Type 1 Cannabinoid Receptor Ligands Display Functional Selectivity in a Cell Culture Model of Striatal Medium Spiny Projection Neurons*

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Background: To understand the differential response to cannabinoids, we examined the functional selectivity of type 1 cannabinoid receptor (CB1) agonists in a cell model of striatal neurons.

Results: 2-Arachidonylglycerol, Δ⁹-tetrahydrocannabinol, and CP55,940 were arrestin²-selective; endocannabinoids and WIN55,212-2 activated Goᵦₒ, Goᵦγ, and Goᵦᵦ; and cannabinoid-activated Goᵦᵦ independent of CB1.

Conclusion: Cannabinoids displayed functional selectivity.

Significance: CB₁ functional selectivity may be exploited to maximize therapeutic efficacy.

Modulation of type 1 cannabinoid receptor (CB₁) activity has been touted as a potential means of treating addiction, anxiety, depression, and neurodegeneration. Different agonists of CB₁ are known to evoke varied responses in vivo. Functional selectivity is the ligand-specific activation of certain signal transduction pathways at a receptor that can signal through multiple pathways. To understand cannabinoid-specific functional selectivity, different groups have examined the effect of individual cannabinoids on various signaling pathways in heterologous expression systems. In the current study, we compared the functional selectivity of six cannabinoids, including two endocannabinoids (2-arachidonylglycerol (2-AG) and anandamide (AEA)), two synthetic cannabinoids (WIN55,212-2 and CP55,940), and two phytocannabinoids (cannabidiol (CBD) and Δ⁹-tetrahydrocannabinol (THC)) on arrestin², Goᵦₒ, Goᵦγ, Goᵦᵦ, and Goᵦᵦ-mediated intracellular signaling in the mouse STHdh²⁷/²⁷ cell culture model of striatal medium spiny projection neurons that endogenously express CB₁. In this system, 2-AG, THC, and CP55,940 were more potent mediators of arrestin² recruitment than other cannabinoids tested. 2-AG, AEA, and WIN55,212-2, enhanced Goᵦₒ and Goᵦγ signaling, with 2-AG and AEA treatment leading to increased total CB₁ levels. 2-AG, AEA, THC, and WIN55,212-2 also activated Goᵦᵦ-dependent pathways. CP55,940 and CBD both signaled through Goᵦᵦ, CP55,940, but not CBD, activated downstream Goᵦᵦ pathways via CB₁ targets. THC and CP55,940 promoted CB₁ internalization and decreased CB₁ protein levels over an 18-h period. These data demonstrate that individual cannabinoids display functional selectivity at CB₁, leading to activation of distinct signaling pathways. To effectively match cannabinoids with therapeutic goals, these compounds must be screened for their signaling bias.

Cannabinoids are a structurally diverse group of compounds that are broadly classified as endogenous cannabinoids (endocannabinoids) (e.g., 2-arachidonylglycerol (2-AG)⁴ and anandamide (N-arachidonylethanolamine (AEA))), phytocannabinoids (e.g., Δ⁹-tetrahydrocannabinol (THC) and cannabidiol (CBD)), and synthetic cannabinoids (e.g., WIN55,212-2 (WIN) and CP55,940 (CP)) (1).

Cannabinoids mediate their effects through several receptors, including the type 1 cannabinoid receptor (CB₁), which has been studied intensively for its neuromodulatory activity. Many cannabinoids, including 2-AG, AEA, and THC, induce analgesic responses, and their use for chronic and acute pain conditions such as arthritis and migraine is being actively explored (2–4). Cannabinoids evoke hypolocomotor responses via CB₁ and may be useful in the treatment of movement disorders such as tremor, ataxia, Tourette syndrome, Parkinson disease, and Huntington disease (5). CB₁, acting independently of CB₁, has been shown to have therapeutic potential as an anti-epileptic and anti-inflammatory agent (6–8). Modulation of CB₁ activity in the central nervous system and periphery also affects appetite and glucose and fat metabolism (9). Cannabinoids may play a therapeutic role in the management of metabolic syndrome.

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4 The abbreviations used are: 2-AG, 2-arachidonylglycerol; AEA, anandamide; BRET², bioluminescence resonance energy transfer; CB₁, type 1 cannabinoid receptor; CBD, cannabidiol; CP, CP55,940; CTx, cholela toxin; FAAH, fatty acid amide hydrolase; FRET, fluorescence resonance energy transfer; MAGL, monoacylglycerol lipase; pFPNK, preproenkephalin; PTx, pertussis toxin; Rluc, Renilla luciferase; THC, Δ⁹-tetrahydrocannabinol; WIN, WIN55,212-2; GTP·S, guanosine 5’-O-(thiotriphosphate); HERG, human ether-a-go-go-related gene; qRT, quantitative reverse transcriptase; ANOVA, analysis of variance; GPCR, G protein-coupled receptor; CREB, cAMP-response element-binding protein.
Functional Selectivity of CB₁ Agonists
diabetes, and lipodystrophies (9). Additionally, it is important
to understand how psychoactive cannabinoids, such as THC,
affect neuronal activity via CB₁, and other effectors within the
context of substance abuse and addiction (1, 10–12).

Cannabinoids differ in their affinity for CB₁, and their
potency and efficacy of action via CB₁ (1, 2, 13). The classical
view of CB₁ activation was that a correlation exists between
binding affinity at CB₁ and the potencies of cannabinoids to
induce the in vivo tetrad responses of anti-nociception, hypo-
activity, hypothermia, and catalepsy (2, 13–15). Because of this
correlation, individual cannabinoids were expected to be simi-
larly potent in all four tetrad responses (2). However, this is not
the case for many cannabinoids. THC and WIN, for example,
are more potent inducers of hypolocomotion than of catalepsy
or hypothermia (16, 17). Similarly, AEA and THC differ in their
potencies for anti-nociception and hypolocomotion in the ICR
strain of mice (18) and their ability to evoke tolerance and
dependence in fatty acid amide hydrolase (FAAH) knock-out
mice (19). Long et al. (20) and Schlosburg et al. (21) observed
that selective blockade of AEA or 2-AG catabolism results in
sustained analgesia or disruption of analgesia and cross-toler-
ance to other CB₁ agonists, respectively. CBD, unlike other can-
nabinoids, does not evoke the tetrad responses (8). CBD dem-
onstrates low affinity for CB₁, and the in vivo effects of CBD,
including its anti-inflammatory properties, appear to be CB₁-
independent (6–8, 12). Differences in the potency and efficacy
of cannabinoids to evoke various responses in vivo may be
exploited in the application of these compounds as therapies.
Although these distinctions may result from pharmacokinetic
differences, it is also possible that in vivo responses to cannabi-
noids may be mediated through the different effects of individ-
ual cannabinoids.

Distinct agonists appear to modulate the signaling specificity
of CB₁ through the coupling of different G proteins (2, 22). CB₁
agonist-selective coupling to Go₅, Go₆, and Go₇ has been dem-
onstrated in cell lines overexpressing CB₁ treated with WIN,
CP, and other synthetic cannabinoids (23–25). The potency of
AEA, CP, WIN, and other cannabinoids to stimulate
[35S]GTPγS has been evaluated in rat cerebellar membranes
(10) and N1E-115 cells overexpressing CB₁ (14). In these and
subsequent studies, WIN and CP were found to be full agonists
of Go₁/γo whereas AEA and, to a lesser extent, THC were partial
agonists (2, 13–15). It is thought that WIN and CP stabilize
functionally different active conformations of CB₁ resulting in a
differential interaction and activation of G proteins (26, 27). In
silico modeling of CB₁-cannabinoid interactions suggests that
each cannabinoid interacts with a different subset of residues
on the third and fourth transmembrane helices of CB₁ (28–30).
Based on these data, Varga et al. (29) proposed that ligand-
specific changes in CB₁ conformation may enhance the binding
of different G proteins (e.g. Go₁/γo versus Go₂) or arrestins, which
would in turn facilitate the activation of different signaling
pathways downstream of CB₁. Glass and Northup (22) used STF
9 cell membrane preparations containing CB₁ and various G pro-
teins to differentiate the Go₁- and Go₅-mediated effects of CB₁.
In their study, the synthetic cannabinoid HU210, WIN, and
AEA were full agonists of Go₅, whereas THC acted as a partial
agonist, and WIN, AEA, and THC were all partial agonists of
Go₅, relative to HU210 (22). Similar to Varga et al. (29), Glass
and Northup (22) concluded that distinct agonists induce
unique receptor conformations resulting in ligand-specific
CB₁-dependent G protein signaling. The data presented in their
studies suggest that the pharmacological activity of cannabin-
noids acting through G proteins depends on their affinity for
CB₁, as well as the signaling bias of specific cannabinoids.

Beyond G proteins, the recruitment of arrestin1 and -2 to
CB₁, has also been examined (31–33). These studies report that
CB₁ interacts weakly with arrestin2, which facilitates internal-
ization upon stimulation with WIN or CP in HEK cells, AtT20
immortalized mouse anterior pituitary cells, and U2OS human
osteosarcoma cells stably expressing CB₁ (31–33). WIN and CP
have been shown to be differentially efficacious activators of
tyrosine hydroxylase transcription, ERK1/2 phosphorylation,
and JNK activation in neuroblastoma cells (14, 34, 35). Although
these observations were not related to agonist-specific
coupling, the authors (14, 34, 35) suggest that the differ-
cences between WIN and CP support functional selectivity of
cannabinoids at CB₁. Other cannabinoids, such as CBD, have
been shown to have some CB₁ modulatory activity but act
largely via CB₁-independent effectors (6–8). To complicate
matters, the functional selectivity of cannabinoid ligands may
be cell type-specific because reports of efficacy have varied
across model systems and tissues (2). Therefore, individual can-
nabinoids may stabilize specific CB₁ receptor conformations,
resulting in a cell- and tissue-specific response (32, 33). This is
interesting because it may be possible for cannabinoids to be
designed that bias receptor signaling toward desirable effects
and away from undesirable ones.

In this study, we sought to characterize the ligand bias of
several cannabinoid ligands in an in vitro model of neurons that
express CB₁. To directly compare cannabinoid ligand bias, the
downstream functional selectivity of two compounds from
two classes of cannabinoids was examined in the STHdhQ7/Q7
cell culture model of striatal medium spiny projection neurons.
This cell culture model was chosen to characterize cannabinoid
ligand bias because these cells model the major output of the
indirect motor pathway of the striatum where CB₁ levels
are highest relative to other regions of the brain (36, 37).
STHdhQ7/Q7 cells endogenously express CB₁ and FAAH (36, 37),
as well as the dopamine D₁ receptor enkephalin and other mark-
ers of striatal neurons, making this in vitro model system ideally
suited to studying cannabinoid signaling in a physiologically
relevant context. The endocannabinoids AEA and 2-AG, the
phytocannabinoids CBD and THC, and the synthetic can-
nabinoids WIN and CP were compared for their ability to activate
arrestin2 (β-arrestin1)-, Go₁/γo-, Gαs-, and Gα₁-dependent
pathways in STHdhQ7/Q7 cells. Based on the existing in vitro
and in vivo data for cannabinoid ligands, we hypothesized that
endocannabinoids, phytocannabinoids, and synthetic cannabin-
oids would differentially bias CB₁-dependent signaling.

EXPERIMENTAL PROCEDURES
Cannabinoids used in this study included 2-AG, AEA, WIN,
CP, CBD, THC, and the CB₁-selective antagonist O-2050. All
cannabinoids were purchased from Tocris Bioscience (Bristol,
United Kingdom) with the exception of THC, which was pur-
chased from Sigma-Aldrich (Oakville, ON). Pertussis and cholera toxins (PTx and CTx) were purchased from Sigma-Aldrich. The Gβγ modulator gallein was purchased from EMD Millipore (Billerica, MA). Cannabinoids and gallein were dissolved in dimethyl sulfoxide (final concentration of 0.1% in assay media for all assays) and added directly to the media at the concentrations and times indicated. No effects of vehicle alone were observed compared with assay media alone. PTx and CTx were dissolved in dH2O (50 ng/ml) and added directly to the media 24 h prior to cannabinoid treatment. Pretreatment of cells with PTx and CTx inhibits Goi and Goi', respectively (38). In the case of CTx, this occurs via down-regulation of Goi' following ADP-ribosylation (38, 39). All experiments included a vehicle treatment control.

STHdiQ7/Q7 cells are a cell line derived from the conditionally immortalized striatal progenitor cells of embryonic day 14 C57Bl/6 mice (Coriell Institute, Camden, NJ) (36). Cells were grown at 33 °C, 5% CO2 in DMEM supplemented with 10% FBS, 2 mM l-glutamine, 104 units/ml penicillin-streptomycin, and 400 μg/ml Geneticin. Twenty-four hours of serum deprivation promotes the differentiation of STHdiQ7/Q7 cells into an adult neuron-like phenotype characterized by increased neurite outgrowth, GABA release, and increased expression of CB1, dopamine D2 receptors, preproenkephalin, and dopamine and cAMP-related phosphoprotein 32 kDa (DARPP-32), typical of mature medium spiny projection neurons of the indirect motor pathway of the striatum (36, 37, 40). These cells are ideally suited for the characterization of cannabinoid ligand bias in vitro because they model a neuronal cell type that expresses CB1 at high levels compared with other cell types in the central nervous system. The striatum is a major site of action of centrally acting cannabinoid-based therapies (41, 42).

Plasmids—Human CB1 and arrestin2 (β-arrestin1) were cloned and expressed as either green fluorescent protein2 (GFP2) or Renilla luciferase (Rluc) fusion proteins at the intracellular C terminus. CB1-GFP2 and arrestin2-GFP2 were generated using the pGFP2-N3 plasmid (PerkinElmer Life Sciences) as described previously (43). CB1-Rluc and arrestin2-Rluc were generated using the pRluc-N3 plasmid (PerkinElmer Life Sciences). The human ether-a-go-go-related gene-C terminus GFP2 and Rluc fusion constructs (HERG-GFP2 and HERG-Rluc), GFP2-Rluc fusion construct, and Rluc plasmids have been described previously (40, 44, 45). The Goi' dominant negative mutant (Glu-209Asp) and Goi' dominant negative mutant (Val-53Asp) pcDNA3.1 plasmid was obtained from the Missouri S&T cDNA Resource Center (Rolla, MO) (25). The arrestin2 dominant negative mutant (Val-53Asp) and arrestin2 dominant negative mutant (Val-53Asp) pcDNA3.1 plasmid has been described previously (45). The arrestin2-β-arrestin2 fusion protein (RFP) was provided by Dr. Denis Dupre’ (Dalhousie University).

Bioluminescence Resonance Energy Transfer 2 (BRET2)—Direct interactions between CB1 and arrestin2 were quantified via BRET2 (46). Cells were grown in a 6-well plate and transfected with the indicated GFP2 and Rluc constructs. Forty-eight hours post-transfection, the cells were washed twice with cold 0.1 M PBS and suspended in 90 μl of 0.1 M PBS supplemented with glucose (1 mg/ml), benzamidine (10 mg/ml), leupeptin (5 mg/ml), and a trypsin inhibitor (5 mg/ml). Cells were dispensed into white 96-well plates and treated as indicated (PerkinElmer Life Sciences). Coelenterazine 400a substrate (50 μM; Biotium, Hayward, CA) was added, and light emissions were measured at 405 nm (Rluc) and 510 nm (GFP2) using a Luminoskan Ascent plate reader (Thermo Scientific, Waltham, MA) with an integration time of 10 s and a photomultiplier tube voltage of 1200 V. BRET efficiency (BRET2) was determined using previously described methods (47) such that Rluc alone was used to calculate BRETmin and the Rluc-GFP2 fusion protein was used to calculate BRETmax.

Fluorescence Resonance Energy Transfer (FRET)—Receptor dimerization was visually assessed via FRET according to the methods of Wu et al. (48). Cells were grown in a 6-well plate and transfected with the indicated GFP2 and RFP constructs. Forty-eight hours post-transfection cells were moved to coverslips and grown for an additional 24 h. Cells were treated as indicated and visualized on a Zeiss 510 upright laser scanning microscope with 20 × and 63 × objective lenses. Images were captured using Zen Image Capture 2009 edition (Carl Zeiss Canada). The following excitation/emission filters were used to directly visualize fluorescence: for GFP2, 492 nm/510 nm; and for RFP, 543 nm/565 nm. For FRET, GFP2 was excited 488 nm, separated by a 488/564 dichromatic mirror, with emitted fluorescence detected between 502 and 651 nm (48). To measure the endogenous association between CB1, and arrestin, paraformaldehyde-fixed cells were used for the immunocytochemical detection of CB1 with a C-terminal CB1 primary antibody (1:500; catalog No. 10006590, Cayman Chemical Co., Ann Arbor, MI) and Alexa Fluor 488 secondary antibody (donor) and detection of arrestin2 with an arrestin1/2 primary antibody (1:250; Santa Cruz Biotechnology, Santa Cruz, CA) and Cy3 secondary antibody (acceptor), as described by Knowles et al. (49). Cells were grown on coverslips and treated as indicated. Cells were washed with 0.1 M PBS, fixed with 4% paraformaldehyde, and washed three times with 0.1 M PBS for 5 min each. Cells were incubated with blocking solution (0.1 M PBS and 5% normal goat serum in dH2O) for 1 h at room temperature. Cells were incubated with primary antibody solutions directed against C-CB1 (1:500) and arrestin1/2 (1:250) diluted in antibody dilution buffer (0.1 M PBS, 1% (w/v) BSA, in dH2O) overnight at 4 °C. Cells were washed three times with 0.1 M PBS for 5 min each. Cells were incubated in Alexa Fluor 488 (1:500) and Cy3 (1:500) (Rockland Immunocchemicals, Gilbertsville, PA) for 1 h at room temperature. Cells were then washed three times with 0.1 M PBS for 5 min each. Microscopy and FRET were then conducted using the same methodology described for FRET in transfected cells. The specificity of the C-terminal CB1 and arrestin1/2 primary antibodies was confirmed using blocking peptide controls (1:500) (Cayman Chemical Co. and Santa Cruz Biotechnology). FRET efficiency was calculated in ImageJ by dividing the average pixel intensity at 565 nm for any given image by the intensity at 522 nm for that image after background subtraction. FRET was represented visually by mapping a pseudo-color lookup table (16 colors, ImageJ) onto the resulting image (48).

In- and On-cell™ Western Analyses and Immunocytochemistry—for On-cell™ Western analyses, cells were fixed for 10 min at room temperature with 4% paraformaldehyde and washed three times with 0.1 M PBS for 5 min each. Cells were
incubated with blocking solution (0.1 M PBS and 5% normal goat serum in dH\textsubscript{2}O) for 1 h at room temperature. Cells were incubated with primary antibody solutions directed against N-CB\textsubscript{1} (1:500; catalog No. 101500, Cayman Chemical Co.) diluted in antibody dilution buffer (0.1 M PBS and 1% (w/v) BSA in dH\textsubscript{2}O) overnight at 4 °C. Cells were washed three times with 0.1 M PBS for 5 min each. Cells were incubated in IR CW/800 dye (1:500; Rockland Immunochemicals) for 1 h at room temperature. Cells were then washed three times with 0.1 M PBS for 5 min each. Cells were allowed to air-dry overnight. On-cell\textsuperscript{TM} data were then collected using the Odyssey imaging system and software (version 3.0; Li-Cor, Lincoln, NE). These data represent the fraction of CB\textsubscript{1} detected on the plasma membrane. The same cells were then used to quantify total CB\textsubscript{1} protein levels using the In-cell\textsuperscript{TM} Western technique. The On-cell\textsuperscript{TM} CB\textsubscript{1} levels were divided by the In-cell\textsuperscript{TM} (total) CB\textsubscript{1} levels to determine the fraction plasma membrane CB\textsubscript{1}. In-cell\textsuperscript{TM} Western analyses and immunocytochemistry were conducted as described above except that 0.3% Triton X-100 was added to the blocking and antibody dilution solutions. Primary antibody solutions were: N-CB\textsubscript{1} (1:500), pERK1/2 (Tyr-205/Tyr-185) (1:200), ERK1/2 (1:200), pCREB (Ser-133) (1:500), CREB (1:500), pPLC\textsubscript{3} (Ser-537) (1:500), PLC\textsubscript{3} (1:1000), pAkt (Ser-473) (1:500), panAkt (1:1000), arrestin1/2 (1:250), and β-actin (1:2000; Santa Cruz Biotechnology). pERK1/2 (Tyr-205/Tyr-185), pAkt (Ser-473), pCREB (Ser-133), and pPLC\textsubscript{3} (Ser-537) were chosen because phosphorylation at these sites demonstrates activation of the ERK, PI3K/Akt, CREB, and G\textsubscript{αq} pathways, respectively. Secondary antibody solutions were IR\textsuperscript{CW/700} and IR\textsuperscript{CW/800} dyes (1:500; Rockland Immunochemicals). In-cell\textsuperscript{TM} Western analyses were then conducted using the Odyssey imaging system and software (version 3.0; Li-Cor). All experiments measuring CB\textsubscript{1} included an N-CB\textsubscript{1} blocking peptide (1:500) control, which was incubated with N-CB\textsubscript{1} antibody (1:500). Immunofluorescence observed with the N-CB\textsubscript{1} blocking peptide was subtracted from all experimental replicates.

**Quantitative Reverse Transcription PCR—**RNA was harvested from cells using the TRIzol\textsuperscript{®} (Invitrogen) extraction method according to the manufacturer’s instruction. Reverse transcription reactions were carried out with SuperScript III\textsuperscript{®} reverse transcriptase (+RT; Invitrogen) or without (−RT) as a negative control in subsequent PCR experiments according to the manufacturer’s instructions. Two micrograms of RNA was used per RT reaction. qRT-PCR was conducted using the LightCycler\textsuperscript{®} system and software (version 3.0; Roche Applied Science). Reactions were composed of a primer-specific concentration of MgCl\textsubscript{2} (Table 1), forward and reverse primers at 0.5 μM each (Table 1), 2 μl of LightCycler\textsuperscript{®} FastStart SYBR Green I reaction mix, and 1 μl of cDNA to a final volume of 20 μl with dH\textsubscript{2}O (Roche Applied Science). The PCR program was as follows: 95 °C for 10 min, 50 cycles of 95 °C 10 s, a primer-specific annealing temperature (Table 1) for 5 s, and 72 °C for 10 s. Experiments always included sample-matched −RT controls, a no-sample dH\textsubscript{2}O control, and a standard control containing product-specific cDNA of a known concentration. cDNA abundance was calculated by comparing the cycle number at which a sample entered the logarithmic phase of amplification (crossing point) with a standard curve generated by amplification of cDNA samples of known concentration (LightCycler software, version 4.1; Roche Applied Science). qRT-PCR data were normalized to the expression of β-actin (50).

**Statistical Analyses—**These were conducted by one- or two-way analysis of variance (ANOVA), as indicated, using GraphPad (version 5.0, Prism). Post-hoc analyses were performed using Bonferroni’s or Tukey’s test as indicated. Homogeneity of variance was confirmed using Bartlett’s test. The level of significance was set to p < 0.001, < 0.01, or < 0.05, as indicated, and all results are reported as the mean ± S.E. from at least four independent experiments. To improve the readability of the data, many of the figures are subdivided as endocannabinoids (AEA and 2-AG), phytocannabinoids (CBD and THC), and synthetic cannabinoids (CP and WIN).

**RESULTS**

**Interactions between CB\textsubscript{1}, and Arrestin2 (β-arrestin1)** Are Ligand-specific—Initially we wanted to determine whether the interaction between CB\textsubscript{1} and arrestin2 differed among CB\textsubscript{1} agonists. To do this, BRET\textsubscript{eff} was measured between CB\textsubscript{1}-GFP\textsuperscript{2} and arrestin2-Rluc. Arrestin2 was chosen because it is endogenously expressed by STHdh\textsuperscript{Q7/Q7} cells (Fig. 1A). The amount of donor and acceptor plasmid used and the ratio of donor to acceptor plasmids were optimized using a BRET saturation curve at 400 ng of CB\textsubscript{1}-GFP\textsuperscript{2} to 200 ng of arrestin2-Rluc (2:1; Fig. 1B). Basal BRET\textsubscript{eff} between CB\textsubscript{1}-GFP\textsuperscript{2} and arrestin2-Rluc was ~0.2 and greater than BRET\textsubscript{eff} between HERG-GFP\textsuperscript{2} and arrestin2-Rluc (Fig. 1B). We also verified that BRET\textsubscript{eff} was independent of time and plasmid expression level for CB\textsubscript{1}-GFP\textsuperscript{2} and arrestin2-Rluc (Fig. 1C). Cells were treated with 1 μM AEA, 2-AG, CBD, THC, CP, or WIN or with 500 nM O-2050, for 0–30 min (Fig. 2, A–C). Treatment with AEA, 2-AG, THC, CP, or WIN increased BRET\textsubscript{eff} within 10 min compared with vehicle treatment, and BRET\textsubscript{eff} was stable over 30 min (Fig. 2, A–C).

**TABLE 1**

| Target | Oligonucleotide sequence (5’-3’) | Annealing temperature (°C) | MgCl\textsubscript{2} (mM) | Source |
|--------|---------------------------------|-----------------------------|-----------------------------|--------|
| CB\textsubscript{1} | GGGCAAAATCTCTTTAAGCA | 58 | 1 | Ref. 39 |
| ppENK | GCGTCACTGACTGAGAAA | 57 | 3 | This study |
| β-Actin | AGGCCAAACTGAAAAGAT | 59 | 2 | Ref. 39 |
| Arrestin2 | CAGCCACCCCTGACACTCC | 59 | 2 | This study |
| Arrestin3 | AGGTGAGCTGGTGTTCC | 59 | 2 | This study |

**TABLE 2**

| PCR primers used in this study | Source |
|--------------------------------|--------|
| AAGGCCAACGTCGAAAAGGT | This study |
| pERK1/2 (Tyr-205/Tyr-185) | This study |
| pCREB (Ser-133) | This study |
| PLC\textsubscript{3} (Ser-537) | This study |
| panAkt (1:1000) | This study |
| arrestin1/2 (1:250) | This study |
| β-actin (1:2000) | This study |
| AAGGCCAACGTCGAAAAGGT | This study |
| GCGTCACTGACTGAGAAA | This study |
| AGGCCAAACTGAAAAGAT | This study |
| CAGCCACCCCTGACACTCC | This study |
| AGGTGAGCTGGTGTTCC | This study |
CBD and O-2050 treatment did not change BRET$_{\text{eff}}$ relative to vehicle treatment. In addition, BRET$_{\text{eff}}$ between CB$_1$-GFP$^2$ and arrestin2-Rluc was greater in cells treated with 2-AG compared with AEA by 15 min, with THC compared with CBD by 10 min, and with CP compared with WIN by 5 min (Fig. 2, A–C). The ligand-specific differences in CB$_1$–arrestin2 association were further analyzed by measuring BRET$_{\text{eff}}$ in cells treated with 0.01–5.00 μM AEA, 2-AG, CBD, THC, CP, or WIN in the absence or presence of 500 nM O-2050 for 10 (Fig. 2, D–F) or 30 min (Fig. 2, G–I). At 10 min, BRET$_{\text{eff}}$ between CB$_1$-GFP$^2$ and arrestin2-Rluc was not different in AEA- and 2-AG-treated cells (Fig. 2D), whereas THC and CP were more potent and efficacious ligands than CBD and WIN, respectively (Fig. 2, E and F). At 30 min, 2-AG was a more efficacious ligand than AEA (Fig. 2G). As observed at 10 min, THC and CP were more potent and efficacious ligands than CBD and WIN, respectively (Fig. 2, H and I). AEA-, 2-AG, THC-, WIN-, and CP-mediated recruitment of arrestin2 to CB$_1$ was inhibited by co-treatment of cells with the CB$_1$ antagonist O-2050, as demonstrated by a significant rightward shift in the BRET$_{\text{eff}}$ dose-response curves (Fig. 2, D–I). The EC$_{50}$ Hill slope, and $E_{\text{max}}$ values generated from these dose-response relationships were also compared (Table 2). THC was more potent than AEA and WIN at 10 min and more potent than AEA, 2-AG, and WIN at 30 min (Table 2). The flat dose-response relationship observed with CBD demonstrates that this ligand had very little effect on the interaction between CB$_1$-GFP$^2$ and arrestin2-Rluc because the basal BRET$_{\text{eff}}$ is not significantly different from the $E_{\text{max}}$ (Fig. 2, E and H). 2-AG (30 min), THC (30 min), and CP (10 and 30 min) were more efficacious ligands and CBD (10 and 30 min) less efficacious than AEA for BRET$_{\text{eff}}$ between CB$_1$-GFP$^2$ and arrestin2-Rluc (Table 2). The BRET$_{\text{eff}}$ $E_{\text{max}}$ values were greater at 30 min than at 10 min when cells were treated with 2-AG or THC (Table 2). No statistically significant changes in the Hill slope were observed. Based on these data, we concluded that 1) with the exception of CBD, each ligand promoted interactions between CB$_1$ and arrestin2; 2) 2-AG, THC, and CP displayed higher maxima than the other cannabinoid ligands tested for enhancing CB$_1$-arrestin2 interactions; and 3) in the assay, THC and CP were more potent than the other cannabinoid ligands tested for enhancing CB$_1$-arrestin2 interactions. A final concentration of 1 μM was used for all subsequent experiments because this dose consistently produced a response that approximated the $E_{\text{max}}$ observed for BRET$_{\text{eff}}$ for all cannabinoids tested.

Because BRET assays quantify the level of interaction between two proteins but do not provide data on the localization of protein complexes, FRET analyses were conducted to determine the localization CB$_1$ and arrestin2 complexes within STHdh$^{Q7/Q7}$ cells in the presence of the cannabinoids studied. FRET was used to study the interaction between CB$_1$-GFP$^2$ and arrestin2-RFP or endogenous CB$_1$ and arrestin2 detected via fluorescent antibodies. A photobleaching experiment was conducted as a control for FRET (48). Cells were transfected with CB$_1$-GFP$^2$ and arrestin2-RFP. As expected, direct excitation of RFP at 543 nm for 5 min eliminated the fluorescent signal at 565 nm in a small, cytoplasmic region of interest, and the GFP2 signal in that area was enhanced, whereas the RFP and GFP2 signals in a non-photobleached region of interest were unchanged (Fig. 3, A and B) (48). The specificity of the anti-CB$_1$ and anti-arrestin1/2 antibodies was analyzed via immunohistochemistry in the absence and presence of CB$_1$- and arrestin1/2 antibody-blocking peptides (Fig. 3C). Fluorescence intensity was ~60-fold greater than in the absence of blocking peptide for both CB$_1$ and arrestin1/2 antibodies (Fig. 3D) FRET was qualitatively higher in cells treated with all cannabinoids tested (1 μM, 30 min) except CBD, indicating that interactions between CB$_1$ and arrestin2 had increased in transfected cells overexpressing CB$_1$-GFP$^2$ and arrestin2-RFP (Fig. 4A) and cells endogenously expressing CB$_1$ and arrestin2 (Fig. 4B). Quantification of total FRET for cells expressing CB$_1$-GFP$^2$ and arrestin2-RFP revealed that FRET was greater in cells treated with 1 μM AEA, 2-AG, THC, CP, or WIN for 30 min than in cells treated with vehicle (Fig. 4C, dotted line). Similarly, total FRET
between Alexa Fluor 488-conjugated antibodies (CB1) and Cy3-conjugated antibodies (arrestin2) was greater in cells treated with 1 μM AEA, 2-AG, THC, CP, or WIN for 30 min than in cells treated with vehicle (Fig. 4C, solid line). Total FRET between Alexa Fluor 488 and Cy3 was reduced in cells treated with 1 μM CBD for 30 min relative to vehicle-treated cells (Fig. 4C). We also observed that total FRET was greater in cells treated with 2-AG (Alexa Fluor 488 and Cy3 only), THC, or CP and less in cells treated with CBD than in cells treated with AEA (Fig. 4C). At the plasma membrane, FRET was greater in cells treated with AEA, 2-AG, or WIN (Alexa Fluor 488 and Cy3 only) and less in cells treated with CBD (Alexa Fluor 488 and Cy3 only) or CP compared with vehicle-treated cells (Fig. 4D).

Moreover, FRET was reduced in cells treated with CBD, WIN (CB1-GFP2 and arrestin2-RFP only), or CP relative to AEA-treated cells (Fig. 4D). Within the cytoplasm, FRET was greater in cells treated with AEA (CB1-GFP2 and arrestin2-RFP only), 2-AG, THC, WIN (CB1-GFP2 and arrestin2-RFP only), or CP and less in cells treated with CBD (Alexa Fluor 488 and Cy3 only) than in vehicle-treated cells (Fig. 4E). FRET within the cytoplasm was also greater in 2-AG (CB1-GFP2 and arrestin2-RFP only)-, THC-, and CP-treated cells and less in CBD-treated cells (Alexa Fluor 488 and Cy3 only) than in AEA-treated cells (Fig. 4E). A further comparison of FRET between the plasma membrane (Fig. 4D) and cytoplasm (Fig. 4E) demonstrates although 2-AG, THC, and CP all enhanced arrestin2 recrui-
ment to CB1 to a greater extent than other ligands tested, THC and CP biased CB1-arrestin2 complexes toward internalization to a greater extent than 2-AG.

Analysis of FRET at 10 min revealed no significant difference between 10 and 30 min of treatment with any cannabinoid tested (data not shown). Quantification of FRET in the nucleus

**TABLE 2**

| BRET<sub>eff</sub> potencies and efficacies of cannabinoid ligands (BRET between CB<sub>1</sub>-GFP<sup>2</sup> and arrestin2-Rluc) |
|---|---|---|---|---|
|  | AEA |  | 2-AG |  |
|  | 10 min | 30 min | 10 min | 30 min |
| EC<sub>50</sub> (µM) (95% CI) | 0.49 (0.39 - 0.52) | 0.55 (0.44 - 0.69) | 0.34 (0.29 - 0.48) | 0.54 (0.46 - 0.73)† |
| Hill Slope (± SEM) | 1.20 ± 0.21 | 1.40 ± 0.39 | 1.40 ± 0.24 | 1.71 ± 0.31 |
| E<sub>max</sub> (BRET<sub>eff</sub>) (± SEM) | 0.55 ± 0.05 | 0.52 ± 0.05 | 0.59 ± 0.05 | 0.80 ± 0.04** |
| CBD |  |  |  |  |
|  | 0.28 (0.13 - 0.59) | 0.09 (0.01 - 0.17)<sup>##</sup> | 0.17 (0.12 - 0.24)<sup>##</sup> | 0.25 (0.18 - 0.34)<sup>##</sup> |
| Hill Slope (± SEM) | 1.00 ± 0.64 | 1.30 ± 0.08 | 1.32 ± 0.40 | 1.97 ± 0.62 |
| E<sub>max</sub> (BRET<sub>eff</sub>) (± SEM) | 0.24 ± 0.03<sup>*</sup> | 0.25 ± 0.04<sup>*</sup> | 0.63 ± 0.03 | 0.83 ± 0.09† |
| THC |  |  |  |  |
|  | 0.65 (0.51 - 0.79)<sup>##</sup> | 0.57 (0.45 - 0.72)<sup>##</sup> | 0.37 (0.32 - 0.41)<sup>##</sup> | 0.35 (0.28 - 0.43) |
| Hill Slope (± SEM) | 0.90 ± 0.49 | 1.10 ± 0.57 | 1.10 ± 0.41 | 1.00 ± 0.31 |
| E<sub>max</sub> (BRET<sub>eff</sub>) (± SEM) | 0.57 ± 0.04 | 0.59 ± 0.13 | 0.91 ± 0.05<sup>*</sup> | 0.86 ± 0.04<sup>*</sup> |
| WIN 55,212-2 |  |  |  |  |
|  |  |  |  |  |
| CP 55,940 |  |  |  |  |
|  |  |  |  |  |

<sup>*</sup>, p < 0.001 compared with AEA within the time point; <sup>##</sup>, p < 0.001 compared with 2-AG within the time point; <sup>##</sup>, p < 0.001 compared with CBD within the time point; <sup>##</sup>, p < 0.001 compared with THC within the time point; <sup>##</sup>, p < 0.001 compared with WIN within the time point; †, p < 0.001 compared with 10-min within-drug treatment as determined via two-way ANOVA followed by Bonferroni’s post-hoc test (n = 6).
and dendrites of cells revealed no difference among treatments (data not shown). The cell diameter, cell area, projection length, and projection number were not different between treatment groups (n/H11005 = 50; data not shown). Overall, these data demonstrate that THC and CP appear to bias CB1 toward arrestin2-mediated internalization to a greater degree than the other cannabinoid ligands tested.

Cannabinoid Ligands Biased Intracellular Signaling—Because we had observed ligand-specific differences in CB1-arrestin2 interactions, we wanted to determine whether intracellular signaling differed among cannabinoids. Treatment with AEA or 2-AG for 10 min resulted in a PTx- and O-2050-sensitive increase in ERK phosphorylation compared with vehicle (Fig. 5, A and B). By 30 min, AEA-mediated ERK phosphorylation was not detectable, whereas O-2050-sensitive ERK phosphorylation persisted in 2-AG-treated cells and was no longer PTx-sensitive compared with vehicle-treated cells or with treatment at 10 min (Fig. 5, A and B). AEA and 2-AG treatment did not change the levels of CREB phosphorylation (Fig. 6, A and B). AEA and 2-AG treatment did increase O-2050 and PTx-sensitive Akt phosphorylation at 10 and 30 min compared with vehicle treatment (Fig. 7, A and B). AEA and 2-AG also increased the CB1- and Gαq-dependent phosphorylation of PLCβ3 at 10 min compared with vehicle treatment (Fig. 8, A and B). Treatment with CBD did not change ERK, Akt, or PLCβ3 phosphorylation but did increase CTx-sensitive CREB phosphorylation at 30 min compared with vehicle treatment and compared to treatment for 10 min with CBD (Figs. 5C, 6C, 7C, and 8C). CBD-mediated CREB phosphorylation was CB1-independent because it was not inhibited by O-2050. Therefore, CBD may enhance CREB activation via other cannabinoid receptors, GPCRs, or GPCR-independent mechanisms (7, 12). Unlike 2-AG, THC treatment did not increase ERK phosphorylation at 10 min or Akt phosphorylation at 10 and 30 min (Figs. 5D, 6D, 7D, and 8D). WIN and CP treatment for 10 min resulted in a PTx- and O-2050-sensitive increase in ERK phosphorylation (Fig. 5, F and F) and CB1- and Gαq-dependent phosphorylation of

![Figure 4. 2-AG, THC, and CP treatment enhanced FRET between CB1-GFP2 and arrestin2-Rluc.](image-url)
PLCβ3 at 10 min (Fig. 8, E and F), relative to vehicle treatment. As with AEA, ERK phosphorylation was not detected in cells treated with WIN for 30 min (Fig. 5E). CP treatment for 30 min resulted in CB₁-dependent, PTx-insensitive ERK phosphorylation compared with vehicle treatment, as observed with 2-AG and THC (Fig. 5F). WIN treatment did not alter CREB phosphorylation, but CP treatment for 30 min did increase O-2050- and CTx-sensitive CREB phosphorylation relative to vehicle treatment and 10-min treatment with CP (Fig. 6, E and F). CP-dependent CREB phosphorylation was less than CB₁-dependent CREB phosphorylation (Fig. 6, C and F). Both WIN and CP treatment for 10 and 30 min increased Akt phosphorylation compared with vehicle treatment but was less than either AEA or 2-AG (Fig. 7, A, B, E, and F). Therefore, AEA, 2-AG, WIN, and CP treatment resulted in Gα<sub>I</sub>-dependent transient ERK phosphorylation (51), Gα<sub>q</sub>-dependent transient PLCβ3 phosphorylation, and persistent Akt phosphorylation. THC treatment also resulted in Gα<sub>q</sub>-dependent transient PLCβ3 phosphorylation. Further, treatment with 2-AG, THC, and CP, the ligands that enhanced BRET<sub>eff</sub> and FRET between CB₁ and arrestin2 more than the other cannabinoids tested, resulted in persistent (30 min) Gα<sub>I</sub>-independent ERK phosphorylation. Finally, CBD and CP treatment enhanced Gα<sub>i</sub>-mediated CREB phosphorylation, although CBD did so independent of CB₁. AEA, 2-AG, THC, WIN, and CP increased the phosphorylation of ERK, CREB, Akt, or PLCβ3 via CB₁, because these effects were blocked by the CB₁-selective antagonist O-2050 (1). Therefore, the functional selectivity between the cannabinoids tested here is the result of ligand bias at CB₁ receptors.

Sustained and Gα<sub>i</sub>-independent ERK phosphorylation occurs via arrestin2 (47). We tested this possibility by treating cells overexpressing an arrestin2 dominant negative mutant
We observed that PTx-insensitive ERK phosphorylation was sustained at each time point above the levels observed before drug treatment in cells treated with 2-AG (Fig. 9A) and for 12 h in cells treated with THC (Fig. 9B) or CP (Fig. 9C). However, the levels of phosphorylated ERK did not differ from basal levels (0 h) in cells expressing arrestin2 V53D. Based on these data, the sustained ERK phosphorylation observed with 2-AG, THC, and CP occurred via arrestin2-mediated signaling.

We also observed PTx-sensitive Akt phosphorylation in cells treated with AEA or 2-AG for 10 or 30 min. Akt activation was observed in AEA-, 2-AG-, WIN-, and CP-treated cells and not in THC- and CBD-treated cells. We hypothesized that this increase in CB1 levels was unique to those cannabinoids that increased Akt phosphorylation. To test this hypothesis, cells were treated with 1 μM AEA, 2-AG, CBD, THC, WIN, or CP with or without 50 ng/ml PTx or 500 nM O-2050 for 18 h, and CB1 mRNA levels were quantified relative to β-actin (Fig. 10A). AEA and 2-AG,
and to a lesser extent WIN, increased CB₁ mRNA levels relative to vehicle treatment, whereas CBD, THC, and CP treatment did not change CB₁ mRNA levels (Fig. 10A). The increase in CB₁ mRNA levels may have been less in WIN-treated cells and absent in CP-treated cells because Akt phosphorylation was lower in WIN- and CP-treated cells relative to AEA- and 2-AG-treated cells (Fig. 7), resulting in insufficient activation of this signaling pathway. In addition, the increase in CB₁ mRNA levels was blocked by treatment with O-2050 or PTx and therefore occurred through CB₁ and G<sub>i/o</sub> (Fig. 10A). Therefore, AEA, 2-AG, and WIN treatment biased CB₁ signaling toward activation of G<sub>i/o</sub> signaling, resulting in increased CB₁ mRNA levels.

CBD and CP treatment increased CREB phosphorylation (Fig. 6, C and F). Therefore, we wanted to know whether treatment with CBD or CP would increase preproenkephalin (ppENK) expression, which is known to be CREB-dependent (54, 55). ppENK mRNA levels were quantified in cells treated with 1 μM AEA, 2-AG, CBD, THC, WIN, or CP with or without 50 ng/ml PTx or CTx or 500 nM O-2050 for 18 h. AEA, 2-AG, and WIN treatment were associated with a CB₁-dependent decrease in ppENK mRNA levels, whereas CBD treatment increased ppENK mRNA levels compared with vehicle treatment (Fig. 10B). The CBD-mediated increase in ppENK mRNA levels was CB₁-independent, because it was not inhibited by
O-2050 (Fig. 10B). CP treatment did not affect ppENK mRNA levels (Fig. 10B). CBD treatment resulted in greater CREB phosphorylation than CP treatment (Fig. 6, C and F). Therefore, CP treatment may have failed to increase ppENK mRNA levels because the magnitude of CREB phosphorylation was too low. Based on these data, AEA-, 2-AG-, and WIN-dependent activation of G\textsubscript{q}/H9251 inhibited CREB-mediated gene expression, whereas CBD-mediated, CB1-independent activation of G\textsubscript{q} increased CREB-mediated gene expression.

Increased CB\textsubscript{1} mRNA levels translated to increased CB\textsubscript{1} protein abundance, as determined by On- and In-cell\textsuperscript{TM} Western analyses. Treatment with 1\(\mu\)M AEA or 2-AG resulted in increased CB\textsubscript{1} levels within 3 h compared with the 0 h measurement or with vehicle treatment within the time point, and this increase was still observed at 18 h (Fig. 11A). In contrast, treatment with 1\(\mu\)M THC or CP resulted in decreased CB\textsubscript{1} levels by 6 h (THC) and 12 h (CP) compared with the 0 h measurement or vehicle control (Fig. 11, B and C). Treatment with 1\(\mu\)M CBD or WIN did not change CB\textsubscript{1} protein levels (Fig. 11, B and C). CB\textsubscript{1} localization was also analyzed over an 18-h treatment period. The fraction of CB\textsubscript{1} receptors at the membrane of AEA- and 2-AG-treated cells was decreased between 0.5 and 3 h compared with the 0 h time point or vehicle-treated cells, which returned to basal levels by 6 h (Fig. 11D). A decrease in the fraction of CB\textsubscript{1} receptors at the membrane was also observed in THC- and WIN-treated cells between 1 and 12 h (THC) and at 1 h (WIN) compared with the 0 h time point or vehicle-treated cells, which returned to basal levels by 18 h (THC) and 3 h (WIN) (Fig. 11, E and F). Treatment with CBD increased the fraction of CB\textsubscript{1} receptors at the membrane between 3 and 18 h relative to the 0 h time point or vehicle-treated cells (Fig. 11E). In contrast, treatment with CP resulted in a sustained decrease in the fraction of CB\textsubscript{1} receptors at the membrane beginning at 0.5 h and persisting to 18 h, as com-

**FIGURE 8.** Cannabinoid ligands biased CB\textsubscript{1}-dependent PLC\textsubscript{3} signaling. PLC\textsubscript{3} (pPLC\textsubscript{3}(Ser-537)/total PLC\textsubscript{3}) was quantified via In-cell\textsuperscript{TM} Western assays in cells treated with 1\(\mu\)M AEA (A), 2-AG (B), CBD (C), THC (D), WIN (E), or CP (F) for 10 or 30 min with or without 500 nM O-2050 or expressing G\textsubscript{q} dominant negative (G\textsubscript{q} DN). *, \(p < 0.001\) compared with vehicle treatment within time point; ∧, \(p < 0.001\) compared with No Toxin treatment within time point; #, \(p < 0.001\) compared with 10 min within drug and toxin treatment as determined via two-way ANOVA followed by Bonferroni’s post-hoc test; \(n = 4\).
pared with the 0 h time point and vehicle-treated cells (Fig. 11F). CB₁ receptor localization was also examined via confocal microscopy in cells expressing CB₁-GFP² that were treated with 1 μM AEA, THC, or CBD for 10 or 30 min or 1, 3, 6, 12, or 18 h. Similar to the observations made in Fig. 11D, CB₁-GFP² localization shifted from the plasma membrane to the cytoplasm and back to the plasma membrane in cells treated with AEA for 18 h (Fig. 12). In contrast to the AEA-treated cells, CB₁-GFP² was first internalized and subsequently degraded, as indicated by decreased fluorescence, in THC-treated cells (Fig. 12). In CBD-treated cells CB₁-GFP² fluorescence at the plasma membrane gradually increased over the 18 h observation period (Fig. 12). Therefore, although AEA and THC treatment affected CB₁ signaling and internalization, CBD did not affect CB₁ internalization and CBD-mediated signaling was CB₁-independent.

In conclusion, the endocannabinoids, AEA and 2-AG, facilitated an increase in CB₁ mRNA and protein via Gαi/o and Gβγ. In contrast to the AEA-treated cells, CB₁-GFP² was first internalized and subsequently degraded, as indicated by decreased fluorescence, in THC-treated cells (Fig. 12). In CBD-treated cells CB₁-GFP² fluorescence at the plasma membrane gradually increased over the 18 h observation period (Fig. 12). Therefore, although AEA and THC treatment affected CB₁ signaling and internalization, CBD did not affect CB₁ internalization and CBD-mediated signaling was CB₁-independent.

In conclusion, the endocannabinoids, AEA and 2-AG, facilitated an increase in CB₁ mRNA and protein via Gαi/o and Gβγ.
WIN also activated $\mathrm{G}_\alpha_{i/o}$ and $\mathrm{G}_\beta\gamma$ signaling but to a lesser extent than AEA and 2-AG. Treatment with the phytocannabinoid THC and the synthetic cannabinoid CP did not alter CB1 mRNA levels but did lead to a decrease in CB1 protein levels over the 18-h time period analyzed. CP also enhanced $\mathrm{G}_i/o$ signaling via CB1.

CBD-mediated $\mathrm{G}_i/o$ signaling occurred independent of CB1 as observed elsewhere (8).

**DISCUSSION**

**CB1-mediated Intracellular Signaling Was Ligand-specific—**

The goal of this study was to compare the CB1-mediated functional selectivity of six cannabinoids in a cell line that models striatal medium spiny projection neurons endogenously expressing CB1. Each ligand displayed functional selectivity for a subset of intracellular signaling pathways (see summary in Fig. 13). With the exception of CBD-dependent $\mathrm{G}_\alpha_s$ signaling, this functional selectivity was CB1-dependent.

2-AG, THC, and CP enhanced the interaction between CB1 and arrestin2 to a greater extent than other cannabinoids tested, suggesting a high degree of interaction between the population of CB1 and arrestin2 molecules in the *in vitro* system following 30 min of treatment with these compounds. The relative BRET$_{eff}$ was a conservative estimate of the interaction between CB1 and arrestin2, because endogenous CB1 and arrestin2 would have competed with their labeled counterparts in ST$_{Hdh}^{Q7/Q7}$ cells in the BRET assays. These observations differ from previous reports that CB1 interacts weakly with arrestins in U2OS, CHO, and HEK cell heterologous expression systems treated with WIN or CP for 5 min (33) or 2 h (33). Previous studies also observed that recruitment of arrestins to CB1 occurs over a wider range of ligand concentrations ($1 \times 10^{-10}$ to $1 \times 10^{-5}$ M) (32, 33) than that observed here ($1 \times 10^{-8}$ to $1 \times 10^{-5}$ M). The variability between our results and previous reports may reflect differences between the functionality of CB1 in ST$_{Hdh}^{Q7/Q7}$ cells and CB1 overexpression in U2OS, CHO, and HEK cells (33). In addition, BRET$^2$, used in this study, is a more sensitive assay for detecting protein-protein interactions compared with the Tango and PathHunter reporter assays used previously (32). Moreover, previous studies examined the recruitment of arrestin3 ($\beta$-arrestin2) in HEK cells (33) and not arrestin2.

At 1 $\mu$M, AEA, 2-AG, WIN, and CP biased CB1 signaling toward $\mathrm{G}_\alpha_{i/o}$-mediated ERK phosphorylation to a greater degree than other cannabinoids tested. The consequence of this functional selectivity is that transient ERK signaling was enhanced by endocannabinoids compared with other cannabinoids tested, whereas sustained ERK signaling from 10 to 30...
min through arrestin2 was enhanced by 2-AG, THC, and CP and not by AEA, CBD, or WIN. Other studies have also reported that transient ERK activation occurs via Goqio, in the N18TG2 mouse neuronal cell line (56) and in HEK 293 cells stably expressing CB1 (51). In our studies CB1 receptors recruited arrestin2, leading to sustained ERK signaling, whereas previous studies have found that sustained ERK signaling is receptor tyrosine kinase-dependent (56).

AEA and 2-AG activated Akt via Goqio- and Gβγ-dependent pathways. This resulted in increased CB1 mRNA and protein levels. Although WIN and CP treatment also resulted in Goqio-dependent ERK phosphorylation and Gβγ-dependent Akt phosphorylation, the magnitude of Gβγ activation was less following WIN and CP treatment than that observed following treatment with AEA and 2-AG. The net result was that WIN and CP treatment did not lead to significantly increased CB1 mRNA and protein levels.

In addition to the activation of Goqio, AEA, 2-AG, WIN, THC, and CP enhanced transient Goq coupled PLCβ3 phosphorylation. Few studies have examined direct coupling of CB1 to Goq (25, 57). Coupling of CB1 to Goq has been reported in HEK 293 cells stably expressing CB1 (25) and human trabecular meshwork cells (58). In these studies, the authors observed transient, Goq-dependent Ca2+ efflux following stimulation of CB1 with WIN (25, 57), which is an indirect measure of Goq coupling. In support of studies that have indirectly measured CB1 coupling to Goq via Ca2+ efflux, the work here measured PLCβ3 activation, which is a direct effect of Goq. Because Goq signaling may affect cellular function, future studies examining CB1 signaling should determine whether CB1 couples to Goq in other model systems.

CBD treatment resulted in Goαs- and CREB-dependent expression of ppENK (7, 12). CBD signaling was independent of CB1, as demonstrated previously by the inability of the direct antagonist O-2050 to block agonist-dependent Goαq signaling (8). Although CBD has a relatively low affinity for CB1 (6), CBD has been shown to act as an agonist and antagonist at the type 2 cannabinoid receptor, an adenosine A2A agonist, a 5HT1A agonist, and a modulator of FAAH and monoacylglycerol lipase activity (MAGL) (6–8, 58). STHdhQ7/Q7 cells express adenosine A2A and 5HT1A receptors and FAAH (36, 37). The inability of O-2050 to block Goαq signaling indicates that CBD acted at non-CB1 targets in STHdhQ7/Q7 cells. In our assays, CBD treatment increased CB1 levels at the plasma membrane but did not affect CB1-dependent signaling through Goqio or Goαq. CBD is being investigated for its utility as an anti-epileptic (8) and an anti-inflammatory (12) and for its neuromodulatory activities in vivo (7, 8). CBD has a relatively safe side effect profile compared with THC and other cannabinoids (7, 8). Our work suggests that CBD has little effect on CB1-dependent signaling. The fact that we observed that CBD selectively increased CREB-dependent gene expression may also have therapeutic potential in neurodegenerative diseases, where CREB-dependent gene expression is dysregulated (6, 8, 37).

Unlike CBD, CP-dependent CREB phosphorylation occurred via CB1. Although CB1 does not typically signal through Goαq, CP may have promoted a conformational change in the receptor that favored Goαq binding. Alternatively, CP treatment may promote the dimerization of CB1 with other GPCRs that signal through Goq. CB1 is known to homodimerize with CB1 and CB1 splice variants (40) and heterodimerize with other receptors including the dopamine D2 receptor (37, 43, 59). STHdhQ7/Q7 cells express adenosine A2A and 5HT1A receptors and FAAH (36, 37). The inability of O-2050 to block Goαq signaling indicates that CBD acted at non-CB1 targets in STHdhQ7/Q7 cells. In our assays, CBD treatment increased CB1 levels at the plasma membrane but did not affect CB1-dependent signaling through Goqio or Goαq. CBD is being investigated for its utility as an anti-epileptic (8) and an anti-inflammatory (12) and for its neuromodulatory activities in vivo (7, 8). CBD has a relatively safe side effect profile compared with THC and other cannabinoids (7, 8). Our work suggests that CBD has little effect on CB1-dependent signaling. The fact that we observed that CBD selectively increased CREB-dependent gene expression may also have therapeutic potential in neurodegenerative diseases, where CREB-dependent gene expression is dysregulated (6, 8, 37).

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cells express dopamine D₂ receptors and CB₁-D₂ dimerization may contribute to the actions of CP in these cells (59). Together, these data demonstrate that CB₁ is a receptor that couples to multiple G proteins within a single cell type (pleiotropic).

**CB₁-mediated Signaling Had Immediate and Sustained Components**—In addition to showing cannabinoid ligand bias, CB₁ signaling was also time-dependent. Gαᵩs and Gαᵩq signaling was transient, being detected at 10 min and returning to basal levels at 30 min. Transient CB₁-dependent activation of Gαᵩo and Gαᵩq signaling has been observed elsewhere in HEK 293 cells stably expressing CB₁ (25, 51). In contrast, Gαᵩq signaling was not detected before 30 min. The association between CB₁ and arrestin2 peaked shortly after 10 min for all ligands tested and remained high for 30 min relative to vehicle control, demonstrating that the pleiotropically coupled CB₁ receptor switches between G protein signaling and arrestin signaling within ~30 min of ligand administration and that this switch is ligand-specific. Over an 18-h treatment period, THC, CP, and 2-AG treatment resulted in CB₁ receptor internalization (beginning at 30 min), but only THC and CP treatment resulted in decreased CB₁ receptor protein levels (beginning at 12 h). This difference may be due to the higher affinity of THC and CP for CB₁ compared with 2-AG (60), which implies that 2-AG is a “fast-off” cannabinoid relative to THC or CP (30–32). **In vivo**, 2-AG is ~1000 times more abundant than AEA (60–62). 2-AG is likely to have a greater effect on the arrestin-mediated recycling of CB₁ between the membrane and intracellular space relative to AEA. Overall, the biased agonism displayed by the six cannabinoids tested here in an *in vitro* model of striatal neurons supports the hypothesis that individual ligands promote unique conformational changes in the CB₁ receptor leading to functionally divergent intracellular effects such as G protein-coupled signaling, arrestin recruitment, receptor trafficking, and gene expression (22, 29, 31–33).

**The Effect of Cannabinoids Is Brain Region- and Agonist-specific**—We observed increased CB₁ mRNA and protein levels following 18 h of treatment with 2-AG or AEA in an *in vitro* cell culture model of striatal neurons. **In vivo**, CB₁ receptor binding does not differ between FAAH knock-out mice and wild-type littermates in the striatum, hippocampus, or cerebellum when treated with vehicle or AEA for 5 consecutive days (19). Further, CB₁ receptor binding decreases in these brain regions of FAAH knock-out mice treated with THC for 5 consecutive days (19). MAGL knock-out mice and mice treated for 6 days with the MAGL inhibitor JZL184 displayed decreased CB₁ receptor binding in the cortex, hippocampus, and periaqueductal gray but no difference in the striatum (21). **In vivo** then, the alteration of CB₁ level depends on the brain region, animal genotype, and duration of treatment, as well as the cannabinoid ligand (19–21). Inhibition of MAGL (5 days), the principle regulator of 2-AG levels, results in functional antagonism of CB₁, whereas inhibition of FAAH, the principle regulator of AEA levels, maintains CB₁ signaling (19–21). Subchronic or chronic exposure to exogenous cannabinoids, such as THC, and high potency cannabinoids decreases CB₁ receptor binding (19–21). The down-regulation and desensitization of CB₁ receptor following repeated THC or WIN treatment are more pronounced in the hippocampus compared with the striatum (63, 64). CB₁ desensitization and arrestin3 (β-arrestin2) recruitment also vary widely among brain regions (65). FAAH knock-out mice show less CB₁ down-regulation and desensitization following AEA treatment compared with THC-treated FAAH knock-out mice (19). CB₁ internalization is also promoted by WIN more than methanandamide in primary rat hippocampal neurons (66). THC-mediated desensitization is faster than WIN-, CP-, and 2-AG-mediated desensitization in HEK 293 cells stably expressing CB₁ and primary neuronal cultures (67). In contrast, AEA-mediated CB₁ desensitization is slower than that of WIN-, CP-, and 2-AG (67). Endogenous cannabinoids, specifically AEA, may have a different effect on CB₁ levels than other cannabinoids. We do not yet know whether there is a difference in the brain region-specific effect on CB₁ mRNA and protein levels under various treatment regimens *in vivo*.

There is the potential to exploit biased agonism at CB₁. Effects such as receptor internalization via arrestin2, Gαᵩo-mediated increases in CB₁, Gαᵩq-mediated modulation of Ca²⁺ release, Gαᵩ-mediated CREB activation, and CB₁ protein down-regulation could be selected or avoided according to their usefulness in different disease states. This could lead to the development of therapeutics that avoid the psychoactive effects of cannabinoids and promote their neuroprotective effects.

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