HHS Public Access

Author manuscript

Biochem Pharmacol. Author manuscript; available in PMC 2018 March 15.

Published in final edited form as:

Biochem Pharmacol. 2017 March 15; 128: 1–11. doi:10.1016/j.bcp.2016.11.014.

Functional selectivity at G-protein coupled receptors: Advancing cannabinoid receptors as drug targets

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Abstract

The phenomenon of functional selectivity, whereby a ligand preferentially directs the information output of a G-protein coupled receptor (GPCR) along (a) particular effector pathway(s) and away from others, has redefined traditional GPCR signaling paradigms to provide a new approach to structure-based drug design. The two principal cannabinoid receptors (CBRs) 1 and 2 belong to the class-A GPCR subfamily and are considered tenable therapeutic targets for several indications. Yet conventional orthosteric ligands (agonists, antagonists/inverse agonists) for these receptors have had very limited clinical utility due to their propensity to incite on-target adverse events. Chemically distinct classes of cannabinergic ligands exhibit signaling bias at CBRs toward individual subsets of signal transduction pathways. In this review, we discuss the known signaling pathways regulated by CBRs and examine the current evidence for functional selectivity at CBRs in response to endogenous and exogenous cannabinergic ligands as biased agonists. We further discuss the receptor and ligand structural features allowing for selective activation of CBR-dependent functional responses. The design and development of biased ligands may offer a pathway to therapeutic success for novel CBR-targeted drugs.

Graphical abstract

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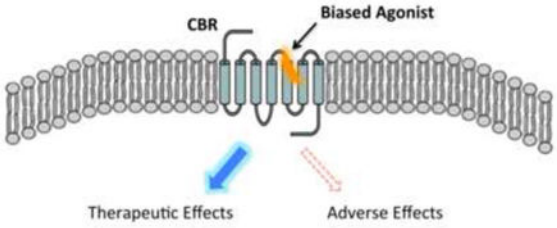
Conflict of interest: The authors declare no conflicts of interest regarding the subject of this paper.



Keywords

GPCR; Biased agonism; Drug discovery; Effector Pathways

1. Pharmacological and drug-discovery implications of functional selectivity

According to the tenets of the traditional “two-state” model of G protein-coupled receptor (GPCR) function, a GPCR acts as a ligand-controlled “on-off” switch, eliciting, when activated by an agonist, a cascade of cellular signals and effects through specific transducers such as G proteins without any particular directionality to the ensuing information output [1]. Based upon data from an array of biophysical and biochemical studies, this outmoded paradigm has been elaborated to a so-called “multi-state” model that encompasses a variety of GPCR conformations along an activity continuum influenced by bound ligands, effector proteins, and other cellular molecules (Na^+, lipids, etc.) [2,3]. Furthermore, it is now well-recognized that GPCRs can mediate cellular signaling through both G protein-dependent (through four major G-protein sub-classes: G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$) [4] and -independent pathways involving, for example, arrestins [5], G-protein receptor kinases (GRKs) [6], ion channels [7], and Src kinases [8]) (Fig. 1).

Traditional GPCR agonists bind to the receptor at the site that engages endogenous ligands, the so-called orthosteric site. Although orthosteric agonists may activate multiple, G protein-dependent and -independent downstream signaling networks, some preferentially activate one (or a few select) effector pathways. This phenomenon, termed “biased agonism” or “functional selectivity,” has been demonstrated for an ever increasing number of GPCRs including 5-HT2A, 5-HT2C, and 5-HT1A serotonin receptors [9,10]; the mu-opioid receptor (MOR) [11]; dopamine D1, D2, and D3 receptors [12-14]; chemokine receptor 7 [15]; melanocortin 4 receptor [16]; α-1a adrenergic receptor [17]; angiotensin type 1 receptor [18]; gonadotrophin-releasing hormone receptor [19]; and type 1 parathyroid hormone receptor (PTH1R) [20]. Comprehensive review articles may be consulted for a global appreciation of the large number and variety of GPCRs to which biased signaling has been attributed [21-23]. Regardless of the specific GPCR involved, current receptor theory holds that biased agonists preferentially promote certain intracellular signaling circuits by selectively stabilizing a subset of possible receptor conformations in different proportions than would an unbiased ligand, leading to pathway-selective biological responses [4,5,11,21]. Biased agonism is typically recognized as an alteration in the balance between

GPCR G-protein dependent and -independent signaling mechanisms, the latter often involving recruitment of the multifunctional scaffold protein, β-arrestin2 [5,22].

As detailed elsewhere [21,23,24], functional selectivity carries important implications for drug discovery, especially with respect to populating the developed chemical space around therapeutic GPCRs lacking novel candidate drugs. Biased agonism offers an opportunity for the development of signaling pathway-selective (rather than just receptor-selective) therapies biased toward salutary effector pathways and away from those associated with on-target adverse events. This is particularly important for therapeutic GPCRs whose pharmacologically active ligands carry adverse effects that have restricted, if not obviated, the medical exploitation of those GPCRs as drug targets. The principal cannabinoid GPCRs are two such receptors.

2. Cannabinoid receptors

2.1. Receptor-mediated cannabinoid physiology

The endogenous cannabinoid ("endocannabinoid") system includes two principal class-A GPCRs: cannabinoid receptor 1 (CB1R), predominantly expressed in the brain [25] and to a lesser extent in the periphery [26], and cannabinoid receptor 2 (CB2R), expressed mainly in immune cells and during inflammatory injury in the central nervous system (CNS) [27]. CB2R has 44% overall sequence identity with CB1R and shows comparatively greater interspecies heterogeneity. Some 25 years ago, identification and initial molecular characterization of CB1R and CB2R were promoted by attempts to understand the mechanism by which the plant-derived cannabinoid (phytocannabinoid) and principal psychoactive constituent of marijuana, Δ^9-tetrahydrocannabinol (THC) (**1**, Fig. 2), exerts its (patho)physiological effects [28]. Other receptors, including GPR55 and GPR119 and members of the transient receptor potential (TRP) family, also bind select endogenous and pharmacologically active synthetic cannabinergic ligands [29,30].

Cannabinoid receptors have been implicated in the regulation of a variety of central and peripheral physiological processes including neurogenesis [31], neuromodulation [32], energy balance and metabolism [33,34], immune-system activity [35], thermoregulation [36], and reproduction [37]. It is thus unremarkable that aberrant CB1R- or CB2R-dependent signaling is implicated in a number of disease states that represent major unsolved medical problems for which CBRs are considered key drug targets. CB1R-selective ligands are currently of interest as potential treatments for overweight/obesity, cardiometabolic and substance-use disorders, and neuropathic pain [38,39]. CB2R-selective agents hold promise for treating neuro-inflammatory diseases such as multiple sclerosis and amyotrophic lateral sclerosis [38,40]. Nonetheless, typical orthosetric CBR ligands (especially CB1R agonists) have a propensity to induce adverse psychobehavioral responses that have limited their therapeutic utility and circumscribed the indications accessible to salutary pharmacotherapeutic CB1R/CB2R modulation [39,41]. At present, although some synthetic CB1R/CB2R orthosteric ligands have utility as pharmacologically-active tool compounds for the laboratory, only two have received regulatory approval. Cesamet® (nabilone) (**2**, Fig. 2) is a synthetic cannabinoid and potent CB1R and CB2R agonist approved to treat chemotherapy-induced nausea and emesis [42]. Rimonabant® (SR141716)

(**3**, Fig. 2), a CB1R antagonist/inverse agonist approved in 2006 the European Union for weight loss, was withdrawn by the manufacturer in 2009 due to its unacceptable risk:benefit ratio [43]. This situation has promoted the quest for efficacious small molecules with novel molecular-pharmacology phenotypes that express the benefits of CB1R/CB2R modulation with less side-effect risk than conventional CBR (ant)agonists [39,44].

2.2. CBR-dependent signaling

Both CB1R and CB2R preferentially couple to $G_{i/o}$ -type G proteins, thereby inhibiting the activity of adenylyl cyclase (AC) and decreasing cellular cyclic adenosine monophosphate (cAMP) accumulation (Fig. 3) [45]. Under certain conditions, some cannabinergic agonists activate CBRs coupled to G_s [46] or G_q [47] G-proteins. Through a G-protein dependent mechanism, both CB1R and CB2R can activate different members of the mitogen-activated protein kinase (MAPK) family, including extracellular kinase-1 and -2 (ERK1/2), p38 and p42/p44 MAPKs, and c-Jun N-terminal kinase (JNK) [45,48-50]. CB1Rs can also negatively couple to N- and P/Q-type voltage-gated Ca^{2+} channels (VGCCs) [51] and positively couple to A-type and inward-rectifying K^+ channels [52]. CB1Rs can also activate phospholipase C-b (PLC-b) to elicit an increase in intracellular Ca^{2+} as well as phosphatidylinositol 3-kinase/protein kinase B (PI3K/PKB) to stimulate glycolysis and modulate cell proliferation [53] (Fig. 3). In contrast, CB2Rs are associated with a more circumscribed repertoire of downstream effector pathways and do not modulate PI3K/PKB signaling, inward rectifying K^+ channels, or Ca^{2+} channels [54]. Both CB1R and CB2R increase ceramide levels either by increasing sphingomyelin hydrolysis or *de novo* ceramide synthesis and can modulate gene transcription [55].

Certain ligands that engage CBRs are functionally biased in that they can activate preferentially one (or more) downstream signaling pathways (Tables 1 and 2; Fig. 2). Multiple lines of evidence supporting this proposition come from both cellular overexpression models and biological systems that express endogenous CB1R or CB2R, the latter suggesting the physiological significance of signaling bias at these receptors and inviting the opportunity for developing targeted, pathway-selective ligands that modulate GPCR-mediated cannabinergic signaling as pharmacotherapeutics. Those data will now be discussed with respect to key structure-function correlates of biased cannabinergic ligands. Particular emphasis will be placed upon findings that inform the design of functionally biased ligands as potential drugs targeted to these two endocannabinoid-system GPCRs at their respective orthosteric sites, at which naturally-occurring endocannabinoid lipid mediators bind.

3. Functional selectivity at CB1R

All endocannabinoid signaling lipids, including the principal, arachidonic acid-derived mediators found in mammals, anandamide (AEA) (**4**, Fig. 2) and 2-arachidonoylglycerol (2-AG) (**5**, Fig. 2), preferentially activate $G_{i/o}$ G proteins at CB1R (Table 1). Certain structurally distinct cannabinergic ligands, upon binding to CB1R, can elicit the receptor's differential interaction with G_i and/or G_o G-protein subtypes. HU210 (**6**, Fig. 2) activates both G_i and G_o proteins to maximal efficacy, whereas WIN55212 (**7**, Fig. 2) and AEA are

biased towards G_i [56]. WIN55212 and SR141716 behave as an agonist and inverse agonist, respectively, at all G_i subtypes ($G_{i1,\ 2\ and\ 3}$). Desacetyl-levonantradol (**8**, Fig. 2) is an agonist for G_{i1} and G_{i2} and an inverse agonist for G_{i3}, whereas methanandamide (**9**, Fig. 2) behaves as an agonist at only G_{i3} and as an inverse agonist at G_{i1} and G_{i2} [57].

Although CB1R preferentially couples to G_i, under certain circumstances cannabinoid agonists such as HU210, WIN55212-A, and CP55940 (**10**, Fig. 2) can activate adenylyl cyclase through G_s-linked G-protein pathways [46,58,59]. Similarly, WIN55212 couples to $G_{q/11}$ G protein and activates Ca^{2+} channels to increase intracellular calcium [47]. Other agonists such as HU210, THC, and CP55940 do not signal through $G_{q/11}$ [47]. Taken together, these data demonstrate that CB1R exhibits complex, ligand-dependent signaling effects at the G-protein level that potentially govern the overall *in vivo* efficacy of these agents. At CB1R, preferential ligand bias through β-arrestin has also been observed, albeit less frequently than at different G proteins: In a murine cell-culture model of striatal medium spiny projection neurons endogenously expressing CB1R, THC and CP55940 promoted CB1R internalization through biased β-arrestin2 recruitment [59].

Endogenous cannabinoids may also show distinct signaling biases at CB1R. Unlike 2-AG, which displays no preference between adenylyl cyclase- and pERK1/2-dependent signaling pathways, AEA exhibits a 7-fold bias towards the former. Similar to 2-AG, WIN55212 does not exhibit any bias between adenylyl cyclase inhibition and pERK1/2 activation. However, THC and synthetic cannabinoids such as CP55940, HU210, and methanandamide all have significant bias towards cAMP inhibition over pERK1/2 activation [60]. Signaling bias has also been observed in further downstream events such as regulation of tyrosine hydroxylase (TH) transcription: in a murine neuroblastoma cell line, CB1R agonists (including THC and WIN55212) stimulate, whereas CP55940 inhibits, TH transcription [61].

4. Functional selectivity at CB2R

Reminiscent of CB1R, CB2R can be activated by cananbinergic agents that signal differentially through various intracellular information pathways in a ligand-dependent manner (Table 2). CB2R preferably interacts with G_i over G_o, the latter not widely expressed in the peripheral tissues where CB2R expression is high. Furthermore, at CB2R, HU210 produces a maximal G_i response unlike AEA, which produces a partial response [56]. At CB2R, low concentrations of CP55940 inhibit adenylyl cyclase and stimulate ERK1/2 phosphorylation, whereas low concentrations of 2-AG behave similarly to CP55940 and stimulate ERK1/2 activity. Significantly greater 2-AG concentrations are required to inhibit CB2R-dependent adenylyl cyclase activity [62].

The specificity for downstream signaling pathways from CB2R may be generalized based on different classes of cannabinergic ligands [63]. In experiments with rat CB2R, non-classical cannabinoid agonists such as HU308 (**11**, Fig. 2) and CP55940 induced β-arrestin recruitment followed by receptor internalization. In contrast, aminoalkylindoles such as the nonselective agonist WIN55212 and the CB2R-selective agonist AM1241 (**12**, Fig. 2) [64] did not themselves elicit CB2R internalization, but rather antagonized CP55940-induced receptor internalization in a concentration-dependent manner. However, WIN55212 still

promoted ERK1/2 activation and β-arrestin recruitment to the plasma membrane. In cAMP inhibition assays, racemic (R)- and (S)-AM1241 was an agonist at human CB2R, but an inverse agonist at rat and mouse CB2R [65]. Cannabilactones such as AM1710 (**13**, Fig. 2) were found to cause β-arrestin2 recruitment and CB2R internalization, but only weakly activated MAPK, and did not affect voltage-gated calcium channel function. The endocannabinoid AEA, a weak CB2R partial agonist, did not elicit CB2R internalization [66].

Signaling bias was also observed with antagonists/inverse agonists at CB2R [67]. The CB2R-selective inverse agonists AM630 (**14**, Fig. 2) and SR144528 (**15**, Fig. 2) reversed the inhibition of cAMP accumulation caused by cannabinoid agonists, but only SR144528 antagonized 2-AG-induced Ca^{2+} accumulation. AM630 did not influence CB2R-dependent, 2-AG-induced Ca^{2+} accumulation. In contrast, 4-O-methylhonokiol (**16**, Fig. 2), a synthetic CB2R-specific ligand that acts as an inverse agonist on 2-AG-induced cAMP inhibition, further potentiated agonist-induced Ca^{2+} ion flux. Similarly, SR144528 reversed agonist-induced CB2R internalization, leading to augmented CB2R levels on the cell surface, whereas AM630 acted as a neutral antagonist, displaying no detectable inverse-agonist efficacy.

5. Structural basis for signaling bias at cannabinoid GPCRs

Exponential progress in atomic-level structure determination of membrane proteins in the last few years has enabled the crystal structures of some 30 unique GPCRs, and over 119 different GPCR structures have been solved [68]. These structures have improved our understanding of the GPCR conformational changes associated with (especially orthosteric) ligand engagement as they pertain to various activity states. The large outward displacement of transmembrane helix (TMH) 6 and inward movement of TMH7 upon agonist binding appear characteristic of GPCR activation. Other hallmark structural accommodations associated with GPCR activation include rearrangements in the side chains of residues in conserved D(E)RY and NPxxY motifs in TMH3 and TMH7, respectively [68]. These activation-associated changes in GPCR TMH topology modulate interactions between the GPCR and intracellular effector molecules such as G-proteins, GRKs, and β-arrestin.

Diverse techniques have been used to interrogate the structure-function correlates of GPCR functional selectivity, as detailed elsewhere [69-71]. In this regard, β-adrenergic and serotonin (5-HT2) receptor subtypes have been used most extensively as model systems, especially in X-ray crystallographic studies. Emerging data indicate that conformational changes in TMHs 3, 5, and 6 are associated with G-protein activation [72], whereas TMH7, carboxy-terminal cytoplasmic helix 8, and intracellular loop (ICL) 2 play particularly critical roles in β-arrestin-mediated signaling [73]. Crystal structures of the liganded β1-adrenergic receptor suggest specific interactions between β-arrestin biased ligands and receptor residues in TMH7 and extracellular loop (ECL) 2 not associated with unbiased ligands [74]. Crystals of the 5-HT1B receptor bound to the strongly biased ligand ergotamine exhibited a classical, agonist-induced active-state conformation, whereas those of the ergotamine-bound serotonin 2B (5-HT2B) receptor showed characteristics of both active- or inactive-state conformations: the NPxxY motif and TMH7 exhibited pronounced active-state features, while the D(E)RY

motif and TMH6 exhibited inactive-state features, indicating a β-arrestin bias consistent with biochemical data [75]. Similarly, a series of polar amino-acid interactions extending from its extracellular loops to the transmembrane helical bundle has been implicated in the signaling bias of the glucagon-like peptide 1 receptor (GLP-1R) [76].

Although a crystallized receptor construct of human CB1R (hCB1R) in complex with a stabilizing antagonist has recently been described [77], the data for that inactive liganded receptor state cannot provide structural details as to the features of ligand docking poses or CB1R interactions with G-proteins or β-arrestin that determine biased agonism at hGPCR. An X-ray crystal structure of CB2R has not yet been reported. Mutational studies, often in conjunction with *in-silico* modeling, have shed some light on the structural features of activation at these cannabinoid receptors. Most of the residues critical for CBR activation are within highly conserved GPCR functional motifs. These amino acid residues include TMH2 D2.50 (of the SLAxAD motif); TMH3 R3.50 (of the D(E)RY motif); TMH6 D6.30 (of the TMH3-7 salt bridge) and P6.50 (of the CWxP motif); and TMH7 P7.50 (of the NPxxY motif). As with other class-A GPCRs, disruption of the ionic interactions between D2.50 of the SLAxAD motif and N7.49 of the NPxxY motif, and between D3.49 of the DRY motif and D6.30 of TMH6, is critical for CBR activation [78].

A number of structural features unique to CBRs have been associated with receptor activation. CB1R sequence analysis revealed the presence of a relatively lengthy extracellular N-terminal end and the absence of the highly conserved proline residue in TMH5. It has been speculated that the long N-terminal tail may play a role in chaperone-mediated regulation of CB1R synthesis, folding, maturation and trafficking [79]. ECL2 connecting TMH4 and TMH5 is considered to be important to the stability of and ligand engagement by most GPCRs. In CB1R, $C256^{ECL2}$ and $C264^{ECL2}$ form an intra-loop disulfide bond critical for receptor stabilization and activation, but not CP55940 binding [80]. Furthermore, CBRs lack a cysteine at the N-terminal end of TMH3, which prevents formation of a disulfide bond with the distal cysteine of the ECL2 that is present in most other GPCRs [81]. Similarly, most class-A GPCRs feature a CWxP motif (including residue W6.48) in their binding pocket that serves as a “toggle switch” for receptor activation upon agonist binding. However, both CB1R and CB2R lack an analogous, critical TMH6 aromatic residue at 6.52 position. Mutation studies suggest that $W258^{6.48}$ pairs with the $F117^{3.36}$ residue to form the toggle switch in CBR1, with consequent loss of aromatic stacking, leading to receptor activation [82]. In contrast to other GPCRs that engage hydrophilic ligands, lipophilic cannabinergic ligands may access CBR binding pockets through the membrane bilayer via an intramembranous portal between TMH6 and TMH7 [83].

Overall, CBRs exhibit high conformational flexibility, as suggested by their high basal activity [84], making them interesting candidates for studying the structural basis of GPCR signaling bias. Yet few experimental studies have interrogated the structural basis of functional selectivity in CBRs. Biochemical/mutation data with cannabinergic ligands and information on the structures of other class-A GPCRs have been used to identify candidate residues potentially involved in ligand functional selectivity at CBRs. Similar to other GPCRs, double mutation of $D213^{3.49}$ and $R214^{3.50}$ in the highly conserved aspartic acid-arginine-tyrosine (“DRY”) motif that plays a pivotal role in regulating GPCR

conformational/activity states caused CB1R to bias towards β-arrestin signaling and away from G-protein activation without significant loss in binding affinity of cannabinoid agonists [85]. It was also observed that $C355^{6.47}$ of the CWxP motif was critical for binding of CP55940 and other classical cannabinoids that are known to induce receptor internalization through β-arrestin-mediated pathways [85]. However, WIN55212, an aminoalkylindole derivative that does not induce receptor internalization, maintained high affinity for CB1R, even when $C355^{6.47}$ had been mutated. These data invited speculation that ligands interacting with $W356^{6.48}$ by aromatic stacking do not induce receptor internalization [82]. Unlike classical agonists, inverse agonists such as the biarylpyrazole SR141716 bind very poorly to CB1R when $C386^{7.41}$ is mutated to a bulky group [81]. The reduced affinity can be explained by the loss of critical aromatic interactions involving $W356^{6.48}$ and $F200^{3.36}$ within the binding pocket. Thus, the unique biochemical characteristics of WIN55212 can be explained by the presence of both inactive- and active-state structural features, reminiscent of the situation with ergotamine in 5-HT2B receptor [75]. In-depth experimental studies are required to understand and confirm the structural features associated with the functional selectivity of specific cannabinoid ligands at CB1R and CB2R.

6. Pharmacotherapeutic implications of biased signaling at CBRs

As a component of its overall pharmacological profile, the ability of a GPCR-targeted therapeutic candidate to activate differentially specific intracellular effector pathways carries critical implications for drug discovery. For example, niacin acts therapeutically as an antilipolytic agent by activating GPR109A receptor-mediated G-protein signaling. However, it also activates the G protein-independent β-arrestin1 pathway responsible for cutaneous flushing and other adverse effects [86]. Similarly, in kappa opioid receptors, activation of ERK signaling while retaining the G-protein signaling bias over β-arrestin2 pathway is effective in relieving pain without inducing adverse effects such as dysphoria, sedation, and diuresis associated with β-arrestin signaling [87,88]. The pharmacological profiles of specific ligands for other GPCRs, including dopamine [89], glucagon-like peptide [90], opioid [91], and angiotensin II [92] receptors, show selective activation of particular signaling pathway(s) that could potentially support a clinically relevant therapeutic effect *vs.* inciting other, deleterious signaling cascades.

Knockout studies have shown that the anti-nociceptive effect of CB1R cannabinergic agonists is not significantly affected by β-arrestin2. However, in β-arrestin2 knockout animals, development of tolerance to antinociception was attenuated following repeated THC administration, an effect attributed to a decrease in receptor desensitization for G-protein-dependent signaling. In contrast, β-arrestin2 knockout resulted in an increased tolerance with respect to cannabinoid dependence characteristics such as catalepsy and also potentiated the hypothermia response to cannabinoid agonists [93]. Therefore, developing CB1R-targeted biased agonists with attenuated β-arrestin2 recruitment may not only improve their anti-nociceptive efficacy, but also reduce the potential for unwanted catalepsy and drug tolerance. This view is supported by demonstration that β-arrestin2-null mice evidence enhanced sensitivity to the principal psychoactive phytocannabinoid in marijuana, THC [94]. In contrast, CB2R-selective antagonists/inverse agonists that preferentially activate the β-arrestin2 pathway incite cytoskeletal rearrangements in immune cells [95,96]

and in lung airway [97] to modulate chemotaxis and invasion of immune and cancerous cells. Although these studies illustrate the important role of β-arrestin in normal, G-protein-dependent GPCR desensitization mechanisms and do not exemplify β-arrestin-biased signaling *per se*, the results suggest that the degree of β-arrestin bias *in vivo* is an important characteristic of potential, CBR-targeted therapeutics.

7. Future research directions

Although it is well established that most GPCR ligands may exhibit pluridimensional efficacies with respect to different signaling pathways, challenges remain in the study of CBR functional selectivity. Experimental nuances such as kinetics of response, response read-out bias, cell/tissue-specific variations, and the system-dependency of the observed pharmacological effects may influence the qualitative nature of biased signaling and its quantification [98]. Stereochemical [65] and species-dependent [99] pharmacological effects among orthosteric cannabinergic ligands and differences in their ability to elicit receptor oligomers that actively signal [100,101] may further extend the pharmacological scope of biased signaling at CBRs.

Over the past decade, novel biased ligands have been profiled preclinically for some GPCRs including the β2-adrenergic [102], kappa-opioid [103], mu-opioid [104], angiotensin II (Type 1) [105], parathyroid hormone [106], and serotonin [107] receptors. A few such ligands are in various stages of clinical development. TRV130, a G protein-biased ligand that targets the mu opioid receptor, provides significantly better pain relief than either placebo or morphine in a phase-II clinical study for treating severe acute pain [108]. Furthermore, TRV130 did not elicit hypercapnia-induced respiratory drive or severe nausea at equi- (or greater) analgesic doses than morphine in healthy volunteers [109]. However, TRV027, a β-arrestin-biased ligand targeting the angiotensin II type 1 receptor, failed to meet both primary and secondary endpoints for treating acute heart failure in a phase-II clinical study [110].

Optimal therapeutic exploitation of functionally selective CB1R and CB2R ligands requires several aspects of their pharmacology to be detailed. Primarily, for a thorough analysis of the molecular mechanisms of signaling bias within the endocannabinoid system, novel biased molecules need to be designed, synthesized, and profiled in both over-expressed and native cell lines under similar experimental conditions to avoid system/context-specific pharmacological activity that could reflect, for example, the relative prevalence of G protein-related and -independent signaling partners across cell-based assays rather than true physiological ligand bias [111,112]. The extent to which a biased phenotype for any given GPCR ligand may directly translate into the systems physiology of a living organism *in vivo* remains of great concern, given that GPCR ligand bias has been most consistently demonstrated at heterologously expressed receptors in functional assays employing cultured-cell or isolated-tissue backgrounds (e.g., Tables 1 and 2 for CBRs)-- not in complex biological systems [113,114]. This concern is exacerbated by the fact that delineation of the relationships between apparent ligand bias defined by cell- or tissue-based screening criteria *in vitro* and therapeutically-relevant biological responses *in vivo* is crucial to drug discovery. More generally, the mechanism and biological/therapeutic significance of cannabinergic

signaling pathways need to be better characterized in both *in vitro* studies and *in vivo* animal models exhibiting normal physiology and relevant disease phenotypes. In this regard, studies of functional selectivity using cells or tissues from patients with clearly defined disease conditions could provide valuable insight into the biological and pathological significance of CBR signaling bias. Ultimately, these efforts may result in cannabinergic drugs with improved therapeutic profiles and less risk of clinically significant adverse events. Such knowledge could also help inform the application of cannabis extracts as well as THC and other plant-derived cannabinoids in medical practice so as to leverage any potential clinical benefit and militate against undesirable adverse events, a subject of much current controversy and speculation for public health [115].

Aside from biased orthosteric modulators, other ligands with pharmacological properties distinct from conventional, unbiased agonists and antagonists/inverse agonists are being studied for their ability to modulate therapeutic CBR signaling. The potential for the inverse-agonist property of CB1R antagonists/inverse agonists to elicit adverse events by influencing physiologically important CB1R constitutive (i.e., ligand-independent) signaling has raised interest in so-called “neutral” or “silent” CB1R antagonists that display little, if any, intrinsic inverse-agonist efficacy [116]. CBR ligands that engage sites functionally and topographically distinct from the binding pockets of endogenous ligands (“allosteric ligands”) are gaining considerable pharmacological and therapeutic attention, since allosteric GPCR modulation is associated with several theoretical advantages that could make for safer, more efficacious pharmacotherpaeutics, especially at GPCRs such as CB1R where orthosteric agonists carry significant adverse-event risk [117,118]. Perhaps most intriguingly, CB1R-selective allosteric modulators may themselves bias receptor-dependent information output along particular signaling circuits [119,120]. As with functionally-selective orthosteric CB1R and CB2R ligands, the underlying molecular mechanisms biasing allosteric effector pathways will hopefully emerge in sufficient detail to help realize the potential of CBR-targeted drug discovery in the clinic. Indeed, this cutting-edge proposition is already driving the discovery and profiling of proprietary biased CB1R allosteric modulators with demonstrable preclinical therapeutic efficacy while free of endogenous inverse-agonist activity [121,122].

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Abbreviations

THC	Δ^9-tetrahydrocannabinol
5-HT2B	serotonin 2B receptor
AC	adenylyl cyclase
AEA	anandamide
Akt	protein kinase B
cAMP	cyclic adenosine monophosphate
CB1R	cannabinoid receptor 1
CB2R	cannabinoid receptor 2
CNS	central nervous system
ECL	extracellular loop

ERK1/2	extracellular kinase-1 and -2
GIRK	G protein-coupled inwardly-rectifying potassium channel
GLP-1R	glucagon-like peptide 1 receptor
GPCR	G-protein coupled receptors
GRKs	G-protein receptor kinases
hCB1R	human cannabinoid receptor 1
JNK	c-Jun N-terminal kinase
MAK	mitogen activated kinase
MAPK	mitogen activated protein kinase
MOR	mu-opioid receptors
mTor	mammalian target of rapamycin
PI3K/PKB	phosphatidylinositol 3-kinase/protein kinase B
PKC	protein kinase C
S1PR	sphingosine 1 receptor
TH	tyrosine hydroxylase
TMH	transmembrane helix
TRP	transient receptor potential

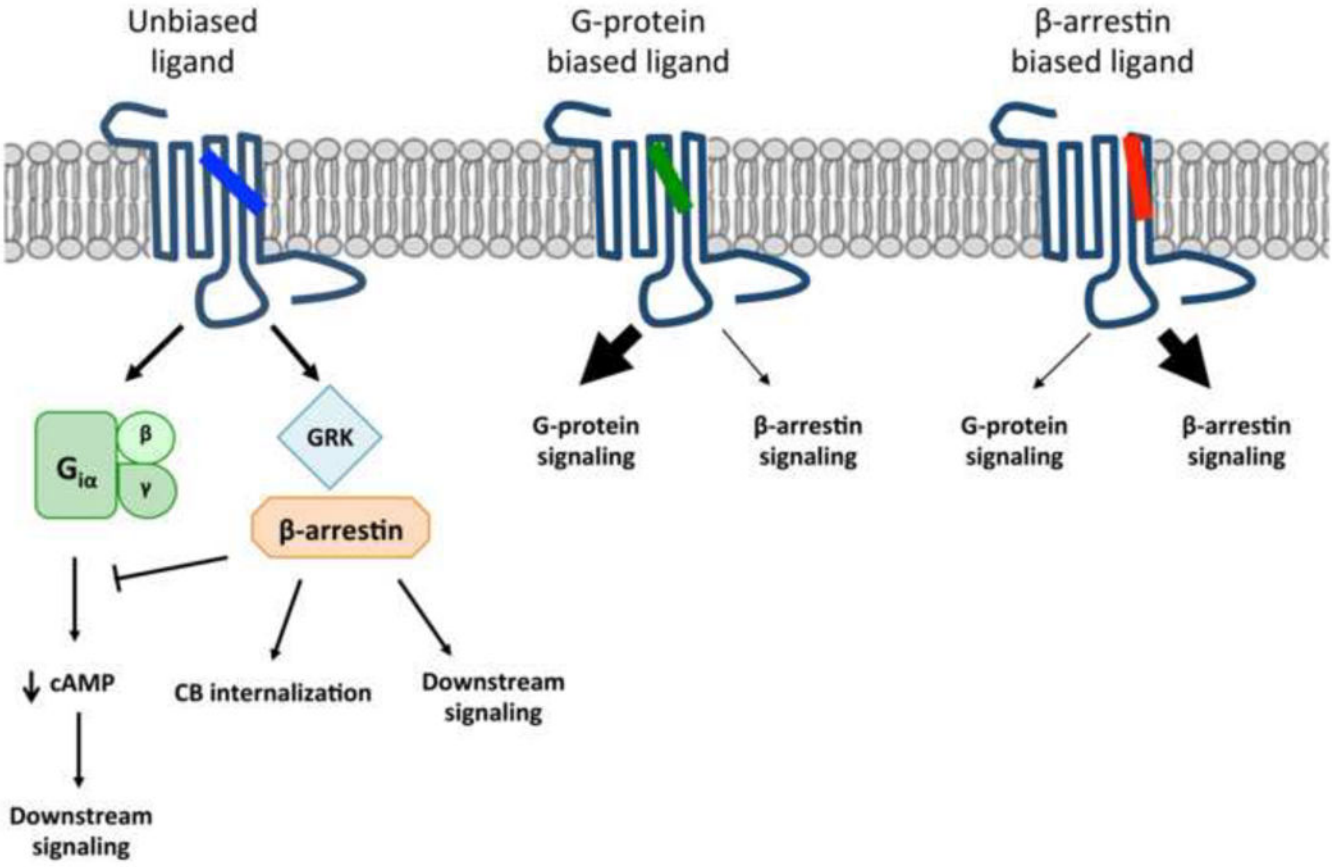

Fig. 1.

Diagrammatic representation of biased signaling at a G-protein coupled receptor (GPCR). In this example, an unbiased agonist is depicted that engages the GPCR and activates both G protein-dependent [i.e., adenylyl cyclase/cyclic adenosine monophosphate (cAMP)] and -independent (i.e., β-arrestin) signaling pathways. A biased agonist engages the receptor to generate a GPCR-ligand conformational ensemble that preferentially activates one or the other signaling cascade. If the preferentially activated signaling pathway were associated with a therapeutic effect *vs.* a harmful response from the non-preferred signaling pathway, the resulting signal bias could generate a pharmacologically improved therapeutic with less risk of on-target adverse events as compared to the unbiased ligand.

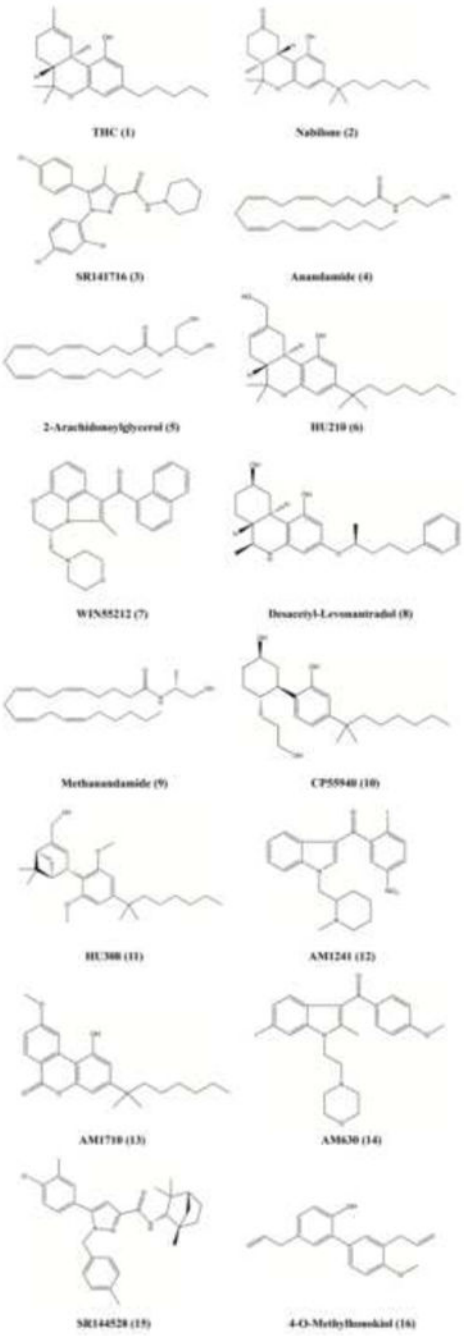

Fig. 2.

Chemical structures of cannabinoid receptor ligands discussed in the text. A summary of the molecular pharmacology of those ligands that display functional selectivity at CB1R and/or CB2R may be found in Tables 1 and 2, with details in references [47,56-63,65,67].

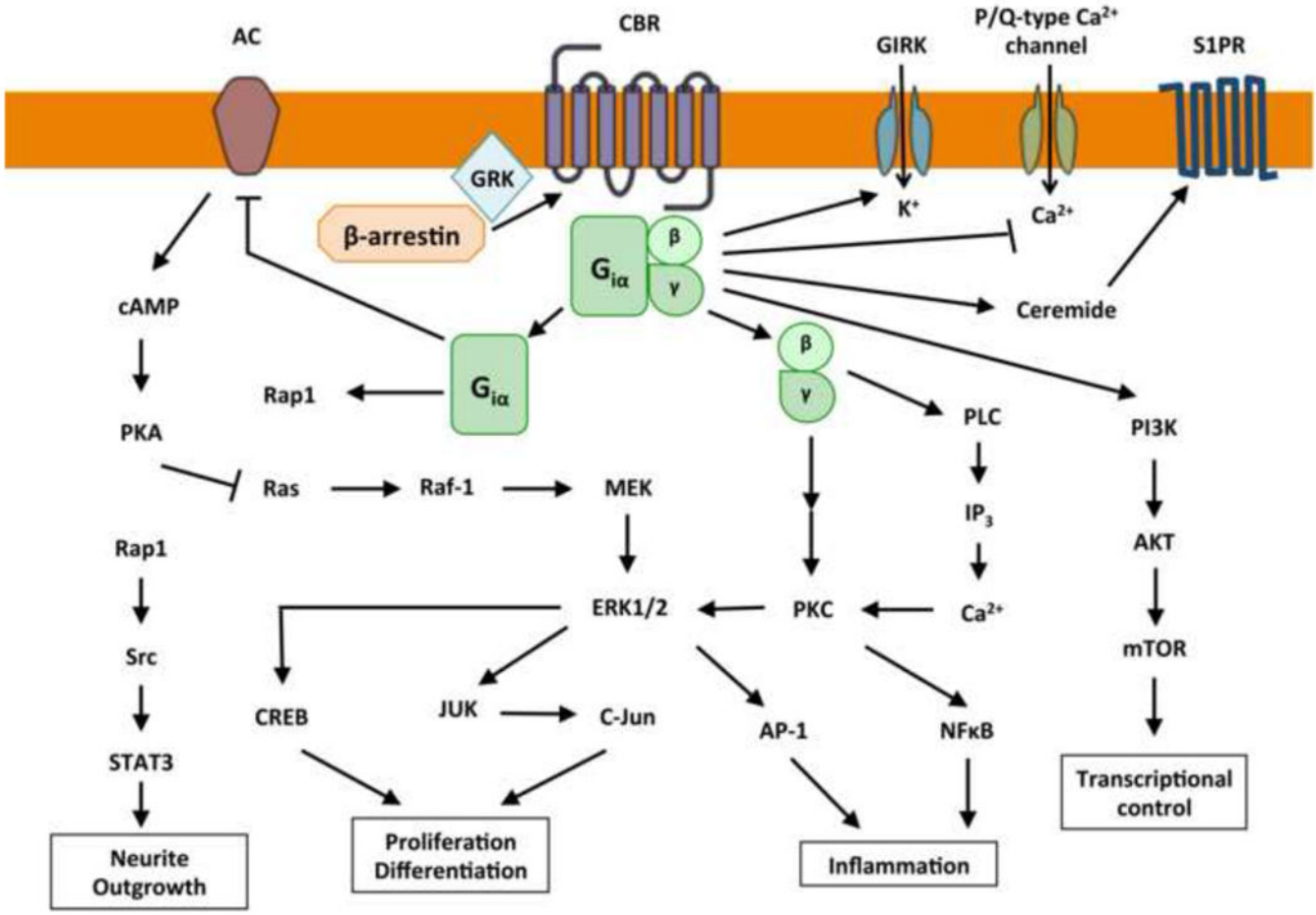

Fig. 3.

Schematic representation of canonical cannabinoid receptor (CBR)-mediated, G protein-dependent signaling pathways. Upon activation, CBRs preferentially couple to $G_{i/o}$-type G proteins and activate a series of downstream signaling cascades through which adenylyl cyclase (AC) is inhibited and mitogen-activated kinase (MAK) and extracellular kinase-1 and -2 (ERK1/2) are activated. Through the G-protein $G_{\beta\gamma}$ subunit, CB1R activation can stimulate phospholipase C (PLC), leading to an increase in intracellular Ca^{2+} and activation of protein kinase C (PKC). Through $G_{i/o\ \alpha\beta\gamma}$, the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) intracellular signaling pathway can be stimulated. In addition, CB1R also modulates N- and P/Q-type voltage-gated Ca^{2+} channels, A-type and inward-rectifying K+ channels (GIRKs), and the sphingosine 1 receptor (S1PR) through which ceramide levels are regulated. These cell signaling circuits can modulate such diverse functions as neurite growth, cell proliferation and differentiation and inflammation and can also control gene transcription.

Table 1

Evidence of CB1R functional selectivity

Ligand	Functional selectivity	Experimental approach	Reference
Endocannabinoids			
2-AG	No bias towards cAMP inhibition over pERK 1/2	Alphascreen cAMP and SureFire assay with CB1R-expressing CHO cells	[60]
	Increases CB1R surface expression through Gβγ signaling	cAMP accumulation assay in CB1R-expressing CHO cells.	[58]
Anandamide	Biased towards Gi over Go	CB1R urea-extracted from sf9 membranes and reconstituted with varying Ga and Gβγ subunits.	[56]
	Biased towards Gi over Gs and doesnot signal through Gq	cAMP accumulation assay in CB1R-expressing CHO cells.	[58]
	Biased towards cAMP inhibition over pERK 1/2	Alphascreen cAMP and SureFire assay with CB1R-expressing CHO cells	[60]
Eicosanoids			
Methanandamide	Inverse agonist at Gi1 and Gi2; Agonist at Gi3	CB1R-G protein complex were CHAPS-extracted from N18TG2 cell membrane and quantified by coimmunoprecipitation	[57]
	Biased towards cAMP inhibition over pERK 1/2	Alphascreen cAMP and SureFire assay with CB1R-expressing CHO cells	[60]
Classical Cannabinoids			
THC	Biased towards cAMP inhibition over pERK 1/2	Alphascreen cAMP and SureFire assay with CB1R-expressing CHO cells	[60]
	Induces expression of Tyrosine Hydrooxylase	mRNA and Protein analysis in mouse neuroblastoma cells N1E-115	[61]
	Induced receptor internalization through β-arrestin recruitment	CREB phosphorylation in mouse striatal medium spiny projection neurons (STHdhQ7/Q7)	[59]
Desacetyl-Levonantradol	Agonist at Gi1 and Gi2; Inverse agonist at Gi3	CB1R-G protein complex were CHAPS-extracted from N18TG2 cell membrane and quantified by coimmunoprecipitation	[57]
HU210	Non-selective between Gi, Go and Gs pathways	cAMP accumulation assay in CB1R-expressing CHO cells.	[58]
	Biased towards cAMP inhibition over pERK 1/2	Alphascreen cAMP and SureFire assay with CB1R-expressing CHO cells	[60]
	Decreases Tyrosine Hydroxylase expression unlike CP55940	Tyrosine hydroxylase expression in N1E-115 Neuroblastoma cells	[61]
Non-classical Cannabinoids			
CP55940	Activates Gs pathway	CREB phosphorylation in mouse striatal medium spiny projection neurons (STHdhQ7/Q7)	[59]
	Biased towards Gi over Gs and does not signal through Go and Gq	cAMP accumulation assay in CB1R-expressing CHO cells.	[58]
	Induced receptor internalization through β-arrestin recruitment	CREB phosphorylation in mouse striatal medium spiny projection neurons (STHdhQ7/Q7)	[59]
Aminoalkylindole			

Ligand	Functional selectivity	Experimental approach	Reference
WIN55212	Activates Gq pathway	Intracellular calcium accumulation in CB1R-expressing HEK293 cells and cultured hippocampal neurons.	[47]
	Non-selective between Gi1, 2 and 3 subtypes	CB1R-G protein complex were CHAPS-extracted from N18TG2 cell membrane and quantified by coimmunoprecipitation	[57]

Table 2

Evidence of CB2R functional selectivity

Ligand	Functional selectivity	Experimental approach	Reference
Endocannabinoids			
2-AG	Actiavted ERK-MAPK pathway with good potency but required higher concentrations for adenylyl cyclase inhibition and intracellular calcium release	Western Blotting analysis of ERK-MAPK phosphorylation and fluorescence-based calcium assay in human CB2R-expressing CHO cells	[62]
Anandamide	Did not induce receptor internalization even in the presence of FAAH inhibitors	Antibody-based analysis of cell-surface CB2R in HEK293 cells	[63]
Classical Cannabinoids			
THC	Did not induce receptor internalization unlike CP55940	Antibody-based analysis of cell-surface CB2R in HEK293 cells	[63]
Cannabilactone			
AM1710	Robustly internalized CB2R and recruited β-arrestin2, but weakly activated MAPK and did not affect VGCCs	Antibody-based analysis of cell-surface CB2R in HEK293 cells	[63]
Aminoalkylindole			
WIN55212	Induced cAMP and MAPK pathways but not receptor internalization and VGCC activation	Antibody-based analysis of cell-surface CB2R in HEK293 cells	[63]
AM1241	Agonist at human CB2R, but an inverse agonist at rat and mouse CB2R	HitHunter cAMP XS assay with CB2R-expressing CHO-K1 cells	[65]
AM630	Inverse agonist at Gi/o pathway but neutral antagonist at CB2R internalization and β-arrestin2 recruitment	Antibody-based analysis of cell-surface CB2R in HEK293 cells	[63]
	Inverse agonist at Gi/o but no effect on 2-AG induced release of intracellular calcium	cAMP assay and FLIPR calcium assay in CB2R-expressing CHO-K1 cells	[67]
Natural Product			
4′-*O*-methylhonokiol	Inverse agonist at Gi/o but induces CB2R-mediated release of intracellular calcium	cAMP assay and FLIPR calcium assay in CB2R-expressing CHO-K1 cells	[67]