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Structural determinants governing β-arrestin2 interaction with PDZ proteins and recruitment to CRFR1

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ABSTRACT

β-Arrestins are multifunctional adaptor proteins best know for their vital role in regulating G protein coupled receptor (GPCR) trafficking and signaling. β-arrestin2 recruitment and receptor internalization of corticotropin-releasing factor receptor 1 (CRFR1), a GPCR whose antagonists have been shown to demonstrate both anxiolytic- and antidepressant-like effects, have previously been shown to be modulated by PDZ proteins. Thus, a structural characterization of the interaction between β-arrestins and PDZ proteins can delineate potential mechanism of PDZ-dependent regulation of GPCR trafficking. Here, we find that the PDZ proteins PSD-95, MAGI1, and PDZK1 interact with β-arrestin2 in a PDZ domain-dependent manner. Further investigation of such interaction using mutational analyses revealed that mutating the alanine residue at 175 residue of β-arrestin2 to phenylalanine impairs interaction with PSD-95. Additionally, A175F mutant of β-arrestin2 shows decreased CRF-stimulated recruitment to CRFR1 and reduced receptor internalization. Thus, our findings show that the interaction between β-arrestins and PDZ proteins is key for CRFR1 trafficking and may be targeted to mitigate impaired CRFR1 signaling in mental and psychiatric disorders.

1. Introduction

The Corticotropin-releasing factor receptors, CRFR1 and CRFR2, belong to secretin family of G protein-coupled receptors (GPCRs), couple $G_{\alpha s}$ and share 70% amino acid sequence homology, but have distinct cell and tissue expression patterns [1,2]. CRFR1 is mainly expressed in cerebral cortex, cerebellum, medial septum, and anterior pituitary while CRFR2 is mainly expressed in heart and skeletal muscle [1,3]. The neuropeptide CRF displays a ten-fold higher affinity for CRFR1 over CRFR2 [4] and is mainly released from hypothalamus in response to stressors to trigger the release of adrenocorticotropic hormone (ACTH) and increase blood cortisol levels [5,6]. Although CRF is crucial for coping with stress responses [7], elevated CRF levels has been shown to correlate with anxiety disorders and depression. This was supported by elevated CRF levels detected in post-mortem brains of depressed suicide victims [8–11]. Moreover, CRFR1 KO mice show an impaired stress response and CRFR1 antagonists have demonstrated anxiolytic and antidepressant-like effects [5,12–14]. Interestingly, stimulation of CRFR1 in both cell cultures and mouse prefrontal cortical neurons resulted in enhanced signaling of the serotonin 2A receptor (5-HT_{2A}R) [15], another GPCR that regulates anxiety and depressive-like behaviors [16]. Taken together, these findings suggest that CRFR1 can be a viable target for treatment of mood and anxiety disorders and further investigation of CRFR1-scafolding proteins that modulate signaling cascade and trafficking of the receptor is essential.

Postsynaptic density protein of 95 kilodaltons (PSD-95), disc large, zona occludens (PDZ) domain-containing proteins are one of the most abundant GPCR-interacting proteins and are important regulators of

Abbreviations: 5-HT_{2A}R, serotonin 2A receptor; ACTH, adrenocorticotropic hormone; CAL, cystic fibrosis transmembrane conductance regulator-associated ligand; CRF, corticotropin releasing factor; CRFR, Corticotropin-releasing factor receptor; ERK, extracellular signal-regulated kinase; GK, guanylate kinase–like; GPCR, G protein-coupled receptor; HA, hemagglutinin; HEK293, human embryonic kidney 293; MAGI, membrane-associated guanylate kinase protein; PDZ, PSD95/Disc Large/Zona Occluden; PDZK1, PDZ domain–containing kidney protein 1; PSD-95, Postsynaptic density protein of 95 kDa; RMSD, root mean square deviation; ROCK, Rho-associated coiled coil-forming kinase.; SAP97, synapse-associated protein 97; SH3, SRC homology 3; SNX27, PDZ protein sorting nexin 27; Vps26, Vacuolar protein sorting-associated protein 26

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GPCR trafficking, signaling, and cellular distribution [17–19]. Interestingly, PSD-95 has previously been linked to the psychiatric disorder schizophrenia [20,21] and has been recently characterized as one of CRFR1-interacting proteins that regulate trafficking and signaling properties of CRFR1 [22]. Overexpression of PSD-95 reduced CRF-stimulated β-arrestin2 recruitment and CRFR1 internalization while shRNA knockdown of PSD-95 increases β-arrestin2 recruitment and receptor endocytosis. Deletion of the PDZ binding motif, TAV motif, in CRFR1 increased CRFR1 internalization and blocked PSD-95-dependent regulation of CRFR1 internalization [22]. Thus, PSD-95 can antagonize CRFR1 internalization by preventing β-arrestin interactions required for receptor endocytosis. In another study, immunoprecipitation of 5-HT_{2A}R from the frontal cortex of agonist treated WT mice revealed a displacement of PSD-95 from the receptor complex and increased βarrestin2 binding. Interestingly, PSD-95 was not displaced from the 5-HT_{2A}R in response to agonist in β-arrestin2 KO mice [23]. This suggested that the interplay between βarrestin2 and PSD-95 modulates β-arrestin recruitment and 5-HT2AR endocytosis.

In addition to PSD-95, CRFR1 has been shown to interact with the PDZ containing proteins membrane-associated guanylate kinase protein 1 (MAGI1) and PDZ domain-containing kidney protein 1 (PDZK1) in a PDZ motif-dependent manner [17,24]. Overexpression of PDZK1 does not affect CRFR1 endocytosis, but results in increased CRFR1-stimulated ERK1/2 phosphorylation without affect in CRFR1-mediated cAMP formation [24]. In contrast, either the overexpression or the knockdown of MAGI-1 results in significant attenuation of CRFR1 internalization and alterations in MAGI-1 expression can affect β-arrestin recruitment to CRFR1 [25]. Taken together, the interaction between β-arrestin and PDZ proteins appears to be key for the regulation of trafficking and signaling of GPCRs. It is worth noting that β-arrestin interaction with Multi-PDZ Domain-containing Protein MPZ-1 in c. elegance has been described, however it remains elusive whether this interaction is evident in higher organisms/mammalian system [26]. Thus, understanding the topography of interaction between PDZ proteins and β-arrestin2 can provide insights into novel approaches to modulate GPCR signaling to treat various disorders, specifically mood and psychiatric disorders in case of CRFR1.

Here, we confirm that β-arrestin2 is capable of interacting with multiple PDZ proteins including PSD95, MAGI1 and PDZK1 and this interaction is mediated by the PDZ domain. We then employed structural alignment and mutagenesis analysis to determine the putative amino acid residues in β-arrestin2 required for interaction with PDZ proteins. Our findings indicate the alanine residue 175 of β-arrestin2 is crucial for interaction with PSD95. Moreover, A175 residue was essential for agonist-stimulated recruitment of β-arrestin2 to CRFR1 receptor. Specifically, mutating alanine residue 175 of β-arrestin2 to another amino acid with a longer or aromatic side chain leucine or phenylalanine respectively, inhibited the recruitment of β-arrestin2 to CRFR1 and reduced receptor internalization. Taken together, these results indicate that the formation β-arrestin/PDZ protein complexes may have an essential role in regulating the GPCR endocytosis and signaling transduction.

2. Materials and methods

2.1. Materials

Western blotting 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 [27]. 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 (K34Q, K34A, V54D, R170E, Q173L, Q173A, F174L, F174A, A175G, A175F, and A175L) were generated using site-directed mutagenesis with the Q5 site-directed mutagenesis kit (New England Biolabs).

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 for co-immunoprecipitation experiments 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 [28]. 18 h post-transfection, cells were washed with phosphate-buffered saline (PBS) and resuspended with trypsin, 0.25% EDTA. All experiments were conducted 48 h after the initial transfection.

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 4 °C. Samples were collected 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 2–4 h at 4 °C with anti-FLAG beads. After incubation, beads were washed three 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) and YFP-tagged (rabbit anti-GFP, 1:1000) protein expression in lysates prepared before incubation with beads [22].

2.5. Western blot

Eluted proteins were applied to 10% SDS-polyacrylamide gel electrophoresis. Separated proteins were then transferred to nitrocellulose membranes that were blocked with 10% powdered milk in TBS containing 0.05% Tween 20 (TBST) for 1 h. Membranes were then blotted overnight by incubation with rabbit anti-GFP at 4 °C. Membranes were then washed three times with 1× TBST, and further incubated with a horseradish peroxidase- conjugated secondary antibody (1:10,000) for 1 h. Membranes were finally washed again with 1× TBST three times before being incubated with enhanced chemiluminescence Western blotting detection reagents (Biorad) and visualized using a Chemidoc Imaging System (Biorad) [29].

2.6. Bioluminescent resonance energy transfer

HEK 293 cells were co-transfected with the indicated cDNA using Lipofectamine 2000 (Thermo Fisher) into 96 well plates. β-arrestin2 WT and β-arrestin2 mutants were tagged with Renilla luciferase (Rluc) and used as the energy donor while CRFR1 was tagged with yellow fluorescent protein (YFP) and used as the energy acceptor. The reaction was started, after 48 h of 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 (BioTek) 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 [30].

2.7. Receptor endocytosis

HEK 293 cells were reseeded into 12 well plates following transfection. Cells were serum-starved for 1 h at 37 °C in HBSS and then stimulated for 30 min with 500 nM CRF in HBSS at 37 °C. Cells were then 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 (Thermo fisher) (1:1000) for 45 min on ice. Cells were washed again 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 then 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. Less fluorescence corresponded with less membrane CRFR1 [25,30].

2.8. 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 software (www.pymol.org).

2.9. Statistical analysis

Densitometric data were normalized first for protein expression and the maximum value was set to 1, with all other values displayed as percentage thereof. All measurements are represented as mean ± SEM. Comparisons were performed using a one-way analysis of variance test (ANOVA) that was followed by a post-hoc Dunnett's multiple comparisons test to determine significance on GraphPad prims 7. *Indicates a P values $< .05$ and is considered significant.

3. Results

3.1. β-arrestin2 interaction with PDZ proteins is PDZ domain-dependent

CRFR1 endocytosis has previously shown to be regulated, in PDZ-motif dependent manner, by PDZ proteins synapse-associated protein 97 (SAP97), MAGI-1, PSD95 and cystic fibrosis transmembrane conductance regulator-associated ligand (CAL) [22,23,25,31,32]. Although PSD95 and MAGI proteins regulate β-arrestin-mediated internalization of CRFR1, the specific PDZ domain(s) required for interaction between β-arrestin2 and PDZ proteins remains unclear. Thus, we performed a series of co-immunoprecipitation experiments between β-arrestin2 and various PDZ proteins that each contained a different assortment of protein interaction domains.

We first confirmed that GFP-PSD95 co-immunoprecipitated with β-arrestin2 in HEK 293 cells co-transfected with FLAG-β-arrestin2 (Fig. 1a). As a negative control, we verified that FLAG-β-arrestin2 did not interact with either peGFP or GFP-Rab8. We next assessed whether YFP-MAGI1 could be co-immunoprecipitated with β-arrestin2 when co-transfected with FLAG-β-arrestin2 (Fig. 1b). PSD-95 contains three PDZ domains, a SRC homology 3 (SH3) domain, and a guanylate kinase-like (GK) domain while MAGI1 contains six PDZ domains, a GK domain, and two tryptophan-tryptophan (WW) domains but lacks SH3 domain [17]. YFP-MAGI1 also co-immunoprecipitated with FLAG-β-arrestin2 indicating that β-arrestin2 can be co-immunopreciptated with a PDZ protein lacking an SH3 domain-independent. We then tested PDZK1 since the only protein interaction domains in this PDZ protein are four PDZ domains [17]. We detected an interaction between YFP-PDZK1 with β-arrestin2 in HEK 293 cells co-transfected with FLAG-β-arrestin2 (Fig. 1c) that suggested that the interaction between PDZ proteins and β-arrestin2 is mediated through the PDZ domains.

3.2. PDZ1 domain is sufficient for β-arrestin2 interaction with PDZK1

To further characterize the interaction between β-arrestin2 and PDZ proteins, we assessed whether FLAG-β-arrestin2 could be co-immunoprecipitated with any of the four isolated PDZ domains of the PDZK1 (YFP-PDZ1, YFP-PDZ2, YFP-PDZ3 or YFP-PDZ4). When compared to full-length PDZK1, FLAG-β-arrestin2 effectively interacted with only the first PDZ domain of PDZK1 (Fig. 2a and b), indicating that this domain is important for the interaction between β-arrestin2 and PDZK1. Our findings not only show that multiple PDZ proteins are capable of interacting with β-arrestin-2, but also highlight the key role of PDZ1 domain in mediating this interaction for PDZK1.

3.3. Structural alignment reveals potential residues for β-arrestin-2 interaction with PDZ proteins

We attempted to determine the amino acid residues of β-arrestin-2 that mediate β-arrestin/PDZ interactions based on the structural homology between the vacuolar protein sorting-associated protein 26 (Vps26A) and β-arrestin family members. Our rationale for using Vps26A to model β-arrestin2/PDZ domain interactions stemmed from the fact that it interacts with the PDZ protein sorting nexin 27 (SNX27) and the crystal structure for this interaction was resolved (Fig. 3a) [33,34]. Despite of low sequence homology, structural alignment revealed a high degree of similarity between mouse Vps26A and β-arrestin family members (arrestin 1, 2, 3 and 4). The highest degree of structural similarity was detected between Vps26A and β-arrestin-2, with an average root mean square deviation (RMSD) of 1.94 Å. Thus, it is possible that β-arrestin2 interacts with the PDZ domain of SNX27 similar to other PDZ proteins. To explore this further, the crystal structures of mouse Vps26A bound to the PDZ domain of rat SNX27 was docked with the crystal structure of β-arrestin2 (Fig. 3b). The docking revealed that residues 44D, 153Q, 154Q and 155A within mouse Vps26A were key for the interaction with rat SNX27 (Fig. 3c). The structurally analogous amino acids within β-arrestin2 were K34, Q173, F174 and A175.

3.4. A175 residue is key in mediating β-arrestin2 interaction with PSD95

We assessed which of the β-arrestin-2 amino acid residues determined by structural docking is crucial for interaction with PDZ proteins by mutating K34, Q173, F174 and A175 residues of β-arrestin2. Co-immunoprecipitation experiment were performed using β-arrestin2 mutants (K34Q, Q173L, F174L, and A175F) and PDZ protein PSD95. For reference, we also included the β-arrestin2 dominant-negative mutant, V54D [35], and the constitutively active β-arrestin2 mutant, R170E [36]. PSD95 was chosen for this experiments as it harbors three PDZ domain, co-immunoprecipitates with CRFR1 and regulates its trafficking without altering canonical $G_{\alpha s}$-mediated signaling [22]. Substitution mutants V54D and R170E showed significantly increased interaction with PSD95 compared to wild-type β-arrestin2 (Fig. 4a and b). Substitution mutants K34Q, Q173L, and F174L similar affinity to PSD95 compared to wild-type β-arrestin2 (Fig. 4a and b). Interestingly, substitution mutant β-arrestin2 A175F exhibited a significantly lower interaction with PSD-95 compared to wild-type (WT) β-arrestin2 (Fig. 4a and b). These findings indicated residue A175 is important for mediating β-arrestin2 interaction with

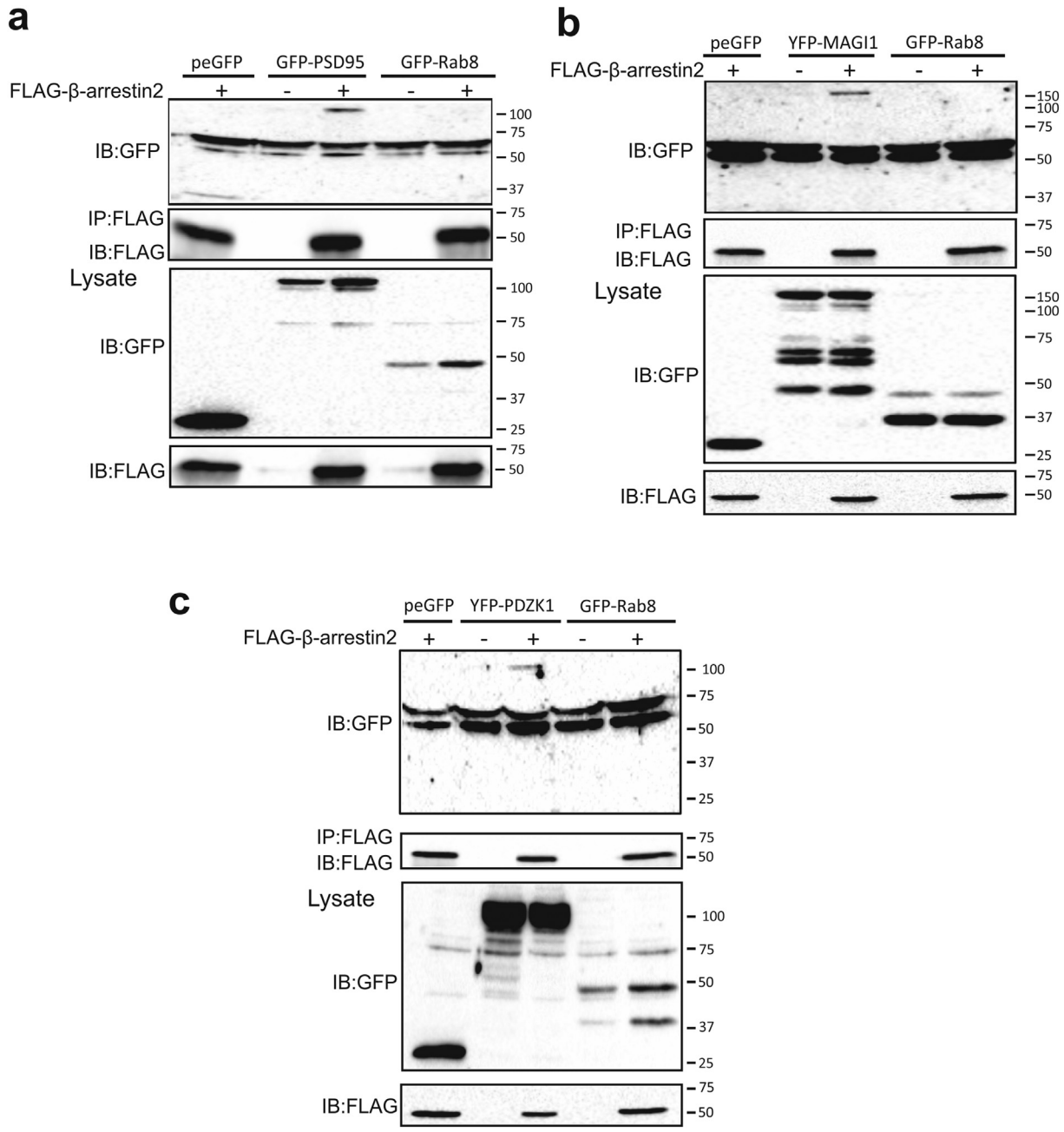


Fig. 1. β-arrestin2 interaction with PSD95 is PDZ domain-dependent and SH3 domain-independent. Representative immunoblot of FLAG-β-arrestin2 co-immunoprecipitated (IP) with (a) GFP-PSD95, (b) YFP-MAG1 and (c) YFP-PDZK1 in transiently-transfected HEK cells. GFP-Rab8 was used as negative control. Samples were run using SDS-PAGE and immunoblotted (IB) with rabbit anti-GFP and rabbit anti-FLAG. Data are representative of three independent experiments.

PSD95.

3.5. Disruption of β-arrestin-PDZ interaction impairs β-arrestin2 recruitment to CRFR1

We have previously showed that CRFR1 internalization is dependent on β-arrestin2 recruitment and can be antagonized by multiple PDZ proteins [22,25,31,32]. Therefore, we tested whether disruption of β-arrestin2 interaction with PDZ proteins can alter its recruitment to CRFR1. We tested the ability of previously described β-arrestin2 mutants to be recruited to the CRFR1 following agonist stimulation by BRET (Fig. 5a). We found that while mutants K43Q and F174L had a similar maximal response for CRF-stimulated β-arrestin2 translocation compared to WT, mutants Q173L and A175F reduced maximal response of β-arrestin2 translocation following CRF stimulation (Fig. 5b and c). It is worth noting that CRF EC_{50} values for β-arrestin translocation were not significantly different between all mutant forms. When compared at similar concentration of CRF, mutants Q173L and A175F reduced CRF-stimulated β-arrestin2 recruitment to HA-CRFR1 over time, while mutants K43Q, and F174L had comparable levels of CRF-stimulated β-arrestin2 recruitment to HA-CRFR1 compared to control (Fig. 5b and c). Since β-arrestin2 substitution mutant A175F impairs the binding to PSD-95, it is possible that this decrease in recruitment of mutant A175F to CRFR1 is due to impaired interaction between β-arrestin2 and PDZ proteins.

3.6. A175 residue of β-arrestin2 dictates recruitment to CRFR1

To further validate our findings, we recreated mutations of the same residues (K34, Q173, F174 and A175) with different amino acid

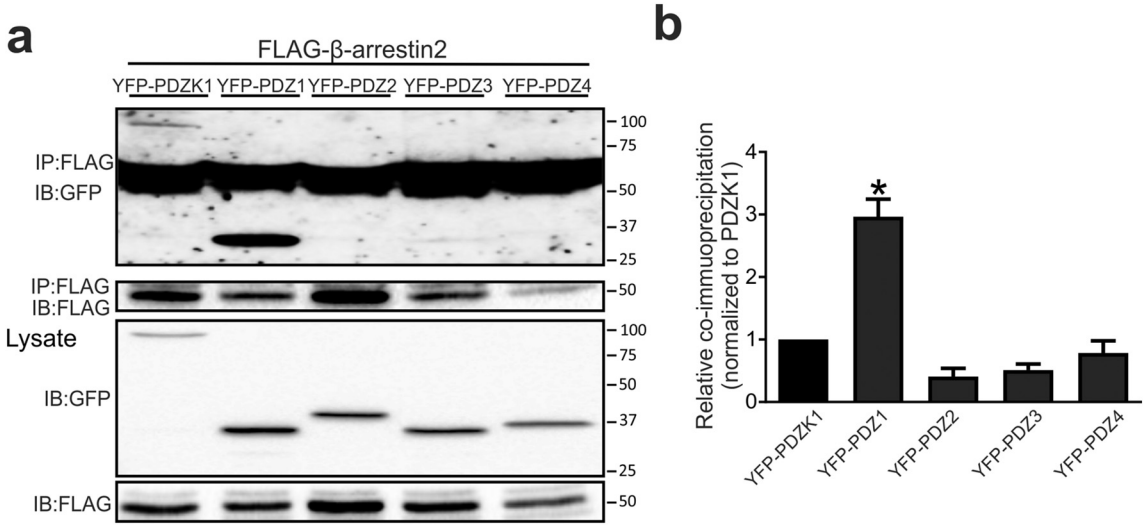


Fig. 2. PDZ1 domain is required for β-arrestin2 interaction with PDZ proteins. (a) Representative immunoblot of FLAG-β-arrestin2 co-immunoprecipitated (IP) with YFP-PDZK1 and YFP-PDZ1 but not YFP-PDZ2, YFP-PDZ3 or YFP-PDZ4 in transiently-transfected HEK cells. Samples were run using SDS-PAGE and immunoblotted (IB) with rabbit anti-GFP and rabbit anti-FLAG. (b) Densiometric quantification of relative co-immunoprecipitation of PDZ domains compared to full length PDZK1. Data represent mean ± SEM of three independent experiments. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple comparisons post hoc test (*$p < .05$ compared to YFP-PDZK1).

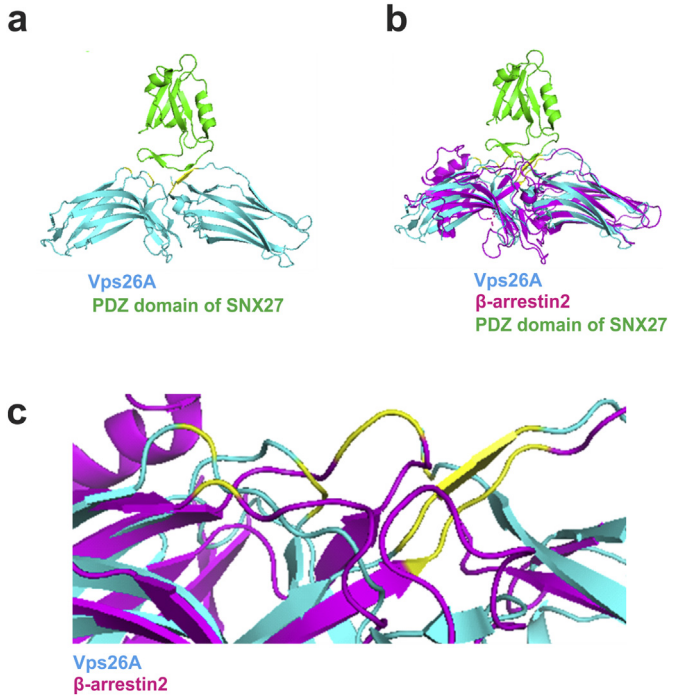
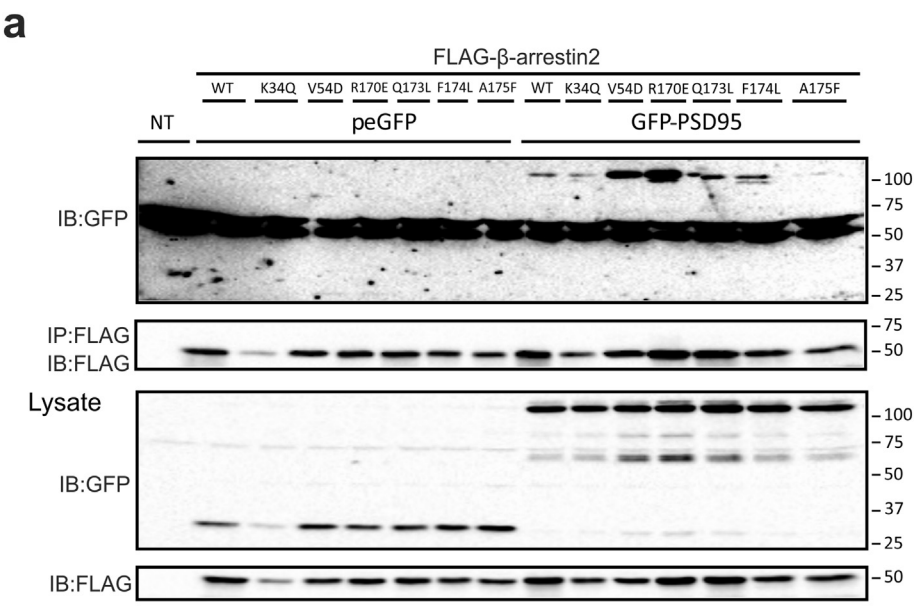


Fig. 3. Crystal structure of amino acid residues within mouse VPS26A important for interaction with rat SNX27 and the structurally analogous residues within bovine β-arrestin2. Vps26A is shown in a ribbon model in blue, β-arrestin-2 is shown in a ribbon model in magenta, and the PDZ domain of SNX27 is 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 for this interaction. (b) The crystal structure of mouse Vps26A bound to the PDZ domain of rat SNX27 is structurally aligned with bovine β-arrestin-2. Highlighted in yellow are the residues within Vps26A thought to be important for the interaction with SNX27 as well as structurally analogous residues within β-arrestin-2. (c) A close-up image of the residues highlighted in yellow for both Vps26 and β-arrestin2 in panel (b). Figures were generated using PyMOL software (www.pymol.org). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

substitutions to confirm that any detected changes in β-arrestin2 mutants function is not due protein misfolding. Specifically, we created both glycine and leucine substitution mutants for A175 site and created alanine substitution mutants for 34K, 173Q and 174F sites. We found that mutants K34Q and K34A, showed no changes in CRF-stimulated β-arrestin2 recruitment to HA-CRFR1 compared to control (Fig. 6a). While mutant Q173L reduced the recruitment to HA-CRFR1 over time, as well as the maximal response, mutant Q173A exhibited no alterations in its recruitment to HA-CRFR1 (Fig. 6b). In addition, similar to what was observed for K34A and K34Q mutants, the F174L and F174A β-arrrestin2 mutants showed little change in recruitment to CRFR1 when compared to wild-type β-arrestin2 (Fig. 6c). Interestingly, mutant A175G exhibited no changes in recruitment to HA-CRFR1 but mutants A175L and A175F decreased the recruitment to HA-CRFR1 compared to wild-type β-arrestin2 control (Fig. 6d). More so, faster kinetics of recruitment in response to CRF was detected with A175G mutant (Fig. 6d). Our findings indicate the size of the sidechain in β-arrestin2 mutant of the site A175 is not only critical for PDZ interaction, it also plays a key role in agonist-stimulated β-arrestin2 recruitment to CRFR1.

3.7. A175 residue of β-arrestin2 is crucial of CRFR1 internalization

β-arrestin2 recruitment to CRFR1 is key for receptor internalization [17,22]. Thus, we tested whether the detected alterations in β-arrestin/PDZ interaction and β-arrestin2 recruitment in some of our mutants will be reflected on HA-CRFR1 endocytosis. We employed flow cytometry to quantify the plasma membrane expression of HA-CRFR1 when expressed with β-arrestin2 mutants K34Q, Q173L, F174L, A175L, A175G and A175F and WT β-arrestin2. Co-transfection of CRFR1 with all β-arrestin2 mutants did not alter receptor surface expression compared to WT indicating that β-arrestin2 mutants that we tested did not alter CRFR1 trafficking to plasma membrane (Fig. 7a). Agonist activation with 500 nM CRF for 30 min resulted in internalization of 34 ± 4.9% of cell surface CRFR1 in wild-type- β-arrestin2 expressing cells (Fig. 7b). The extent of CRFR1 internalization was not altered in β-arrestin2-K34Q cells and was only modestly attenuated in either β-arrestin2-Q173L, F174L or A175G expressing HEK 293 cells (Fig. 7b). In contrast, CRFR1 internalization in response to CRF treatment was severely attenuated in βarrestin2-A175L and -A175F expressing cells (Fig. 7b and c). These findings indicate that A175 of β-arrestin2 is key



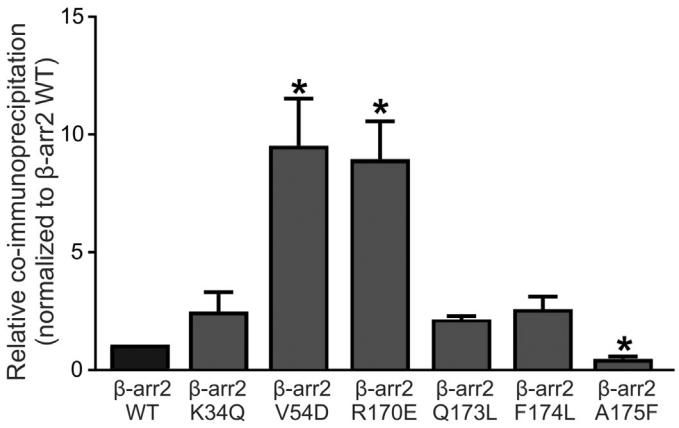


Fig. 4. A175 residue is essential in mediating β-arrestin2 interaction with PSD95. (a) Representative immunoblot of GFP-PSD95 co-immunoprecipitated (IP) with FLAG-β-arrestin2 and mutants forms of FLAG-β-arrestin2 with substitutions K34Q, V54D, R170E, Q173L, F174L, and A175F. Transient transfections were performed in HEK 293 cells. Samples were run using SDS-PAGE and immunoblotted (IB) with rabbit anti-GFP and rabbit anti-FLAG. (b) Densiometric quantification of relative co-immunoprecipitation of PSD-95 with β-arrestin2 mutants compared to WT β-arrestin2. Data represent mean ± SEM of four independent experiments. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple comparisons post hoc test ($^{*}p < .05$ compared to WT β-arrestin2).

for stabilizing the interaction with PDZ proteins and β-arrestin2 recruitment to CRFR1 and receptor endocytosis.

4. Discussion

Although the regulation of GPCR trafficking by PDZ proteins and β-arrestins have been widely documented, the functional and molecular basis of interaction between PDZ proteins and β-arrestin is not fully characterized. In this study we focused on delineating some of the structural determinants required for PDZ/β-arrestin2 interactions and β-arrestin2 recruitment to CRFR1 required for endocytosis. We find that multiple PDZ domain containing proteins are capable of interacting with β-arrestin2 and that PDZ1 domain is key for such interaction. We also show that substitution of alanine residue 175 in β-arrestin2 with a bulky side chain amino acid impairs interaction with PSD-95, thus highlighting its importance in mediating PDZ/β-arrestin2 interactions. We finally determine that modifying β-arrestin2/PDZ interactions impairs agonist-stimulated β-arrestin2 recruitment to CRFR1 and receptor internalization.

We have previously demonstrated that CRFR1 interacts with multiple PDZ proteins including PSD95, CAL, PDZK1 and SAP97 in HEK293 cells [22,24,25,31]. Previous studies showed β-arrestin2 recruitment to CRFR1 following agonist stimulation is essential for receptor endocytosis [22,37]. Here we demonstrate the novel association of β-arrestin2 and the PDZ proteins PSD-95, MAGI1, and PDZK1. Since the only shared protein interaction domain between all three PDZ proteins is the PDZ domain [17], this indicates that PDZ/β-arrestin interaction is most likely mediated through the PDZ domain. We then employed the four PDZ domains of PDZK1 to show that the only the first PDZ domain of PDZK1 is required for interaction with β-arrestin2. These findings indicate that the interaction between PDZ proteins and β-arrestin2 occurs through a single domain and not multiple domains. It is noteworthy that direct interaction between β-arrestin and a PDZ domain containing protein have recently been described for the Endothelin A receptor. Specifically, a direct interaction between β-arrestin1 and PDZ domain containing protein PDZ-RhoGEF was required for activation of the RhoC GTPase and downstream ROCK (Rho-associated coiled coil-forming kinase) pathway to support motility and metastasis in ovarian carcinoma [38,39]. These findings suggest that the interactions between β-arrestins and PDZ proteins can be of functional significance.

We find that the alanine residue 175 of β-arrestin2 is key for interaction with PSD95. Structural alignment of Vps26A with all members of β-arrestin family revealed a high degree of structural similarity with β-arrestin2. This structural similarity was of importance since the crystal structure of Vps26A interaction with the PDZ domain of SNX27 was previously resolved [34]. Thus, we created several point mutations in β-arrestin2 based on the structural alignment results and tested the abilities of the mutants to bind to PDZ proteins by comparing levels of

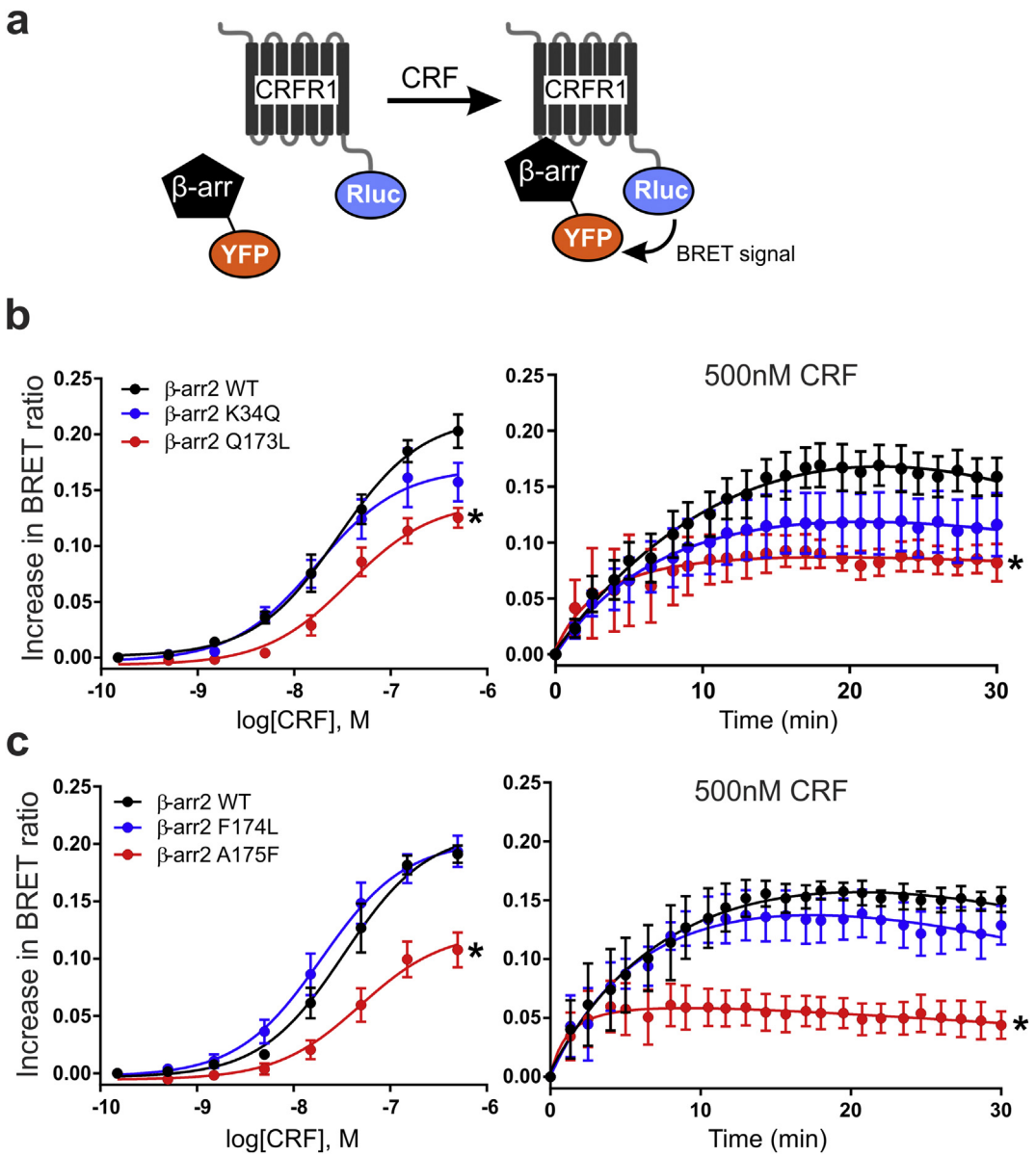


Fig. 5. Disruption of β-arrestin-PDZ interaction impairs β-arrestin2 recruitment to CRFR1. (a) Schematic representation of the principal of Bioluminescent Resonance Energy Transfer (BRET). β-arrestin was tagged with YFP and CRFR1 was tagged with Rluc domain. CRF-stimulated recruitment of β-arrestin to the receptor allows intermolecular interaction between YFP and Rluc domains to generate BRET signal. BRET was employed to quantify the recruitment of WT β-arrestin2 and mutant forms (b) K34Q, Q173L and (c) F174L and A175F to CRFR1. (Left) Dose-response curves for the increase in BRET ratios at 20 min post stimulation with increasing concentration of CRF and (right) Time-response curves for the increase in BRET ratios following exposure to 500 nM CRF. The data are representative of mean ± SEM of four independent experiments. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple comparisons post hoc test (*$p < .05$ compared to WT β-arrestin2).

co-immunoprecipitation with PSD-95. Out of the four putative interaction sites on β-arrestin2 detected by structural alignment, only A175F mutant was capable of reducing the interaction with PSD-95 compared to WT β-arrestin2. This alanine residue at position 175 in β-arrestin-2 is conserved in multiple other species as well as in Vps26A, thus indicating its potential importance. A155, the structurally analogous residue to A175 in Vps26A, is located in loop 10 and is 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 [34]. Moreover, this residue is also a part of Vps26A's short linker region that connects the two domains of Vps26A, the N-terminal domain and C-terminal domain. This flexible interdomain linker consists of a 15-residue loop, from 149 to 163, and corresponds with amino acids from position 173 to 185 in β-arrestin2 [33,34]. 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 is also possible that A175 residue supports the conformational changes in β-arrestin2 structure required for proper scaffolding function. Interestingly, we detected an enhanced binding of mutants dominant negative V54D and constitutively active R170E mutant to PSD95 that further emphasizes β-arrestin binding to PDZ protein is not the only interaction governing β-arrestin function and downstream signaling. However, this increased interaction could be due to higher expression of the V54D and R170E mutants compared to the other mutants that were relatively comparable. This might affect the stochiometric interaction of those two mutants with PSD95 and warrant further investigation.

We also show that PDZ proteins interaction with β-arrestin2 is also key for regulating β-arrestin2 recruitment to CRFR1 and receptor signaling. Only Q173L and A175F β-arrestin2 mutant reduced CRF-

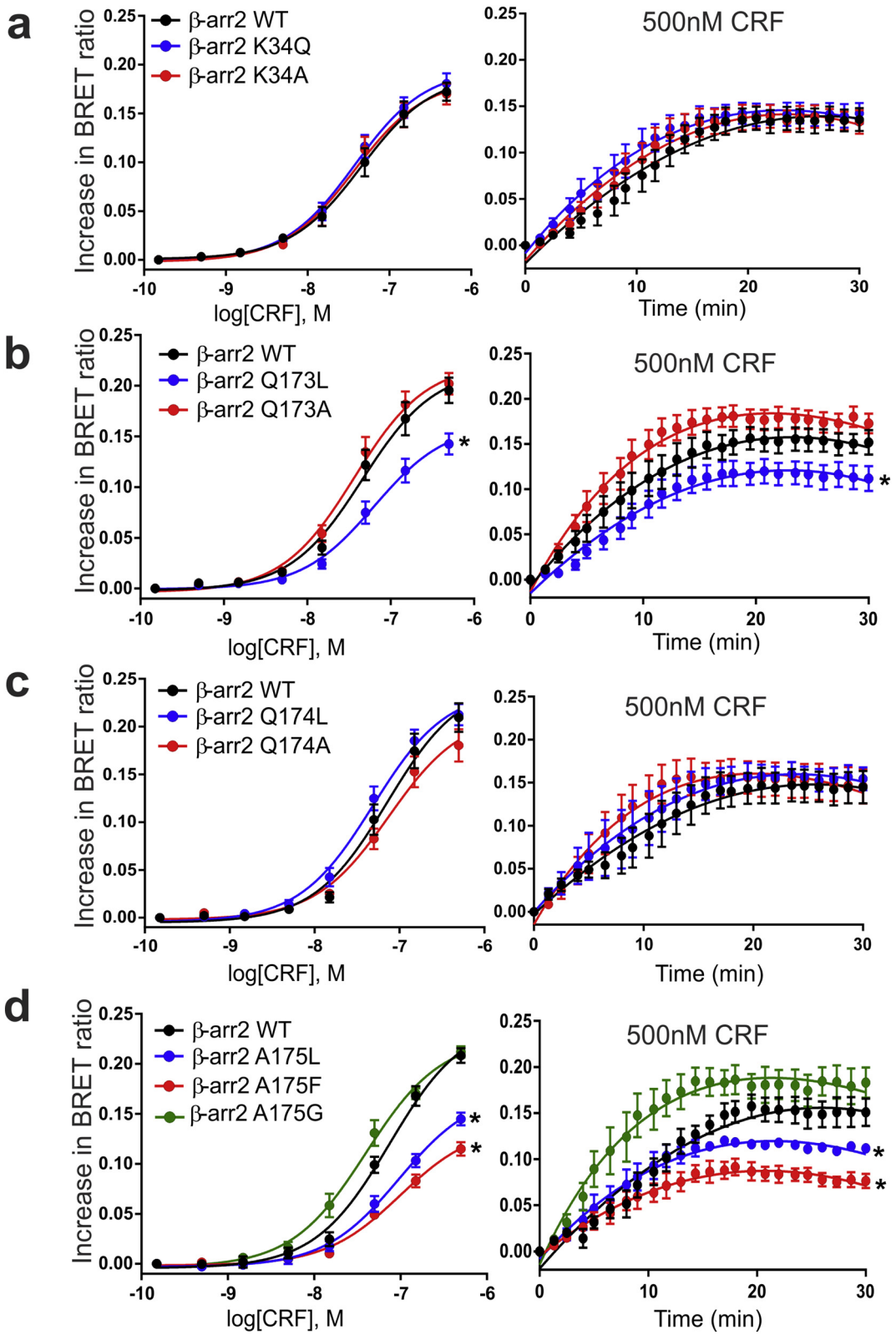


Fig. 6. β-arrestin2 mutants A175F and A175L, but not A175G, show impaired CRF-stimulated recruitment to CRFR1. Bioluminescent Resonance Energy Transfer (BRET) was employed to quantify the recruitment of WT β-arrestin2 and mutant forms (a) K34Q and K34A, (b) Q173L and A173A, (c) Q174L and Q174A, (d) A175L, A175F ad A175G to CRFR1. (Left) Dose-response curves for the increase in BRET ratios at 20 min post stimulation with increasing concentration of CRF and (right) Time-response curves for the increase in BRET ratios following exposure to 500 nM CRF. The data are representative of mean ± SEM of four independent experiments. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple comparisons post hoc test (*$p < .05$ compared to WT β-arrestin2).

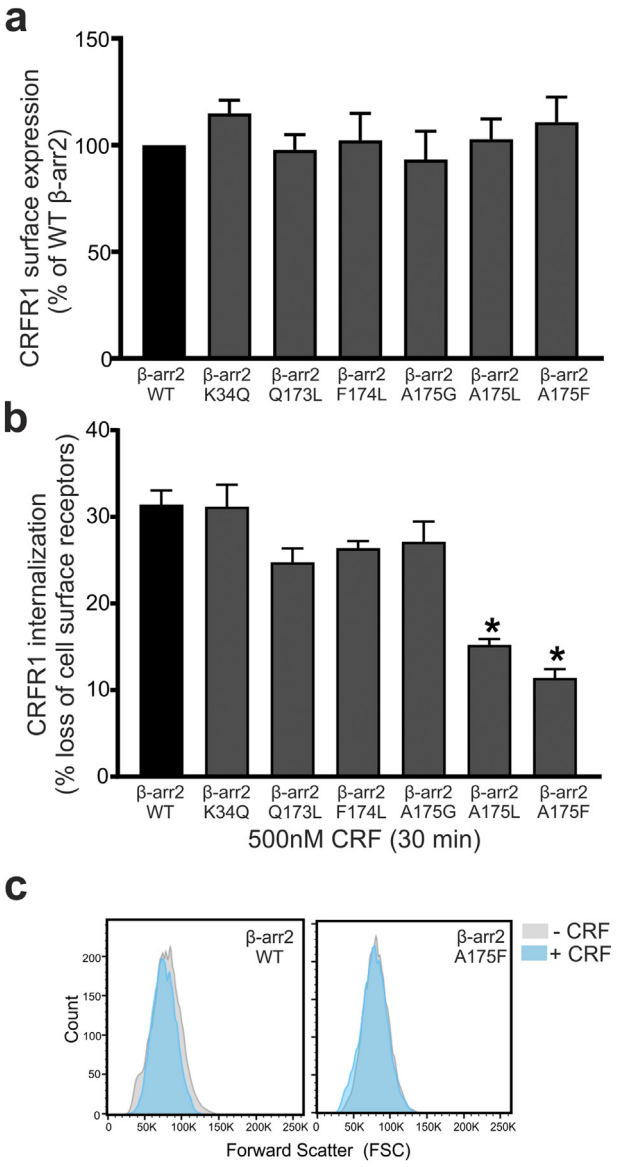


Fig. 7. A175 residue of β-arrestin2 is essential for CRFR1 internalization. (a) basal levels of cell surface expression of HA-CRFR1 co-transfected with WT β-arrestin2 or mutant forms K34Q, Q173L, Q174L, A175G, A175L and A175F as measured by flow cytometry. (b) Agonist-stimulated internalization (% loss of cell surface receptors) of HA-CRFR1 following 30 min of 500 nM CRF treatment in HEK cells co-transfected with WT β-arrestin2 or mutant forms K34Q, Q173L, Q174L, A175G, A175L and A175F. The data represent mean ± SEM of five independent experiments. Statistical significance is assessed by one-way ANOVA followed by Dunnett's multiple comparisons post hoc test (*$p < .05$ compared to WT β-arrestin2). (c) Representative histogram for flow cytometric analysis of HEK cells co-transfected with CRFR1 and either WT β-arrestin2 or A175F mutant before and after 30 min-treatment with 500 nM CRF.

stimulated β-arrestin2 recruitment to CRFR1, maximal response for CRF-stimulated β-arrestin translocation and receptor endocytosis. Combined with our data that show a decreased interaction of PSD-95 with β-arrestin2 A175F, it indicates that residue A175, possibly in combination with other nearby amino acids, are integral for β-arrestin2 interaction with PDZ proteins and thereby modulating β-arrestin2 recruitment and GPCR endocytosis.

To further validate the β-arrestin2 putative interaction sites with PDZ proteins, we performed alanine screening of residues K34, Q173, F174 and detected no differences in β-arrestin2 recruitment to CRFR1. We also created glycine and leucine mutants for A175 site to verify that any functional changes seen with mutant A175F are not due to disruption of the protein backbone of β-arrestin-2. While mutant A175G showed no changes in β-arrestin2 recruitment or CRFR1 internalization, a similar reduction in recruitment level and receptor internalization of A175L was detected when compared to A175F. It is possible that a change in recruitment was not evident with A175G because the short side chain of glycine was not capable of inducing changes in β-arrestin2 recruitment. These findings further support the functional significance of alanine residue at 175 site of β-arrestin2 for receptor signaling and trafficking.

We believe that the binding of PDZ proteins to β-arrestins can dictate the stability of the receptor/β-arrestin complex that will be reflected on the trafficking and signaling functions of the receptor. GPCRs exhibit different patterns of agonist-induced β-arrestin interactions, some receptors form stable complexes with β-arrestin and internalize to endosomes while others bind β-arrestin transiently and dissociate after internalization and recycle back to the plasma membrane [40,41]. Thus, the stability of the receptor/β-arrestin interaction might dictate the fate of the internalized receptor [42]. It is possible that PDZ protein binding to β-arrestins regulates stability of the receptor/β-arrestin complex to modulate CRFR1 recycling and degradation.

The activation and binding of arrestin to GPCRs induce inter-domain rearrangements and movement of the N- and C-terminals in β-arrestin [43,44]. The hinge loop that connects the N- and C-extremities of β-arrestin is necessary for maintaining receptor/β-arrestin complexes in a stable conformation [45]. Therefore, it is possible that binding of PDZ proteins to the flexible linker region sterically inhibits conformational changes of β-arrestin that hinder stable receptor interactions and internalization. In fact, this could also explain why mutant A175G shows no changes in recruitment to CRFR1 compared to mutants A175L and A175F. The side chain of glycine is small and closely-related to alanine that allows for changes in conformation that could be hindered by other longer or aromatic side chains in leucine and phenylalanine, respectively. Although we have shown previously that PSD-95 has been shown to antagonize the recruitment of β-arrestin2 to CRFR1 [22], it is possible that A175F mutant will affect the interaction of β-arrestin2 with other PDZ proteins that may support β-arrestin2 recruitment to the receptor. It is worth noting that while our study was performed in HEK293 cell, we have previously demonstrated that both endogenous PSD95 and SAP97 interacts with CRFR1 in adult mouse cortex [22,31] that further supports the functional significance of our findings.

5. Conclusion

We describe a novel structural association between β-arrestin2 and PDZ domain containing proteins. More specifically, this interaction occurs via PDZ domains and regulates internalization for CRFR1 through modulation of β-arrestin2 recruitment. Rather than modulating CRF release or signaling, generating pharmacological tools that target the interactions between β-arrestin2 and the PDZ proteins could have potential for the development of novel strategies to modulate impaired GPCR signaling associated with a spectrum of mental illnesses including anxiety, depression, and schizophrenia.

Competing interests

The author(s) declare no competing interest.

Authors contributions

S.G, H.A.D and S.S.G.F were responsible for the conception and design of all experiments. S.G and A.A performed the experiments. S.G, K.S.A, H.A.D, A.A and S.S.G.F analyzed the data. S.G, K.S.A and S.S.G.F wrote the manuscript. S.S.G.F supervised the study. All authors have approved the final article.

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Data availability

All data generated or analyzed during this study are included in this published article.

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