



Cannabinoid CB1 and CB2 Receptor-Mediated Arrestin Translocation: Species, Subtype, and Agonist-Dependence

Mikkel Søs Ibsen^{1,2}, David B. Finlay³, Monica Patel^{1,2}, Jonathan A. Javitch^{4,5}, Michelle Glass^{3*†} and Natasha Lillia Grimsey^{1,2*†}

¹ Department of Pharmacology and Clinical Pharmacology, School of Medical Sciences, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand, ² Centre for Brain Research, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand, ³ Department of Pharmacology and Toxicology, University of Otago, Dunedin, New Zealand, ⁴ Department of Psychiatry and Pharmacology, Columbia University Vagelos College of Physicians and Surgeons, New York, NY, United States, ⁵ Division of Molecular Therapeutics, New York State Psychiatric Institute, New York, NY, United States

OPEN ACCESS

Edited by:

Vsevolod V. Gurevich,
Vanderbilt University, United States

Reviewed by:

Sudarshan Rajagopal,
Duke University Health System,
United States
Mary Abood,
Temple University, United States

*Correspondence:

Michelle Glass
michelle.glass@otago.ac.nz
Natasha Lillia Grimsey
n.grimsey@auckland.ac.nz

† These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Experimental Pharmacology
and Drug Discovery,
a section of the journal
Frontiers in Pharmacology

Received: 07 February 2019

Accepted: 21 March 2019

Published: 10 April 2019

Citation:

Ibsen MS, Finlay DB, Patel M,
Javitch JA, Glass M and Grimsey NL
(2019) Cannabinoid CB1 and CB2
Receptor-Mediated Arrestin
Translocation: Species, Subtype,
and Agonist-Dependence.
Front. Pharmacol. 10:350.
doi: 10.3389/fphar.2019.00350

Arrestin translocation and signaling have come to the fore of the G protein-coupled receptor molecular pharmacology field. Some receptor–arrestin interactions are relatively well understood and considered responsible for specific therapeutic or adverse outcomes. Coupling of arrestins with cannabinoid receptors 1 (CB₁) and 2 (CB₂) has been reported, though the majority of studies have not systematically characterized the differential ligand dependence of this activity. In addition, many prior studies have utilized bovine (rather than human) arrestins, and the most widely applied assays require reporter-tagged receptors, which prevent meaningful comparison between receptor types. We have employed a bioluminescence resonance energy transfer (BRET) method that does not require the use of tagged receptors and thereby allows comparisons of arrestin translocation between receptor types, as well as with cells lacking the receptor of interest – an important control. The ability of a selection of CB₁ and CB₂ agonists to stimulate cell surface translocation of human and bovine β-arrestin-1 and -2 was assessed. We find that some CB₁ ligands induce moderate β-arrestin-2 translocation in comparison with vasopressin V₂ receptor (a robust arrestin recruiter); however, CB₁ coupling with β-arrestin-1 and CB₂ with either arrestin elicited low relative efficacies. A range of efficacies between ligands was evident for both receptors and arrestins. Endocannabinoid 2-arachidonoylglycerol stood out as a high efficacy ligand for translocation of β-arrestin-2 via CB₁. Δ⁹-tetrahydrocannabinol was generally unable to elicit translocation of either arrestin subtype via CB₁ or CB₂; however, control experiments revealed translocation in cells not expressing CB₁/CB₂, which may assist in explaining some discrepancy with the literature. Overexpression of GRK2 had modest influence on CB₁/CB₂-induced arrestin translocation. Results with bovine and human arrestins were largely analogous, but a few instances of inconsistent rank order potencies/efficacies between bovine and human arrestins raise the possibility that subtle differences in receptor conformation stabilized by these ligands manifest in

disparate affinities for the two arrestin species, with important potential consequences for interpretation in ligand bias studies. As well as contributing important information regarding CB₁/CB₂ ligand-dependent arrestin coupling, our study raises a number of points for consideration in the design and interpretation of arrestin recruitment assays.

Keywords: cannabinoid receptor 1 (CB₁), cannabinoid receptor 2 (CB₂), G protein-coupled receptor (GPCR), arrestin, cannabinoid, signaling bias, signaling, vasopressin

INTRODUCTION

Cannabinoid receptor 1 (CB₁) and cannabinoid receptor 2 (CB₂) are seven transmembrane domain G protein-coupled receptors (GPCRs). CB₁ regulates neurotransmission and a range of peripheral functions, whereas CB₂ regulates immune and inflammatory pathways (Pertwee et al., 2010).

Many cannabinoid ligands demonstrate activity at both CB₁ and CB₂. These compounds show a remarkable degree of chemical diversity, and comprise at least four distinct classes: eicosanoids, including all the known endocannabinoids [such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG)]; classical [*Cannabis sativa*-derived Δ^9 -tetrahydrocannabinol (THC) and THC-like]; non-classical (CP55,940); and aminoalkylindole (WIN55,212-2).

The cannabinoid receptors (CBRs) are well-characterized in their signaling via several G proteins to influence the activity of adenylyl cyclase and induce activation of the mitogen-activated protein kinase (MAPK) pathway, among other effects (Felder et al., 1995; Glass and Northup, 1999; Stamer et al., 2001; Bash et al., 2003; Finlay et al., 2017; Ibsen et al., 2017). However, they are less characterized as β -arrestin-coupled receptors. Two β -arrestins are relevant to CB signaling: β -arrestin-1 and β -arrestin-2. It is believed that the downstream effects of β -arrestin-coupled signaling can be isolated from the G protein-mediated pathways and that the non-canonical β -arrestin signaling pathways may be exploitable in the future by the development of novel pharmaceutical agents (Donthamsetti et al., 2018). For this reason, the ability of different agonists to preferentially activate specific intracellular signaling pathways over others – known as “functional selectivity” or “biased signaling” – has recently gained increasing attention (Kenakin and Christopoulos, 2013).

The CBRs have long been considered to hold promise as drug targets. However, to date, only cannabis-derived products have reached the market. The μ -opioid receptor (MOR), which activates G α_i -type G proteins and recruits arrestins (like both CB₁ and CB₂), provides an example of the therapeutic potential of biased agonism. Studies of MOR suggest that activation of G protein-coupled signaling over β -arrestin-coupled signaling leads to fewer adverse effects (Raehal et al., 2005; DeWire et al., 2013; Manglik et al., 2016), and G protein-biased agonists are now in clinical trial (Viscusi et al., 2016; Singla et al., 2017). In the same manner, it is hypothesized that drugs could target the CBRs but only activate the signaling pathways that elicit desirable effects (Ibsen et al., 2017).

Arrestin–GPCR interactions are thought to follow receptor C-terminal or intracellular loop phosphorylation by a G

protein-coupled receptor kinase (GRK) after agonist binding (Moore et al., 2007; Shukla et al., 2013; Zhou et al., 2017). The phosphorylation of GPCRs by GRKs has given rise to the “barcode” hypothesis, wherein GRK isoforms differentially recognize various receptor conformations stabilized by different types of agonists and then phosphorylate the receptors on different sites. These distinctive phosphorylation patterns would then form a “bar code,” which may be recognized by a β -arrestin. Thus, a specific agonist will determine the receptor conformation and subsequent repertoire of canonical signaling responses, the intracellular receptor phosphorylation “bar code,” and thereafter the isoform and conformation of the arrestin that is recruited and direct the downstream effectors (Nobles et al., 2011). For example, depending on the particular agonist used to stimulate the β_2 adrenergic receptor, either GRK2 or GRK6 phosphorylates the receptor at distinct sites resulting in structurally and functionally distinct β -arrestin-2 conformations (Nobles et al., 2011). Recent observations suggest that CB₁ signaling is also regulated by different GRK isoforms leading to distinct downstream effects (Delgado-Peraza et al., 2016).

Following GRK-mediated receptor phosphorylation, the binding of arrestin has traditionally been thought to mask the domains interacting with G proteins and thereby prevent further G protein-mediated signaling. As such, arrestin signaling has generally been thought to occur in the absence of bound G protein (Shukla et al., 2014; Kang et al., 2015), although this has been disputed (Thomsen et al., 2016). However, arrestin-mediated signaling likely occurs as a result of different ligand/receptor conformation(s) from those involved in G α interactions (Kenakin and Christopoulos, 2013).

Both β -arrestin-1 and -2 have been reported to be involved in CB₁ and CB₂ signaling. The binding of β -arrestin-2 to CB₁ has been indicated to cause desensitization and internalization (Jin et al., 1999; Daigle et al., 2008a,b). However, only subtle differences in behavior have been observed between wild-type versus β -arrestin-2 knock-out mice following THC treatment, with enhanced analgesia and hypothermia but no change in catalepsy (Breivogel et al., 2008; Nguyen et al., 2012). The same studies suggested enhanced G protein activation in response to THC in some brain regions of the β -arrestin-2 knock-out mice, but, in contrast, a further study on these mice demonstrated decreased efficacy of THC signaling in brain membranes (Breivogel et al., 2013).

Recruitment of β -arrestin-2 to CB₂ has been demonstrated using the PathHunter DiscoverX enzyme complementation assay (McGuinness et al., 2009; Dhopeswarkar and Mackie, 2016; Soethoudt et al., 2016, 2017) and by measuring the translocation of fluorescently tagged β -arrestin-2 to the cell

membrane (Atwood et al., 2012; Nogueras-Ortiz et al., 2017). The downstream effects of β -arrestin-2 interacting with CB₂ are also suggested to cause internalization and desensitization (Chen et al., 2014). CB₂ undergoes agonist-mediated C-terminal phosphorylation, followed by a decrease in signaling and surface receptor levels (Bouaboula et al., 1999; Derocq et al., 2000; Chen et al., 2014). Furthermore, agonist-mediated internalization of CB₂ has been widely observed (Carrier et al., 2004; Shoemaker et al., 2005; Grimsey et al., 2011; Atwood et al., 2012).

Some reports have found little-to-no β -arrestin-1 recruitment to CB₁ (Gyombolai et al., 2013, 2015; Delgado-Peraza et al., 2016). Others find that β -arrestin-1 is recruited to CB₁ (Laprairie et al., 2014, 2016), a finding that is corroborated by structural studies of β -arrestin-1 interaction with a synthesized CB₁ C-terminus (Bakshi et al., 2007; Singh et al., 2011). There is only one recent report suggesting β -arrestin-1 recruitment to CB₂ (Nogueras-Ortiz et al., 2017), whereas a prior study indicated lack of recruitment or functional involvement in internalization (Chen et al., 2014).

There is also limited evidence indicating the downstream effects of β -arrestin-1 binding to the CBRs. Generally, in binding to GPCRs β -arrestin-1 serves as a scaffold for a pathway leading to the phosphorylation of MAPK extracellular signal-regulated kinase (ERK) (DeWire et al., 2007). There is evidence to support this function of β -arrestin-1 after binding to CB₁ (Flores-Otero et al., 2014; Delgado-Peraza et al., 2016) and CB₂ (Nogueras-Ortiz et al., 2017).

The interaction between a GPCR and a β -arrestin is often observed via bioluminescence resonance energy transfer (BRET) or enzyme complementation assays. A potential issue with these types of assays is that protein “modules” are typically fused to both the cytoplasmic tail of the receptor and to the arrestin to enable quantification of the interaction using optical techniques. The signaling capacity of these chimeric proteins is not usually verified, and so it is not obvious as to whether the interactions being reported faithfully reflect those of the native receptor and effector. Imaging-based approaches, on the other hand, involve monitoring of the translocation of a fluorescently tagged arrestin by confocal microscopy. This approach offers the advantage that the receptor of interest need not be tagged, but it is highly labor-intensive, and as a result typically very few cells per experimental treatment are sampled. Consequently, it is highly impractical to perform thorough concentration responses utilizing multiple ligands and/or receptors.

Considering these factors, a novel BRET approach has been developed where the receptor remains untagged, and this approach has been recently utilized to study the dopamine receptor D₂ (D₂R) (Clayton et al., 2014; Donthamsetti et al., 2015). In this design, the β -arrestin is fused to the BRET donor modified *Renilla* Luciferase (Rluc8), and the BRET acceptor Citrine is fused to a doubly palmitoylated fragment of GAP43, which anchors it to the plasma membrane. As such, the assay output is not a direct measure of the distance between the receptor and β -arrestin but rather the proximity of β -arrestin to the plasma membrane. Hence, the assay is not a β -arrestin recruitment assay and is instead a

translocation assay. The membrane translocation of β -arrestins can generally be interpreted as recruitment to the receptor. Using the native receptor circumvents the risk of tags fused to the cytoplasmic tail of the receptor introducing aberrant conformational states, and it ensures that receptor signaling and trafficking are unaffected. Furthermore, the assay allows for comparisons of β -arrestin recruitment capabilities between different receptors. This study has utilized this assay (with receptors minimally modified with an extracellular epitope-tag to facilitate measurement of expression) to investigate the ability of CB₁ and CB₂ to induce β -arrestin-1 and -2 plasma membrane translocation in response to stimulation with a range of cannabinoid ligands. Furthermore, we have compared the CB₁- and CB₂-mediated activity profiles of human and bovine β -arrestins.

MATERIALS AND METHODS

Plasmids and Cloning

We used the human form of all receptor constructs and transiently expressed transgenes to ensure high expression levels. The ppls-3HA-hCB₁ pEF4a construct has been described previously (Finlay et al., 2017). The use of the preprolactin signal sequence (ppls) chimera of CB₁ was necessary to ensure sufficient expression levels. The 3HA-hCB₂ pEF4a construct has been described previously (Grimsey et al., 2011).

The 3HA-hV₂R pcDNA3.1 construct and 3HA-hD₂R pcDNA3.1 construct were both purchased from cDNA Resource Center (#AVR020TN00 and #DRD020TN01, respectively; www.cdna.org, Bloomsburg, PA, United States).

The Rluc8-b β -arrestin-2-Sp1 pcDNA3.1 (bovine β -arrestin-2) and mem-linker-Citrine-SH3 pcDNA3.1 were described in the original papers detailing the β -arrestin translocation assay (Clayton et al., 2014; Donthamsetti et al., 2015). Rluc8-b β -arrestin-1-Sp1 was synthesized and cloned into pcDNA3.1 commercially, using the restriction sites HindIII and NotI (GenScript, Piscataway, NJ, United States).

The Rluc8-h β -arrestin-2-Sp1 pcDNA3.1 (human β -arrestin-2) construct was generated by Restriction-Free Cloning (van den Ent and Lowe, 2006). In brief, the entire human β -arrestin-2 gene except the stop codon was amplified by PCR from the originally sourced pcDNA3.1 plasmid (#ARRB200001; cDNA Resource Center). The primers used for this reaction created overhangs complementary for the proximal Rluc8 and Sp1 regions of the target vector. The reaction product was treated with DpnI (Roche, Basel, Switzerland) to destroy template DNA, and then electrophoresed on a 1% agarose gel and gel extracted. Using the Rluc8-b β -arrestin-2-Sp1 pcDNA3.1 construct (see above) as a template, a second PCR reaction was performed using the purified PCR product as the primer (with terminal additions complementary for Rluc8 and Sp1 regions) such that the extant b β -arrestin-2 gene would be replaced with h β -arrestin-2. This reaction product was DpnI-treated and then transformed into ultracompetent *Escherichia coli*, purified by commercial miniprep kit and sequence verified. Rluc8-h β -arrestin-1-Sp1 pcDNA3.1 was generated by

commercial Gibson Assembly kit (New England Biolabs, Ipswich, MA, United States). In brief, PCR reactions were performed to generate products of: the entire human β -arrestin-1 gene (except the stop codon) with 5' and 3' overhangs complementary for the proximal Rluc8 and Sp1 regions of the target vector (amplified from plasmid #ARRB100002, cDNA Resource Center); and the backbone of the target vector, including both Rluc8 and Sp1 fragments but with the original β -arrestin-2 fragment removed. Both reaction products were electrophoresed on a 1% agarose gel and gel extracted, and then combined with Gibson Assembly reaction components in a 10 μ l final volume. A Gibson Assembly reaction was performed in accordance with the manufacturer's instructions, and the product was transformed into ultracompetent *E. coli*, purified by commercial miniprep kit, and sequence verified.

The bGRK2 pcDNA3 plasmid was a kind gift from Associate Professor Kevin Pflieger (Harry Perkins Institute of Medical Research, The University of Western Australia, Perth, Western Australia, Australia). Empty pcDNA3.1+ was purchased from Thermo Fisher Scientific (#V79020, Waltham, MA, United States).

Cell Culture and Transfection

HEK-293 cells (ATCC #CRL-1573, Manassas, VA, United States) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (HyClone, GE Life Sciences, Logan, UT, United States) supplemented with 10% (v/v) fetal bovine serum (FBS) (Moregate Biotech, Bulimba, Australia) in a humidified incubator at 37°C and 5% CO₂. The transfection protocol was modified from a previously described protocol (Donthamsetti et al., 2015). 4 \times 10⁶ HEK-293 cells were seeded in a 10-cm dish and cultured for approximately 24 h in 8 ml of media. For each 10-cm dish to be transfected, 300 ng Rluc8- β -arrestin-Sp1 pcDNA3.1, 12 μ g mem-linker-Citrine-SH3 pcDNA3.1, receptor construct (9.6 μ g ppls-3HA-hCB₁ pEF4a, 9.6 μ g 3HA-hCB₂ pEF4a, 2.4 μ g 3HA-hV₂R pcDNA3.1, or 4.8 μ g 3HA-hD₂R pcDNA3.1), and either 0 or 6 μ g bGRK2 pcDNA3.1 were combined. Empty pcDNA3.1 vector (without an insert) was added to a total mass of 27.9 μ g DNA. The DNA was mixed in Opti-MEM (Thermo Fisher Scientific, Waltham, MA, United States) to a final volume of 500 μ l. In a separate tube, 42 μ l PEI MAX (1 μ g/ μ l) (Polysciences, Warrington, PA, United States) was mixed with Opti-MEM to a total volume of 500 μ l. The 500 μ l of DNA in Opti-MEM and 500 μ l of PEI MAX in Opti-MEM were mixed (mass ratio of 1:1.5 DNA:PEI MAX) and incubated for 20 min at room temperature (RT). During the incubation the 8 ml of cell media was replaced. The transfection mix was added drop-wise to the 10-cm dish and incubated for approximately 24 h.

Bioluminescence Resonance Energy Transfer Arrestin Assay

Transfected cells were lifted from the 10-cm dishes by trypsinization and plated in poly-D-lysine (Sigma-Aldrich, St. Louis, MO, United States) coated, white 96-well Costar® plates

(Corning, NY, United States) at a density of 6 \times 10⁴ cells/well in 100 μ l of DMEM with 10% (v/v) FBS and cultured overnight. Cells were equilibrated in Hank's Balanced Salt Solution (HBSS) (Gibco, Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 1 mg/ml bovine serum albumin (BSA) (ICPBio, Auckland, New Zealand) for 30 min. Coelenterazine H (NanoLight Technologies, Pinetop, AZ, United States) was added to a final concentration of 5 μ M for 4 min prior to dispensing drugs and initiating luminescence reading at 475 \pm 30 nm (Rluc8) and 535 \pm 30 nm (Citrine) on a LUMIstar® Omega luminometer (BMG Labtech, Ortenberg, Germany) to obtain baseline BRET ratios. Stimulating drugs were then added and BRET signals were detected in real-time for 25 min. All drugs (including coelenterazine H) were prepared in HBSS with 1 mg/ml BSA, and all incubations and stimulations were performed at 37°C.

To account for small differences in basal BRET ratios between replicates, post-drug addition data were normalized by subtracting the average individual pre-drug addition (baseline) read. To obtain Δ BRET ratios, the mean vehicle trace was subtracted from each drug condition at matched time points. The 25 min Δ BRET time course data were analyzed using net area under the curve (AUC) analysis (GraphPad Prism v7; GraphPad Software Inc., La Jolla, CA, United States) and the AUC values were used to generate concentration–response curves. All arrestin translocation data therefore have the units of Δ BRET ratio.second (Δ BRET.sec). A subset of the h β -arrestin-2 experiments were undertaken with a different biosensor expression level than the primary set of experiments. This influenced the overall Δ BRET.sec response magnitude measured. Importantly, response potency was unaffected. Internal controls with equivalent stimulation conditions between the two datasets with different biosensor expression levels were utilized to normalize the data such that they could be meaningfully compared on the same Δ BRET.sec scale. Concentration–response parameters were obtained by fitting three-parameter (Hill coefficient constrained to 1) nonlinear regression curves. No-receptor controls were performed using cells not transfected with receptor (otherwise transfected in the same manner) but stimulated with the same drug concentrations and analyzed in the same way. The no-receptor measurements were subtracted at each concentration point to eliminate non-receptor-mediated effects.

Drugs

WIN55,212-2 and BAY59-3074 (BAY) were purchased from Tocris Bioscience (Bristol, United Kingdom). CP55,940, AEA, 2-AG, JWH-015, and JWH-133 were purchased from Cayman Chemical Company (Ann Arbor, MI, United States). (–)-*trans*-THC was purchased from THC Pharm GmbH (Frankfurt, Germany). Dopamine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, United States). Arginine vasopressin (AVP) was a kind gift from Dr. Mark Oliver (The University of Auckland, Auckland, New Zealand).

Drug stocks were prepared in absolute ethanol (CP55,940, WIN55,212-2, AEA, 2-AG, THC, BAY, JWH-133) or DMSO

(JWH-015) and were stored in aliquots at -80°C prior to use. Dopamine and AVP were made up in H_2O immediately prior to use. Drug aliquots used for experiments involving serial dilutions were always single-use. Vehicle controls for serial dilutions were maintained constant within experiments.

Immunocytochemistry

Transfected cells were lifted from the dish with trypsin, and plated 6×10^4 per well in a poly-D-lysine-treated clear 96-well plate (Nunc, Roskilde, Denmark) and incubated for approximately 24 h. The wells were aspirated and the cells were fixed in 4% (w/v) paraformaldehyde buffered with phosphate pH 7.4 for 10 min. Following fixation, the cells were washed with phosphate buffered saline (PBS). Cells were then treated with 90% (v/v) methanol for 10 min at -20°C . The methanol solution was aspirated and the remaining methanol evaporated for 10 min at RT before the cells were washed in PBS with 0.2% (v/v) Triton X-100 (PBS-T).

The hemagglutinin (HA) tag was detected by incubation with mouse anti-HA IgG (Biolegend, San Diego, CA, United States) diluted 1:1000 in immunobuffer (PBS-T, 1% v/v goat serum, and 0.4 mg/ml Thiomersal) for 3 h at RT with rocking. The primary antibody was aspirated, and the cells were washed in PBS-T. Secondary antibody, Alexa Fluor 647 goat anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA, United States), was diluted 1:400 in immunobuffer, and incubated overnight at 4°C with rocking. Following staining with the secondary antibody, the cells were washed in PBS-T. The nuclei were stained with Hoechst 33258 (4 mg/ml in H_2O diluted 1:500 in PBS-T) for 30 min at RT with rocking. Following the nuclei staining the cells were washed in $100 \mu\text{l}$ PBS-T and stored in PBS-T with 0.4 mg/ml Thiomersal. The image acquisition was performed on an ImageXpress Micro XLS (Molecular Devices, Sunnyvale, CA, United States). Quantitative analysis was performed with MetaXpress[®] software (Molecular Devices, Sunnyvale, CA, United States) using an in-house journal, which measures staining intensity from receptor-positive cells, as previously described (Finlay et al., 2016).

Data and Statistical Analysis

All statistical analyses were performed using data from at least three biological (independent) replicates. All plots and curve-fits were obtained using GraphPad Prism v7. Data presented in figures are either a single representative experiment (mean \pm SD of technical replicates) or mean (\pm SEM) from independent experiments (as indicated in the figure legends). Parameters noted in the text and recorded in tables are the means from independent experiments (\pm SEM).

Statistical tests were executed with SigmaPlot (v.13.0.0.83, Systat Software, Chicago, IL, United States). One-way ANOVA were utilized when comparing more than two groups with one independent variable, and two-way ANOVA for more than two groups with two independent variables. Normality and equality of variance assumptions were verified with Shapiro–Wilk and Brown–Forsythe tests, respectively. If these tests did not pass, data were transformed appropriately to satisfy parametric test assumptions. When overall ANOVA results suggested a

significant difference, groups were compared with Holm–Šidák *post hoc* tests (either all pairwise, or versus vehicle, as indicated in the section “Results”). In tabular representations, * indicates a *P*-value of <0.05 .

RESULTS

Bovine β -Arrestin-2 ($\text{b}\beta$ -Arrestin-2) Translocation

We began our investigation using bovine β -arrestin-2 ($\text{b}\beta$ -arrestin-2). The receptor-independent nature of the β -arrestin translocation BRET assay we employed allows for comparison of β -arrestin translocation between receptors. Human vasopressin receptor 2 (V_2R) is a known efficient recruiter of β -arrestin-2 (Oakley et al., 1999; Charest and Bouvier, 2003). We used V_2R expressed in HEK293 cells stimulated with AVP to validate the expression and translocation of $\text{b}\beta$ -arrestin-2 (Figure 1A). $\text{b}\beta$ -Arrestin-2 was translocated to the plasma membrane upon V_2R stimulation with a *p*EC50 of 8.30 ± 0.04 , similar to that previously reported (Table 1; Armstrong et al., 2013).

To investigate $\text{b}\beta$ -arrestin-2 plasma membrane translocation following CB_1 activation, we stimulated the receptor with a panel of six well-known CB_1 agonists: CP55,940, 2-AG, AEA, BAY, THC, and WIN55,212-2 (Figure 1C). CP55,940 was found to be the most potent agonist, while 2-AG was the most efficacious (Table 2). THC and BAY did not elicit a response, indicating that these two ligands do not detectably induce translocation of $\text{b}\beta$ -arrestin-2 to CB_1 within the concentration range tested (not significantly different from vehicle at $10 \mu\text{M}$, $p = 0.84$; one-way ANOVA).

The β -arrestin translocation BRET assay was initially developed to investigate $\text{b}\beta$ -arrestin-2 translocation to the human D_2 dopamine receptor (D_2R) (Clayton et al., 2014; Donthamsetti et al., 2015). The same work suggests that $\text{b}\beta$ -arrestin-2 is more efficaciously translocated to D_2R when GRK2 is over-expressed (Clayton et al., 2014). As the maximum efficacy of translocation of the most efficacious ligand (2-AG) for CB_1 was only approximately 57% of the vasopressin response, we investigated whether GRK2 potentiates the translocation of $\text{b}\beta$ -arrestin-2 to CB_1 . Thus, we co-expressed GRK2 and stimulated with CP55,940 and 2-AG (Figure 1D). We chose to utilize only these two ligands because CP55,940 had high potency, while 2-AG had high efficacy. The co-expression of GRK2 did not significantly alter the efficacy of translocation of $\text{b}\beta$ -arrestin-2 to CB_1 when stimulating with CP55,940 or 2-AG (CP55,940: $p = 0.15$, 2-AG: $p = 0.25$; two-way ANOVA), however, both ligands appeared to translocate arrestin with slightly increased potency, reaching statistical significance for CP55,950 which was shifted by half a log unit ($p = 0.036$; two-way ANOVA) (Table 2). We verified that we could replicate the prior finding by assaying D_2R expressed with or without co-expressed GRK2 and stimulated with dopamine. As previously reported, in the presence of co-expressed GRK2, $\text{b}\beta$ -arrestin-2 was translocated with a significantly greater efficacy ($p < 0.001$; two-way ANOVA)

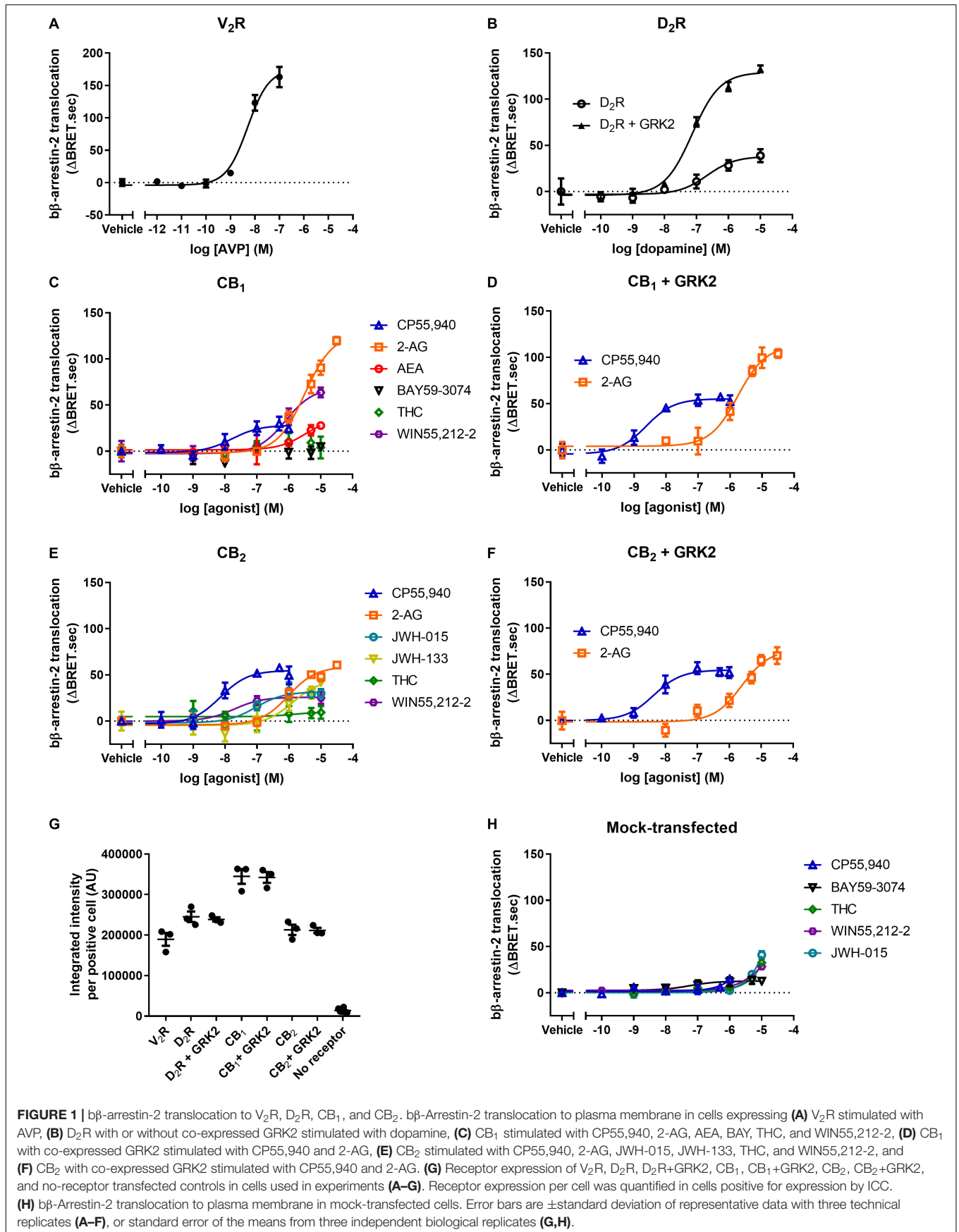


TABLE 1 | Bovine β -arrestin-2 translocation to V_2R and D_2R .

	$b\beta$ -Arrestin-2					
	V_2R		D_2R		$D_2 + GRK2$	
	logEC50 \pm SEM (M)	Emax \pm SEM (Δ BRET.sec)	logEC50 \pm SEM (M)	Emax \pm SEM (Δ BRET.sec)	logEC50 \pm SEM (M)	Emax \pm SEM (Δ BRET.sec)
AVP	-8.30 \pm 0.04	193.6 \pm 11.2	X	X	X	X
Dopamine	X	X	-6.85 \pm 0.16	40.6 \pm 3.1	-7.22 \pm 0.13 ^Δ	129.0 \pm 2.1 ^Δ

X, not determined. ^ΔIndicates value is significantly different in the presence of GRK2 compared to the absence of GRK2 for the same ligand.

TABLE 2 | Bovine β -arrestin-2 translocation to CB_1 and CB_2 .

	$b\beta$ -Arrestin-2							
	CB_1		$CB_1 + GRK2$		CB_2		$CB_2 + GRK2$	
	logEC50 \pm SEM (M)	Emax \pm SEM (Δ BRET.sec)	logEC50 \pm SEM (M)	Emax \pm SEM (Δ BRET.sec)	logEC50 \pm SEM (M)	Emax \pm SEM (Δ BRET.sec)	logEC50 \pm SEM (M)	Emax \pm SEM (Δ BRET.sec)
CP55,940	-7.99 \pm 0.20	35.2 \pm 7.7	-8.45 \pm 0.12 ^Δ	58.9 \pm 9.0	-8.28 \pm 0.14	45.4 \pm 6.2	-8.44 \pm 0.04	70.9 \pm 8.7 ^Δ
2-AG	-5.33 \pm 0.08	116.2 \pm 13.3	-5.65 \pm 0.09 ^Δ	91.7 \pm 11.4	-6.10 \pm 0.05	45.0 \pm 7.1	-5.63 \pm 0.09	75.7 \pm 8.3 ^Δ
WIN55,212-2	-6.15 \pm 0.06	72.0 \pm 9.2	X	X	-7.71 \pm 0.09	25.6 \pm 1.9	X	X
AEA	-5.75 \pm 0.16	32.1 \pm 2.7	X	X	X	X	X	X
THC	NA [^]	0.54 \pm 3.7 [^]	X	X	NA [^]	2.0 \pm 5.2 [^]	X	X
BAY	NA [^]	2.4 \pm 2.8 [^]	X	X	X	X	X	X
JWH-015	X	X	X	X	-6.85 \pm 0.11	26.7 \pm 2.6	X	X
JWH-133	X	X	X	X	-5.71 \pm 0.20	49.7 \pm 4.1	X	X

X, not determined; NA, not active. [^]A concentration–response curve could not be fitted, therefore efficacy at 10 μ M is noted instead of Emax. ^ΔValue is significantly different in the presence of GRK2 compared to the absence of GRK2 for the same ligand.

and we also observed that potency was increased ($p = 0.038$; two-way ANOVA) (**Figure 1B** and **Table 1**).

To investigate the translocation of $b\beta$ -arrestin-2 to CB_2 , we stimulated the receptor with a panel of six well-known CB_2 agonists: CP55,940, 2-AG, JWH-015, JWH-133, THC, and WIN55,212-2 (**Figure 1E**). CP55,940 was the most potent agonist at inducing $b\beta$ -arrestin-2 translocation via CB_2 . CP55,940, 2-AG, and JWH-133 were similarly and maximally efficacious, while THC was again inactive (not significantly different from vehicle at 10 μ M, $p = 0.72$; t -test) (**Table 2**). All ligands with measurable responses were very low efficacy in comparison with AVP at the vasopressin receptor, ranging from approximately 13–26% of the maximum signal observed with this V_2R .

The effect of co-expressing GRK2 on $b\beta$ -arrestin-2 translocation to CB_2 was assayed following stimulation with CP55,940 and 2-AG (**Figure 1F**). The co-expression of GRK2 significantly increased the translocation of $b\beta$ -arrestin-2 to CB_2 when stimulating with either ligand (CP55,940: $p = 0.046$, 2-AG: $p = 0.022$; two-way ANOVA) (**Table 2**).

To confirm receptor expression, and particularly to verify similar expression levels between conditions with and without GRK2, we carried out immunocytochemistry (ICC) and quantified the expression levels (**Figure 1G**). Reassuringly, the receptor expression levels between conditions with and without GRK2 co-expression were similar. Overall, the expression of the V_2R , D_2R , and CB_2 were very similar, with CB_1 levels being

generally higher. Thus, the low efficacy of interactions between CB_1 or CB_2 and the arrestin is unlikely to be due to low receptor expression levels.

For each ligand assayed, $b\beta$ -arrestin-2 translocation was also measured in mock-transfected cells lacking the receptor of interest (**Figure 1H**). Any such “off-target” responses were subtracted from the data discussed above prior to analysis. CB_1 or CB_2 mRNA is undetectable in HEK-293 cells (Atwood et al., 2011), and absence of CBRs is also supported by a lack of response with 2-AG (which when CB_1 and CB_2 were present induced arrestin translocation with the greatest efficacy of the set of ligands tested; **Table 2** and **Figures 1C,E**). JWH-015, WIN55,212-2, and THC induced the greatest non- CB_1 / CB_2 -mediated responses; JWH-015 and WIN55,212-2 induced translocation similar to the CB_2 -mediated response (i.e., the maximal response in CB_2 -containing cells would have been approximately twice the magnitude shown in **Figure 1E** prior to subtracting the off-target response), and THC producing a response equivalent to that in CB_1 - or CB_2 -expressing cells (hence our conclusion above that THC does not induce $b\beta$ -arrestin-2 translocation to CB_1 - or CB_2). These non- CB_1 - or CB_2 -mediated responses were all low potency and only significantly different from vehicle at or above 3 μ M ($p = < 0.001$ – 0.003 ; two-way ANOVA). CP55,940 and BAY also induced small but statistically significant responses at or above 1 μ M. The remainder of the ligands studied did not induce

any measurable β -arrestin-2 translocation in the absence of an introduced receptor (data not shown).

Human β -Arrestin-2 (h β -Arrestin-2) Translocation

Bovine β -arrestin-2 has often been used when measuring β -arrestin recruitment to various human GPCRs, presumably due mainly to the convenience of utilizing existing historically available assay constructs. However, given that bovine β -arrestin has been used to make inferences about human physiology, it is necessary to validate that b β -arrestin-2 functions analogously to the human (h) β -arrestin-2. AVP-induced h β -arrestin-2 translocation to V₂R with an equivalent EC₅₀ compared to b β -arrestin-2 (**Figure 2A** and **Table 3**). However, the apparent efficacy of translocation was elevated for h β -arrestin-2 (Δ BRET.sec ratio 194 versus 306; **Figure 2A**), possibly reflecting increased translocation of h β -arrestin-2 in comparison with b β -arrestin-2, though it is possible that this may be due to differences in biosensor expression or orientation.

The translocation of h β -arrestin-2 to D₂R was approximately half a log unit less potent than that of b β -arrestin-2 but the co-expression of GRK2 again significantly increased the efficacy and potency of translocation (both $p < 0.001$, two-way ANOVA) (**Figure 2B**). Of the six agonists assayed, WIN55,212-2 was the only ligand found to induce translocation of b β -arrestin-2 and h β -arrestin-2 with significantly different potencies, being approximately 0.4 log units less potent at translocating b β -arrestin-2 ($p = 0.02$, two-way ANOVA) (**Table 4**). The rank order of efficacies was also very similar, with 2-AG the most efficacious, and THC and BAY again failing to translocate the arrestin (**Figure 2C**) ($p = 0.059$, one-way ANOVA).

Co-expression of GRK2 did not alter the efficacy of translocation of h β -arrestin-2 to CB₁ when stimulated with CP55,940 ($p = 0.114$, two-way ANOVA), though potency was increased by approximately 0.7 log units ($p < 0.001$, two-way ANOVA) (**Figure 2D**). Surprisingly, when stimulating with 2-AG, the efficacy was significantly lower when co-expressing GRK2 ($149.9 \pm 17.6 \Delta$ BRET.sec with GRK2 versus $235.6 \pm 17.9 \Delta$ BRET.sec without; $p = 0.002$ two-way ANOVA) (**Figure 2D** and **Table 4**), despite similar receptor expression (**Figure 2G**) and slightly increased potency for inducing translocation ($p = 0.002$, two-way ANOVA).

WIN55,212-2-, JWH-133-, and CP55,940-induced h β -arrestin-2 translocation to CB₂ occurred with significantly greater potencies in comparison with b β -arrestin-2 translocation ($p < 0.001$ – 0.048 , two-way ANOVA) (**Figure 2E** and **Table 4**). All ligands tested exhibited similar maximal efficacy to each other, except for THC which was again ineffective ($p = 0.43$, *t*-test).

As was the case for b β -arrestin-2, when stimulating with either CP55,940 or 2-AG, the efficacy of translocation was significantly enhanced in the presence of co-expressed GRK2 ($p < 0.001$ – 0.002 , two-way ANOVA), however potency was unchanged ($p = 0.78$ – 0.98 , two-way ANOVA) (**Figure 2F**).

The expression of CB₁, CB₂, D₂R, and V₂R with or without co-expressed GRK2 was measured by ICC confirming that the co-expression did not affect receptor expression (**Figure 2G**). Again, all receptors exhibited similar expression levels except CB₁, which was generally higher than the other receptors.

Off-target h β -arrestin-2 translocation was assessed in mock-transfected cells. All but one of the cannabinoids produced statistically significant non-CB₁ or CB₂-mediated translocation at high concentrations; generally, 10 μ M ($p = 0.001$ – 0.038 ; except 2-AG, $p = 0.059$), though significant translocation was also evident for CP55,940 at 1 μ M and 500 nM ($p = 0.004$, 0.001, respectively; **Figure 2H**). While the magnitudes of these translocations were small in comparison with some of the robust CB₁-mediated translocations (namely WIN55,212-2, CP55,940, 2-AG), translocations induced by THC and BAY were equivalent to those measured in CB₁-expressing cells leading to our conclusion above that these ligands do not induce CB₁-mediated arrestin translocation. Similarly, for CB₂, off-target THC response accounted for the entirety of the translocation measured in receptor-transfected cells. For the remainder of the ligands tested at CB₂, at the highest tested ligand concentration, off-target arrestin translocations were approximately half the maximal measured genuine CB₂-mediated h β -arrestin-2 translocation. Neither vasopressin nor dopamine induced measurable off-target h β -arrestin-2 translocation (data not shown).

Bovine β -Arrestin-1 (b β -Arrestin-1) Translocation

Having investigated the translocation of bovine and human β -arrestin-2, we were interested in comparing these to the translocation of β -arrestin-1. We validated that b β -arrestin-1 is efficiently translocated to V₂R with a potency similar to that previously reported (Tenenbaum et al., 2009), confirming that the β -arrestin translocation assay can be used to detect β -arrestin-1 translocation (**Table 5** and **Figure 3A**).

Similarly to β -arrestin-2, the efficacy of translocation of b β -arrestin-1 to D₂R was significantly potentiated when co-expressing GRK2 ($p = 0.001$, two-way ANOVA) (**Table 5** and **Figures 3B,C**). Generally, we found that cannabinoids induce little β -arrestin-1 translocation compared to β -arrestin-2 to both CB₁ and CB₂ and complete concentration–response curves could not be generated. Hence, we opted to only measure responses with a single high concentration of each agonist. The poor translocation of β -arrestin-1 was not caused by lack of expression of the arrestin, as the Rluc8 signal was comparable to the signal obtained using β -arrestin-2. Utilizing a high concentration of each ligand, only 10 μ M WIN55,212-2, 31.6 μ M 2-AG, and 1 μ M CP55,940 produced statistically significant b β -arrestin-1 translocation to CB₁ above vehicle (WIN55,212-2 and 2-AG: $p < 0.001$, CP55,940: $p = 0.026$; one-way ANOVA) (**Table 6** and **Figures 3D,E**). No significant response was observed for the other ligands at CB₁.

The co-expression of GRK2 significantly elevated the translocation of b β -arrestin-1 after stimulation with both CP55,940 and 2-AG (CP55,940: $p = 0.007$, 2-AG: $p = 0.046$, two-way ANOVA), although the change in efficacy was

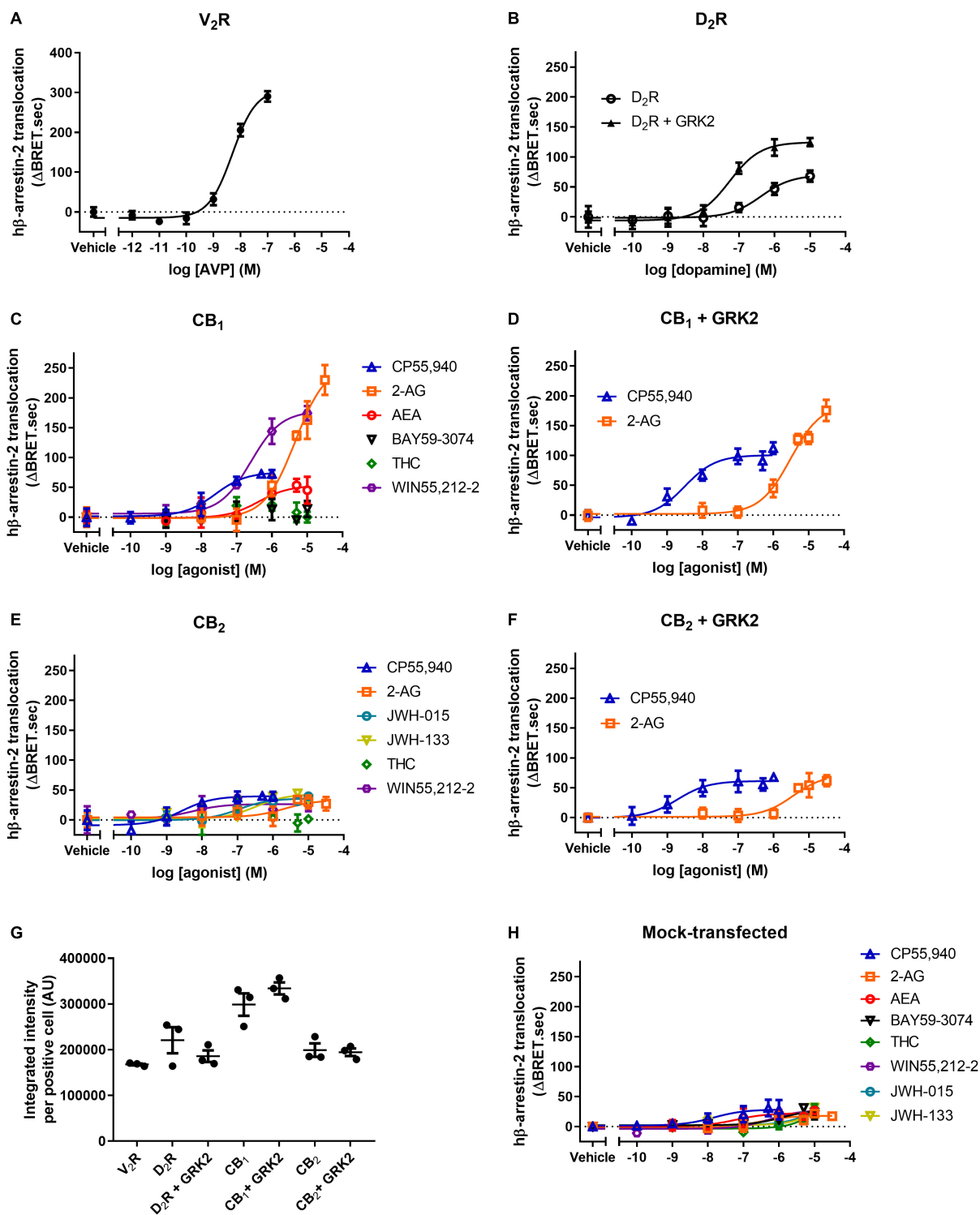


FIGURE 2 | hβ-arrestin-2 translocation to V₂R, D₂R, CB₁, and CB₂. hβ-Arrestin-2 translocation to plasma membrane in cells expressing (A) V₂R stimulated with AVP, (B) D₂R with or without co-expressed GRK2 stimulated with dopamine, (C) CB₁ stimulated with CP55,940, 2-AG, AEA, BAY, THC, and WIN55,212-2, (D) CB₁ with co-expressed GRK2 stimulated with CP55,940 and 2-AG, (E) CB₂ stimulated with CP55,940, 2-AG, JWH-015, JWH-133, THC, and WIN55,212-2, and (F) CB₂ with co-expressed GRK2 stimulated with CP55,940 and 2-AG. (G) Receptor expression of V₂R, D₂R, D₂R+GRK2, CB₁, CB₁+GRK2, CB₂, and CB₂+GRK2 in cells used in experiments (A–G). Receptor expression was quantified in cells positive for expression by ICC. (H) hβ-Arrestin-2 translocation to plasma membrane in mock-transfected cells. Error bars are ±standard deviation of representative data with three technical replicates (A–F), or standard error of the means from three independent biological replicates (G,H).

TABLE 3 | Human β -arrestin-2 translocation to V_2R and D_2R .

	h β -Arrestin-2					
	V_2R		D_2R		$D_2R + GRK2$	
	logEC50 (\pm SEM)	Emax (\pm SEM) (Δ BRET.sec)	logEC50 (\pm SEM)	Emax (\pm SEM) (Δ BRET.sec)	logEC50 (\pm SEM)	Emax (\pm SEM) (Δ BRET.sec)
AVP	-8.27 \pm 0.05	305.9 \pm 30.8	X	X	X	X
Dopamine	X	X	-6.40 \pm 0.03	72.9 \pm 1.5	-7.27 \pm 0.04 ^Δ	132.0 \pm 10.9 ^Δ

X, not determined. ^ΔValue is significantly different in the presence of GRK2 compared to the absence of GRK2 for the same ligand.

TABLE 4 | Human β -arrestin-2 translocation to CB_1 and CB_2 .

	h β -Arrestin-2							
	CB_1		$CB_1 + GRK2$		CB_2		$CB_2 + GRK2$	
	logEC50 \pm SEM (M)	Emax \pm SEM (Δ BRET.sec)	logEC50 \pm SEM (M)	Emax \pm SEM (Δ BRET.sec)	logEC50 \pm SEM (M)	Emax \pm SEM (Δ BRET.sec)	logEC50 \pm SEM (M)	Emax \pm SEM (Δ BRET.sec)
CP55,940	-7.81 \pm 0.13	83.7 \pm 8.6	-8.53 \pm 0.03 ^Δ	120.0 \pm 12.5	-8.70 \pm 0.12 [§]	37.4 \pm 6.2	-8.71 \pm 0.06	70.4 \pm 7.1 ^Δ
2-AG	-5.35 \pm 0.09	235.6 \pm 17.9	-5.69 \pm 0.10 ^Δ	149.9 \pm 17.6 ^Δ	-5.73 \pm 0.17	31.3 \pm 3.9	-5.68 \pm 0.16	68.7 \pm 2.5 ^Δ
WIN55,212-2	-6.60 \pm 0.13 [§]	164.2 \pm 19.9	X	X	-8.56 \pm 0.20 [§]	24.4 \pm 1.4	X	X
AEA	-6.09 \pm 0.19	47.9 \pm 3.6	X	X	X	X	X	X
THC	NA [^]	5.9 \pm 3.1 [^]	X	X	NA [^]	-3.8 \pm 4.3 [^]	X	X
BAY	NA [^]	15.3 \pm 4.6 [^]	X	X	X	X	X	X
JWH-015	X	X	X	X	-7.07 \pm 0.21	34.0 \pm 2.6	X	X
JWH-133	X	X	X	X	-6.55 \pm 0.04 [§]	37.8 \pm 2.3	X	X

X, Not determined; NA, Not active. [^]A concentration–response curve could not be fitted; therefore, efficacy at 10 μ M is noted instead of Emax. [§]Value is significantly different to bovine β -arrestin-2 for the same ligand. ^ΔValue is significantly different in the presence of GRK2 compared to the absence of GRK2 for the same ligand.

considerably smaller than that conferred via D_2R (Table 6 and Figures 3D,F).

For CB_2 , the translocation of β -arrestin-1 is weak but WIN55,212-2 and CP55,940 stimulated translocation significantly differently from vehicle (Both: $p = 0.008$, one-way ANOVA; Figures 3G,H). Co-expression of GRK2 did not alter the response significantly ($p = 0.137$), though a slightly greater mean response and smaller between-experiment variability resulted in 2-AG inducing a measurable β -arrestin-1 translocation in the presence of GRK2, which was not detected when GRK2 was absent (with GRK2: $p < 0.001$, without GRK2: $p = 0.088$; Table 6 and Figures 3G,I).

The receptor expression was equivalent between all the experiments involving β -arrestin-1 as measured by ICC (Figure 3J). As per our procedure for h β -arrestin-2, off-target β -arrestin-1 translocation was assessed in mock-transfected cells. Only 10 μ M JWH-015 induced a detectable signal above the vehicle control (22.4 \pm 4.3 Δ BRET.sec; $p = 0.005$, one-way ANOVA).

Human β -Arrestin-1 (h β -Arrestin-1) Translocation

We wanted to compare our findings with β -arrestin-1 to h β -arrestin-1. Again, we used V_2R to validate the translocation of h β -arrestin-1 and this was indeed recruited with similar efficacy and potency as β -arrestin-1 (Figure 4A). As was

also expected from our β -arrestin-1 data, the translocation of h β -arrestin-1 to D_2R was significantly potentiated when GRK2 was co-expressed ($p = 0.001$, two-way ANOVA) (Table 7 and Figures 4B,C).

Also in contrast to β -arrestin-1, the co-expression of GRK2 did not significantly alter the translocation of h β -arrestin-1 after stimulation with CP55,940 or 2-AG ($p = 0.467$), although a slightly greater mean response and smaller between-experiment variability resulted in CP55,940 inducing a measurable β -arrestin-1 translocation in the presence of GRK2 ($p = 0.008$; Table 8 and Figures 4D,F).

For CB_2 , h β -arrestin-1 translocation was significantly different from vehicle when stimulating with 10 μ M JWH-015, 1 μ M CP55,940, 31.6 μ M 2-AG, and 10 μ M THC ($p = 0.001$ –0.012; Table 8 and Figure 4H) but not for 10 μ M WIN55,212-2 or JWH-133 (both: $p = 0.46$), however, generally the translocation is weak and not affected by the co-expression of GRK2 ($p = 0.48$; Figures 4G,I). Receptor expression was confirmed by ICC (Figure 4J), and no ligand-induced h β -arrestin-1 translocation was detected in mock-transfected cells ($p = 0.13$, one-way ANOVA).

DISCUSSION

This study set out to systematically characterize the ability of a structurally diverse range of ligands to drive the translocation

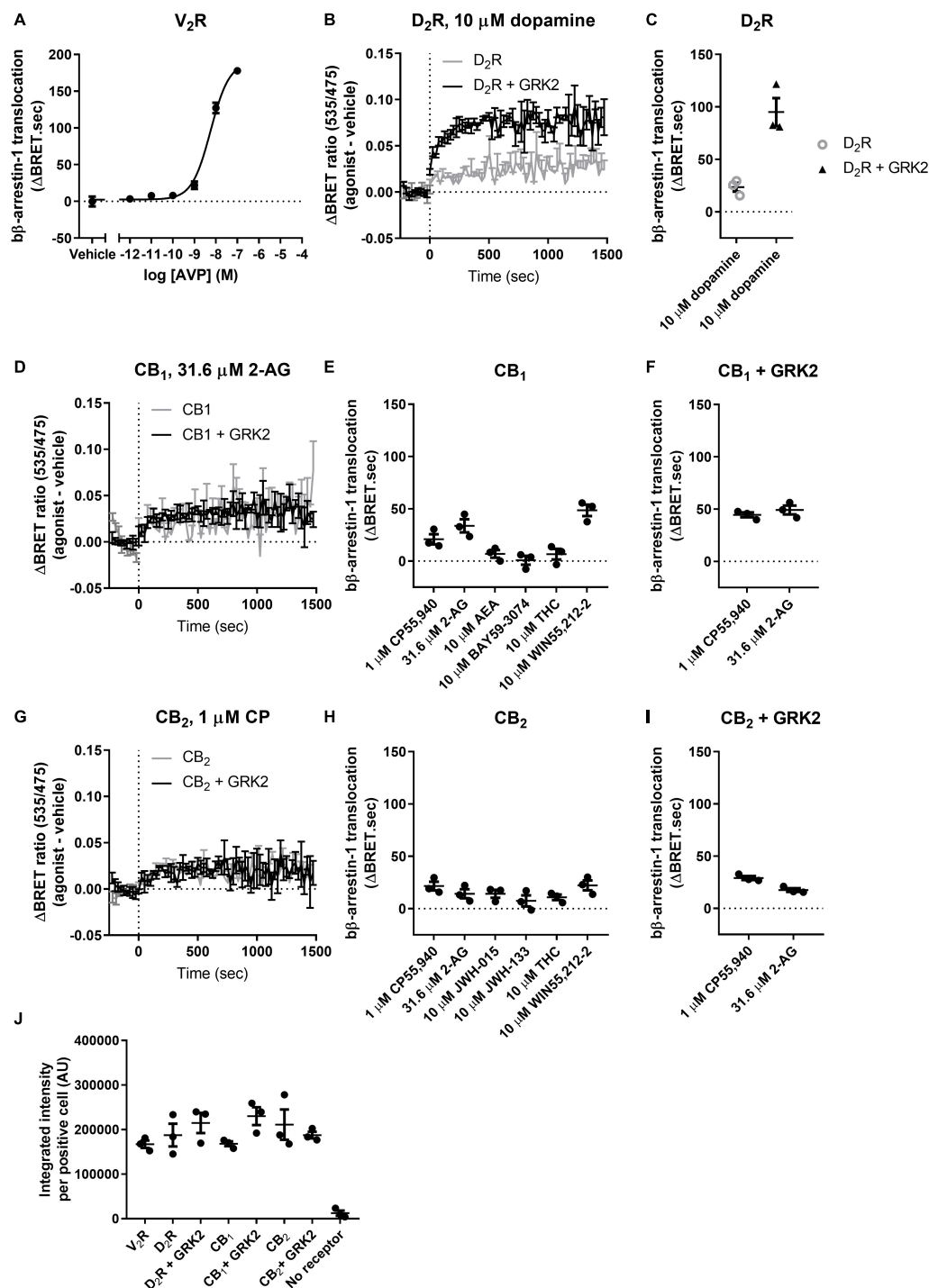


FIGURE 3 | β -Arrestin-1 translocation to V_2R , D_2R , CB_1 , and CB_2 . **(A)** β -Arrestin-1 translocation to V_2R stimulated with AVP. **(B)** Δ BRET ratio curves of β -arrestin-1 translocation to D_2R with or without co-expressed GRK2 stimulated with 10 μ M dopamine. **(C)** AUC quantification of translocation to D_2R with or without co-expressed GRK2 stimulated with 10 μ M dopamine as shown in **(B)**. **(D)** Δ BRET ratio curves of β -arrestin-1 translocation to CB_1 with or without co-expressed GRK2 stimulated with 31.6 μ M 2-AG. **(E)** AUC of β -arrestin-1 translocation to CB_1 stimulated with 1 μ M CP55,940, 31.6 μ M 2-AG, 10 μ M AEA, 10 μ M BAY, 10 μ M THC, and 10 μ M WIN55,212-2. **(F)** AUC of β -arrestin-1 translocation to CB_1 with co-expressed GRK2 stimulated with 1 μ M CP55,940 and 31.6 μ M 2-AG. **(G)** Δ BRET ratio curves of β -arrestin-1 translocation to CB_2 with or without co-expressed GRK2 stimulated with 1 μ M CP55,940. **(H)** AUC of β -arrestin-1 translocation to CB_2 stimulated with 1 μ M CP55,940, 31.6 μ M 2-AG, 10 μ M JWH-015, 10 μ M JWH-133, 10 μ M THC, and 10 μ M WIN55,212-2. **(I)** AUC of β -arrestin-1 translocation to CB_2 with co-expressed GRK2 stimulated with 1 μ M CP55,940 and 31.6 μ M 2-AG. **(J)** Receptor expression of V_2R , D_2R , $D_2R+GRK2$, CB_1 , CB_1+GRK2 , CB_2 , and CB_2+GRK2 in cells used in experiments **(A–G)**. Receptor expression was quantified in cells positive for expression by ICC. Error bars represent \pm standard deviation of representative data with three technical replicates **(A, B, D, G)** or standard error of the means from three independent biological replicates **(C, E, F, H, I, J)**.

TABLE 5 | Bovine β -arrestin-1 translocation to V_2R and D_2R .

	β-Arrestin-1			
	V_2R		D_2R	$D_2R + GRK2$
	logEC50 \pm SEM (M)	Emax \pm SEM (ΔBRET.sec)	Response \pm SEM (ΔBRET.sec)	Response \pm SEM (ΔBRET.sec)
AVP	-8.237 \pm 0.04	176.9 \pm 10.9	X	X
10 μ M dopamine	X	X	23.4 \pm 4.1	95.0 \pm 13.3 ^A

X, not determined. ^AValue is significantly different in the presence of GRK2 compared to the absence of GRK2 for the same ligand.

TABLE 6 | Bovine β -arrestin-1 translocation to CB_1 and CB_2 .

	β-Arrestin-1			
	CB_1	$CB_1 + GRK2$	CB_2	$CB_2 + GRK2$
	Response \pm SEM (ΔBRET.sec)	Response \pm SEM (ΔBRET.sec)	Response \pm SEM (ΔBRET.sec)	Response \pm SEM (ΔBRET.sec)
1 μ M CP55,940	20.8 \pm 4.9 ^S	44.4 \pm 2.3 ^{S,A}	21.5 \pm 4.2 ^S	29.0 \pm 1.9 ^S
31.6 μ M 2-AG	33.7 \pm 6.2 ^S	49.1 \pm 4.2 ^{S,A}	14.2 \pm 4.3	17.5 \pm 1.7 ^S
10 μ M WIN55,212-2	48.5 \pm 5.5 ^S	X	22.2 \pm 4.7 ^S	X
10 μ M AEA	6.9 \pm 3.6	X	X	X
10 μ M THC	6.5 \pm 5.1	X	10.8 \pm 2.6	X
10 μ M BAY	0.7 \pm 4.2	X	X	X
10 μ M JWH-015	X	X	14.2 \pm 3.7	X
10 μ M JWH-133	X	X	7.4 \pm 5.3	X

X, not determined. ^SValue is significantly different from vehicle control. ^AValue is significantly different in the presence of GRK2 compared to the absence of GRK2 for the same ligand.

of β -arrestin-1 and -2 through CB stimulation. We have further extended the study to investigate whether bovine and human arrestins function equivalently in this assay. To this end we utilized a BRET assay monitoring arrestin translocation to the plasma membrane that does not require modification of the receptor(s) of interest, initially developed to investigate interactions between D_2R and arrestins (Clayton et al., 2014; Donthamsetti et al., 2015). We have thus utilized D_2R and the robust arrestin recruiter V_2R (Oakley et al., 1999; Charest and Bouvier, 2003) as controls to validate the assay in our laboratory.

Initial studies focused on the ability of CB_1 activation to drive the translocation of bovine β -arrestin-2 in response to a structurally diverse range of agonists. The results showed a wide range of agonist efficacies, with the endogenous agonist 2-AG being the most efficacious, whereas the phytocannabinoid THC and partial synthetic agonist BAY did not significantly activate this pathway. The rank order of potencies for the effective ligands was CP55,940 \gg WIN55,212-2 $>$ AEA, 2-AG.

We further sought to investigate whether the recruitment mediated by CBRs was similar between bovine and human arrestins. The relative ability of ligands to induce arrestin translocation in the presence of h CB_1 expression was generally very similar between bovine and human β -arrestin-2, with the same rank order of efficacy. WIN55,212 was moderately (approximately half a log unit) more potent in recruiting bovine than human β -arrestin 2.

Similarly, to β -arrestin-1, the translocation of h β -arrestin-1 was significantly different from vehicle only when

stimulating CB_1 with 2-AG and WIN55,212-2 (both: $p < 0.001$, one-way ANOVA), however, unlike the bovine counterpart CP55,940 did not induce translocation of h β -arrestin-1 to a statistically measurable degree ($p = 0.097$; **Table 8** and **Figures 4D,E**).

We recently characterized the same group of ligands as studied here for CB_1 -mediated arrestin translocation in the context of modulation of cAMP production via CB_1 (Finlay et al., 2017). As well as inhibiting the production of cAMP via $G\alpha_i$, CB_1 is known to also couple to the stimulation of cAMP through a putative $G\alpha_s$ mechanism under some circumstances (Glass and Felder, 1997; Finlay et al., 2017). It is interesting to note that the ligands' potencies for inducing β -arrestin-2 recruitment at CB_1 are well correlated with their potencies for driving increases in cAMP (as revealed when cells were pertussis toxin-treated), whereas $G\alpha_i$ -mediated cAMP inhibition was induced with between 10 and 100-fold greater potency. The low potency for inducing β -arrestin-2 recruitment is unlikely to be due to low receptor expression as the transient transfection of p $plss$ -tagged h CB_1 is known to result in high expression levels (Finlay et al., 2017). Furthermore, we previously observed a similar pattern and rank order of efficacy for $G\alpha_s$ -mediated stimulation of cAMP synthesis as seen here for β -arrestin-2 recruitment (including lack of efficacy for THC and BAY, and moderate efficacy for CP55,940 which is widely assumed to be a full agonist), whereas all ligands were similarly effective at inhibiting cAMP production via $G\alpha_i$.

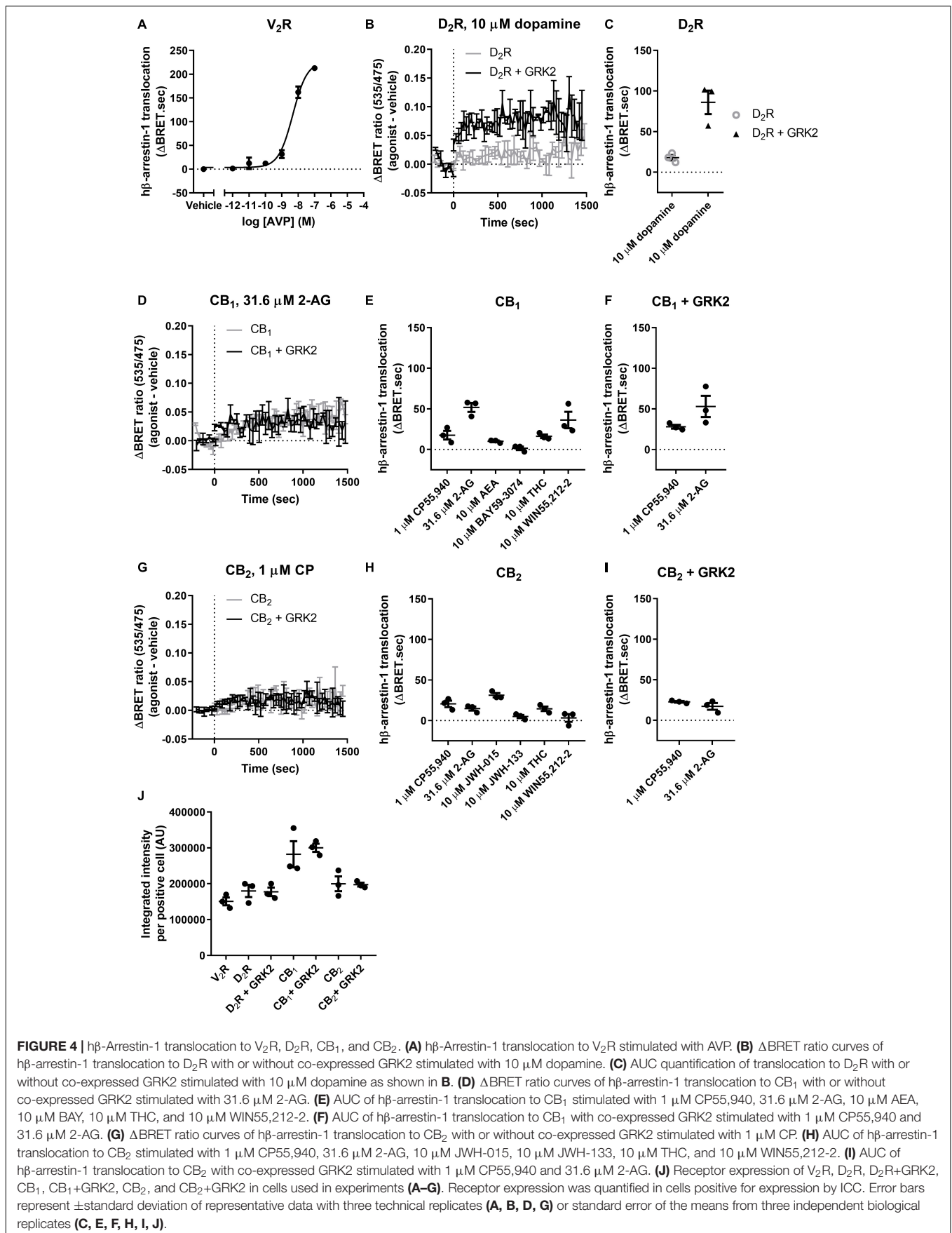


TABLE 7 | Human β -arrestin-1 translocation to V_2R and D_2R .

	h β -Arrestin-1			
	V_2R		D_2R	$D_2R + GRK2$
	logEC50 \pm SEM (M)	E _{max} \pm SEM (Δ BRET.sec)	Response \pm SEM (Δ BRET.sec)	Response \pm SEM (Δ BRET.sec)
AVP	-8.33 \pm 0.03	246.3 \pm 21.8	X	X
10 μ M dopamine	X	X	17.9 \pm 3.3	86.1 \pm 14.5 ^A

X, not determined. ^AValue is significantly different in the presence of GRK2 compared to the absence of GRK2 for the same ligand.

TABLE 8 | Human β -arrestin-1 translocation to CB_1 and CB_2 .

	h β -Arrestin-1			
	CB_1	$CB_1 + GRK2$	CB_2	$CB_2 + GRK2$
	Response \pm SEM (Δ BRET.sec)	Response \pm SEM (Δ BRET.sec)	Response \pm SEM (Δ BRET.sec)	Response \pm SEM (Δ BRET.sec)
1 μ M CP55,940	17.6 \pm 5.3	28.1 \pm 2.3 ^S	20.5 \pm 3.9 ^S	22.7 \pm 1.1 ^S
31.6 μ M 2-AG	51.8 \pm 5.6 ^S	53.0 \pm 13.1 ^S	14.7 \pm 2.5 ^S	17.2 \pm 4.2 ^S
10 μ M WIN55,212-2	36.2 \pm 10.2 ^S	X	3.2 \pm 4.7	X
10 μ M AEA	10.0 \pm 1.0	X	X	X
10 μ M THC	16.2 \pm 2.2	X	14.4 \pm 2.8 ^S	X
10 μ M BAY	1.4 \pm 2.1	X	X	X
10 μ M JWH-015	X	X	31.2 \pm 2.6 ^S	X
10 μ M JWH-133	X	X	4.9 \pm 2.0	X

X, not determined. ^SValue is significantly different from vehicle control. ^AValue is significantly different in the presence of GRK2 compared to the absence of GRK2 for the same ligand.

These observations are consistent with CB_1 having a low relative efficiency for coupling to both the $G\alpha_s$ and β -arrestin-2 effector pathways in comparison with $G\alpha_i$. While we have not interrogated this directly, perhaps the CB_1 receptor conformational state(s) requisite for β -arrestin-2 recruitment are more closely related to those required for $G\alpha_s$ activation, than for $G\alpha_i$. We are also aware that our model system (the CB_1 -expressing HEK cell line and associated assays utilized) exhibits “receptor reserve” for the $G\alpha_i$ pathway, wherein the system’s maximal response is reached at low receptor occupancy, and the potential ability to exert greater efficacy via occupation of more receptors is manifested as increases in potency because the system maximum effect has already been reached. In comparison, perhaps the $G\alpha_s$ and β -arrestin-2 pathways are able to report a relatively greater dynamic range of efficacy, and thereby the efficacies and potencies indicate more directly the true intrinsic efficacy (“stimulus per receptor”) of the ligands (Kenakin, 2013). Indeed, it has been suggested that β -arrestin recruitment is less likely than downstream pathways to be subject to receptor reserve due to less signal amplification (Smith and Rajagopal, 2016).

The maximum observed efficacy of CB_1 β -arrestin-2 recruitment was substantially lower than that generated by V_2R , but was similar to the efficacy of D_2R in recruiting β -arrestin-2 in response to dopamine. In the case of D_2R , co-expression of GRK2 has been shown to enhance the potency and efficacy of this interaction (Clayton et al., 2014), a finding we were able to replicate, and thus we investigated if GRK2 expression could

also enhance the ability of the cannabinoid ligands to drive this pathway. In contrast to D_2R however, the co-expression of GRK2 did not enhance the efficacy of the response to either of the cannabinoids tested (CP55,940 and 2-AG) but did enhance the potency of the ligands (although this only reached statistical significance for CP55,940). This relatively subtle change was surprising, as previous studies have suggested an interaction between GRK2, β -arrestin-2, and CB_1 . For example, the presynaptic expression of dominant negative GRK2 or β -arrestin-2 reduced desensitization of CB_1 receptor-mediated presynaptic inhibition of glutamatergic neurotransmission in rat hippocampal neurons (Kouznetsova et al., 2002). This could reflect that the endogenous level of GRK2 expression in these cells is sufficient for CB_1 -mediated interactions with β -arrestin-2, although the pronounced effect of GRK2 on dopaminergic signaling would suggest that this is not the case. The endogenous expression of GRKs in HEK cells is not clear; Atwood et al. (2011) reported GRK3-5 were expressed but not GRK1 or 2, however two previous papers had identified GRK2 in these cells (Hasbi et al., 2004; Zidar et al., 2009).

The inability to detect an h CB_1 -mediated response to THC in this assay was also surprising in light of prior reports. THC has been shown to produce greater antinociception and hypothermia in β -arrestin-2^(-/-) mice compared to wild-type mice, yet no differences were observed in either assay for CP55,940 (Breivogel et al., 2008). Meanwhile, two *in vitro* studies have suggested that THC induces β -arrestin-2 recruitment via CB_1 , though with considerably different reported potencies (Soethoudt et al.,

2017; Navarro et al., 2018). The disparity in findings may reflect differential GRK or other adaptor protein [such as CRIP1a (Blume et al., 2017)] expression between cell types and lines; Soethoudt et al. (2017) performed their assays on Chinese Hamster Ovary (CHO) cells and Navarro et al. (2018) utilized the HEK-293 “T” subclone, which is known to harbor considerable genomic differences in comparison with the parental HEK-293 line which we utilized in our experiments (Lin et al., 2014). Further studies could address the dependence of THC-stimulated arrestin recruitment on various GRK isoforms and arrestin- and/or CB₁-associated signaling adaptor proteins. If this were the case, it would imply that THC induces a substantially different conformation of the receptor, which in turn is phosphorylated differently to the other agonists.

It is also important to consider differences which may have arisen due to the arrestin assays used; both Soethoudt et al. (2017) and Navarro et al. (2018) monitored translocation of tagged arrestin to tagged receptor. The former study utilized a PathHunter assay, which calls for a “ProLink tagged receptor,” and thus we wonder whether changes in assay signal may be at least partially reflective of a change in conformation or activation of the receptor rather than the actual capacity of the native receptor to recruit arrestin. Another comparative advantage of our approach, in which arrestin translocation to the plasma membrane is measured without the requirement for the receptor of interest to be tagged, is the ability to carry out assays in the absence of receptor to determine whether a portion of the signal is not mediated by the receptor of interest. The source of this non-specific signal is not clear, but with all fluorescent/luminescent proteins it is possible that changes in conformation could result in quenching or unquenching of the signal produced. With cannabinoid ligands being highly lipophilic and able to cross the plasma membrane there is more potential for this to occur than with other non-cell-permeable small molecules. Importantly, non-CB₁/CB₂-mediated effects of cannabinoids may occur via other putative CBRs and other targets, particularly when applied at high concentrations (Felder et al., 1992; Soethoudt et al., 2017). All the receptor-mediated arrestin translocation data shown in this paper is following the subtraction of the non-receptor-of-interest mediated component of the signal; the measured non-receptor-mediated signals are shown separately. Although these non-CB-mediated responses were low potency relative to the receptor-mediated responses, these have clear potential to influence the apparent receptor-mediated concentration–response curve if not accounted for. Tellingly, the entire β -arrestin-2 response we observed for THC or BAY appeared to be non-CB_{1/2}-mediated. Thus, the difference between our findings and those described above could also be due to the inability of prior assays to distinguish between receptor and non-receptor-mediated signal.

While the presence versus absence of THC response was the most striking difference in finding in comparison with prior *in vitro* studies, for effective agonists we also observed some differences in rank maximum efficacy; our data indicated 2-AG > WIN55,212-2 > CP55,940, whereas Soethoudt et al. (2017) observed CP55,940, WIN55,212-2 > 2-AG, despite measured potencies being similar. We can only speculate that

a combination of mechanisms described above may contribute to the differences observed. The possibility that differential expression of signaling adaptors might give rise to completely altered CB₁-mediated arrestin recruitment patterns is intriguing and is indicative of system factors influencing the measurement of ligand bias. This also reinforces that when studying ligand bias it is important to perform the various signaling assays for comparison under as closely analogous conditions as possible.

We continued by examining a range of ligands at hCB₂ for bovine and human β -arrestin-2. All ligands tested except THC were able to translocate bovine and human β -arrestin-2, although in general the maximum efficacy for all ligands was low. Interestingly, bovine β -arrestin-2 was translocated with greater efficacy (relative to V₂R and CB₁) than human β -arrestin-2; however, human β -arrestin-2 responses were more potent for three of the five agonists with measurable responses.

Our finding of a lack of efficacy for THC again differs from Soethoudt et al. (2017), wherein THC was able to recruit β -arrestin-2 to CB₂ utilizing a PathHunter assay in CHO cells. However, the response reported was only partial and did not reach a plateau by 10 μ M. Furthermore, an earlier study reported measurable but extremely slight THC-induced arrestin recruitment; again, a concentration–response curve could not be drawn (Dhopeswarkar and Mackie, 2016). We suspect a considerable contributor to the differences between these studies is whether or not non-receptor-of-interest-mediated arrestin assay signal was controlled for. However, other than THC, our relative CB₂ efficacy results align very closely with Soethoudt et al. (2017) and an earlier study which included a smaller subset of ligands in HEK cells with the PathHunter assay system (McGuinness et al., 2009). In contrast, a 2016 study (Dhopeswarkar and Mackie, 2016) of mouse CB₂ in HEK cells could not detect 2-AG induced β -arrestin-2 recruitment and measured relatively lower efficacy responses for WIN55,212-2, JWH-015, and JWH-133. We suggest the disparity of the latter study with ours and the other two noted studies were likely due to the utilization of rodent CB₂; indeed, important ligand binding and signaling disparities between mouse, rat, and human CB₂ have been reported (Griffin et al., 2000; Mukherjee et al., 2004).

In contrast to β -arrestin-2, very few cannabinoid ligands generated a statistically significant bovine β -arrestin-1 response through either CB₁ (WIN55,212-2 > 2-AG > CP55,940) or CB₂ (WIN55,212-2, CP55,940), despite robust translocation induced by V₂R. Controls for receptor expression suggested very similar expression of CB₁ and CB₂ relative to these receptors, thus it seems unlikely that the low efficacy of responses was due to insufficient receptor expression. The small response window prevented reliable assessment of concentration–response curves and therefore the data reported are for the response generated at maximal tested concentrations. Similarly, only 2-AG and WIN55,212-2 induced h β -arrestin-1 translocation to CB₁ significantly differently from vehicle. In contrast to b β -arrestin-1, the translocation of h β -arrestin-1 to CB₂ could be significantly induced by JWH-015, CP55,940, 2-AG, and THC. Interestingly, this was the only condition in our study under which we detected CB-mediated THC-induced arrestin translocation. Our observations are consistent with a weak

interaction between β -arrestin-1 and CB₁ and CB₂ as reported by some (Gyombolai et al., 2013, 2015) and corroborated by the fact that β -arrestin-2, but not β -arrestin-1, could not be immunoprecipitated with CB₁ (Delgado-Peraza et al., 2016). Nonetheless, a direct interaction between β -arrestin-1 and CB₁, and β -arrestin-1-mediated signaling have been observed in some studies, although most notably with non-orthosteric ligands (Laprairie et al., 2014; Delgado-Peraza et al., 2016; Laprairie et al., 2016). It is therefore possible that β -arrestin-1 has only weak affinity for CB₁ and CB₂ or is only very transiently interacting with these receptors, and therefore not efficiently captured by this assay. Complementation assays may be more sensitive to very transient signals as they are non-reversible, essentially acting as an accumulation assay. We also note the possibility that β -arrestin-1 could bind with a different modality/conformation to CB₁ and CB₂ versus V₂R and D₂R which may be sufficient to reduce BRET signal even though translocation may still be present. Although we feel that this likelihood is small, it would be interesting to carry out imaging experiments to test this possibility. GRK2 potentiated the translocation of both species of β -arrestin-1 to D₂R, but CB₁ and CB₂ β -arrestin-1 translocation was only enhanced very slightly.

Although previously used in connection with D₂R, we have optimized a receptor-independent β -arrestin-2 translocation BRET assay for CB₁, CB₂, and V₂R and added tools for measuring β -arrestin-1 and human β -arrestin-1 and 2. This assay represents a new tool for drug discovery which is amenable to high-throughput screening and is therefore particularly useful for discovery and characterization of biased ligands targeting GPCRs. Given the conflicts already in the literature and findings presented here, this work highlights that there can be different outcomes between widely used β -arrestin recruitment assays and the non-receptor-tagged translocation assay used for this work. These disparities are likely due to system factors, such as co-expression of requisite accessory proteins and whether or not

non-receptor-of-interest arrestin assay signal is appropriately controlled. Furthermore, this study has revealed potentially important differences between utilization of bovine versus human arrestins. These issues should be taken into consideration by future studies investigating arrestin recruitment to CBRs to avoid misplaced conclusions.

AUTHOR CONTRIBUTIONS

MI designed and performed the majority of the experiments, and wrote the first draft of the manuscript. DF designed and made the human arrestin constructs and contributed to the data analysis. MP contributed experimental datasets. JJ provided the arrestin assay and technical expertise. MI, MG, and NG conceived of the experiments. MG and NG oversaw all elements of the project, obtained the funding, and contributed to experimental design and analysis. All authors reviewed and edited the manuscript.

FUNDING

This research was supported by funding from the Auckland Medical Research Foundation and the Health Research Council of New Zealand (to NG). Support for MI salary and consumables was received from the School of Medical Science, University of Auckland (to MG). Development of the arrestin assay was supported by National Institutes of Health grant MH54137 (to JJ).

ACKNOWLEDGMENTS

We thank Associate Professor Kevin Pflieger for kindly providing the bGRK2 pcDNA3 plasmid and for general advice and discussion on BRET, and Mr. Jamie Manning for helpful discussions and provision of data from one experiment.

REFERENCES

- Armstrong, S. P., Seeber, R. M., Ayoub, M. A., Feldman, B. J., and Pflieger, K. D. G. (2013). Characterization of three vasopressin receptor 2 variants: an apparent polymorphism (V266A) and Two Loss-of-Function Mutations (R181C and M311V). *PLoS One* 8:e65885. doi: 10.1371/journal.pone.0065885
- Atwood, B. K., Lopez, J., Wager-Miller, J., Mackie, K., and Straiker, A. (2011). Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC Genomics* 12:14. doi: 10.1186/1471-2164-12-14
- Atwood, B. K., Wager-Miller, J., Haskins, C., Straiker, A., and Mackie, K. (2012). Functional selectivity in CB(2) cannabinoid receptor signaling and regulation: implications for the therapeutic potential of CB(2) ligands. *Mol. Pharmacol.* 81, 250–263. doi: 10.1124/mol.111.074013
- Bakshi, K., Mercier, R. W., and Pavlopoulos, S. (2007). Interaction of a fragment of the cannabinoid CB1 receptor C-terminus with arrestin-2. *Febs Lett.* 581, 5009–5016. doi: 10.1016/j.febslet.2007.09.030
- Bash, R., Rubovitch, V., Gafni, M., and Sarne, Y. (2003). The stimulatory effect of cannabinoids on calcium uptake is mediated by Gs GTP-binding proteins and cAMP formation. *Neurosignals* 12, 39–44. doi: 10.1159/000068915
- Blume, L. C., Patten, T., Eldeeb, K., Leone-Kabler, S., Ilyasov, A. A., Keegan, B. M., et al. (2017). Cannabinoid Receptor Interacting Protein 1a Competition with beta-Arrestin for CB1 Receptor Binding Sites. *Mol. Pharmacol.* 91, 75–86. doi: 10.1124/mol.116.104638
- Bouaboula, M., Dussosoy, D., and Casellas, P. (1999). Regulation of peripheral cannabinoid receptor CB2 phosphorylation by the inverse agonist SR 144528. Implications for receptor biological responses. *J. Biol. Chem.* 274, 20397–20405. doi: 10.1074/jbc.274.29.20397
- Breivogel, C. S., Lambert, J. M., Gerfin, S., Huffman, J. W., and Razdan, R. K. (2008). Sensitivity to Delta 9-tetrahydrocannabinol is selectively enhanced in beta-arrestin2^{-/-} mice. *Behav. Pharmacol.* 19, 298–307. doi: 10.1097/Fbp.0b013e328308f1e6
- Breivogel, C. S., Puri, V., Lambert, J. M., Hill, D. K., Huffman, J. W., and Razdan, R. K. (2013). The influence of beta-arrestin2 on cannabinoid CB1 receptor coupling to G-proteins and subcellular localization and relative levels of beta-arrestin1 and 2 in mouse brain. *J. Recept. Signal. Transduct. Res.* 33, 367–379. doi: 10.3109/10799893.2013.838787
- Carrier, E. J., Kearn, C. S., Barkmeier, A. J., Breese, N. M., Yang, W., Nithipatikom, K., et al. (2004). Cultured rat microglial cells synthesize the endocannabinoid 2-arachidonylglycerol, which increases proliferation via a CB2 receptor-dependent mechanism. *Mol. Pharmacol.* 65, 999–1007. doi: 10.1124/mol.65.4.999
- Charest, P. G., and Bouvier, M. (2003). Palmitoylation of the V2 vasopressin receptor carboxyl tail enhances beta-arrestin recruitment leading to efficient

- receptor endocytosis and ERK1/2 activation. *J. Biol. Chem.* 278, 41541–41551. doi: 10.1074/jbc.M306589200
- Chen, X., Zheng, C., Qian, J., Sutton, S. W., Wang, Z., Lv, J., et al. (2014). Involvement of beta-arrestin-2 and clathrin in agonist-mediated internalization of the human cannabinoid CB2 receptor. *Curr. Mol. Pharmacol.* 7, 67–80. doi: 10.2174/1874467207666140714115824
- Clayton, C. C., Donthamsetti, P., Lambert, N. A., Javitch, J. A., and Neve, K. A. (2014). Mutation of three residues in the third intracellular loop of the dopamine D-2 receptor creates an internalization-defective receptor. *J. Biol. Chem.* 289, 33663–33675. doi: 10.1074/jbc.M114.605378
- Daigle, T. L., Kearns, C. S., and Mackie, K. (2008a). Rapid CB1 cannabinoid receptor desensitization defines the time course of ERK1/2 MAP kinase signaling. *Neuropharmacology* 54, 36–44. doi: 10.1016/j.neuropharm.2007.06.005
- Daigle, T. L., Kwok, M. L., and Mackie, K. (2008b). Regulation of CB(1) cannabinoid receptor internalization by a promiscuous phosphorylation-dependent mechanism. *J. Neurochem.* 106, 70–82. doi: 10.1111/j.1471-4159.2008.05336.x
- Delgado-Peraza, F., Ahn, K. H., Noguera-Ortiz, C., Mungro, I. N., Mackie, K., Kendall, D. A., et al. (2016). Mechanisms of biased beta-arrestin-mediated signaling downstream from the cannabinoid 1 receptor. *Mol. Pharmacol.* 89, 618–629. doi: 10.1124/mol.115.103176
- Derocq, J. M., Jbilo, O., Bouaboula, M., Segui, M., Clere, C., and Casellas, P. (2000). Genomic and functional changes induced by the activation of the peripheral cannabinoid receptor CB2 in the promyelocytic cells HL-60. Possible involvement of the CB2 receptor in cell differentiation. *J. Biol. Chem.* 275, 15621–15628. doi: 10.1074/jbc.275.21.15621
- DeWire, S. M., Ahn, S., Lefkowitz, R. J., and Shenoy, S. K. (2007). Beta-arrestins and cell signaling. *Annu. Rev. Physiol.* 69, 483–510. doi: 10.1146/annurev.ph.69.013107.100021
- DeWire, S. M., Yamashita, D. S., Rominger, D. H., Liu, G., Cowan, C. L., Graczyk, T. M., et al. (2013). A G protein-biased ligand at the mu-opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. *J. Pharmacol. Exp. Ther.* 344, 708–717. doi: 10.1124/jpet.112.201616
- Dhopeswarkar, A., and Mackie, K. (2016). Functional Selectivity of CB2 cannabinoid receptor ligands at a canonical and noncanonical pathway. *J. Pharmacol. Exp. Ther.* 358, 342–351. doi: 10.1124/jpet.116.232561
- Donthamsetti, P., Gallo, E. F., Buck, D. C., Stahl, E. L., Zhu, Y., Lane, J. R., et al. (2018). Arrestin recruitment to dopamine D2 receptor mediates locomotion but not incentive motivation. *Mol. Psychiatry* doi: 10.1038/s41380-018-0212-4 [Epub ahead of print].
- Donthamsetti, P., Quejada, J. R., Javitch, J. A., Gurevich, V. V., and Lambert, N. A. (2015). Using Bioluminescence Resonance Energy Transfer (BRET) to Characterize Agonist-Induced Arrestin Recruitment to Modified and Unmodified G Protein-Coupled Receptors. *Curr. Protoc. Pharmacol.* 70, 2.14.11–2.14.14. doi: 10.1002/0471141755.ph0214s70
- Felder, C. C., Joyce, K. E., Briley, E. M., Mansouri, J., Mackie, K., Blond, O., et al. (1995). Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol. Pharmacol.* 48, 443–450.
- Felder, C. C., Veluz, J. S., Williams, H. L., Briley, E. M., and Matsuda, L. A. (1992). Cannabinoid agonists stimulate both receptor- and non-receptor-mediated signal transduction pathways in cells transfected with and expressing cannabinoid receptor clones. *Mol. Pharmacol.* 42, 838–845.
- Finlay, D. B., Cawston, E. E., Grimsey, N. L., Hunter, M. R., Korde, A., Vemuri, V. K., et al. (2017). Galphas signalling of the CB1 receptor and the influence of receptor number. *Br. J. Pharmacol.* 174, 2545–2562. doi: 10.1111/bph.13866
- Finlay, D. B., Joseph, W. R., Grimsey, N. L., and Glass, M. (2016). GPR18 undergoes a high degree of constitutive trafficking but is unresponsive to N-Arachidonoyl Glycine. *PeerJ* 4:e1835. doi: 10.7717/peerj.1835
- Flores-Otero, J., Ahn, K. H., Delgado-Peraza, F., Mackie, K., Kendall, D. A., and Yudowski, G. A. (2014). Ligand-specific endocytic dwell times control functional selectivity of the cannabinoid receptor 1. *Nat. Commun.* 5:4589. doi: 10.1038/Ncomms5589
- Glass, M., and Felder, C. C. (1997). Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. *J. Neurosci.* 17, 5327–5333. doi: 10.1523/JNEUROSCI.17-14-05327.1997
- Glass, M., and Northup, J. K. (1999). Agonist selective regulation of G proteins by cannabinoid CB(1) and CB(2) receptors. *Mol. Pharmacol.* 56, 1362–1369. doi: 10.1124/mol.56.6.1362
- Griffin, G., Tao, Q., and Abood, M. E. (2000). Cloning and pharmacological characterization of the rat CB(2) cannabinoid receptor. *J. Pharmacol. Exp. Ther.* 292, 886–894.
- Grimsey, N. L., Goodfellow, C. E., Dragunow, M., and Glass, M. (2011). Cannabinoid receptor 2 undergoes Rab5-mediated internalization and recycles via a Rab11-dependent pathway. *Biochim. Biophys. Acta* 1813, 1554–1560. doi: 10.1016/j.bbamcr.2011.05.010
- Gyombolai, P., Boros, E., Hunyady, L., and Turu, G. (2013). Differential beta-arrestin2 requirements for constitutive and agonist-induced internalization of the CB1 cannabinoid receptor. *Mol. Cell. Endocrinol.* 372, 116–127. doi: 10.1016/j.mce.2013.03.013
- Gyombolai, P., Toth, A. D., Timar, D., Turu, G., and Hunyady, L. (2015). Mutations in the "DRY" motif of the CB1 cannabinoid receptor result in biased receptor variants. *J. Mol. Endocrinol.* 54, 75–89. doi: 10.1530/JME-14-0219
- Hasbi, A., Devost, D., Laporte, S. A., and Zingg, H. H. (2004). Real-time detection of interactions between the human oxytocin receptor and G protein-coupled receptor kinase-2. *Mol. Endocrinol.* 18, 1277–1286. doi: 10.1210/me.2003-0440
- Ibsen, M. S., Connor, M., and Glass, M. (2017). Cannabinoid CB1 and CB2 Receptor Signaling and Bias. *Cannabis Cannabinoid Res.* 2, 48–60. doi: 10.1089/can.2016.0037
- Jin, W. Z., Brown, S., Roche, J. P., Hsieh, C., Celver, J. P., Koo, A., et al. (1999). Distinct domains of the CB1 cannabinoid receptor mediate desensitization and internalization. *J. Neurosci.* 19, 3773–3780. doi: 10.1523/JNEUROSCI.19-10-03773.1999
- Kang, Y., Zhou, X. E., Gao, X., He, Y., Liu, W., Ishchenko, A., et al. (2015). Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. *Nature* 523, 561–567. doi: 10.1038/nature14656
- Kenakin, T. (2013). New concepts in pharmacological efficacy at 7TM receptors: IUPHAR review 2. *Br. J. Pharmacol.* 168, 554–575. doi: 10.1111/j.1476-5381.2012.02223.x
- Kenakin, T., and Christopoulos, A. (2013). Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat. Rev. Drug Discov.* 12, 205–216. doi: 10.1038/nrd3954
- Kouznetsova, M., Kelley, B., Shen, M., and Thayer, S. A. (2002). Desensitization of cannabinoid-mediated presynaptic inhibition of neurotransmission between rat hippocampal neurons in culture. *Mol. Pharmacol.* 61, 477–485. doi: 10.1124/mol.61.3.477
- Laprairie, R. B., Bagher, A. M., Kelly, M. E., and Denovan-Wright, E. M. (2016). Biased type 1 cannabinoid receptor signaling influences neuronal viability in a cell culture model of Huntington disease. *Mol. Pharmacol.* 89, 364–375. doi: 10.1124/mol.115.101980
- Laprairie, R. B., Bagher, A. M., Kelly, M. E. M., Dupre, D. J., and Denovan-Wright, E. M. (2014). Type 1 cannabinoid receptor ligands display functional selectivity in a cell culture model of striatal medium spiny projection neurons. *J. Biol. Chem.* 289, 24845–24862. doi: 10.1074/jbc.M114.557025
- Lin, Y. C., Boone, M., Meuris, L., Lemmens, I., Van Roy, N., Soete, A., et al. (2014). Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations. *Nat. Commun.* 5:4767. doi: 10.1038/ncomms5767
- Manglik, A., Lin, H., Aryal, D. K., McCorvy, J. D., Dengler, D., Corder, G., et al. (2016). Structure-based discovery of opioid analgesics with reduced side effects. *Nature* 537, 185–190. doi: 10.1038/nature19112
- McGuinness, D., Malikzay, A., Visconti, R., Lin, K., Bayne, M., Monsma, F., et al. (2009). Characterizing cannabinoid cb2 receptor ligands using discoverx pathhunter (TM) beta-Arrestin assay. *J. Biomol. Screen.* 14, 49–58. doi: 10.1177/1087057108327329
- Moore, C. A. C., Milano, S. K., and Benovic, J. L. (2007). Regulation of receptor trafficking by GRKs and arrestins. *Annu. Rev. Physiol.* 69, 451–482. doi: 10.1146/annurev.physiol.69.022405.154712
- Mukherjee, S., Adams, M., Whiteaker, K., Daza, A., Kage, K., Cassar, S., et al. (2004). Species comparison and pharmacological characterization of rat and human CB2 cannabinoid receptors. *Eur. J. Pharmacol.* 505, 1–9. doi: 10.1016/j.ejphar.2004.09.058
- Navarro, G., Reyes-Resina, I., Rivas-Santisteban, R., Sanchez de Medina, V., Morales, P., Casano, S., et al. (2018). Cannabidiol skews biased agonism

- at cannabinoid CB1 and CB2 receptors with smaller effect in CB1-CB2 heteroreceptor complexes. *Biochem. Pharmacol.* 157, 148–158. doi: 10.1016/j.bcp.2018.08.046
- Nguyen, P. T., Schmid, C. L., Raehal, K. M., Selley, D. E., Bohn, L. M., and Sim-Selley, L. J. (2012). Beta-arrestin2 regulates cannabinoid CB1 receptor signaling and adaptation in a central nervous system region-dependent manner. *Biol. Psychiatry* 71, 714–724. doi: 10.1016/j.biopsych.2011.11.027
- Nobles, K. N., Xiao, K., Ahn, S., Shukla, A. K., Lam, C. M., Rajagopal, S., et al. (2011). Distinct phosphorylation sites on the beta(2)-adrenergic receptor establish a barcode that encodes differential functions of beta-arrestin. *Sci. Signal.* 4:ra51. doi: 10.1126/scisignal.2001707
- Nogueras-Ortiz, C., Roman-Vendrell, C., Mateo-Semidey, G. E., Liao, Y. H., Kendall, D. A., and Yudowski, G. A. (2017). Retromer stops beta-arrestin 1-mediated signaling from internalized cannabinoid 2 receptors. *Mol. Biol. Cell* 28, 3554–3561. doi: 10.1091/mbc.E17-03-0198
- Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (1999). Association of beta-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J. Biol. Chem.* 274, 32248–32257. doi: 10.1074/jbc.274.45.32248
- Pertwee, R. G., Howlett, A. C., Abood, M. E., Alexander, S. P., Di Marzo, V., Elphick, M. R., et al. (2010). International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB(1) and CB(2). *Pharmacol. Rev.* 62, 588–631. doi: 10.1124/pr.110.003004
- Raehal, K. M., Walker, J. K., and Bohn, L. M. (2005). Morphine side effects in beta-arrestin 2 knockout mice. *J. Pharmacol. Exp. Ther.* 314, 1195–1201. doi: 10.1124/jpet.105.087254
- Shoemaker, J. L., Ruckle, M. B., Mayeux, P. R., and Prather, P. L. (2005). Agonist-directed trafficking of response by endocannabinoids acting at CB2 receptors. *J. Pharmacol. Exp. Ther.* 315, 828–838. doi: 10.1124/jpet.105.089474
- Shukla, A. K., Manglik, A., Kruse, A. C., Xiao, K. H., Reis, R. I., Tseng, W. C., et al. (2013). Structure of active beta-arrestin-1 bound to a G-protein-coupled receptor phosphopeptide. *Nature* 497, 137–141. doi: 10.1038/nature12120
- Shukla, A. K., Westfield, G. H., Xiao, K., Reis, R. I., Huang, L. Y., Tripathi-Shukla, P., et al. (2014). Visualization of arrestin recruitment by a G-protein-coupled receptor. *Nature* 512, 218–222. doi: 10.1038/nature13430
- Singh, S. N., Bakshi, K., Mercier, R. W., Makriyannis, A., and Pavlopoulos, S. (2011). Binding between a Distal C-Terminus Fragment of Cannabinoid Receptor 1 and Arrestin-2. *Biochemistry* 50, 2223–2234. doi: 10.1021/bi1018144
- Singla, N., Minkowitz, H. S., Soergel, D. G., Burt, D. A., Subach, R. A., Salamea, M. Y., et al. (2017). A randomized, Phase IIb study investigating oliceridine (TRV130), a novel micro-receptor G-protein pathway selective (mu-GPS) modulator, for the management of moderate to severe acute pain following abdominoplasty. *J. Pain Res.* 10, 2413–2424. doi: 10.2147/JPR.S137952
- Smith, J. S., and Rajagopal, S. (2016). The beta-Arrestins: multifunctional Regulators of G Protein-coupled Receptors. *J. Biol. Chem.* 291, 8969–8977. doi: 10.1074/jbc.R115.713313
- Soethoudt, M., Grether, U., Fingerle, J., Grim, T. W., Fezza, F., de Petrocellis, L., et al. (2017). Cannabinoid CB2 receptor ligand profiling reveals biased signalling and off-target activity. *Nat. Commun.* 8:13958. doi: 10.1038/ncomms13958
- Soethoudt, M., van Gils, N., van der Stelt, M., and Heitman, L. H. (2016). Protocol to Study beta-Arrestin Recruitment by CB1 and CB2 Cannabinoid Receptors. *Methods Mol. Biol.* 1412, 103–111. doi: 10.1007/978-1-4939-3539-0_11
- Stamer, W. D., Golightly, S. F., Hosohata, Y., Ryan, E. P., Porter, A. C., Varga, E., et al. (2001). Cannabinoid CB(1) receptor expression, activation and detection of endogenous ligand in trabecular meshwork and ciliary process tissues. *Eur. J. Pharmacol.* 431, 277–286. doi: 10.1016/S0014-2999(01)01438-8
- Tenenbaum, J., Ayoub, M. A., Perkovska, S., Adra-Delenne, A. L., Mendre, C., Ranchin, B., et al. (2009). The constitutively active v2 receptor mutants conferring NSIAD are weakly sensitive to agonist and antagonist regulation. *PLoS One* 4:e8383. doi: 10.1371/journal.pone.0008383
- Thomsen, A. R. B., Plouffe, B., Cahill, T. J., Shukla, A. K., Tarrasch, J. T., Dosey, A. M., et al. (2016). GPCR-G protein-beta-arrestin super-complex mediates sustained G protein signaling. *Cell* 166, 907–919. doi: 10.1016/j.cell.2016.07.004
- van den Ent, F., and Lowe, J. (2006). RF cloning: a restriction-free method for inserting target genes into plasmids. *J. Biochem. Biophys. Methods* 67, 67–74. doi: 10.1016/j.jbbm.2005.12.008
- Viscusi, E. R., Webster, L., Kuss, M., Daniels, S., Bolognese, J. A., Zuckerman, S., et al. (2016). A randomized, phase 2 study investigating TRV130, a biased ligand of the mu-opioid receptor, for the intravenous treatment of acute pain. *Pain* 157, 264–272. doi: 10.1097/j.pain.0000000000000363
- Zhou, X. E., He, Y. Z., de Waal, P. W., Gao, X., Kang, Y. Y., Van Eps, N., et al. (2017). Identification of Phosphorylation Codes for Arrestin Recruitment by G Protein-Coupled Receptors. *Cell* 170, 457–469.e13. doi: 10.1016/j.cell.2017.07.002
- Zidar, D. A., Violin, J. D., Whalen, E. J., and Lefkowitz, R. J. (2009). Selective engagement of G protein coupled receptor kinases (GRKs) encodes distinct functions of biased ligands. *Proc. Natl. Acad. Sci. U.S.A.* 106, 9649–9654. doi: 10.1073/pnas.0904361106

Conflict of Interest Statement: Columbia University has applied for a patent for a related arrestin translocation assay, and the technology has been licensed; JJ is an inventor. In all other respects, the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Ibsen, Finlay, Patel, Javitch, Glass and Grimsey. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.