Misawa et al., 2001). Interestingly, synaptic transmission appears unaltered in these transgenic mice despite targeted double-knockout of MALS1 and MALS2 (Misawa et al., 2001). However, this double-knockout resulted in a significant upregulation of MALS3 protein, suggesting MALS3 could compensate for the loss of MALS1 and MALS2 to maintain synaptic function (Misawa et al., 2001). It is possible that PSD-95, SAP97 and/or other PDZ domain-containing proteins could exhibit a similar degree of functional redundancy in regards to the CRFR1-mediated enhancement of 5-HT_{2A}R signaling. Therefore, a future strategy could include utilizing multiple different shRNA constructs against combinations of PDZ domain-containing proteins to examine if any combination of knocked-down PDZ domain-containing proteins could abolish the CRFR1-mediated enhancement of 5-HT_{2A}R-mediated IP₃ signalling.

5.5 Implications to Mental Health

The CRFR1-mediated enhancement of 5-HT_{2A}R-mediated IP₃ signalling has been a proposed molecular mechanism for stress-induced anxiety and depressive disorders (Magalhaes et al., 2010). Although neither SAP97 nor PSD-95 shRNA knockdown were successful in abolishing the CRFR1-mediated enhancement of 5-HT_{2A}R signaling, the maximum efficacy following CRF-pretreatment was still significantly lower during SAP97

shRNA knockdown when compared to scrambled shRNA, and trended to be lower during PSD-95 shRNA knockdown (Dunn et al., 2014; Dunn et al., submitted). These observations are likely due to effects primarily on 5-HT_{2A}R. However, they still have significance to the field of mental health research. 5-HT_{2A}R has been demonstrated to be upregulated in the postmortem brain tissue of schizophrenic individuals, and the efficacy of atypical anti-psychotics has been demonstrated to be dependent upon the ability to downregulate 5-HT_{2A}R-mediated signalling and upregulate mGluR2 signalling (Catapano and Manji, 2007; Fribourg et al., 2011). Furthermore, the action of atypical anti-psychotics and mood-altering hallucinogens via 5-HT_{2A}R appear dependent upon the endogenous expression of PSD-95 (Abbas et al., 2009). Therefore, it is foreseeable that SAP97 may be of similar importance to PSD-95 in the regulation of mood through their regulation of 5-HT_{2A}R, as well as CRFR1 (Dunn et al., 2014; Dunn et al., 2013; Xia et al., 2003; Dunn et al., submitted).

Like 5-HT_{2A}R, CRFR1 has been recognized as a molecular target for the treatment of mental illness and inhibitors of CRFR1 function have been demonstrated to have anxiolytic and antidepressant-like qualities (Mansbach et al., 1997; Catapano and Manji, 2007). Additionally, the HPA axis stress response is initiated by the hypothalamic release of CRF into the anterior pituitary by acting on CRFR1 and CRFR2 receptors (Tsigos and Chrousos, 2002; Janssen and Kozicz, 2013). The ability of SAP97 and PSD-95 to regulate CRFR1 and 5-HT_{2A}R function may provide some explanation to their associations to psychiatric disease (Toyooka et al., 2002; Clinton et al., 2003; Clinton & Meador-Woodruff, 2004; Toro and Deakin, 2005; Clinton et al., 2006; Kristiansen, 2006; Tsai et al., 2007; Sato et al., 2008; Funk et al., 2009). Pharmacologically targeting the interactions

between receptors 5-HT_{2A}R or CRFR1 with SAP97, PSD-95, or other candidate PDZ domain-containing proteins could lead to specific regulation strategies for these receptors and the psychiatric disorders they underlay. Rather than modulating neurotransmitter availability, as is the case with selective serotonin-reuptake inhibitors, or SSRIs, we can begin to not only target specific receptors activated by these neurotransmitters, but modulate specific signalling pathways or receptor trafficking processes that underlie a spectrum of mental illnesses.

5.6 Contribution to the Molecular Physiology of GPCRs and PDZ Domain-Containing Proteins

These studies mark the first documentations of specific PDZ domain-containing proteins regulating CRFR1 function, and the identification of a second PDZ domain-containing protein capable of regulating 5-HT_{2A}R (Dunn et al., 2013; Dunn et al., submitted; Dunn et al., 2014). Additionally, these are only the second and third GPCRs shown to have their trafficking and/or signalling properties regulated by SAP97, and the fifth by PSD-95 (Magalhaes et al., 2012). Considering it is estimated that 20% of GPCRs have PDZ-binding motifs, and over 800 GPCRs have been identified in the human genome, it is safe to assume that this field is still in its infancy (Lee and Zheng, 2010; Fredriksson et al., 2003). Notably, this is also the first indication that SAP97 could play an integral role in ERK1/2 phosphorylation, and this appears to go beyond its interaction with GPCRs (Dunn et al., 2013; Dunn et al., 2014). Although this result was unexpected and outside the scope of the designed studies, it is one of the most curious due to its implications on

general MAPK signalling and as a consequence cellular growth, proliferation, and apoptosis.

GPCRs are influential in the regulation of every aspect of human physiology; therefore, any advancement in the understanding of how they can be regulated could contribute to the design and development of new pharmacological treatment and prevention strategies for a multitude of human pathologies (Bockaert et al., 2010; Heng et al., 2013). Dozens of studies have been published on the role of specific PDZ domain-containing proteins in regulating GPCRs; however, it is unclear whether each PDZ domain-containing protein has specific functional consequences on GPCRs, or whether this regulatory role is dependent upon the receptor in question or the cell-type in which the interaction takes place. In regards to PSD-95 and SAP97, it now appears these proteins act generally on a subset of GPCRs to promote their membrane localization by antagonizing receptor internalization and/or promoting receptor recycling. Their ability to suppress receptor internalization may be a consequence of competitive binding to the receptor with β -arrestins. PSD-95 and SAP97 do not appear to have a general role in G α s-mediated signalling; however, they may provide a bias towards G α q-coupling and activation. Future studies will look to elucidate the specific roles of PSD-95, SAP97, and other PDZ domain-containing proteins in regulating the diversity of GPCR-mediated signalling pathways and trafficking mechanisms in hopes of developing new treatment strategies for human disease.

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Appendices

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August 19, 2011

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October 28, 2014

Dear Henry Dunn:

This is to grant you permission to include the following article in your thesis entitled "Regulation of CRFR1 and 5-HT_{2A}R by SAP97 and PSD-95" for the University of Western Ontario:

Henry A. Dunn, Cornelia Walther, George Y. Yuan, Fabiana A. Caetano, Christina M. Godin, and Stephen S. G. Ferguson, Role of SAP97 in the Regulation of 5-HT_{2A}R Endocytosis and Signaling, *Mol Pharmacol* September 2014 86:275-283

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Journals Director

HENRY A. DUNN

EDUCATION:

PhD: Physiology

September 2008-December 2014

J. Allyn Taylor Centre for Cell Biology, Molecular Brain Research Group,
Molecular Medicine, Robarts Research Institute;
Department of Physiology and Pharmacology, Schulich School of Medicine &
Dentistry, University of Western Ontario.
Under Supervision of Dr. Stephen S.G. Ferguson

Graduate School Average: **87.4%**

Honours Bachelor of Medical Science

September 2003-April 2008

Double Major in Physiology and Medical Science
Department of Physiology and Pharmacology, Schulich School of Medicine &
Dentistry, University of Western Ontario.

Senior-Level Coursework:

*Regulatory Neurophysiology, Immunology, Cellular & Molecular
Neurobiology, Fetal Physiology, Motor Neurophysiology, Mammalian
Histology, Physiology of the Senses, Systemic Human Anatomy,
Gastrointestinal Physiology, Cellular Physiology, Advanced Cellular
Physiology, Mammalian Physiology, Physiology Laboratory,
Pharmacology of Drug Action.*

Dean's Honor List

September 2007-April 2008

Final Year Average: **81.5%**

Second Program:

Certificate in Writing:

September 2003-April 2008

Writing, Rhetoric, and Professional Communication

Graduated with Distinction

Faculty of Arts & Humanities, University of Western Ontario.

PUBLICATIONS:

Henry A. Dunn, Cornelia Walther, George Y. Yuan, Fabiana Caetano, Christina M. Godin, and Stephen S.G. Ferguson. *Role of SAP97 in the regulation of 5HT_{2A}R endocytosis and signaling.* ***Molecular Pharmacology***, 2014.

Henry A. Dunn, Cornelia Walther, Christina M. Godin, Randy A. Hall, and Stephen S.G. Ferguson. *Role of SAP97 in regulation of corticotropin-releasing factor receptor 1 endocytosis and ERK1/2 signalling.* ***Journal of Biological Chemistry***, 2013.

Citations: 4

Ana C Magalhaes, **Henry Dunn**, and Stephen S.G. Ferguson. *Review: Regulation of G protein-coupled receptor activity, trafficking and localization by GPCR-interacting proteins.* ***British Journal of Pharmacology***, 2012.

Citations: 98

MANUSCRIPTS IN PROGRESS:

Henry A. Dunn, Harpreet S. Chahal, George Y. Yuan, Kevin Holmes, Ruchi Parikh, Fabiana Caetano, Cornelia Walther, and Stephen S.G. Ferguson. *Role of PSD-95 in regulation of corticotropin-releasing factor receptor 1 endocytosis and β -arrestin2 recruitment.* Submitted to ***Molecular Pharmacology***.

Chakravarthi Narla, **Henry A. Dunn**, Stephen S.G. Ferguson, and Michael O. Poulter. *Suppression of piriform cortex activity by Corticotropin Releasing Factor 1 and Serotonin 2A/C receptors.* Submitted to ***Journal of Physiology***.

Cornelia Walther, Fabiana A. Caetano, **Henry A. Dunn**, and Stephen S.G. Ferguson. *PDZK1/NHERF3 Differentially Regulates Corticotropin-releasing Factor Receptor 1 and Serotonin 2A Receptor Signaling and Endocytosis.* Submitted to ***Cellular Signalling***.

RECENT ACCOLADES:

Awarded Best Poster Presentation Prize

2014 Gairdner Symposium:

Shedding New Light on Monoaminergic Signalling & Neuropsychiatric Disorders
Edmonton, Alberta.

May 29-30, 2014.

1st Place: Graduate Student Poster Presentation

Neurosciences Category: Charles W. Gowdey Research Day (UWO)
 London, Ontario.
 November 4, 2013.

Awarded Poster Prize

14th Annual Joint Meeting of the Great Lakes GPCR Retreat
 Cleveland, Ohio.
 October 17-19, 2013.

Awarded 2013 Jonathan & Joshua Memorial Graduate Scholarship

Schulich School of Medicine & Dentistry Internal Graduate Award:
Mental Health Research
 September 2013-August 2014.

Nominated for 2013 Drs. Madge & Charles Macklin Fellowship

Schulich School of Medicine & Dentistry Internal Graduate Award:
Teaching and Research
 Representative of Department of Physiology & Pharmacology (UWO)
 May 21, 2013.

Nominated for 2013 Drs. Madge & Charles Macklin Fellowship

Schulich School of Medicine & Dentistry Internal Graduate Award:
Significant Publication
 Representative of Department of Physiology & Pharmacology (UWO)
 May 21, 2013.

Nominated for Graduate Student Teaching Award

Society of Graduate Studies (UWO)
 May 21, 2013.

Awarded Poster Prize

13th Annual Joint Meeting of the Great Lakes GPCR Retreat
 London, Ontario
 October 17-19, 2012.

Awarded 2012 Jonathan & Joshua Memorial Graduate Scholarship

Schulich School of Medicine & Dentistry Internal Graduate Award:
Mental Health Research
 September 2012-August 2013.

Awarded Honourable Mention

CIHR National Student Research Poster Competition
 Canadian Student Health Research Forum
 Winnipeg, Manitoba
 June 12-14, 2012.

Nominated for CIHR National Student Research Poster Competition

Canadian Student Health Research Forum
 Winnipeg, Manitoba
 June 12-14, 2012.

Selected for Platform Competition (Invited Lecture)

Placed in top 10% of abstracts among Schulich students (UWO)
 London Health Research Day
 March 20, 2012.

2nd Place: Graduate Student Poster Presentation

Molecular Physiology & Pharmacology Category
 Department of Physiology and Pharmacology Annual Research Day (UWO)
 London, Ontario
 November 9, 2010.

Awarded CIHR Strategic Training Fellowship

Vascular Biology
 September 2009-August 2011.

Awarded Schulich Tuition Scholarship

January 2009-August 2013.

TEACHING EXPERIENCE:**Lead Teaching Assistant**

September 2010-April 2013

Physiology 1020 “Human Physiology”

Lecturer/Tutorial Leader for entire class (~250 students/year)
 Collaborative Nursing Program
 University of Western Ontario & Fanshawe College
Cumulative Lecture Hours: ~200

Teaching Assistant

September 2009-April 2010

Physiology 3130y “Physiology Laboratory”

Tutorial Leader for groups of 10-12 students per section

Department of Physiology & Pharmacology

University of Western Ontario

SUPERVISION EXPERIENCE:**Sarah Gupta**

April 2014-August 2014

Summer Student

Co-Supervisor (with Primary Supervisor Dr. Christina Godin)

Thesis project: Role of SAP97 in ERK1/2 phosphorylation

George Y. Yuan

September 2013-August 2014

4th Year Physiology Student & Summer Student

Primary Supervisor

Thesis project: Role of PSD-95 and SAP97 in Barrestin2 recruitment to CRFR1 and 5HT2AR

Harpreet S. Chahal

May 2012-April 2013

Summer Student & 4th Year Physiology Student

Primary Supervisor

Thesis project: Role of PSD-95 in CRFR1 trafficking and signalling

Ruchi Parikh

September 2011-April 2012

4th Year Physiology Student

Primary Supervisor

Thesis project: Role of PSD-95 in CRFR1-mediated cAMP signalling

Aditi Ghandi

September 2010-April 2011

4th Year Biology Student

Co-Supervisor (with Primary Supervisor Dr. Ana Magalhaes)

Thesis project: PSD-95 interaction with CRFR1 class I PDZ-binding motif

INVITED LECTURES:

Henry A. Dunn, Harpreet S. Chahal, Cornelia Walther, Kevin Holmes, Ruchi Parikh, Randy A. Hall, and Stephen S.G. Ferguson. *Regulation of CRF receptor 1 and 5HT2A receptor by PDZ domain-containing proteins*. Department of Physiology and Pharmacology Seminar Series, University of Western Ontario, November 11, 2013.

Henry A. Dunn, Cornelia Walther, Christie Godin, Randy A. Hall, and Stephen S.G. Ferguson. *Role of SAP97 in regulation of corticotropin-releasing factor receptor 1 endocytosis and ERK1/2 signalling*. Data Club presentation for the Molecular Brain Research Group at the Robarts Research Institute, March 20, 2013.

Henry A. Dunn, Ana C. Magalhaes, and Stephen S.G. Ferguson. *SAP97 regulates CRFR1 trafficking and ERK1/2 phosphorylation through a novel interaction with the CRFR1 class I PDZ-binding motif*. Invited Platform Presentation at the London Health Research Day in London, Ontario. March 20, 2012.

POSTER PRESENTATIONS:

Henry A. Dunn, Harpreet S. Chahal, Kevin Holmes, George Y. Yuan, Ruchi Parikh, and Stephen S.G. Ferguson. *PSD95 regulates CRFR1-mediated endocytosis and β arrestin2 recruitment*. 15th Annual Joint Meeting of the Great Lakes GPCR Retreat in Bromont, Quebec. October 2-4, 2014.

Maha M. Hammad, Cornelia Walther, **Henry A. Dunn**, and Stephen S.G. Ferguson. *The role of CAL in the regulation of corticotropin-releasing factor receptor 1 function*. 15th Annual Joint Meeting of the Great Lakes GPCR Retreat in Bromont, Quebec. October 2-4, 2014.

Henry A. Dunn, Cornelia Walther, George Y. Yuan, and Stephen S.G. Ferguson. *Role of SAP97 in regulating 5HT2AR endocytosis and signaling*. Gairdner Symposium: Shedding New Light on Monoaminergic Signalling and Neuropsychiatric Disorders in Edmonton, Alberta. May 29-30, 2014.

George Y. Yuan, **Henry A. Dunn**, and Stephen S.G. Ferguson. *Regulation of β -arrestin2 recruitment by PDZ domain-containing proteins*. 4980 Physiology Poster Day, April 7, 2014.

Maha M. Hammad, Cornelia Walther, **Henry A. Dunn**, and Stephen S.G. Ferguson. *The role of PDZ proteins in regulating the corticotrophin-releasing-factor receptor 1 and the serotonin 2A receptor*. 6th International Conference on Drug Discovery & Therapy in Dubai, United Arab Emirates. February 9-12, 2014.

Cornelia Walther, **Henry A. Dunn**, and Stephen S.G. Ferguson. *Identification of novel PDZ proteins as CRF receptor 1 and 5HT_{2A} receptor interactors*. GPCR Workshop 2013 in Maui, Hawaii. December 1-5, 2013.

Henry A. Dunn, Cornelia Walther, and Stephen S.G. Ferguson. *SAP97 regulates 5HT_{2A}R-mediated signaling pathways*. Charles W. Gowdey Research Day in London, Ontario. November 4th, 2013.

Maha M. Hammad, Cornelia Walther, **Henry A. Dunn**, and Stephen S.G. Ferguson. *The role of PDZ proteins in regulating the corticotrophin-releasing-factor receptor 1 and the serotonin 2A receptor*. Charles W. Gowdey Research Day in London, Ontario. November 4th, 2013.

Cornelia Walther, **Henry A. Dunn**, and Stephen S.G. Ferguson. *PDZK1 differentially regulates CRF-receptor 1 and 5HT_{2A}-receptor function*. Charles W. Gowdey Research Day in London, Ontario. November 4th, 2013.

Henry A. Dunn, Harpreet S. Chahal, Kevin Holmes, Ruchi Parikh, and Stephen S.G. Ferguson. *PSD95 regulates CRFR1-mediated endocytosis and β arrestin2 recruitment*. 14th Annual Joint Meeting of the Great Lakes GPCR Retreat in Cleveland, Ohio. October 17-19, 2013.

Henry A. Dunn, Cornelia Walther, and Stephen S.G. Ferguson. *SAP97 regulates 5HT_{2A}R-mediated signaling pathways*. 14th Annual Joint Meeting of the Great Lakes GPCR Retreat in Cleveland, Ohio. October 17-19, 2013.

Maha M. Hammad, Cornelia Walther, **Henry A. Dunn**, and Stephen S.G. Ferguson. *The role of PDZ proteins in regulating the corticotrophin-releasing-factor receptor 1 and the serotonin 2A receptor*. 14th Annual Joint Meeting of the Great Lakes GPCR Retreat in Cleveland, Ohio. October 17-19, 2013.

Cornelia Walther, **Henry A. Dunn**, and Stephen S.G. Ferguson. *PDZK1 differentially regulates CRF-receptor 1 and 5HT_{2A}-receptor function*. 14th Annual Joint Meeting of the Great Lakes GPCR Retreat in Cleveland, Ohio. October 17-19, 2013.

Henry A. Dunn, Cornelia Walther, Christina M. Godin, Randy A. Hall, and Stephen S.G. Ferguson. *Identification of novel PDZ proteins as CRF receptor 1 and 5HT2A receptor interactors*. Gordon Research Conference in Molecular Pharmacology: New Opportunities with G-protein Coupled Receptors: From Single Molecules and Signaling Complexes to Physiology and Therapeutic Interventions. Lucca (Barga), Italy. April 28 - May 3, 2013

Harpreet S. Chahal, **Henry A. Dunn**, and Stephen S.G. Ferguson. *PSD-95 regulates CRFR1 trafficking via a novel interaction with a class I PDZ-binding motif*. 4980 Physiology Poster Day, April 2013.

Henry A. Dunn, Harpreet S. Chahal, and Stephen S.G. Ferguson. *SAP97 regulates CRFR1 trafficking and CRF-mediated ERK1/2 phosphorylation*. London Health Research Day in London, Ontario. March 19, 2013.

Henry A. Dunn, Harpreet S. Chahal, and Stephen S.G. Ferguson. *SAP97 regulates CRFR1 trafficking and CRF-mediated ERK1/2 phosphorylation*. 13th Annual Joint Meeting of the Great Lakes GPCR Retreat in London, Ontario. October 17-19, 2012.

Henry A. Dunn, Ana C. Magalhaes, and Stephen S.G. Ferguson. *SAP97 regulates CRFR1 trafficking and ERK1/2 phosphorylation*. CIHR National Student Research Poster Competition at the Canadian Student Health Research Forum in Winnipeg, Manitoba. June 12-14, 2012.

Ruchi Parikh, **Henry A. Dunn**, and Stephen S.G. Ferguson. *PSD-95 interacts and co-localizes with CRFR1 via a novel interaction with a class I PDZ-binding motif*. 4980 Physiology Poster Day, April 2012.

Henry A. Dunn, Ana C. Magalhaes, and Stephen S.G. Ferguson. *SAP97 regulates CRFR1 trafficking and ERK1/2 phosphorylation through a novel interaction with the CRFR1 class I PDZ-binding motif*. Poster Presentation at the Charles W. Gowdey Research Day in London, Ontario. November 8, 2011.

Henry A. Dunn, Ana C. Magalhaes, and Stephen S.G. Ferguson. *SAP97 regulates CRFR1 trafficking and ERK1/2 phosphorylation through a novel interaction with the CRFR1 class I PDZ-binding motif*. Poster Presentation at the 12th annual joint meeting of the Great Lakes G Protein-Coupled Receptor Retreat in Montebello, Quebec. October 27-29, 2011.

Magalahaes AC, **Dunn H**, and Ferguson SS. *PDZ proteins involved in the modulation of 5-HT2a/c receptors by CRF peptide*. Poster Presentation at the 12th annual joint meeting of the Great Lakes G Protein-Coupled Receptor Retreat in Montebello, Quebec. October 27-29, 2011.

Magalhaes, AC, Dunn, H and Ferguson, SS. *Identifying PDZ proteins involved in scaffolding the serotonin/CRFR1 receptors complex*. Poster Presentation at the 23rd biennial International Society for Neurochemistry in Athens, Greece. August 28-September 1, 2011.

Henry Dunn, Ana Magalhaes, and Stephen S.G. Ferguson. *Role of PDZ-Protein SAP97 in CRF1 α & 5HT2a Receptor Trafficking and Signaling*. Poster Presentation at the Department of Physiology & Pharmacology (UWO) Annual Research Day in London, Ontario. November 9, 2010.

Henry Dunn, Ana Magalhaes, and Stephen S.G. Ferguson. *Role of PDZ-Protein SAP97 in CRF1 α & 5HT2a Receptor Trafficking and Signaling*. Poster Presentation at the 11th annual joint meeting of Great Lakes G Protein-Coupled Receptor Retreat in King City, Ontario. October 21-23, 2010.

Henry Dunn, Ana Magalhaes, and Stephen S.G. Ferguson. *Role of PDZ-protein SAP97 in CRF1 α —5HT2a receptor signalling pathway*. Poster presentation at the 10th annual joint meeting of Great Lakes G Protein-Coupled Receptor Retreat in Rochester, New York. October 15-17, 2009.

Magalhaes AC, Holmes KD, Dale LB, Drysdale L, Comps-Agrar L, Lee D, Dunn H, Poulter MO, Pin JP, Anisman H and Ferguson SSG. *Modulation of 5HT receptor activity by CRF receptor activation*. Poster presentation at the 10th annual joint meeting of Great Lakes G Protein-Coupled Receptor Retreat in Rochester, New York. October 15-17, 2009.