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CB_1 & CB_2 Receptor Pharmacology

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Abstract

The CB_1 and CB_2 cannabinoid receptors (CB_1R, CB_2R) are members of the G protein coupled receptor (GPCR) family that were identified over 20 years ago. CB_1Rs and CB_2Rs mediate the effects of Δ^9-tetrahydrocannabinol (Δ^9-THC), the principal psychoactive constituent of marijuana and subsequently identified endogenous cannabinoids (endocannabinoids) anandamide and 2-arachidonoyl glycerol. CB_1Rs and CB_2Rs have both similarities and differences in their pharmacology. Both receptors recognize multiple classes of agonist and antagonist compounds and produce an array of distinct downstream effects. Natural polymorphisms and alternative splice variants may also contribute to their pharmacological diversity. As our knowledge of the distinct differences grows, we may be able to target select receptor conformations and their corresponding pharmacological responses. This chapter will discuss their pharmacological characterization, distribution, phylogeny and signaling pathways. In addition, the effects of extended agonist exposure and how that affects signaling and expression patterns of the receptors is considered.

Keywords

Cannabinoid; GPCR; G-protein; polymorphism; splice variant; human; rodent; tissue selectivity; biased agonism

Introduction

The CB_1 cannabinoid receptor was discovered (Devane, Dysarz, Johnson, Melvin, & Howlett, 1988) and subsequently cloned (Matsuda, Lolait, Brownstein, Young, & Bonner, 1990) on the basis of its responsiveness to (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC). Δ^9-THC is the primary psychoactive constituent in *Cannabis* (a.k.a. marijuana), hence the name "cannabinoid" receptor. CB_1 is a member of the Gprotein coupled receptor (GPCR) family. An arachidonic acid metabolite, N-arachidonylethanolamide was shown to activate CB_1, and named "anandamide" from the Sanskrit word for "bliss" (Devane et al., 1992), and this was followed by the identification of a second metabolite 2-arachidonoylglycerol (2-AG)

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Conflict of Interest

The authors have no conflicts of interest to declare.

(Mechoulam et al., 1995; Sugiura et al., 1995). The identification of endogenous ligands and the availability of novel ligands with cannabinoid receptor activity led to subsequent breakthroughs elucidating an “endocannabinoid system” (Di Marzo, Melck, Bisogno, & De Petrocellis, 1998). A second cannabinoid receptor (CB_2) was isolated by a PCR-based strategy designed to isolate GPCRs in differentiated myeloid cells (Munro, Thomas, & Abu-Shaar, 1993). The CB_2 receptor shares 44% amino acid homology with CB_1, and a distinct yet similar binding profile, thus representing a receptor subtype. The most current nomenclature for cannabinoid receptors has been reported by a subcommittee of the International Union of Basic and Clinical Pharmacology (IUPHAR)(Pertwee et al., 2010).

Pharmacological Characterization

A range of pharmacological and genetic tools have been developed and used to delineate “cannabinoid receptor”-mediated activity. Five structurally distinct classes of cannabinoid compounds have been identified: the classical cannabinoids (e.g., Δ^9-THC, Δ^8-THC-dimethylheptyl (HU210)); bicyclic cannabinoids (e.g., CP-55,940); indole-derived cannabinoids (e.g., WIN 55,212), eicosanoids (e.g., the endogenous ligands; e.g., anandamide, 2-arachidonylglycerol) and antagonist/inverse agonists (e.g., SR141716A for CB_1, SR145528 for CB_2) (Devane et al., 1992; Eissenstat et al., 1995; Howlett, 1995; Mechoulam & Fride, 1995; Rinaldi-Carmona et al., 1994; Rinaldi-Carmona et al., 1998; Xie, Melvin, & Makriyannis, 1996). While many of the agonists show little selectivity between the CB_1 and CB_2 receptors, the antagonist compounds are highly selective (>1000 fold selective for CB_1 vs. CB_2 and vice versa with nanomolar affinity at the relevant receptor). The selectivity of these antagonists allows the discrimination of CB_1- vs CB_2-mediated effects in vitro and in vivo. There are some very selective CB_1 and CB_2 agonists. One example is arachidonyl-2′-chlorethylamide (ACEA) (Kearn, Greenberg, DiCamelli, Kurzawa, & Hillard, 1999), which is highly selective for CB_1 (nanomolar affinity at CB_1 and >1000 fold selectivity for CB_1 vs. CB_2). HU-308, a Δ^9-THC analog, is a highly selective CB_2 agonist with nanomolar affinity at CB_2 and >1000 fold selectivity for CB_2 vs. CB_1 (Hanus et al., 1999). Several other compounds show >100 fold selectivity and are generally classified as selective agonists. However, these compounds are used at micromolar concentrations in vitro, and therefore may be acting at both receptors (see (Pertwee et al., 2010) for more examples). Thus additional controls should be performed to ensure the site of action of these compounds.

Natural Polymorphisms and Alternative Splice Variants

Natural polymorphisms have been identified in both the CB_1 and CB_2 receptors. In addition, alternative splice variants have been identified for both receptors. This literature is summarized below.

The CB_1 receptor gene (CNR1) is located on human chromosome 6q14-15 (Bonner, 1996). Several human CB_1 receptor polymorphisms have been identified. The initial polymorphism found was a restriction fragment length polymorphism (RFLP) in the intron preceding the coding exon of the receptor (Caenazzo et al., 1991). The CB_1 receptor gene is intronless in its coding region, but possesses an intron 5′ to the coding exon with three putative upstream

exons (Bonner, 1996; Zhang et al., 2004). The genomic structure of the human CB_1 receptor has been reported (Zhang et al., 2004). In this study, three exons upstream of the coding exon were identified (a total of 4 exons), with a variation in the first exon. Five distinct variant exonic structures were demonstrated.

A positive association between a microsatellite polymorphism $((AAT)_n)$ in the CB_1 gene and IV drug abuse has been described (Comings et al., 1997). This polymorphism has subsequently been localized 3′ to the coding exon of the CB_1 receptor (Zhang et al., 2004). Although there are differences between populations, the CB_1 $(AAT)_n$ polymorphism has also been associated with schizophrenia (Ujike et al., 2002) as well as with depression in Parkinson's disease (Barrero et al., 2005), providing genetic evidence for a role of the cannabinoid system in these disorders. A recent systematic review of this and other polymorphisms in addictive disorders showed a significant association with illicit substance dependence but only in the Caucasian population samples and using a risk allele definition of ≥ 16 repeats (Benyamina, Kebir, Blecha, Reynaud, & Krebs, 2011).

Zhang and colleagues studied several polymorphisms in control and drug-abusing individuals from European, African and Japanese ethnicities and found association with a 5′ "TAG" haplotype that was highly associated with substance abuse in all three populations (Zhang et al., 2004). Analysis of mRNA levels from post-mortem brain samples of individuals with the TAG haplotype showed reduced expression for individuals expressing this allele.

The rs806371 polymorphism in the CNR1 promoter is a common functional variant associated with high-density lipoprotein cholesterol levels (Feng et al., 2013). Using 1% of 100,000 BioVU subject records claiming European Ancestry for further study (50% female and 50% male subjects), as well as functional assays, this polymorphism was found to alter HDL-C level in humans by generating a novel regulatory DNA-binding site capable of reducing CNR1 expression.

The rs2180619 polymorphism in the CNR1 promoter has recently been found to be associated with working memory in a Mexican-mestito population (Ruiz-Contreras et al., 2016), where the G allele was associated with a decrement. A previous report found that the G allele was more frequent in subjects with polysubstance abuse (Zhang et al., 2004). The GG genotype of the rs2180619 in combination with the SS genotype of a polymorphism in the promoter of the 5-HTTLPR gene (which encodes a serotonin transporter) was associated with higher anxiety compared with other genotypes (Lazary et al., 2009). This polymorphism is, therefore, associated with a variety of symptoms, but further work is needed to confirm these reports.

The first polymorphism in the coding exon described was a silent mutation in T453 (G to A), a conserved amino acid present in the C terminal region of the CB_1 and CB_2 receptors, that was a common polymorphism in the German population (Gadzicki, Muller-Vahl, & Stuhrmann, 1999). While this mutation is silent, analysis of several human sequences present in the database reveals that CB1K5 (accession #AF107262), a full length sequence, contains 5 nucleotide changes, three of which result in amino acid differences.

Coincidentally, two amino acid differences are in the third transmembrane domain, F200L and I216V. The third variant is in the fourth transmembrane domain, V246A. A report by the group that submitted the sequence to the database revealed that this was a somatic mutation in an epilepsy patient; i.e., DNA obtained from their blood was unaltered, but DNA from the hippocampus showed the mutation (Kathmann, Haug, Heils, Nothen, & Schlicker, 2000). The presence of a somatic mutation rather than a polymorphism is generally indicative of the disease process in cancers (e.g. mutant p53 or APC expression in tumors but not normal tissues (Baker et al., 1989; Lamlum et al., 2000)). CB_1 receptor polymorphisms may affect responsiveness to cannabinoids.

Shortly after its molecular cloning, splice variants of the human CB_1 receptor were identified. A PCR amplification product was isolated that lacked 167 base pairs of the coding region of the human CB_1 receptor (Shire et al., 1995). This alternative splice form (CB_{1a}) is unusual in that it is generated from the mRNA encoding CB_1, and not from a separate exon (Shire et al., 1995). When expressed, the CB_{1a} clone would translate to a receptor truncated by 61 amino acid residues with 28 amino acid residues different at the amino-terminal. A second splice variant of the coding region has been reported in which a 99 base portion of the coding exon is spliced out of the human mRNA leading to an in-frame deletion of 33 amino acids (Ryberg et al., 2005). This hCB_{1b} cDNA was isolated while cloning the previously reported splice variant. Both the CB_{1a} and CB_{1b} variants showed altered ligand binding and [^{35}S]GTPγS binding activity compared with CB_1 when the cDNAs were expressed in HEK293 cells (Ryberg et al., 2005). Of the six cannabinoids tested, only 2-AG showed significant affinity for hCB_{1b}; furthermore, 2-AG acted as an inverse agonist at both variants. Anandamide was able to activate the variants at concentrations > 10 μM. However, Δ^9-THC, CP55940, WIN55212, HU210 and SR141716 exhibited good affinity and [^{35}S]GTPγS binding activity with the variants. hCB_{1a} and hCB_{1b} expression has been detected at very low levels in many human tissues by RT-PCR; less than 5% of hCB_1 (Ryberg et al., 2005; Shire et al., 1995; Xiao et al., 2008). However, a subsequent study found no differences in the pharmacology of the variants with respect to the wild-type receptor when each was expressed in CHO cells (Xiao et al., 2008). Also, when the splice variants were expressed in mouse hippocampal neurons cultured from CB_1 null mice, yet a different profile arose (Straiker, Wager-Miller, Hutchens, & Mackie, 2012). In this expression system, the splice variants were less efficacious than the full-length version in producing the measured response, which was depolarization-induced suppression of excitation. Neither splice variant is present in rat or mouse, because the splice consensus sequence is absent in these genes (Bonner, 1996). The presence of the splice variants was reported in human and macaque brains using a commercially available antibody (Bagher, Laprairie, Kelly, & Denovan-Wright, 2013). These authors found that each splice variant could form heterodimers with hCB_1 and increase its cell surface expression, when the constructs were co-expressed in HEK293 cells. These data suggest that the splice variants may play an important physiological role as regulators of the endocannabinoid system. In sum, the genomic studies implicate the CB_1 receptor in drug addiction and disease.

Polymorphisms in the CB_2 receptor have also been associated with disease phenotypes (Karsak et al., 2005; Sipe, Arbour, Gerber, & Beutler, 2005). The human CB_2 gene (CNR2) is located at chromosome 1p36. Polymorphisms of the human CB_2 gene are linked to

osteoporosis in several studies (Karsak et al., 2005; Karsak et al., 2009; Yamada, Ando, & Shimokata, 2007). Karsak et al examined CB_1 and CB_2 receptor DNA in a sample of French post-menopausal patients and female controls. The authors report that certain changes in CB_2 receptor, but not the CB_1 receptor, were strongly associated with osteoporosis (Karsak et al., 2005). A second study replicated these findings in a group of pre- and post-menopausal Japanese women (Yamada et al., 2007). In contrast, a recent study has found only nominally significant correlations with CB_2 polymorphisms and osteoporosis in a Chinese population; the role of the CNR2 gene in the etiology of Chinese osteoporosis thus requires further study in larger samples (Huang, Li, & Kung, 2009).

A related study examined the role of CB_2 DNA or genes on hand bone strength (Karsak et al., 2009). The authors analyzed radiographic images and DNA samples from a Chevashian population, an ethnically homogeneous population of people of Bulgaric ancestry that live along the Volga River. Several SNPs (small nucleotide polymorphisms) were significantly associated with certain bone phenotypes as previously reported (Karsak et al., 2005). Two of the associated SNPs were in adjacent nucleotides (“double SNP” rs2502992–rs2501432) within the coding region of CB_2 and result in a non-conservative missense variant (Gln63Arg, also referred to as the Q63 variant and the CB2-63 nonsynonymous polymorphism). This Q63 variant is probably functionally relevant as demonstrated by a differentially endocannabinoid-induced inhibition of T lymphocyte proliferation (Sipe et al., 2005). A less functional form of the CB_2 receptor appears to lead to weak hand bone strength and is associated with osteoporosis.

Because the CB_2 receptor is associated with immunomodulation, many studies have investigated a link between CNR2 polymorphisms and various immune disorders. The most widely studied is the Q63 variant, which has been found to be associated with hepatitis (Coppola et al., 2015), as well as other immune mediated disorders (Coppola et al., 2016), such as chronic child immune thrombocytopenia (Mahmoud Gouda & Mohamed Kamel, 2013). Intriguingly, this polymorphism along with two others in the CNR2 gene have been associated with schizophrenia in a Japanese population (Ishiguro, Horiuchi, et al., 2010). These authors also found reduced responsiveness of the R63 variant when it was heterologously expressed, confirming the earlier report (Sipe et al., 2005). These same authors found an association with eating disorders and this CNR2 polymorphism (Ishiguro, Carpio, et al., 2010) as well as with depression in a Japanese population (Onaivi, Ishiguro, Gong, Patel, Meozzi, Myers, Perchuk, Mora, Tagliaferro, Gardner, Brusco, Akinshola, Hope, et al., 2008; Onaivi, Ishiguro, Gong, Patel, Meozzi, Myers, Perchuk, Mora, Tagliaferro, Gardner, Brusco, Akinshola, Liu, et al., 2008).

Another study has reported an association between bipolar disorder and the 524A/C (Leu133Ile, rs41311993) polymorphism in an Italian population (Minocci et al., 2011). This residue is present in the third transmembrane domain and has been suggested to be important for the stability and/or the functionality of the receptor, but this has not been directly examined (Xie, Chen, & Billings, 2003). Although the presence of CB_2 in the normal brain has been controversial, there is a consensus that CB_2 is expressed on microglia during neuroinflammation, and a neuroimmunological etiology of bipolar disorder has been

suggested (Minocci et al., 2011), thereby providing a link between CB_2, neuroinflammation and psychiatric diseases.

Phylogeny

Comprehensive reviews of cannabinoid receptor phylogeny have been published (Elphick, 2012; McPartland, 2004); we provide here a brief summary of their pharmacology. The CB_1 receptors are highly conserved among vertebrate species and have also been found in some invertebrates (Elphick & Egertova, 2001; McPartland & Glass, 2003; Murphy et al., 2001). The cannabinoid receptor was originally cloned from rat (Matsuda et al., 1990); shortly thereafter, isolation of a human CB_1 receptor cDNA was reported (Gerard, Mollereau, Vassart, & Parmentier, 1991). The human CB_1 receptor has one less amino acid in the N-terminus as compared to the other mammalian species (472 amino acids vs. 473 amino acids). The rat and human receptors are highly conserved, 93% identity at the nucleic acid level and 97% at the amino acid level. Similarly, the mouse and rat clones have 95% nucleic acid identity (100% amino acid identity) and the mouse and human clones have 90% nucleic acid identity (97% amino acid identity) (M.E. Abood, Ditto, Noel, Showalter, & Tao, 1997; Chakrabarti, Onaivi, & Chaudhuri, 1995; Ho & Zhao, 1996). A meta-analysis of the literature examining cannabinoid ligand binding affinity revealed subtle interspecies differences for the binding affinities of some ligands (Δ^9-THC, CP55,940, WIN55,212-2, SR141716A) for rat vs. human CB_1 receptors (McPartland, Glass, & Pertwee, 2007).

The sequence diversity of the CB_1 receptor showed a variance from 0.41–27% in 62 mammalian species using a molecular phylogenetic analysis (Murphy et al., 2001). In addition to mammals, the CB_1 receptor has been isolated from birds (Soderstrom, Leid, Moore, & Murray, 2000), fish (Yamaguchi, Macrae, & Brenner, 1996), amphibia (Cottone, Salio, Conrath, & Franzoni, 2003; Soderstrom et al., 2000), and an invertebrate, *Ciona intestitinalis* (Elphick, Satou, & Satoh, 2003), among others. This deuterostomian invertebrate CB receptor contains 28% amino acid identity with CB_1, and 24% with CB_2 (Elphick et al., 2003). Since a CB receptor ortholog has not been found in *Drosophila melanogaster* or *Caenorhabditis elegans*, it has been suggested that the ancestor of vertebrate CB_1 and CB_2 receptors originated in a deuterostomian invertebrate (Elphick et al., 2003).

The CB_2 receptor was initially isolated from HL60 cells, a human promyelocytic leukemic cell line (Munro et al., 1993). In addition to the human CB_2 receptor, clones have been isolated from mouse (Shire et al., 1996; Valk et al., 1997), rat (Griffin, Tao, & Abood, 2000) (Brown, Wager-Miller, & Mackie, 2002; Q. R. Liu et al., 2009), dog (Ndong, O'Donnell, Ahmad, & Groblewski, 2011), the puffer fish *Fugu rubripes* (Elphick, 2002) as well as zebrafish (McPartland, Glass, Matias, Norris, & Kilpatrick, 2007). There is also information in the GenBank database on additional species. The CB_2 receptor shows less homology between species than does CB_1; for instance the human and mouse CB_2 receptors share 82% amino acid identity (Shire et al., 1996), and the mouse and rat 93% amino acid identity. The human, rat and mouse sequences diverge at the C-terminus; the mouse sequence is 13 amino acids shorter, whereas the rat clone is 50 amino acids longer than the human CB_2 (Brown et al., 2002).

The first evidence for alternative splice forms of CB_2 was in the C-terminus of the rat CB_2 receptor (Brown et al., 2002; Griffin et al., 2000). That this may give rise to rat-specific pharmacology of the CB_2 receptor was suggested by differences in ligand recognition with a number of compounds at the rat CB_2 receptor compared to the human CB_2 receptor in transfected cells (Griffin et al., 2000). The clone described in these studies was amplified from genomic DNA rat CB_2; however this isoform has subsequently been shown to be the major splice form of rat CB_2 (Q. R. Liu et al., 2009). Now, variants of the human and mouse CB_2 receptors have been reported as well (Q. R. Liu et al., 2009).

In summary, from what we know so far, the diversity in the regulatory regions of the CB_1 and CB_2 genes may provide extensive flexibility in gene regulation of these receptors in health and disease. A ‘clinical endocannabinoid deficiency syndrome’ resulting from defects in the endocannabinoid system (i.e. receptor mutations, alterations in endocannabinoid production), has already been proposed to underlie certain diseases including treatment resistant conditions (Russo, 2008). To date a mutation is yet to be identified in the human cannabinoid receptor that results in conclusive alteration of ligand-receptor interactions; however, we have discovered amino acids residues important for selective ligand recognition and maintaining receptor-ligand interactions *in vitro* (Kapur, Samaniego, Thakur, Makriyannis, & Abood, 2008; Song & Bonner, 1996). The efficacy of future cannabis-based clinical trials could be enhanced by developing patient screening methods for polymorphisms or mutations in genes associated with the endocannabinoid system.

Distribution

The CB_1 receptor is one of the most abundant GPCRs in the brain; it is highly expressed in the basal ganglia nuclei, hippocampus, cortex and cerebellum (Glass, Dragunow, & Faull, 1997; Herkenham et al., 1990) (Tsou, Brown, Sanudo-Pena, Mackie, & Walker, 1998) (reviewed in (Howlett et al., 2002)). The distribution of this receptor within the central nervous system correlates with its role in the control of motor function, cognition and memory, and analgesia. CB_1 receptors are primarily localized to the terminals of central and peripheral neurons, where they mediate inhibition of neurotransmitter release (reviewed in (Szabo & Schlicker, 2005)). CB_1 receptors are found at significantly higher levels on GABAergic than glutamatergic neurons in various brain regions (Katona et al., 2001; Katona et al., 1999; Puighermanal et al., 2009). CB_1 receptors are also present on astrocytes, where they are expressed at much lower levels than on neurons; but where they have been shown to modulate synaptic transmission and plasticity (Han et al., 2012)(reviewed in (Oliveira da Cruz, Robin, Drago, Marsicano, & Metna-Laurent, 2016)). There has been some controversy regarding CB_1 receptor expression in other glial subtypes *in situ* (Stella, 2010).

The CB_1 receptor is also expressed throughout the periphery, albeit at much lower levels than in the CNS (reviewed in (Howlett et al., 2002)). Early after its identification, the CB_1 receptor was detected in a variety of circulating immune cells (Galiegue et al., 1995) (Bouaboula et al., 1993). Furthermore, the level of CB_1 expression appears to be increased or decreased during immune cell activation (reviewed in (Klein, 2005)). This is also the case with CB_2 expression as described below. CB_1 is expressed in numerous peripheral tissues,

including the adrenal gland, heart, lung, prostate, liver, uterus, ovary, testis, vas deferens, bone marrow, thymus and tonsils (Galiegue et al., 1995).

The CB_2 receptor is abundantly expressed in peripheral organs with immune function, including macrophages, spleen, tonsils, thymus, and leukocytes, as well as the lung and testes (Brown et al., 2002; Galiegue et al., 1995; Munro et al., 1993). Initial studies suggested that CB_2 receptors were absent from the healthy brain (Brown et al., 2002; Griffin et al., 1999). Subsequently, studies have now shown CB_2 receptor expression in diseased brain cells, including astrocytomas (Ellert-Miklaszewska, Grajkowska, Gabrusiewicz, Kaminska, & Konarska, 2007; Sanchez et al., 2001), microglia and astrocytes in Alzheimer's disease (Benito et al., 2003; Esposito et al., 2007), and T cells, microglia and astrocytes in multiple sclerosis (Benito et al., 2007). These studies and others indicate that the CB_2 receptor is up-regulated in response to immune cell activation and inflammation (Klein, 2005; Stella, 2010). More recently, CB_2 receptor expression has been reported in the healthy CNS (Van Sickle et al., 2005), although its presence in adult native brain tissue remains somewhat controversial (Atwood & Mackie, 2010; Soethoudt et al., 2017).

Cannabinoid Receptor Signaling Pathways associated with Differentiated Tissues

The CB_1 cannabinoid receptor was originally discovered based upon its signaling as a GPCR coupled to the Gi/o α proteins that inhibit adenylyl cyclase thereby reducing cellular cAMP levels (for overview, see (Howlett, 1990, 1995). For an overview, please consult the following excellent reviews that have highlighted CB_1 signal transduction (Console-Bram, Marcu, & Abood, 2012; Howlett, 2005; Howlett et al., 2002; McAllister & Glass, 2002; Turu & Hunyady, 2010). The CB_2 receptor cellular signaling has been characterized as Gi/o-coupled signaling, although Gi/o inhibits cAMP production with varying efficacy depending upon experimental model and agonist used (reviewed in (Dhopeshwarkar & Mackie, 2014; Turcotte, Blanchet, Laviolette, & Flamand, 2016)). In addition to Gi/o-mediated signaling, CB_1 and CB_2 receptors are phosphorylated by G protein receptor kinases (GRKs) and subsequently associate with β-arrestin1 or β-arrestin2 (Breivogel et al., 2013; Chen et al., 2014), which can serve as a scaffold for interaction with proteins that divert signaling along β-arrestin-mediated pathways. Both CB_1 and CB_2 receptors stimulate extracellular signal regulated kinase (ERK)1 and 2, involving either Gβγ or β-arrestin interactions. We are just beginning to appreciate the cellular signaling pathways that make up the phenotype for healthy differentiated cell types as well as significant modifications in signaling pathways in states of disease. Some of these pathways are exemplified herein.

Signaling in smooth muscle cells

The ability of cannabinoid agonists to attenuate contraction of vas deferens smooth muscle was among the first bioassays for this pharmacological class (Howlett et al., 2002) and now extends to clinical relevance for diseases associated with smooth muscle regulation. Several signal transduction pathways have been identified that are regulated by the CB_1 receptor to attenuate smooth muscle cell contraction. Smooth muscle contraction requires a Ca^{2+}-mediated pathway leading to phosphorylation of myosin light chain. Pathways by which

stimulation of CB_1 receptors can signal, culminating in the interference of contraction, have been identified in several model systems.

Vas deferens—In Syrian hamster vas deferens smooth muscle DDT1MF-2 cells, Δ^9-THC evoked a large capacitative Ca^{2+} influx accompanied by a small release of intracellular Ca^{2+} (Filipeanu, de Zeeuw, & Nelemans, 1997). These responses were demonstrated to be due to CB_1 pathways by sensitivity to SR141716, and the capacitative Ca^{2+} mechanism by sensitivity to the sarcoplasmic/endoplasmic reticulum Ca^{2+} pump inhibitor thapsigargin. The capacitative Ca^{2+} influx was in part responsible for a CP55940-stimulated CB_1 receptor and Gi/o-mediated activation of a large conductance Ca^{2+}-dependent K^+ channel that was dependent upon both an inhibition of cAMP production as well as the activation of ERK1/2 (Begg, Baydoun, Parsons, & Molleman, 2001). It is likely that the ERK1/2 effects on channel regulation occur via a pathway involving a PLA_2-mediated release of arachidonic acid, which activates a non-capacitative Ca^{2+} entry (Demuth et al., 2005).

Vascular arterioles—Studies of isolated vascular components have identified a cellular mechanism that occurs in vascular smooth muscle cells isolated from cat cerebral microvessels which express the CB_1 receptor (Gebremedhin, Lange, Campbell, Hillard, & Harder, 1999). A nifedipine-sensitive L-type Ca^{2+} current was attenuated by either anandamide or WIN55212-2. The CB_1 and Gi/o-dependence of the response was demonstrated by evidence that it was antagonized by SR141716 and precluded by pertussis toxin. These results correlate with the vasorelaxation of serotonin-constricted cat cerebral arterioles by anandamide or WIN55212-2, suggesting that the reduction in Ca^{2+} influx via the L-type channels can account for the vasorelaxation in vascular smooth muscle cells.

Gastric smooth muscle—The signaling pathway utilized by CB_1 receptors in gastric smooth muscle cells to attenuate acetylcholine (M3 muscarinic)- and Gq-mediated contraction was comprehensively described by Mahavadi and colleagues (Mahavadi, Sriwai, Huang, Grider, & Murthy, 2014). In dispersed or cultured rabbit gastric smooth muscle cells, anandamide stimulation of the CB_1 receptor activated predominantly Gi2, and inhibited cAMP accumulation. However, unlike for other Gi-coupled receptors in these cells, the $G\beta\gamma$ released by CB_1 receptor stimulation failed to initiate PLC-mediated phosphatidylinositol hydrolysis required for contraction of the cells. Rather, anandamide attenuated the acetylcholine-mediated contraction by a signaling pathway occurring via GRK5-phosphorylation of the CB_1 receptor and recruitment of β-arrestins, leading to activation of ERK1/2 and Src kinases for a two-pronged attenuation process. The ERK1/2 phosphorylated the regulator of G protein signaling 4 (RGS4) to promote inactivation of $G\alpha q$ and subsequent reduction in acetylcholine-mediated phosphatidylinositol hydrolysis that initiates contraction. The Src kinase promoted interaction of Rho1 with RhoA-myosin phosphatase1 interacting protein, thereby inhibiting Rho kinase as well as activating myosin light chain phosphatase, leading to net dephosphorylation of myosin light chain and inhibition of the sustained contraction.

Myometrium—Human myometrial strips, obtained as biopsies during C-section deliveries, express CB_1 receptors and respond to anandamide or Δ^9-THC with an SR141716-sensitive

relaxation of oxytocin-induced contractions (Dennedy et al., 2004). Cellular signaling characterized in human myometrial smooth muscle ULTR cells (Brighton et al., 2009) and non-pregnant human myometrial cells in primary culture (Brighton, Marczylo, Rana, Konje, & Willets, 2011) indicated that stimulation of the CB_1 receptor by anandamide or methanandamide could promote an early phase ERK1/2 phosphorylation in response to the sequential activation of Gi/o, phosphatidylinositol-3-kinase (PI3K), and a Src kinase. In ULTR cells, the role of CB_1 receptors (but not CB_2 receptor or TRP channels) was established (Brighton et al., 2009). Interestingly, the desensitization of the cAMP inhibition response in primary myometrial cells was entirely abolished by transfection with siRNA to negate translation of β-arrestin2. However, after that same β-arrestin2-knockdown, the ERK1/2 phosphorylation was augmented and sustained (Brighton et al., 2011). These findings suggests that β-arrestin2 mediates desensitization of Gi/o-driven responses, but that β-arrestin1 mediates processes associated with a prolonged ERK1/2 activation in myometrial smooth muscle.

Signaling in metabolic regulation and disease

Liver development and function—Studies of zebra fish (Danio Rerio) embryonic development demonstrated that liver differentiation (but not heart, pancreas or kidney differentiation) requires functional CB_1 and CB_2 receptor signaling (L. Y. Liu et al., 2016). Receptor knockout or antagonism resulted in defective biliary morphogenesis, developmental reduction of hepatocyte proliferation and liver mass, as well as a functional reduction in gene expression of liver-specific enzymes, a protective metabolic response in $CB_1{-}/{-}$ embryos to the physiological insults of either ethanol or “high fat” egg yolk, and an increased appearance of steatosis in CB_2 $-/-$ adults (L. Y. Liu et al., 2016). The deficits induced by cannabinoid receptor deficiency were the result of reduced sterol regulatory element-binding transcription factor(s) (Srebf) expression that persists into adulthood (Jeong et al., 2008; Pai et al., 2013). The decreased Srebf led to reduced methionine pathway intermediates, findings that were also observed in livers from $CB_1{-}/{-}$ mice (Liu et al., 2016). This resulted in a generalized pattern of reduced methylation of proteins (Liu et al., 2016), implicating a reduction in S-adenosylmethionine as a methyl donor for nucleic acid, phospholipid, and protein methylation critical for epigenetic regulation. The aberrant hepatogenesis could be overcome by overexpression of Srebf1 during development, but not entirely overcome by methionine replacement, suggesting that cannabinoid receptors and Srebf exert additional developmental regulatory functions not involving methylation.

The endocannabinoid system plays an integral role in mediating homeostasis in metabolic regulation, as has become evident in pathological states that require adjustment such as high fat or chronic alcohol diets (as described in (Tam et al., 2011). In such perturbations, CB_1 receptors are increased in hepatocytes and contribute to the ensuing insulin resistance and dyslipidemia. CB_1 receptors in stellate cells are engaged in fibrogenic activity in diseased states, and CB_2 receptors are induced in response to pathological states including fatty liver disease and liver fibrosis. Both Δ^9-THC and the CB_2-selective agonist JWH-133 reduced the proliferation rate and promoted apoptosis of stellate cells and myofibroblasts, thereby serving a hepatoprotective function (Tam et al., 2011). Thus, cannabinoid receptor signaling in liver may be uniquely targeted toward attempts to regain metabolic homeostasis.

Liver hepatocytes—Human hepatocytes express predominantly isoform CB_{1b}, which differs from the CB_1 isoform expressed in the brain, and exhibits an efficacious inhibition of adenylyl cyclase in response to CB_1-selective agonist ACEA (Gonzalez-Mariscal et al., 2016). Note that we refer to the more generic term CB_1 for the remainder of this discussion because these isoforms do not appear in rodents. CB_1 receptors are induced in human or mouse hepatocytes under conditions of high fat or alcohol diets, and this may involve a 2-AG-stimulated, CB_1 receptor-mediated "autoinduction" via the retinoid A receptor γ (RARγ) (Mukhopadhyay et al., 2010; Osei-Hyiaman et al., 2005). The 2-AG required to stimulate hepatocyte CB_1 receptors is generated by neighboring stellate cells in ethanol-induced fatty liver, and this paracrine regulation is required for the ensuing lipogenesis and suppression of fatty acid oxidation by the hepatocytes (Jeong et al., 2008; Osei-Hyiaman et al., 2008).

Increased anandamide or 2-AG levels in response to high fat diet in obese mice stimulated CB_1 receptor signaling that directs lipogenic gene expression, as CB_1 antagonists could block this pathway (Jourdan et al., 2010; Mukhopadhyay et al., 2010; Osei-Hyiaman et al., 2005). CB_1 receptor activation by 2-AG initiated signaling to induce mRNA for sterol regulatory element binding protein 1c (SREBP1c) and thereby induce fatty acid synthase (FAS), leading to increased plasma triglyceride-rich apolipoproteins (Ruby et al., 2008). Similarly, a chronic ethanol diet led to increased expression of lipogenic FAS, an effect that was precluded in hepatic CB_1 (−/−) mice (You, Fischer, Deeg, & Crabb, 2002). *In vitro* experiments in hepatoma cells indicated that this was due to the metabolite acetaldehyde initiating signaling to increase levels of SREBP1 which activated an SRE promoter (You et al., 2002).

Lipogenesis in hepatocytes is stimulated for liver's production of fatty acids for storage in an anabolic state, or inhibited when lipids are needed for energy in a catabolic state. This process is regulated by cAMP activation of PKA, which phosphorylates and inhibits the transcription factor liver X receptor-α (LXRα). When dimerized with retinoic X receptor (RXR), LXRα is responsible for inducing SREBP-1c expression. SREBP-1c is the master regulatory transcription factor that promotes expression of lipogenic genes coding for FAS as well as for acetyl co-A carboxylase (ACC), and stearoyl-CoA desaturase-1 (SCD-1). Under conditions in which fatty acids are needed for energy rather than storage, a physiological response to adrenergic stimulation would activate the PKA, which would directly phosphorylate a serine on LXRα, which inhibits the SREBP-1c transcription. Conversely, the CB_1 receptor-Gi/o complex can promote the activation of SREBP-C1 in a pertussis toxin-sensitive manner (Wu, Yang, & Kim, 2011). As described in the report by Wu and colleagues (Wu et al., 2011), the CB_1 receptor-stimulated, Gi/o-mediated inhibition of cAMP and subsequent reduction in PKA could be correlated with two separate mechanisms that attenuated SREBP-1c expression. By one mechanism, under conditions of CB_1-mediated reduction in cAMP, the PKA would no longer be activated, such that LXRα serine would be unphosphorylated and would be able to induce SREBP-1c expression. Wu and colleagues showed that when the CB_1 receptor was antagonized by SR141716, PKA could phosphorylate the LXRα-serine and inhibit transcription of SREBP-1c (in much the same way as initiating an adrenergic response). By a second (delayed) mechanism

demonstrated by Wu and colleagues (Wu et al., 2011), PKA can initiate a sequential phosphorylation of liver kinase B1 (LKB1), which phosphorylates AMP kinase (AMPK), which phosphorylates a threonine on LXRα to attenuate the induction of SREBP-1c. This delayed pathway would also be attenuated by a CB_1-mediated reduction in cAMP and PKA activity, and was shown to be augmented by the antagonism of the CB_1 receptor by SR141716. In summary, the SREBP-1c transcriptional program leading to lipogenesis can be promoted in pathological states under conditions of increased endocannabinoid-stimulated CB_1 receptor signaling via Gi/o and cAMP inhibition. The competitive antagonism of the CB_1 receptor by SR141716 can intervene to curtail that lipogenic program.

High fat or ethanol diets also reduced mitochondrial respiration and decreased mitochondrial fatty acid β-oxidation due to reduced entry of fatty acids into the mitochondria via the rate-limiting enzyme carnitine palmitoyltransferase 1 (CPT1) (Flamment et al., 2009; Osei-Hyiaman et al., 2008; Tam et al., 2010). This signaling pathway is mediated by agonist-stimulated CB_1 receptors reducing CPT1 activity. Studies by Tedesco and colleagues investigated the effects of stimulating the CB_1 receptors in liver after six weeks of high fat diet. The diet-induced increased body mass and adiposity could be further augmented by chronic (four weeks) treatment with the CB_1-selective agonist ACEA (Tedesco et al., 2008). The signaling pathway for this augmentation involved an increase in p38 MAPK phosphorylation and a reduction in AMPK phosphorylation in ACEA-treated mice. ACEA treatment exacerbated the reduction in endothelial nitric oxide synthase (eNOS) mRNA in the liver of the obese mice. Similar exacerbation was observed for the high fat diet-induced decrements in total mitochondrial DNA as well as mRNA for mitochondrial functional proteins PPARγ-coactivator-1α (PGC-1α), nuclear respiratory factor-1 (NRF-1) and mtDNA transcription factor A (MTfam), protein levels of cytochrome C oxidase IV and cytochrome C, and the activity of citrate synthase. These findings demonstrate that the ACEA treatment down-regulates mitochondrial biogenesis in the liver of high fat diet-induced obese mice. However, one caveat is that these experiments failed to include a group treated with ACEA plus a CB_1 receptor-selective antagonist in order to confirm that these responses were occurring solely as the result of a CB_1 receptor mechanism.

High fat diet induces insulin resistance at the level of hepatocytes, and this is believed to be initiated by Endoplasmic Reticulum (ER) stress in mice expressing hepatic CB_1 receptors but not $CB_1{-}/{-}$ mice (J. Liu et al., 2012). The injection of anandamide promoted the same markers of ER stress, confirming a role for the endocannabinoid system. CB_1 receptor involvement in high fat diet-induced pathology began with pertussis toxin-sensitive Gi/o signaling through a pathway that led to the phosphorylation of Insulin Receptor Substrate 1 (IRS1) at ser307 (J. Liu et al., 2012). This stimulated induction of the ser/thr phosphatase, *PH* domain leucine-rich repeat protein phosphatase-1 (Phlpp1), thereby reversing insulin-stimulated phosphorylation of protein kinase B (also known as akt-2), which increased glycogen phosphorylase a activity, culminating in insulin-resistant glycogenolysis. The IRS1 phosphorylation also resulted in suppressed expression of hepatic insulin degradation enzyme (IDE), resulting in reduced insulin clearance and a consequent hyperinsulinemia (Liu et al., 2012).

Gluconeogenesis is regulated in the liver by the CB_1 receptor via the induction of the liver-specific, ER-bound transcription factor cAMP-responsive element binding protein H (CREBH). CREBH is an ER stress-associated liver-specific transcription factor (Chanda et al., 2011). In studies of primary cultures of rat or human hepatocytes, 2-AG-stimulation of CB_1 receptors promoted phosphorylation of jun N-terminal kinase (JNK) and ERK1/2. JNK could phosphorylate c-Jun, allowing formation of Activating Protein 1 (AP-1). An AP-1 activation of its binding site on the CREBH promoter would lead to induction of CREBH (Chanda et al., 2011). CREBH in turn, promotes the induction of gluconeogenic genes (phosphoenolpyruvate carboxykinase (Pepck), glucose-6-phosphatase catalytic subunit (G6pc), and PGC1), leading to glucose production. The CB_1 antagonist AM251 mimicked the response to insulin to reduce CREBH gene expression and attenuate gluconeogenesis in cultured hepatocytes (Chanda et al., 2011). Interestingly, in these same studies, 2-AG also induced CB_1 receptor gene expression in cultured hepatocytes (Chanda et al., 2011), which has the potential to augment subsequent responses.

Liver stellate cells, myofibroblasts and bile duct epithelial cholangiocytes— CB_1 receptors were induced in stellate cells and myofibroblasts in human cirrhosis and mouse models of fibrosis, and antagonism by SR141716 could ultimately decrease fibrogenesis. Stimulation of these augmented CB_1 receptors by anandamide could increase TGFβ1 levels, leading to proliferation and cytoprotection of myofibroblast fibrogenic cells, and increased fibrogenesis (Teixeira-Clerc et al., 2006).

CB_2 receptors were induced in hepatic stellate cells and myofibroblasts in conditions of fatty liver disease and liver fibrosis (Julien et al., 2005; Mendez-Sanchez et al., 2007). Anandamide stimulation of CB_2 receptors in cholangiocytes initiated a signaling pathway via induction of AP-1 and thioredoxin 1 (also known as redox factor 1), leading to production of reactive oxygen species and cell death (DeMorrow et al., 2008).

White adipocytes—Adipocyte differentiation is accompanied by an increased expression of the CB_1 receptor and by increased mitochondrial biogenesis, as an important means of regulating metabolic function (Bensaid et al., 2003; Engeli et al., 2005). The CB_1 receptor plays a key role in reducing energy utilization and increasing adiposity by suppressing mitochondrial mass and function (Tedesco et al., 2008; Tedesco et al., 2010). The cellular signaling mechanisms can be inferred from studies of mouse primary white adipocytes in culture, in which the antagonism of the CB_1 receptor by SR141716 led to a persistent increase in AMPK phosphorylation and activity (Tedesco et al., 2008; Tedesco et al., 2010). AMPK can activate eNOS by phosphorylation at Ser1177, leading to NO production (Morrow et al., 2003). In the process of mitochondrial biogenesis, NO regulation of NO-sensitive guanylyl cyclase stimulates cGMP production, PKG activation, and gene expression of signaling enzymes such as PGC-1α (Nisoli & Carruba, 2006). This process can be inhibited by the pro-inflammatory cytokine TNFα released from white and brown fat stores in obese rodents, which induced iNOS and inhibited eNOS expression (Merial-Kieny et al., 2003; Valerio et al., 2006). The observations that SR141716 increased mitochondrial DNA, and mRNA for key enzymes that regulate mitochondrial biogenesis (PGC-1α, NRF-1 and MTfam) in cultured adipocytes suggests that the CB_1 receptor must exert an inhibitory

role in limiting mitochondrial biogenesis (Tedesco et al., 2008; Tedesco et al., 2010). SR141716 increased mitochondrial oxidative phosphorylation functions by increasing cyclooxygenase IV and cytochrome c protein levels, citrate synthase activity, and oxygen consumption (Tedesco et al., 2008; Tedesco et al., 2010), as would be expected if the CB_1 receptor precluded mitochondrial expansion. The responses to CB_1 antagonism in cultured adipocytes were recapitulated in $CB_1-/-$ mice placed on standard or high-fat diets, lending credence to a role for the CB_1 receptor in impairment of this metabolic signaling pathway *in vivo* (Tedesco et al., 2008; Tedesco et al., 2010).

High fat diet alters adipocyte functions in an effort to adjust to the anabolic state. In this fed state, serum endocannabinoid levels are increased (Engeli et al., 2005; Matias et al., 2006). In high fat diet, epididymal white adipose tissue levels of phosphorylated AMPK (Thr172) and AMPK activity are reduced in WT mice but not in $CB_1-/-$ mice (Tedesco et al., 2008), implicating the CB_1 receptor in the processes associated with this metabolic adjustment. In cultured adipocytes, SR141716 increased AMPK phosphorylation within 10 minutes, and this phosphorylation was sustained for at least two days under the influence of SR141716 (Tedesco et al., 2008). Outcomes that result from SR141716 treatment of mouse 3T3F442A adipocytes include an induction of the beneficial cytokine adiponectin (also known as Acrp30) mRNA and protein (Bensaid et al., 2003), whereas CB_1 receptor stimulation decreases adiponectin expression (Matias et al., 2006). CB_1 receptor stimulation increases fat storage determined as lipid droplets in cultured 3T3-F442A adipocytes (Matias et al., 2006).

Cannabinoid Receptor Signaling in neuronal cells

Cellular signaling in neurons has been described as several prototypical signal transduction pathways. Excellent reviews have described the neurophysiology associated with retrograde short-term or long-term regulation of neurotransmitter release by CB_1 receptors (Kano, 2014; Lu & Mackie, 2016), signaling to the nucleus to regulate neuronal differentiation, migration and neurite extension in neurodevelopment (Diaz-Alonso, Guzman, & Galve-Roperh, 2012; Gaffuri, Ladarre, & Lenkei, 2012; Maccarrone, Guzman, Mackie, Doherty, & Harkany, 2014) and synapse remodeling (Busquets Garcia, Soria-Gomez, Bellocchio, & Marsicano, 2016), and the CB_1 and CB_2 receptor functions associated with neuroprotection (Fernandez-Ruiz, Moro, & Martinez-Orgado, 2015; Navarro et al., 2016). We will briefly describe two examples of cellular signaling that extend from CB_1 receptor-Gi/o stimulation that have been investigated in model neuronal systems and brain.

CB_1 receptor regulation of Focal Adhesion Kinase and Integrin signaling for actin cytoskeleton organization and cell adhesion—CB_1 receptor signaling is important for cellular matrix interactions at the focal adhesions, actin cytoskeletal reorganization, and the scaffolding to multiple proteins via the tyrosine phosphorylation of pp125 Focal Adhesion Kinase (FAK). FAK is a non-receptor tyrosine kinase that acts as a scaffolding protein within focal adhesions to participate in organization of the actin cytoskeleton, migration, and cell adhesion (Franchini, 2012; Schaller, 2010). CB_1 receptor-Gi/o signaling promoted phosphorylation of tyrosines on FAK in hippocampal slices (Derkinderen et al., 1996; Derkinderen et al., 2001). Gi/o-mediated inhibition of cAMP

synthesis and decreased PKA activity were required for FAK tyrosine phosphorylation (Derkinderen et al., 1996). The FAK autophosphorylation site (tyrosine 397) initiates FAK activation, followed by Src family kinases binding to phospho-tyrosine 397 to phosphorylate additional tyrosine residues. The phosphotyrosines serve as scaffolds for proteins that regulate cell adhesion, migration, and survival. In the N18TG2 neuroblastoma model, CB_1 receptor-stimulated FAK tyrosine397 phosphorylation was low in magnitude and dependent upon Src. Once phosphorylated, FAK tyrosine397 bound Src, which phosphorylated tyrosines576/577 to obtain full FAK catalytic activity. FAK tyrosine576/577 phosphorylation was governed by reduced PKA activation, which leads to protein tyrosine phosphatase (PTP1B, Shp1/Shp2)-mediated Src activation. Src-mediated phosphorylation at tyrosine925 creates an SH2 binding site for the adaptor protein Grb2 to initiate the ERK1/2 signaling cascade Ras-Raf-MEK-ERK1/2 (Dalton, Peterson, & Howlett, 2013).

CB_1 receptor signaling to FAK is also dependent upon extracellular matrix engagement by integrins. In the N18TG2 cell, fibronectin (α5β1) and laminin (α6β1, α7β1) integrin receptors are endogenously expressed (Dalton et al., 2013). Cells attached to fibronectin or laminin surfaces exhibited significantly higher basal FAK tyrosine397 and tyrosine576/577 phosphorylation compared with suspended cells, and this phosphorylation could be augmented by CB_1 agonists. The RGDS peptide integrin antagonist significantly reduced CB_1-mediated FAK phosphorylation in adherent N18TG2 cells, and α5 integrin silencing with siRNA also decreased FAK tyrosine576/577 phosphorylation (Dalton et al., 2013). RGDS peptide disrupted CB_1-mediated hippocampal FAK activation, demonstrating that the results from the neuronal model could also be observed in a brain preparation (Karanian, Brown, Makriyannis, & Bahr, 2005).

CB_1 Receptor regulation of gene expression in neurite elongation—Neurite elongation in the N2A neuroblastoma model is regulated by CB_1 receptor signaling via Gαi/o (Bromberg, Iyengar, & He, 2008; He et al., 2005; He, Neves, Jordan, & Iyengar, 2006; Jordan et al., 2005). CB_1 receptor-mediated Gαi/o attenuated the ability of the Rap1-GTPase activating protein (GAP) to terminate Rap1activation by facilitating the ubiquitination of Rap1-GAP, thereby promoting its degradation by proteasomes (Jordan et al., 2005). Active Rap1-GTP signals to small G protein Ral, which led to phosphorylation and activation of Src (He et al., 2005; Jordan et al., 2005). HU210-stimulated CB_1 receptor evoked a sustained (hours) phosphorylation of Src kinase and transcription factor signal transducer and activator of transcription3 (Stat3) (He et al., 2005). Activation of Stat3 required both a direct phosphorylation at tyrosine by Src kinase, as well as an indirect activation of the small G protein Rac-GTP by Src kinase, thereby activating JNK to phosphorylate a serine on Stat3. Both Src and JNK were required for the CB_1 receptor-mediated Stat3 activation. Phosphorylated and dimerized Stat3 could enter the nucleus to promote transcription necessary for neurite elongation. This pathway could be reversed by activation of the phosphotyrosine phosphatase SHP2 which dephosphorylated and thereby inactivated Stat3 (Zorina, Iyengar, & Bromberg, 2010).

Extended Agonist Exposure

Cannabinoid tolerance develops in the absence of pharmacokinetic changes (Martin, Dewey, Harris, & Beckner, 1976); therefore, biochemical and/or cellular changes are responsible for this adaptation. One hypothesis for tolerance development is that receptors lose function during chronic agonist treatment leading to diminished biological responses. The phenomenon of receptor down-regulation has been observed in many brain receptor systems. While an early study failed to detect changes in either receptor number or mRNA levels in whole brains from mice tolerant to Δ^9-THC (M. E. Abood, Sauss, Fan, Tilton, & Martin, 1993), brain region specific changes are observed (Breivogel et al., 1999; McKinney et al., 2008; Oviedo, Glowa, & Herkenham, 1993; Rodriguez de Fonseca, Gorriti, Fernandez-Ruiz, Palomo, & Ramos, 1994; Romero et al., 1997). A comprehensive study examining the time course of changes in cannabinoid-stimulated [^{35}S]GTPγS binding and cannabinoid receptor binding in both rat brain sections and membranes, following daily Δ^9-THC treatments for 3, 7, 14, and 21 days found time-dependent decreases in both [^{35}S]GTPγS binding and [^{3}H]WIN 55212-2 and [^{3}H]SR141716 binding in cerebellum, hippocampus, caudate-putamen, and globus pallidus, with regional differences in the rate and magnitude of down-regulation and desensitization (Breivogel et al., 1999). In a parallel study, the time course and regional specificity of expression of the CB_1 receptor was examined (Zhuang et al., 1998). Interestingly, receptor desensitization was found to be greater in brain sections than in brain membranes (Breivogel et al., 1999). These data suggest that cellular components important for desensitization (e.g., soluble kinases or β-arrestins) may be lost in the process of preparation of membranes. Indeed, in a comparison between β-arrestin2 knockout mice and WT mice, distinct regional differences were observed following chronic Δ^9-THC administration (Nguyen et al., 2012). These studies and others suggest that β-arrestin2 regulates CB_1 receptor signaling and adaptation in a central nervous system region-dependent manner (Kendall & Yudowski, 2016; Nguyen et al., 2012).

CB_1 receptor down-regulation following chronic cannabis exposure in humans has also been reported using positon emission tomography (D'Souza et al., 2016; Hirvonen et al., 2012). Interestingly, regional specificity of down-regulation was also observed in cannabis-dependent people, with reduction in cortical areas but not in non-cortical areas (Hirvonen et al., 2012). The receptor down-regulation correlated with years of cannabis smoking and was reversible upon cessation (D'Souza et al., 2016; Hirvonen et al., 2012). The authors concluded that cortical CB_1 cannabinoid receptor downregulation is a neuroadaptation that may promote cannabis dependence in human brain.

The CB_2 receptor is also desensitized and internalized following agonist treatment *in vitro* (Atwood, Wager-Miller, Haskins, Straiker, & Mackie, 2012; Bouaboula, Dussossoy, & Casellas, 1999; Carrier et al., 2004; Grimsey, Goodfellow, Dragunow, & Glass, 2011). The first studies were conducted in human CB_2-transfected CHO cells and demonstrated that phosphorylation at S352 appears to play a key role in the loss of responsiveness of the CB_2 receptor to CP-55,940 (Bouaboula et al., 1999). Furthermore, SR144528 could regenerate the desensitized CB_2 receptors by activating a phosphatase that dephosphorylated the receptor (Bouaboula et al., 1999). A subsequent study demonstrated that this process was dependent on Rab-5 (Grimsey et al., 2011). Interestingly, in another study, marked

functional selectivity of cannabinoid receptor internalization was observed, where WIN55,212-2 did not produce internalization; nor did most of the aminoalkylindoles tested (Atwood et al., 2012). They reported that Δ^9-THC did not produce any internalization of HEK-293 cells expressing rat CB_2, but compounds that are structurally similar to Δ^9-THC notably JWH133, THCV, and HU210 did (Atwood et al., 2012). One study utilized cultured rat microglia cells, where chronic exposure to 2-AG increased CB_2 receptor internalization (Carrier et al., 2004). Hence, the pharmacological properties and phosphorylation state of the CB_2 receptor can be regulated by both agonists and antagonists, but this appears to be agonist-selective. Whether this is also true in vivo remains to be determined.

Agonist-Biased Signaling: targeting receptor conformations leading to selective pharmacological responses

Early pharmacological studies have identified differences between the CB_1 and CB_2 receptors in their signaling via agonists that can bind to both receptors. A very relevant example is the response to Δ^9-THC, which in COS or CHO cells expressing either receptor exogenously, serves as a partial agonist for CB_1 receptors, but a weak partial agonist or antagonist at CB_2 receptors to inhibit adenylyl cyclase activity (Bayewitch et al., 1996). Drug design in the new millennium has significantly advanced development of novel ligands that can select for either CB_1 or CB_2 receptors. The endocannabinoids anandamide and 2-AG and their structural analogs have the capacity to interact with other receptors, thereby extending the network of cellular signaling pathways beyond the two cannabinoid receptors (see other reviews in this series).

Beyond selectivity based upon receptor type, the next level of selectivity is based upon the receptor's ability to couple to various signaling pathways. The protein(s) with which the receptor interact can provide the initial platform directing signaling along a pathway that can lead to preferred outcomes. For 7-transmembrane receptors, the primary divergence in signaling occurs at the selection between pathways generated via G proteins, versus pathways generated by β-arrestins (see Figure 1). In response to the endogenous agonists, the determinants between these two signaling outcomes are very likely dependent upon the time course and the strength of the stimulus. Prototypically, the receptor will initiate a pathway determined by the G protein-effector-second messenger-kinase(s) interactome, which can be unique to the differentiated functions of the cell. As the stimulus progresses or strengthens, the phosphorylation by one or several GRKs can direct the interaction with a β-arrestin, which can serve as a scaffold to couple to other signaling proteins, creating one or more signalosomes associated with differentiated functions of the cell. The next level of selectivity is which G protein or which β-arrestin (1 or 2) can be favored, which may depend upon the availability of these proteins due to co-translational synthesis and trafficking of signaling proteins, intracellular compartmentalization of the receptors and signaling complexes, or the membrane organization (e.g. partitioning to lipid rafts, or scaffolding by protein-protein interactions).

Functional selectivity in CB_1 or CB_2 receptor signaling

Advances in cannabinoid receptor-based pharmacotherapies are being sought based upon cannabinoid receptor interactions favoring either Gi/o (or other G protein) versus β-arrestin (1 or 2), referred to as “functional selectivity” or “biased agonism”. The notion that drugs can be designed to initiate signaling via one of these primary pathways is based upon our understanding that the CB_1 receptor can adopt multiple unique conformations depending upon agonist occupancy (Bosier, Muccioli, Hermans, & Lambert, 2010; Georgieva et al., 2008; Khajehali et al., 2015; Laprairie, Bagher, Kelly, & Denovan-Wright, 2016).

One of the first series of studies to investigate functional ligand selectivity identified a differential regulation by HU210 versus CP55940 in tyrosine hydroxylase gene expression in N1E115 neuroblastoma cells and rat striatum (Bosier et al., 2012; Bosier, Tilleux, Najimi, Lambert, & Hermans, 2007). Further investigations in the N1E115 cells identified cellular signaling pathway divergence by which HU210 stimulated ERK1/2 phosphorylation, whereas CP55940 stimulated JNK phosphorylation preferentially (Bosier, Lambert, & Hermans, 2008).

Investigations have identified agonist biased signaling in a non-Huntington’s disease phenotype generated by expressing STHdh(Q7/Q7) in mouse striatal medium spiny neurons. 2-AG, Δ^9-THC, and CP55940 were more potent mediators of β-arrestin2 recruitment than other agonists, whereas 2-AG, anandamide, and WIN55212-2 preferred Gi/o signaling (Laprairie, Bagher, Kelly, Dupre, & Denovan-Wright, 2014). In the Huntington’s disease phenotype of STHdh cells that have been genetically engineered to express the (Q111/Q111 Huntingtin) (Laprairie, Bagher, Kelly, et al., 2016), WIN55212-2, 2-AG and anandamide stimulated Gi/o pathways, whereas 2-AG, Δ^9-THC and CP55940 stimulated β-arrestin pathways concurrently with a reduction in CB_1 and reduced cell viability (Laprairie, Bagher, & Denovan-Wright, 2016; Laprairie, Bagher, Kelly, et al., 2016).

Other recent studies have used model systems that express cannabinoid receptors exogenously in model systems that have been developed to recognize either G protein signaling or β-arrestin mobilization. Example studies have provided predictive clues regarding pathway-preferring ligands for both CB_1 receptors (Baillie et al., 2013; Delgado-Peraza et al., 2016) and CB_2 receptors (Dhopeshwarkar & Mackie, 2016).

Conclusion

The cannabinoid receptors are expressed throughout the human body and have been shown to play critical roles in nearly all tissues examined. The diversity of signaling pathways, modulation by chronic exposure, and presence of splice variants contributes to their unique pharmacology and physiology. While selective agonists and antagonists have been discovered, it can be predicted that more extensive investigations will be appearing in the future that can guide drug design and development based upon conformational selection by agonists and perhaps also antagonist ligands that promote cellular signaling pathways.

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Abbreviations

ACC	acetyl co-A carboxylase
ACEA	arachidonyl-2-chloroethanolamide
AMPK	AMP-activated protein kinase
AP-1	Activating Protein 1
CREBH	cAMP-responsive element binding protein H
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FAK	Focal Adhesion Kinase
FAS	fatty acid synthase
GAP	GTPase activating protein
G6pc	glucose-6-phosphatase catalytic subunit
GPCR	G protein coupled receptor
GRK	G protein receptor kinase
IRS1	Insulin Receptor Substrate 1
LKB1	liver kinase B1
LXRα	liver X receptor-α
MAPK	mitogen-activated protein kinase
MTfam	mtDNA transcription factor A
NOS	nitric oxide synthase
NRF-1	nuclear respiratory factor-1
Pepck	phosphoenolpyruvate carboxykinase
PI3K	phosphatidylinositol-3-kinase
PPAR	peroxisome proliferator-activated receptor
PGC-1α	PPARγ-coactivator-1α
Phlpp1	*PH* domain leucine-rich repeat protein phosphatase-1

RARγ	retinoid A receptor γ
SCD-1	stearoyl-CoA desaturase-1
SREBP1c	sterol regulatory element binding protein 1c
SRE	sterol response element
Δ^9-THC	delta9-tetrahydrocannabinol

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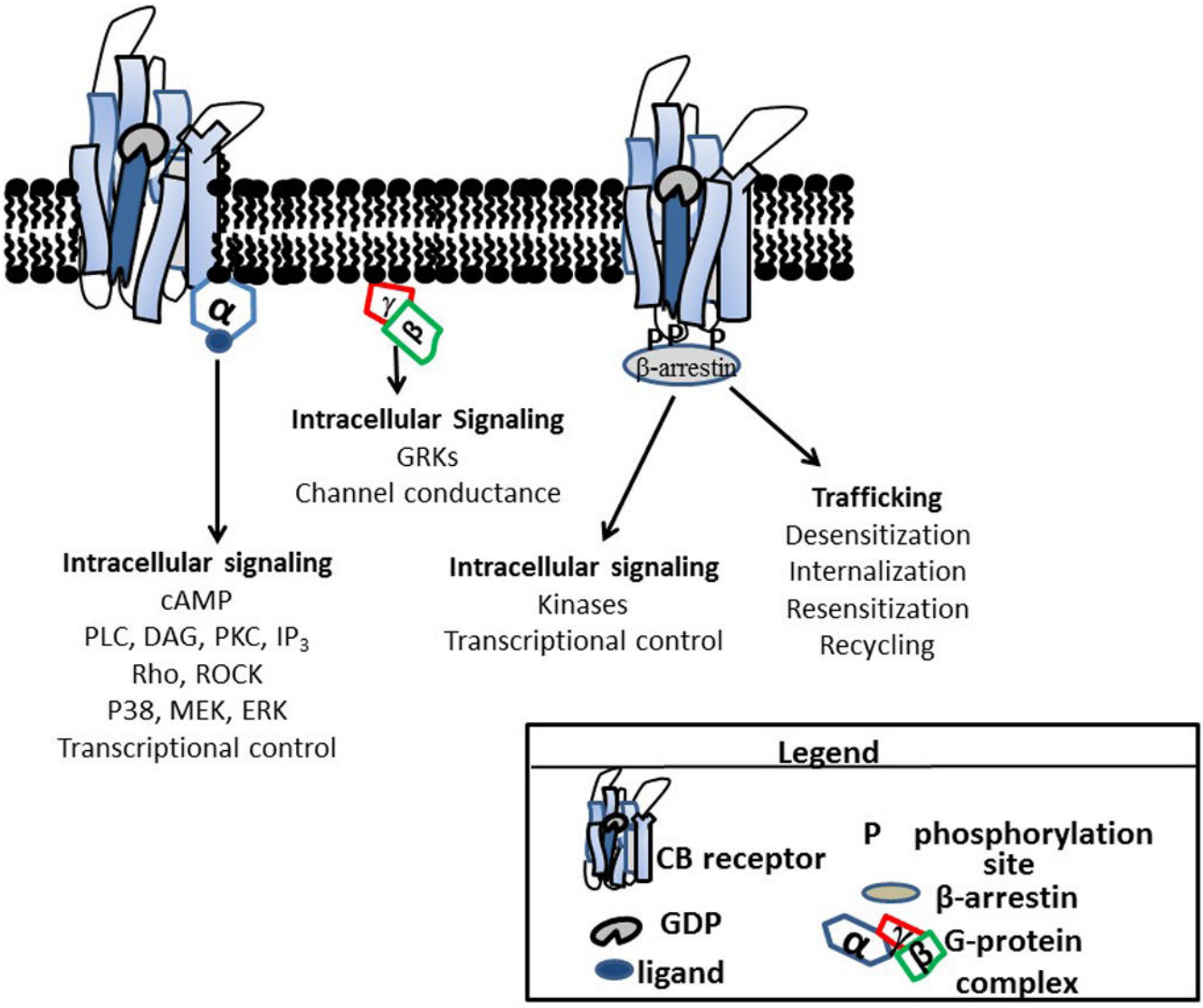

Figure 1. Biased agonism

Upon agonist binding, G-proteins dissociate into α and βγ subunits and intracellular signaling pathways commence. Phosphorylation of the receptor (by one or more GRKs, not shown) recruits β-arrestin, which, in addition to directing internalization, can also initiate intracellular signaling.