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Delineating the interactions between the cannabinoid CB_2 receptor and its regulatory effectors; β-arrestins and GPCR kinases

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Background and Purpose: The cannabinoid CB_2 receptor (CB_2) is a promising therapeutic target for modulating inflammation. However, little is known surrounding the mechanisms underpinning CB_2 desensitisation and regulation, particularly the role of GPCR kinases (GRKs). Here, we evaluated the role of six GRK isoforms in β-arrestin recruitment to CB_2. Mutagenesis of several distal C-terminal aspartic acid residues was also performed in an attempt to delineate additional structural elements involved in the regulation of CB_2.

Experimental Approach: In CB_2-expressing HEK 293 cells, β-arrestin translocation was measured using real-time BRET assays. G protein dissociation BRET assays were performed to assess the activation and desensitisation of CB_2 in the presence of β-arrestin 2.

Key Results: Overexpression of GRK isoforms 1–6 failed to considerably improve translocation of either β-arrestin 1 or β-arrestin 2 to CB_2. Consistent with this, inhibition of endogenous GRK2/3 did not substantially reduce β-arrestin 2 translocation. Mutagenesis of C-terminal aspartic acid residues resulted in attenuation of β-arrestin 2 translocation, which translated to a reduction in desensitisation of G protein activation.

Conclusion and Implications: Our findings suggest that CB_2 does not adhere to the classical GPCR regulatory paradigm, entailing GRK-mediated and β-arrestin-mediated desensitisation. Instead, C-terminal aspartic acid residues may act as phospho-mimics to induce β-arrestin activation. This study provides novel insights into the regulatory mechanisms of CB_2, which may aid in our understanding of drug tolerance and dependence.

KEYWORDS
cannabinoid CB_2 receptor, GPCR kinase, β-arrestin

1 | INTRODUCTION

The endocannabinoid system and its receptors have been implicated in a broad spectrum of physiological and pathological processes. In

Abbreviations: 2-AG, 2-arachidonoylglycerol; CB_1, cannabinoid CB_1 receptor; CB_2, cannabinoid CB_2 receptor; D_2, dopamine D_2 receptor; GRK, GPCR kinase; HA, haemagglutinin; PEI, polyethylenimine; THC, (−)-trans-Δ^9-tetrahydrocannabinol.

particular, the **cannabinoid CB_2 receptor** (CB_2) has emerged as a promising drug target for immunomodulation, inflammatory conditions and neuropathic pain (Bie et al., 2018; Tabrizi et al., 2016). The predominant expression of CB_2 in peripheral tissue presents a notable therapeutic advantage over the **cannabinoid CB_1 receptor** (CB_1) (Atwood & Mackie, 2010). Ligands targeting CB_2 will be devoid of the deleterious psychotropic side effects associated with CB_1, which is primarily expressed in the CNS (Glass et al., 1997), thus impelling attempts to develop CB_2-selective ligands.

Manipulating the function and regulation of **GPCRs**, such as the cannabinoid receptors, remains an important approach in the development of novel therapeutic agents. Increasingly, studies have focused on understanding the interaction between GPCRs and β-arrestins due to the consequential effects on receptor desensitisation and tolerance. Agonist-activated GPCRs recruit one or both isoforms of β-arrestin (β-arrestin 1/arrestin 2 and β-arrestin 2/arrestin 3), which sterically inhibit G protein coupling, resulting in attenuation or loss of signalling, while also facilitating clathrin-mediated endocytosis of the receptor. Understanding of β-arrestins has evolved, however—added to their role in terminating G protein signalling is a capacity to scaffold proteins that modulate various intracellular signalling pathways (Peterson & Luttrell, 2017). This understanding has introduced the possibility of designing drugs that elicit preferential activation of β-arrestin-mediated processes linked to desired therapeutic effects (ligand bias), as demonstrated in the characterisation of biased agonism at the **angiotensin AT_1 receptor** (Rajagopal et al., 2006; Strachan et al., 2014; Violin et al., 2010; Wingler et al., 2020).

Recruitment of β-arrestins is classically preceded by phosphorylation of the receptor by **GPCR kinases** (GRKs). GRKs constitute a family of seven kinases that phosphorylate serine/threonine residues on the C-terminus and/or third intracellular loop of activated GPCRs (Komolov & Benovic, 2018). Engagement of different GRK subtypes is suggested to dictate the pattern of phosphorylation (so-called phosphorylation barcode) on GPCR intracellular domains, thus modulating the β-arrestin isoform recruited, its conformation when bound and its consequent activity (Butcher et al., 2011; Nobles et al., 2011). In light of this, GRKs have emerged as crucial regulators of biased signalling, as distinct ligands may stabilise unique receptor conformations specific for phosphorylation by particular GRKs. Studies utilising pharmacological inhibition or genetic deletion of GRKs suggest that GRK isoforms differentially contribute to receptor desensitisation, endocytosis and signalling (Butcher et al., 2011; Kim et al., 2005; Matti et al., 2020; Møller et al., 2020; Nobles et al., 2011; Ren et al., 2005).

The mechanisms underpinning CB_2 desensitisation remain largely unexplored. We have previously found that β-arrestin translocation to CB_2 is relativity inefficient compared with CB_1 and the **vasopressin V_2 receptor**, with the **GRK2** subtype only marginally improving the response (Ibsen et al., 2019). Further insight into the regulatory mechanisms of CB_2 may contribute to the development of ligands with improved efficacy and prolonged activity by modulating receptor desensitisation and down-regulation. Hence, this study aimed to delineate the regulatory effects of several GRK isoforms on β-arrestin recruitment to CB_2. To further elucidate the molecular determinants of β-arrestin interaction with CB_2, the influence of alternative receptor structural elements was also evaluated by mutagenesis of several C-terminal aspartic acid residues.

What is already known

- GRKs are involved in GPCR phosphorylation, desensitisation and β-arrestin recruitment.
- Interaction of GRKs with the cannabinoid CB_2 receptor is poorly understood.

What does this study add

- GRKs have only a small contribution to agonist-mediated β-arrestin translocation to CB_2.
- C-terminal aspartic acid residues may be involved in the desensitisation of CB_2.

What is the clinical significance

- CB_2 may follow a non-classical mechanism of desensitisation that may not necessitate phosphorylation by GRKs.
- Non-classical mechanisms of CB_2 regulation may have implications for drug development.

2 | METHODS

2.1 | Drugs

2-Arachidonoylglycerol (2-AG), AMB-FUBINACA and **CP55940** were purchased from Cayman Chemical Company (Ann Arbour, MI, USA); **(−)-trans-Δ^9-tetrahydrocannabinol** (THC) was purchased from THC Pharm GmbH (Frankfurt, Germany); and **compound 101** was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). Drug stocks were prepared in absolute ethanol (2-AG, CP55940 and THC) or DMSO (AMB-FUBINACA and compound 101) and stored in single-use aliquots at −80°C prior to use. Drug vehicles were controlled for serial dilutions and were maintained at a constant level (0.1%) across all experiments.

2.2 | Plasmids and cloning

Human GRK pcDNA3.1+ plasmids were purchased from VectorBuilder Inc. (Santa Clara, CA, USA). The pplss-3HA-hCB_2 pEF4a construct was generated by terminal addition and overlap extension (fusion) PCR. In brief, the DNA sequence for pplss-3HA was amplified

by high fidelity, blunt cloning polymerase (Vita High Fidelity Enzyme Mix, Procomcure Biotech GmbH, Thalgau, Austria) from an existing construct. The human CB_2 gene (63R single-nucleotide polymorphism) was also amplified by PCR from an existing construct. The hCB_2 forward primer for this reaction was designed with an overhang complementary to the end of the pplss-3HA sequence. The purified reaction products were assembled using overlap extension PCR with Vita LongRange Enzyme Mix (Procomcure Biotech GmbH, Thalgau, Austria). The purified pplss-3HA-hCB_2 product was ligated into a pEF4a plasmid using restriction enzymes *Kpn*I and *Xba*I. Following transformation into XL10 Gold Ultracompetent cells (Agilent Technologies, Santa Clara, CA, USA), clones were sequence verified prior to use in experiments (Otago Genetic Analysis Services). C-terminal mutants of pplss-3HA-hCB_2 63R were generated using a modified QuikChange® (Stratagene, San Diego, CA, USA) site-directed mutagenesis approach, using KAPA HiFi Hotstart Polymerase (KAPA Biosystems, Roche, Basel, Switzerland). The pplss-3HA-hCB_2 plasmids utilised in this study are available by request.

2.3 | Cell culture

Human Embryonic Kidney 293 (HEK 293; RRID:CVCL_0045) cells were cultivated in high-glucose DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (New Zealand origin, Moregate Biotech, Brisbane, Australia) and cultured in 5% CO_2 at 37°C in a humidified incubator.

2.4 | β-Arrestin translocation assay

β-Arrestin translocation assays were performed as previously described in Ibsen et al. (2019), with modifications described in Finlay et al. (2019). Briefly, HEK 293 wild-type cells were seeded at an appropriate density in six-well plates or 10-cm culture dishes to achieve a confluency of 40–50% for transfection. Following overnight culture, medium was replaced, and transfection mixtures were prepared in Opti-MEM reduced serum medium (Thermo Fisher Scientific, Waltham, MA, USA), containing 2-μg mem-Linker-Citrine-SH3 pcDNA3.1+, 50-ng Rluc8-β-arrestin pcDNA3.1+ (human β-arrestin 1 or 2), 1.6-μg pplss-3HA-hCB_2 63R pEF4a (wild type or mutants) and 350-ng empty pcDNA3.1+ or 350-ng hGRK pcDNA3.1+, with a total mass of 4 μg for 10-cm dish transfections. DNA amount for each plasmid was appropriately scaled according to surface area for six-well plate transfections. Transfection mixture was combined in a 1:9 ratio (DNA:polyethylenimine [PEI]) with PEI MAX (1 $\mu g \cdot \mu l^{-1}$; Polysciences, Warrington, PA, USA), incubated for 20 min at room temperature and then added dropwise to cells. Following overnight incubation, transfected cells were lifted and seeded at a density of 30,000–60,000 cells per well in poly-D-lysine (0.05 $mg \cdot ml^{-1}$, PDL; Sigma-Aldrich, St. Louis, MO, USA)-coated, white 96-well CulturPlate plates (PerkinElmer, Waltham, MA, USA) and cultured overnight. For assaying, culture medium was aspirated, and cells were washed with PBS and equilibrated for approximately 30 min in phenol red-free DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 1-$mg \cdot ml^{-1}$ fatty acid-free BSA (ICPBio, Auckland, New Zealand) and 10-mM HEPES (Thermo Fisher Scientific, Waltham, MA, USA) ('assay medium'). For experiments with compound 101, cells were incubated in the presence of 30-μM compound 101 (as previously utilised by Leff et al., 2020; Lowe et al., 2015; Miess et al., 2018) or vehicle for 30 min. Cells were then incubated with 5-μM coelenterazine-h (NanoLight Technologies, Pinetop, AZ, USA) and luminescence at 475 and 535 nm was read simultaneously for 5 min at 37°C in a LUMIstar® Omega luminometer (BMG Labtech, Ortenberg, Germany) to establish a baseline BRET ratio. Serial dilutions of drugs were added, with luminescence detected in real time at 37°C for approximately 25 min. BRET ratios (535/475 nm) were exported from Omega MARS software and analysed in GraphPad Prism v8 (GraphPad Software Inc., La Jolla, CA, USA). To account for potential differences in basal BRET ratio, all raw BRET ratios were normalised to the average BRET values of the pre-drug (coelenterazine-h) incubation at matched time points. Mean 'vehicle' BRET ratios were then subtracted from all drug responses to obtain ΔBRET ratios, with net AUC obtained for concentration–response analysis, expressed as ΔBRET ratio seconds (ΔBRET.s).

2.5 | G protein dissociation assay

G protein dissociation BRET assays were performed utilising a pIRES Gβ2A-cpVenus-Gγ2-Gαi1-Nluc, initially described in Matti et al. (2020). In brief, HEK 293 wild-type cells were seeded at an appropriate density in six-well plates to achieve a confluency of 40–50% for transfection. Following overnight culture, medium was replaced, and transfection mixtures were prepared in Opti-MEM reduced serum medium, containing 450-ng pIRES Gβ2A-cpVenus-Gγ2-Gαi1-Nluc (see Matti et al., 2020, for G protein construct design), 150-ng pplss-3HA-hCB_2 63R pEF4a (wild type or mutant) or 3HA-hD_2 pcDNA3.1+, 200-ng human β-arrestin 2 pcDNA3.1+ and/or 100-ng hGRK pcDNA3.1+, with a total DNA mass of 900 ng. Additional empty pcDNA3.1+ vector was added to ensure total mass was consistent between all conditions. Plasmids were combined in a 1:9 ratio (DNA: PEI) with PEI MAX and incubated at room temperature for 20 min before dropwise addition to cells. Following overnight incubation, transfected cells were lifted and seeded at a density of 30,000–50,000 cells per well in PDL-treated, white 96-well CulturPlate (PerkinElmer) plates. Cells were cultured overnight prior to assay detection. For assaying, cells were washed with PBS and serum starved in assay medium for approximately 30 min. Cells were then treated with 5-μM coelenterazine-h and equilibrated for 5 min with luminescence at 475 and 535 nm detected simultaneously at 37°C in the LUMIstar Omega luminometer (BMG LABTECH). Serial dilutions of drugs were then added, and luminescence was detected in real time for approximately 30 min at 37°C. BRET ratios (535/475 nm) were exported from Omega MARS software and analysed in GraphPad Prism v8. For concentration–response analysis, average BRET ratios

for the vehicle conditions were subtracted from all drug responses to afford ΔBRET ratios and net AUC determined to obtain ΔBRET.s.

2.6 | Immunocytochemistry

The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology*. Transfected HEK 293 wild-type cells from β-arrestin and G protein dissociation experiments were plated into PDL-coated, Costar clear 96-well culture plates (Corning®, Corning, NY, USA) at a density of 30,000–60,000 cells per well and incubated overnight. For detection of surface receptor expression, medium was aspirated from wells and washed with assay medium. Primary mouse monoclonal anti-HA.11 IgG (clone 16B12, BioLegend, San Diego, CA, USA; Cat# 901503; RRID:AB_2565005), diluted 1:500 in assay medium, was dispensed and incubated for 30 min at room temperature. Primary antibody was aspirated, and cells were washed with assay medium and fixed in 4% (w/v) paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO, USA) in 0.1-M phosphate buffer for 10 min. For quantification of total receptor expression, medium was aspirated from wells and cells were fixed in PFA for 10 min. Following fixation, cells were washed with PBS, and primary mouse anti-HA.11 diluted 1:1000 in immunobuffer (PBS with 1% goat serum, 0.2% Triton X-100 and 0.4-mg·ml^{-1} merthiolate) was dispensed and incubated for 3 h at room temperature or overnight at 4°C. Primary antibody was aspirated, and cells were washed with PBS containing 0.2% Triton X-100 (PBS-T).

Secondary antibody, Alexa Fluor® 594 goat anti-mouse IgG (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA; Cat# A11032; RRID:AB_2534091) was diluted 1:400 in immunobuffer and incubated for 3 h at room temperature or overnight at 4°C for both surface and total receptor expression. Secondary antibody was removed, and cells were washed with PBS-T. Nuclei were then stained with Hoechst 33258 (4 mg·ml^{-1} in MilliQ water; Sigma-Aldrich, St. Louis, MO, USA), diluted 1:500 in PBS-T, for approximately 20 min at room temperature. Following nuclei staining, cells were washed twice with PBS-T. Cells were stored and imaged in PBS-T supplemented with 0.4-mg·ml^{-1} merthiolate. Image acquisition was performed using ImageXpress® Micro XLS (Molecular Devices, San Jose, CA, USA). Quantitative immunocytochemistry analysis was performed with MetaXpress® software (Molecular Devices, San Jose, CA, USA) as previously described (Finlay et al., 2016; Grimsey et al., 2008).

2.7 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). For each BRET experiment, randomisation was performed for drugs and/or different transfection conditions to minimise potential effects arising from plate layout. As all assays were performed in 96-well plates, blinding was unfeasible for all experimental and data analysis procedures.

To control for unwanted sources of variability, data from BRET experiments were normalised to their matched vehicle response. Data presented are either representative data from a single experiment (performed in technical duplicate or triplicate, expressed as mean ± SD) or averaged (combined) data from at least five biological (independent) replicates (expressed as mean ± SEM) (Cumming et al., 2007). Inconsistencies in the number of biological replicates are clarified in the figure legends or table titles. Statistical analyses were performed only on collated data from biological replicates with $n \geq 5$ (where n refers to independent replicates) using GraphPad v8. The Shapiro–Wilk and Brown–Forsythe tests were performed to ensure datasets met the assumptions of normality and equality of variance, respectively. Statistical significance ($P < 0.05$) was assessed using paired t tests, one-way ANOVA or two-way ANOVA, as appropriate. Groups were further analysed with a Holm–Šídák post hoc multiple comparisons test where F achieved statistical significance in ANOVA.

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Christopoulos, et al., 2021; Alexander, Fabbro, et al., 2021).

3 | RESULTS

3.1 | GRK overexpression failed to substantially increase CB_2-mediated β-arrestin translocation

In order to evaluate the role of GRKs in CB_2 desensitisation, the influence of different GRK isoforms on β-arrestin translocation was assessed using real-time BRET assays (Figures 1 and 2). The agonists CP55940, 2-AG, AMB-FUBINACA and THC were employed to represent the different 'classes' of cannabinoid ligands—non-classical, endocannabinoid, synthetic cannabinoid receptor agonist and phytocannabinoid, respectively, allowing for potential ligand-specific effects to be revealed. Stimulation of CB_2 with CP55940, 2-AG and AMB-FUBINACA induced concentration-dependent translocation of both β-arrestins 1 and 2 in the absence of exogenous GRKs, albeit with varying efficacies and potencies. Translocation of β-arrestin 2 was considerably more robust when compared with β-arrestin 1 for all ligands, indicating a CB_2 system preference for β-arrestin 2, in line with our previous findings for the cannabinoid receptors (Finlay et al., 2019; Ibsen et al., 2019; Patel et al., 2020). 2-AG and CP55940 were equally efficacious for each β-arrestin pathway, although CP55940 was over ∼70-fold more potent. AMB-FUBINACA exhibited submaximal translocation of both β-arrestins to CB_2, despite being the most potent ligand examined. As previously reported, the phytocannabinoid THC elicited negligible β-arrestin translocation at CB_2, with the magnitude of the response too small to accurately

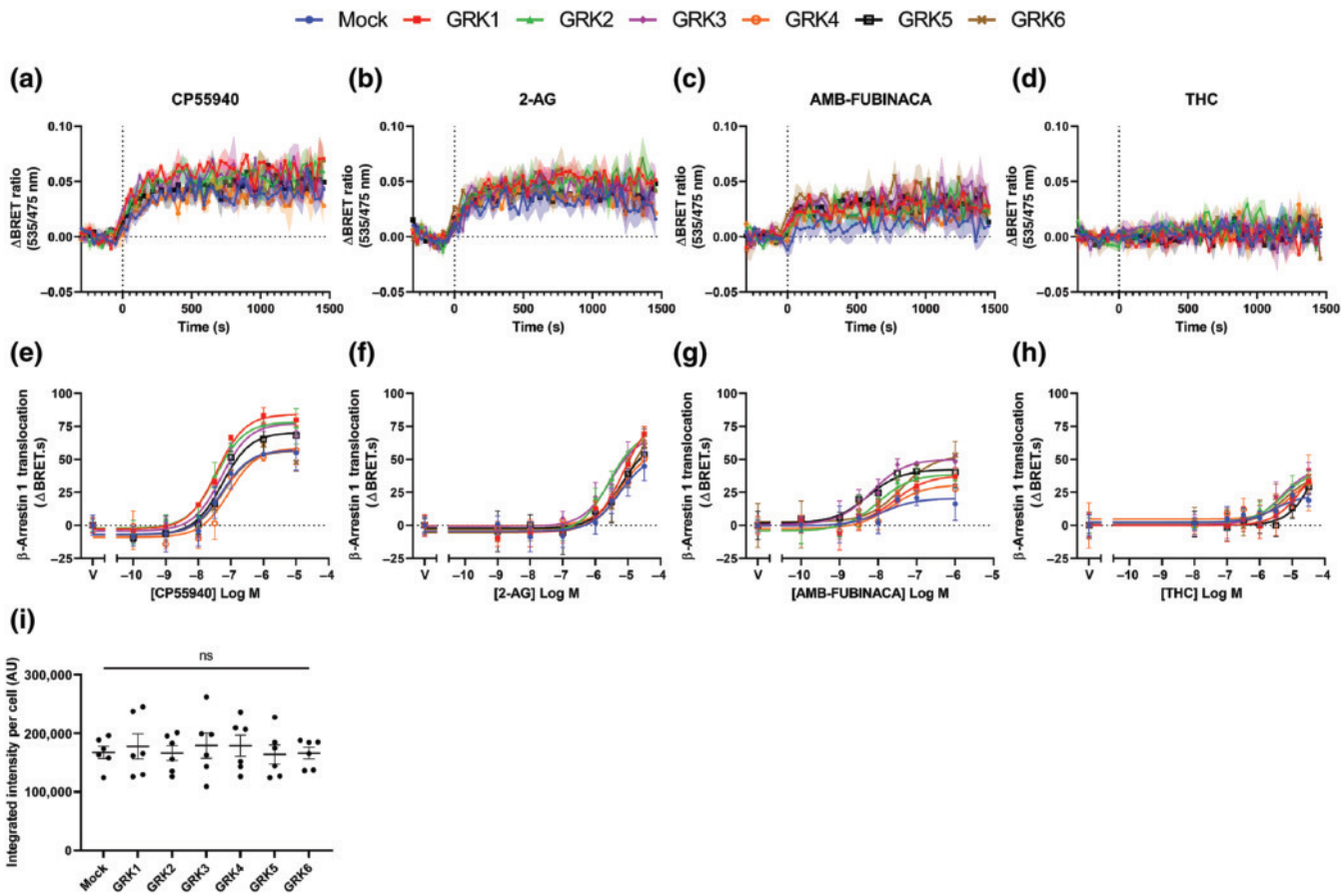

FIGURE 1 Influence of various GRK isoforms on β-arrestin 1 translocation to CB_2 in HEK 293 cells. Representative kinetic traces of β-arrestin 1 translocation to CB_2 at 1-μM CP55940 (a), 31.6-μM 2-AG (b), 1-μM AMB-FUBINACA (c) and 1-μM THC (d) normalised to vehicle (0 ΔBRET) in the absence (mock) or presence of GRK1, GRK2, GRK3, GRK4, GRK5 or GRK6. Concentration–response curves for β-arrestin 1 translocation to CB_2 coexpressed without (mock) or with GRK1–6 in response to CP55940 (e), 2-AG (f), AMB-FUBINACA (g) or THC (h). Total receptor expression in mock-transfected and GRK cells, quantified by immunocytochemistry with statistical significance assessed using repeated measures one-way ANOVA (i). Data presented are mean ± SD of technical triplicates (a–h) or mean ± SEM from six independent biological replicates (i)

obtain efficacy and potency values in either pathway (Ibsen et al., 2019). Responses above 1-μM THC are non-specific (Ibsen et al., 2019); hence, only responses at 1 μM were considered in this study (Tables 1 and 2).

Coexpression of GRKs revealed only modest changes to β-arrestin efficacy for all ligands. GRK1, GRK2 and GRK3 significantly improved β-arrestin 1 translocation for CP55940, 2-AG and AMB-FUBINACA between 20% and 60%, with GRK1 possessing the greatest effect. GRK5 and GRK6 also potentiated the 2-AG-mediated translocation of β-arrestin 1 (Figure 1 and Table 1). Surprisingly, all six GRK isoforms either had no effect or slightly reduced the extent of β-arrestin 2 translocation to CB_2. The efficacies of CP55940 and 2-AG-induced β-arrestin 2 translocation were significantly lower upon coexpression of GRK2, GRK3 and GRK5 by approximately 10–20% (Figure 2 and Table 2). Additionally, GRK6 significantly decreased translocation of β-arrestin 2 for 2-AG alone, suggesting that GRK activity may be ligand specific. GRK4 appeared to be the least active at CB_2, demonstrating no significant impact on β-arrestin efficacy for all compounds tested. All GRK isoforms also failed to improve translocation of either β-arrestin for THC and β-arrestin 2 for AMB-FUBINACA. Furthermore, overexpression of GRKs 1–6 resulted in no or minimal shifts in potency for all agonists in either β-arrestin pathways (Tables 1 and 2). All significant potency changes were within 0.5 log units.

To ensure any alterations in efficacy or potency were not attributable to changes in receptor number between conditions with and without GRKs, total receptor expression was quantified using immunocytochemistry. Expression of CB_2 was found to be equivalent between all conditions across β-arrestin 1 and β-arrestin 2 experiments, indicating the effects observed were not due to altered receptor expression (Figures 1i and 2i).

3.2 | Endogenous GRK2 and GRK3 contribute negligibly to β-arrestin 2 translocation to CB_2

In comparison with other GPCRs we have previously examined, such as CB_1 and dopamine D_2 receptor (D_2) (Ibsen et al., 2019), the impact

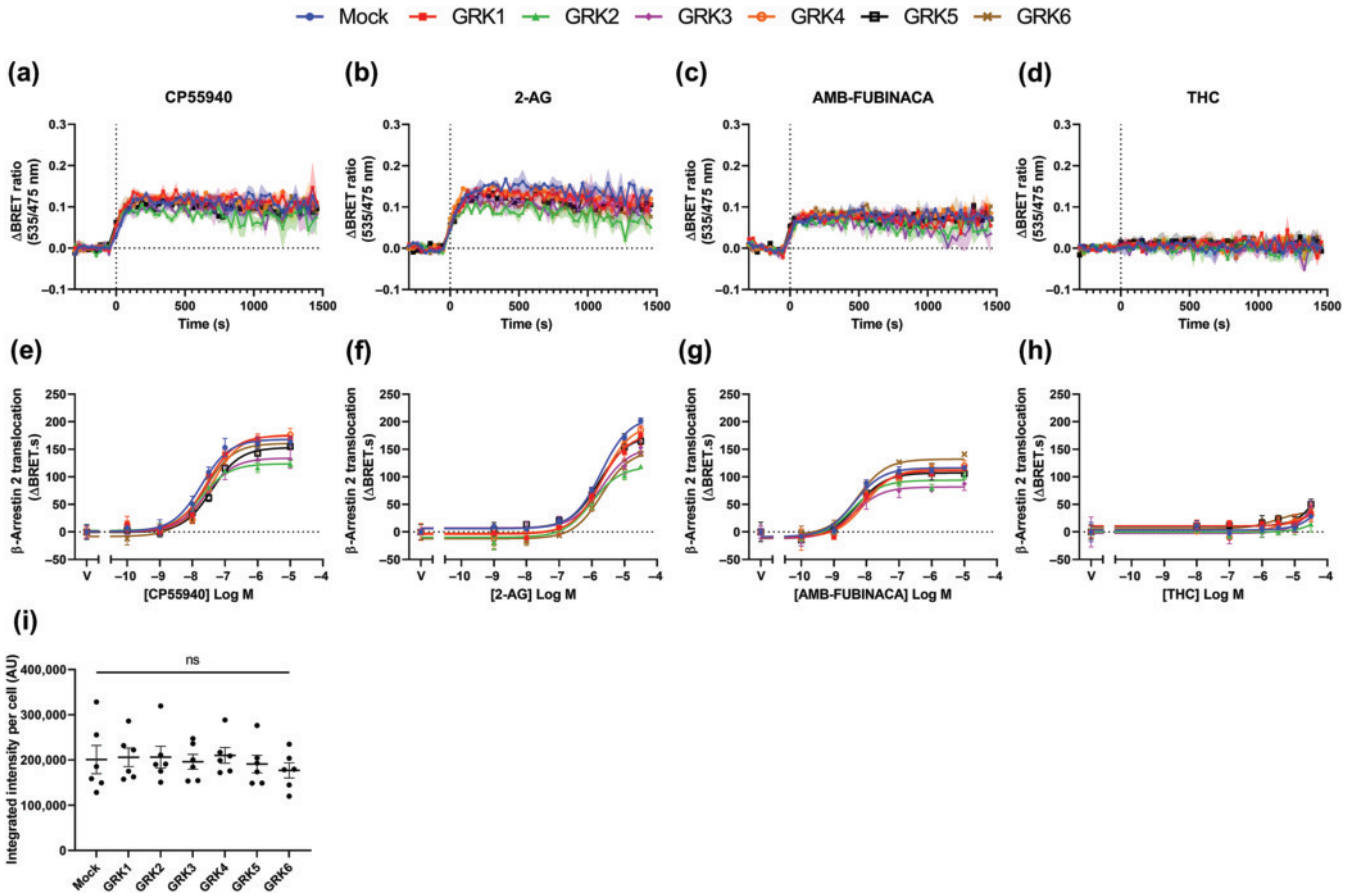

FIGURE 2 Influence of various GRK isoforms on β-arrestin 2 translocation to CB_2 in HEK 293 cells. Representative kinetic traces of β-arrestin 2 translocation to CB_2 at 1-μM CP55940 (a), 31.6-μM 2-AG (b), 1-μM AMB-FUBINACA (c) or 1-μM THC (d) normalised to vehicle (0 ΔBRET) in the absence (mock) or presence of GRK1, GRK2, GRK3, GRK4, GRK5 or GRK6. Concentration–response curves for β-arrestin 2 translocation to CB_2 coexpressed without (mock) or with GRK1–6 in response to CP55940 (e), 2-AG (f), AMB-FUBINACA (g) or THC (h) in HEK 293 cells. Total receptor expression in mock-transfected and GRK cells, quantified by immunocytochemistry with statistical significance assessed using repeated measures one-way ANOVA (i). Data presented are mean ± SD of technical triplicates (a–h) or mean ± SEM from six independent biological replicates (i)

TABLE 1 Potencies and efficacies for cannabinoid ligands in β-arrestin 1 translocation to CB_2 without and with coexpression of various GRK isoforms[a]

	CP55940		2-AG		AMB-FUBINACA		THC	
	pEC_{50}	Span (ΔBRET.s)	pEC_{50}	Span (ΔBRET.s)	pEC_{50}	Span (ΔBRET.s)	pEC_{50}	Span (ΔBRET.s)[b]
Mock	7.31 (0.06)	63.07 (4.10)	5.45 (0.11)	56.14 (1.52)	7.94 (0.10)	29.34 (2.43)	–	−0.71 (2.15)
GRK1	7.26 (0.10)	86.32 (2.74)*	5.49 (0.08)	83.57 (2.82)*	7.97 (0.13)	47.12 (3.44)*	–	2.51 (2.44)
GRK2	7.27 (0.10)	79.90 (3.34)*	5.64 (0.06)	71.73 (3.39)*	8.16 (0.09)	45.12 (2.90)*	–	1.96 (2.84)
GRK3	7.29 (0.08)	79.51 (2.33)*	5.70 (0.10)	68.57 (2.99)*	8.26 (0.07)*	46.69 (1.39)*	–	1.72 (2.33)
GRK4	7.14 (0.07)	64.47 (1.90)	5.54 (0.09)	62.00 (2.72)	7.98 (0.18)	35.06 (2.32)	–	0.36 (3.07)
GRK5	7.15 (0.08)	70.53 (2.52)	5.44 (0.06)	65.96 (1.69)*	7.97 (0.11)	38.17 (1.55)	–	−0.95 (2.25)
GRK6	7.13 (0.08)	66.73 (3.12)	5.48 (0.14)	74.27 (4.00)*	8.01 (0.18)	44.72 (3.21)*	–	6.99 (1.45)

[a]Data shown are means (±SEM) from six independent biological replicates.
[b]Response at 1-μM THC due to the presence of non-specific effects at higher concentrations.
*Statistically significant differences compared with experimentally matched mock (no GRK) conditions performed in GraphPad Prism using repeated measures two-way ANOVA, followed by Holm–Šídák post hoc multiple comparisons test, with $P < 0.05$.

TABLE 2 Potencies and efficacies for cannabinoid ligands in β-arrestin 2 translocation to CB_2 without and with coexpression of various GRK isoforms[a]

	CP55940		2-AG		AMB-FUBINACA		THC	
	pEC_{50}	Span (ΔBRET.s)	pEC_{50}	Span (ΔBRET.s)	pEC_{50}	Span (ΔBRET.s)	pEC_{50}	Span (ΔBRET.s)[b]
Mock	7.54 (0.06)	190.2 (12.38)	5.64 (0.04)	204.9 (14.67)	8.15 (0.06)	123.9 (8.36)	—	5.57 (2.44)
GRK1	7.44 (0.03)	183.2 (11.32)	5.78 (0.03)	198.6 (8.08)	8.03 (0.06)	134.2 (11.15)	—	7.79 (5.58)
GRK2	7.51 (0.07)	164.3 (16.29)*	5.92 (0.05)*	165.5 (10.92)*	8.28 (0.05)*	123.2 (9.45)	—	13.50 (5.85)
GRK3	7.52 (0.04)	155.0 (9.91)*	5.87 (0.07)	164.7 (9.83)*	8.17 (0.11)	119.0 (9.10)	—	13.38 (9.09)
GRK4	7.36 (0.03)*	176.9 (8.00)	5.67 (0.05)	191.3 (6.31)	8.13 (0.03)	126.6 (9.03)	—	10.33 (5.13)
GRK5	7.40 (0.02)	156.0 (6.71)*	5.82 (0.05)*	157.1 (4.07)*	8.07 (0.07)	114.6 (5.98)	—	10.77 (5.61)
GRK6	7.38 (0.06)*	160.6 (8.71)	5.83 (0.04)*	158.6 (6.39)*	8.12 (0.04)	125.8 (10.37)	—	5.02 (5.78)

[a]Data shown are means (±SEM) from five (CP55940) or six (2-AG, AMB-FUBINACA and THC) independent biological replicates. A single biological replicate for CP55940 was excluded from this dataset due to drug degradation and consequent change in response.
[b]Response at 1-μM THC due to the presence of non-specific effects at higher concentrations.
*Statistically significant differences compared with experimentally matched mock (no GRK) conditions performed in GraphPad Prism using repeated measures two-way ANOVA, followed by Holm–Šídák post hoc multiple comparisons test, with $P < 0.05$.

of GRKs on β-arrestin translocation to CB_2 was minimal. We therefore hypothesised that the presence of endogenous GRKs in our HEK 293 cell line may be generating a maximum response in the absence of exogenously expressed GRKs. To investigate the role of endogenous GRKs on β-arrestin 2 translocation to CB_2, we utilised the small molecule, selective GRK2/3 inhibitor, compound 101 (Thal et al., 2011), and a kinase-deficient dominant-negative variant of GRK2, GRK2 K220R (Kong et al., 1994) (Figure 3). Treatment of cells with compound 101 modestly but significantly reduced the extent of β-arrestin 2 translocation for 10-μM CP55940, 31.6-μM 2-AG and 1-μM AMB-FUBINACA by approximately 15%. Coexpression of dominant-negative GRK2 K220R alone did not alter the maximum β-arrestin 2 responses for all ligands, in comparison with mock-transfected cells. Simultaneous application of compound 101 and GRK2 K220R significantly attenuated β-arrestin 2 translocation for CP55940 by 15% when compared with mock-transfected cells, although this effect could be entirely attributed to compound 101. Total CB_2 expression was similar with and without coexpression of GRK2 K220R (Figure 3d), signifying that changes in β-arrestin translocation are unlikely the result of differences in receptor expression levels.

3.3 | C-terminal aspartic acid residues regulate β-arrestin 2 interactions with CB_2

Given the minimal enhancement in β-arrestin translocation with GRKs and the relatively small contribution of endogenous GRK2/3, we explored additional phosphorylation-independent structural features that may be determinants of β-arrestin 2 interactions with CB_2. In general, β-arrestins display high affinity for phosphorylated GPCRs, with recent structural studies on **rhodopsin** and visual arrestin proposing a common phosphorylation motif required for arrestin binding (Mayer et al., 2019; Zhou et al., 2017). However, negatively charged acidic amino acids, such as aspartates and glutamates, may serve as phosphate mimics by promoting interaction with the positively charged pockets on the surface of β-arrestin (Seyedabadi et al., 2021). The C-terminus of CB_2 is rich in aspartic acid residues, which may enable the activated receptor to interact directly with β-arrestin, independent of GRK phosphorylation. Therefore, mutagenesis of several distal C-terminal aspartic acid residues was performed to further probe the interaction between CB_2 and β-arrestin 2.

Double mutants of the residues D^{351}/D^{354} and D^{356}/D^{359} were generated, which obey the 'key site' phosphorylation motif (PxxP, where P represents a phosphorylatable or phospho-mimetic residue) recently proposed by Zhou et al. (2017) and Mayer et al. (2019). Substitution of aspartic acid (D) residues to alanine (A) residues significantly decreased CP55940-induced translocation of β-arrestin 2, with the $D^{351}A/D^{354}A$ and $D^{356}A/D^{359}A$ mutants possessing 41% and 24% reductions in efficacy, respectively. Removal of all four aspartic acid residues ($D^{351}A/D^{354}A/D^{356}A/D^{359}A$) failed to further abrogate β-arrestin 2 translocation, reducing the response by only 36% when compared with wild-type CB_2 (Figure 4a). To assess whether elimination of phospho-mimetic sites may unmask a GRK effect at CB_2, we measured CP55940-mediated translocation of β-arrestin 2 in the presence of GRK2 and GRK3 with each mutant (Figure 4b–d). In accordance with our initial findings, the addition of exogenous GRK2 and GRK3 attenuated β-arrestin 2 translocation to wild-type CB_2. β-Arrestin 2 efficacy was slightly improved following overexpression of GRK2 and GRK3 for $D^{351}A/D^{356}A$ by approximately 11% when compared with mock-transfected cells, although statistical significance was only achieved for GRK2 (Figure 4b). Neither GRK2 nor GRK3 significantly influenced the β-arrestin 2 response of the $D^{356}A/D^{359}A$ mutant. Intriguingly, coexpression of the GRKs almost completely restored CP55940-mediated β-arrestin 2 translocation for $D^{351}A/D^{354}A/D^{356}A/D^{359}A$, increasing the response by 36% and 26% for GRK2 and GRK3, respectively (Figure 4e and Table 3). Potency remained unchanged for all mutants, across both GRK conditions, with

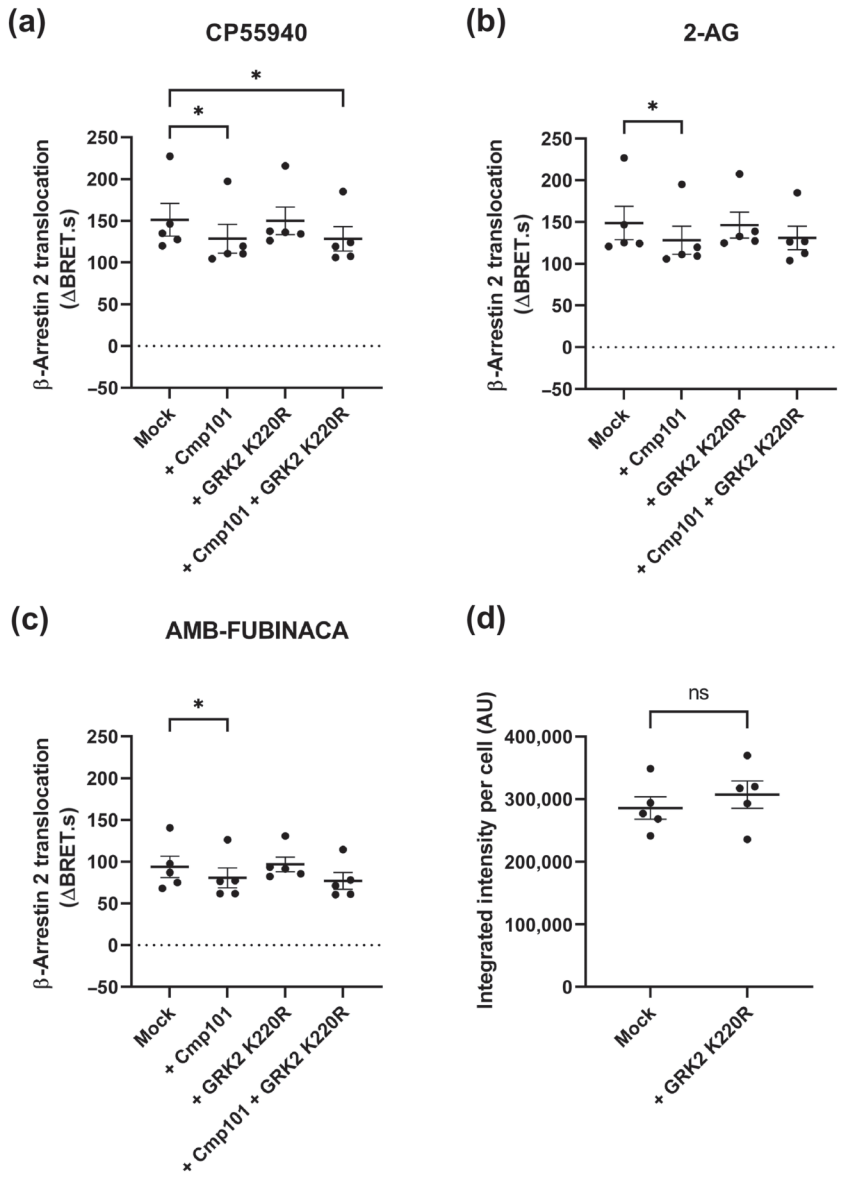

FIGURE 3 Contribution of endogenous GRK2 and GRK3 on β-arrestin 2 translocation to CB_2 in HEK 293 cells. β-Arrestin 2 translocation in response to stimulation of CB_2 by 10-μM CP55940 (a), 31.6-μM 2-AG (b) or 1-μM AMB-FUBINACA (c) in the presence of 30-μM compound 101 (Cmp101) and/or coexpression of dominant-negative GRK2 (GRK2 K220R). Statistical significance determined from repeated measures two-way ANOVA, followed by Holm–Šídák post hoc multiple comparisons test when compared with mock, with *P* values indicated as *<0.05 (a–c). Total receptor expression in mock and GRK2 K220R cells, quantified by immunocytochemistry with statistical significance assessed using a paired *t* test (d). Data represent mean ± SEM from five independent experiments

the exception of a slight increase in potency for the D^{351}/D^{354} and D^{356}/D^{359} mutants following the addition of GRK3 (Table 3).

To determine whether these changes in β-arrestin translocation were influenced by differences in receptor number, immunocytochemistry was performed to quantify total receptor expression levels for each mutant (Figure 4e,f). For experiments conducted with GRK2, receptor expression levels for $D^{351}A/D^{354}A$, $D^{356}A/D^{359}A$ and $D^{356}A/D^{359}A$ plus GRK2 were lower than wild-type CB_2 levels. Similarly, all mutants in the absence and presence of GRK3 demonstrated lower receptor expression compared with wild-type CB_2. However, correlation of fluorescence intensity with β-arrestin 2 efficacy for wild-type CB_2 did not reveal a convincing relationship when all experiments performed under matched immunocytochemistry conditions were considered (Figure 5). Given the range of fluorescence intensities obtained, it suggests that the removal of C-terminal aspartic acids was responsible for the reductions in β-arrestin 2 translocation observed, despite discrepancies in receptor expression.

3.4 | C-terminal aspartic acid residues influence CB_2 G protein desensitisation

In order to assess potential functional consequences of reduced β-arrestin 2 translocation to CB_2, we employed G protein dissociation BRET assays to measure the desensitisation capacity for each C-terminal aspartic acid mutant. To corroborate the effects of receptor desensitisation on G protein dissociation, the impact of β-arrestin 2 and GRK2 was initially evaluated for D_2, as the regulation profile for this GPCR has been extensively characterised (reviewed in Gurevich et al., 2016) (Figure 6a,b). Stimulation with **quinpirole** induced robust, concentration-dependent dissociation of $G\alpha_i$ from the $G\beta\gamma$ subunit, signifying G protein activation. The addition of β-arrestin 2 failed to change the extent of G protein dissociation for D_2, aligning with the receptor's poor β-arrestin 2 recruitment ability (Ibsen et al., 2019). Conversely, overexpression of GRK2 alone significantly reduced G protein dissociation, which was further attenuated upon coexpression

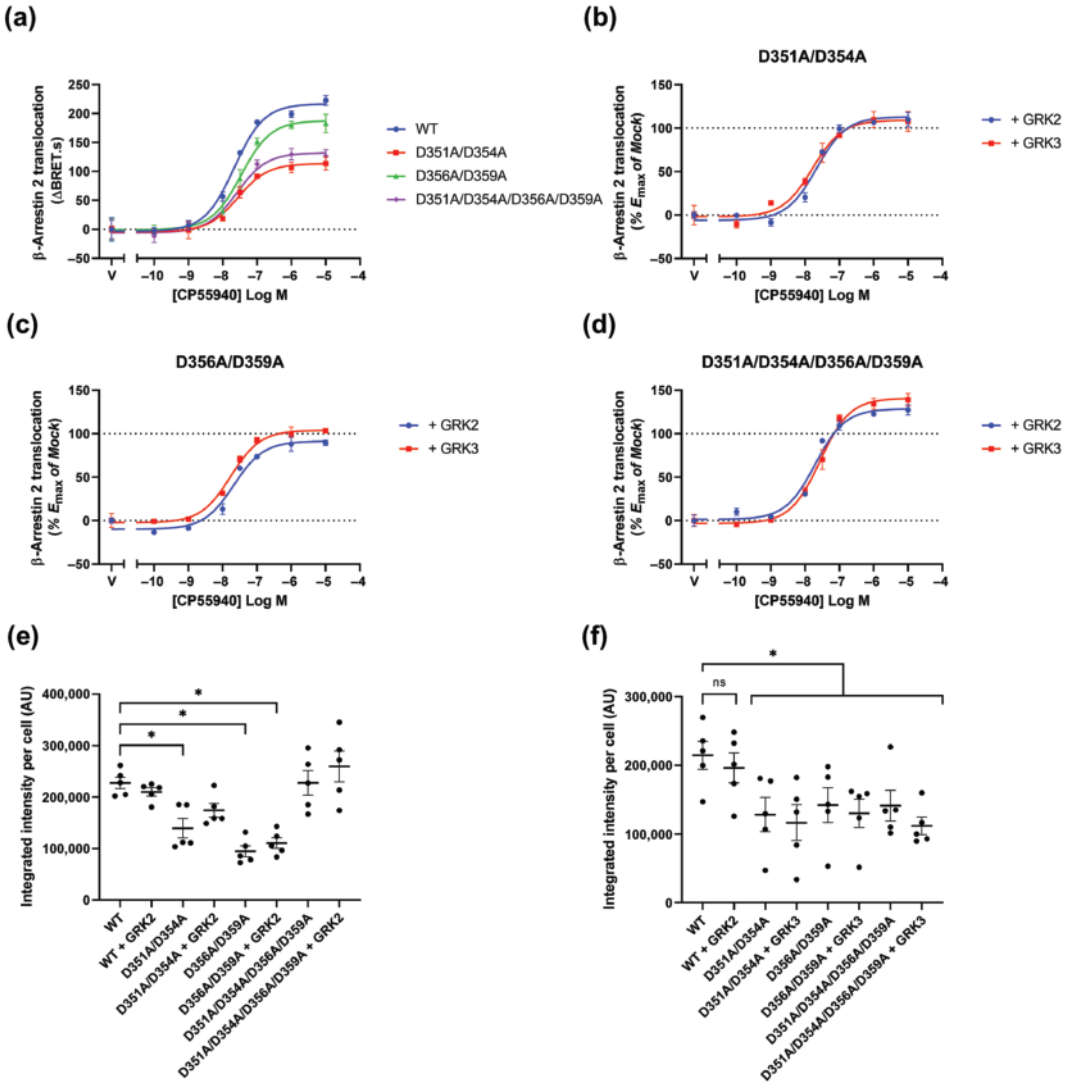

FIGURE 4 Influence of CB_2 C-terminal aspartic acid residues on β-arrestin 2 translocation in HEK 293 cells. Representative concentration–response curves for β-arrestin 2 translocation in response to CP55940 obtained from HEK 293 cells expressing wild-type (WT) CB_2 or CB_2 mutants that contain alanine substitutions for C-terminal aspartic acid residues (a). Representative concentration–response curves for CB_2 mutants D351A/D354A (b), D356A/D359A (c) and D351A/D354A/D356A/D359A (d) when coexpressed with GRK2 or GRK3, with responses normalised to E_{max} (span) of no GRK (mock). Total receptor expression in wild-type and mutant CB_2 cell lines for GRK2 (e) and GRK3 (f) experiments, quantified by immunocytochemistry, with *P* values indicated as *<0.05 determined from repeated measures one-way ANOVA, followed by Holm–Šídák post hoc multiple comparisons test when compared with wild-type. Data presented are mean ± SD of technical duplicates or triplicates (a–d) or mean ± SEM from five independent biological replicates (e,f)

of β-arrestin 2 and GRK2—likely reflecting GRK-mediated receptor phosphorylation and subsequent β-arrestin 2 recruitment. This is consistent with previous studies showing that GRK2 significantly improves β-arrestin 2 translocation to D_2 (Clayton et al., 2014; Ibsen et al., 2019; Kim et al., 2001; Namkung et al., 2009) and suggests that receptor desensitisation in this assay will manifest as a reduction in the extent of G protein dissociation, as opposed to a progressive loss of efficacy and gradual return to baseline.

As expected, CP55940 stimulation of wild-type CB_2 led to concentration-dependent G protein dissociation. All mutants exhibited functional G protein activation to an equivalent or greater extent as wild-type CB_2, with the exception of the $D^{356}A/D^{359}A$ mutant, which was significantly less efficacious. Potencies were also similar between all receptors in this pathway (Figure 6d and Table 4). The addition of β-arrestin 2 attenuated G protein dissociation by over 50% for the wild-type, $D^{351}A/D^{354}A$ and $D^{356}A/D^{359}A$ CB_2 variants following stimulation with 1-μM CP55940 (Figure 6e–h). By contrast, CP55940-induced dissociation was significantly higher for the $D^{351}A/D^{354}A/D^{356}A/D^{359}A$ mutant than wild-type CB_2 in the presence of β-arrestin 2, indicative of reduced G protein desensitisation. Coexpression of β-arrestin 2 and GRK3 also reduced G protein dissociation for this mutant to a similar extent (Figure 6h). Interestingly, overexpression of GRK3 alone potentiated G protein activation for $D^{351}A/D^{354}A/D^{356}A/D^{359}A$ but not wild-type CB_2, when compared

TABLE 3 Potencies and efficacies for CP55940-mediated β-arrestin 2 translocation for wild-type CB_2 and various CB_2 mutants[a]

	Wild-type		D351A/D354A		D356A/D359A		D351A/D354A/D356A/D359A	
	pEC_{50}	Span (ΔBRET.s)	pEC_{50}	Span (ΔBRET.s)	pEC_{50}	Span (ΔBRET.s)	pEC_{50}	Span (ΔBRET.s)
Mock	7.63 (0.03)	222.91 (6.17)	7.56 (0.03)	132.61 (6.99)◊	7.50 (0.02)◊	170.18 (6.70)◊	7.50 (0.04)◊	142.57 (8.39)◊
GRK2	7.63 (0.07)	174.54 (11.90)*	7.63 (0.04)	147.34 (2.60)*	7.56 (0.04)	161.84 (5.06)	7.55 (0.07)	194.18 (13.37)*
GRK3	7.72 (0.04)	210.00 (2.53)*	7.79 (0.08)*	147.06 (10.88)	7.67 (0.04)*	188.04 (8.90)	7.63 (0.06)	180.16 (13.23)*

[a]β-Arrestin 2 translocation in response to CP55940 in HEK 293 cells expressing wild-type (WT) CB_2 and several CB_2 mutants that contain alanine substitutions for C-terminal aspartic acid residues, without (mock) and with coexpression of GRK2 or GRK3. Data shown are means (±SEM) from ten (mock conditions) or five (GRK conditions) independent biological replicates. Discrepancy in the number of replicates as GRK2 and GRK3 experiments were performed separately under matched wild-type conditions.

◊Statistical significance determined using repeated measures two-way ANOVA, followed by Holm–Šídák post hoc multiple comparisons test performed in GraphPad Prism, with $P < 0.05$ when compared with WT.

*Statistical significance determined using repeated measures two-way ANOVA, followed by Holm–Šídák post hoc multiple comparisons test performed in GraphPad Prism, with $P < 0.05$ when compared with 'mock' of the receptor variant concerned.

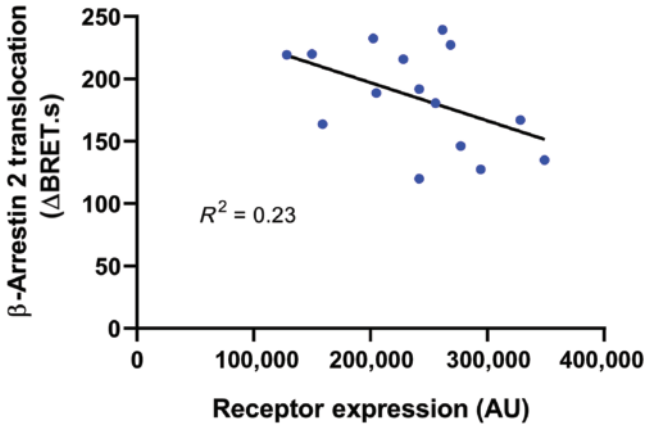

FIGURE 5 Correlation between receptor expression and efficacy in β-arrestin 2 translocation assays. Receptor expression quantified using immunocytochemistry and correlated with the matched E_{max} (span) of CP55940-mediated β-arrestin 2 translocation in HEK 293 cells expressing wild-type CB_2. Linear regression modelled using GraphPad Prism. Each data point represents mean of two or three technical replicates for both β-arrestin translocation and receptor expression

with the mock-transfected conditions. The β-arrestin 2-mediated reductions in G protein dissociation for each receptor mutant were preserved when assessed as a proportion of the mock-transfected response (Table 4). However, this signified only a small (~20%) but significant difference in the desensitisation magnitude between the $D^{351}A/D^{354}A/D^{356}A/D^{359}A$ mutant and wild-type receptor. Interestingly, surface and total expression for the $D^{351}A/D^{354}A/D^{356}A/D^{359}A$ mutant appeared to be higher than wild-type CB_2 (Figure 6i). Although this failed to reach statistical significance, it may account for the improved G protein dissociation efficacy of the $D^{351}A/D^{354}A/D^{356}A/D^{359}A$ mutant and the different extent of its desensitisation.

For the other CB_2 variants and D_2, total and cell surface expression confirmed equivalent expression levels to the wild-type mock-transfected condition, with the exception of three CB_2 conditions (Figure 6c,i). Surface expression for CB_2 was significantly different for wild-type receptor coexpressing β-arrestin 2 and GRK3, the $D^{351}A/D^{354}A$ mutant and the $D^{351}A/D^{354}A$ mutant coexpressing β-arrestin 2. However, given the range of fluorescence intensities and consistency in efficacies within all receptor conditions, together with the poor correlation between receptor expression and efficacy (Figure 5), the small differences in surface expression are unlikely to have influenced the responses obtained. Furthermore, the expression pattern for surface and total receptor was generally consistent across all conditions, indicating that measurement of total receptor expression was sufficient to capture any discrepancies that may have arisen in surface receptor levels for the β-arrestin experiments. Coexpression of β-arrestin 2 and/or GRK3 also did not alter receptor expression when compared with mock-transfected conditions within each receptor variant.

4 | DISCUSSION AND CONCLUSIONS

The application of cannabinoid agonists has been shown to elicit a range of effects through the activation of CB_1 and CB_2. However, like many drugs, the therapeutic effectiveness of cannabinoid ligands has been marred by on-target side effects, including the development of tolerance and drug dependence. Hence, events involved in the disruption or termination of receptor signalling have gained interest, specifically the mechanisms within the receptor desensitisation and internalisation pathways. The emergence of β-arrestins and GRKs as key regulators of GPCRs has presented a unique and viable therapeutic strategy to overcome issues inherent in traditional GPCR-targeting approaches. In this present study, we have utilised real-time BRET assays to gain novel insights into the short-term regulatory mechanisms of CB_2, focusing on the role of GRKs and β-arrestins.

The current canonical GPCR regulation paradigm proposes that distinct GRKs imprint specific receptor phosphorylation patterns to dictate β-arrestin recruitment (Butcher et al., 2011; Nobles et al., 2011). Given the low efficacy β-arrestin recruitment by CB_2 in HEK 293 cells compared with other GPCRs, we hypothesised that the addition of specific GRK isoforms may enable efficient recruitment of

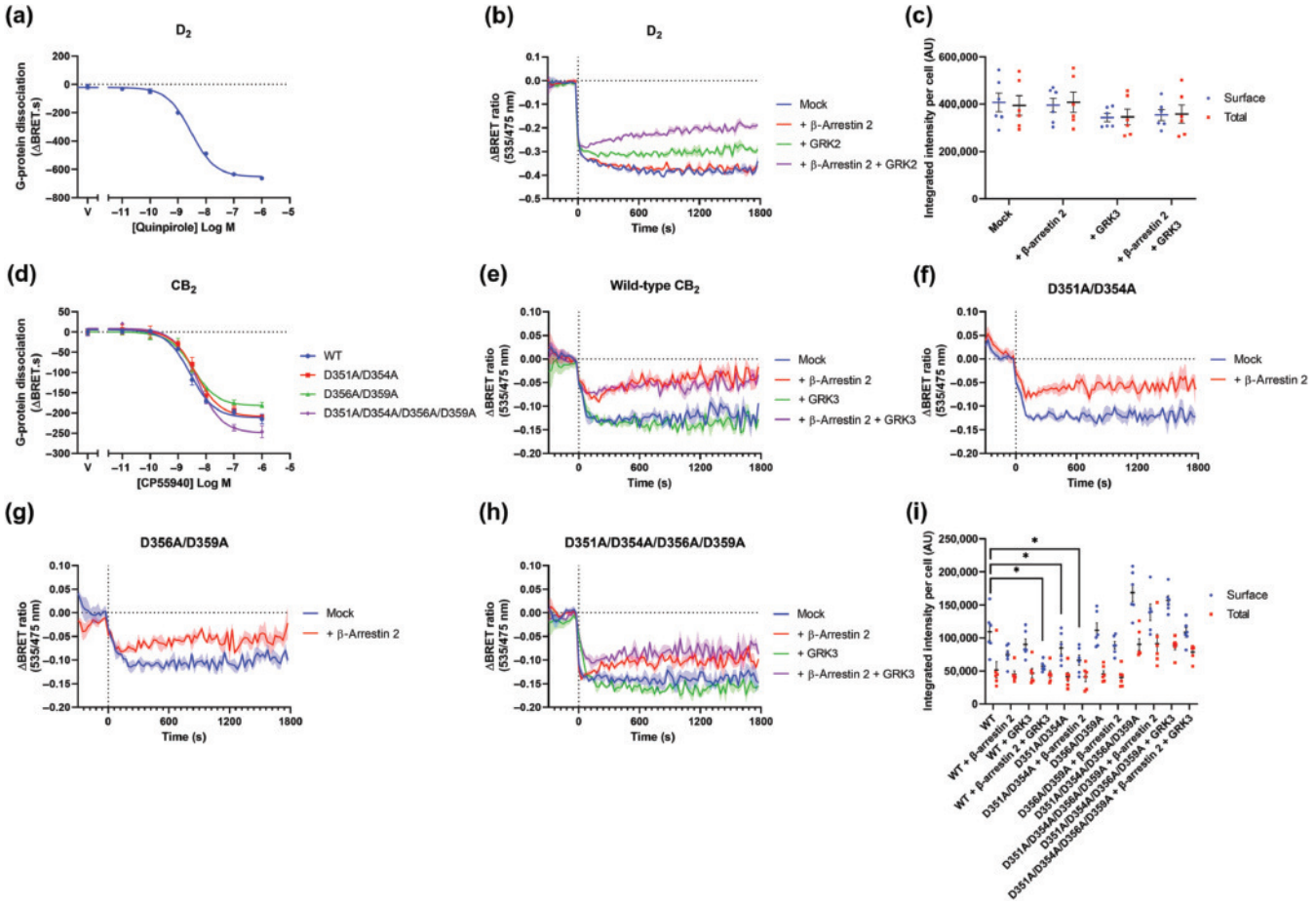

FIGURE 6 Effect of CB_2 C-terminal aspartic acid residues on G protein dissociation in HEK 293 cells. Representative concentration–response curves for wild-type D_2 in response to quinpirole (a) or wild-type (WT) CB_2 and CB_2 C-terminal mutants, D351A/D354A, D356A/D359A and D351A/D354A/D356A/D359A, in response to CP55940 (d). Representative kinetic traces of G protein dissociation in response to 1-μM quinpirole for wild-type D_2 when coexpressed with β-arrestin 2 and/or GRK2 (b). Kinetic traces of G protein dissociation in response to 1-μM CP55940 for wild-type (e), D351A/D354A (f), D356A/D359A (g) and D351A/D354A/D356A/D359A (h) CB_2 when coexpressed with β-arrestin 2 and/or GRK3. Surface and total receptor expression for wild-type D_2 (c) or wild-type CB_2 and CB_2 mutant cell lines (i), quantified by immunocytochemistry with *P* values indicated as *<0.05 determined from repeated measures one-way ANOVA, followed by Holm–Šídák post hoc multiple comparisons test when compared with matched mock or wild type. Data represent mean ± SD of technical duplicates or triplicates (a,b d–h) or mean ± SEM from six independent biological replicates (c,i)

β-arrestin to CB_2, as previously observed for D_2 (Gurevich et al., 2016). Overexpression of GRK isoforms 1–6 were shown to differently influence β-arrestin translocation to CB_2. In particular, GRK1, GRK2 and GRK3 were found to marginally improve β-arrestin 1 translocation, whereas GRK2, GRK3 and GRK5 slightly reduced β-arrestin 2 translocation following stimulation with the agonists CP55940, 2-AG and AMB-FUBINACA. Although these results may infer potential functional differences between the GRK subtypes, the failure of any subtype to substantially elevate β-arrestin translocation strikingly contrasts with studies examining the role of GRKs across an array of GPCRs, including CB_1 (Ibsen et al., 2019; Jin et al., 1999; Kim et al., 2005; Kouznetsova et al., 2002; Mahavadi et al., 2014; Møller et al., 2020; Nobles et al., 2011; Ren et al., 2005). However, CB_2 has demonstrated the capacity to undergo phosphorylation (Bouaboula et al., 1999; Derocq et al., 2000), with more recent findings supporting an interaction between CB_2, GRK5 and β-arrestin 2 in cannabinoid-mediated up-regulation of the 5-HT_{2A} and D_2 receptors (Franklin et al., 2021; Franklin & Carrasco, 2013). It should be noted that Bouaboula et al. (1999) found that CB_2 undergoes constitutive phosphorylation, which may have obscured GRK effects in this study. However, constitutive CB_2 phosphorylation was enhanced upon stimulation with CP55940 (Bouaboula et al., 1999), suggesting that potential GRK-mediated influences on β-arrestin translocation should remain detectable following agonist stimulation. Therefore, GRK-mediated reductions in the β-arrestin 2 response could simply be consequences of GRK overexpression, which may have improved the rate of β-arrestin translocation, enhanced receptor desensitisation/internalisation and thus reduced surface receptor expression. Although kinetic traces were too variable to accurately quantify rates in this study, the relatively small magnitude of the GRK-mediated reductions in response would likely lack biological importance if indeed accurate.

TABLE 4 Potencies and efficacies for G protein dissociation for wild-type D_2, wild-type CB_2 and various CB_2 mutants[a]

	Mock		+ β-arrestin 2		+ GRK2/3	+ β-arrestin 2 + GRK2/3
	pEC_{50}	Span (ΔBRET.s)	1 μM quinpirole or CP55940 (ΔBRET.s)	% mock	1 μM quinpirole or CP55940 (ΔBRET.s)	1 μM quinpirole or CP55940 (ΔBRET.s)
Wild-type D_2	8.54 (0.02)	−620.63 (21.91)	−623.94 (26.22)	100.42 (0.93)	−489.35 (24.99)†	−381.93 (16.96)†
Wild-type CB_2	8.42 (0.05)	−216.50 (8.58)	−97.47 (3.67)	45.28 (2.15)	−226.64 (5.63)	−91.65 (4.04)
CB_2 D351A/ D354A	8.37 (0.04)	−204.02 (5.54)	−97.62 (4.27)	47.86 (1.69)	—	—
CB_2 D356A/ D359A	8.38 (0.06)	−186.32 (4.70)*	−91.91 (4.94)	49.37 (2.63)	—	—
CB_2 D351A/ D354A/D356A/ D359A	8.31 (0.06)	−246.10 (6.75)*	−165.31 (10.19)◊	67.29 (4.02)◊	−267.49 (4.62)‡	−155.77 (3.82)◊

[a]G protein dissociation measured in HEK 293 cells for wild-type D_2 with and without coexpression of β-arrestin 2 and/or GRK2 in response to quinpirole or for wild-type CB_2 and several CB_2 mutants that contain alanine substitutions for C-terminal aspartic acid residues, with and without coexpression of β-arrestin 2 and/or GRK3 in response to CP55940. Data shown are means (± SEM) from six independent biological replicates.

*Statistical significance determined using repeated measures one-way ANOVA performed in GraphPad Prism, with $P < 0.05$ when compared with wild-type CB_2. ◊Statistical significance determined using repeated measures one-way ANOVA performed in GraphPad Prism, with $P < 0.05$ when compared with wild-type CB_2 in the presence of β-arrestin 2.

†Statistical significance determined using repeated measures one-way ANOVA performed in GraphPad Prism, with $P < 0.05$ when compared with mock-transfected hD_2.

‡Statistical significance ($P < 0.05$) for the D351A/D354A/D356A/D359A mutant plus GRK3 when compared with mock-transfected condition determined using a paired *t* test performed in GraphPad Prism.

The ability of some GRKs to drive subtle improvements in β-arrestin 1 translocation may reflect a weak interaction between β-arrestin 1 and CB_2, which is improved upon receptor phosphorylation. The efficiency of arrestin–receptor interactions purportedly correlates with the occurrence of C-terminal phosphorylation codes (Oakley et al., 2000). Interestingly, the receptor C-terminal tail and transmembrane helical core of rhodopsin were found to contribute differentially to the activation of β-arrestins 1 and 2, with the C-terminus binding more robust with β-arrestin 1 in the presence of key phosphorylation sites (Mayer et al., 2019). This may account for the reduced capability of CB_2 to recruit β-arrestin 1, as the C-terminus of Class A GPCRs generally lack the 'ideal' spacing between phosphorylation sites for effective β-arrestin 1 binding (Oakley et al., 2000). Class A GPCRs are proposed to interact more tightly with β-arrestin 2 as receptor phosphorylation is considered less significant for β-arrestin 2 activation and binding, aligning with the underlying system preference for β-arrestin 2 for the cannabinoid receptors (Finlay et al., 2019; Ibsen et al., 2019).

Despite the confounding GRK effect on β-arrestin translocation, GRK2 and GRK3 were found to significantly influence efficacy in both β-arrestin pathways. GRK2 and GRK3 have been previously shown to improve the efficiency of agonist-dependent β-arrestin recruitment and receptor endocytosis; unlike GRK5 and GRK6 (Kim et al., 2005; Ren et al., 2005). This may pertain to C-terminal divergences between the GRK subfamilies. The pleckstrin homology domain contained within the C-terminal region of GRK2/3 binds acidic phospholipids and free Gβγ subunits, which specifically targets cytosolic GRK2/3 to the receptor following G protein activation (Komolov & Benovic, 2018). Conversely, the GRK4/5/6 subfamily indiscriminately associates with the membrane via palmitoylation of their C-terminal cysteines and/or interaction with membrane phospholipids via an amphipathic α-helix, with GRK1/7 achieving constitutive membrane localisation through post-translational prenylation at their C-termini. Although palmitoylation and prenylation ensure GRK1/4/5/6/7 remain in close proximity to unstimulated GPCRs (Komolov & Benovic, 2018), emerging research on the subcellular localisation of GPCRs into membranous compartments may complement the lack of prominent GRK effects in this study, as these membrane confinements may render CB_2 inaccessible to GRK1/4/5/6 but not GRK2/3 (Lobingier & von Zastrow, 2019).

Alternatively, the absence of a notable GRK effect may reflect that the endogenous levels of GRKs in our cell line are sufficient to induce maximal translocation of β-arrestin to CB_2. Inhibition of endogenous GRK2/3 with compound 101 (but not dominant-negative GRK2 K220R) partially but significantly reduced agonist-stimulated β-arrestin 2 translocation to CB_2, supporting the idea that GRK2/3 participates in a weak interaction with CB_2. One recent study has suggested that concentrations ≥30 μM of compound 101 (as used in this study) may have off-target effects in HEK 293 cells (Møller et al., 2020). However, this is unlikely to explain its *lack* of effect in this study, considering the internal consistency between our compound 101, dominant-negative GRK2 and GRK overexpression data. Furthermore, CB_2 phosphorylation has shown to be pertussis toxin insensitive (Bouaboula et al., 1999), congruent with the modest contribution of the Gβγ-dependent kinases, GRK2/3 in this study. The lack of availability of pharmacological inhibitors limited our evaluation of other endogenous GRK isoforms. The use of CRISPR/Cas9 genomic editing or acute knockdown by siRNA to individually deplete GRK

subtypes would allow for further validation of their regulatory effects. The endogenous expression of GRKs in HEK 293 cells remains inconclusive (Atwood et al., 2011; Hasbi et al., 2004; Zidar et al., 2009). Nevertheless, in the same assay system and cell line, we have previously demonstrated that overexpression of GRK2 could enhance β-arrestin 2 translocation at D_2 (Ibsen et al., 2019), suggesting that responses were not impeded by the attainment of system maximum. Other common kinases, such as **protein kinase C** and **JNK**, have also been implicated in GPCR phosphorylation, which should be examined in order to fully delineate the mechanisms of CB_2 desensitisation (Busillo et al., 2010; Feng et al., 2011; Illing et al., 2014; Leff et al., 2020).

While attempting to further define the structural determinants of β-arrestin interactions with CB_2, we demonstrated that four C-terminal aspartic acids, D^{351}, D^{354}, D^{356} and D^{359}, were involved but not essential for β-arrestin 2 translocation. The importance of acidic, negatively charged residues in receptor phosphorylation and regulation has only been established for a handful of GPCRs (Butcher et al., 2014; Galliera et al., 2004; Jewell-Motz & Liggett, 1995; Lee et al., 2000; Mukherjee et al., 2002). Aspartates and glutamates can successfully serve as phosphate mimics (Kang et al., 2020; Lin et al., 1997, 2002; Luan et al., 2005; Paradis et al., 2015). Recent structural studies have offered insights into the intramolecular mechanisms by which acidic amino acids may activate β-arrestins (Min et al., 2020; Staus et al., 2020; Yin et al., 2019; Zhou et al., 2017). The C-terminus of the receptor is proposed to bind to positively charged crevices on the surface of the β-arrestin N-domain, triggering an integrated set of structural changes that stabilise an active β-arrestin conformation by interdomain twisting. The negatively charged residues on the receptor are purported to neutralise the positively charged residues of β-arrestin through the formation of an electrostatic interaction interface.

It has been posited that bulk negative charge, as opposed to a precise motif of phosphorylatable or phospho-mimetic residues, may be sufficient to satisfy the requirements for β-arrestin/receptor binding. However, the lack of an apparent additive reduction in β-arrestin 2 translocation with the $D^{351}A/D^{354}A/D^{356}A/D^{359}A$ mutant, compared with the individual $D^{351}A/D^{354}A$ and $D^{356}A/D^{359}A$ mutants, argues against the importance of a bulk negative charge on the C-terminus. Indeed, discrepancies in β-arrestin 2 efficacy between the $D^{351}A/D^{354}A$ and $D^{356}A/D^{359}A$ mutants may reinforce the necessity for specific phosphorylation sites or sequences, as described for other receptors (Butcher et al., 2011; Inagaki et al., 2015; Mayer et al., 2019; Nobles et al., 2011; Yang et al., 2015; Zhou et al., 2017; Zidar et al., 2009). Failure to completely abrogate β-arrestin 2 translocation with the C-terminal mutants in this study further suggests that alternative key sites or phosphorylation motifs within CB_2 may be present. Alternatively, removal of aspartic acid residues may have impeded the activity of acidotropic kinases, and subsequent CB_2 phosphorylation and β-arrestin binding (Bouaboula et al., 1999). It has been proposed that the negative charges on phosphate groups may not be solely liable for the consequential effects of phosphorylation, with the introduction of steric hindrance or conformational change exerting a greater influence (Paleologou et al., 2008). Therefore, multiple receptor structural elements, such as acidic amino acids and phosphates, may operate in concert to recruit β-arrestin to CB_2, as previously illustrated for the **free fatty acid receptor 4** (Butcher et al., 2014). Notably, the restoration of GRK2/3 activity with the $D^{351}A/D^{354}A/D^{356}A/D^{359}A$ mutant implies that aspartic acid residues interfere with GRK-mediated phosphorylation of wild-type CB_2. Simultaneous mutagenesis of aspartic acid and serine/threonine residues may better outline the complete structural determinants for β-arrestin activation.

Given the impact of the C-terminal aspartic acid mutations on β-arrestin 2 translocation, we sought to evaluate the potential implications of altered β-arrestin translocation on normal (wild-type) CB_2 function (Figure 6). G protein activation for wild-type CB_2 was impaired in the presence of β-arrestin 2, indicative of increased receptor desensitisation. However, CB_2 desensitisation was not further potentiated by coexpression of a GRK, unlike D_2, in agreement with the β-arrestin 2 translocation data (Figure 2). Notably, all receptor mutants possessed similar functionality in G protein activation to wild-type CB_2 but diverged in the presence of β-arrestin 2. Although the $D^{351}A/D^{354}A$ and $D^{356}A/D^{359}A$ mutants retained similar desensitisation capabilities as wild-type CB_2, the CB_2 mutant lacking all four aspartic acid residues demonstrated a slightly impaired capacity to desensitise, even upon coexpression of GRK3 (Figure 6 and Table 4). This conflicted with our β-arrestin 2 findings, where $D^{351}A/D^{354}A$ showed the greatest reduction in β-arrestin translocation and GRK3 potentiated β-arrestin 2 translocation for $D^{351}A/D^{354}A/D^{356}A/D^{359}A$. Interestingly, our data showed that GRK3 alone slightly increased G protein activation for the $D^{351}A/D^{354}A/D^{356}A/D^{359}A$ mutant, perhaps reflecting an improved efficiency in G protein coupling for the phosphorylated variant of the receptor. It is possible that this could mask the amplifying actions of GRK3 on β-arrestin-mediated desensitisation.

Taken together, our data suggest compensation by other phospho-mimetic or phosphorylation sites that still facilitate β-arrestin binding. Congruently, studies have indicated that β-arrestin activation is controlled by multiple phosphorylation moieties across the GPCR C-terminus (Mayer et al., 2019; Nobles et al., 2011; Yang et al., 2015). These particular motifs may shape the structural and functional consequences of β-arrestin activation. For example, β-arrestin 1 has been shown to adopt two conformations: a 'core' conformation, whereby β-arrestin engages with the receptor intracellular core via its finger-loop region; or a 'tail' conformation, whereby β-arrestin interacts solely with the receptor C-terminus. When bound in this tail conformation, β-arrestin exhibited canonical signalling and internalisation capabilities but was unable to mediate G protein desensitisation of the vasopressin V_2 receptor (Cahill et al., 2017; Kumari et al., 2016). It could therefore be speculated that the CB_2 mutants possess distinct interaction points with β-arrestin, thus driving a specific β-arrestin conformation that differentially regulates G protein desensitisation. Changes in receptor conformation and/or interactions with effectors following C-terminal mutagenesis should also be considered.

Differences in receptor expression should be considered in the interpretation of findings from this study. The expression levels of several mutants were significantly lower in β-arrestin and G protein assays compared with wild-type receptor, possibly influencing the responses obtained. However, our data suggest that the relationship between total receptor expression and β-arrestin efficacy is non-linear within this range of receptor expression (Figure 5). Therefore, normalisation of data to adjust for receptor expression would likely lead to inaccurate representations of agonist efficacy and potency. It is also worth noting that the G protein pathway may exhibit receptor reserve, which would enable maximal responses in spite of lower receptor occupancy. This could explain the comparable efficacies in G protein activation for the mutants in instances of reduced receptor expression.

In summary, we have demonstrated that GRKs contribute little to agonist-stimulated β-arrestin translocation to CB_2. However, C-terminal phosphate-mimic—aspartic acid—residues were found to be important for β-arrestin translocation and G protein desensitisation. The relatively small contribution of GRKs and aspartic acid residues in β-arrestin translocation and desensitisation also alludes to alternative effectors or processes involved in the regulation of CB_2. Nevertheless, this work complements the few studies on phospho-mimetic residues and GPCR regulation, which warrants further study in order to comprehensively define 'phosphorylation barcodes' or patterns of phosphorylation pertinent to the activation of β-arrestins—particularly in the light of our data that suggest that these proposed determinants of response specificity may not contribute to regulating β-arrestin recruitment to CB_2.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

M.P. designed and performed the experiments, analysed the data and wrote the paper. C.M. and D.F.L. generated and kindly provided the pIRES Gβ2A-cpVenus-Gγ2-Gαi1-Nluc construct for the G protein dissociation assays. N.L.G. designed and developed the original wild-type CB_2 63R receptor construct and contributed to obtaining funding and aspects of experimental design. J.A.J. provided and advised on the arrestin assay. D.B.F. and M.G. designed the experiments, obtained funding and reviewed drafts of the paper. All authors reviewed and approved the final version of the paper.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis and Immunoblotting and Immunochemistry and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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