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PDZ Protein Regulation of β-arrestin Recruitment and GPCR Trafficking

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Abstract

β-arrestins are versatile adaptor proteins that play a vital role in regulation of G protein coupled receptor (GPCR) trafficking and signalling properties. PDZ proteins have previously been shown to modulate β-arrestin2 recruitment and receptor internalization for many GPCRs including Corticotropin-Releasing Factor Receptor 1 (CRFR1), a receptor whose antagonists have been shown to demonstrate both anxiolytic- and antidepressant-like effects. Further characterization of the interplay between β-arrestins and PDZ proteins may aid in determining a potential mechanism for PDZ protein regulation of GPCR trafficking. Our findings suggest that PDZ proteins PSD-95, MAGI1, and PDZK1 complex with β-arrestin2 by interacting via the PDZ domain. Using a proteomic approach, mutational analyses were used to reveal that the β-arrestin2 A175F mutant impairs interaction with PSD-95. Additionally, this mutant form of β-arrestin2 shows decreased CRF-stimulated recruitment to CRFR1. Thus, investigating how β-arrestins and PDZ proteins interact could provide further insight into GPCR trafficking properties and the development of novel therapeutics.

Key Words: G protein-coupled receptors, CRFR1, β-arrestins, PDZ, PSD-95, MAGI1, PDZK1, endocytosis, trafficking, mental disorders, anxiety, depression

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List of Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
5-HT2AR	5-HT2A receptor
AC	Adenylyl Cyclase
ACTH	adrenocorticotropin hormone
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride
ANOVA	analysis of variance
β1-AR	β1-adrenergic receptor
CAL	cystic fibrosis transmembrane conductance regulator-associated ligand
CaMK	calmodulin kinase-like
cAMP	cyclic adenosine monophosphate
Cdc42	Cell division control protein 42 homolog
CNS	Central Nervous System
CRF	corticotropin-releasing factor
CRFR1	CRF receptor 1
CRFR2	CRF receptor 2
CRIB	Cdc42- and Rac-interactive binding
DAG	diacylglycerol
DLGs	discs larges
DOI	2,5-Dimethoxy-4-iodoamphetamine
Dvl	Dishevelled
EBP50	ERM Binding Protein 50
ECLs	extracellular loops
ERK1/2	extracellular signal-regulated kinase
GASPs	GPCR-associated sorting proteins
GAPs	GTPase-activating proteins
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GIP	GPCR-interacting protein
GIPC	Gα-Binding Protein Interacting Protein Carboxyl-Terminus
GK	guanylate kinase-like
GPCR	G protein-coupled receptor
GRIP1	Glutamate Receptor Interacting Protein 1
GRK	G protein-coupled receptor kinase
HA	hemagglutinin
HBSS	HEPES-buffered saline solution
HEK	293 human embryonic kidney 293
HPA	Hypothalamo-Pituitary-Adrenal Axis
HtrA	high temperature requirement protease A
IKEPP	intestinal and kidney-enriched PDZ protein
IP_3	Inositol trisphosphate
JNK	c-Jun N-terminal kinase
Kir2.3	Inward rectifier K+ channel

MAGI	MAGUK with Inverted orientation PDZ
MAGUK	Membrane Associated Guanylate Kinase
MAPK	mitogen activated protein kinase
MRP2	Multidrug resistance-associated protein 2
NHERF	Na+/H+ Exchanger Regulatory Factor
nNOS	neuronal nitric oxide synthase
PBS	phosphate-buffered saline
PDB	Protein Data Bank
PDZ	PSD95/ Disc Large/ Zona Occludens
PDZK1	PDZ Domain Containing 1
P13	Phosphoinositide 3-kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PLCβ	phospholipase Cβ
PSD-95	postsynaptic density 95
PTB	phosphotyrosine binding
PVN	paraventricular nucleus
RA	Ras-associated
Rab	Ras-related protein
RAMPs	receptor activity modifying proteins
RGS	regulators of G-protein signaling
Rluc	Renilla luciferase
RMSD	root mean square deviation
SAP97	synapse-associated protein 97
SH2	Src-homology 2
SH3	Src homology 3
SHANK	SH3 and multiple ankyrin repeat domains protein
shRNA	short hairpin RNA
SNX1	Sorting Nexin 1
SNX2	Sorting Nexin 2
SNX27	Sorting Nexin 27
SSRIs	Selective Serotonin Reuptake Inhibitors
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TGN	Trans-Golgi Network
Vps26	Vacuolar protein sorting-associated protein 26
Vps29	Vacuolar protein sorting-associated protein 29
Vps35	Vacuolar protein sorting-associated protein 35
YFP	yellow fluorescent protein
ZO-1	Zonula occludens 1

Chapter 1: Introduction

1.1 G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs) constitute the single largest and most diverse class of transmembrane receptor proteins encoded by the mammalian genome (Oldham & Hamm, 2008). Structural features common to all GPCRs include seven transmembrane helical domains, an extracellular N terminus, and an intracellular C terminus (Kobilka, 2007). GPCRs can be activated upon binding of a wide variety of ligands including peptides, hormones, amino acids, neurotransmitters, odorants and photons, among others (Kroeze et al., 2003). Activation of GPCRs by an agonist leads to a conformational change in the α-subunit of the heterotrimeric G protein such that it dissociates from the Gβγ subunit. This is caused by cycling of the α-subunit between an inactive GDP-bound state and an active GTP-bound conformation that can then regulate various aspects of downstream signaling pathways (Oldham & Hamm, 2008). Each GPCR can be bound by multiple ligands that can induce activation of a specific signaling pathway.

GPCRs regulate a myriad of physiological processes and are heterogeneously distributed in many different tissues (Tautermann, 2014). This versatility, coupled with the convenience of being able to manipulate an entire intracellular signaling cascade with a single target, makes GPCRs excellent pharmaceutical targets. This is of particular importance when considering nearly 40% of all current drugs in clinical use target GPCRs, thus marking their importance for the development of novel therapeutics (Tautermann, 2014).

1.1.2 GPCR interacting Proteins

GPCRs can interact with numerous proteins that function not only to attenuate their signalling, but also to couple these receptors to heterotrimeric G-protein-independent signalling pathways (Magalhaes et al., 2012). In addition, intracellular and transmembrane proteins associate with GPCRs and regulate their processing in the endoplasmic reticulum, trafficking to the cell surface, compartmentalization to plasma membrane microdomains, endocytosis and trafficking between intracellular membrane compartments (Magalhaes et al., 2012). Examples of proteins that can bind to GPCRs include β-arrestin, receptor activity-modifying proteins (RAMPS), regulators of G-protein signalling (RGS), GPCR-associated sorting proteins (GASPs), Homer proteins, small GTPases, PDZ domains, spinophilin, protein phosphatases, calmodulin, optineurin and Src homology 3 (SH3) containing proteins (Magalhaes et al., 2012).

1.2 Arrestins

Arrestins are a predominantly cytoplasmic and versatile family of proteins. In mammals, the family consists of four members: arrestin 1 (visual or rod arrestin), arrestin 2 (β-arrestin1), arrestin 3 (β-arrestin2), and arrestin 4 (cone or X-arrestin) (Han et al., 2001). Arrestin 1 and arrestin 4 are exclusively expressed in the visual system, either in rod or cone cells. Conversely, β-arrestin1 and β-arrestin2 are ubiquitously expressed in virtually all cell types and within a variety of tissues (Han et al., 2001).

1.2.1 Structure of Arrestins

Structurally, arrestins are commonly characterized as having an elongated shape consisting of two domains (N-domain and C-domain) made up of β-sheets that are

connected by a short hinge (Moore et al., 2007). Embedded between the two domains of the molecule is a central polar core of buried salt bridges that functions to regulate affinity of interaction with phosphorylated receptors. The proteins also contain an extended C-terminal tail that that is connected to the body of the C-domain by a flexible linker (Moore et al., 2007). It has been proposed that while GPCRs interact with arrestins on their concave side, other arrestin interacting proteins bind on their convex side (Gurevich & Gurevich, 2013). When a receptor binds it may cause a substantial conformational rearrangement of arrestin involving movement of the two domains relative to each other (Vishnivetskiy et al., 2002). This process may be dependent on the length of the hinge connecting both domains since it has been shown that decreasing the length of the hinge also reduces the amount of active receptor-bound arrestin (Vishnivetskiy et al., 2002).

1.2.2 β-arrestin Interaction With GPCRs

Arrestins are multifunctional adaptor proteins that bind to GPCRs to exert a regulatory role in which they can mediate different forms of ligand directed functional signaling as well as internalization of the GPCR, a mechanism of GPCR desensitization (Fig. 1.1). Upon agonist binding and activation, in addition to activation of multiple signaling transduction pathways, agonist activation also promotes phosphorylation of the receptor by G-protein coupled receptor kinases (GRKs) (Park et al., 2016). This promotes the translocation and binding of β-arrestins, which serves to uncouple receptors from heterotrimeric G-proteins and terminate signalling. β-arrestins can then function as adaptors, linking the receptor to components of clathrin endocytic machinery such as

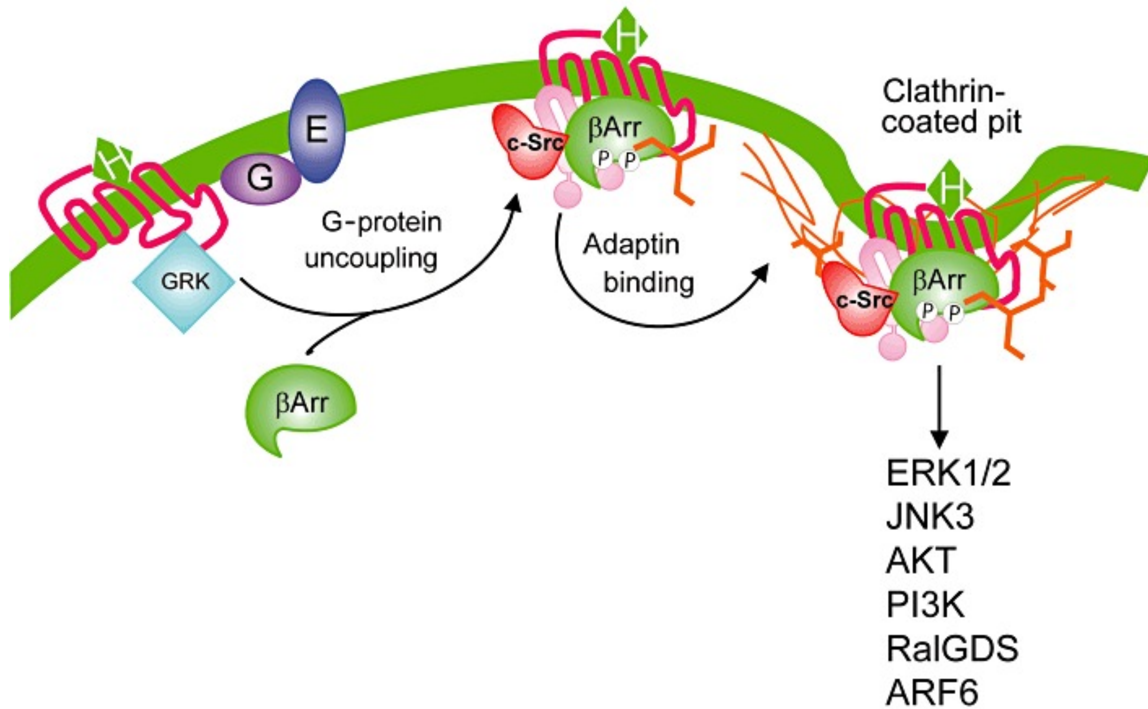

Figure 1.1 Schematic illustration of β-Arrestin-dependent endocytosis and signalling of GPCRs. Agonist activation promotes the GRK2-mediated phosphorylation that promotes the translocation and binding of β-arrestins, which serves to uncouple receptors from heterotrimeric G-proteins. β-Arrestins function as adaptor proteins that interact with both clathrin and β2-adaptin promoting the clathrin coated vesicle-mediated endocytosis of many GPCRs. β-Arrestin interactions with a variety of proteins allows for the coupling of GPCRs to a variety of different signal transduction pathways whose activation may be independent of heterotrimeric G-proteins. (Adapted from Magalhaes et al., 2012)

clathrin and adaptin, thereby promoting clathrin coated vesicle-mediated endocytosis of the receptor (Ferguson 2001). The internalized receptor may then be targeted for degradation to the lysosome or in some cases undergo receptor dephosphorylation and be sent back to the cell surface for reactivation as a fully functional receptor (Ferguson 2001). Almost all cell membrane receptors, including GPCRs, exhibit these common properties for internalization, from the plasma membrane to the cytoplasm, in response to ligands.

Studies done over the past few years have demonstrated that β-arrestins not only induce GPCR desensitization, but also can function as GPCR signal transducers. They can form complexes by binding to several signaling proteins, including Src-family tyrosine kinases and components of the ERK1/2 and JNK3 MAP cascades (Moore et al., 2007). In addition to ERK activation, they have also been shown to be able to scaffold numerous other proteins important for regulation of signalling including AKT, PI3 kinase, phosphodiesterases, transcription factors, and small GTPases (Magalhaes et al., 2012). Evidence now suggests that GPCRs can selectively activate signalling pathways that are predominantly β-arrestin mediated. This is partly dependent on the nature of the ligand; interaction with the receptor determines the conformational state of the ligand and preference for binding either heterotrimeric G-proteins or β-arrestins (Magalhaes et al., 2012).

1.2.3 Vacuolar Protein Sorting-Associated Protein 26 (VPS26)

The retromer complex is a large multimeric complex that consists of the five subunits SNX1, SNX2, Vps26, Vps29 and Vps35 (Shi et al., 2006). In humans, this complex is thought to mediate retrograde transport of proteins from endosomes to the

trans-Golgi network (TGN) as well as recycling of cargo proteins from the endosome to the plasma membrane (Shi et al., 2006). The Vps26 subunit of this complex has two isoforms within the mouse genome, Vps26A and Vps26B, that can both interact with other retromer subunits (Gallon et al., 2014). Vps26 has previously been shown to be required for embryonic development in mice (Lee et al., 1992). It has also been demonstrated that Vps26 protein expression is decreased in patients with Alzheimer's disease (Small et al., 2005). Therefore it is an important protein in mammalian physiology.

The structure of Vps26A has recently been solved using x-ray crystallography and determined to be a structural relative of the arrestin family of proteins (Shi et al., 2006). Though Vps26 shares little sequence homology with the arrestin family, it shows a high degree of structural similarity and shares the same overall protein fold. Interestingly, an unusual characteristic of the arrestin family is the polar core embedded between its two domains (N-domain and C-domain) (Shi et al., 2006). This characteristic is also shared by Vps26, which also contains a polar core, though there is little sequence homology between the polar residues (Shi et al., 2006).

1.2.3 β-arrestin Gene Knockouts

Considering the number of distinct GPCRs expressed in the mammalian genome, the fact that there are only two β-arrestin subtypes expressed ubiquitously is rather remarkable and demonstrates their physiological importance (Gurevich & Gurevich, 2013). Therefore, it can be expected that the genetic deletion of arrestins give a distinct phenotype. While β-arrestin1 knockout mice and β-arrestin 2 knockout mice exhibit altered functional phenotypes, a β-arrestin1/2 double knockout has been shown to be

embryonically lethal (Bohn et al., 1999; Conner et al., 1997; Pierce & Lefkowitz, 2001). This is similarly seen in Drosophila, where a knockout of the β-arrestin analog Kurtz results in a broad lethal phase during embryogenesis (Roman et al., 2000). Since the double knockout of β-arrestin1/2 is embryonically lethal, and the individual knockouts of either β-arrestin lack an extreme phenotype, it can be implied that there is a functional redundancy between the two proteins. This is further supported by the similarity in tissue expression of the two genes (Sterne-Marr et al., 1993), as well as by data suggesting that both proteins bind a range of receptors with similar levels of affinity (Gurevich et al., 1995).

1.3 PDZ Proteins

PDZ domains are highly abundant protein-protein interaction domains of approximately 80-90 amino acids residues (Fan & Zhang, 2002). They were originally discovered in, and thereby named after, the proteins postsynaptic density 95 (PSD-95), disks-large-1 (DLG), and Zonula occludens 1 (ZO-1) (Kennedy, 1995). Approximately 400 PDZ domains have been recognized, in over 200 proteins, within the human genome (Wang et al., 2010). A large majority of PDZ domain containing proteins serve primarily scaffolding functions, although some also have catalytic functions. While some PDZ proteins contain only PDZ domains, such as PDZ domain containing protein PDZK1 which contains 4 PDZ domains, other PDZ proteins contain additional functional domains (Figure 1.2). These domains can include Src-homology 2 (SH2) domains, phosphotyrosine binding (PTB) domains, calmodulin kinase-like (CaMK) and Ras associated (RA) domains, among others (Dunn & Ferguson, 2015; Harris & Lim, 2001; Magalhaes et al., 2012). The MAGUK proteins are a family of PDZ proteins that, in

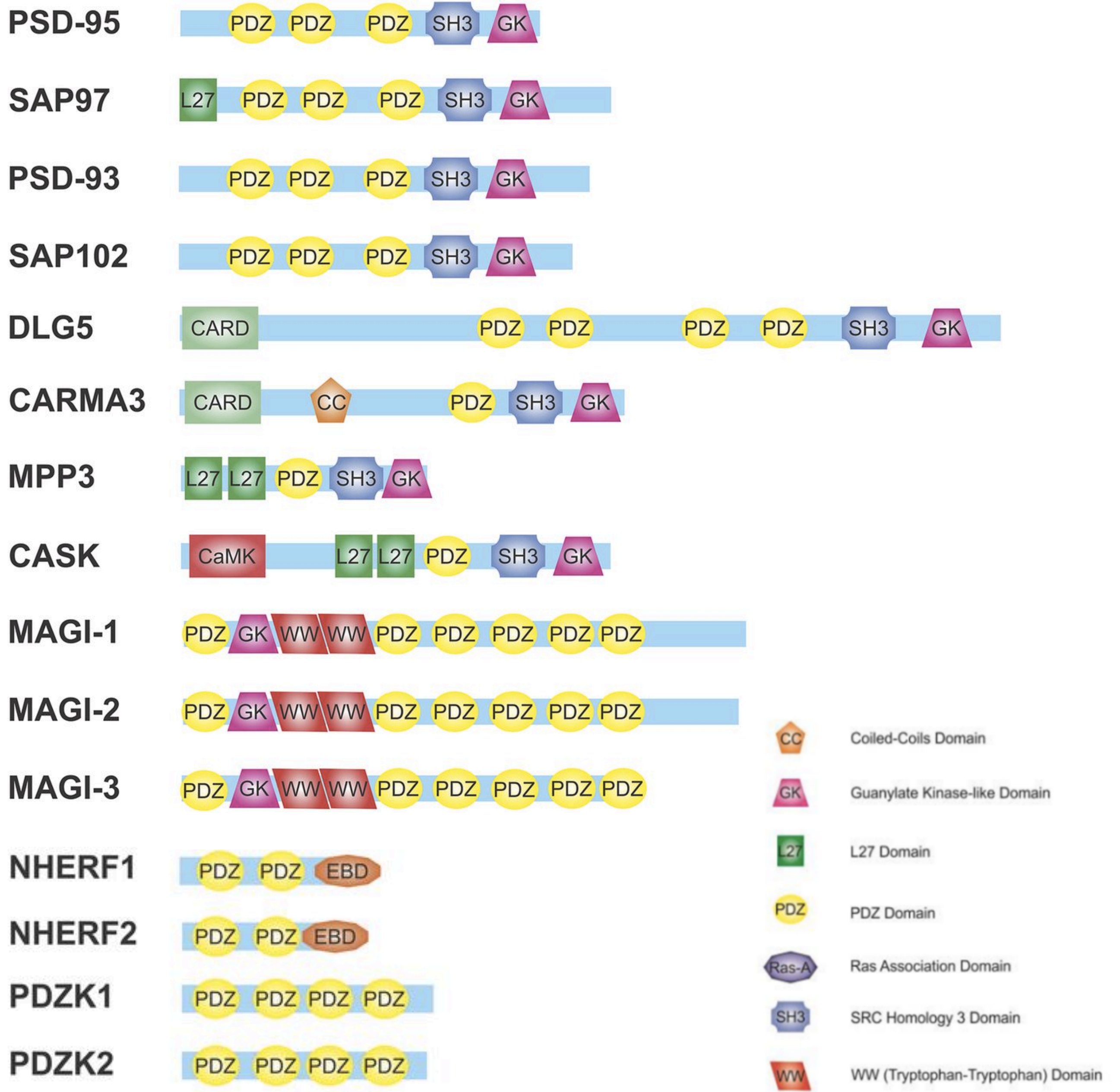

Figure 1.2 Molecular topology of PDZ domain-containing proteins. (Adapted from Dunn & Ferguson, 2015)

addition to one to three PDZ domains, are characterized as containing a Src- homology 3 (SH3) domain and a catalytically inactive guanylate kinase-like (GK) domain (Anderson, 1996; Dimitratos et al., 1999; Zheng et al., 2011).

Combining PDZ domains with other functional domains allows PDZ domain containing proteins to mediate many cellular functions such as epithelial cell polarity (Bilder et al., 2003), neuronal signal transduction (Kim & Sheng, 2004), protein trafficking and recycling (Roche et al., 2001), cytoskeleton dynamics related cell growth (Voltz et al., 2001), and hair cell stereociliar development (Reiners et al., 2006).

1.3.1 Structure of PDZ Domains

Since first solving the structure of PDZ3 in PSD95 (Doyle et al., 1996), the structures of hundreds of PDZ domains have been crystalized. Most PDZ domains share similar globular structures that are characterized as containing two α-helices and six β-strands. The β-sheets together form a partially open barrel that is covered by an α-helix on each opening (Doyle et al., 1996; Fanning & Anderson, 1996; Lee & Zheng, 2010). In most PDZ domains the N- and C- terminals lie in close proximity and opposite to the hydrophobic ligand binding groove. This cup-like binding pocket contains the highly conserved Gly-Leu-Gly-Phe (GLGF) motif (Harris & Lim, 2001). This motif is unique to PDZ domains and is thought to facilitate hydrogen bond formation and binding of ligands to the domain. This normally occurs through the amide side chains of the GLGF motif and a carboxylate group on the ligand (Sheng & Sala, 2001). In some PDZ domains, residues such as serine, threonine, or phenylalanine can replace the first glycine residue of the GLGF motif (Sheng & Sala, 2001). Differences in the domains have also been seen with the second and fourth hydrophobic amino acid residues in the motif, which can be

valines or isoleucines. This variation within the GLGF motif contributes to specificity in PDZ domain interactions (Garner et al., 2000; Harris & Lim, 2001; Sheng & Sala, 2001; Subbaiah et al., 2011).

1.3.2 Characterization of PDZ-mediated Interactions

PDZ binding peptide screening studies have established that many PDZ domain containing proteins have the ability to bind multiple targets with comparable affinities. This ability is likewise seen with PDZ-binding motifs, which have been shown to be able to interact with a wide variety of PDZ domain containing proteins (Songyang et al., 1997; Zhang et al., 2006). The promiscuity of PDZ interactions is unexpected given the importance of PDZ domain containing proteins in multiple diverse cellular processes and within a variety of living organisms. However, this phenomenon may in part be defined by limited and specific spatial distributions of certain PDZ motif- and domain- containing proteins (Songyang et al., 1997; Zhang et al., 2006).

PDZ domains interact with specific sequences of three amino acids, called PDZ-binding motifs, often found on carboxyl terminal targets (Kim & Sheng, 2004; Lee & Zheng, 2010). PDZ domains have different affinities for PDZ-binding motifs and therefore are traditionally classified into three classes: Class I PDZ domains preferentially bind to the S/T-X-Φ motif, Class II domains selectively bind to the Φ-X-Φ motif, while Class III domains prefer the Ψ-X-Φ motif (where Φ indicates any hydrophobic amino acid, Ψ represents any acidic amino aid residue, and X stands for any amino acid) (Doyle et al., 1996; Dunn & Ferguson, 2015; Songyang et al., 1997). However, it is worth mentioning that there are still some PDZ domains that cannot be categorized into any of these specific classes.

Although the majority of PDZ domain containing proteins bind to a PDZ binding motif found on the carboxyl terminal of protein targets, there is accumulating evidence that suggests some PDZ domains may bind to internal PDZ binding motifs contained within target proteins (Harris & Lim, 2001; Mu et al., 2014; Trejo, 2005). An example of this type of interaction has been demonstrated previously to occur with both PDZ proteins syntropin and PSD-95 to the internal β-hairpin finger structure found in neuronal nitric oxide synthase (nNOS) (Christopherson et al., 1999). This β-hairpin binds PDZ domains by replacing the conventional c-terminal carboxylate group with a sharp β-turn (Christopherson et al., 1999). Another example of this is the interaction between PDZ protein Par-6 and the internal binding motif found in Pals1 protein (Penkert et al., 2004). In this complex, the Pals1 binding motif forms an extended conformation similar to the one seen in the nNOS-PDZ domain interaction. The binding of Pals1 induces a conformational change in the ligand binding groove of Par-6, which may occur due to salt bridges formed between the two proteins (Penkert et al., 2004). Other examples of internal PDZ binding motifs interactions described in the literature include: the interaction between PDZ protein Dvl and internal motifs found in Frizzled and Idax, PDZ binding of nNOS to Vac14, and the interaction of HtrA1/2/3 with internal sequences of misfolded polypeptides (Lemaire & McPherson, 2006; London et al., 2004; Runyon et al., 2007; Wong et al., 2003). It has yet be be determinded if proteins containing an internal PDZ binding motif undergo a shift in conformation upon binding to a PDZ domain (Lee & Zheng, 2010).

Though most PDZ proteins studied thus far have been monomers, it has been established that certain PDZ domains dimerize into either homodimers or heterodimers,

often without disruption of peptide binding (Hillier et al., 1999; Lau & Hall, 2001; Xu et al., 1998). It is thought that as many as 30% of PDZ domains dimerize with low micromolar affinities that appear to be more specific than PDZ-ligand binding, therefore influencing the specific make up of protein complexes (Chang et al., 2011). Studies have shown that these interactions can occur through multiple mechanisms. For example, the PDZ domain of shank1 and GRIP1 PDZ6 form back-to-back homodimers through interactions between their conserved β2-β3 loops and N-terminal β1-strands, while concurrently leaving their peptide binding sites open for interaction (Im, Lee, et al., 2003; Im, Park, et al., 2003). A novel form of dimerization occurs with the second PDZ domain of ZO-1 that forms homodimers through symmetrical swapping of β-strands in the domains. The ZO-1 homodimer arranges such that the peptide binding site still remains accessible, for both domains, on either side of the dimer (Chen et al., 2008; Fanning et al., 2007). Although there are exceptions, it seems PDZ domain dimers often leave the peptide binding site available, thus encouraging binding partners while also forming another means of regulation for these interactions.

PDZ domains are largely characterized as protein-protein interaction modules, however it has been shown that some PDZ domains can also bind to lipids such as phosphoinositides (PIPs) (Zimmermann et al., 2002). As one of the phospholipid components of mammalian cell membranes, phosphatidylinositol can serve either as a precursor for second messengers or as a signaling molecule on the membrane that regulates the localization of protein complexes (Di Paolo & De Camilli, 2006). PIPs can control a broad range of cellular processes, such as the activity of ion channels and transporters, membrane and actin dynamics, cell growth and differentiation, and vesicular

trafficking (Balla et al., 2009). Therefore, interactions between PDZ proteins and PIPs may provide an essential method of regulation for PDZ complexes, further illustrated by recent estimates that as much as 40% of all PDZ domains interact with phospholipids (Chen et al., 2012; Gallardo et al., 2010). These associations have been shown to occur through multiple mechanisms such as electrostatic interactions, membrane penetration, and specific PIP binding with basic clusters found in the PDZ domains (Gallardo et al., 2010). However, there is still no consensus on where the PIP binding site lies in PDZ domains. While some studies suggest a synergistic interaction, others suggest competitive binding between PIPs and the peptide ligand (Ivarsson et al., 2011; Wu et al., 2007; Zimmermann et al., 2002). Further studies are required to fully understand the interplay between lipid-PDZ domain interactions, along with the consequential physiological implications.

1.3.3 Regulation of PDZ Proteins

PDZ interactions are regulated in several ways, as can be expected from their critical functions in assembling protein complexes. One form of regulation includes phosphorylation of serine, threonine, or tyrosine resides within the PDZ binding motif (H.-J. Lee & Zheng, 2010). Phosphorylation of the PDZ binding motif, in some cases, can disrupt and reduce PDZ domain binding. This is seen in the interaction between the Kir2.3 channel and the PDZ domains of PSD-95 (Cohen et al., 1996). However, in other cases, phosphorylation has also been shown to increase affinity for PDZ domain binding, such as in the binding of PDZ proteins PDZK1, IKEPP or EBP50 to the C-terminal of MRP2 (Hegedüs et al., 2003). Thus, phosphorylation of PDZ binding motifs may serve as

a functional switch, allowing for different conformations that each optimally bind to a different PDZ domain (Ivarsson, 2012).

Another method of regulation includes allosteric regulation, which can modulate the binding preference of PDZ domains (H.-J. Lee & Zheng, 2010). For example, binding of the small GTPase Cdc42 to a CRIB domain adjacent to the PDZ domain of Par6 can change the dynamics of the protein, from a fluid low-affinity state to a rigid high-affinity state with an increased affinity for its carboxy-terminal ligand (Peterson et al., 2004). Some PDZ domains can even self regulate through the process of autoinhibition. Many PDZ domains containing proteins also contain a PDZ binding motif at their distal C-terminal tail, which can fold back to bind with the PDZ domain, and thus prevent binding of other ligands (Ye & Zhang, 2013). This mechanism has been seen in PDZ proteins such as X11α, tamalin and NHERF1 (H.-J. Lee & Zheng, 2010; Ye & Zhang, 2013). Finally, it has been shown that PDZ interactions can also be regulated by environmental changes in pH or ionic strength (Harris et al., 2003; Chi et al., 2006).

1.3.4 Regulation of GPCRS by PDZ Proteins

Numerous GPCRs contain a PDZ binding motif on their C-terminal tail and thus can interact with PDZ proteins to form multiprotein complexes. Since PDZ domain containing proteins are one of the most abundant types of GPCR-interacting proteins, it comes as no surprise that they have been shown to be important regulators of various properties including receptor trafficking, signalling, and cellular distribution (Table 1.1) (Dunn & Ferguson, 2015; Magalhaes et al., 2012). GPCRs can bind multiple PDZ proteins and vice versa with different regulatory mechanisms, in fact studies show PDZ

PDZ Protein	Trafficking Function	GPCR
PSD-95	↓ Endocytosis ↑ Recycling ↑ Membrane localization ↑ Endocytosis	β_1AR, 5-HT_{2A}R D_1R GPR30 5-HT_{2C}R, D_1R
SAP97	↓ Endocytosis ↑ Recycling	CRFR1, 5-HT_{2A}R β_1AR
SAP102	↓ Mobility	A_{2A} receptor
MAGI-2	↓ Endocytosis ↑ Endocytosis ↑ Membrane localization	VPAC1 β_1AR mGluR1a
NHERF1	↓ Endocytosis ↑ Recycling ↑ Membrane localization ↑ Endocytosis	β_2AR, TPβ β_2AR, human κopioid receptor SSTR5, PTH1R CCR5, platelet-activating factor receptor, $P2Y_{12}$R
PDZK1	↓ Endocytosis ↑ Membrane localization	5-HT_{2A}R hIPR
PDZK2	↑ Membrane localization	hIPR
Spinophilin	↓ Endocytosis ↑ Endocytosis	α_2AR μOR
MUPP1	↑ Membrane localization ↑ Tight junction localization	5-HT_{2A}R SSTR3
SNX27	↑ Recycling	β_2AR, β_1AR, SSTR5
CAL	↓ Membrane localization ↓ Recycling ↑ Golgi localization	β_1AR, SSTR5 β_1AR SSTR5

Table 1.1 Effect of PDZ proteins on GPCR trafficking. (Modified from Dunn & Ferguson, 2015)

proteins can have distinct functions dependent on the specific GPCR-PDZ interaction in question (Dunn & Ferguson, 2015). For example, it has been documented that GPCR β1-adrenergic receptor (β1AR) can be regulated by various PDZ proteins in various different manners. While PDZ domain containing protein PSD-95 has been shown to promote membrane localization of the β1AR through inhibition of receptor internalization, PDZ protein SAP97 seems to do so by promoting receptor recycling to the membrane (Hu et al., 2000; Gardner et al., 2007). However, PDZ proteins MAGI-2 and Cal have both been shown to decrease cell surface expression of the β1AR, though through different mechanisms. While MAGI-2 promotes receptor internalization into endocytic vesicles, Cal retains the receptor within the Golgi apparatus (Xu et al., 2001; He et al., 2004). Thus, PDZ proteins are important regulators of GPCR function and a greater understanding of these interactions will not only further our understanding of GPCR function, but also potentially aid in the development of new pharmaceuticals.

1.4 Corticotropin-releasing factor receptors (CRFRs)

The corticotropin releasing factor receptors, CRFR1 and CRFR2, are class B GPCRs that share 70% amino acid sequence homology (Bale et al., 2002; Grigoriadis et al., 1996). However these receptors display differences in expression profiles, while CRFR1 is mainly expressed in the brain within regions such as the cerebral cortex, cerebellum, medial septum, and anterior pituitary, CRFR2 is mainly expressed in peripheral regions such as the heart and skeletal muscle (Bale et al., 2002). Thus, CRFR1 seems to be predominantly involved in regulating the physiological functions of the central nervous system.

Upon CRF activation, both receptors primarily signal through coupling via Gαs, which activates adenylyl cyclase, leading to increases in cAMP, and downstream activation of protein kinase A (PKA). The activation of PKA can lead to the activation of a diverse range of signalling molecules such as: guanylyl cyclase, transcription factor NF-kB, glycogen synthase kinase-3 and the Wnt/β-catenin pathway (Aggelidou et al., 2002; Khattak et al., 2010; Zhao & Karalis, 2002). Importantly, CRFR1 has also been shown to activate the MAPK/ERK signalling pathway (Grammatopoulos, 2012).

It has been shown CRFR1 internalization is initiated by binding of β-arrestin to GRK-phosphorylated receptors, which also acts to attenuate signalling of the receptor (Holmes et al., 2006). Upon internalization, the CRFR1-β-arrestin complex has been shown to act differently in various experimental settings. While some studies show that the two proteins form a stable complex which internalizes as one unit into endocytic vesicle, others show that β-arrestin dissociates and remains at plasma membrane, where it can then act as a scaffolding protein to facilitate signalling pathways such as the MAPK cascade (Holmes et al., 2006; Markovic et al., 2006; Perry et al., 2005; Punn et al., 2006; Rasmussen et al., 2004) Some studies have also suggested that internalization of CRFR1 can be independent of β-arrestin recruitment (Rasmussen et al., 2004). This indicates that the mechanisms of CRFR1 internalization are diverse; furthermore this versatility indicates that internalization of the receptor is not solely dependent on receptor activation and signalling. Upon internalization, CRFR1 initially colocalizes with GTPase Rab5 in early endosomes, and then transitions to Rab4 positive endosomes to allow the recycling of the receptor (Holmes et al., 2006).

CRFR1 has been shown to have a ten-fold higher affinity for CRF than CRFR2

(Dautzenberg & Hauger, 2002). CRF is a neuropeptide, commonly released as a response to stressors, which initiates the hypothalamic-pituitary-adrenal (HPA) axis (Bale & Vale, 2004). Any actual or perceived stressor causes the release of CRF from neurons, within the paraventricular nucleus in the hypothalamus, into the hypophyseal portal vein system towards the anterior pituitary. Here CRF acts on CRFRs to activate release of adrenocortictoropic hormone (ACTH) secretion into systemic circulation, which subsequently causes the adrenal cortex to synthesize and release glucocorticoids such as cortisol into the bloodstream (Bale & Vale, 2004). Following exposure to stress, cortisol acts to regulate glucose levels, ion transport, and the immune response (Tsigos & Chrousos, 2002). CRF is therefore important for coping with stressors and restoring homeostasis in the body. However, elevation in levels of CRF and chronic exposure to cortisol has been shown to correlate with anxiety disorders and depression, supported by evidence of increased levels of CRF within extra-hypothalamic brain regions in the post-mortem brains of depressed suicide victims (Arborelius et al., 1999; Austin et al., 2003; Gold et al., 1996; Holsboer, 1999). Thus, targeting the CRF system seems may be important for the treatment of mood disorders.

Perhaps, one of the most promising pharmacological targets for treatment of mood disorders includes the CRFR1. Antagonists for this receptor have been shown to have anxiolytic effects by decreasing the HPA response (Dautzenberg & Hauger, 2002; Mansbach et al., 1997). Furthermore, knockout studies done in mice show that deletion of the CRF receptors causes distinct phenotypes (Bale & Vale, 2004; Janssen & Kozicz, 2013). Mice deficient in CRFR1 display decreased levels of anxiety-like behavior and have an impaired stress response. Conversely, mice lacking the CRFR2 seemed to display

increased anxiety-like behavior and were hypersensitive to stress (Bale & Vale, 2004; Janssen & Kozicz, 2013).

The 5-HT2AR is another GPCR that has been implicated in regulation of mood disorders and mental illness, particularly depression (Catapano & Manji, 2007). Interestingly, it has previously been shown that the stimulation of CRFR1 with CRF results in enhanced signalling of the 5-HT_{2A}R, in both cell cultures and mouse prefrontal cortical neurons (Magalhaes et al., 2010). Specifically, infusion of both CRF and DOI, a 5-HT2AR agonist, in mice led to a significant increase in anxiety-related behavior that was not seen with DOI alone (Magalhaes et al., 2010). This sensitization of 5-HT_{2A}R signalling seems to be dependent on CRFR1 receptor endocytosis into endosomes, which allows for recruitment of an intracellular pool of 5-HT_{2A}R and thereafter rapid recycling to the membrane. Blocking these processes seems to eliminate receptor crosstalk (Magalhaes et al., 2010). Thus, this is a biochemical mechanism that relates CRF mediated stress with enhanced anxiety behaviours mediated through the 5-HT_{2A}R (Magalhaes et al., 2010).

While CRFR2 does not contain a PDZ binding motif, CRFR1 and 5-HT_{2A}R both contain PDZ binding motifs on their distal C-terminal tails (Romero et al., 2011). Crosstalk between the two receptors, which allows for CRFR1-mediated sensitization of 5-HT2R IP3 signalling, seems to be mediated through PDZ protein interactions (Magalhaes et al., 2010). When the PDZ binding motif on either receptor is deleted, it attenuates the CRFR1-dependent increases of 5-HT_{2A}R at the cell surface as well sensitization of 5-HT_2R signalling (Magalhaes et al., 2010). However, the identity of the PDZ protein mediating this crosstalk still remains unknown. Nonetheless, this

phenomenon seems to form a link between CRF-mediated stress responses to 5-HT_2R-mediated anxiety and depression (Magalhaes et al., 2010; Magalhaes et al., 2012).

This is supported by findings that suicide victims with depression also seemed to possess enhanced levels of CRF in serotonergic neurons within the raphe nuclei (Austin et al., 2003). This is of importance since it has also been found that CRF can activate serotonergic neurons in the raphe nuclei that contain efferents to the medial prefrontal cortex, to ultimately initiate anxiety-like behaviour (Meloni et al., 2008). Therefore, CRFR1 activation sensitizes 5-HT_2R-mediated anxiety behaviours in response to stress, and PDZ proteins may be important regulators of this process (Magalhaes et al., 2010). Taken together, PDZ protein regulation of CRFR1 may prove to play an integral role in initiation of mental illness, and therefore may foreseeably be a promising pharmacological target when treating psychiatric disease.

1.5 Regulation of CRFR1 and 5-HT_2R by PDZ proteins

It has thus far been determined that PDZ proteins are important regulators of GPCR signaling and trafficking properties. However, while the evidence for this is increasing, still very little is known about the mechanism behind this mode of regulation. Interestingly, it has previously been shown that PDZ protein PSD95 regulates trafficking properties of the GPCR CRFR1 (Dunn et al., 2016). While overexpression of GFP-PSD-95 in HEK293 cells reduces CRF-stimulated CRFR1 internalization, shRNA knockdown of endogenous PSD-95 expression increases CRFR1 endocytosis (Dunn et al., 2016). Deletion of the PDZ binding motif in CRFR1 (consisting of the three amino acids TAV), such that the receptor can no longer bind PDZ domain containing proteins, causes an

increase in CRFR1 internalization and prevents PSD-95-dependent regulation of HA-CRFR1 internalization (Dunn et al., 2016).

In the same study it was also shown that PSD95 antagonizes β-arrestin2 recruitment to CRF-agonist stimulated CRFR1 (Dunn et al., 2016). It was shown, through use of BRET, that overexpressing PSD95 decreases CRF-stimulated Barr2 recruitment to CRFR1 and reduces the maximal response for CRF-stimulated β-arrestin translocation. Conversely, shRNA knockdown of PSD95 was shown to increase β-arrestin2 recruitment to CRFR1 and enhance the maximal response of β-arrestin translocation (Dunn et al., 2016). Thus, it appears that PSD-95 antagonizes CRFR1 internalization by preventing β-arrestin interactions required for the endocytosis of the receptor, thereby providing a potential mechanism for PDZ protein regulation of GPCR trafficking (Dunn et al., 2016).

This form of PDZ protein regulation of GPCRs is also seen in a study done by Schmid and Bohn (2010), this time with the 5-HT2AR. In this study, 5-HT2AR was immunoprecipitated from the frontal cortex of both WT and βarr2-KO mice following 5-HTP agonist treatment. In WT mice, PSD-95 was displaced from the 5-HT2AR in response to agonist treatment and 5-HT2AR increased associations with βarrestin2 (Schmid & Bohn, 2010). However, for the Barr2 KO mice, in the absence of βarrestin2, PSD-95 was not displaced from the 5-HT2AR in response to agonist (Schmid & Bohn, 2010). This suggests that the interplay between βarrestin2 and PDZ protein PSD-95 may determine whether the receptor is internalized or remains on the cell surface through regulation of β-arrestin recruitment (Schmid & Bohn, 2010). Additionally, perhaps this does not only apply to GPCRs CRFR1 and 5-HT2AR, but is a general mechanism of regulation by PDZ proteins for GPCR trafficking and signalling.

1.6 Hypothesis and Objectives

This thesis will explore the hypothesis that PDZ domain-containing proteins interact with β-arrestin-2 to modulate arrestin recruitment and receptor trafficking. To test this hypothesis, our objectives were:

1. Determine if PDZ proteins are capable of interacting with β-arrestin2.
2. Determine the mechanism of interaction between PDZ proteins and β-arrestin2. What amino acid residues or protein interaction domains are involved for both proteins?
3. Determine whether this interaction is important for regulation of β-arrestin2 recruitment to GPCRs and trafficking of GPCRs.

Chapter 2: Materials and Methods

2.1 Materials

ECL Western blotting detection reagents were purchased from Biorad (Mississauga, ON, Canada). Rabbit anti-GFP antibody was obtained from Invitrogen/Life Technologies (Burlington, ON, Canada). Anti-Flag M2 Affinity Gel, rabbit anti-Flag antibody, and all other biochemical reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.2 Plasmids

HA-tagged CRFR1 was described previously (Holmes et al., 2006). GFP-PSD-95 was provided by Dr. Gregory Dekaban (Robarts Research Institute). His-MAGI-1 was provided by Dr. Randy Hall (Emory University, School of Medicine). YFP-PDZK1 was described previously (Walther et al., 2015). All FLAG and YFP tagged β-arrestin2 mutants were generated using site-directed mutagenesis with the Q5 site-directed mutagenesis kit (New England Biolabs). Primers used are outlined in Table 2.1.

2.3 Cell Culture and Transfection

Human embryonic kidney (HEK293) 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. Transfections were performed using a modified calcium phosphate method. Empty pcDNA3.1 vector was used to equalize the total amounts of plasmid cDNA used to transfect cells. 18 h post-transfection, cells were washed with phosphate-buffered saline (PBS). All experiments were conducted 48 h after the initial transfection.

Isoform	Forward Primer (5'→3')	Reverse Primer (5'→3')
K34Q	TCACTTGGACcaaGTGGATCCTG	TCCACAAAGTCACGCTTG
K34A	TCACTTGGACgcaGTGGATCCTG	TCCACAAAGTCACGCTTG
V54D	GGACCGGAAAgacTTTGTGACCCTC	TTCAAGTAGTCAGGATCC
R170E	GCTTATCATCgaaAAGGTACAGTTTGC	CGCACGGAGTTCCTTTTG
Q173L	CAGAAAGGTActgTTTGCTCCTG	ATGATAAGCCGCACGGAG
Q173A	CAGAAAGGTAgcgTTTGCTCCTGAGACAC	ATGATAAGCCGCACGGAG
F174L	AAAGGTACAGttgGCTCCTGAGAC	CTGATGATAAGCCGCACG
F174A	AAAGGTACAGgctGCTCCTGAGAC	CTGATGATAAGCCGCACG
A175F	GGTACAGTTTtttCCTGAGACACC	TTTCTGATGATAAGCCGC
A175G	GGTACAGTTTggtCCTGAGACAC	TTTCTGATGATAAGCCGCAC
A175L	GGTACAGTTTcttCCTGAGACACC	TTTCTGATGATAAGCCGC

Table 2.1. **Primers used for site directed mutagenesis of β-arrestin2.**

2.4 Co-immunoprecipitation

Transfected HEK 293 cells were seeded on 10 cm dishes the day before the experiment. Cells were lysed in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10 mg/ml leupeptin, and 5 mg/ml aprotinin) for 20 min on a rocking platform at 4ºC. Samples were collected into 1.5 mL Eppendorf tubes and centrifuged at 15,000 g for 15 min at 4 ºC to pellet insoluble material. A Bronsted-Lowry protein assay was performed and 400 μg of protein was incubated for 2-4 h at 4ºC with anti-FLAG beads. After incubation, beads were washed 3 times with cold lysis buffer and eluted with 100 μl of SDS loading buffer containing β-mercaptoethanol before being stored overnight at -20 °C. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted to identify co-immunoprecipitated GFP or YFP tagged PDZ proteins (rabbit anti-GFP, 1:1000). An additional Western blot was performed to examine FLAG-βarrestin2 (rabbit anti-FLAG, 1:1000) protein expression.

2.5 Western Blot Analysis

Eluted proteins were applied to 10% SDS-polyacrylamide gel electrophoresis (30% acrylamide mix, 1.5 M tris-HCl, 20% SDS, 10% ammonium persulfate and TEMED). Separated proteins were then transferred to nitrocellulose membranes that were blocked with 10% powdered milk in TBS for 1h. Membranes were then blotted overnight by incubation with the appropriate antibody at 4°C. Membranes were then washed at least three times with 1X TBS containing 0.05% Tween 20, and further incubated with a

horseradish peroxidase- conjugated secondary antibody (1:10,000) for 1h. Membranes were finally washed again with 1X TBS containing 0.05% Tween 20 three times before being incubated with enhanced chemiluminescence Western blotting detection reagents and visualized using a Chemidoc Imaging System.

2.6 Bioluminescent Resonance Energy Transfer

HEK 293 cells were co-transfected with the indicated cDNA using Lipofictamine 2000 into 96 well plates. β-arrestin was tagged with Renilla luciferase (Rluc) and used as the energy donor while CRFR1 was tagged with YFP and used as the energy acceptor. The reaction was then started, 48h after transfection, by the addition of Coelenterazine to each well at a final concentration of 5 μM. Cells were also treated with 500 nM CRF and the BRET ratio was determined over time. Furthermore, multiple concentrations of CRF were employed to create a dose–response curve of β-arrestin2 recruitment following 20 min stimulation. Signals were collected on a Synergy Neo2 plate reader (Thermo Fisher) using 460/40-nm (luciferase) and 540/25-nm (YFP) band pass filters. The BRET ratio was determined by calculating the ratio of light that passed by the 540/25 filter to that passed by the 460/40 filter.

2.7 Protein Alignments and Figures

Structural and sequential alignments of proteins were determined using the web program, protein BLAST with the Molecular Modeling Database (MMDB) (http://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdb.shtml). Cn3D 4.3 (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3dmac.shtml) was used to display the structures. All structural figures were generated using PyMOL (www.pymol.org).

2.8 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. All measurements are represented as mean ± SEM. Comparisons were performed using either a two-tailed student's t test or a one-way analysis of variance test (ANOVA) that was followed by a post-hoc Dunnett's multiple comparisons test to determine significance. * indicates a P values less than 0.05 and is considered to be significant.

Chapter 3: Results

3.1 PDZ proteins interact with β-arrestin2

Many PDZ proteins have been documented to regulate GPCR trafficking properties (Dunn & Ferguson, 2015). It has also previously been shown that internalization of many receptors is β-arrestin dependent, including CRFR1 (Holmes et al., 2006; Moore et al., 2007). The PDZ protein PSD-95 has been shown to regulate β-arrestin2 recruitment and thereby receptor trafficking properties for GPCRs CRFR1 and 5-HT2AR (Dunn & Ferguson, 2015; Dunn et al., 2016; Schmid & Bohn, 2010). CRFR1 endocytosis has also previously shown to be antagonized, in PDZ-motif dependent manner, by PDZ proteins SAP97 and CAL (Dunn et al., 2013; Hammad et al., 2015). Meanwhile, 5-HT2AR endocytosis has previously been shown to be supressed by PDZ proteins PSD95, SAP97 and PDZK1 (Dunn et al., 2014; Walther et al., 2015; Xia et al., 2003). Therefore, to further understand PDZ protein regulation of GPCRs, and whether this is mediated by interactions with β-arrestin2, we sought to further characterize the interaction between the two protein families. To do this, we performed a series of co-immunoprecipitation experiments in HEK293 cells. In these studies, we looked at the interaction between β-arrestin2 and various PDZ proteins that each contained a different assortment of protein interaction domains.

First, we assessed whether GFP-PSD95 could by co-immunoprecipitated with β-arrestin2 in HEK 293 cells that were co-transfected with FLAG-β-arrestin2 (Fig. 3.1). PSD-95 contains several protein interaction domains including three PDZ domains, an

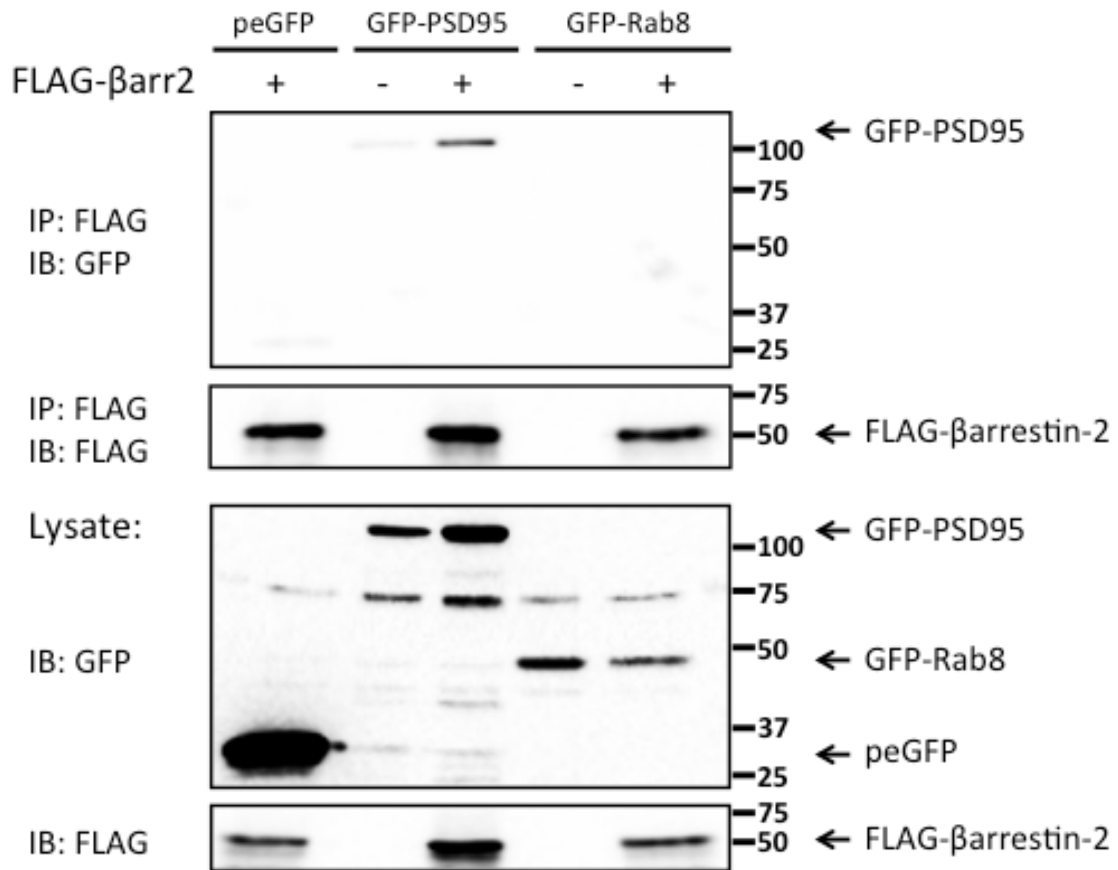

Figure 3.1: **GFP-PSD95 co-immunoprecipitates with FLAG-β-arrestin2 in HEK293 cells.** Representative immunoblot of FLAG-β-arrestin2 co-immunoprecipitated (IP) with GFP-PSD95 but not peGFP or GFP-Rab8. Transient transfections were performed in HEK 293 cells as labeled. Samples were run using SDS-PAGE and immunoblotted (*IB*) with rabbit anti-GFP and rabbit anti-FLAG. Data are representative of three independent experiments.

SH3 domain, and a GK domain. We found that GFP-PSD95 interacted with FLAG-β-arrestin2 and therefore concluded one of the domains within PSD95 mediated the interaction. As a negative control, we assessed whether FLAG-β-arrestin2 could interact with peGFP or GFP-Rab8 and found it did not interact with either.

We next assessed whether YFP-MAGI1 could by co-immunoprecipitated with β-arrestin2 in HEK 293 cells that were co-transfected with FLAG-β-arrestin2 (Fig. 3.2). MAGI1 also contains several protein interaction domains including six PDZ domains, a GK domain, and two WW domains. While MAGI1 contains PDZ domains and a GK domain similar to PSD-95, it does not contain an SH3 domain. We found that YFP-MAGI1 also interacted with FLAG-β-arrestin2 and therefore concluded that PDZ protein interaction with β-arrestin2 must not be mediated through the SH3 domain. FLAG-β-arrestin2 did not interact with negative controls peGFP or GFP-Rab8.

Finally, we assessed whether YFP-PDZK1 could by co-immunoprecipitated with β-arrestin2 in HEK 293 cells that were co-transfected with FLAG-β-arrestin2 (Fig. 3.3). The only protein interaction domains in PDZK1 are PDZ domains, to be exact four of them. We found that YFP-PDZK1 also interacted with FLAG-β-arrestin2. Since PDZK1 only contains PDZ domains, this suggests that that the interaction between PDZ proteins and β-arrestin2 is mediated through the PDZ domain. FLAG-β-arrestin2 did not interact with negative controls peGFP or GFP-Rab8.

To further determine and characterize the interaction between β-arrestin2 and PDZ proteins, which seemed to be mediated through the PDZ domain, we assessed whether FLAG-β-arrestin2 could co-immunoprecipitated with any of the four isolated

Figure 3.2. **YFP-MAGI1 co-immunoprecipitates with FLAG-β-arrestin2 in HEK293 cells.** Representative immunoblot of FLAG-β-arrestin2 co-immunoprecipitated (IP) with YFP-MAGI1 but not peGFP or GFP-Rab8. Transient transfections were performed in HEK 293 cells as labeled. Samples were run using SDS-PAGE and immunoblotted (*IB*) with rabbit anti-GFP and rabbit anti-FLAG. Data are representative of three independent experiments.

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Figure 3.3. **YFP-PDZK1 co-immunoprecipitates with FLAG-β-arrestin2 in HEK293 cells.** Representative immunoblot of FLAG-β-arrestin2 co-immunoprecipitated (IP) with YFP-PDZK1 but not peGFP or GFP-Rab8. Transient transfections were performed in HEK 293 cells as labeled. Samples were run using SDS-PAGE and immunoblotted (*IB*) with rabbit anti-GFP and rabbit anti-FLAG. Data are representative of three independent experiments.

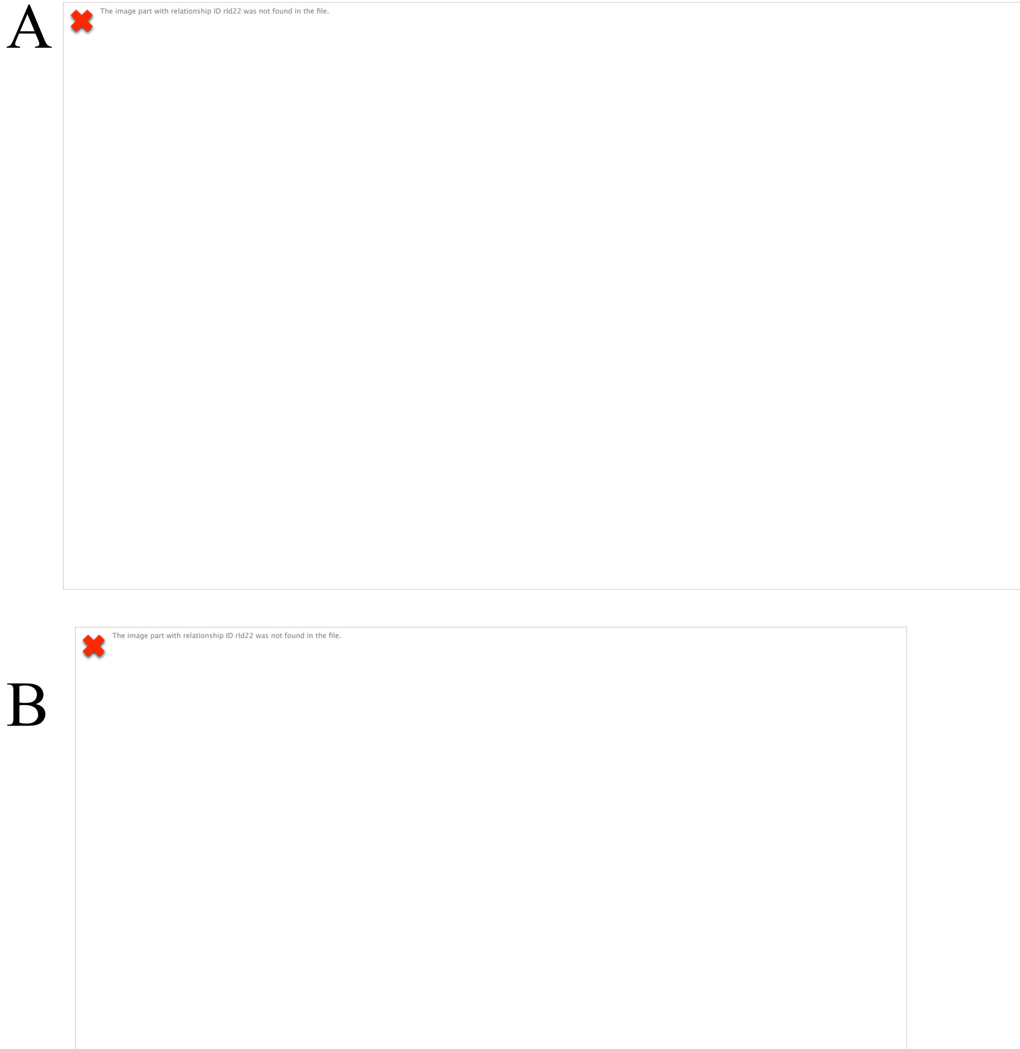


Figure 3.4. **The first PDZ domain of YFP-PDZK1 co-immunoprecipitates with FLAG-β-arrestin2 in HEK293 cells.** (A) Representative immunoblot of FLAG-β-arrestin2 co-immunoprecipitated (IP) with YFP-PDZK1 and YFP-PDZ1 but not YFP-PDZ2, YFP-PDZ3 or YFP-PDZ4. Transient transfections were performed in HEK 293 cells as labeled. Samples were run using SDS-PAGE and immunoblotted (*IB*) with rabbit anti-GFP and rabbit anti-FLAG. (B) Immunoblots were analyzed by densitometry. Co-immunopreciptated PDZ domains were compared to full length PDZK1. Statistical significance is assessed by one-way ANOVA followed by Dunnett's multiple comparisons post hoc test (*$p < 0.05$). Data are averaged means ± S.E.M. of three independent experiments.

YFP-tagged PDZ domains within PDZK1 (Fig. 3.4). This would not only further confirm that this interaction occurs through the PDZ domain, but also determine the specificity of the interaction and if it occurs through multiple domains. We found that FLAG-β-arrestin2 interacted with only the first PDZ domain of PDZK1, indicating that this domain may be important in mediating the interaction between β-arrestin2 and PDZK1. Therefore, we have shown that multiple PDZ proteins are capable of interacting with β-arrestin-2, and that PDZ proteins may mediate this interaction via the PDZ domain.

3.2 Characterization of residues within β-arrestin2 important for PDZ-arrestin interactions

After previously looking at PDZ proteins, we next tried to determine what regions or amino acids of β-arrestin-2 mediate arrestin-PDZ interactions. It has previously been described that while there is little sequence similarity between Vps26 and arrestin family members, the two protein families are structurally homologous (Shi et al., 2006). To further explore the extent of similarity between Vps26A and arrestin family members, we performed sequential and structural alignments summarized in Table 3.1. Structural similarity was defined by average root mean square deviation (RMSD) in Angstroms (Å), while sequence similarity was defined by % of similar amino acid residue identity. Alignment of the amino acid sequence of mouse Vps26A showed low sequence homology with bovine β-arrestin-2, β-arrestin-1, and S-arrestin as well as with cone arrestin from *ambystoma tigrinum*. However, all arrestin family members showed a high degree of structural similarity to mouse Vps26A, indicated by a low RMSD. The highest degree of structural similarity seemed to be between Vps26A and β-arrestin-2, with a RMSD of 1.94 Å (Table 3.1).

Arrestin Proteins aligned with VPS26A (PDB: 4P2A)	**PDB ID**	**RMSD**	**Sequence Homology**
β-arrestin-2 (arrestin-3)	3P2D	1.94 Å	11%
Cone arrestin (arrestin-4)	1SUJ	2.16 Å	10%
S-arrestin (arrestin-1)	3UGX	2.27 Å	9%
β-arrestin-1 (arrestin-2)	1G4R	2.31 Å	11%

Table 3.1. **Structural and sequential alignment of mouse Vps26A protein with arrestin family members.**

PDZ protein sorting nexin 27 (SNX27) was previously been shown to mediate sorting of transmembrane cargo from endosomes to the plasma membrane (Lauffer et al., 2010). The crystal structure of this protein determined that the PDZ domain of SNX27 bound the retromer subunit Vps26A (Gallon et al., 2014). Since Vps26A bound to the PDZ domain of SNX27, and β-arrestin2 had close structural to Vps26A, it could be extrapolated that perhaps β-arrestin2 also interacted with the PDZ domain of SNX27, as well as other PDZ proteins. To explore this further, we did a structural alignment between Vps26A and β-arrestin2, the most structurally similar arrestin to Vps26A. Shown in Figure 3.5 are the crystal structures of mouse Vps26A bound to the PDZ domain of rat SNX27 (PDB: 4P2A) and docked with the crystal structure bovine β-arrestin2 (PDB: 3P2D). Summarized in Table 3.2 are important residues within mouse VPS26A thought to be important for interaction with rat SNX27 (Gallon et al., 2014). Also summarized are the structurally analagous amino acids within bovine β-arrestin2, determined from the structural alignment. These residues as observed in Figure 3.6 A-C, are highlighted in yellow within the crystal structures of the two proteins.

To determine which β-arrestin-2 residues were important for interaction with PDZ proteins, we created β-arrestin-2 substitution mutants of the residues determined to be structurally analogous to Vps26A residues important for interaction with SNX27 (Table 3.2). To assess how these mutations affected β-arrestin2 interactions with PDZ proteins, we performed co-immunoprecipitation experiment with the β-arrestin2 mutants and PDZ protein PSD95 (Fig. 3.7). Along with β-arrestin2 substitution mutants K34Q, Q173L, F174L, and A175F presumed to mediate PDZ domain interactions, we also tested

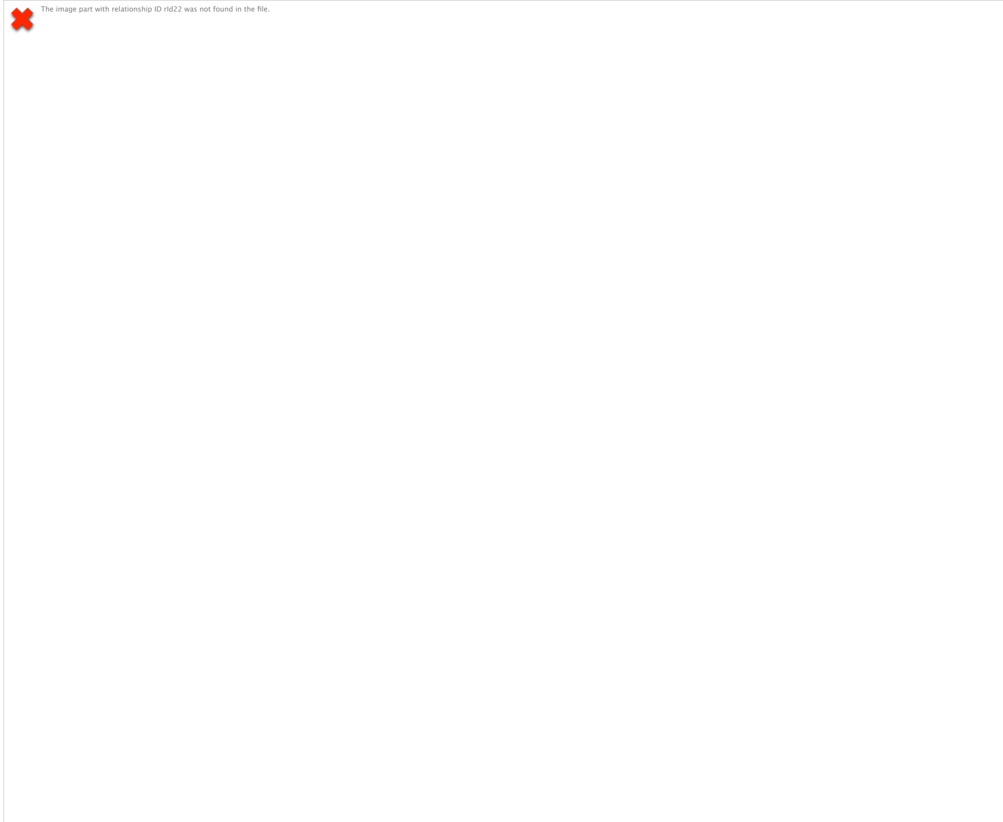


Figure 3.5. **Structural similarities between mouse Vps26 and bovine β-arrestin2.** Vps26A is shown in a ribbon model in blue, β-arrestin-2 is shown in a ribbon model in magenta, and the PDZ domain of SNX27 is shown in a ribbon model in green. Mouse Vps26A bound to the PDZ domain of rat SNX27 was structurally aligned with bovine β-arrestin-2. All structural figures with generated with Pymol (www.pymol.org).

Amino acid residues in mouse Vps26A important for interaction with rat SNX27	Structurally analogous amino acid residues within bovine β-arrestin2
44 D	34 K
153 Q	173 Q
154 L	174 F
155 A	175 A

Table 3.2. **Summary of important residues within mouse Vps26A important for interaction with rat SNX27 and the structurally analogous amino acids within bovine β-arrestin2.**

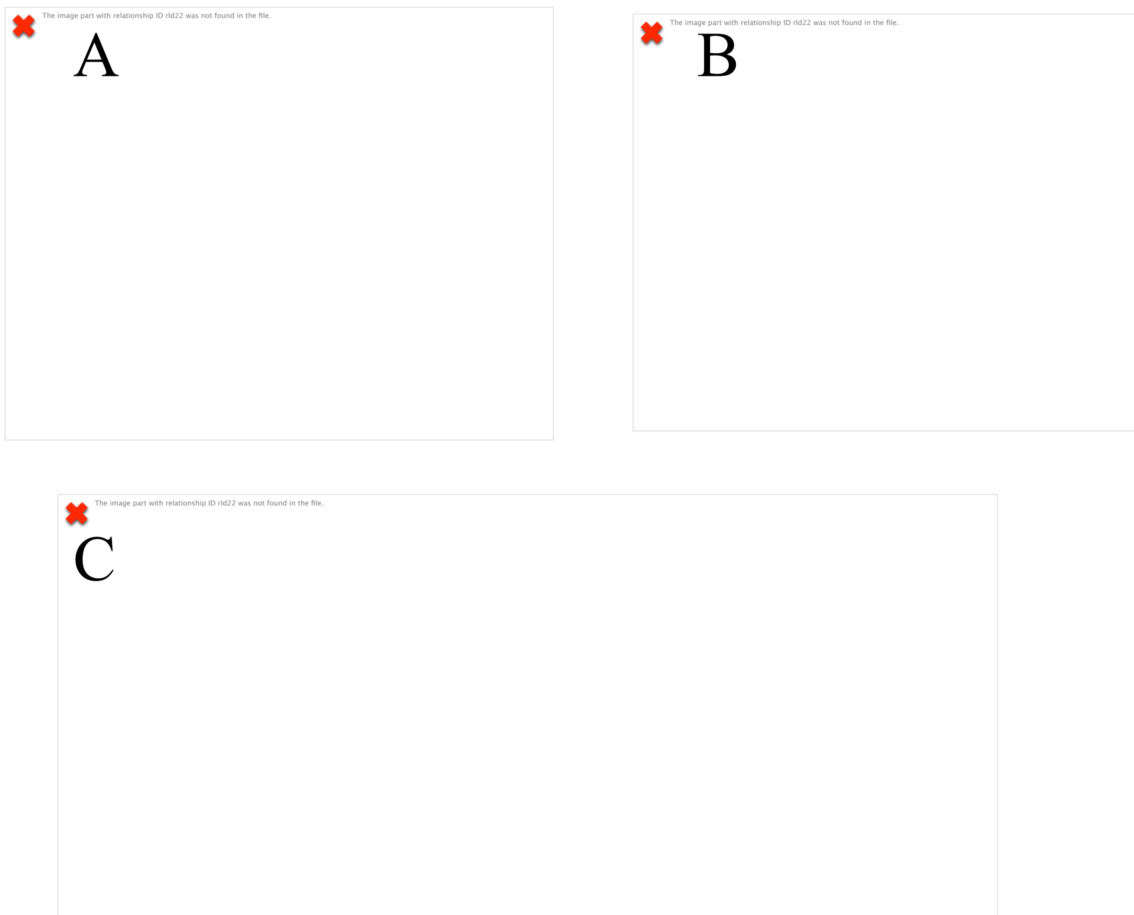
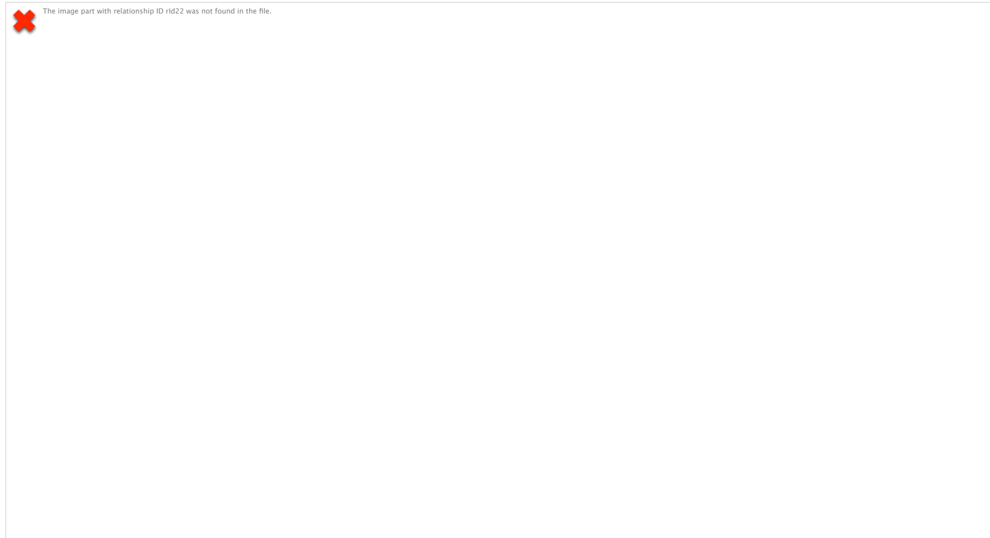


Figure 3.6. **Crystal structure of amino acid residues within mouse VPS26A important for interaction with rat SNX27 and the structurally analogous residues within bovine β-arrestin2.** Vps26A is shown in a ribbon model in blue, β-arrestin-2 is shown in a ribbon model in magenta, and the PDZ domain of SNX27 is shown in a ribbon model in green. **(A)** Crystal structure of mouse Vps26A bound to the PDZ domain of rat SNX27. Highlighted in yellow are the residues within Vps26A thought to be important to mediate this interaction (outlined in Table 2). **(B)** The crystal structure of mouse Vps26A bound to the PDZ domain of rat SNX27 is structually aligned with bovine β-arrestin-2. Highlighted in yellow are the residues within Vps26A thought to be important to mediate the interaction with SNX27 as well as structually analogous residues within β-arrestin-2 (outlined in Table 2). **(C)** A close up image of the residues highlighted in yellow for both Vps26 and β-arrestin2.

A



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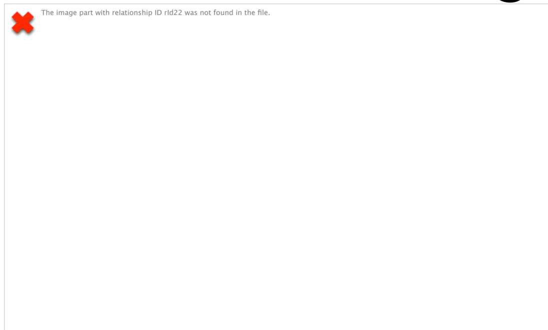

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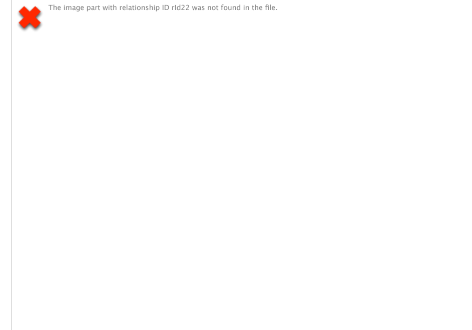

Figure 3.7. **FLAG-β-arrestin2 mutants V54D and R170E increase co-immunoprecipitation of GFP-PSD95.** (A) Representative immunoblot of GFP-PSD95 co-immunoprecipitated (IP) with FLAG-β-arrestin2 and mutants forms of FLAG-β-arrestin2 with substitutions K34Q, V54D, R170E, Q173L, F174L, and A175F. Transient transfections were performed in HEK 293 cells as labeled. Samples were run using SDS-PAGE and immunoblotted (*IB*) with rabbit anti-GFP and rabbit anti-FLAG. (B and C) Immunoblots were analyzed by densitometry to quantify amount of PSD-95 co-immunoprecipitated to β-arrestin2 mutants compared to wild type (WT) β-arrestin2. Data represents the mean ± SD of four independent experiments. *, $p < 0.05$.

β-arrestin2 mutants previously documented in literature for a point of reference. This includes β-arrestin2 V54D, a dominant negative form of β-arrestin2 (Ferguson et al., 1996), and β-arrestin2 R170E which is a constitutively active form of β-arrestin2 (Kovoor et al., 1999). Substitution mutants V54D and R170E showed significantly increased interaction with PSD95 compared to wild-type β-arrestin2. Substitution mutants K34Q, Q173L, and F174L showed no significant differences in interaction with PSD95. Although substitution mutant A175F didn't show a significant difference in co-immunoprecipitation with PSD95, there appeared to be a trend towards a decrease (Fig. 3.7B). This was confirmed when follow-up statistical testing with an unpaired two-tailed t-test revealed that β-arrestin2 A175F significantly decreased interaction with PSD-95 compared to wild-type β-arrestin2 (Fig. 3.7C). This indicated residue A175 was important for mediating β-arrestin2 interaction with PSD95.

3.3 Modifying arrestin-PDZ interactions impairs β-arrestin2 recruitment to CRFR1

It was previously demonstrated that while CRFR1 internalization was β-arrestin-dependent, multiple PDZ proteins could antagonize endocytosis (Dunn et al., 2016, 2013; Hammad et al., 2015). This included PDZ protein PSD95, which was demonstrated to antagonize β-arrestin2 recruitment to CRFR1 (Dunn et al., 2016). Therefore, our studies looked to further explore whether PDZ interacts with β-arrestin2 contributed to the regulation of β-arrestin2 recruitment to CRFR1. To do this we tested the previously described β-arrestin2 mutants ability to interact with the CRFR1. We tested how CRF agonist stimulation would affect both recruitment over time and the maximal response. It was found that while mutants V54D, R170E, Q173L, and A175F decreased CRF-

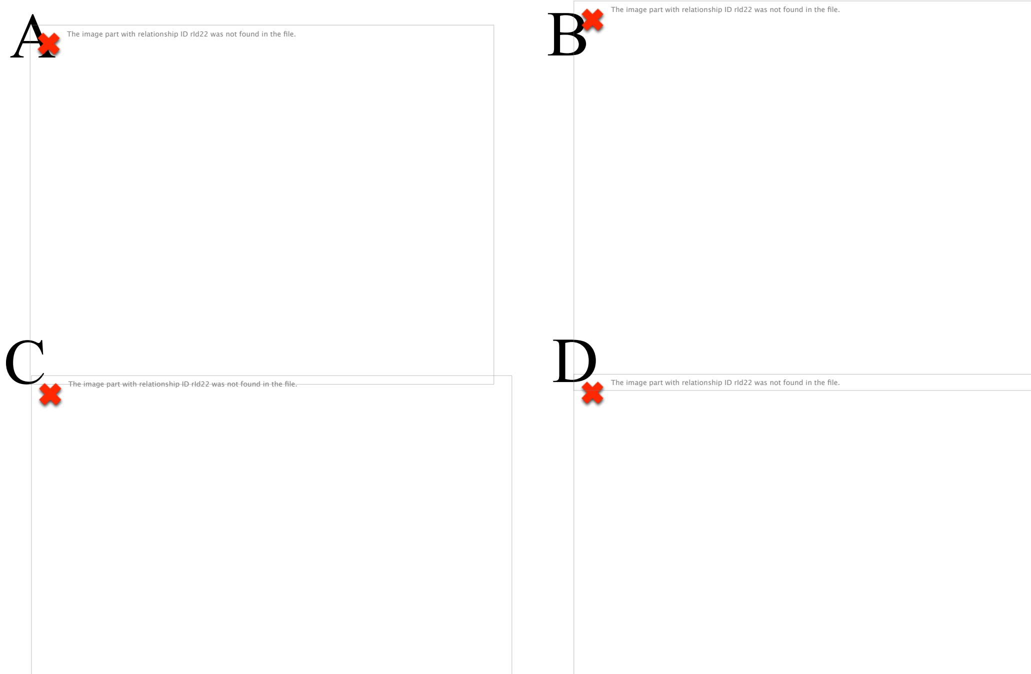

Figure 3.8. **Modifying arrestin-PDZ interactions impairs agonist-stimulated β-arrestin2 recruitment to CRFR1. (A)** Bioluminescent Resonance Energy Transfer (BRET) was employed to quantify the recruitment of β-arrestin2 as well as mutant forms K34Q, V54D, and R170E to CRFR1. An initial BRET ratio was calculated followed by various concentrations of CRF. BRET ratios are shown at 20 minutes post stimulation. The data are representative of the mean ± SEM of 4 independent experiments. **(B)** BRET readings correspond to the recruitment of β-arrestin2 as well as mutant forms K34Q, V54D, and R170E to CRFR1. An initial BRET ratio was calculated followed by 500 nM CRF stimulation. BRET ratios were repeatedly calculated following CRF stimulation and plotted over time. The data are representative of the mean ± SEM of 4 independent experiments. **(C)** BRET readings correspond to the recruitment of β-arrestin2 as well as mutant forms Q173L, F174L, and A175F to CRFR1. An initial BRET ratio was calculated followed by various concentrations of CRF. BRET ratios are shown at 20 minutes post stimulation. The data are representative of the mean ± SEM of 4 independent experiments. **(D)** BRET readings correspond to the recruitment of β-arrestin2 as well as mutant forms Q173L, F174L, and A175F to CRFR1. An initial BRET ratio was calculated followed by 500 nM CRF stimulation. BRET ratios were then repeatedly calculated following CRF stimulation and plotted over time. The data are representative of the mean ± SEM of 4 independent experiments.

stimulated β-arrestin2 recruitment to HA-CRFR1 over time, mutants K43Q, and F174L had similar levels of CRF-stimulated β-arrestin2 recruitment to HA-CRFR1 compared to control (Figure 3.8 B and D). To further validate our findings, we assessed translocation of these β-arrestin2 mutants in response to increasing concentrations of CRF under the same conditions. We found that while mutants K43Q and F174L had a similar maximal response for CRF-stimulated β-arrestin translocation compared to control, mutants V54D, R170E, Q173L, and A175F reduced the maximal response for CRF-stimulated β-arrestin translocation (Figure 3.8 A and C). Therefore, since β-arrestin2 substitution mutant A175F was previously also shown to have impaired binding to PSD-95, perhaps the decrease in recruitment to CRFR1 is due to impaired interaction with PDZ proteins.

To further validate the previously created β-arrestin2 mutants K34Q, Q173L, F174L, and A175F, we recreated the mutants with different amino acid substitutions and made sure any functional changes seen with the mutants couldn't be attributed to disruptions in protein folding. For mutant A175F, we also created substitutions with glycine and leucine to make A175G and A175L. For mutants K34Q, Q173L, and F174L we substituted all of them with alanine to create K34A, Q173A, and F174A. We then tested how CRF agonist stimulation would affect both recruitment over time and maximal response of all the new mutants. We found that mutants K34Q and K34A, as well as F174L and F174A, showed no changes in CRF-stimulated β-arrestin2 recruitment to HA-CRFR1 over time and no changes in maximal response for CRF-stimulated β-arrestin translocation compared to control (Fig 3.9 A, B, E, F). While mutant Q173A also showed no changes in recruitment to HA-CRFR1 over time or maximal response, mutant Q173L

seemed to decrease recruitment to HA-CRFR1 over time as well as the maximal response (Fig 3.9 C, D). While mutant A175G also showed no changes in recruitment to HA-CRFR1 over time or maximal response, mutants A175L and A175F seemed to decrease recruitment to HA-CRFR1 over time as well as the maximal response (Fig 3.9 G, H). Therefore β-arrestin2 mutants A175F and A175L, but not A175G, show impaired agonist-stimulated recruitment to CRFR1.

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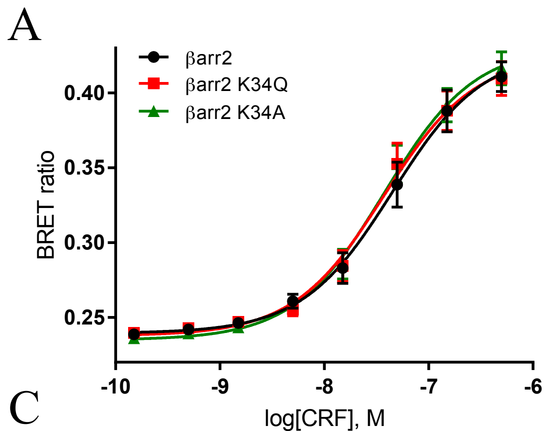

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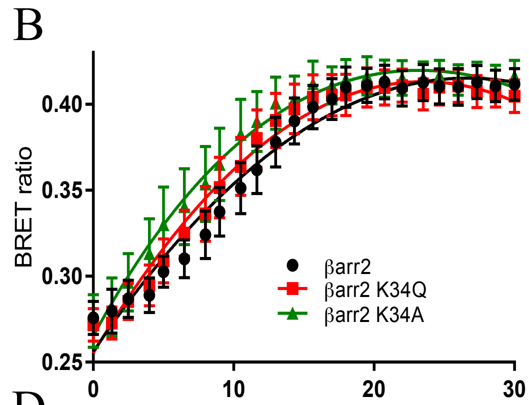

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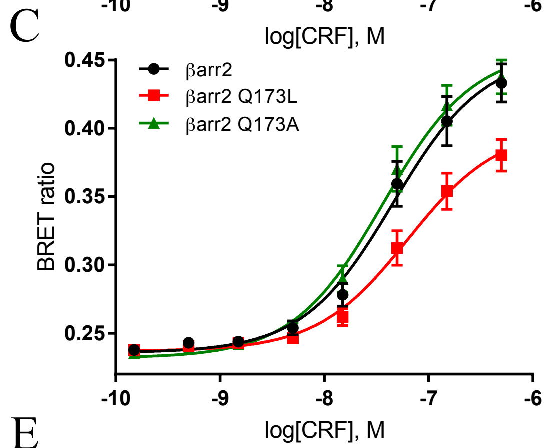

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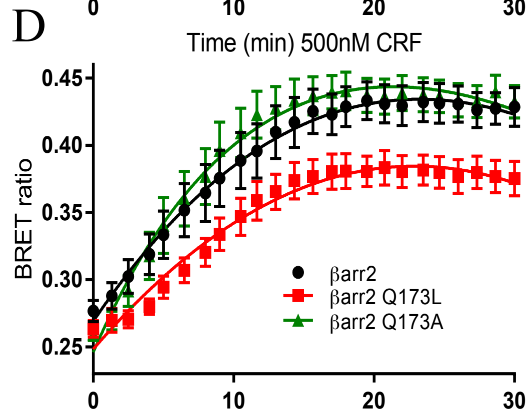

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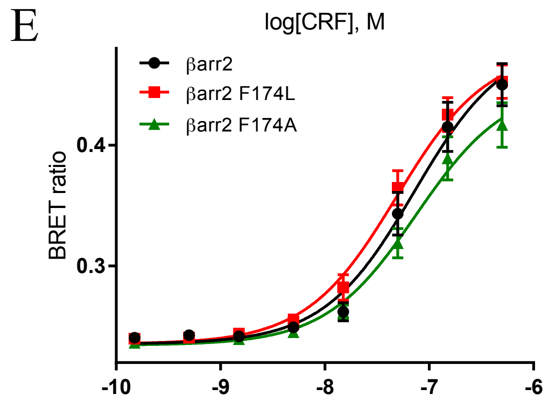

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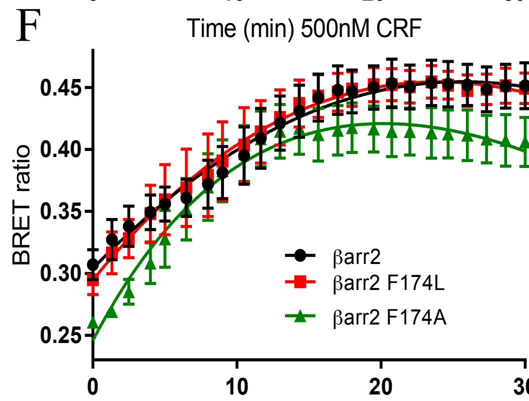

G

H

Figure 3.9. **β-arrestin2 mutants A175F and A175L, but not A175G, show impaired agonist-stimulated recruitment to CRFR1. (A and B)** Bioluminescent Resonance Energy Transfer (BRET) was employed to quantify the recruitment of β-arrestin2 as well as mutant forms K34Q and K34A to CRFR1. The data are representative of the mean ± SEM of 4 independent experiments. **(C and D)** BRET readings correspond to the recruitment of β-arrestin2 as well as mutant forms Q173L and Q173A to CRFR1. The data are representative of the mean ± SEM of 4 independent experiments. **(E and F)** BRET readings correspond to the recruitment of β-arrestin2 as well as mutant forms F174L and F174A to CRFR1. The data are representative of the mean ± SEM of 4 independent experiments. **(G and H)** BRET readings correspond to the recruitment of β-arrestin2 as well as mutant forms A175G, A175L, and A175F to CRFR1. The data are representative of the mean ± SEM of 4 independent experiments. **(A, C, E, G)** An initial BRET ratio was calculated followed by various concentrations of CRF. **(B, D, F, H)** An initial BRET ratio was calculated followed by 500 nM CRF stimulation. BRET ratios were repeatedly calculated following CRF stimulation and plotted over time.

Chapter 4: Discussion

GPCRs form an important family of proteins due to their widespread distribution in the body and essential role in regulating many different physiological functions that control homeostasis within the body. It is thus crucial to understand the signalling and trafficking properties of each receptor, as well as the proteins responsible for regulating these processes. Though much research has been dedicated to understanding GPCRs, many questions have not yet fully been answered. Although the regulation of GPCR trafficking properties by scaffolding proteins such as PDZ proteins and arrestins have been widely documented, the complex relationship between the two protein families has yet to be fully characterized. In addition, the mechanism of PDZ protein regulation for GPCR trafficking properties is not fully understood. This project thus focuses on further understanding how PDZ proteins regulate GPCR trafficking and whether it occurs through interactions with β-arrestin2. This is of importance since a further characterization of this interaction could lead to the development of novel pharmacological targets. The first key finding of this study is that multiple PDZ domain containing proteins are capable of interacting with β-arrestin2 via the PDZ domain. Secondly, it was determined that β-arrestin2 substitution mutant A175F shows impaired interaction with PDZ protein PSD-95, thus highlighting the importance of the residue in mediating PDZ-arrestin interactions. Finally, it was found that modifying arrestin-PDZ interactions impairs agonist-stimulated β-arrestin2 recruitment to CRFR1.

4.1 Association between PDZ proteins and β-arrestin2

This present thesis demonstrates the novel association of β-arrestin2 with PDZ proteins PSD-95, MAGI1, and PDZK1. These interactions were confirmed through co-

immunoprecipitation experiments in HEK293 cells (Figure 3.1, 3.2, and 3.3). Since the only shared protein interaction domain between all three PDZ proteins is the PDZ domain, this suggested that PDZ-arrestin interactions are mediated through the PDZ domain. This was further confirmed with our next experiment that looked at the interaction between β-arrestin2 and the four PDZ domains of PDZK1 (Figure 3.4). We demonstrated that only the first PDZ domain of PDZK1 interacts with β-arrestin2 and thus may be important for mediating the interaction. Furthermore, at least for PDZK1, this implies that the interaction occurs through a single domain and not multiple domains. Taken together, these findings provide new functional significance for the interactions between β-arrestin proteins and PDZ proteins. It has recently been shown that the Endothelin A receptor (ET_AR) regulates formation of invadopodia in cancer cells through RhoC activity, which in turn is regulated by a direct interaction between β-arrestin1 and PDZ domain containing protein PDZ-RhoGEF (Bagnato & Rosanò, 2016; Semprucci et al., 2016). However, the mechanism of interaction remains unclear and it remains to be determined what role the PDZ domain plays in mediating this interaction (Bagnato & Rosanò, 2016; Semprucci et al., 2016). Nevertheless, this provides further evidence for direct PDZ protein interactions with β-arrestins.

Beyond the bounds of this current thesis, we would like to further characterize these novel interactions. Although it now seems likely that arrestin-PDZ interactions are mediated through the PDZ domain, future experiments could look at which amino acids or motifs within PDZ domains meditate this interaction. Of particular interest would be to determine the involvement of the GLGF motif present in PDZ domains. This motif has previously shown to facilitate hydrogen bond formation and binding of many ligands to

PDZ domains (Kalyoncu et al., 2010; Sheng & Sala, 2001). Another future means of characterizing arrestin-PDZ interactions would be to confirm whether this is a direct or indirect interaction. Since co-immunoprecipitation experiments can reveal both direct and indirect interactions, it is still possible that the interaction is in fact mediated through bridging proteins. Future experiments could use techniques such as GST-pull downs or FRET to confirm a direct interaction (Phizicky & Fields, 1995; Rao et al., 2014). Finally, it would be interesting to replicate these co-immunoprecipitation experiments in mouse brain tissue and test if these interactions can also take place with endogenously expressed proteins in physiologically relevant tissue.

4.2 Mechanism of interaction between PDZ proteins and β-arrestin2

It has previously been found that though Vps26A shows low sequence homology with the arrestin family, there is a high degree of structural similarity with arrestin proteins (Shi et al., 2006). We showed that, of all arrestin family members, β-arrestin2 showed the highest degree of structural similarity with Vps26A (Table 3.1 & Figure 3.5). This structural similarity is of importance because Vps26A has also been shown to interact with the PDZ domain of SNX27 (Gallon et al., 2014). Since Vps26A binds to the PDZ domain of SNX27, and is closely structurally aligned with β-arrestin2, we hypothesized that perhaps β-arrestin2 interacts with PDZ domain containing proteins in a similar manner. We tested this, by using its structural similarities to Vps26A, to create several point mutations in β-arrestin2 (Table 3.2 and Figure 3.6). We tested the ability of these mutants to bind PDZ proteins by comparing levels of co-immunoprecipitation with PSD-95 (Fig. 3.7). While mutants V54D and R170E immunoprecipitated with PSD-95 at

higher amounts compared to wild type β-arrestin2, A175F showed decreased interaction with PSD-95. This alanine residue at position 175 in bovine β-arrestin-2 is conserved in multiple other species as well as in Vps26A, thus indicating its potential importance. Gallon et al., (2014) describe that the structurally analogous residue of A175 in Vps26A, A155, is in L10 and involved in main chain contacts through stretches of intermolecular β-sheets with residues 65–66 preceding the β3-β4 hairpin within the SNX27 PDZ domain. This residue is part of Vps26A's short linker region that connects the two domains of Vps26A, the N-terminal domain (N domain) and C-terminal domain (C domain). This interdomain linker consists of a 15-residue loop, from 149 to 163, and has been found to be flexible as well as relatively mobile (Gallon et al., 2014; Shi et al., 2006). It has been determined that in visual arrestin this region is at position 179 to 191 Vishnivetskiy et al., 2002). This corresponds with amino acids from position 173 to 185 in β-arrestin2, and it contains A175. Since β-arrestin2 A175F did not completely abolish interaction with PSD-95, perhaps it is part of several amino acids in this region important for mediating interactions with PDZ proteins. It would be interesting in future studies to create double and triple amino acid mutant forms of β-arrestin2 and observe how that would effect PDZ protein binding.

Although β-arrestin2 does not contain a canonical PDZ binding motif, it is possible it contains an internal PDZ binding motif. Evidence suggests that some PDZ domains may bind to internal PDZ binding motifs contained within target proteins (Harris & Lim, 2001; Trejo, 2005). Though the sequence of the internal PDZ binding motifs remains to be determined, it has been shown that many of them possess a sharp β-turn similar in structure to canonical motifs often found on the C-terminals of ligands

(Paasche et al., 2005). Therefore, further experiments are needed to determine if β-arrestin uses an internal PDZ binding motif to bind PDZ domains.

4.3 Role of arrestin-PDZ interactions in β-arrestin2 recruitment to CRFR1

Due to the potential role of arrestin-PDZ interactions in mediating PDZ protein regulation of GPCR trafficking, we next moved on to explore the effects of arrestin-PDZ interactions on β-arrestin2 recruitment to CRFR1. We also wanted to further characterize the previously created β-arrestin2 substitution mutants and how they would affect arrestin-PDZ interactions. β-arrestin2 mutants K34Q and F174L showed no change in CRF-stimulated β-arrestin2 recruitment to CRFR1 and no change in the maximal response for CRF-stimulated β-arrestin translocation (Figure 3.8). However, mutants V54D, R170E, Q173L, and A175F all showed a decrease in both CRF-stimulated β-arrestin2 recruitment to CRFR1 and maximal response for CRF-stimulated β-arrestin translocation (Figure 3.8). Therefore these residues may be important for β-arrestin2 recruitment to CRFR1. Combined with data that shows decreased interaction of PSD-95 with β-arrestin2 A175F (Figure 3.7), it implicates that residue A175, perhaps in combination with other nearby amino acids, might be integral for association of β-arrestin2 with PDZ proteins and thereby modulates β-arrestin2 recruitment and GPCR endocytosis.

To further investigate the β-arrestin2 mutants K34Q, Q173L, F174L, and A175F, we recreated additional mutants with different amino acid substitutions (Figure 3.9). While mutant A175G showed no changes in its recruitment to CRFR1, mutants A175L and A175F both showed decreased recruitment to CRFR1. A similar recruitment level to

CRFR1 between A175L and A175F verifies that any functional changes seen with mutant A175F are due to change in amino acid and not disruption of the protein backbone of β-arrestin-2. It is possible that no change in recruitment was seen with A175G because glycine is much smaller and closer to alanine, and therefore wasn't a big enough change from alanine to show any changes in arrestin recruitment. Alanine screening of residues K34 and F174 show no differences in β-arrestin2 recruitment to CRFR1, thus further confirming the validity mutants K34Q and F174L. However, while mutant Q173L showed decreased recruitment to CRFR1, Q173A showed no changes in recruitment to CRFR1. It is possible substituting the polar glutamine for a hydrophobic leucine somehow disrupted regular function of β-arrestin2, however further studies are needed.

It has previously been proposed that activation and binding of arrestin to GPCRs induces a conformation change in arrestin and movement of the N- and C- terminals (Gurevich and Gurevich 2004). These conformational rearrangements suggest that arrestin binds the receptor from the concave domains, while the convex side of arrestin is left to bind other various binding partners may that regulate trafficking and downstream signalling of GPCRs (Lohse & Hoffmann, 2014). In addition to inter-domain rearrangements, multiple movements in the arrestin loops have also been detected that are in part responsible for activation of arrestin and binding to the receptor (Shukla et al. 2013). The hinge loop that connects the N- and C- extremities of arrestin has been shown to be necessary for maintaining receptor/arrestin complexes in a stable conformation (Vishnivetskiy et al. 2002). This was shown when sequential deletion in the hinge region of visual arrestin resulted in a decreased ability to bind rhodopsin (Vishnivetskiy et al. 2002). While some studies propose arrestins change conformation in a "clamshell"-like

manner to wrap around the receptors (Gurevich and Gurevich 2004), in contrast, more recent studies suggest that the N- and C- terminal domains of the activated arrestin undergo a 20° rotation from the central axis and this twisting facilitates interaction with the receptor (Fig 4.1 and Fig 4.2) (Shukla et al. 2013). Either way, it seems flexibility and movement of the two domains is required for interaction with the receptor. Therefore, it is possible that binding of PDZ proteins in the flexible linker region may sterically inhibit any spatial movements or changes in conformation of β-arrestin. This also may serve to prevent β-arrestin interaction with GPCRs, and thereby receptor internalization (Fig 4.2C and D). This would also provide an alternative explanation for why mutant A175G shows no changes in recruitment to CRFR1 compared to mutants A175L and A175F. Glycine, though small and similar to alanine, is unusually flexible and allows for changes in conformation generally not allowed by other amino acids. Therefore, it is possible changing alanine to glycine didn't prevent the conformational changes in β-arrestin that changing to leucine or phenylalanine did.

Alternatively, other than conformational changes, it is also possible that binding of PDZ proteins to β-arrestins dictates the stability of the receptor–β-arrestin complex. GPCRs exhibit different patterns of agonist-induced β-arrestin interactions where, while some receptors form stable complexes with β-arrestin and internalize as a unit to

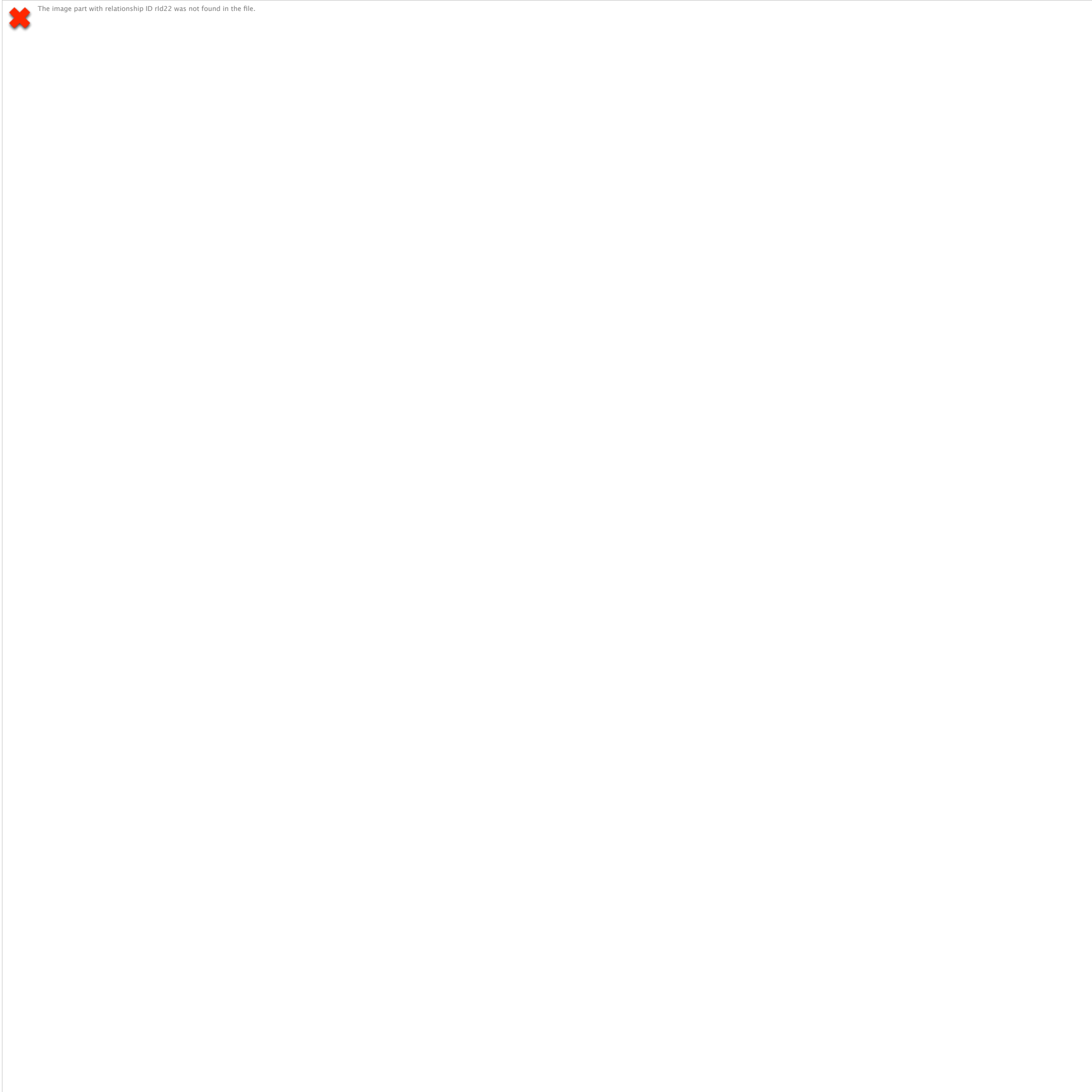

Figure 4.1 Conformational changes associated activation of β-arrestin-1.

(A) Shows the structural features of the finger, middle and lariat loops of the inactive (grey) form of arrestin and the conformational rearrangements associated to its active form (orange). (B) Representation of the 20° rotational movement of the arrestin when switching from an inactive (grey) to active (orange) form. (C) View of arrestin movement from the C-terminal domain (Modified from Shukla et al., 2013).

Figure 4.2 Proposed schematic of how PDZ protein and β-arrestin interactions regulate GPCR endocytosis. (A) β-arrestin does not bind GPCRs at the plasma membrane in its basal inactive conformation. (B) When switching to an active conformation, β-arrestin undergoes a 20° rotation along its central axis which allows it to bind GPCRs and cause receptor internalization. (C) It is possible that binding of PDZ proteins to β-arrestin prevents any spatial movements or changes in the conformation of β-arrestin such that it can no longer bind GPCRs. (D) It is thus possible that upon binding to GPCDs, PDZ proteins prevent GPCR endocytosis, through binding of β-arrestins and preventing their interaction with GPCRs.

endosomes, others bind β-arrestin transiently and dissociate after internalization of the receptor recycle back to the plasma membrane (Oakley et al., 2000; Zhang et al., 1999). The stability of the receptor–β-arrestin interaction might dictate the fate of the internalized receptor. Receptors that transiently bind β-arrestin seem to be more likely to recycle back to the plasma membrane whereas receptors that form stable interactions with β-arrestin seem to be more likely to be targeted to the lysosome for degradation (Luttrell & Lefkowitz, 2002). It is possible PDZ protein binding to β-arrestins regulates stability of the receptor–β-arrestin complex to modulate receptor recycling and degradation.

For future studies, it will be important to fully explore the how PDZ proteins and β-arrestins interact to regulate β-arrestin recruitment and receptor trafficking. There have been conflicting results as to what effect PDZ protein/β-arrestin interactions have on β-arrestin recruitment to receptors. PSD-95 has been shown to antagonize the recruitment of β-arrestin2 to GPCRs CRFR1, β_1AR, and 5-HT$_{2A}$R (Dunn et al., 2016; Hu et al., 2000; Xia et al., 2003). However, PDZ proteins may also facilitate the recruitment of β-arrestins to the receptor as has previously been demonstrated with PDZ protein NHERF1 interactions with CC chemokine receptor 5 (Hammad et al., 2010). This may be explained by receptor-specific functions or even variances due to cell-specific roles. It would be interesting in future studies to look at how the β-arrestin2 mutants affect recruitment to GPCRs other than CRFR1. Additionally, future experiments could use flow cytometry to look at how all the β-arrestin2 mutants affect agonist stimulated receptor endocytosis. Overall, further studies are required to better understand the relationship between these two important regulators of receptor trafficking.

4.4 Summary

This thesis elucidates the novel association between β-arrestin and PDZ domain containing proteins. More specifically, this interaction occurs via PDZ domains and regulates trafficking properties for GPCRs, including CRFR1, through modulation of β-arrestin2 recruitment. Pharmacologically targeting the interactions between β-Arrestin2 and the PDZ proteins could lead to specific regulation strategies for the numerous disorders associated with congenital or acquired disregulation of GPCR signaling. Rather than modulating neurotransmitter availability, we can begin to not only target specific receptors activated by these neurotransmitters, but also modulate specific signalling pathways or receptor trafficking processes that underlie a spectrum of mental illnesses including, though not limited to, anxiety, depression, and schizophrenia.

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