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Approaches to Assess Biased Signaling at the CB1R Receptor

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Abstract

G protein-coupled receptors, such as the cannabinoid type 1 receptor (CB1R), have been shown to interact with multiple binding partners to transmit signals. In both transfected cell systems and in endogenously expressing cell lines, CB1R signaling has been described as multifaceted. The question remains as to how this highly widely expressed receptor signals in a given cell at a given time in vivo. The concept of functional selectivity, or biased agonism, describes the ability of an agonist to engage the receptor in a manner that preferentially engages certain signaling interactions (e.g., G proteins) over others (e.g., β -arrestins), presumably by stabilizing certain receptor conformations. There is growing interest in using such properties of ligands to direct signaling downstream of CB1R toward desirable therapeutic outcomes and to avoid adverse side effects. While it is not currently clear what pathways should be engaged and which should be avoided, the development of biased agonist tool compounds will aid in answering these questions. In this chapter, we discuss the approaches and caveats to assessing biased agonism at the CB1R.

1. INTRODUCTION

The endocannabinoid system is composed of the cannabinoid receptors, their endogenous ligands—2-arachidonoylglycerol (2-AG) and anandamide (AEA), and the anabolic and catabolic enzymes of these endogenous ligands (Pertwee, 2008). The canonical cannabinoid receptors are the type 1 cannabinoid receptor (CB1R) and type 2 cannabinoid receptor (CB2R); both of which are G protein-coupled receptors (GPCR) (Bayewitch et al., 1995; Pertwee, 2008; Pinto et al., 1994). Cannabinoid receptor activity is modulated by a structurally diverse array of endogenous, synthetic, and plant-based ligands (Pertwee, 2008; Pinto et al., 1994). The CB1R is expressed throughout the periphery and is the most abundant GPCR in the central nervous system (Bouaboula et al., 1995; Pertwee, 2008; Pinto et al., 1994). Although the CB1R is classically considered a $G_{i/o}$ -coupled GPCR, it has also been shown to couple with G_{α_s} and $G_{\alpha_{q/11}}$; moreover, CB1R interactions with β -arrestins have been established (Felder et al., 1995; Glass & Northup, 1999; Hudson, Hébert, & Kelly, 2010; Mukhopadhyay & Howlett, 2005). In cell culture assay systems, activation of CB1R has been shown to activate multiple downstream pathways as well, including regulation of cyclic adenosine monophosphate (cAMP) accumulation, phosphorylation of extracellular-regulated kinase (ERK) and CREB, and fluxuations in intracellular Ca^{2+} , among other effects (Glass & Northup, 1999; Hudson et al., 2010; Lauckner, Hille, & Mackie, 2005; Mukhopadhyay & Howlett, 2005; and “Protocols and Good Operating

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Practices in the Study of Cannabinoid Receptors” by Consroe-Bram et al.; “Real-Time Measurement of Cannabinoid Receptor-Mediated cAMP Signaling” by Hunter et al.; “Assessing Allosteric Modulation of CB1 at the Receptor and Cellular Levels” by Scott and Kendall). In rodent studies, CB1R activation produces multiple physiological responses, including inhibition of neurotransmitter release, antinociception, hypoactivity, catalepsy, hypothermia, increased appetite and insulin sensitivity, reduced gastrointestinal transit, and antiinflammatory effects (Felder et al., 1995; Izzo & Coutts, 2005; Motaghedi & McGraw, 2008; “Modeling Neurodegenerative Disorders for Developing Cannabinoid-Based Neuroprotective Therapies” by Fernández-Ruiz et al.). Given this wide spectrum of cellular and physiological effects, CB1R is considered a potential therapeutic target for the treatment of a wide range of ailments, including: chronic and acute pain; addiction; metabolic disorders and diabetes; epilepsy; multiple sclerosis, anxiety; and neurodegenerative disorders such as Huntington’s and Parkinson’s disease (Ranieri, Laezza, Bifulco, Marasco, & Malfitano, 2016).

Given that signaling at CB1R can propagate diverse and multifaceted physiological responses, a question arises as to whether the effects of cannabinoids could be harnessed to improve desirable therapeutic aspects while limiting unwanted side effects. Indeed, diverse signaling profiles have been identified downstream of CB1R in cell culture systems, yet a question also remains as to how CB1R expressed in different tissues respond in the endogenous system. Evidence for signaling bias at CB1R in vivo has been presented by several groups as separation between the potency of CB1R agonists to produce antinociceptive, hypothermic, and cataleptic effects (Breivogel, Lambert, Gerfin, Huffman, & Razdan, 2008; Grim et al., 2016; Nguyen et al., 2012; Wiley, Smith, Razdan, & Dewey, 2005). Therefore, if certain signaling pathways are associated with certain physiologies, then there may be opportunities to refine CB1R signaling and preserve one pathway while avoiding another (Breivogel et al., 2008; Grim et al., 2016; Nguyen et al., 2012; Wiley et al., 2005). This idea, however, only works when the desirable effects are mediated by different pathways than the adverse effects, which is not necessarily going to be the case.

The concept of inducing preferential signaling to one pathway over another has been termed functional selectivity or ligand bias (Kenakin, 2010; Kenakin & Christopoulos, 2013; Luttrell, Maudsley, & Bohn, 2015; Fig. 1). In this paradigm, the chemical structure of the ligand is proposed to interact with the receptor in a manner that preferentially promotes or stabilizes a conformation of receptor that favors the interaction with certain intracellular effectors (e.g., G proteins) more than another (e.g., β -arrestin) (Christopoulos, 2014; Kenakin, 2010; Kenakin & Christopoulos, 2013). As such a G protein-biased agonist would be expected to produce more signaling to G protein pathways than observed in a β -arrestin recruitment assay. However, the key here is the term “more,” which emphasizes that determinations of bias are measures of relative performance and that “more” is a term of comparison. Comparison in the case of agonist pharmacology requires a standard, or a reference agonist, to which is assigned the characterization of displaying “balanced” signaling, or the ability to stimulate the two pathways of interest with high efficacy. As such, bias is intrinsically context dependent—as it will rely on the biological system and upon the properties of the reference agonist. Therefore, in pharmacological assessments, the

determination of bias serves as a means to rank and compare properties of new agonists as compared to the performance of a reference agonist between multiple assays.

For CB1R, there are an increasing number of publications describing the different performance of ligands relative to reference ligands across many assays, or, biased agonism (Delgado-Peraza et al., 2016; Khajehali et al., 2015; Laprairie, Bagher, Kelly, & Denovan-Wright, 2016). The benefit of developing biased agonists has been more apparent for some GPCRs, including the angiotensin IIa receptor (Christensen et al., 2010), kappa opioid receptor (Brust et al., 2016), and the mu opioid receptor (Luttrell et al., 2015; Rankovic, Brust, & Bohn, 2016; Zhou & Bohn, 2014). Indeed, the first clinical application of a biased mu opioid receptor agonist, oliceridine[®] is in late-stage clinical trials with the goal of preserving pain relief while avoiding respiratory side effects (DeWire et al., 2013; Soergel et al., 2014). For the CB1R, it is not immediately clear what benefit there will be to developing CB1R-biased agonists. However, given the wide distribution of expression of this receptor, and the diverse number of signaling cascades it has potential with which to engage, the concept of pharmacologically harnessing its downstream signaling potential may open new therapeutic avenues for controlling CB1R-mediated physiologies. Therefore, a considerable effort is ongoing for developing biased CB1R ligands to promote specific receptor-dependent signaling responses in hopes of avoiding undesirable physiologies (Kenakin & Christopoulos, 2013; Khajehali et al., 2015; Laprairie et al., 2016).

The property of bias is intrinsically context dependent; and therefore, it is highly necessary to limit the influence of the cell-based assay when comparing the performance of an agonist to a reference agonist. The adaptation of mathematical modeling allows for such intra- and intersystem normalization to be weighted when comparing dose–response derived potencies and efficacies. In this chapter we will briefly review the quantification of bias of CB1R ligands using the operational model first described by Black and Leff (1983). We will then describe assay systems that can be used to collect data for bias analyses and consider the caveats and limitations therein.

2. QUANTIFYING CB1R LIGAND BIAS

Past attempts to understand CB1R ligand bias or functional selectivity have relied on qualitative comparisons using “bias plots” or direct comparisons of maximum response achievable from an applied drug ($E_{\max}$), half maximal effective concentration (EC_{50}) (Rajagopal et al., 2011). The determination and rank order comparison of EC_{50} and/or $E_{\max}$ are extremely useful as tools to highlight instances when the two-state model does not adequately describe the data (Clarke & Berg, 2010). However, these approaches do not provide quantitative measures of ligand activity and are less useful at minimizing differences in assay system, cell type, receptor density, or assay kinetics. Due to these limitations, rank order comparison should not be used to determine “bias” (Ehlert, 2015; Luttrell, 2014; Rajagopal et al., 2011; Stahl, Zhou, Ehlert, & Bohn, 2015). Instead, the operational model (Black & Leff, 1983; Eq. 1) more appropriately handles the complexities of intrasystem comparison (assay system, cell type, receptor density, etc.) and is well suited to be applied in a straightforward manner to the familiar concentration-response data.

$$E = \frac{E_{\max}[A]^n \tau^n}{[A]^n \tau^n + ([A] + K_A)^n} \quad (1)$$

In the operational model $E_{\max}$ is the maximum response, A represents the agonist concentration, n the Hill slope, τ the agonist efficacy, and K_A the agonist affinity (Black & Leff, 1983). A useful reparameterization of this model has been elaborated and employed over the past couple years. The transduction coefficient, defined as the (τ/K_A) or synonymously the $\log R$, provides a composite parameter value that can be produced and used to compare agonist activity between different systems (Kenakin, 2010). The transduction coefficient is not a descriptive parameter value (i.e., potency or efficacy) but rather a relative measure of the ability of a ligand to activate the receptor in the target system (Kenakin, 2010; Kenakin & Christopoulos, 2013). A reference agonist is commonly run in parallel in these experiments to provide a method of direct normalization across systems. The reference agonist is chosen based on the appreciable view that it is commonly used, widely available, and acts as a well-behaved full agonist in each system of study (Kenakin & Christopoulos, 2013; Rajagopal et al., 2011). The reference agonist should be a full agonist in each of the assay systems being used (Kenakin & Christopoulos, 2013; Rajagopal et al., 2011). For many receptors, the first choice is, therefore, the endogenous ligand(s) (Khajehali et al., 2015). CB1R may be unique in this point because AEA and 2-AG are not consistent full agonists of the receptor (Pertwee, 2008). Instead, the compound CP55,940 is a commonly used and generally agreed upon full agonist at the CB1R. Based on the concentration-response curve of CP55,940, the relative agonist activity of test agonists can be determined when fit to the operational model (Eq. 1). A useful normalization of this analysis is to produce the relative activity of each test agonist. This normalization is produced by calculating the difference between the transduction coefficient of a test agonist and the reference agonist and results in a relative transduction coefficient ($\log R$) within an assay (Eq. 2).

$$\Delta \log R = \log (\tau / K_A)_{\text{Test compound}} - \log (\tau / K_A)_{\text{Ref compound}} \quad (2)$$

To best-fit data to this model and derive $\log R$, we recommend sharing $\log R$, n , and $E_{\max}$ across datasets and constraining $\log K_A$ between 0 and -15 (femtomolar, i.e., an upper bound that described as extremely high-affinity ligand) (Ehlert, 2015; Stahl et al., 2015).

$\log R$ values can then be compared for a test compound(s) between assays to yield a system-independent bias factor for that compound(s), $\log R$ (Eq. 3; Ehlert, 2015; Stahl et al., 2015).

$$\begin{aligned} \log \text{bias} &= \Delta \Delta \log R = \Delta \Delta \log (\tau / K_A)_{R1 - R2} \\ &= \Delta \log (\tau / K_A)_{R1} - \Delta \log (\tau / K_A)_{R2} \end{aligned} \quad (3)$$

The antilog of $\log R$ is the bias factor of the test compound (Kenakin, Watson, Muniz-Medina, Christopoulos, & Novick, 2012; Stahl et al., 2015). $\log R$ values from multiple,

independent, experiments can be used to calculate mean bias factors with associated error (Stahl et al., 2015). These data can then be compared using standard statistical analyses.

3. ASSAYS USED TO EXAMINE CB1R LIGAND BIAS

Several cellular effects can be compared in order to quantify ligand bias using the operational model, and multiple assays may exist to measure a cellular effect. Here, we describe some of the common downstream effectors of CB1R and the assays available to quantify their responsiveness, as well as advantages and disadvantages of each.

3.1 G Protein Coupling

One of the most proximal effectors of GPCR activation are their namesakes—the G proteins. CB1R has classically been described as a $G_{\alpha_{i/o}}$ -coupled GPCR (Felder et al., 1995). Quantification of G protein coupling to a GPCR can be conducted using the [35 S]GTP γ S assay. Agonist binding to the GPCR results in the exchange of guanosine diphosphate for guanosine triphosphate (GTP). Guanosine 5'-O-[gamma-thio]triphosphate (GTP γ S) is a nonhydrolyzable form of GTP. In this assay, a radiolabeled form of GTP γ S, [35 S]GTP γ S, is incubated with dissociated membranes from cultured cells (e.g., cells stably expressing CB1R) or tissue homogenate and the compounds being tested. Compound concentration-dependent coupling of the alpha subunit of G protein (G_{α}) to the receptor results in the binding of [35 S]GTP γ S. Bound [35 S]GTP γ S is then separated from free [35 S]GTP γ S by filtration, and G_{α} -bound [35 S]GTP γ S is quantified (Bohn, Zhou, & Ho, 2015). [35 S]GTP γ S binding can be quantified using cell lines stably expressing CB1R or tissue from mouse brain homogenates (Bohn et al., 2015; Hua et al., 2016; Janero et al., 2015). We have described the methodology of these assays in detail (Bohn et al., 2015).

[35 S]GTP γ S binding reveals the efficiency of coupling to the most prevalent G protein signal available in the system, and different types of G_{α} protein binding cannot be readily determined or distinguished (Bohn et al., 2015). In cell models the predominant G protein is often $G_{\alpha_{i/o}}$, but in endogenous settings these may be less-characterized G_{α} proteins, such as G_{α_Z} (Hinton et al., 1990; Wettschureck et al., 2005). Additional experiments, such as immunoprecipitation and western blot, are required to determine G protein type (Hinton et al., 1990; Wettschureck et al., 2005). A major advantage [35 S]GTP γ S binding is that the signal is measured proximal to the receptor and can therefore be considered a more direct measurement of agonist-directed receptor signaling than other output measures discussed later (Kenakin et al., 2012).

3.2 β -Arrestin Recruitment

CB1R interacts with both β -arrestin1 and β -arrestin2 (Delgado-Peraza et al., 2016; Laprairie et al., 2016). Like G protein coupling, β -arrestin recruitment is proximal to the receptor and therefore a direct measurement of agonist-induced changes in receptor conformation. Several approaches have been developed to quantify β -arrestin recruitment, but the most widely used rely on bioluminescence resonance energy transfer (BRET), enzyme complementation, and transcriptional activation.

BRET, like FRET, relies on the transfer of energy between a donor protein and an acceptor protein that are present within close enough proximity for energy transfer to occur (James, Oliviera, Carmo, Iaboni, & Davis, 2006). Unlike in FRET, the donor protein used in BRET assays is a form of the *Renilla* luciferase protein that only emits blue light upon addition of its substrate, coelenterazine (James et al., 2006). Therefore, energy transfer to the acceptor protein can only occur upon addition of coelenterazine when it is quantified within the assay. BRET is advantageous because it can be used to examine kinetic effects, it is highly sensitive, adaptable to high-throughput screening, and is adaptable to measure any protein–protein interaction aside from GPCRs and β -arrestin (James et al., 2006). However, effective use of BRET requires the use of rigorous controls including BRET positive (donor–acceptor fusion proteins), BRET negative (donor protein alone), noninteracting protein controls, and determination of appropriate donor: acceptor ratios, among others (Hudson et al., 2010; James et al., 2006; Laprairie et al., 2016). Cloned BRET constructs must be in the correct orientation to facilitate energy transfer but not disrupt normal protein function (James et al., 2006), and BRET cannot be conducted in an endogenous system because it requires the expression of heterologous fusion proteins (James et al., 2006).

Enzyme fragment complementation assays utilize CB1R modified with a β -galactosidase fragment coexpressed in the same cell as β -arrestin conjugated to the remainder of β -galactosidase (van der Lee et al., 2009). Recruitment of β -arrestin produces a complete enzyme whose activity can be quantified by addition of chemiluminescent substrate, such that the amount of chemiluminescence produced is proportional to β -arrestin recruitment. This popular assay format is utilized in the PathHunter® assays commercially available from DiscoverX and others (van der Lee et al., 2009). Enzyme complementation assays are advantageous because they are highly sensitive and produce large signal over background and are designed for high-throughput screening (van der Lee et al., 2009). Two disadvantages of these assays are (1) that they are irreversible upon addition of the substrate and are therefore not suitable for quantifying kinetic effects, and (2) it requires modification of the C-terminus of the receptor and cannot be used to quantify β -arrestin recruitment in endogenous systems.

The Tango assay utilizes transcriptional activation following β -arrestin translocation (Barnea et al., 2007). GPCRs of interest are fused to a transcription factor by a peptide containing a TEV protease cleavage sequence and β -arrestin is fused to the TEV protease (Barnea et al., 2007; Kroeze et al., 2015). Association of the GPCR with β -arrestin results in proteolytic cleavage of the transcription factor, which consequently activates transcription of a reporter gene, such as luciferase (Barnea et al., 2007; Kroeze et al., 2015).

As a result, a transient interaction between GPCR and β -arrestin is amplified and stabilized. Tango assay systems are now openly available for a wide swath of GPCRs, making this system amenable to screening compounds at many different receptor systems (Kroeze et al., 2015). As with enzyme complementation assays, Tango is advantageous because of its sensitivity and large signal over background. However, modification of the both β -arrestin and GPCR means that it cannot be used to quantify β -arrestin recruitment in endogenous systems.

3.3 Inhibition of cAMP Accumulation

Beyond proximal interactions between CB1R and G protein, and CB1R and β -arrestin, $G\alpha_{i/o}$ proteins inhibit adenylate cyclases and reduce the concentration of cAMP. Measurements of cAMP can be made from cultured cell systems (Cawston, Connor, Di Marzo, Silvestri, & Glass, 2015; Hua et al., 2016; Khajehali et al., 2015) and dissociated cell membranes from animals (Brust et al., 2017), using the many quantification assays currently available. The earliest studies of the CB1R modulation of adenylyl cyclase were performed by measuring the conversion of α -[^{32}P]ATP to [^{32}P]cAMP (Meschler & Howlett, 2001).

For higher throughput studies avoiding radioactivity, assay kits based on fluorophores have become available. Homogeneous time-resolved fluorescence resonance energy transfer (FRET) (HTRF) can be used to measure changes in cAMP level (Cawston et al., 2015; Hua et al., 2016). The cAMP HTRF assay utilizes an anti-cAMP antibody conjugated to a donor fluorophore and a fluorescent acceptor molecule conjugated to exogenous cAMP (Degorce et al., 2009). Excitation of the bound donor-conjugated antibody results in emission of fluorescence that then excites the acceptor molecule to emit a different wavelength of light (Degorce et al., 2009). The ratio of acceptor to donor fluorescence is FRET (Degorce et al., 2009). HTRF uses a delay between quantification of donor emission and acceptor emission ($\sim 150 \mu\text{s}$) to enhance resolution between the two protein's spectra, which reduces background signal allows for quantification (Degorce et al., 2009). Endogenous cAMP produced by the cell/tissue being measured competes with the acceptor molecule for antibody binding, and so FRET is inversely proportional to cAMP concentration (Degorce et al., 2009). HTRF is advantageous over other assay systems because it is amenable to measurements of cAMP accumulation ($G\alpha_s$ signaling at CB1R; Glass & Northup, 1999) and inhibition of cAMP ($G\alpha_{i/o}$ signaling at CB1R; Mukhopadhyay & Howlett, 2005), it can be used to examine kinetic effects of cAMP modulation (Cawston et al., 2015), it is highly sensitive (Degorce et al., 2009), and assay systems are relatively easy to use. Although quantification of cAMP is less proximal to the receptor than G protein coupling, cAMP represents a major cellular effector of CB1R and is therefore important to measure.

Other approaches to measure cAMP include enzyme-linked immunosorbent assays (EIA) that utilize fluorophore-conjugated anti-cAMP anti-bodies (Jiang et al., 2007), similar to HTRF; BRET-based assays that utilize the fusion protein "cAMP sensor-using YFP-Epac-Rluc" (Cawston et al., 2015; Jiang et al., 2007); transcriptional activation in cells that express a reporter gene (e.g., luciferase) under the regulatory control of cAMP-response elements (Zhaung & Liu, 2006). EIA is similar to HTRF but is difficult to use to examine kinetic effects (Jiang et al., 2007). BRET-based quantification of cAMP is highly sensitive and amenable to kinetic experiments, but must be done in heterologous systems (Cawston et al., 2015; Jiang et al., 2007). Transcriptional assays are irreversible and are cannot be used quantifying kinetic effects, and must be conducted in heterologous systems, but provide a high signal relative to background (Zhaung & Liu, 2006). cAMP signaling is discussed further in Chapter 3 of this volume ("Real-Time Measurement of Cannabinoid Receptor-Mediated cAMP Signaling" by Hunter et al.).

3.4 Changes in Ca^{2+}

In addition to $\text{G}\alpha_{i/o}$, CB1R couples $\text{G}\alpha_{q/11}$ to modulate intracellular Ca^{2+} levels (Lauckner et al., 2005). Ca^{2+} modulation may be monitored through electrophysiological approaches (Lauckner et al., 2005) or fluorescence-based approaches (Vandevoorde, Lambert, Smart, Jonsson, & Fowler, 2003). Electrophysiological techniques provide for high spatiotemporal resolution and can be easily utilized in in vivo and ex vivo neuronal preparations; however, they are not amenable to the screening of large numbers of test compounds (Lauckner et al., 2005). The FLIPR Ca^{2+} detection assays utilize a dye that fluoresces upon binding Ca^{2+} (Sirenko et al., 2013). FLIPR was specifically designed for high-throughput applications and provides some degree of spatiotemporal resolution (Sirenko et al., 2013). Due to the high cost associated with this assay, it is not a feasible approach for ex vivo neuron preparations, but can be used for cell systems whether they endogenously or heterologously express CB1R.

3.5 Receptor Internalization

Following ligand binding, CB1R is internalized through β -arrestin-mediated mechanisms and subsequently recycled or degraded. Internalization of the unmodified receptor can be detected with a fluorescent protein conjugate (e.g., β -arrestin-green fluorescent protein (GFP)) or fluorescent-conjugated antibody. Internalized receptors associated with β -arrestin-GFP are visible within the cell as puncta. The number of puncta per cell is related to the concentration of compound used in a concentration-dependent manner. To conduct this assay, cells are grown in glass-bottomed plates, treated with test compound and images of cells are captured and analyzed for the number of puncta/cell (Gasparri, 2009). This assay is advantageous because kinetics can be determined easily, they can produce large signal over background, and they are amenable to high-content analysis and compound screening (Gasparri, 2009). Because this assay relies on high-content image analysis, it provides qualitative information on the movement and localization of receptors following compound treatment. Other systems have been generated to quantify receptor internalization such as the PathHunter[®] assays (DiscoverX) which can be useful if imaging systems are not available. In these assays, the enzyme fragment is localized to the endosome and so complementation and signal detection occurs at the endosome upon receptor internalization (van der Lee et al., 2009). These assays have similar advantages and disadvantages as the PathHunter[®] β -arrestin recruitment assays (van der Lee et al., 2009).

3.6 Protein Phosphorylation and Changes in Gene Expression

CB1R activation effects the phosphorylation and activation of signaling proteins, including ERKs, protein kinase B (Akt), activator protein 1 (AP-1), and others, downstream of changes in cAMP or Ca^{2+} . Protein phosphorylation can be measured through standard western blot techniques using cell lysates or tissue homogenates (Bouaboula et al., 1995; Howlett et al., 2000). Although western blots are widely used, can be conducted using native tissues or nontransfected cell cultures, and an effective means of measuring protein, they are not amenable to high-throughput screening. Protein phosphorylation can also be measured by the In-cell western assay (Hudson et al., 2010; Laprairie et al., 2016, 2017; Redmond et al., 2016). In an In-cell western, cells are cultured in multiwell plates and treated with the

compounds of interest. Cells are subsequently fixed, permeablized, and incubated with antibody directed against the proteins of interest (phosphoprotein and total protein), followed by a second incubation with infrared-conjugated secondary antibodies (e.g., green for phosphoprotein and red for total protein). Immunocytochemical staining is quantified using an Odyssey imaging system (Li-Cor), and protein phosphorylation is expressed relative to total protein (Hudson et al., 2010; Laprairie et al., 2016, 2017; Redmond et al., 2016). In-cell western assays can be adapted for use as a medium-throughput screen in heterologous and endogenous receptor expression systems. However, antibody conditions and specificity must be verified through standard western blot approaches before being applied in this assay.

Several groups have demonstrated that CB1R activation produces quantifiable changes in transcription factor activity (Bosier, Hermans, & Lambert, 2008; Bouaboula et al., 1995) and gene expression (Bosier et al., 2008; Laprairie et al., 2016), which can be measured by chromatin immunoprecipitation and quantitative reverse transcriptase-PCR (qRT-PCR), respectively. As with immunocytochemical approaches, quantification of transcription factor activity and qRT-PCR is amenable to medium- to high-throughput assay development and can be applied to both heterologous and endogenous receptor systems. However, the interpretation of agonist bias several steps removed from the receptor is difficult, as discussed later. Therefore, data from these assays may be physiologically relevant to the overall actions of the drug, but less relevant with regard to elucidating mechanisms of bias (Kenakin et al., 2012). Furthermore, extensive computational power may be necessary for managing and interpreting extensive datasets that can arise from large-scale transcriptome evaluation studies (Luttrell et al., 2015).

4. IMPORTANT CONSIDERATIONS AND CAVEATS

Bias is a relative term, requiring the comparison of one effect to another. As such, ligand bias describes the ability of an agonist to perform differently compared to another agonist when both agonists are examined in parallel across multiple assays. As such, some degree of bias may appear in nearly every direction examined for nearly every ligand. The key is to discern what is truly the anomaly and whether such divergences from the normality will correspond to fundamental differences in receptor function. In this regard, advances will be made as we begin to understand more about the active-, inactive-, and differently active-state structures that GPCRs assume when bound to different ligands. While the number of structures for GPCRs explode and the resolution of refined binding poses comes further into focus, we can foresee a time of structure-guided ligand design. However, the question we will still need to address is what kind of bias do we want to impart in our compounds to control what physiologies in vivo? Therefore, the pursuit of compounds with diverse pharmacological signatures will be useful for starting to answer that question in animal models. Meanwhile, to facilitate ranking our compounds and defining the differences by which they act, quantitative studies of CB1R signaling should include analyses of bias, as has been done for other GPCRs (Luttrell, 2014; Rajagopal et al., 2011).

4.1 Cell Type and Receptor Density

Unlike other approaches to quantify ligand bias, such as comparison of pEC_{50} or relative activity, the transducer coefficient used to determine bias (τ/K_A) is a linear parameter in relation to receptor density (Kenakin et al., 2012). This is critical because it allows for comparisons to be made between most agonists as well as between tissue and cell types, which may be used to conduct various assays (Kenakin et al., 2012). Therefore, the operational model as first described by Black and Leff (1983) (see earlier) can be used in most cases so long as the compounds being studied produce an appreciable response in the assay used. If a compound(s) does not produce an appreciable response in all assays, then an operational-competitive model can be applied to determine bias (Stahl et al., 2015). The competitive model can be applied by taking advantage of the competitive nature of partial agonists in the presence of a full agonist (Ehlert, 2015; Stahl et al., 2015). This method was developed and is described in detail by Stahl et al. (2015).

In all cases, the quality of the concentration-response curves used to assess bias is critically important. Compound solubility, preparation of compound plates, and evaporation may all effect the observed response and consequently the calculated bias of a compound. For cannabinoids, as highly lipophilic compounds, the use of a carrier protein such as BSA is important because solubility can be improved and compounds do not stick to vessel walls (Howlett et al., 2002). Compound plates should be set up immediately prior to use to limit compound degradation and solvent evaporation. For live cell assays, cell number and serum deprivation should be optimized and considered across assay platforms; and protein concentration should be optimized for membrane-based assays. Early protocol optimization will improve the resolution of the assays and produce reliable concentration-response curves that can be used to determine bias.

4.2 Proximal vs Distal Effects to the Receptor

GPCR agonists facilitate cellular effects upon binding to their receptor and promoting a conformational change in that population of receptors that is conducive to signaling (Kenakin & Christopoulos, 2013). In the case of “balanced” agonists, the net effect is that of pluripotent signaling through the available repertoire of effector proteins (Kenakin et al., 2012; Luttrell et al., 2015). For biased agonists, however, a specific conformation of the receptor is favored among the ensemble of conformations and it is this shift toward a specific conformation that results in preferential signaling through specific effector proteins and therefore a biased receptor population (Luttrell et al., 2015). As a consequence of this, bias-driven effects that occur at effectors proximal to the receptor (e.g., G protein-coupling and β -arrestin recruitment) are likely representative of a change in receptor conformation producing a cellular change (Kenakin et al., 2012; Luttrell et al., 2015). As a specific signal is propagated from the GPCR to other proteins that signal is amplified.

Therefore, agonist bias becomes more pronounced at secondary and tertiary effector proteins (e.g., adenylate cyclase or G protein-coupled inwardly rectifying K^+ channels) relative to primary effectors (Kenakin & Christopoulos, 2013; Kenakin et al., 2012). However, cross talk between signals results in overlap between different pathways and signal bias may be misinterpreted as the consequence of one pathway rather than another (Luttrell et al., 2015).

This is particularly true in the case of ERK and Akt, whose activation is a convergence point for multiple CB1R signaling pathways including $G\alpha_{i/o}$ and β -arrestin (Khajehali et al., 2015; Laprairie et al., 2016). Another example is transcription factors such as AP-1 and Jnk that are activated through multiple CB1R-mediated signaling pathways (Bosier et al., 2008). Pathway-specific effects can be determined in such cases through careful examination of signaling kinetics (Herenbrink et al., 2016) as well as ligand potencies in different assays (Kenakin et al., 2012). Ultimately, agonist bias must be quantified such that the regulatory pathway is known and the quantified signals are amenable to bias analysis.

4.3 Ligand Kinetics

Increasing evidence from studies of other GPCRs, such as the dopamine D2 receptor, supports the hypothesis that an agonist's dissociation rate has a profound effect on its observed bias (Herenbrink et al., 2016). Herenbrink et al. (2016) observed that the apparent bias of D2 agonists varied widely depending on the time at which assay measurements were made. The authors were able to relate these differences in bias to the dissociation rate of agonists and concluded that slowly dissociating agonists were more likely to display β -arrestin bias than rapidly dissociating agonists (Herenbrink et al., 2016). Although this has yet to be directly demonstrated for agonists of CB1R, Cawston et al. (2015) observed that changes in signal transduction following treatment with the CB1R allosteric modulator Org27569 changed dramatically as a function of time. Khajehali et al. (2015) reported that high-affinity CB1R agonists CP55,940 and HU-210 displayed bias for cAMP relative to pERK but did not relate these effects to ligand dissociation. The role that ligand kinetics play in bias is important to understand for two reasons: (1) it represents a possible source for discrepancy between model systems and assays in interpreting bias, and (2) it may be that biased ligands can be designed for CB1R on the basis of their dissociation rate. With respect to future studies of CB1R agonist bias, the contribution of kinetics to bias must be considered when designing experiments and interpreting data (Cawston et al., 2015; Herenbrink et al., 2016).

4.4 Applying Measurements of Bias to Allosteric Ligands

In addition to biased CB1R orthosteric agonists, allosteric modulators of CB1R can alter the signaling bias of orthosteric CB1R agonists (Khajehali et al., 2015; Laprairie et al., 2017). Khajehali et al. (2015) suggest that the negative allosteric modulatory activity of Org27569 for pERK1/2 may be due to biased allosterism and probe dependence. Laprairie et al. (2017) observed that the allosteric compound GAT228 is moderately biased for β -arrestin1, whereas its enantiomer GAT229 is biased for inhibition of cAMP. Given the growing interest in developing allosteric modulators of CB1R, evaluations of their potential bias will become increasingly important, and with it the need to establish reliable analytical approaches.

4.5 Crystal Structures of CB1R

To date two crystal structures of CB1R in inactive conformations have been described (Hua et al., 2016; Shao et al., 2016). Hua et al. (2016) first crystallized CB1R bound to the rimonabant (SR141716A) derivative, and irreversible CB1R antagonist, AM6538. The development of irreversible antagonists and subsequent crystallization of hCB1R is described in Chapter 10 of this volume ("Ligand-Assisted Protein Structure (LAPS): An

Experimental Paradigm for Characterizing Cannabinoid-Receptor Ligand-Binding Domains” by Janero et al.). From these first glimpses into the structure of CB1R we can begin to understand the ligand binding landscape and form hypotheses about residues within the agonist binding pocket that may be important for mediating changes in receptor conformation and consequently bias. The next hurdle to overcome in this field will be to obtain a crystal structure of CB1R in an active, agonist-bound state. If agonist-bound crystals were obtained using differentially biased agonists, it may even be possible to observe conformational differences in receptor related to bias.

5. CONCLUSIONS

CB1R is considered an important pharmacological target because modulation of this receptor produces wide-ranging physiological effects—appetite, locomotion, mood, analgesia—that from a clinical perspective appear to be applicable to many different pathological conditions. At the receptor level, CB1R couples to multiple effector proteins and facilitates a myriad of intra- and intercellular effects. The current state of CB1R research, as described here, indicates that biased ligands can selectively enhance certain receptor-mediated effects, to a limited degree. The present questions for studying ligand bias at CB1R are (1) what is the possible extent of ligand bias for the receptor? and (2) does ligand bias facilitate physiologically relevant effects that could be therapeutically useful? The application of tools, such as the operational model, receptor crystal structures, irreversible antagonists, and a better understanding of kinetic and allosteric effects, previously not applied to CB1R or not available, will permit a more thorough understanding of CB1R pharmacology than was previously possible. From these tools we can begin to understand the importance of CB1R ligand bias.

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ABBREVIATIONS

2-AG	2-arachidonoylglycerol
AEA	anandamide
Akt	protein kinase B
AP-1	activator protein 1
BRET	bioluminescence resonance energy transfer
cAMP	cyclic adenosine monophosphate
CB1R	type 1 cannabinoid receptor
CB2R	type 2 cannabinoid receptor
EC₅₀	half maximal effective concentration

$E_{\max}$	maximum response achievable from an applied drug
ERK	extracellular-regulated kinase
FRET	Förster resonance energy transfer
Gα	alpha subunit of G protein
GFP	green fluorescent protein
GPCR	G protein-coupled receptors
GTP	guanosine triphosphate
GTPγS	guanosine 5'-O-[gamma-thio]triphosphate
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction

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Fig. 1. CB1R-mediated signaling. (A) CB1R interacts with multiple effector proteins to produce different intracellular signals. These effector proteins include: $G_{\alpha_{i/o}}$, G_{α_s} , $G_{\alpha_{q/11}}$, $G_{\alpha_{12/13}}$, $G\beta\gamma$, and β -arrestins. (B) The binding of different agonists to CB1R stabilizes the receptor in conformations that preferentially enhance coupling to certain effector proteins and diminish coupling to other effector proteins. Consequently, cellular signaling pathways are effectively enhanced or diminished; this describes ligand bias. Here, three theoretical agonists bind CB1R and each facilitates a unique receptor conformation and unique signaling outputs.